Biotechnological production of rosmarinic acid and ruscogenins. Scaling up the optimized processes to benchtop bioreactors

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BIOTECHNOLOGICAL PRODUCTION OF ROSMARINIC ACID AND RUSCOGENINS. SCALING-UP THE OPTIMIZED PROCESSES TO BENCHTOP BIOREACTORS

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Dedication

To the human brain

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Keywords:

- Caspases
- Ciclodextrines
- Coronatine
- HepG2 cancer cell line
- MCF-7 cancer cell line
- Methyl jasmonate
- Neoruscogenin
- Plant cell cultures
- Root-rhizome cultures
- Rosmarinic acid
- Ruscogenin
- *Ruscus aculeatus*
- *Satureja khuzistanica*

Abstract:

Rosmarinic acid (RA), an ester of caffeic acid and 3,4-dihydroxyphenyl lactic acid, is widely distributed in the plant kingdom, including *Satureja khuzistanica*, a threatened Iranian plant species. Interest in RA is growing due to its biological activities, including cognitive-enhancing effects, slowing the development of Alzheimer's disease, cancer chemoprotection and anti-inflammatory activity. In order to meet the increasing demand for this compound and preserve its natural sources, a new biotechnological platform based on cell suspension cultures of *S. khuzistanica* was developed. The results show that cell suspensions of this plant species synthesized high amounts of RA, which accumulated mainly inside the cells. In order to improve the system's biotechnological production, the effect of three elicitors, methyl jasmonate (MeJA), cyclodextrin (CD) and coronatine (COR) were tested. The obtained results demonstrate the effectiveness of MeJA and COR in improving the RA production of the *S. khuzistanica* cell cultures and also that the time course of growth and RA production can change with the age of the cell line and the number of subcultures. In contrast, the effects of CD were found to be insignificant. In this pipeline, the scale up of the biotechnological system to a benchtop bioreactor demonstrated the adaptability of the *S. khuzistanica* cell cultures to the scaling-up process and that the elicitors MeJA and COR also effectively improved the production at bioreactor level. Concerning the biological activity of *S. khuzistanica*, we demonstrated that an enriched RA extract was able to reduce the viability of cancer cell lines, increasing the sub-G0/G1 cell population and the activity of caspase-8 in MCF-7 cells, which suggests that *S. khuzistanica* extracts can induce apoptosis of MCF-7 cells through activation of the extrinsic pathway. In addition, our findings indicate that other compounds of the extract may act synergistically to potentiate the anticancer activity of RA.

Similarly to *S. khuzistanica*, *Ruscus aculeatus* is a threatened medicinal plant. Its main bioactive components, the steroid saponins ruscogenin and neoruscogenin, have long been used in the treatment of hemorrhoids and varicose veins, but recently demonstrated activity against some types of cancer have stimulated interest in *R. aculeatus* extracts. Thus, plant cell biofactories could constitute an alternative to the whole plant as a source of these bioactive compounds. In our work, despite the *in vitro* recalcitrance of *R. aculeatus*, after many attempts in which we tested more than 70 different culture media, we developed friable *R. aculeatus* calli and their derived plant cell suspensions. Their ruscognenin 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 COR, as in other plant *in vitro* cultures. The final total ruscogenin content was higher than 1.8 mg g DW^{-1}, after 4 weeks of culture. 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, because production was higher in the whole plant than in the root-rhizome cultures. The most productive biotechnological system, COR-elicited root rhizome cultures, was scaled up to a benchtop bioreactor without losing its capacity for biomass and ruscogenin production.

Resumen:

El ácido rosmarínico (RA), un éster del ácido caféico y el ácido 3,4-dihidroxifenil-láctico, se distribuye ampliamente en el reino vegetal, incluida *Satureja khuzistanica*, una especie vegetal iraní amenazada. El interés por el RA está incrementando actualmente debido a sus actividades biológicas, incluidos los efectos de mejora cognitiva y la desaceleración del desarrollo de la enfermedad de Alzheimer, quimioprotección del cáncer y su actividad antiinflamatoria, entre otros. Con el fin de satisfacer la creciente demanda de este compuesto y preservar sus fuentes naturales, se ha desarrollado una nueva plataforma biotecnológica basada en cultivos de células en suspensión de *S. khuzistanica*. Los resultados muestran que las suspensiones celulares de esta especie vegetal sintetizan altas cantidades de RA, que se acumula principalmente en el interior de las células. Para mejorar la producción biotecnológica del sistema, se probó el efecto de tres elicitores, jasmonato de metilo (MeJA), ciclodextrina (CD) y coronatina (COR). Los resultados obtenidos demostraron la efectividad de MeJA y COR para incrementar la producción de RA de los cultivos celulares de *S. khuzistanica* y también que el curso de crecimiento y la producción de RA podrían cambiar con la edad de la línea celular y el número de subcultivos. Contrariamente, los efectos del CD no fueron relevantes. El escalado del sistema biotecnológico a un biorreactor de sobremesa demostró la adaptabilidad de los cultivos celulares de *S. khuzistanica* a esta escala y la efectividad de los elicitores, MeJA y COR para incrementar la producción también a nivel de biorreactor. Por otro lado, y en relación con la actividad biológica de *S. khuzistanica*, demostramos que un extracto del cultivo celular enriquecido en RA fue capaz de reducir la viabilidad, aumentar la población de células sub-G0/G1 y la actividad de la caspasa-8 en la línea celular cancerígena MCF-7, lo que sugiere que los extractos de *S. khuzistanica* pueden activar la vía extriínseca de inducción a la apoptosis de las células MCF-7. Además, nuestros resultados también indican que otros compuestos del extracto de *S. khuzistanica* pueden actuar de manera sinérgica para potenciar la actividad anticancerígena del RA.

De forma similar a *S. khuzistanica*, *Ruscus aculeatus* también es una planta medicinal amenazada cuyos principales componentes bioactivos, las saponinas esteroídicas ruscogenina y neoruscogenina, se han usado clásicamente en el tratamiento de hemorroides y varices, pero recientemente estos compuestos han demostrado actividad frente algunos tipos de cáncer incrementando el interés por los extractos *de R. aculeatus*. En este contexto, las biofactorías vegetales podrían constituir una alternativa

al cultivo de la planta como fuente alternativa para la producción de estos importantes compuestos bioactivos. En nuestro trabajo y a pesar de que *R. aculeatus* es una planta recalcitrante a su cutltivo *in vitro*, después de muchos intentos en los que probamos más de 70 medios de cultivo diferentes, obtuvimos cultivos de callo friables de *R. aculeatus* y suspensiones celulares vegetales derivadas de ellos. La producción de ruscogneninas de estos sistemas se comparó con la de cultivos de plántulas y de raíces-rizomas también cultivados *in vitro*. El sistema raíz-rizoma mostró una mayor capacidad para la producción de biomasa y ruscogeninas que las suspensiones celulares y los rendimientos mejoraron en gran medida mediante la elicitación de los cultivos con COR. El contenido final total de ruscogeninas fue superior a 1,8 mg g^{-1} de PS, después de 4 semanas de cultivo. Aunque las ruscogeninas se acumulan en las plantas principalmente a nivel de rizomas, también se demostró que la parte aérea podría desempeñar un papel importante en su biosíntesis, porque la producción fue mayor en la planta entera que en los cultivos de raíz-rizoma. El sistema biotecnológico más productivo, los cultivos de raíz-rizoma elicitados con COR fue escalado a nivel de biorreactor de sobremesa sin pérdida de su capacidad para producir biomasa y ruscogeninas.

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1. Index of abbreviations

1-D: one-dimensional

2,4-D: 2,4-dichlorophenoxyacetic acid

2-D: two-dimensional

3-D: three-dimensional

4CL: 4-coumaric acid CoA-ligase

4C-pHPL: 4-coumaroyl-4'-hydroxyphenyllactic acid

4PU-30: N- (2-chloro-4-pyridyl) -N'- phenylurea

AtPAP1: anthocyanin pigment transcription factor

B5: Gamborg culture medium

BA: benzyladenine

BAP: benzylaminopurine

BMP_{CFW}: biomass productivity: CFW/t

BSA: bovine serum albumin

C3′H: coumarate 3′-hydroxylase

C4H: cinnamate 4-hydroxylase

CCoAMT: caffeoyl-CoA O-methyltransferase

CCR: cinnamoyl-CoA reductase

CD: cyclodextrin

cDNA-AFLP: cDNA-amplified fragment length polymorphism analysis

CDW: cell dry weight

CFD: computational fluid dynamics

CFW: cell fresh weight

CFWt0: initial cell fresh weight

CHPL: 4-coumaroyl-CoA:4’-hydroxyphenyllactic acid 4-coumaroyltransferase

COMT: caffeic acid O-methyltransferase

COR: coronatine

D: darkness

DMAPP: dimethylallyl diphosphate

DMSO: dimethyl sulfoxide

DO: dissolved oxygen

DTT: dithiothreitol

DW: dry weight

FW: fresh weight

GA_3: gibberellic acid

GI: growth index [(DW harvested-DW inoculum)/DW inoculum]

GT: glycosyl transferase

HepG2: human hepatoma-derived

HCT: hydroxycinnamoyl transferase

HPPR: hydroxyphenylpyruvate reductase

IAA: indolacetic acid

IBA: indol-3-butyric acid

IpPP: Isopentenyl diphosphate

Kin: kinetin

k_La: volumetric oxygen transfer coefficient

L: light

MeJA: methyl jasmonate

MEP: methyl-D-erythritol-4 phosphate

MCF-7: human breast adenocarcinoma-derived

MS: Murashige and Skoog

MT: meta-topolin

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

M-β-CD: randomly methylated-β-CD

NAA: naphthalenacetic acid

NAD(P)H: Nicotinamide adenine dinucleotide phosphate

NT: non-treated

P/V: power input values

PAL: phenylalanine ammonia lyase

PBS: phosphate-buffered saline

pcv: packed cell volume

PGRs: plant growth regulators.

pHPL: 4-hydroxyphenyllactic acid

pHPP: 4-hydroxyphenylpyruvic acid

Pic: Picloram

PME: plant metabolic engineering

PMSF: phenylmethanesulfonyl fluoride

PSM: plant secondary metabolites

PVP: polyvinyl pyrrolidone

RA: rosmarinic acid

RAS: rosmarinic acid synthase

ROS: reactive oxygen species

SA: salicylic acid

SKE: *Satureja khuzistanica* extract

SF: shaken flasks

TAT: pyridoxalphophate-dependent tyrosine aminotransferase

t_D: doubling time: Ln2/μ_{max}

UK: United Kingdom

WV: working volume

YE: yeast extract

μ_{CFW}: specific growth rate: $(CFW_{highest} – CFW_{to})/t_{highest}$

μ_{max}: maximum-specific growth rate: $(\ln[X]_{DW2}-\ln[x]_{DW1}/(t_2-t_1)$

ZT: zeatin

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4. Objectives

When the natural source of a phytochemical cannot meet the market demand or is becoming increasingly limited because of over-harvesting or habitat deterioration, plant biotechnology can provide an alternative system for its production. Plant cell cultures producing phytochemicals have several advantages over plant cultures in the field: a) the target product can be harvested anywhere in the world, while maintaining strict production and quality control; b) herbicides and pesticides are not required; c) problems related with the climate and ecology are avoided; and d) growth cycles are significantly reduced, taking weeks rather than the years of an intact plant (Rao and Ravishankar, 2002). Yet despite all these advantages, there are still only a few examples of successful bioactive compound production in plant cell factories, including that of taxol, arbutin and ginsenosides (Sharma et al. 2014), probably because too little is known about plant secondary metabolism and its control *in vitro*.

Another factor is that biotechnological platforms are expensive and can only be used in cost-effective processes. This is the case when the product has a high price on the market, production levels are higher than in field-cultured plants, the target compound is easy to recover from the system, production is stable, there are no alternative synthetic processes, and the process can be scaled up to bioreactor level (Bourgaud et al., 2001).

To be successful, the biotechnological production of plant secondary metabolites (PSM) requires the optimization of the system, whether by empirical or rational approaches. Empirical approaches typically involve optimization of the plant culture system by input and output factors. Input factors consist of cell line selection, the composition of the culture medium and conditions, addition of elicitors and precursors, the design of the bioreactor, improvement of down-stream processes, etc., while output factors include growth capacity, nutrient uptake, productivity and release of the target compound to the culture medium (Cusido et al., 2014).

In contrast, a rational approach to plant cell bioprocessing is primarily concerned with identifying the genes, enzymes, and biosynthetic pathways involved in the production of a specific secondary metabolite and how they are controlled (Walker et al., 2002). This cellular and molecular information is then applied to the engineering of plant

secondary metabolism. Biochemical studies using classical methodology have been gradually mapping biochemical and signaling pathways involved in PSM production, including signal transduction, biosynthesis, transport and storage. Nowadays, the rate of discovery of new genes and proteins potentially involved in PSM is being accelerated by state of the art “omics” technology, such as new hypothesis-free high-throughput methods of transcription profiling (microarrays, cDNA-AFLP, SSH cDNA libraries) and proteomics. The functions of candidate sequences are being assessed by new bioinformatics and functional genomics tools (Sabater-Jara et al., 2010a).

Interest in rosmarinic acid (RA) is growing with increasing awareness of its potential benefits for human health as a pharmaceutical or dietary supplement. Among its promising biological activities are cognitive-enhancing and cardioprotective effects, cancer chemoprevention properties and a potential use in the treatment of Alzheimer’s disease. Therefore, a challenge for biotechnologists is the development of highly productive cell cultures that can be scaled up to an industrial level in a cost-effective way (Bulgakov et al., 2012). Many laboratories have already achieved successful results in this field because RA is relatively easy to produce in cell cultures.

On the other hand, *Ruscus aculeatus* extracts are an exclusive source of hexacyclic spirostanol and pentacyclic furostanol saponins, the main aglycones being ruscogenin and neoruscogenin, which have vasoconstrictive and venotonic properties and are currently used to treat conditions such as chronic venous insufficiency, varicose veins and hemorrhoids. It has recently been demonstrated that ruscogenin is an innovatively effective inhibitory agent of hepatocellular carcinoma metastasis (Hua et al., 2018) and in an optimized combination with ginsenoside Rb1 and schisandrin (GRS) exhibits a significant protective effect against myocardial ischemia (Li et al., 2018). These recently discovered pharmacological properties of *Ruscus* steroidal saponins, together with the fact that *R. aculeatus* is an endangered species, has prompted a growing interest in finding new and biosustainable sources of these compounds.

In this scenario, the research for this PhD thesis had a dual objective: to improve the biotechnological production of RA and gain further insight into its potential anticancer activity, as well as to look for new sources of *Ruscus* steroidal saponins based on biotechnological platforms such as *R. aculeatus* root-rhizome and cell cultures.

To achieve the first objective, we used a cell line of *Satureja khuzistanica* derived from calli generated by the group of Prof. H.M. Mirjalili from the Shahid Beheshti University of Tehran (Iran). Given that this cell line accumulated high amounts of RA, but had a doubling time (t_d) higher than 10 days (personal communication), the aim was to

optimize the culture conditions of the *S. khuzistanica* cell suspension in shake flasks and scale up the process to a benchtop bioreactor in order to improve the biotechnological production of RA. In one of the first steps towards achieving our goal, we optimized the inoculum density of the cell cultures for biomass production. Then, by the use of the elicitors methyl jasmonate (MeJA) and coronatine (COR), as well as the permeabilizing agent cyclodextrin (CD), either separately or in combination, we optimized the production of RA without significantly affecting the cell growth. Finally, having established the best conditions for RA biotechnological production in our cell line, the culture system was scaled up to benchtop bioreactor level with a working volume of 1 L. Additionally, to investigate the potential anticancer properties of RA, we carried out several bioassays to confirm the cytotoxic activity of this compound and try to elucidate its mechanism of action.

Regarding the second objective, and in view of the limited success of the few reported examples of ruscogenin biotechnological production, we optimized the conditions for obtaining friable *R. aculeatus* callI, developed two types of biotechnological platforms based on the culture of isolated root-rhizomes and cell suspensions of *R. aculeatus* and tested the effects of elicitation with COR on the production of ruscogenins in both. The final aim was to scale up the most productive system to benchtop bioreactor level.

5. Introduction

5.1. Rosmarinic acid

5.1.1. *Satureja khuzistanica*

The genus *Satureja* L. belongs to the family Lamiaceae, subfamily Nepetoidae, and tribe Mentheae, and comprises approximately 200 species, which are mainly aromatic and range from herbaceous plants to shrubs, with a wide distribution in the Mediterranean area, Asia and boreal America. Fourteen wild species, *S. sahandica, S. edmondi, S. intermedia, S. khuzistanica, S. mutica, S. rechingeri, S. isophylla, S. atropatana, S. spicigera, S. bachtiarica, S. montana, S. macrantha, S. laxiflora and S. hortensis*, grow in northern, northwestern, western, southwestern, and central parts of Iran. *S. khuzistanica* Jamzad, with the common Persian name of “marzeh khuzestani”, is an endemic plant of the southwestern areas of Iran (Fig. 1) (Hadian et al., 2011).

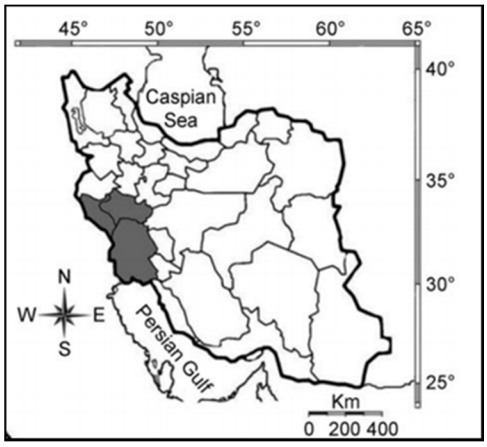

Figure 1. Map of Iran. Regions with wild populations of *S. khuzistanica* are in gray (modified from Hadian *et al*., 2011).

5.1.1.1 Botanical description and habitat

S. khuzistanica is a subshrub with a branched stem and densely leafy, whose leaves are ovate-orbicular shaped and pubescent with short white hairs. The base of the stem

leaves is attenuate and petioliform, while the floral leaves are similar but smaller (Fig. 2). The reproductive verticil has from two to eight violet flowers connected to the stem with a short peduncle (Hadian et al., 2011). *S. khuzistanica* has a strong fragrance and is a popular herb in some parts of Iran where it is used by native populations as a herbal tea and in folk medicine for its analgesic and antiseptic properties. More recent biological activities reported for *S. khuzistanica* include antiviral, antibacterial, antifungal, antispasmodic and antidiarrhea or vasodilatory properties (Abdolllahi et al., 2003; Amanlou et al., 2004, Haeri et al., 2006, Shahsavari et al., 2009; Sadeghi-Nejad et al., 2010).

Figure 2. *S. khuzistanica* Jamzad plants growing in their natural habitat (southwestern areas of Iran) (image courtesy of Prof. H.M. Mirjalili, Shahid Beheshti University, Tehran, Iran).

5.1.1.2 Phytochemical composition

Eighteen compounds have been identified in the essential oil of *S. khuzistanica* populations, representing 97.2–99.3% of the total oil composition (Hadian et al., 2011). The main component in all studied populations is carvacrol, found in a high percentage. Carvacrol is thought to play an important role in the adaptation of *S. khuzistanica* to harsh environmental conditions, such as a hot dry climate and calcareous, stony soils. The carvacrol precursors, *p*-cymene and γ-terpinene, have also been identified but in low concentrations. Carvacrol is a monoterpenoid phenol biosynthesized via the

aromatization of γ–terpinene to *p*-cymene and then hydroxylated to *p*-cymene. This compound, with its two precursors, γ-terpinene and *p*-cymene, are the major components in several essential oils of the Lamiaceae family (e.g., in thyme, oregano, and savory oil). Carvacrol has a wide range of activities including anti-inflammatory, antioxidant, antimicrobial and anticandidal properties (Di Pasqua et al., 2007; De Vincenzi et al., 2004).

The main components of *S. khuzistanica* essential oil in the studied populations are carvacrol (93.9%), eugenol (1.0%), p-cymene (0.8%), and thymol (0.6%) (Farsam et al., 2004). This suggests that the populations of *S. khuzistanica* are fundamentally homogeneous in their chemical composition. The essential oil constituents of other *Satureja* species have also been studied, revealing the existence of chemotypes in these species. For example, Sefidkon et al. (2004) reported a chemical variation in *S. sahandica* oils from different populations and identified thymol (19.6–41.7%), *p*-cymene (32.5–54.9%), and γ-terpinene (1.0–12.8%) as the main oil constituents. Similarly, the essential oil composition of *S. montana* showed significant variations in the concentration of its major components, i.e., carvacrol (5.0–69.0%), linalool (1.0–62.0%), γ-terpinene (1.0–31.0%), and p-cymene (3.0–27.0%), indicating the existence of several chemotypes (Milos et al., 2001). The variability of the essential oil composition of the cultivated accessions of *S. hortensis* has been recently reported (Hadian *et al*., 2010), and carvacrol (42.0–83.3%), γ-terpinene (0.5–28.5%), and *p*-cymene (1.0–17.1%) were identified as the major components.

The caffeic acid ester rosmarinic acid (RA) is known to have cognitive-enhancing effects and can slow down the development of Alzheimer's disease. It also has cancer chemoprotection properties and anti-inflammatory, antibacterial, and antiviral activities (Bulgakow et al., 2012). The content of RA in MeOH extracts of the *S. khuzistanica* samples varies significantly among different populations (Hadian et al., 2011) in contrast with other phenols found in this plant species. Abdanan populations accumulate the highest levels of RA (1.81%), followed by those from Kaver (1.31%), while the lowest value has been obtained from Paalam populations (0.59%). Several authors suggest that RA is synthesized in response to stress produced under harsh environmental conditions or as a defense compound against plant pathogens (Bais et al., 2002; Javanmardi et al., 2002). In accordance with this theory, the higher RA levels found in the Abdanan and Kaver populations may be explained by the very hot and dry conditions where the plants grow.

5.1.2. Rosmarinic acid

Rosmarinic acid (RA) was first isolated from *Rosmarinus officinalis* (Lamiaceae) by Scarpati and Oriente in 1958. RA is an ester of caffeic acid and (R)-(+)-3-(3,4-dihydroxyphenyl) lactic acid (Fig. 1), prevalent in a wide range of plants, and it is considered to be a bioactive component of several medicinal plant species (Petersen et al., 2009). RA exhibits various pharmacological activities, including the prevention of oxidation of low density lipoprotein, and inhibition of murine cell proliferative, cyclooxygenase and anti-allergic activity (Park et al., 2008). The classical biological activities attributed to RA are antibacterial, antiviral, anti-inflammatory as well as antioxidant (Szabo et al., 1999; Hras et al., 2000).

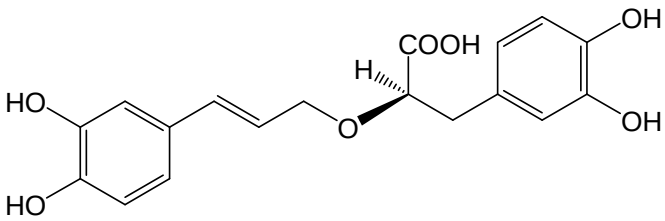

Figure 3. Chemical structure of rosmarinic acid.

5.1.2.1. Natural sources

Based on current knowledge, RA is distributed in 39 plant families, including one of the earliest groups of land plants to evolve (the hornworts) and highly evolved monocotyledonous and eudicotyledonous species. In particular, RA has been isolated from many species of the Lamiaceae (genus: *Ajuga, Agastache, Calamintha, Cedronella, Coleus, Collimsonia, Dracocephalum, Elsholtzia, Glechoma, Hornium, Lavandula, Lycopus, Melissa, Mentha, Micromeria, Monarda, Origanum, Perilla, Perovskia, Plectranthus, Salvia* and *Satureja*) and Boraginaceae (genus: *Cerinthe*, *Echium*, *Heliotropium*, *Lindefolia*, *Lithospermum*, *Nonea*, *Symphytum*, *Hydrophyllum*, *Nemophila* and *Phacelia*), but not all the members of these families accumulate RA. Additionally, RA has also been found in many plants outside the Lamiaceae and Boraginaceae, including several species of the Choranthaceae (genus: *Chloranthus*) or Blechnaceae (genus: *Blechnum*), as well as some orders of the monocotyledonous plants, and the rosids and asterids within the eudicotyledonous plants. However, to date RA has not been reported in any of the gymnosperms (Petersen et al., 2009; Petersen, 2013 and references therein).

5.1.2.2. Biosynthesis

The biosynthetic pathway of RA has been comprehensively elucidated, and eight enzymes involved in the different steps have been characterized (Barberini et al., 2013). The initial precursors of RA are the aromatic amino acids L-phenylalanine and L-tyrosine, which are transformed to the intermediates 4-coumaroyl-CoA and 4-hydroxyphenyllactic acid (pHPL), respectively (Fig. 4). Phenylalanine is converted to 4-coumaroyl-CoA by the enzymes of the phenylpropanoid pathway, phenylalanine ammonia-lyase (PAL), cinnamic acid 4-hydroxylase (C4H) and 4-coumaric acid CoA-ligase (4CL). Tyrosine, with 2-oxoglutarate as a co-substrate, is transaminated by the pyridoxalphophate-dependent tyrosine aminotransferase (TAT) to 4-hydroxyphenylpyruvic acid (pHPP). Hydroxyphenylpyruvate reductase (HPPR) is the enzyme responsible for the NAD(P)H-dependent reduction of pHPP. This enzyme, considered to be the first specific key enzyme responsible for the biosynthesis of RA, was first characterized in cell-free extracts obtained from suspension-cultured cells of *C. blumei* (Petersen and Alfermann, 1988; Häusler et al., 1991) and was then purified and sequenced (Kim et al., 2004).

The two intermediary precursors (pHPL and 4-coumaroyl-CoA) are coupled by ester formation to 4-coumaroyl-4'-hydroxyphenyllactic acid (4C-pHPL), with release of coenzyme A. The condensation reaction is catalysed by 4-coumaroyl-CoA:4'-hydroxyphenyllactic (CHPL) acid 4-coumaroyltransferase from the BAHD acyltransferase family, commonly referred to as rosmarinic acid synthase (RAS). RAS transfers the 4-coumaroyl moiety to the aliphatic OH-group of pHPL (Fig. 4). Two meta-hydroxylations of the 4-coumaroyl moiety in the ester by two distinct (3- and 3'-) cytochrome P450 monooxygenases from the CYP98A family convert CHPL to RA (Fig. 4).

Only four of the enzymatic activities involved in this biosynthetic pathway seem to be specific to RA biosynthesis (Hücherig and Petersen, 2013). The enzymes PAL, C4H, and 4CL belong to the general phenylpropanoid pathway and are highly prevalent in land plants, since they catalyze the precursors for the formation of lignin and other phenolic compounds. TAT is also considered as a primary enzyme because it forms pHPP, which is needed for the biosynthesis of tocopherols and plastoquinones (Douce and Joyard, 1996).

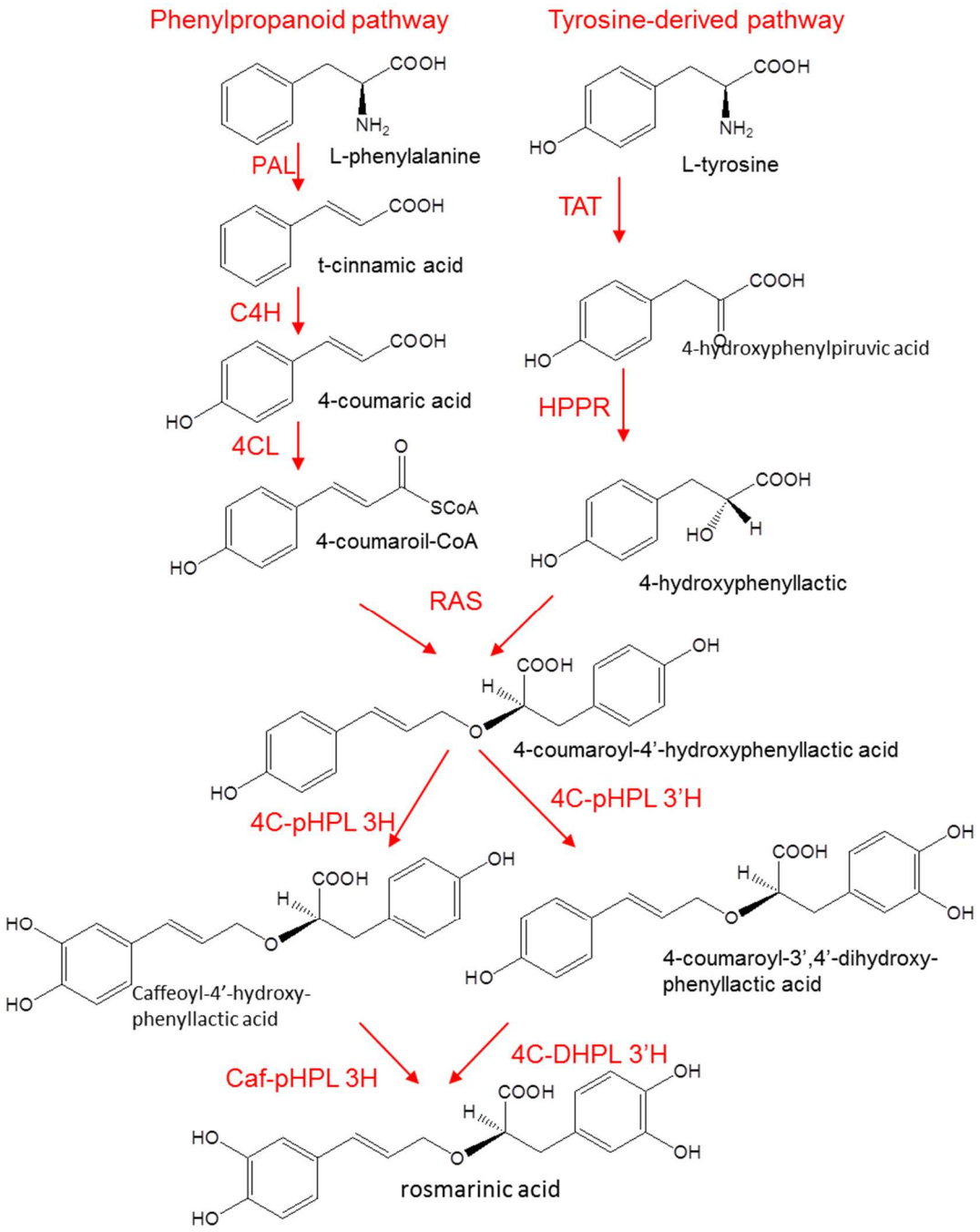

Figure 4. Scheme of the biosynthetic pathway of rosmarinic acid in *Coleus blumei*. PAL: phenylalanine ammonialyase, C4H: cinnamic acid 4-hydroxylase, 4CL: 4-coumarate:coenzyme A ligase, TAT: tyrosine aminotransferase, HPPR: hydroxyphenylpyruvate reductase, HPPD: hydroxyphenylpyruvate dioxygenase, RAS: rosmarinic acid synthase, 4C-pHPL 3/3'-H 4-coumaroyl-4'-hydroxyphenyllactate 3/3'-hydroxylase(s), Caf-pHPL 3H caffeoyl-4'-hydroxyphenyllactate 30-hydroxylase, 4C-DHPL 30H 4-coumaroyl-30,40-dihydroxyphenyllactate 3-hydroxylase (modified from Hücherig and Petersen, 2013).

The conversion of hydroxypyruvate to glycerate by either a NADH-dependent peroxisomal hydroxypyruvate reductase (HPR) or a cytosolic NADPH-dependent HPR2

during photorespiration (Timm et al., 2008) is similar to the stereospecific reduction of pHPP by HPPR. In fact, HPR2 from *Arabidopsis thaliana* heterologously expressed in *E. coli* accepted hydroxyphenylpyruvate as a substrate, even though *A. thaliana* does not biosynthesize RA (Petersen, 2013). It is currently under investigation whether HPRR is related to cytosolic HPR and if it can be strictly considered as a key enzyme in RA synthesis.

The enzymes involved in the last three steps of RA biosynthesis, RAS and the 3- and 3'-hydroxylases, have been characterized in cellular and subcellular preparations of suspension cells of *C. blumei* (Petersen et al., 1993; Petersen, 1997). Although the hydroxycinnamoyl transferases and the meta-hydroxylases show a high level of sequence similarity and thus seem to be closely related, expression studies in heterologous systems showed that the enzymes from *C. blumei* are nevertheless specific for substrates involved in RA biosynthesis (Berger et al., 2006, Eberle et al., 2009).

A list of all the enzymes involved in RA biosynthesis whose full-length cDNA sequence has been described is included in Petersen (2013). The RAS gene has been cloned from *C. blumei*, *Melissa officinalis*, *Lavanda angustifolia* and *Salvia miltiorrhiza*, showing that it has a high homology with genes encoding hydroxycinnamoyl transferases. CYP98A4 has been cloned from *Lithospermun erythrorhizon* (CYP98A46), *Ocium basilicum* (CYP98A13), *C. blumei* (CYP98A414) and *S. miltiorrhiza*.

5.1.2.3. Biological activity and medical applications

The extensive literature available about the bioactivity of RA shows it is a well-studied topic (Bulgakov et al., 2012). RA presents antimicrobial, anti-inflammatory and antioxidant activities, and is used to treat peptic ulcers, arthritis, cataracts, cancer, rheumatoid arthritis and bronchial asthma, among other illnesses (Petersen and Simmonds, 2003). The biological activities of RA have led to another use, mainly in Japan, as a food preservative to extend the shelf life of fresh seafood (Park et al., 2008). The biological activities of RA are summarized in Table1.

Table 1. Biological activities of RA (modified from Bulgakov et al., 2012).

Biological activity	Potential usage
Antioxidant activity and membrane stabilization[a]	Protection against chemically induced chromosome breakage and primary DNA damage
Increase of the physical and oxidative stability of liposomes[a]	
Reduction of the frequency of micronuclei and the extent of DNA damage induced by doxorubicin[a]	
Suppression of UVB-induced alterations to human keratinocytes[a]	Skin protection against UVB light
Reduction of IFN-γ and IL-4 production by activated T cells[b]	Skin protection against atopic dermatitis
Protection of neurons against insults[a]	Rosmarinic acid is a promising neuroprotective compound of potential use at the nutritional/pharmaceutical interface
Cognitive-enhancing effect[b]	
Prevention of the development of Alzheimer's disease[b]	
Attenuation of the degeneration of motor neurons and extension of the life span of model mice[b]	
Anti-angiogenic activity against retinal neovascularization[b]	Treatment of retinopathy
Inhibition of TNF-α-induced ROS generation and NF-κB activation and activation of TNF-α-induced apoptosis[a]	Promising for cancer prevention and treatment of a variety of human cancers that are resistant to chemotherapy
The long-term exposure of animals to RA in the diet is sufficient for cancer chemoprevention[b]	
Inhibition of bone metastasis from breast carcinomas[b]	
Antifibrotic activity[a,b]	Drug candidate for ameliorating liver fibrosis
Dramatic apoptotic activity on potentially pathogenic $CD4^+CD45RO^+$ effector T cells[a]	Treatment of rheumatoid arthritis
Inhibition of caspase-1 activity, mitochondrial apoptotic pathway and activation of NF-κB by cisplatin[a]	Prevention of harmful side effects of anticancer agents in patients undergoing chemotherapy

[a], *in vitro* studies; [b], *in vivo* (animals); [c], *in vivo* (humans)

One of the main properties of RA is its antioxidant activity, which is probably due to the fact that it stabilizes membranes and prevents free radical movement, consequently protecting the membranes against oxidative problems (Pérez-Fons et al., 2010). These results were confirmed when it was shown that RA increases the general stability of the spherical vesicles known as liposomes (Panya et al., 2010). At the same time, Vostálová et al. (2010) showed that RA significantly reduced ROS- (reactive oxygen species) generation activity and decreased the secretion of IL-6 from T cells and macrophages, thereby avoiding the generation of human keratinocytes caused by UVB. An important application of RA as an antioxidant is that it significantly decreases the side

effects involving DNA/chromosome damage induced by the anticancer compound, doxorubicin (Furtado et al., 2010).

Alzheimer's patients are known to have amyloid-B plaques in their brain, so there is considerable interest in finding a way of preventing the aggregation of these peptides. The use of orally administered RA has proved to be effective against different pathways leading to the formation of these plaques (Hamaguchi et al., 2009). Fallarini et al. (2009) showed that RA at low concentrations exerted a protective effect on neurons against one of the most common motor neuron diseases, known as amyotrophic lateral sclerosis, which is a neurodegenerative disease. When RA was administered intraperitoneally in mice, at the pre-symptomatic stage, the motor dysfunction was delayed and their life span extended (Shimojo et al., 2010). Also using mice as an animal model, Kim et al. (2009) showed that RA inhibited angiogenesis in the retinal surface, since this compound clearly inhibits the growth of the cells that participate in this process (retinal endothelial cells). In an *in vitro* angiogenesis test, RA also inhibited the formation of tube-like structures characteristic of this pathological vascularization.

RA also reduced the fibrosis process and improved the biochemical indicators and morphology of the pathological tissues in a rat model with fibrosis in the liver (Li et al., 2010). This was due to RA provoking a reduction of some liver cytokines as well as the expression levels of a related gene. Also, an apoptotic effect of RA has been shown by Hur et al. (2007) and strong evidence has been presented for anticancer action, especially a metastasis inhibitory capacity, after long-term consumption of large amounts of RA in the diet (Paluszczak et al., 2010).

5.1.3. Approaches to the biotechnological production of rosmarinic acid

The high demand for many medicinal plants has led to massive overharvesting and many of them have become endangered species in their original habitats. An important challenge for Plant Biotechnology is to find an alternative source of biologically active PSM. Biotechnological platforms based on plant cell cultures have been developed for several medicinal plant species. Among the methods used to improve the biotechnological production of PSM are screening and selection of highly productive cell lines, as well as optimization of culture conditions and induction of secondary metabolism by the use of elicitors. When undifferentiated cultures such as calli and cell suspensions are used to produce the target compounds, results are often poor.

Currently, it is potentially possible to induce callus cultures from practically all plant species, although some species present more difficulties. Mineral nutrient composition, the type and concentration of plant growth regulators, as well as the source of explants are factors to be considered in callus induction (Fig. 5). *In vitro* techniques can generate somaclonal variation: in this case, the selection of highly productive cell lines is another strategy for the successful production of PSM (Georgiev et al., 2009). Growth and secondary metabolism are often antagonistic processes due to competition for the same precursors. That is why in biotechnological processes it is often necessary to change the conditions that are optimal for growth to achieve high productivity of the target compound. The use of biosynthetic precursors and elicitors are widely used strategies for this purpose.

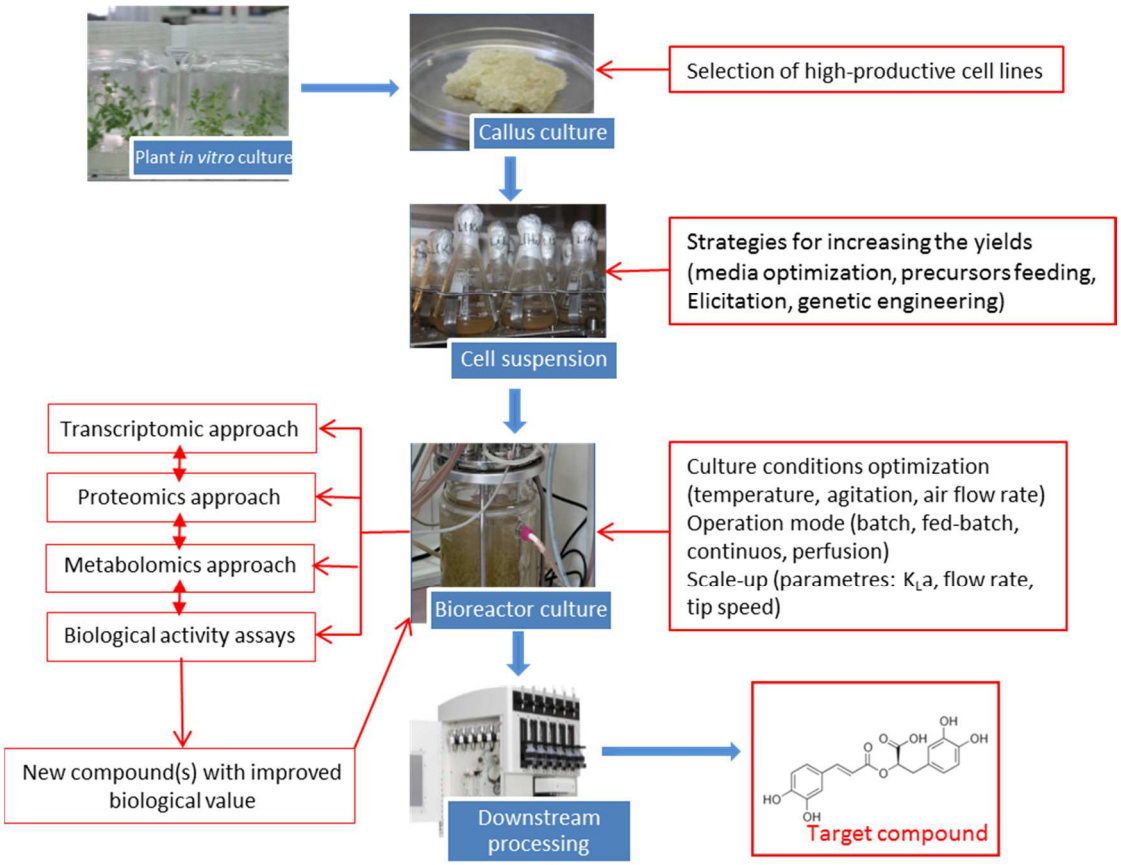

Figure 5. State-of-the-art biotechnological production of plant secondary metabolites in plant cell cultures (modified from Georgiev et al., 2009).

Another particular issue in the development of a biotechnological process is scaling up the culture to bioreactor level. Key factors in this process include the selection of a suitable method for the process mode (batch, feed-batch, perfusion, etc.), and bioreactor type (stirred, airlift, bubble column, wave, etc.).

Recently, functional genomics (including transcriptomics and proteomics) synergically coupled with metabolomics have led to a systems biology approach, which potentially allows a full exploration of the biochemical machinery of plant cells and consequently their biosynthetic capacity can be more efficiently exploited (Goossens and Rischer, 2007). In *Taxus* media cell cultures elicited with methyl jasmonate (MeJA), this approach recently enabled the isolation of 667 gene sequence tags (using cDNA-amplified fragment length polymorphism analysis (cDNA-AFLP)) whose expression was modulated by the elicitor. A new gene was cloned from these tags and expressed *in vitro* and the results allowed the identification of Taximin, a new master regulator of taxane biosynthesis that could be used to improve the biotechnological production of taxol in *Taxus* sp. cell cultures (Onrubia et al., 2014). Combining metabolomics analyses with biological assays (testing for anticancer, anti-inflammatory activities, etc.) will facilitate the discovery of new phytochemicals with improved therapeutic properties.

The current possibilities that plant biotechnology offers for PSM production and the important pharmacological activities of RA have prompted many researchers to try to produce this compound in biotechnological platforms, such as shoots (Françoise et al., 2007), cell suspensions (Petersen and Simons, 2003) and hairy root cultures (Fattahi et al., 2013).

5.1.3.1. Plant cell cultures for RA production

Numerous species have been used to produce RA in plant *in vitro* cultures, such as *Anchusa officinalis*, *Eritrichium sericeum, Lithospermum erythrorhizon* (Boraginaceae), *Agastache rugosa*, *Coleus blumei*, *Hyssopus officinalis*, *Lavandula vera*, *Ocimum basilicum*, *O. sanctum, Salvia officinalis, S. chamelaeagnea, S. fruticosa, S. miltiorrhiza, S. maxima, S. verde, Zataria multiflora* (Lamiaceae) and *Anthoceros agrestis* (Anthocerotaceae), among other species (reviewed in Bulgakov et al., 2012), and more recently in *S. khuzistanica* (Sahraroo et al., 2014). The different stages in the development of RA-producing cell cultures of *S. khuzistanica* are represented in Fig. 6. Biotechnological production of RA in plant cell cultures has achieved high yields,

because this compound belongs to the so-called preformed secondary metabolites in plants, which are persistently biosynthesized (Bais et al., 2002).

The first high RA production in cell suspension cultures was reported in sage (*S. officinalis*) by Hippolyte et al. (1992). The growth capacity and production of RA by these cells were modified by the culture conditions, leading to a 10-fold increase in RA production, and attaining 6.4 g/L under optimal conditions. Suspension cultures of *C. blumei* accumulated high amounts of RA in a medium with elevated sucrose concentrations (Petersen et al., 1994). Sucrose levels also affected RA production in cell suspension cultures of *Anthoceros agrestis* Paton: 2 and 4% sucrose were used, and cell suspensions achieved up to 5.1% dry weight (DW) of RA at day 8 in the medium supplemented with the lower concentration of sucrose (Vogelsang et al., 2006). In contrast, 7% sucrose was the optimal concentration for increasing RA production in cell cultures of *L. vera* (Llieva and Pavlov, 1997). Sugar-feeding experiments have also been carried out in callus cultures of *Zataria multiflora*, and the best source of sugar was 75 g glucose L^{-1} culture medium, when the callus achieved 158.26 mg RA g DW^{-1}, a content 13-fold higher than the maximum reached in *in vitro* micropropagated shoot cultures (Françoise et al., 2007).

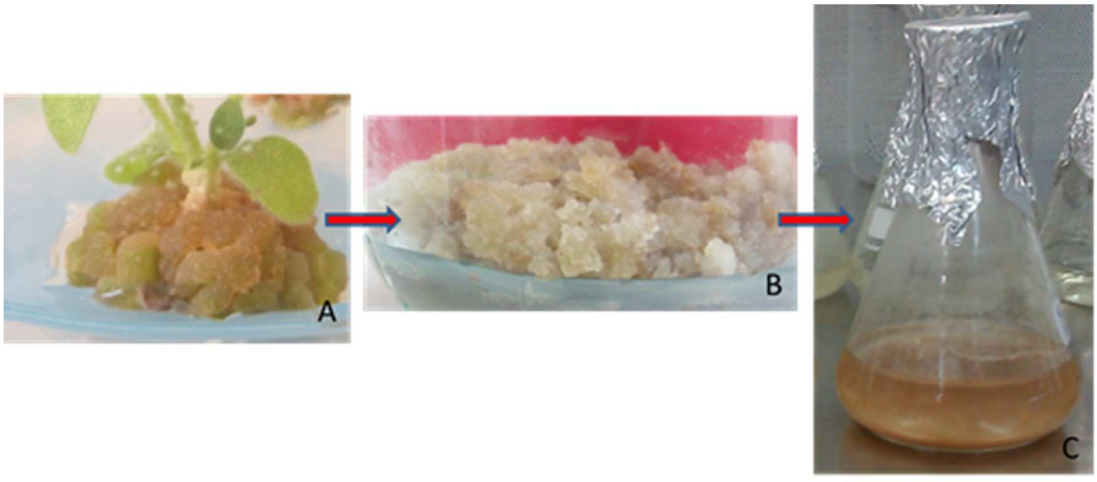

Figure 6. Steps for obtaining cell suspensions of *S. khuzistanica*. (A) Plantlet *in vitro* culture developing callus; (B) Callus culture; (C) Cell suspension (image courtesy of Prof. Mirjalili, Shahid Beheshti University, Tehran, Iran).

Periodic culture perfusion increased cell density and RA production in *Achusa officinalis* cell cultures. In these conditions the maximum RA production was reached with an inoculum size of 4 g DW L^{-1} (Su and Lei, 1993). In order to increase RA levels, a

perfused-batch culture was developed in shake flasks (Su et al., 1993). This strategy involved an intermittent medium exchange and the results showed a 2.3-fold increase in dry biomass and 2.2-fold rise in RA production in comparison with non-perfused cultures.

RA contents of callus cultures of *S. chamelaeagnea* induced on Murashige and Skoog (MS) culture medium containing 1-2 mg of 2,4-dichlorophenoxyacetic acid (2,4-D) were higher than the levels achieved by shoots induced on the same medium supplemented with 1 mg L^{-1} benzyladenine (BA) (Huang and Van, 2002). Biotechnological production of RA in callus, cell suspension, and root cultures of *S. fruticosa* was studied by Karam et al. (2003). The highest production was reached by 5-week-old calli (2.12 mg 100 mg DW^{-1}), being 10-fold higher than in organs of field-grown plants.

Other reported sources of RA are calli and cell suspensions of *A. rugosa* and *E. sericeum*. After 10 days of culture, cell suspensions of *A. rugosa* reached the maximum growth and RA production when cultured in B5 liquid medium together with 2 mg L^{-1} 2, 4-D and 0.1 mg L^{-1} of benzylaminopurine (BAP) (Xu et al., 2008). *E. sericeum* root lines derived spontaneously from calli produced up to 4.50% DW of RA, whereas the original callus line achieved only 2.04% (Fedoreyev et al., 2005).

In plant cell cultures an elicitor can be defined as a factor that, when introduced to a living system, promotes the biosynthesis of secondary compounds with phytoalexinic activities. Classically, elicitors have been classified in two types, abiotic or biotic, according to their chemical nature and their origin as exogenous or endogenous elicitors (Namdeo, 2007). Several elicitors, such as yeast extract (YE), methyl jasmonate (MeJA), salicylic acid (SA) and *Pythium aphanidermatum* extracts, have been used to enhance the RA production in *in vitro* cultures, and their effects have been reviewed (Park et al., 2008; Bulkalov *et al*., 2012). Mizukami et al. (1992) achieved very good results, increasing RA production up to 10-fold, by eliciting cell suspensions of *L. erythrohizon* with 100 μM of MeJA. Abiotic elicitors such as vanadyl sulphate have been utilized successfully to increase RA production in cell cultures of *Lavandula vera* MM (Georgiev et al., 2006). Consequently, it is possible to infer that elicitation is an effective strategy for increasing the RA production in cell cultures, as reported for other secondary compounds. Some examples of the effect of elicitors on RA production are summarized in Table 2. These studies also show that there exists a positive relationship between RA production and the expression level of the main genes involved in its biosynthetic pathway.

Combining the effects of elicitation with feeding experiments has achieved successful results. The effects of different concentrations of sucrose, phenylalanine and the elicitors YE and MeJA at several concentrations were tested with *Ocimum sanctum* cell suspension cultures. Production of RA reached a maximum when the culture medium was supplemented with sucrose at 5.0%, phenylalanine at 0.25 g L^{-1} and the elicitor MeJA (Hakkim et al., 2011).

Little information is currently available regarding the stability of biotechnological production of RA over long-term culture periods. However, Bulkalov *et al*. (2001) have reported that a selected *L. erythrorhizon* callus line presented a highly stable RA production (approximately 1.1% DW) over a 15-year culture period.

Table 2. Attempts to increase the biotechnological production of RA in cell suspensions and hairy root cultures by the use of elicitors and permeabilizing agents.

Culture type	Plant species	Elicitor treatment	RA production % DW	Reference
Cell suspension	*Coleus blumei*	Fungal elicitor	2.1	Szabo et al. 1999
		MeJA	3.3	Szabo et al. 1999
		DMSO	2.9	Bauer et al. 2009
	Eritrichium sericeum	MeJA	5.3	Inyushkina et al. 2009
	Lithospermun erythrorhizon	MeJA	≈4	Mizukami et al. 1993
		YE	1.4	Mizukami et al. 1992
		Cuprum ions	1.5	Yamamoto et al. 2000a, 2000b
	Ortosiphon aristatus	YE	≈7	Sumaryono et al. 1991
	Salvia miltiorriza	SA	-	Dong et al. 2010
	Agastache rugosa	YE	≈8	Kim et al. 2001
Hairy roots	*Coleus blumei*	MeJA	3.5	Bauer et al. 2004
	Eritrichium sericeum	MeJA	6.9	Bulgakov et al. 2005
	Ocium basilicum	Fungal	1.4	Bais et al. 2002
	Salvia miltiorrhiza	MeJA	6.9	Xiao et al. 2009
		YE	2.9	Chen et al. 2001
		YE	7.4	Yan et al. 2006
		Silver ions	2.8	Xiao et al. 2010

MeJA: methyl jasmonate; YE: yeast extract; SA: salicylic acid

Dimethyl sulfoxide (DMSO) is a permeabilizing agent that has been successfully used in cell and root cultures for enhancing the release of secondary metabolites from the cells to the culture medium. The excretion of target compounds to the culture medium facilitates the downstream processes to increase overall production. Cell suspensions of

C. blumei, when maintained with the addition of 0.1% DMSO, presented a cell death rate lower than 15% in relation to the total cells, with a doubling time of 10.7 h and a RA production of 1.0-1.1 g L^{-1}. This shows that this concentration of the permeabilizing agent is not harmful for the cells (Martinez and Park, 1994). The response of the preconditioned cells (previously treated with 0.1% DMSO) was an improved cell growth and RA production when they were later treated with higher DMSO concentrations (0.5%). They reached a maximum of 2.85 g RA 100 g DW^{-1} in the culture medium, which represented 66.4% of the total RA produced. In contrast, the viability of non-preconditioned cell cultures decreased dramatically on treatment with the same DMSO concentration.

Immobilization is a plant cell culture technique that fixes the cells in a suitable matrix and prevents their movement into the culture medium. The first successful immobilization of plant cells was reported by Brodelius et al. (1979), who entrapped *Catharathus roseus* and *Daucus carota* cells in alginate beds. Immobilization was proposed as a strategy to enhance the overall production of secondary metabolites in plant cell culture. Immobilized cell cultures of *C. blumei* were developed to study the effect of the permeabilizing agent DMSO and growth regulators on biomass and RA production. Cells were immobilized in a support matrix composed of the fibrous skeleton of Luffa fruits. Immobilized conditions reduced the growth rate and RA production of cell cultures by half. The absence of growth regulators decreased the cell biomass and did not increase RA production. In this case, preconditioning treatment with 0.1% DMSO did not improve the cell adaptability to higher concentrations (0.5%) of the permeabilizing agent (Martinez and Park, 1994).

5.1.3.2. Hairy root cultures for RA production

Several approaches have been developed to produce RA in hairy root cultures. Transformed root cultures are induced by infection of sterile plant segments with a pathogenic strain of *Agrobacterium rhizogenes*. In most plant species, roots begin to appear in the inoculation sites after a period of 2-4 weeks to more than 6 months (Expósito et al., 2010). Transgenic roots can be cultivated in hormone-free medium, and show a high growth capacity. They typically have numerous branching points, display plagiotropic behavior and above all, they can synthesize the same plant secondary metabolites as the root of the intact plant (Fig. 7) (Srivastava and Srivastava, 2007).

RA production has been reported in hairy root cultures of *Ocimum basilicum* (Tada et al., 1996), *Hyssopus officinalis* (Murakami et al., 1998), *Salvia miltiorrhiza* (Yan

et al., 2006; Zhang et al., 2014a), *Dracocephalum kotschyi* (Fattahi et al., 2013), and *D. moldavica* (Weremczuk-Jezyna et al., 2013), among other plant species. Hairy root cultures of sweet basil, induced by two strains of *A. rhizogenes*, were established and the chemical constituents RA and related phenolic compounds lithospermic acid and lithospermic acid B were determined in the cultures. The RA content in root lines varied, ranging from 5 to 10 % of DW (Tada et al., 1996).

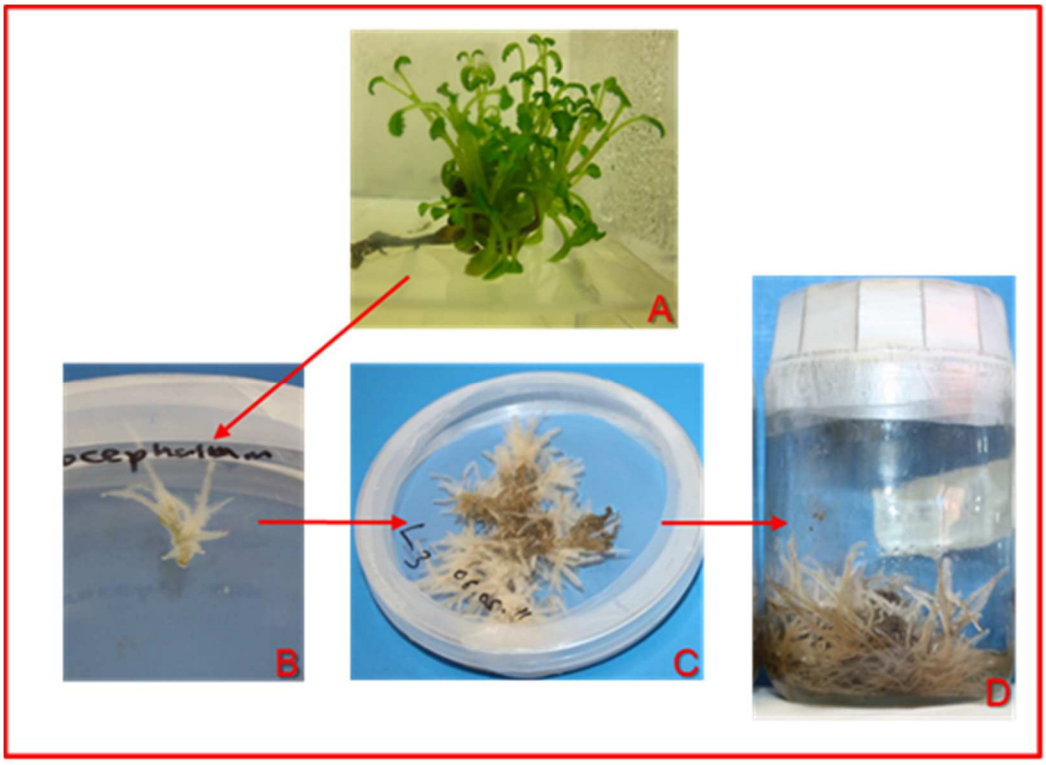

Figure 7. Steps for obtaining RA-producing hairy root lines of D. *kostchyi.* (A) Plantlet *in vitro* culture; (B) stem explant with roots; (C) solid hairy root culture; (D) liquid hairy root culture (image courtesy of Dr. Fattahi. Urmia University, Urmia, Iran).

H. officinalis transformed roots induced by infection with *A. rhizogenes* strain ATCC 15834 grew well, especially in hormone-free Woody Plant liquid medium, and after a growth period of 6 weeks reached an RA content of 8.03 % of DW. This amount was over 8-fold greater than in the leaves of the intact plant (Murakami et al., 1998). The effect of the nutrient basal medium was also investigated in transformed roots of *Coleus forskohlii*, showing that in this plant species RA production was higher in MS-cultures than in B5 medium (Li et al., 2005).

Growth and RA production depend not only on the plant species but also on the *Agrobacterium* strain utilized. In hairy root cultures of *Salvia officinalis*, both parameters (growth and RA levels) were higher in root lines induced with the strain ATCC 15834 than lines induced with the strain A4. In the same cultures, RA content was up to 2.4-fold higher than in untransformed root cultures (Grzegorczyk et al., 2006). Other plant species have been used to develop hairy root cultures with a high RA-producing capacity, such as *Agastache rugosa* (Lee et al., 2008) and *Nepeta cataria* (Lee et al., 2010), etc.

The effect of elicitors has also been successfully used to increase RA production in hairy roots (Table 2). Treatment of *S. miltiorrhiza* hairy root cultures with YE and the abiotic elicitor Ag^+ enhanced production of RA, but YE was more effective than the abiotic elicitor (Yan et al., 2006). In the same plant species, the effect of MeJA was also tested and RA production in elicited cultures underwent a high increase (Xiao et al., 2009; Zhang et al., 2014a). In hairy root cultures of *C. forskohlii*, RA levels were higher in MeJA-elicited roots than in root lines treated with YE or SA (Li et al., 2005).

Although hairy root cultures can grow well in hormone-free media, Liang et al. (2013) investigated the effects of the plant growth regulators, gibberellin, abscisic acid and ethylene, on the biosynthesis of RA in transformed root cultures of *S. miltiorrhiza*. The results showed that all the plant hormones were effective in enhancing production of RA by increasing the activities of some key enzymes involved in RA biosynthesis.

Hairy root lines can display different morphologies, and morphological changes have been associated with the production of PSM (Mallol et al., 2001). *D. kotschyi* transformed root lines, with typical hairy root and callus-like morphologies, showed a high capacity for producing RA. Root line15, which exhibited callus-like morphology, achieved up to 1.5 mg g DW^{-1}, constituting a 15-fold increase compared with the content of plant roots (Fattahi et al., 2013). The effects of light on the biosynthesis of RA in hairy root cultures have been little studied, but recently, Weremczuk-Jezyna et al. (2013) demonstrated that transformed roots of *D. moldavica* L. accumulated up to 78 mg RA g DW^{-1} (Weremczuk-Jezyna et al., 2013) when cultured in light conditions.

5.1.3.3. Biotechnological production of RA at bioreactor level

The last step in the development of a biotechnological process for producing phytochemicals is the scale up from the laboratory to bioreactor level, while ensuring identical process characteristics (Sharma et al., 2014). Bioreactors were originally

designed for the culture of microorganisms, which have different traits from plant cells and involve different processes compared to plant cell cultures (see Table 3). Plant cells have a diameter of 20-50 μm and a length of 100-500 μm, so are significantly larger than bacterial (<1 μm diameter) and fungal (5-10 μm diameter and <100 μm length) cells, with intracellular vacuoles that occupy up to 90% of cell volume. For this reason, they can be considered as "bags of water with thin cell walls" (Verpoorte et al., 2002).

Plant cells are more sensitive to shear forces than microbial cells, and this fact conditions the mechanical stirring inside the tanks of fermenters, which need to meet the oxygen demands of the plant cells (Wilson and Roberts, 2012). The growth capacity of plant cells is slower compared to microorganism cells and during the culture period they often release significant amounts of polysaccharides, increasing the viscosity of the culture medium. Therefore, in most cases the design of the bioreactors has to be modified and adapted to the typical traits of the plant cell cultures in order to overcome these difficulties and implement the systems at an industrial level (see Table 3).

Table 3. The main differences between biotechnological processes based on microorganism and plant cell cultures. PGRs: plant growth regulators.

Main traits of the culture	**Microbial cultures**	**Plant cell cultures**
Size:	Small (1-10 μm)	Big (40-200 μm)
Growth form:	Single cells and clusters	Clusters and isolated cells
Growth rate:	Fast	Slow
Doubling time:	Hours	Days
Tolerance to shear stress:	Low	Moderate
Product accumulation:	Extracellular	Intracellular
Main traits of the process		
Culture medium composition:	Simple (few components)	Complex (Salts, sugars, PGRs, etc.)
Inoculum density:	Low	High (5-10%)
Temperature:	26-36ºC	25ºC
Aeration rate:	High	Low
Culture period:	Days	Weeks

A wide variety of bioreactor designs have been used for the cultivation of plant cells. Traditional bioreactor configurations, such as stirred tank, bubble column or airlift reactors, have been utilized successfully for the production of phytochemicals in plant

cell factories. Bioreactor design depends on whether the biosynthesis of the target compound is growth- or non-growth-associated and where the product is stored, either within the cell or secreted extracellularly. If a plant secondary metabolite is produced during the exponential growth phase, only one reactor is normally enough for growth and product recovery, but if the desired compound is synthesized after the cell growth phase, one reactor can be used during exponential growth to increase cell biomass, and another one for metabolite production during the stationary phase (Wilson and Roberts, 2012). When the secondary compound accumulates inside the cell, the reactor is usually run in batch or feed-batch mode so that the cells can be permeabilized to release the product after the run is completed. If the product is secreted to the media, a continuous reactor can be used and the compounds of interest can be removed as they are synthesized (Wilson and Roberts, 2012).

A bioreactor could be defined as a closed system where the producing organism synthesizes a target product, with guaranteed control of the process conditions. In a classical reusable bioreactor, the cultivation container is made of glass or stainless steel, but single-use and multi-usable disposable bioreactors are being increasingly used (Lehmann et al., 2014). Both kinds of bioreactors, reusable and disposable, have been successful applied for the production of plant secondary metabolites in biotechnological systems (Huang and McDonald, 2012).

The biotechnological production of RA has been carried out using different bioreactor systems. *Anchusa officinalis* cell suspensions were cultivated in a stirred prototype reactor (2.3 L working volume) with an internal cross-flow filter working as an automated perfusion reactor, where fresh medium was added by a perfusion device and the spent medium was removed by a peristaltic pump. In this bioreactor a two-stage culture was successfully performed and after a culture period of 17 days the harvested biomass was 26 g DW L^{-1} and RA productivity 94 mg L^{-1} day, a productivity 3-fold higher than achieved in a batch culture (Su and Humphrey, 1991). The same authors subsequently developed a two-stage perfusion culture. The best results were obtained when an *A. officinalis* high density cell suspension was cultivated in batch mode for 10 days in B5 medium supplemented with 3% sucrose and 0.25 mg L^{-1} NAA, followed by perfusing the culture with B5 medium with the sucrose concentration increased to 6 % at a constant perfusion rate of 0.1 day^{-1} (Su et al., 1995).

In *Lavandula vera* MM cell suspensions, the effect of the temperature (T) on growth and RA production was investigated in a stirred bioreactor of 3 L, showing that a T lower than 26ºC was not suitable for biomass and RA production (Georgiev et al.,

2004). The best production was achieved when cells were cultivated at 30ºC. In the same system, the relationship between dissolved oxygen (DO) and the agitation rate was also investigated. After 12 days of culture, the cell suspension accumulated the highest amounts of biomass (34.8 g L^{-1}) when 50% DO was supplied and the agitation rate was 100 rpm, whereas the highest RA production (1.8 g L^{-1}) was achieved with 30% DO and an agitation speed of 300 rpm (Pavlov et al., 2005a).

The systematic optimization of culture conditions for the biotechnological production of RA has been poorly investigated. One exception is the work carried out by the Pavlov group (Pavlov et al., 2005b), who developed and applied a polynomial regression model to describe the production of RA in a stirred 3 L tank, taking into account the DO concentration, agitation and temperature. This was followed by a statistical optimization using a simple modified method. In optimized conditions, RA productivity was 3.5 g L^{-1}, 2-fold higher than in the shake-flask stage, the optimal culture conditions being 50% air saturation, 400 rpm and 29.9ºC.

5.2. Ruscogenins

5.2.1. *Ruscus aculeatus*

Ruscus aculeatus (Butcher's broom) is a small evergreen shrub widespread in western, southern and south-central Europe (Pignatti, 1982; Webb, 2010). Its underground part consists of a thick, sympodially branched rhizome that develops whitish roots (Fig. 8). The aerial part has multiple tough, erect, and striated stems forming numerous short branches and very rigid leaves that are in fact extensions of the stem (cladodes) and terminate in a single sharp spine. Cladodes functionally replace leaves, which are reduced to triangular scarious scales of up to 5 mm.

Butcher's broom has been described as a dioecious (Hillman, 1979; Warren, 1973), subdioecius (Yeo, 1980), andromonoecious, (Kay and Page, 1985), or subandroecious species (Martínez-Palle and Aronne, 2000), depending on the population. The masculine and feminine flowers are similar: small, whitish and arising from the axil of a small bract in the center of the upper surface of the cladode, each with a short pedicle.

Figure 8. *Ruscus* rhizome

5.2.1.1. Botanical description and habitat

The genus *Ruscus* belongs to the Asparagaceae, although it has previously been included in other families, such as the Ruscaceae, Convallariceae or Liliaceae (Thomas and Mukassabi, 2014). The genus includes 7-10 species distributed mainly in the Mediterranean area of Europe, extending as far as Iran (Yeo 1968). It also includes the ornamental *R. hypoglossum*, which is cultured in the UK and south-eastern Europe. The best-known and most appreciated *Ruscus* species is *R. aculeatus*, which has been used since the Middle Ages as a medicinal agent for disorders involving the venous system. Although primarily a Mediterranean species, it has oceanic tendencies, hence it is also found in sub-Mediterranean and sub-Atlantic regions, reaching as far north as the south of England (Fig. 9). It requires soils with a pH between 3 and 5 and of average moisture retention but can adapt to other types of soil (Hill et al., 2004). Soil fertility is not an important factor, because in England *R. aculeatus* grows well on very fertile as well as infertile soils (Hill *et al*., 2004). It can be generally considered shade-tolerant and drought resistant (Thomas and Mukassabi, 2014).

As mentioned, *R. aculeatus* is a multi-stemmed shrub whose leaves have been functionally replaced by cladodes and photosynthetic stems. Cladodes are 1-4 x 0.4-1 cm in size and are thought to derive from a branch or a leaf on an aborted shoot, according to their growth capacity and venations (Thomas and Mukassabi, 2014). However, Hirayama et al. (2007) showed that the YAB2 genes, associated with leaf morphology, and STM genes, associated with stem morphology, are both expressed in cladodes, suggesting that it is a double organ derived from both.

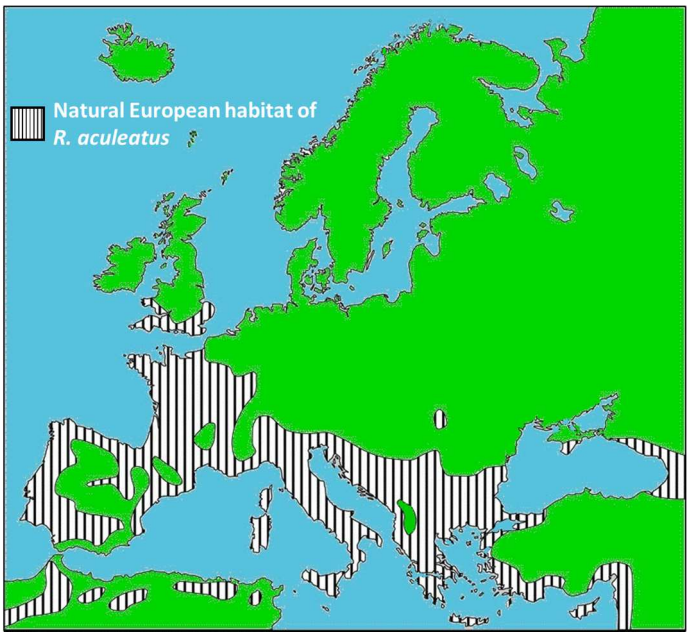

Figure. 9. Distribution of the *Ruscus* genus in Europe (modified from Thomas and Mukassabi, 2014).

R. aculeatus flowers are concentrated in a small number of cladodes. The masculine and feminine flowers, which are found in separate plants, are very similar: small and whitish, and arising from the center of the cladodes. In the male flower, the perianth consists of two whorls, each one composed of three small green tepals (Fig. 10). The three stamens are fused into a dark column, with the anthers forming a crown, and it contains an undeveloped ovary inside. The perianth in the female flower is similar, with the same column structure composed of stamen filaments but with degenerate anthers forming a necklace around a small style. The ovary is unilocular and tricarpelar (Martinez-Pallé and Aronne, 2000).

The flowering period is mainly in winter, although it can extend from September to June, peaking between January and April (Martinez-Pallé and Arone, 2000). It is a non-self-pollinated plant and needs entomological pollination to reproduce. Only one out of five pollinations is successful (Thomas and Mukassabi, 2014). Despite the long flowering period, fruit development usually does not begin until late April, with a long ripening process, so mature fruits normally appear at the end of October (Aronne and Wilcock, 1997). Ripe fruits remain on the plant for a long period of about one or two years (Martinez-Palle and Aronne, 1999).

Ripe fruits are red berries that contain one to four seeds (Fig. 10) usually with a very low germination capacity of less than 50%. Seeds can survive in the soil for more than one year and normally germinate after 11 months. Nevertheless, germination can be improved by stratification or hormonal treatment with gibberellin (Thomas and Mukassabi, 2014). 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 declines in the wild population and currently *R. aculeatus* is a threatened plant species, whose survival depends primarily upon vegetative reproduction from robust rhizomes (Thomas and Mukassabi, 2014). For these reasons, finding new sources of steroidal sponins is a current challenge for plant biotechnology.

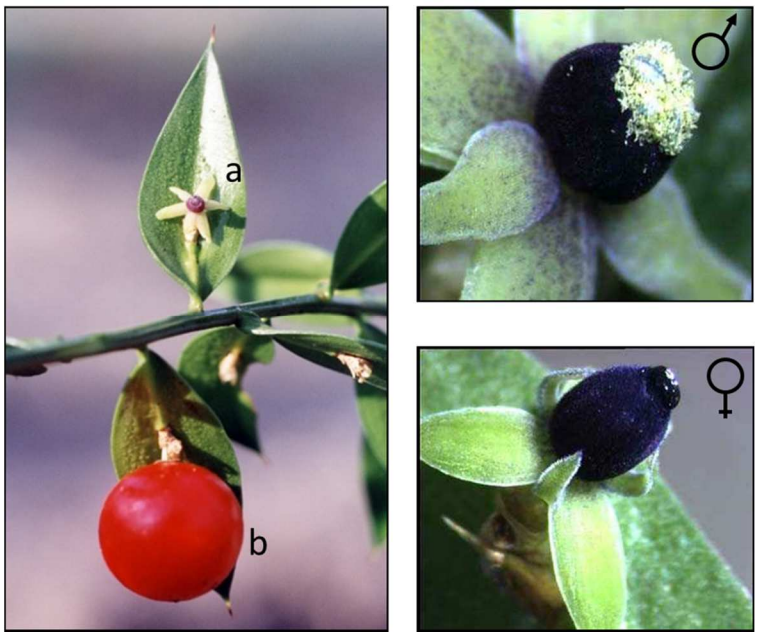

Figure 10. Left: *R. aculeatus* branch with a cladode bearing a flower (a) and ripe berry (b). Right: details of male and female flowers.

5.2.2. Phytochemical composition of *Ruscus* extracts

Butcher's broom has different types of active ingredients: triterpenes, steroids, flavonoids, coumarins, sparteine, triamine and glycolic acid, among others. Two steroid saponins with important biological activity, ruscogenin and neoruscogenin, have been isolated from the rhizomes (de Combarieu et al., 2002), where these molecules have the highest concentration. Ruscogenin and neoruscogenin have two heterocycles with

oxygen bound by a spiroacetal carbon on the D ring of the steroid core. The hydroxyl group of 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 the ruscoside or neoruscoside (Fig. 11).

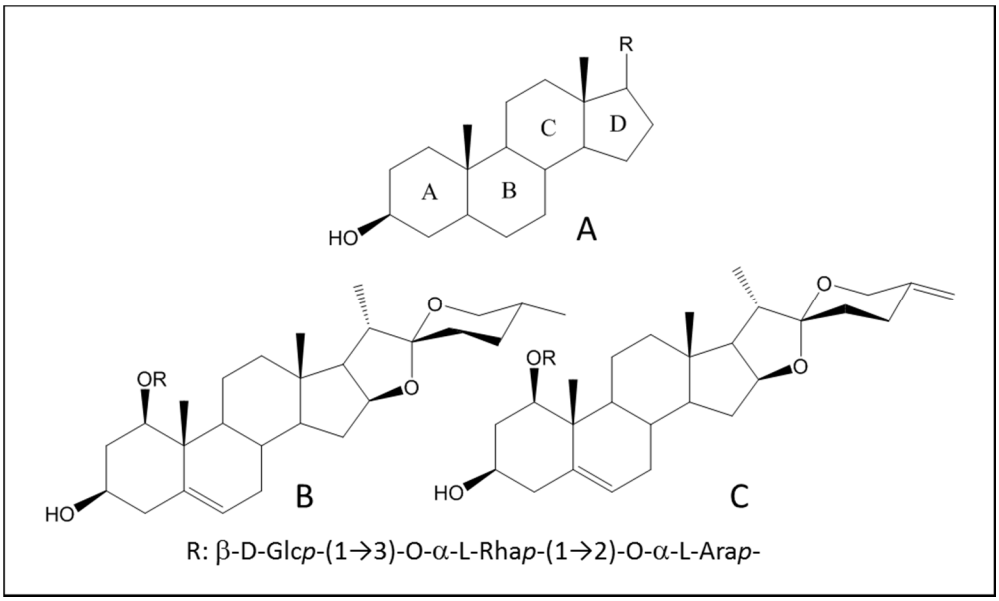

Figure 11. Chemical structure of the steroidal backbone (A), ruscogenin (B) and neoruscogenin (C).

5.2.2.1. Spirostanol saponins from Ruscus spp.

Steroidal saponins are oxidized cholesterol derivatives with a C27 skeleton bearing a variable number of sugars at diferent positions of the steroidal core. In *Ruscus* spp. steroidal saponins can be classified into two different groups, the hexacyclic spirostanol saponins, characterized by the presence of a bicyclic ketal at C-22, and the pentacyclic furostanol saponins, which contain a hemiketal function at C-22 and a single β-D-glucose residue at C-26. Both types bear a sugar chain linked mostly to C-1 (de Combarieu et al., 2002). A series of pregnane glycosides have also been isolated from *R. aculeatus*. In spirostanol and furostanol saponins, C-25 can have either an R or S configuration, and futher structural diversity is generated by differences in the stereochemistry of C-22 and in the cis/trans fusion of the A and B rings of the steroid core (Fig. 12).

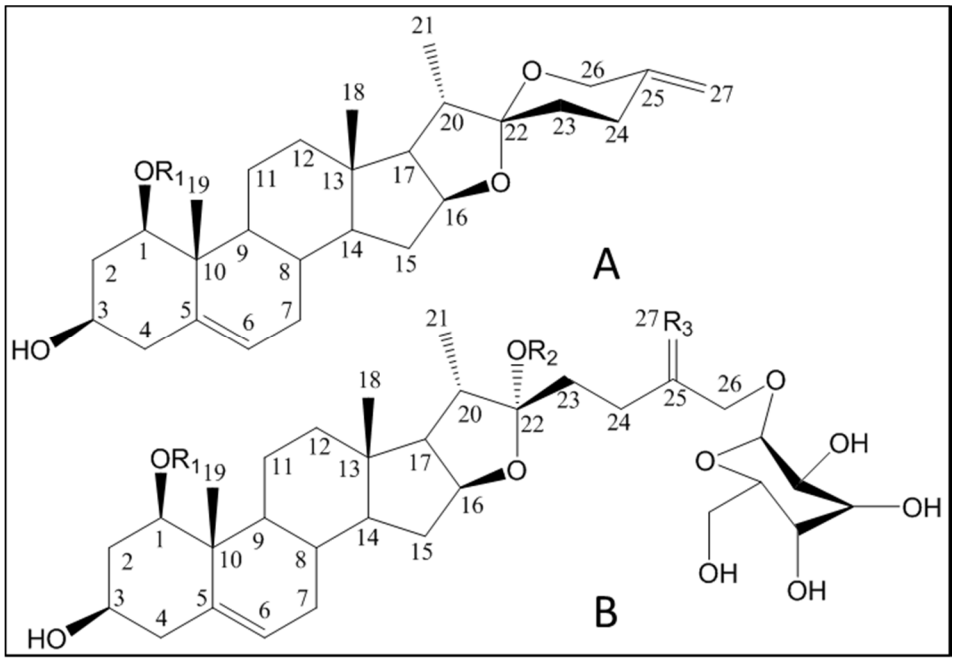

Figure 12. Chemical structure of the spirostanol (A) and furostanol (B) saponins.

Ruscogenin and neoruscogenin were the first spirostanol saponins isolated from *R. aculeatus*, in 1955-57 (Sannie and Lapin, 1957). Their structure is very similar to that of diosgenin, differing in the presence of a β–hydroxyl group at C-1, whereas other structural features, such as the orientation of the C-3 oxygen atom, and junctions between rings B/C (*trans*), C/D (*trans*) and D/E (*trans*), are identical (Masullo et al., 2016). Since the elucidation of the chemical structures of ruscogenin and neoruscogenin, a large family of spirostanol saponins has been isolated, including several neoruscogenin derivatives characterized by a glucosidation pattern with a sugar chain only at C-1. Other more unusual structures are aculeoside A, a bisdesmosidic spirostanol saponine with a 6-deoxy-D-glycero-L-threo-4 hexulose attached to the C-24 hydroxyl group, and aculesoside B, which has three acetyl esters at the inner galactose unit and the glucosyloxy group linked to C-23. Also, a series of ruscogenin glycosides has been isolated from *R. aculeatus* with a diglycoside moiety composed of rhamnose and galactose attached to C-1 (Masullo et al., 2016).

5.2.2.2. Furostanol saponins from Ruscus spp.

Furostanol saponins are pentacyclic compounds and can be divided in two groups, those with a hydroxyl or methoxy moiety at C-22 or those with a Δ20(22)-unsaturation.

Desglucoruscoside and ruscoside, containing a double bond at the C-25-C-27 position, have been isolated from *R. aculeatus* as major furostanol saponin constituents, but two 25,27-dihydro derivatives are also present in minor quantities (de Combarieu et al., 2002). More than 21 other furostanol saponins have been isolated from *R. aculeatus* (Masullo et al., 2016), but they are also found in other *Ruscus* spp. such as *R. colchicus*, *R. hypoglossum* or *R. ponticus*.

5.2.3. Biosynthesis of ruscogenins

It has been established that steroidal saponins arise mainly from the cytoplasmic pathway in which isopentenyl diphosphate (IpPP) derives from mevalonate, although recently it has been suggested that the plastid methyl-D-erythritol-4 phosphate (MEP) pathway may constitute an alternative source of the IpPP involved in steroidal saponin biosynthesis (Upadhyay et al., 2018). Condensation of two units of IpPP with its isomer dimethylallyl diphosphate (DMAPP) leads to farnesyl diphosphate, which is dimerized by the enzymatic activity of squalene synthase to squalene, the universal precursor of all terpenes. Squalene monooxigenase oxidizes squalene to 2,3-oxidosqualene, the precursor of cholesterol and β-sitosterol via cycloartenol. Although the biosynthesis of spirostanol and furostanol saponins is not fully elucidated, it is accepted that both cholesterol and β-sitosterol can act as precursors of diosgenin, a spirostanol saponin structurally very similar to ruscogenin and neoruscogenin (Sahu et al., 2008; Chaudhary et al., 2015). Cholesterol in plants is biosynthesized from cycloartenol in 8 steps via 31-norcycloartenol, cholesta-7-en-3β-ol and 7-dehydrocholesterol (Fig. 13). Cholesterol oxidation at C-16, C-22 and C-26/27 and glucosidation of the hydroxyl group at C-26/27 lead to the formation of furostanol 26-β-D-glucoside (Fig. 14). The latter undergoes spontaneous cyclization after the enzymatic removal of the C-26 glucose moiety to generate the heterocyclic ring of spirostane (Heftmann, 1983).

The biosynthesis of steroidal saponins has also been described from sitosterol via sterol-3β-D-glucoside by the action of a β-glucosidase (Sinh et al., 2017). Sitosterol is also derived from cycloartenol in a multi-step pathway, involving the formation of more than 10 intermediates, including obtusifoliol, methylenlophenol, avenasterol and isofucosterol (Sonawane et al., 2016) (Fig. 13). The final steps in the formation of steroidal saponins in *R. aculeatus* remain unclear, although the enzymes and genes involved are probably similar to those active in the biosynthesis of other more extensively studied saponins such as diosgenin.

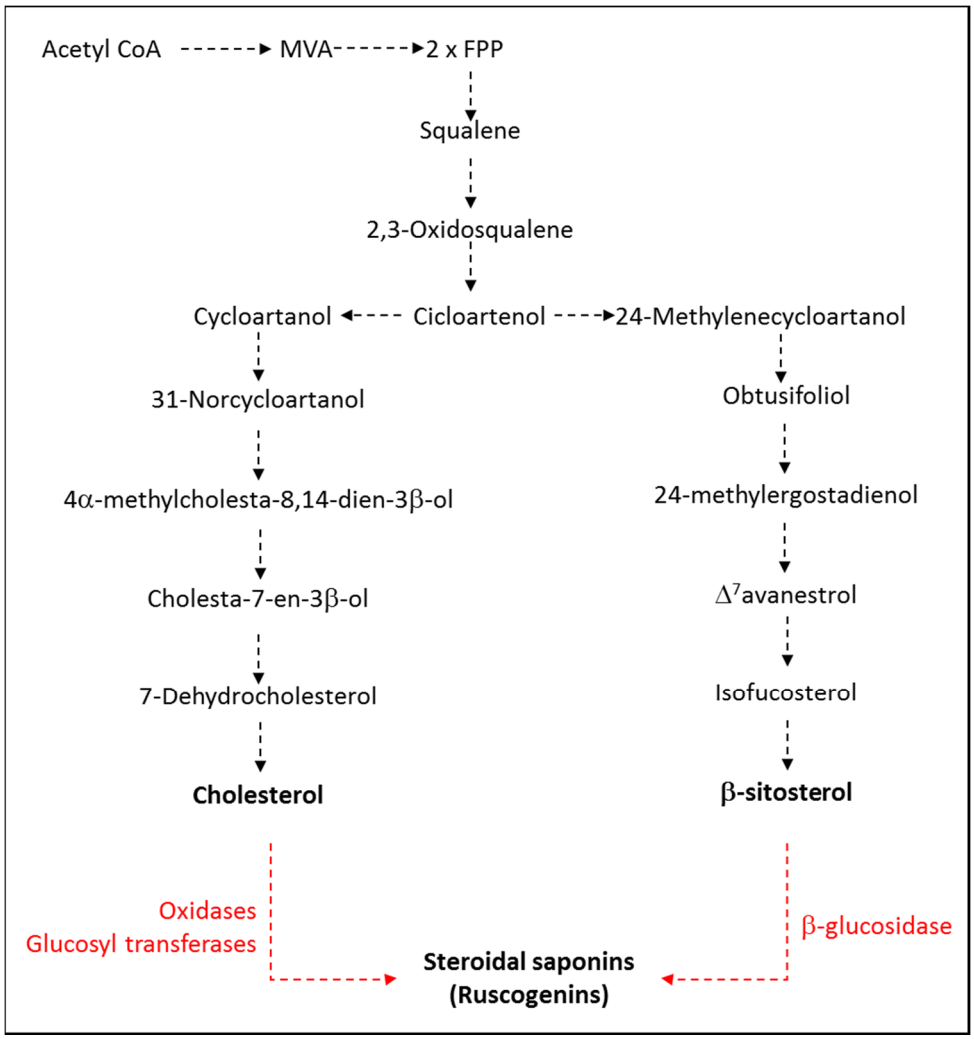

Figure 13. Putative ruscogenin biosynthetic pathway. MVA: mevalonate; FPP: farnesyl diphosphate. In black: established steps in other plant species. In red: unknown steps.

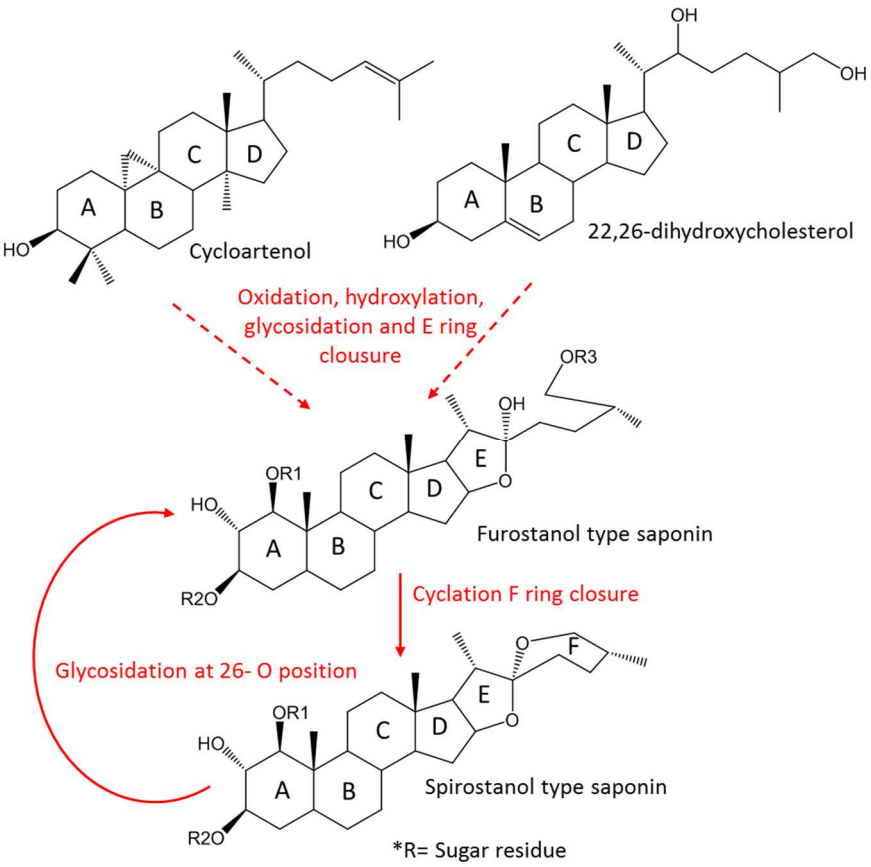

Figure 14. Last steps in the biosynthesis of furostanol and spirostanol saponins (modified from Upadhyay et al., 2018).

5.2.4. Biological activity and medical applications of ruscogenins

The aerial parts of *R. aculeatus* are edible, whereas the root-rhizome has been used in Europe since the Middle Ages to relieve varicose veins and hemorrhoids. The active ingredients of *R. aculeatus* extracts, the steroid saponins, have an anti-inflammatory and anti-oedematous action and a toning effect on veins and blood vessels. They have a notable activity against chronic venous insufficiency, poor blood circulation of inflamed legs, hands and feet, and in the treatment of edema. Other applications include recovery therapy after treatment of phlebitis, the prevention of diabetic retinopathy and the treatment of orthostatic hypotension (Redman, 2000).

The therapeutic use of the steroidal saponins of *R.aculeatus*, 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 (Bousquela et al., 1993, Bouskela and Cyrino, 1994, Bouskela et al., 1994, Svenjo et al., 1997).

In European folk medicine, the aerial part and root-rhizomes of *R. aculeatus* are considered as diuretic and mildly laxative. In Turkey, a root decoction is used as a diuretic and for the treatment of urinary disorders and kidney stones (Masullo et al., 2016). Other traditional uses of *R. aculeatus* extracts are for the treatment of skin diseases, colitis and diarrhea, and inflammation and arthritis (Hadzifejzovic et al., 2013).

Different pharmaceutical forms of *R. aculeatus* are available on the market, such as ointments, capsules or hydroalcoholic extracts. The active components (steroid saponins) are also administered in topical or rectal preparations in combination with other compounds such as bromelain, escin, and esculoside, as well as hydroalcoholic extracts administered orally in association with other venotonic agents such as red currant or horse chestnut. Recently, the activity of the bioactive components (ginsenoside Rb1/ruscogenin/schisandrin) of one of the most famous complex formulas in traditional Chinese medicine called Sheng-Mai-San has been optimized for the treatment of myocardial ischemia; their therapeutical effectiveness is based on their anti-oxidation and anti-inflammation properties, inhibition of apoptosis and enhancing the tolerance of ischemic tissue to hypoxia (Li et al., 2018).

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 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. These latest reports have increased interest in ruscogenins and finding new natural sources of these compounds.

5.2.5. Approaches to *R. aculeatus in vitro* cultures

As mentioned, *Ruscus* extracts are used both in traditional and conventional medicine and the natural resources of *R. aculeatus* plants in Europe and Asia are diminishing dramatically due to uncontrolled and unsustainable harvesting, as well as the naturally low fertility of this plant species and the difficulties in culturing it from seed. Currently, *R. aculeatus* is included in the Directive 92/43/EEC and the European Plant Red List (Ivanova et al., 2017). Conventional vegetative propagation of the plant via cuttings and division of underground rhizomes is not suitable for commercial purposes because only a limited number of plants are produced, which then need rooting. Therefore, *in vitro*

mass micropropagation could represent an alternative system for preserving and reintroducing endangered germplasm into the wild populations. Several *in vitro* micropropagation approaches have been developed with this aim. Essential factors to be taken into account when developing a successful micropropagation method include suitable primary explants, disinfection agents, culture medium and conditions, regeneration rates and acclimatization of the regenerated plantlets (Winarto, 2017).

Segments of rhizomes, young stems or nodes have been utilized as a source of explants for *Rucus* spp. micropropagation, all of which can be regenerated directly from aerial shoots or via callus induction. Direct shoot induction and plant regeneration were achieved from *R. hypophyllum* rhizome segments in MS medium supplemented with several combinations of the plant growth regulators benzylaminopurine (BA) and indolacetic acid (IAA). Plantlet elongation and rooting were induced in the same MS basal medium with different concentrations of indolbutyric acid (IBA) (Purwito et al., 2005). Culture of shoot tips and inflorescence explants of *R. hypophyllum* in shake liquid culture also directly induced both axillary and adventitious shoot proliferation without callus proliferation. Previous treatment with 1 mg L^{-1} of 2,4-dichloroacetic acid (2,4-D) and 6-benzylaminopurine (BAP) and subsequent transfer to to a solid medium with a 5:1 cytokinin: auxin ratio was optimum for shoot development (Ziv, 1983).

In most cases, hormonal treatments aimed at increasing shoot proliferation also provoke callus induction and it is possible to regenerate shoots via indirect organogenesis. Moyano et al. (2006) utilized embryos isolated from immature fruits and aerial buds excised from rhizomes for direct plant regeneration. However, in order to increase the number of regenerated plants from each explant, the same authors treated sterilized segments of rhizomes, stems and cladodes with plant hormones with the aim of obtaining calli for plant regeneration. Two plant growth regulators were tested in this experiment, 2,4-D (0.5 to 2.5 mg L^{-1}), and kinetin (0 to 1.0 mg L^{-1}). According to their results, the best source of explants for obtaining organogenic calli were rhizomes, whereas only very small and non-organogenic pieces of calli were obtained from stems and cladodes. The shoots excised from the organogenic calli were rooted in MS medium with 2 mg L^{-1} of naphtalenacetic acid. More than 60% of the plantlets obtained were successfully transferred from *in vitro* to *ex vitro* conditions.

One of the problems of *in vitro* regenerated plants is their *ex situ* conservation. Ivanova et al. (2015) studied the long-term cloning stability of *R. aculeatus* by investigating ruscogenin biosynthesis, genome-size stability, propagation rate and morpho-physiological responses. The authors compared several clones from different

geographical locations cultivated under the same conditions for 18 months. After this period, *in vitro* clones grew slowly and only three clones had propagation rates above 100 regenerants. Despite the slow growth, regenerants were characterized by genome-size stability. Production of ruscogenins in shoots, rhizomes and roots of fully developed regenerants showed a specific profile for each clone unrelated to their geographical origin and could not be grouped on a geographical basis.

Regarding the ruscogenin content, the same authors (Ivanova et al., 2015) reported a shared profile among the regenerants. Ruscogenin production in the underground organs of all clones was approximately two-fold lower compared to neoruscogenin, but roots were generally more productive than shoots.

There are few reports of ruscogenin production in *R. aculeatus in vitro* cultures. 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 culture media with different hormonal treatments. The plant growth regulators used were the auxins IAA and 2,4-D, and the cytokinins kinetin, N- (2-chloro-4-pyridyl) -N'- phenylurea (4PU-30) and BAP. In several of the media assayed, small pieces of callus appeared after 4–6 weeks of culture, but simultaneously, in the same culture media, a high number of rhizome explants also developed aerial shoots and/or roots. Substitution of the auxin IAA by 2,4-D improved callus induction but did not achieve the total inhibition of direct organogenesis in the explants. When cultured, the isolated pieces of calli also showed morphogenetic activity, developing roots and shoots (Fig. 15). Saponin content was lower in calli without *versus* with organogenesis, and in these callus pieces the saponin contents were higher in the developing aerial shoots than in developing roots. In all cases, the saponin contents of the calli were lower than in the aerial part and roots of the plants.

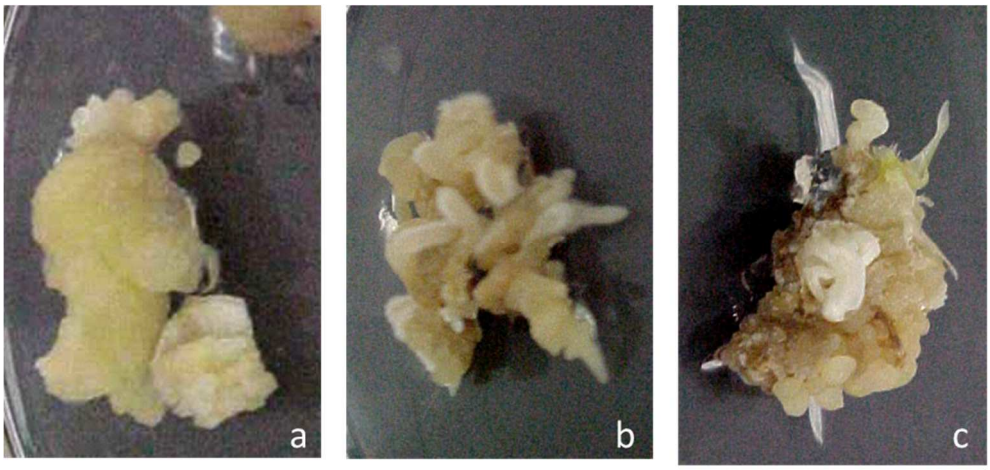

Figure 15. Appearance of *R. aculeatus* calli. (a) Non organogenic, (b) with roots, (c) with shoots.

5.3. Future outlook for rosmarinic acid and ruscogenin production in plant cell factories

Little work has been done on ruscogenin production in plant biofactories to date. As mentioned, very few attempts to produce these compounds *in vitro* have been made, and without much success. The optimization of culture conditions, including testing the effects of classical or new elicitors, etc., still remains to be done. So far, only the effect of the elicitor methyl jasmonate has been tested in *in vitro* plantlets of *R. aculeatus* (Mangas et al., 2006).

In contrast, considerable progress has been made in the biotechnological production of RA, although much more work is still necessary. Key strategies for enhancing the biotechnological production of RA include the implementation of disposable bioreactors in fermentation procedures, the use of new elicitors and/or permeabilizing agents, as well as attempting to develop metabolic engineering approaches for the design of new cell lines with an improved capacity to biosynthesize/accumulate RA.

5.3.1 Disposable plant cell bioreactors

As mentioned in Section 5.1.3, despite the interest of RA and the extensive published work on its biotechnological production in plant cell and hairy root cultures, very few studies have been carried out at bioreactor level, and these studies have mostly been performed in reusable bioreactors. In recent years, reusable bioreactors have been

replaced by disposable bioreactors, and currently plant cell cultures are being grown in disposable bioreactors with volumes up to 200 L for the production of high-value compounds such as proteins and secondary metabolites (Lehmann et al., 2014). These bioreactors have several advantages: they require less sterilization work, are time- and cost-saving, and produce less waste and environmental contamination (Aranha, 2004; Rader and Langer, 2011). These features can be attributed to the plastic materials of the plant cell culture bags, which are made from multilayered films (Barbaroux and Gueneron, 2012; Vanhamel and Masy, 2011). Interestingly, there have been no reports of interactions between the culture media and the inner contact layer of the bags having any negative influence on plant cell growth (Kadarusman et al., 2005). However, a disadvantage of this type of bioreactor is that a new bag has to be used for each bioprocess, which could increase the total cost of the biotechnological process.

Some disposable bioreactors have cultivation containers (bags or rigid vessels) that can be used more than once and are called disposable multi-use bioreactors (Eibl et al., 2012; Georgiev et al., 2013). Compared to single-use containers, disposable multi-usable versions are more complex and more time-consuming to work with, but they are cheaper to manufacture and purchase (Lehmann et al., 2014). Both types have been used for plant cell suspension, hairy root and embryogenic cultures (Georgiev et al., 2013; Weathers et al., 2010; Wilson and Roberts, 2012).

Three main classes of disposable bioreactors have been used in biotechnological platforms to produce plant cell biomass, secondary metabolites and recombinant proteins such as antibodies: (**1**) mechanically driven, (**2**) hydraulically driven and (**3**) pneumatically driven, all of them with filling volumes of up to 400 L (Lehmann et al., 2014). Mechanically driven disposable bioreactors are the most used. Mixing in disposable bioreactors is performed by rotating and tumbling stirrers, vibrating perforated disks, rocking and rising platforms, or orbitally shaken platforms (Fig. 16).

Although all these reactors can be used for the cultivation of plant cells and organs, each type may be more suitable for a specific approach. For example, orbitally shaken Erlenmeyer flasks on a mL-scale are widely used for inoculum production and preliminary screening studies with plant cells, in order to investigate the best culture conditions to further scale up the processes to a bioreactor level. Disposable bioreactors with rotary and tumbling stirrers are rarely used for plant cell cultures, because they were originally developed for animal cell cultivation, but recently, a UniVessel SU Bioreactor has been used for the biomass production of *Corylus avellana* cell cultures and the results were comparable with those obtained with the reusable BIOSTAT®B plus. The

obtained results confirm the suitability of the single-use bioreactor for the culture of *C. avellana* cell suspensions (Gallego et al., 2015). Disposable oscillating bioreactors with one or more vibrating disks are less utilized for plant cell cultures than the other systems. In contrast, disposable wave-mixed systems have been implemented for the culture of plant cell suspensions and hairy roots (Lehmann et al., 2014), and their uses and characteristics are described below.

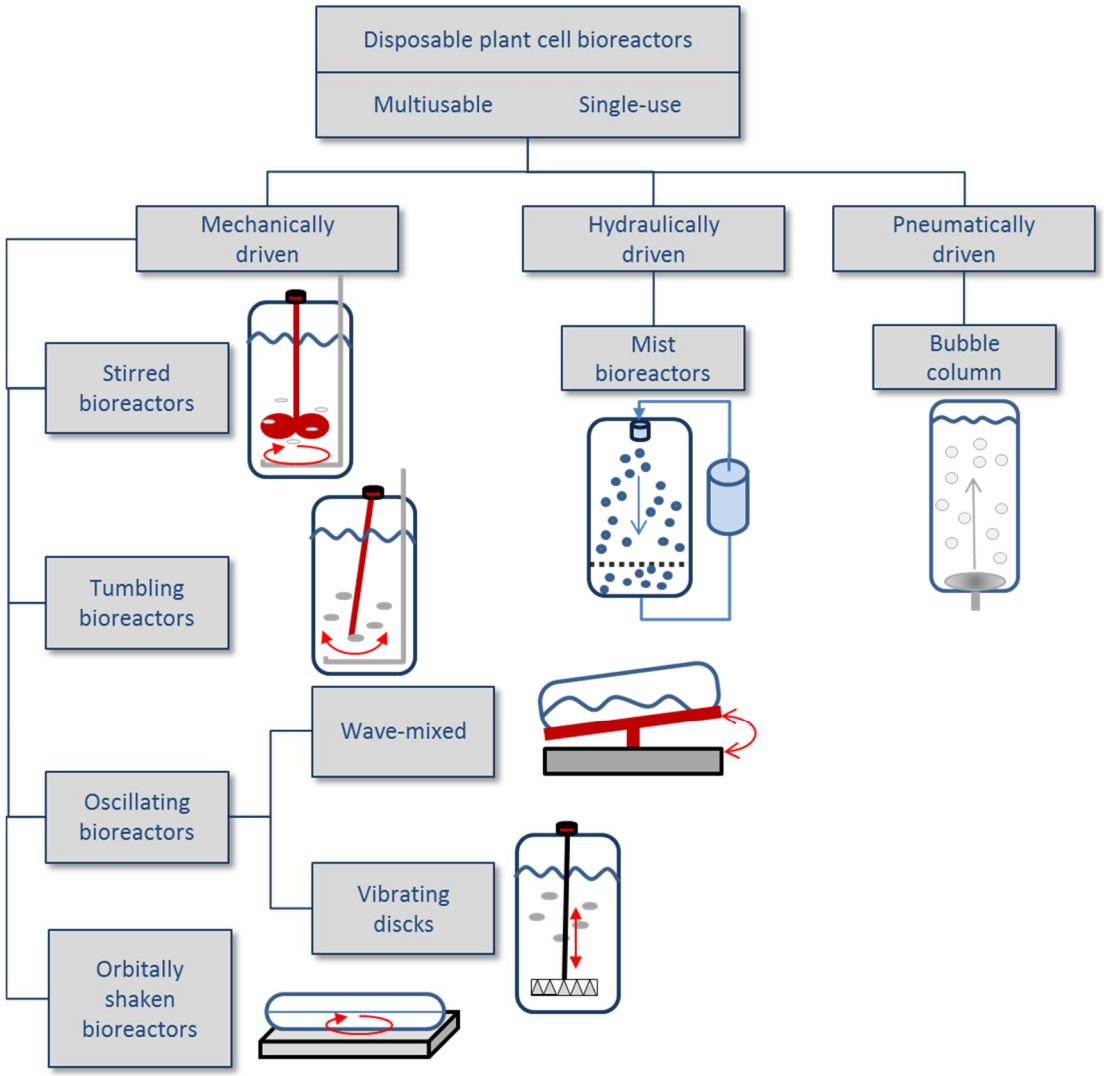

Figure 16. Disposable bioreactors classified according to their driving system (modified from Lehmann et al., 2014).

In addition to the mechanically driven disposable bioreactor types, pneumatically driven bubble columns can be used for plant cell suspension cultures. In the more simply designed disposable bubble columns, mass and heat transfer is achieved by direct

sparging of air/gas into tall cultivation containers. The resulting bubbling causes mixing and fluid circulation of the culture medium. A modification of the bubble column is the airlift bioreactor, which has inner draft tubes to improve the circulation, whereas the bubble column is a simple tower (Georgiev et al., 2014). In the context of RA production, a 5-L disposable pre-sterilized plastic airlift bioreactor has been applied for the culture of *Ocium basilicum shoot cultures* (Kintzios et al., 2004).

Disposable mist bioreactor systems are based on a disposable bag in which a mesh matrix can immobilize cells and support biomass. Nutrient mist reactors are gas-phase reactors that provide *in vitro* plants with small droplets of culture medium fully infused with whatever gas or gas mixture is generated as an aerosol in the growth chamber. These bioreactors are suitable for propagating plant organ cultures such as embryogenic or hairy root cultures. The use of temporary immersion systems reduces tissue hyperhydricity. Vitrification has been classically associated with a complete or partial immersion of plant tissues in liquid medium (Fei and Weathers, 2014).

Disposable wave-mixed bioreactors are composed of an inflated pre-sterilized plastic bag that forms a disposable cell cultivation chamber containing the culture medium and cells. The bag is fixed on an electrically-driven rocking base, whose movement induces a wave, which introduces bubble-free oxygen into the culture medium from the headspace of the bag, and the surface of the medium is continuously renewed. The wave movement sweeps up cells and prevents them from settling in the bioreactor. It also positively influences the fluid flow, mixing time, oxygen mass transfer rate, and reduces shear stress on cells, and thus enhances cell growth and product formation (Lehmann et al., 2014).

Although the different wave-mixed bioreactors are based on an identical working principle, they differ considerably in the culture bag design (bag material, scale and dimension, and the type of employed sensor probes and filters) and in the platform movement (Eibl et al., 2009a).

The motion of wave-mixed bioreactors can be one-, two- or three-dimensional (1-D, 2-D or 3-D). In most of the rocking motion type bioreactors, agitation and mixing are induced by a vertical displacement, which generates a wave in the cell culture (1-D) (Table 4). In these bioreactors, the rocking angle and motion can be controlled (Eibl et al., 2009a). The 2-D disposable bioreactors allow for both vertical and horizontal movement, resulting in relatively high mass-transfer capacities compared with the conventional rocking motion-type bioreactors (Thomassen et al., 2012). The 3-D system rocks on two axes simultaneously (Bi-Axial agitation), producing a low turbulence swirling

pattern within the bag. Under similar agitation conditions, the 3-D rocking system mixes almost 3 times faster than conventional rockers and demonstrates the highest oxygen mass transfer coefficient (k_La) (Löffelholz et al., 2014).

Wave-mixed bioreactors are currently the most favoured for plant cell cultures, even at an industrial level. For example, Mibelle Biochemistry and Sederma have already developed and manufactured bioactive products for cosmetics, such as Phyto Cell Tec Argan, Solar, Vitis, Malus domestica, Alp Rose and RESISTEM. Greenovation also uses this type of bioreactors to produce different therapeutic proteins in *Physcomitrella patents* cell suspensions (Lehmann et al., 2014).

Table 4. Some examples of wave-mixed bioreactors with different oscillatory motion (modified from Löffelholz et al. 2014).

Oscillatory motion	Bioreactor brand	Max. cv (L)	Developed or manufactured by
1-D	**Wave BioreactorTM**	500	GE Healthcare
	BIOSTAT® CultiBag RM	300	Sartorius Stedim Biotech
	AppliFlex	25	Applikon Biotech
2-D	Cell-tainer	125	Cellution Biotech
3-D	XRS Bioreactor System	25	Pall Life Sciences

In bold: bioreactors used for plant cell cultures

To date, only 1-D wave-mixed bioreactors have been used for plant cell and organ cultures, and most of the experiments have been carried out with the BIOSTAT CultiBag RM and its precursor BioWave (Table 4). To characterize the principles of mass and energy transfer in wave-mixed bioreactors for comparison with the stirred systems, the Wave's engineering parameters have been determined, such as in a modified Reynolds number, which was established to describe the fluid flow in the bag with 1-D oscillatory motion. For Newtonian culture broths, other important parameters such as mixing times, residence time distributions, specific power input values (P/V) and volumetric oxygen transfer coefficients (k_La) were mainly dependent on the rocking angle and rocking rate, the type of bag and its geometry and culture volume (Lehmann et al., 2014). For example, a maximum k_La of up to 10 h^{-1}, mixing times between 20-50 s, and P/V between 70 to 180 W m^{-3} have been measured and calculated (Eibl et al., 2009b). All these values are considered adequate for the culture of plant cells. Computational Fluid Dynamics (CFD) simulations showed a more homogeneous energy dissipation and shear stress pattern in BIOSTAT CultiBag RM than in stirred reusable cell culture

bioreactors (Löffelholz et al., 2014), which is probably why wave-mixed bioreactors are more suitable for producing plant cell biomass than stirred bioreactors at benchtop and pilot scale.

CFD simulations have been used to obtain more information about local and temporal parameters such as temperature and velocity distributions, mixing intensities and shear as well as dissipation rates. This information may be used to increase the growth of non-Newtonian plant cell culture broths (Eibl et al., 2009b). A genetically modified BY-2 tobacco cell suspension (MTA) with a packed cell volume (pcv) of 15% and viscosity of 0.0057 Pas initially generates waves very similar to the waves of water and unlimited mass and gas transfer. However, due to the high growth rate of the MTA cells (doubling time of 16 h), the increase of cell density and viscosity (0.41 Pas) over time negatively affects the mass and gas transfer inside the bag. This represents a limiting factor, even when doubling the P/I by adjusting the rocking rate (42 rpm) and rocking angle (10º) at the maximum filling volume (2 L) (Eibl et al., 2009b). The Cell-tainer and XRS Bioreactor System, with 2-D and 3-D oscillatory motion bags, could be a potential solution for high-growth plant cell cultures with non-Newtonian fluid flow behaviour, but no references have been found to date.

Nevertheless, wave-mixed bioreactors like BioWave®, Wave BioreactorTM, and BIOSTAT® CultiBag RM have been successfully used for the culture of, among others, *Vitis vinifera*, *Malus domestica*, *Nicotiana tabacum*, and *Hordeum vulgare* cell suspensions as well as hairy root cultures of *Harpagophytum procumbens*, *Hyoscyamus muticus* and *Panax ginseng* (Eibl et al., 2009b and references therein). This type of bioreactor can therefore be considered as a good option for the cultivation of plant cell suspensions and hairy roots.

5.3.2. Use of new elicitors/permeabilizing agents

Several elicitor treatments have been used to improve the biotechnological production of RA (Table 2), including fungal elicitors, cuprum ions, silver ions, salicylic acid or methyl jasmonate (MeJA) (Bulkalov et al., 2012). Permeabilizing agents like DMSO can also facilitate downstream processes (Park et al., 2008). In this context, cyclodextrins (CD), which are cyclic polymers of D-glucose linked by α-1,4-glycosidic bonds (Saenger, 1980), have been tested in plant cell cultures for the production of bioactive secondary metabolites (Morales et al., 1998). CD have attracted considerable interest recently, because, unlike other permeabilizing agents, they can trigger plant secondary

metabolism in cell cultures by acting as true elicitors, inducing defense responses, as well as increasing the release of the target compounds to the culture medium (Bru et al., 2006; Lijavetzky et al., 2008; Zamboni et al., 2009).

In the case of ruscogenins, very little research has been carried out on enhancing their *in vitro* production with elicitors. In the only study reported to date, Mangas et al. (2006) treated *in vitro* plant cultures of *R. aculeatus* with MeJA at the concentration of 100 μM, but the ruscogenin content remained similar to that of the untreated plantlets in the aerial parts and slowly decreased in roots. These results call for new experiments using other elicitors or biotechnological systems to improve both ruscogenin and neoruscogenin production.

Coronatine (COR) is a pathogenic toxin produced by *Pseudomonas syringae* that has been tested as an elicitor in plant cell cultures of *T. media* and *T. globosa* (Onrubia et al., 2013a, Ramirez-Estrada et al., 2015). It acts as a molecular stimulator of the isoleucine-conjugated form of jasmonic acid (JA-Ile) (Katsir et al., 2008), but being more stable, its mechanism of action is similar to that of the elicitor MeJa (Onrubia et al., 2013b). The addition of COR to cell cultures of *T. media* and *T. globosa* was more effective than MeJA in increasing taxane production, even at much lower concentrations. The taxane yields reached in *Taxus* spp. cell cultures treated with COR have been up to 5.3 times higher than those obtained with MeJA (Onrubia et al., 2013a, Ramirez-Estrada et al., 2015).

Thus, in most cases the combined use of elicitors and permeabilizing agents can enhance the biotechnological production of secondary metabolites (Zhao et al., 2005), and could represent a suitable approach for increasing RA production in plant cell cultures of different plant species. It has been shown that elicitation of plant cell cultures with MeJA and CD enhances the accumulation of other phenolic compounds, such as resveratrol in *Vitis vinifera* (Lijavetzky et al., 2008) and silymarin in *Silybum marianum* (Belchi-Navarro et al., 2011). The production of other types of PSM has also been increased by this joint treatment, for example, aromadendrene in *Capsicum annuun* cell cultures (Sabater-Jara et al., 2010b), taraxasterol in *Solanum lycopersicum* (Briceño et al., 2012), ajmalicine in *C. roseus* (Almagro et al., 2011) and most recently taxol and related taxanes in *T. media* cell cultures (Sabater-Jara et al., 2014). In the same way, the joint application of COR and CD dramatically increased the taxane production in *T. globosa* cell cultures (Ramirez-Estrada et al., 2015). These studies have demonstrated the effectiveness of this combined treatment and suggest it has the potential to improve the production of other PSM such as RA.

5.3.3. Metabolic engineering approaches

Plant metabolic engineering (PME) offers a set of tools for overexpressing or silencing genes that could limit carbon flux to the biosynthesis of a target compound by modulating single steps. Alternatively, the expression of regulatory genes can be modified to establish multiple controls over one or more pathways in the cells (Fig. 17) (Onrubia et al., 2013b). As previously mentioned, the biosynthetic pathway of RA has been well elucidated but its regulation remains unclear, which is probably why only single-step and not holistic approaches have been developed until now for improving RA biotechnological production by means of metabolic engineering tools. In contrast, nothing is known about the last steps of the ruscogenin biosynthetic pathway or the genome of *R. aculeatus.* Therefore, the application of metabolic engineering techniques to improve the biotechnological production of ruscogenins is still far from being a realistic option.

One challenge in PME is to find enzymes that limit the precursor-flow in metabolic pathways and may therefore be suitable targets for engineering. In this regard, *in vitro* plant cultures can constitute an excellent platform for basic studies and to identify bottlenecks in a PSM pathway. As mentioned above, elicitors are used to increase RA production. Moreover, it has been demonstrated that in elicited cell suspensions and hairy root cultures, high RA production is correlated with high transcript accumulation of genes encoding key enzymes involved in RA biosynthesis.

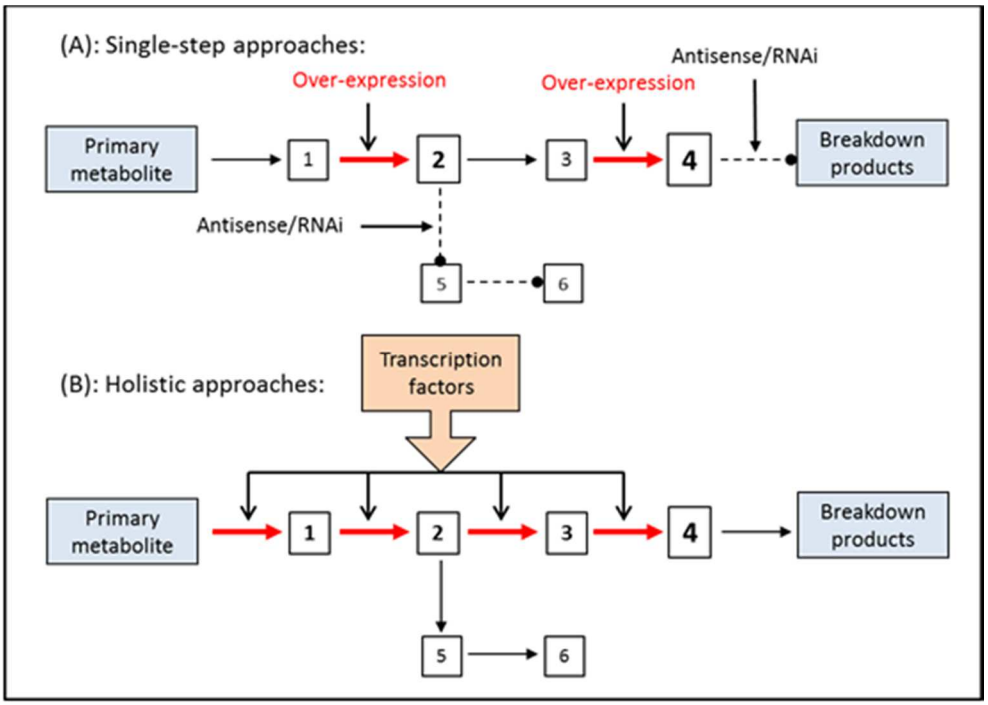

Figure 17. Comparative scheme of single-step and holistic approaches in plant metabolic engineering (modified from Onrubia et al., 2013).

PAL activity has been shown to increase in elicited cells of *C. blumei* as well as the activity of the specific RA biosynthetic enzyme RAS (Szabo et al., 1999). The limiting role of the PAL pathway was further confirmed by Kim et al. (2013), who demonstrated that PAL, 4CL and C4H were more active in *Agastache rugosa* cell cultures elicited with MeJA. Enhanced activities of PAL and TAT have been demonstrated in highly RA productive cell lines of *S. milthorriza* elicited with MeJA (Xing et al., 2013), showing that increasing the precursor of both committed pathways in RA biosynthesis could be a successful strategy to increase the biotechnological production of this compound. On the contrary, in YE-elicited hairy root cultures of *S. miltiorrhiza* TAT activity underwent a notable rise whereas PAL activity dropped rapidly (Yan et al., 2006). In the same system, HPPR gene expression was correlated with increased RA for the first time (Xiao et al., 2009). This was subsequently confirmed by Zhang et al. (2014a), who found that a high RA production is more closely correlated with the activation of the enzymes involved in the tyrosine-derived pathway than with those related with the phenylpropanoid pathway.

In this scenario, Xiao et al. (2011) overexpressed some genes which encode key enzymes involved in the biosynthesis of RA, SmHPPR (hydroxyphenylpyruvate reductase), SmC4H (cinnamic acid 4-hydrolase) and tyrosine aminotransferase (TAT), in hairy root cultures of *Salvia miltiorrhiza* and observed higher RA accumulation in the

transgenic lines. The highest levels of RA (992 mg L^{-1}) were achieved when *hppr* and *tat* genes were co-overexpressed. These results were partially confirmed by Barberini et al. (2013), who demonstrated that in cell suspension cultures of *Salvia officinalis* RA biosynthesis is highly correlated with the expression of the *hppr* gene, suggesting that this step could be a bottleneck for RA production in the cells and consequently a relevant target for new metabolic engineering approaches.

In hairy root cultures of *Coleus blumei*, overexpression of *hppr* and *ras* genes had controversial effects. Three hairy root lines harboring the *hppr* gene under the control of the 35S-promotor were obtained, two lines showing a higher accumulation of the *hppr* transcript than the control (untransformed) lines. High expression levels of the gene were correlated with an increased production of RA, which was up to 176% higher than in the control cultures. In contrast, expression of the *ras* gene in transformed roots carrying the *ras* overexpression construct did not increase and their RA production was generally lower than in the control root lines. The reduced expression of both genes in some of the hairy root clones obtained was attributed by the authors to co-suppression effects (Hüchering and Petersen, 2013).

Transgenic plants of *Perilla frutesces* overexpressing the tyrosine aminotransferase (TAT) gene were obtained by *Agrobacterium*-mediated transformation with the vector pCAMBIA23400-35S, which contains PfTAT under the control of the 35S promoter (Lu et al., 2013). Transgenic plants accumulated increased contents of RA, which was correlated with the transcript level of the PfTAT gene.

A new and very interesting metabolic engineering approach has been designed by the group of Zhang (Zhang et al., 2014b) based on “increasing income and reducing expenditure”. To perform this, the Arabidopsis Production of an Anthocyanin Pigment transcription factor (AtPAP1) under the control of the 35S promoter was overexpressed in the high RA-producing plant species *S. miltorrizha*. AtPAP1 belongs to the MYB family of transcription factors and controls anthocyanin biosynthesis in plant tissues (Fig. 18) (Dubos et al., 2010). Ectopic expression of AtPAP1 increases the accumulation of anthocyanins in *Arabidopsis* (Rowan et al., 2009), tobacco, (Zhou et al., 2008), tomato (Zuluaga et al., 2008), canola (Li et al., 2010) and *Taraxacum* plants (Qui et al., 1014).

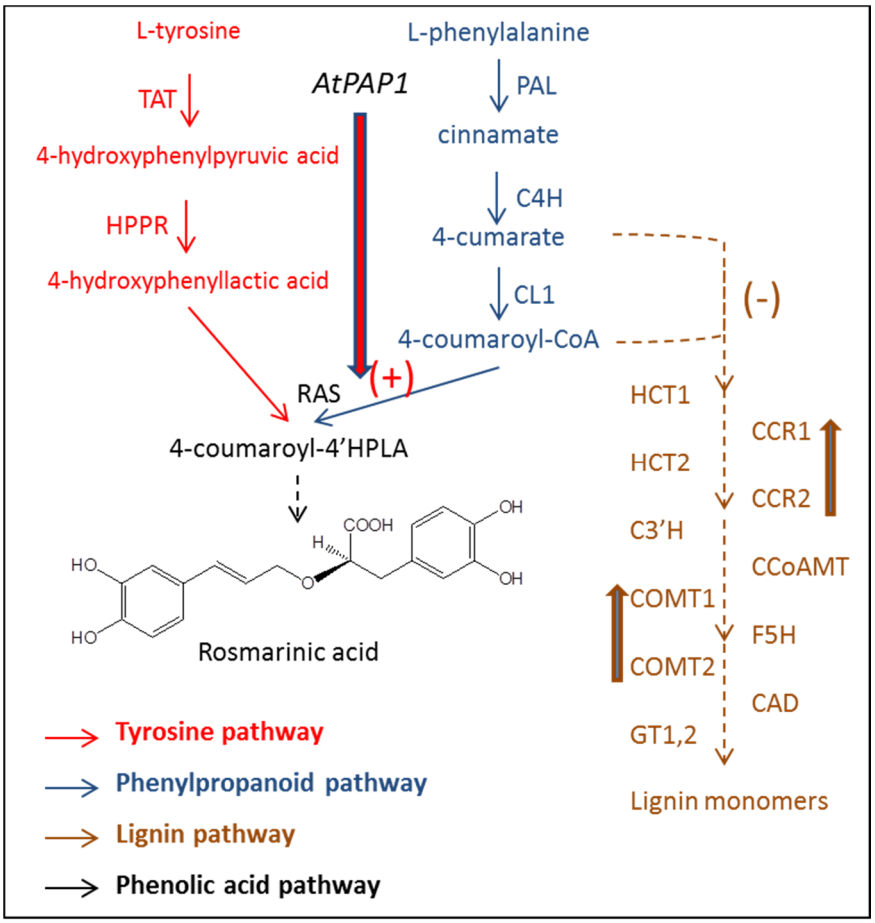

Figure 18. Methodological design based on the biotechnological strategy "increasing income and reducing expenditure" proposed by Zhang et al. (2014) for improving RA production. CCR, cinnamoyl-CoA reductase; CCoAMT, caffeoyl-CoA O methyltransferase; C3′H, coumarate 3′-hydroxylase; C4H, cinnamate 4-hydroxylase; 4CL, hydroxycinnamate-CoA ligase; COMT, caffeic acid O-methyltransferase; GT, glycosyl transferase; HCT, hydroxycinnamoyl transferase; HPPR, hydroxyphenylpyruvate reductase; PAL, phenylalanine ammonia lyase; RAS, rosmarinic acid synthase; TAT, tyrosine aminotransferase.

Zhang's group had previously demonstrated that overexpression of AtPAP1 in *S. milthorrizha* not only increased the production of RA up to two-fold, but also enhanced lignin accumulation (Zhang et al., 2010). As RA and lignins are in competition for the same precursors (Fig. 18), in a later experiment, the same authors increased the biosynthesis of phenolic precursors by the ectopic expression of AtPAP1 and simultaneously decreased lignin biosynthesis via co-supression of two key enzymes involved in this process, cinnamoyl-CoA reductase (SmCCR) and caffeic acid O-methyltransferase (SmCOMT), using chimeric RNAi technology. The obtained transgenic plants accumulated significantly higher levels of RA than the control plants, showing the suitability of this strategy to increase phenolic compounds such as RA.

6. Material and Methods

6.1. Rosmarinic acid production

6.1.1. Material

As starting plant material, we used a friable callus line of *Satureja khuzistanica* routinely subcultured in Gamborg's (B5) (Gamborg et al., 1968) solidified media containing 30 g L^{-1} sucrose, 2 g L^{-1} PVP, 20 mg L^{-1} L-glutamin, 200 mg L^{-1} casein hydrolysate, 5 mg L^{-1} benzyladenine (BA) and 1 mg L^{-1} indole-3-butyric acid (IBA). This callus line was obtained from the basal end of *S. khuzistanica* shoot tip explants, as described by Sahraroo et al. (2014), in the Department of Agriculture, Medicinal Plants and Drugs Research Institute, Shahid Beheshti, and it was kindly provided by Prof. H.M. Mirjalili of the aforementioned Department.

6.1.2. Methods

6.1.2.1. Establishment of suspension culture

To obtain a homogenous cell suspension, 10 g of *S. khuzistanica* calli (routinely subcultured, as described by Sahraroo et al. (2014), every 2 weeks for more than 1 year) were transferred to an Erlenmeyer flask of 1 L with a working volume (WV) of 200 mL of B5 medium supplemented with 30 g L^{-1} sucrose, 20 mg L^{-1} L-glutamine, 200 mg L^{-1} casein hydrolysate, 5 mg L^{-1} BA and 1 mg L^{-1} IBA. B5 liquid medium was prepared according to the instructions of the supplier. Sterilization was carried out by sterile filtration (0.2 μm filters) into autoclaved 1L Schott bottles. The plant growth regulators were added before use by taking an aliquot of a stock solution until the final desired concentration.

6.1.2.2. Culture conditions.

The culture conditions for all the experiments performed on a small-scale (Erlenmeyer flasks) were: temperature 25ºC; shaking frequency 110 rpm; shaking diameter 25 mm; light, dark conditions. These conditions were maintained in all the experiments carried out so far.

6.1.2.3 Subcultures

For the maintenance of the *S. khuzistanica* cell line, the cell suspension was periodically subcultured each week for a period of approximately 16 months. To carry out this task, the system of pouring cell culture was employed (Mustafa et al., 2011). The pcv of the cell suspension was measured and new B5 fresh medium was added to an inoculum density of 10%. The final volume was divided and transferred to a new flask of 1 L with a WV of 200 mL in order to increase the biomass of the cell suspension for further experiments. The suspension culture was maintained, as described previously, in a shaker (INFORS HT) at 25°C in darkness and at a shaker speed of 110 rpm and shaking diameter of 25 mm.

6.1.2.4. Inoculum preparation

In order to investigate the optimum pcv inoculum for a high biomass production, two inoculum pcv (10% and 20%) were used in this experiment. Thus, the pcv of a 2-week-old cell suspension was measured and the adequate volume of new B5 culture medium was added to a final pcv of 10% or 20%, respectively. 200 mL of these suspensions were transferred to 1 L flasks to perform the experiment. Culture conditions was the same as described above. To prepare the inoculum for the rest of the experiments (elicitor experiments and scaling up to bioreactor cultures), the pcv of a one-week-old cell suspension was measured and the adequate volume of new B5 culture medium was added to a final pcv of 10%.

6.1.2.5. Methyl jasmonate and cyclodextrin elicitation

To perform this experiment, flasks of 125 mL with a working volume (WV) of 25 mL and a pcv inoculum of 10% were utilized. After 7 days of culture, randomly methylated-β-CD (M-β-CD) at a final concentration of 40 mM was added to the flasks, and after 2 days MeJA at a final concentration of 100 μM was added to the cultures previously

supplemented with CD and also to control cultures without CD (Fig. 19). As MeJA was dissolved in ethanol (EtOH), 50 µL of EtOH was also added to the cultures (mock conditions). The culture period consisted of 21 days and samples were harvested at 0, 2, 4, 7, 9, 11, 14, 16, 18 and 21 days of growth, in order to determine their pcv, cell fresh weight (CFW), cell dry weight (CDW), pH, conductivity, and RA production.

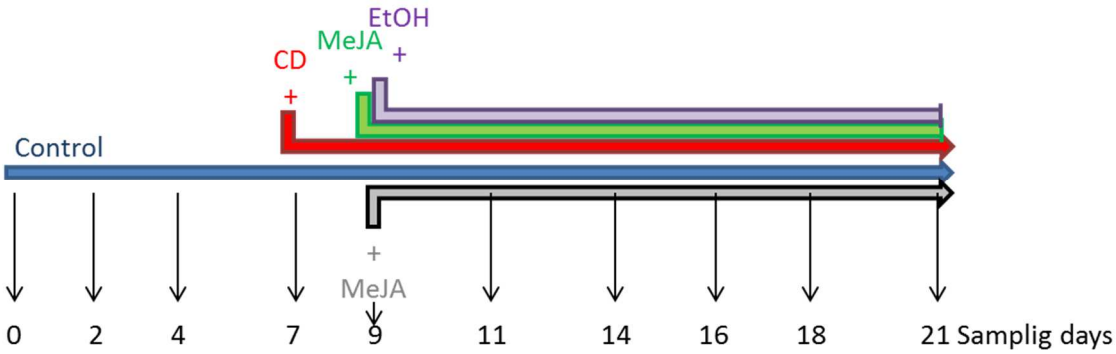

Figure 19. Methodological design of the elicitor experiment. EtOH, ethanol; MeJA, methyl jasmonate, CD, cyclodextrin.

6.1.2.6. Scale up to BIOSTAT CultiBag RM benchtop bioreactor

A disposable mechanically-driven bioreactor (BIOSTAT CultiBag RM) was used to study the RA production of the *S. khuzestanica* cell line on a bench-top scale. Two different sets of experiments were performed at bioreactor level: a control or under optimized elicitation (MeJA) conditions, based on the results we obtained in the small-scale experiments (shake flasks). The culture bags were filled with Gamborg's B5 medium and a *S. khuzistanica* cell suspension with a viability of >95% reaching a pcv of 10% in the CultiBag. For the elicitor experiments, at day 11 a sterile solution of MeJA was added to the culture at a final concentration of 100 µM. All work was carried out in a laminar flow hood. In the BIOSTAT CultiBag RM, culture bags of 2 L (1 L working volume) were used (Fig. 20). The experiments lasted 21 days and were carried out in batch mode in the dark at 25ºC. The pH was not controlled. Wave-mixed culture bags were operated at a rocking angle of 6° and an aeration rate of 0.1 vvm. The BIOSTAT CultiBag RM`s rocker rate was increased from 20 to 30 rpm during all cultures and the initial biomass concentration was 45 g FW L^{-1}. This device also controlled the working temperature (25ºC) of the reaction space, and the culture period finished at day 21. The most important parameters for the BIOSTAT Cultibag RM are summarized in Table 5.

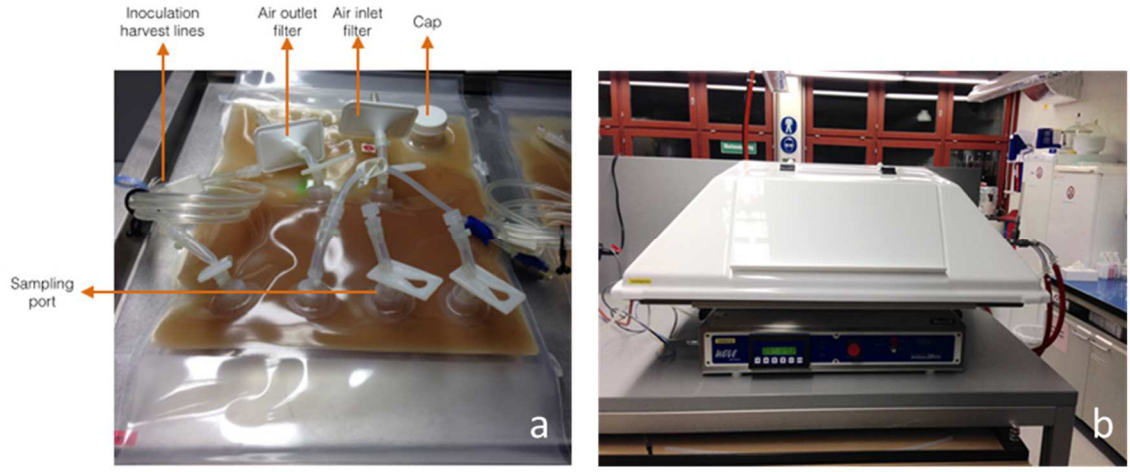

Figure 20. The CultiBag bioreactor fittings (a) and the BIOSTAT Cultibag RM device (b).

Samples were taken every two days under sterile conditions. Sampling was used to determine different in-process control measurements, such as cell fresh weight (CFW), cell dry weight (CDW), pcv, pH and conductivity. 10-mL culture broth was taken every two days from the cultivation bags using sterile pipettes. The pcv was determined by calculating the percentage of the pellet volume over the total volume after centrifugation at 4000 rpm for 10 min. After that, the supernatant was used to measure the conductivity and pH of the culture, and the pellet was utilized to determine the CFW. The pellet was resuspended in demineralized water and transferred to the filtration unit. It was then vacuumed for 3 min and the weight was determined. Both the doubling time (t_d) and specific growth rate (μ_{CFW}) of the cell line was measured based on the exponential phase curve. CDW and RA content of cells were determined after freeze-drying the cell biomass and the culture medium. RA was extracted from the lyophilized biomass and culture medium and determined by HPLC.

6.1.2.7. Coronatine experiment

Shake flask scale. To perform this experiment, 125 mL flasks with WV of 20 mL and a pcv inoculum of 10% from a week-old *S. khuzistanica* cell suspension were utilized. As in the previous experiments, the culture conditions were as follows: T of 25^0C, 110 rpm shaking frequency and darkness. After a growth period of 14 days (near the end of the exponential phase), COR at a final concentration of 1 µM was added as an elicitor to the flasks. The culture period consisted of 24 days and samples were harvested at 0, 2, 4, 7, 11, 14, 16, 18, 21, and 24 days of growth in order to determine in-process control measurements, pcv, CFW, CDW, pH, conductivity, as well as enzymatic activities and

RA production. As previously described, the doubling time (t_d) and specific growth rate (μ_{CFW}) were measured based on the exponential growth curve. Samples were taken in triplicate and the results compared with control conditions (without elicitation).

Table 5. Setpoints and culture parameters of the BIOSTAT CultiBag RM and the CellBag Orbitally Shaken Bioreactor.

Cultivation system	Mixing principle	Culture volume (L)	pcv inoculum (%)	Rocking rate/Shaking seep (rpm)	Rocking angle/shaking diameter	Airation (vvm)	Temperature (°C)
BioWave	Wave-induced motion	1	10	20-30	6º	0.1	25
Shaken CellBag	Orbital shaking motion	1	10	38	50 mm	0.1	25

Benchtop bioreactor scale. In order to scale up the process, a 2 L culture bag (CellBag) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with a working volume of 1 L (Fig. 21), shaken in a Kuhner orbital shaker in the dark at 25ºC and 35-38 rpm, with a shaking diameter of 50 mm, was used. The culture was initiated at 35 rpm and gradually the shaking was increased up to 38 rpm to obtain a good distribution of cell biomass and oxygen transfer without foaming. The sterile airflow was 0.2 L min^{-1} and the inoculum size was 10% (w/v) (Table 5). In previous experiments, these conditions were determined as optimal for the cell line growth and to avoid out-of-phase phenomena (data not shown). After 11 days of culture (near the end of the exponential phase in these conditions), the elicitor 1 µM COR was added. Samples were taken daily in triplicate at days 0, 4, 7, 9, 11, 14, 16, 18 and 21. The experiment was run in duplicate.

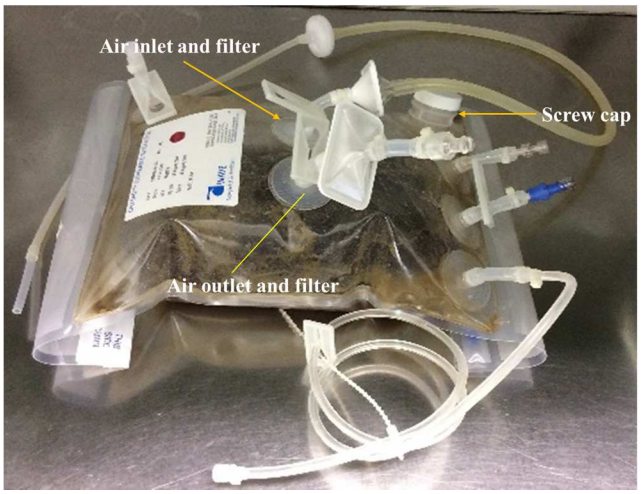

Figure 21. The 2 L CellBag fittings.

6.1.2.8. In-process controls

To study how the time course of the different experiments affected the cell line growth and production, 3 flasks were harvested according with the sampling program and checked for viability, pcv, CFW, CDW, pH, conductivity and RA.

Cell viability. In order to calculate the % of living cells, samples were colored with Evans Blue (4 volumes cell suspension: 1 volume Evans Blue), placed on an objective slide and observed under the microscope (200x). Viability was determined by assessing the ratio of total and living cells.

The pcv: 10 mL of resuspended cell suspension was transferred to 15-mL Falcon tubes. The tubes were put in a centrifuge and spun at 4000 rpm for 10 min. The pcv was read in the tube. The supernatant was transferred to a new 15-mL tube. The tubes were kept at – 20 ºC in the freezer for further analyses.

Cell fresh weight (CFW): 10 mL of the cell suspension was centrifuged at 4000 rpm, the pellet was transferred from the centrifuge tube to a filtration unit, which was connected to a vacuum pump for 3 min. Fresh biomass was weighed (FW) and kept in Petri dishes in the freezer at - 20ºC.

Cell dry weight (CDW): Frozen biomass was freeze-dried for 24 h and weighed, the DW of each sample being registered. Additionally, supernatants were collected and stored in the freezer (-20ºC) until the end of the experiment. Subsequently, supernatants were lyophilized and used to determine extracellular RA production.

pH: pH-meter calibration: the electrode was rinsed with distilled water, the electrode placed in a pH 4.00 buffer solution, and the measure recorded. The electrode was removed from the solution and rinsed with distilled water. The electrode was placed in the second buffer solution (pH, 7.00) and the measure recorded. The electrode was removed from the second buffer solution and rinsed with distilled water. After calibration, the supernatant pH was measured and the measure recorded.

Conductivity: Conductivity meter calibration: the sensor was held in the air and “Cal” was pressed. The sensor was held in the calibration standard and “Cal” pressed. The sensor was rinsed, the electrode placed in the supernatant, “read” was pressed and the measure recorded.

6.1.2.9. Rosmarinic acid extraction and quantification

Extraction and quantification of RA from lyophilized cells and supernatants was performed following the protocol described by Georgiev et al. (2006) with some modifications.

Extraction from freeze-dried biomass: 20 mg dried cells were mixed with 9 mL methanol. After 2 min in vortex, the extract was incubated for 20 min in an ultrasonic bath, and then centrifuged (4000 rpm). The supernatant was dried in a rotary evaporator under reduced pressure, and the residue was dissolved in 1.5 mL of methanol (Fig. 22). Then the samples were passed through a 0.2 µm filter before HPLC analyses.

Extraction from supernatant: 10 mL of filtered culture medium was frozen and lyophilized. Dry extracts were dissolved in 5 mL of methanol (for HPLC), refrigerated for 24 h at 4º C, and then filtered (0.45 or 0.2 µm) to ensure that all sugars had gone (Fig. 22), and simply injected into the HPLC system.

HPLC analyses of rosmarinic acid. An aliquot of 20 µL of the filtrate samples was injected into the HPLC for analysis. 330 nm was selected as the wavelength for UV detection. The HPLC column was a Spherisorb ODS-2 (5 µm) reverse phase 4.6 mm × 250 mm. Elution was carried out at a flow rate of 1.0 mL min^{-1} at 25°C. Two mobile phases, A and B, were used. Mobile phase A was 0.1 % (v/v) formic acid solution in water, while mobile phase B was acetonitrile. A ratio of 88 % A and 12 % B was applied in the first 30 min. After 30 min, a ratio of 80 % A and 20 % B was used for the next 15 min. Finally, 70 % A and 30 % B were used after 45 min for an additional 15 min. The chromatographic peak of RA was confirmed by comparing its retention time with that of the reference

standard. Working standard solutions were injected into the HPLC and peak area responses obtained. Standard graphs were prepared by plotting concentration versus area. Quantification was carried out from integrated peak areas of the samples using the corresponding standard graph.

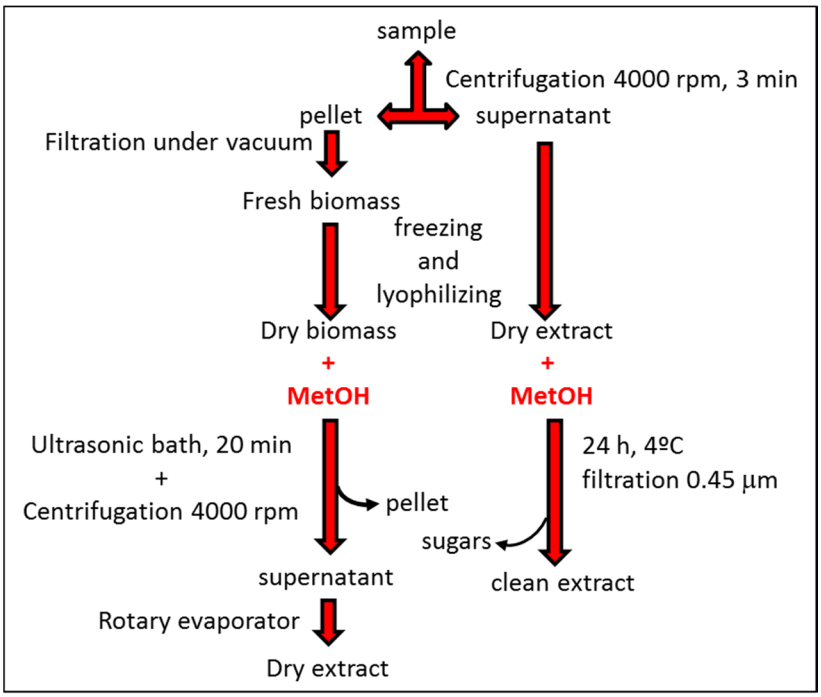

Figure 22. The RA extraction protocol. MetOH, methanol.

6.1.2.10. Determination of enzymatic activities

Determination of PAL and RAS activities

In order to determine the enzymatic activities of phenylalanine ammonialyse (PAL) and rosmarinic acid synthase (RAS), a crude protein extract was obtained from fresh cell material after filtration. Extractions were done at 4°C. After homogenization, each sample consisted of 2-5 g of cells, 1 g polyvinylpyrolidone (Polyclar), 50 µL dithiothreitol 1 mM (DTT), and 5 ml KPi buffer 0.1 M. The extractions were at pH 8.0 and pH 7.5 for PAL and RAS activities, respectively. Samples were vortexed 3 times for 30 seconds and kept on ice. After centrifugation at 5000 rpm for 40 min, the supernatant was filtered through glass wool and used for the spectrometric enzyme assay as well as for the protein quantification (Melotto et al., 2008). Protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

PAL activity was determined by the method described by Zucker with slight modifications (1965). The reaction mixture (1 mL) contained 650 µL of 0.1 M KPi buffer pH 8, 200 µL L-phenylalanine-KPi buffer 0.1 M, and 150 µL of the protein extract. Incubation was performed at 36°C for 30 min and the production of cinnamic acid was measured as the increase in absorbance from the initial time (0 min) to the final (30 min) time of the reaction, against a reagent blank at 290 nm (Yang and Shetty, 1998). The enzyme activity was expressed as pkat/mg protein.

RAS activity was measured as described by Berger et al. (2006). 20 µL of the protein extract was incubated at 30°C in 67.7 µL of 0.1 M KPi buffer pH 7 with 12.5 µL 0.1 M DTT, 10 µL 12.5 mM ascorbic acid, 10 µL 2.5 mM 4-coumaroyl-CoA and 5 µL 20 mM shikimic acid, and the RA production was measured as an increase in absorbance at 333 nm.

Determination of TAT activity.

In order to determine the enzymatic activities of tyrosine aminotransferase (TAT), a crude protein extract was obtained from fresh cell material after filtration. Extractions were done at 4°C. After homogenization, each sample consisting of 2 g of cells was added to a solution of 5 mL total volume containing 1 mM phenylmethanesulfonyl fluoride (PMSF), 1 mM DTT and 0.1 M Tris-HCl (pH 7.2). Samples were then vortexed for 30 sec and kept on ice. After centrifugation at 14000 rpm for 30 min, the obtained supernatant was used for the spectrometric enzyme assay as well as for the protein quantification. Protein concentrations were determined by the Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as the standard.

TAT activity was determined by the method of De-Eknamkul and Ellis (1987). The enzyme extract (50 mL) was added to a reaction solution containing 6.0 mM L-tyrosine, 10 mM α-ketoglutarate, 0.05 mM pyridoxal phosphate, and 42.5 mM glycyglycine at pH 9.0 in a total volume of 1.0 mL, and the mixture was incubated at 30°C for 30 min. The reaction was stopped by adding 20 mL of 10 M KOH, and the TAT activity was determined by measuring the initial (0 min) and final absorbance (30 min) of the reaction solution against a reagent blank at 331 nm.

6.1.2.11. Preparation of the S. khuzistanica extract for biological assays

In order to prepare a *S. khuzistanica* methanolic extract enriched in RA, 10 g of CDW from a 24-old cell suspension treated with COR was distributed in Falcon tubes, each

containing 100 mg of CDW supplemented with 40 mL of methanol. RA was extracted as described above from the tubes and the extracts were dried in a rotary evaporator. Due to the toxicity of methanol for the biological analyses, we utilized the minimum quantity of DMSO to dissolve the dry extract and obtain the *S. khuzistanica* RA-enriched extract. An aliquot of the extract was evaporated and redissolved in methanol for HPLC analyses. The final concentration of RA in the *S. khuzistanica* extract was 5.8±0.2 mg RA mL^{-1}.

6.1.2.12. Human cancer-derived cell lines

Human breast adenocarcinoma-derived MCF-7 (ATCC no. HTB-22) and human hepatoma-derived HepG2 (ATCC no. HB-8065) cells were grown at 37 ºC and 5 % CO_2 in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 2 mM glutamine, 110 mg/l sodium pyruvate, 10 % fetal bovine serum, 100 IU/ml penicillin and 100 µg/ml streptomycin.

6.1.2.13. Cell viability assay

MCF-7 and HepG2 cells were seeded in 24-well plates at a density of 2 x 10^4 cells/well. Twenty-four hours later, different amounts of *S. khuzistanica* extract, RA or vehicle (DMSO) were added to the cell cultures. Forty-eight hours after the addition of the compounds, cell viability was determined by means of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as previously described (Gallego et al., 2017). The cells were incubated in the presence of 0.63 mM of MTT and 18.4 mM of sodium succinate for 3 h at 37 ºC. Following removal of the medium, formazan was resuspended in DMSO supplemented with 0.57 % CH_3COOH and 10 % sodium dodecyl sulphate. Spectrophotometric determinations were performed at 600 nm in a Cobas Mira S analyzer (Hoffman-La Roche, Basel, Switzerland).

6.1.2.14. Cell cycle analysis

Analysis of the cell cycle was performed using the PI/Cell Cycle Analysis Kit (Canvax Biotech, Córdoba, Spain). MCF-7 cells were seeded in 6-well plates at a density of 5 x 10^5 cells/well and left to grow for 24 h. Afterwards, the cells were incubated in the presence of 0.15 mg/ml or 0.6 mg/ml of *S. khuzistanica* extract, 0.1 mM or 0.4 mM of RA or DMSO (vehicle) for 48 h. The cells were harvested, sedimented by centrifugation

(1000 rpm, 5 min), washed with ice cold phosphate-buffered saline (PBS), centrifuged again in the same conditions and fixed in ice cold 70 % ethanol for 45 min. Cells were recovered by centrifugation, washed with PBS, centrifuged again, resuspended with 200 µl of staining solution containing propidium iodide and RNase A, and incubated at 37 ºC for 30 min in the dark. The cells were analyzed by flow cytometry using a Gallios analyzer and Kaluza software (Beckman Coulter, Brea, CA, USA). The percentage of cells in each phase of the cell cycle was calculated and the apoptotic cells were considered to constitute the sub-G0/G1 cell population.

6.1.2.15. Caspase activity assays

Activity assays for caspase-1, -2, -5, -6, -8 and -9 were performed using the Caspase-Family Colorimetric Substrate Set Plus kit (BioVision, Milpitas, CA, USA). Following treatment with 0.6 mg mL^{-1} of *S. khuzistanica* extract and 0.4 mM RA or DMSO (vehicle) for 48 hours, 5 x 10^6 MCF-7 cells were harvested, sedimented by centrifugation, resuspended in 50 µl of chilled cell lysis buffer and incubated on ice for 10 min. After centrifugation at 10,000 *g* for 1 min, the supernatant was recovered to assay the protein concentration and caspase activities. Total protein in cell extracts was assayed with the Bradford method (Bradford, 1976) using BSA as a standard. For each caspase activity assay, the reaction mixture contained 100 µg of cell extract protein, 4.8 mM dithiothreitol and 190 µM of the corresponding caspase *p*-nitroaniline conjugated substrate in a total volume of 52.5 µl. The reaction proceeded at 37 ºC for 2 hours. Spectrophotometric determinations were performed at 30 °C in a Cobas Mira S analyzer (Hoffman-La Roche, Basel, Switzerland) at a wavelength of 405 nm (caspase activity assays) and 600 nm (total protein).

6.1.2.16. Statistical analysis

The statistical analysis was performed with SPSS version 24 (IBM, Armonk, NY). Data were submitted to one-way ANOVA. Significant differences among treatments were determined with the Scheffé *post hoc* test.

6.2. Ruscogenin production

6.2.1 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 we used phylloclades, rhizomes and seeds of these plants.

6.2.2. Methods

6.2.2.1. 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 Fig. 23.

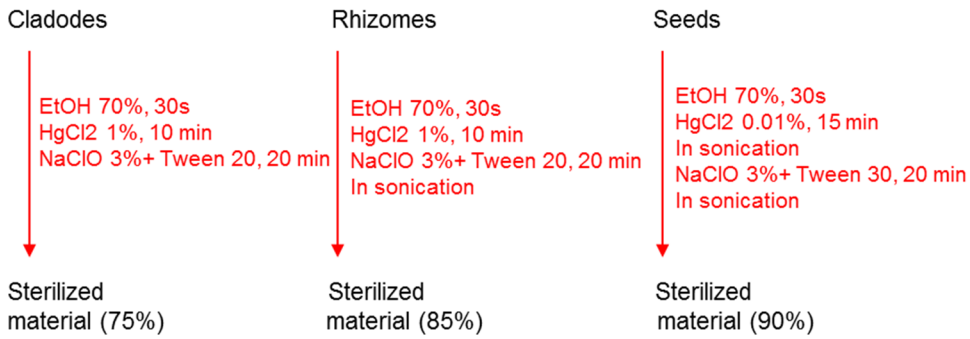

Figure. 23. Summary of optimized protocols for the sterilization of the different types of explants

6.2.2.2. Plantlet in vitro cultures

Plantlet *in vitro* cultures were obtained as previously described by Moyano 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 25ºC in the light 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 (Fig. 24).

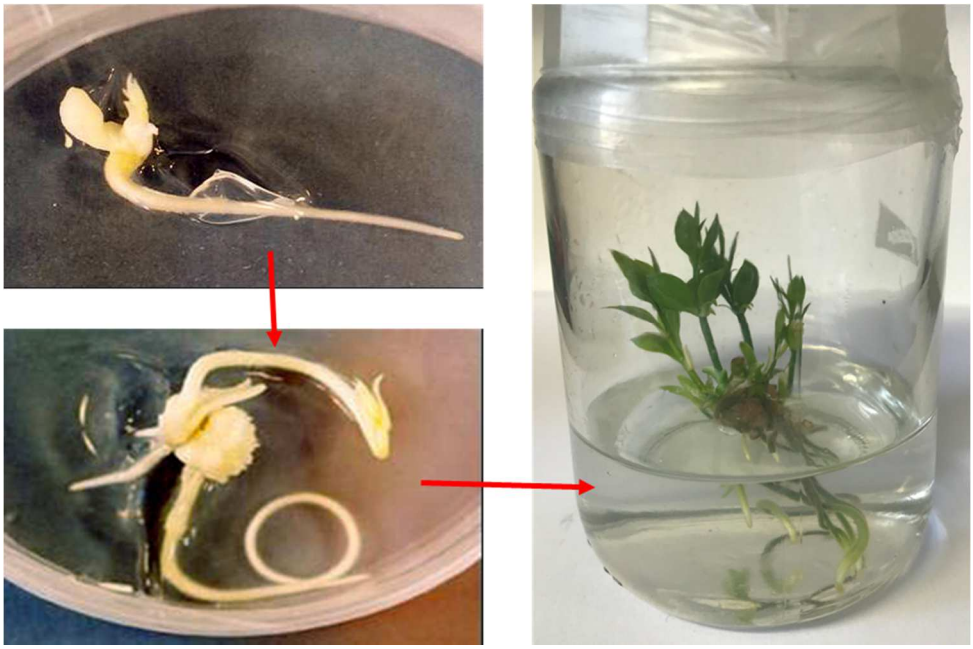

Figure 24. Stages in the development of an *in vitro* plant from a germinated embryo.

6.2.2.3. Callus induction and culture

To obtain friable and non-organogenic calli we used 3 different types of explants: segments of phylloclades, segments of rhizomes, and embryos. Phylloclades and rhizome segments were obtained from 5-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. In the experiments, we tested 2,4.dichlorophenoxyacetic acid (2,4-D), indolbutyric acid (IAA), 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-topoline (MT) and benzyladenine

(BA) as cytokinins, as well as gibberellic acid (GA_3), all of them at several concentrations. Explants were kept in a controlled climate chamber in darkness at 25ºC. After 2, 4 and 6 weeks of culture, the number of explants with calli were recorded (Fig. 25). 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 organogenesis of both roots and shoots; moreover, the callus was hard and did not disintegrate when cultured in liquid medium. Therefore, another set of culture media were tested to obtain friable calli without organogenesis. In this case, we tested the best media for callus induction and several new media based on different combinations of PGRs, such as the new auxins, meta-topolin (MT) and zeatin (ZT). New combinations of vitamins and amino acids were also tested. A total of 28 new combinations were assayed. The calli were routinely subcultured every 4 weeks.

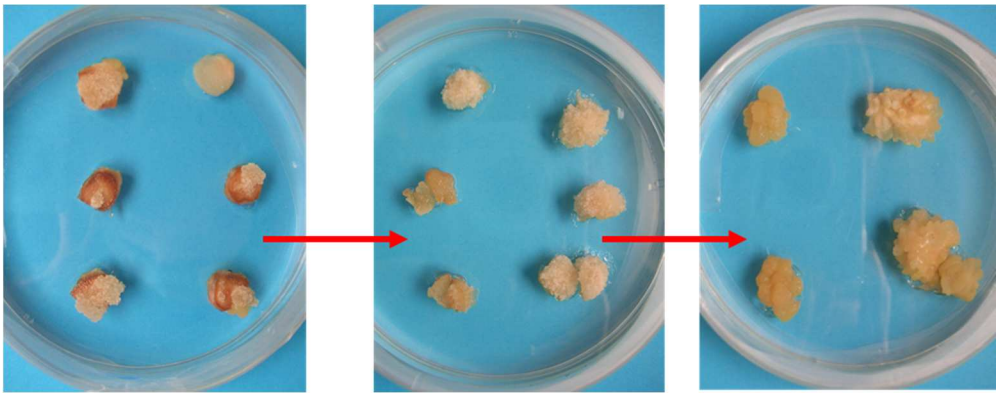

Figure 25. Establishment of *R. aculeatus* callus cultures from rhizomes.

6.2.2.4. Establishment of cell suspensions

2 g pieces of friable calli were transferred to 250 mL flasks containing 20 mL of basal salt liquid media with different combinations of PGRs (Fig. 26). The flasks were cultured in darkness at 25ºC in an incubator shaker (Adolf Kuhner AG, Schweiz) at 100 rpm. The composition of the media assayed is shown in Table 6. Some callus pieces were also incubated in the same media with the addition of 0.4% pectinase for 72 hours. Isolated cells were then recovered by 1000 g centrifugation and resuspended in the same fresh medium without pectinase. The *R. aculeatus* cell line was maintained and periodically subcultured (every 4 weeks) using the pouring cell culture system (Mustafa et al., 2011),

Table 6. Composition of the culture media assayed for callus disintegration.

Mineral nutrition	Auxin	Cytokinin
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})
B5	IBA (1 mg L^{-1})	BA (5 mg L^{-1})
B5	NAA (4 mg L^{-1})	-

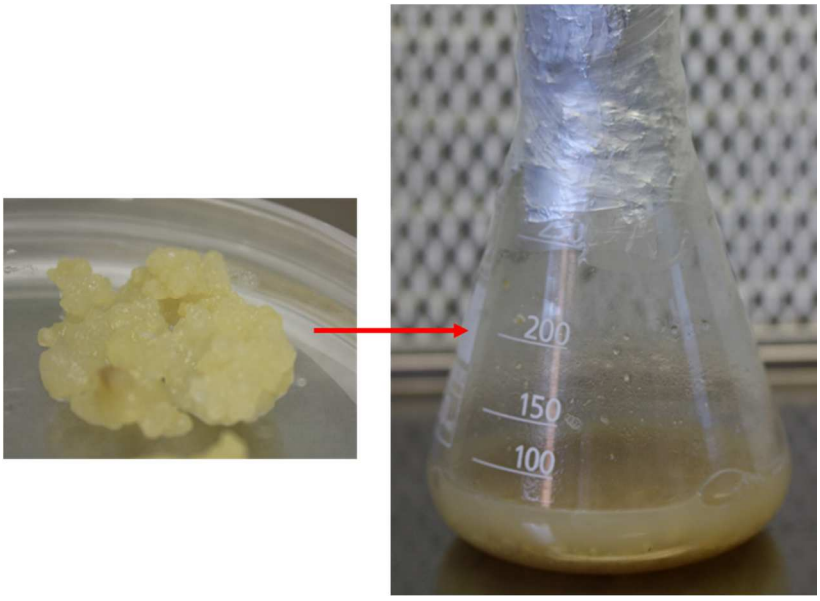

Figure 26. Transfer of the *R. aculeatus* calli from solid to liquid medium and establishment of cell suspension cultures.

6.2.2.5. Root-rhizome cultures

Root-rhizomes from *in vitro* young 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 (Fig. 27). Root-rhizomes were cultured under a 16-hour photoperiod or in darkness and routinely subcultured every 4 weeks.

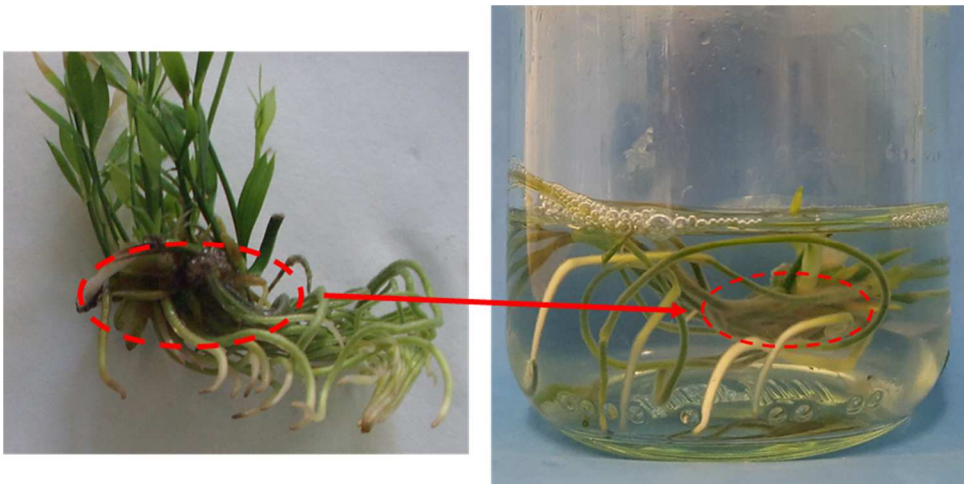

Figure 27. Establishment of the *R. aculeatus* root-rhizome cultures from the *in vitro* plants.

6.2.2.6. 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 the cultures were treated with 1 µM of COR after two weeks of culture. In all the systems, samples were taken in triplicate two weeks after the elicitor treatment. The fresh weight (FW) of the samples was registered and after their lyophilisation the dry weight (DW) was also determined. Dry powder was kept for saponin extraction as described below.

6.2.2.7. Benchtop bioreactor scale

In order to scale up the process, a 2 L culture bag (CellBag) (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with a working volume of 500 mL, shaken in a Kuhner orbital shaker in the dark at 25ºC and 35-38 rpm, with a shaking diameter of 50 mm, was used. The culture was initiated at 33 rpm and gradually the shaking was increased up to 35 rpm to obtain a good distribution of root biomass and oxygen transfer without foaming. The sterile airflow was 0.2 L min^{-1} and the inoculum size was 10% (w/v). After two weeks of culture 1 µM COR was added and samples were taken two weeks later. The experiment was run in duplicate.

6.2.2.8. Saponin extraction and quantification

The quantification of *Ruscus* saponins was based on their conversion to the genins 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 x 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 80ºC, in order to hydrolyse the saponins. The hydrolysate was adjusted to a pH of 8-10 with KOH (10%), and a final volume of 10 mL with methanol. The samples were filtered through a 0.2 μm Millipore filter and analysed by HPLC. Quantification of the sapogenins ruscogenin and neoruscogenin was performed according to Bertani and Forni (1984). 20 μL of the extract was loaded onto a Supelcosil LC8 (i.d. 4.4 x 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.

6.2.2.9. Statistics

Statistical analysis was performed with Excel software. All data are the average of three 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.

7. Results

7.1. Rosmarinic acid production

7.1.1. Inoculum production experiment

The *S. khuzistanica* cell line was maintained and periodically subcultured using the pouring cell culture system (Mustafa et al., 2011), where a certain volume of fresh medium is added periodically into the cell suspension culture and this is distributed among more flasks. Compared with other systems, the pouring technique is simpler (fewer tools used), faster and has less chance of contamination (Mustafa et al., 2011). As previously mentioned, two inoculum densities were tested, 10% and 20%, to optimize the growth capacity of the *S. khuzistanica* cell suspension. In this experiment the culture period was of 21 days and samples were harvested at days 7, 11, 14, 17 and 21. The pcv, pH, conductivity and FW were determined.

The time course of the pcv varied significantly depending on the inoculum density (Fig. 28). When we used an inoculum density of 10%, the pcv increased exponentially until day 11, when the cells probably entered the stationary phase, and then remained practically constant until the end of the experiment, when the pcv reached a maximum value of 36%. This means that the pcv increased 3.6-fold in 21 days. In contrast, when the experiments started with an inoculum density of 20%, the pcv peaked at day 14, achieving a value of approximately 50%, and then decreased to 38% at the end of the culture period. In this case, the pcv at the end of the culture period had increased only 1.9-fold.

Conductivity decreased continuously in both experiments until day 14, remaining practically constant thereafter in the case of 10% inoculum density, but surprisingly, at 20%, the conductivity started to increase until it recovered values similar to those at the beginning of the experiment (Fig. 29). Conductivity is known to be related with nutrients in the culture medium and for this reason decreases during the culture period according to the cell growth. The increase of conductivity in the 20% pcv experiment after day 14 was probably related with a massive cell death and the consequent release of ions and other metabolites from the cells to the culture medium. This hypothesis could be confirmed when considering the aforementioned decrease of pcv of these cultures.

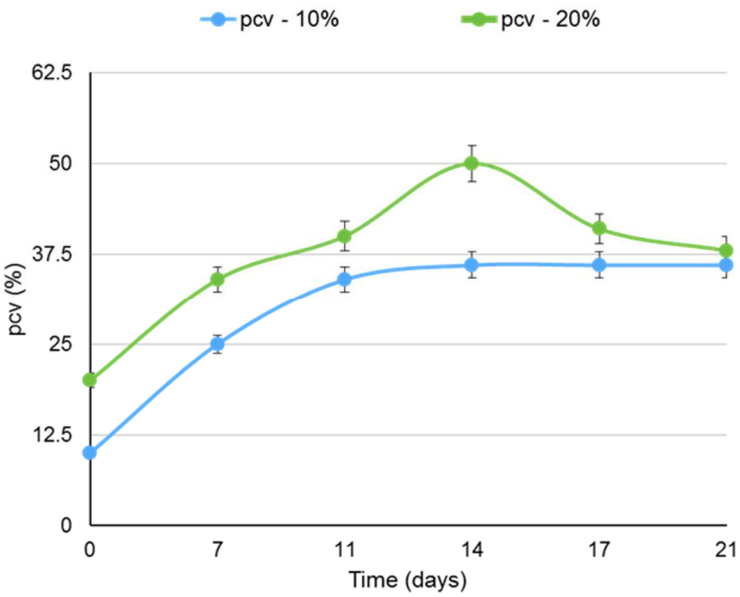

Figure 28. Time course of pcv expressed as %. Each value is the average of 3 determinations ± SD.

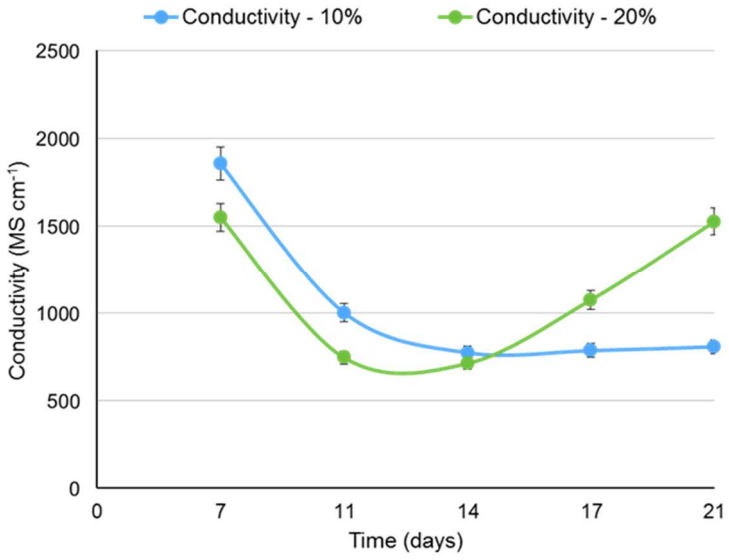

Figure 29. Time course of the conductivity expressed as MS cm^{-1}. Each value is the average of 3 determinations ± SD.

The pH of the culture medium was higher throughout the culture period in the experiment with an inoculum pcv of 20% *versus* 10%. In the latter, the pH increased until day 11 and thereafter remained practically constant until the end of the culture period (Fig. 30). On

the other hand, with an inoculum pcv of 20%, the pH increased until day 17, when it was 6.61, and decreased further until day 21.

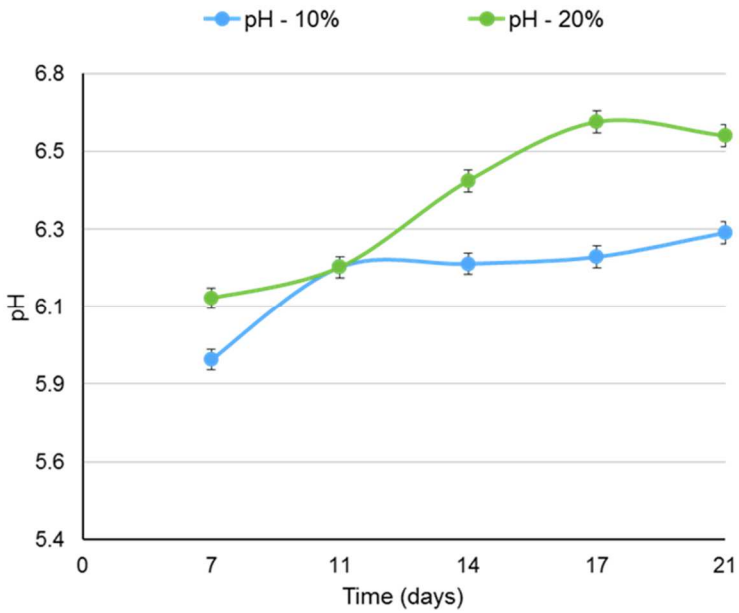

Figure 30. Time course of pH. Each value is the average of 3 determinations ± SD.

Similar to the variations in pcv throughout the culture period, when the inoculum density was 10%, the cell fresh weight (CFW) of the culture reached a maximum (178 g L^{-1}) at day 14 and then remained constant until the end of the experiment (Fig. 31). In the experiment with 20% pcv, the CFW peaked at day 14 and then decreased until day 21, when the experiment finished. The final CFW was very similar to the one achieved when working with an initial pcv of 10%.

The results show that in the *S. khuzistanica* cell suspension, when working with an inoculum density of 20% pcv, a high percentage of the cells died after 14 days of culture. On the other hand, when considering an approximate average doubling time (t_d) of 11.7 and 22 days for 20% pcv and 10% pcv, respectively, calculated on the basis of pcv, our results suggested it was better to work with an inoculum pcv of 10%, which was therefore used in subsequent experiments. In any case, the CFW at the end of the experiment was not very high, reaching a maximum of about 180 g L^{-1} and we decided to substitute the 2-week-old inoculum with the youngest inoculum (1-week-old) in future experiments.

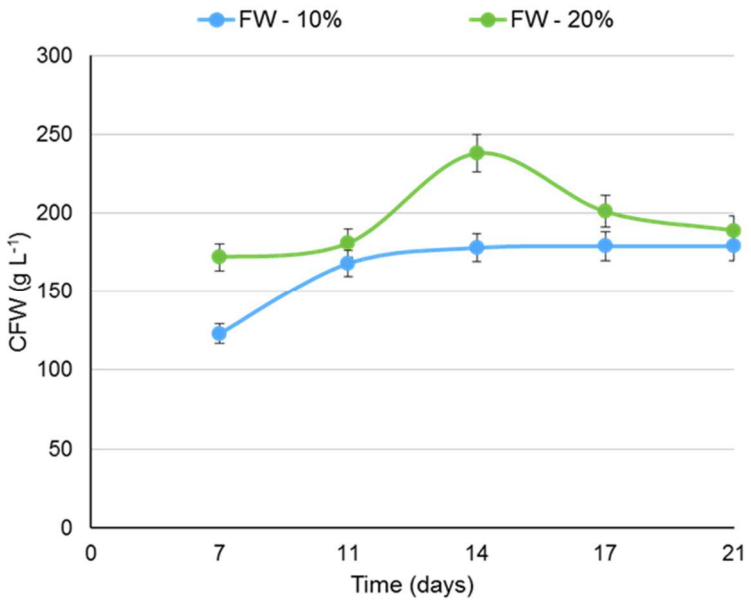

Figure 31. Time course of the growth measured as CFW and expressed as g L^{-1}. Each value is the average of 3 determinations ± SD.

7.1.2. Methyl jasmonate and cyclodextrin experiment

In this experiment we tested the effects of the elicitor MeJA at the concentration of 100 µM and the elicitor/permeabilizing agent M-β-CD at a final concentration of 40 mM (see Fig. 19). Both elicitors have been previously used to increase plant secondary metabolism in other plant cell cultures and in most cases the aforementioned concentrations were determined as optimal for inducing the accumulation of the target compound (Lijavetzky et al. 2008; Hakkim et al. 2011; Bauer et al. 2009).

7.1.2.1. Fresh Weight

An inoculum pcv of 10% (1-week-old) was prepared to optimize the growth capacity of the *S. khuzistanica* cell suspension. At the beginning of the culture, the initial cell fresh weight (CFWt0) was 44.55 g L^{-1}. After a short lag phase of 1 to 2 days, the cultures entered the exponential growth phase, which lasted until day 7 (Fig. 32). At this moment the biomass productivity (BMP_{CFW}) was approximately 24.08 g L^{-1} d^{-1}, and the inoculum doubling time was approximately 2.5 days. These results show that the established culture conditions were optimal to achieve a high production of cell biomass measured as FW. In previous experiments carried out with the same *S. khuzistanica* cell line, the BMP_{CFW} obtained was only about 12 g L^{-1} d^{-1} (Sahraroo et al., 2014). After day 7, a slow

increase of biomass measured as FW was achieved and at day 11 the culture entered the stationary growth phase (Fig. 32). These results also confirm that it is better to work with an inoculum of younger cells (1-week-old), since the final biomass in control conditions (309.5 g L^{-1}) was 1.6-fold higher than that achieved in the previous inoculum experiment (179 g L^{-1}) with a 2-week-old inoculum (Fig. 31).

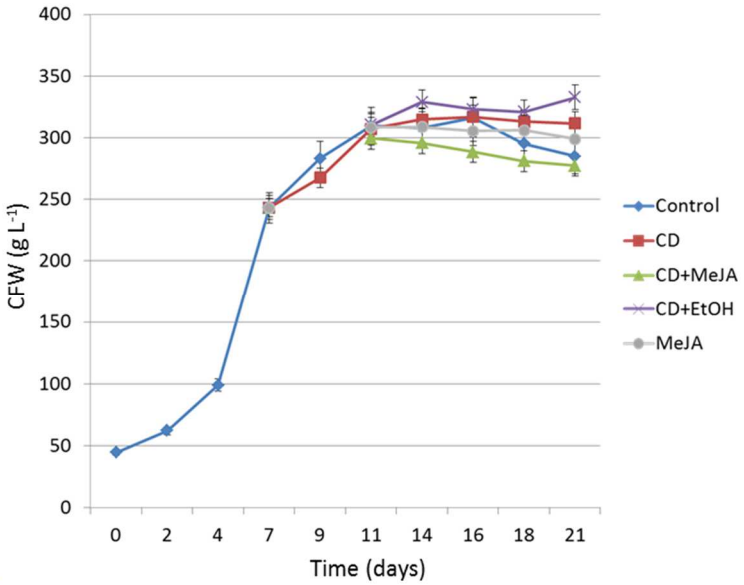

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Figure 32. Time course of the biomass production measured as CFW (g L^{-1}) in a growth period of 21 days. Each result is the average of 3 replicates ± SD.

Elicitor treatments did not significantly affect the biomass production of the culture, measured as CFW. Only at the end of the study (days 18-21) was a small increase of CFW observed in cell cultures treated with CD. On the contrary, the addition of MeJA, alone or together with CD, slowly reduced the biomass concentration, especially at the end of the culture period. During this period, significant differences were observed between the CD and MeJA treatments ($p<0.05$), but not between the other treatments.

7.1.2.2. Dry Weight

The initial DW of 2.45 g L^{-1} increased after one week to 17 g L^{-1}, which represents an approximately 7-fold increase and an average doubling time (t_d) of 2.44 d measured as DW. This means that during the first week of culture the cells doubled their biomass every 2 days (Fig. 33). According to the CDW curves, the stationary phase began at day

7, rather than at day 11 as indicated by the CFW curves. This difference could be explained by the cells absorbing water without gaining DW from days 7 to 11. The growth results

also confirm that in most cases the elicitation did not affect the cell CDW. A significant decrease of CDW was only observed in the cells treated with MeJA, which resulted in the t_d increasing to 6.4 d (Table 7). This negative effect was not observed in the cell culture treated with CD+MeJA, which reached a t_d of 5.8, a very similar value to the untreated cell cultures. These results suggest CD had a positive effect of on the CDW of MeJA-treated *S. khuzistanica* cell suspensions. As in the case of the CFW, the time course of biomass production measured as CDW only presented significant differences ($p<0.05$) when comparing CD- and MeJA-treated cultures at the end of the culture period.

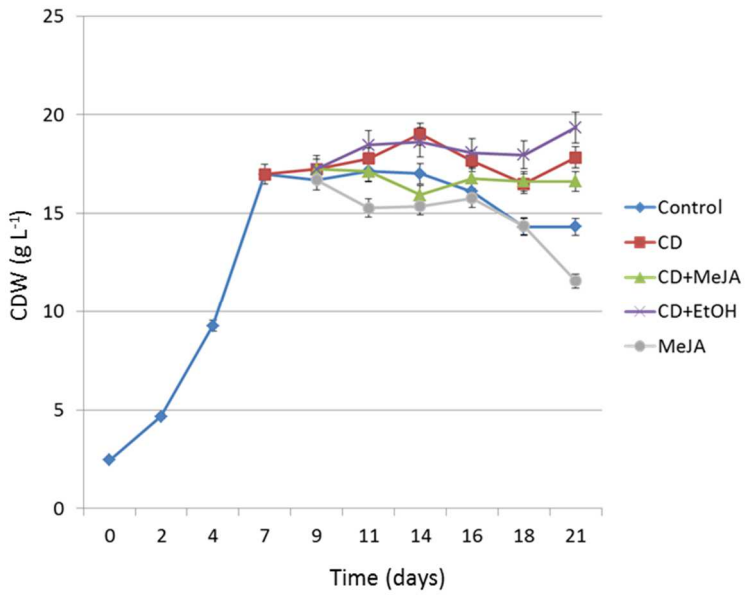

Figure 33. Time course of biomass production measured as CDW (g L^{-1}) under a growth period of 21 days. Each result is the average of 3 replicates ± SD.

7.1.2.3. Conductivity

The conductivity decreased during the first week due to the consumption of the medium salts (nutrients). From days 7 to 9, the conductivity of the CD experiments increased significantly ($p<0.05$), probably due to a direct effect of CD on cell permeability and the release of mineral ions from inside the cells (Fig. 34). After this period, conductivity decreased continuously until day 16, when it increased slightly, especially in control and

CD+MeJA treated cell cultures. This higher conductivity at the end of culture period could be due to cell death and the consequent release of ions from inside the cells. Treatment with MeJA did not significantly affect the cell conductivity of the cultures.

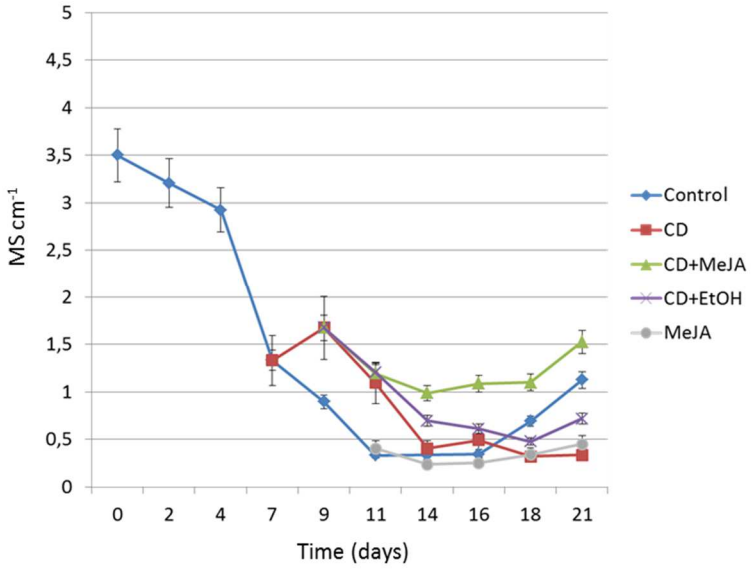

Figure 34. Time course of cell conductivity expressed as MS cm^{-1}. Each result is the average of 3 replicates ± SD.

7.1.2.4. pH

The initial pH of the suspension culture was 5.7, which dropped to 5.15 at day 2, probably due to massive ammonium uptake (Fig. 35). After day 2, the pH started to rise due to nitrate uptake. Elicitation with CD caused the pH to decrease slowly for 2 days, after which it started to increase. In contrast, in the control experiment the pH was stable from days 7 to 11. At the end of the experiment, the pH of CD cultures was lower than under control conditions, probably due to the changes in cell permeability induced by this elicitor. No significant differences among the cultures treated with MeJA and CD+MeJA in relation to the control conditions were found.

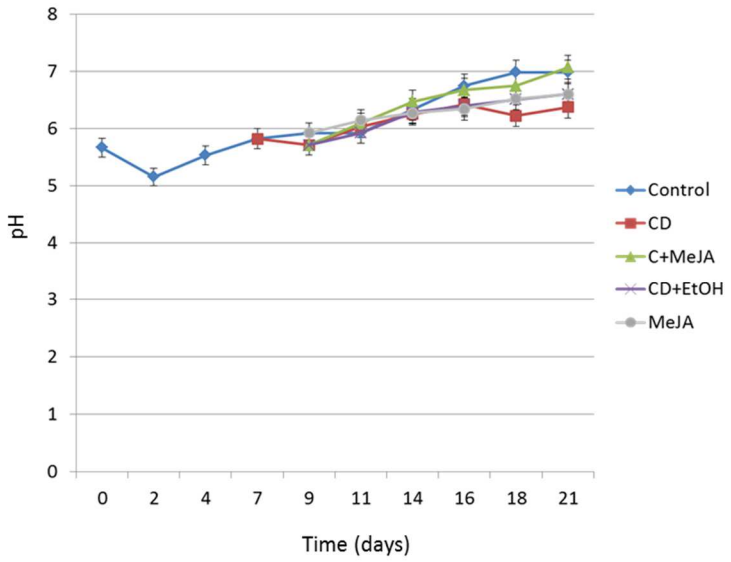

Figure 35. Time course of pH. Each result is the average of 3 replicates ± SD.

7.1.2.5. RA production

In control conditions, RA production in the cells started to increase from the beginning of the experiment, achieving an RA content of 84 mg g DW $^{-1}$ (1201 mg L^{-1}) at day 21 (Fig. 36). MeJA treatment dramatically increased ($p<0.05$) the production of intracellular RA in the cell cultures of *S. khuzistanica*, and after an elicitation period of one week (day 16), RA content peaked at a maximum of 245 mg g DW^{-1} (3858 mg L^{-1}), which represents a significant 3.2-fold increase compared to the control at the same time point. The final productivity of the MeJA-treated cell cultures was 241 mg L^{-1} d^{-1}, 3.5-fold higher than in control conditions (Table 7). The effect of adding the permeabilizing agent CD to the culture medium was more moderate, inducing an increase ($p<0.05$) of RA inside the target cells only after 11 days of treatment (day 18). In the CD-treated cell cultures, the addition of MeJA significantly enhanced the production of RA, which peaked at day 14, when its content in the cells (117 mg g DW^{-1}) was 1.6-fold higher than in the cells treated only with CD and 1.4-fold higher than in the control cultures (Fig. 36). When considering the time factor, the improvement in productivity was even higher, as the cultures elicited with CD+MeJA reached a productivity of 103 mg L^{-1} d^{-1} at day 14, compared to only 68 mg L^{-1} d^{-1} in the control after 21 days (Fig. 37). Notably, the cultures treated with both MeJA and CD reached a higher intracellular RA production than those treated only with MeJA, which reached an RA productivity of 241 mg L^{-1} d^{-1} at day 16 (Table 7).

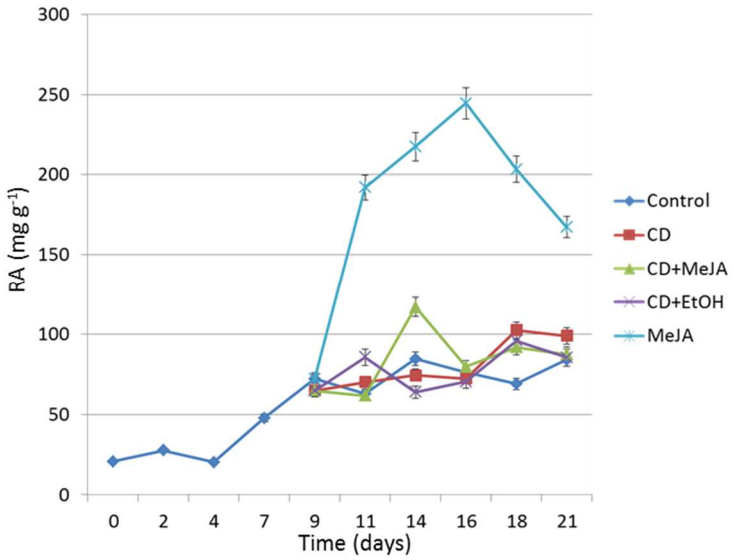

Figure 36. Time course of RA production in cell biomass measured as mg g DW^{-1}. Each result is the average of 3 replicates ± SD.

CD has been utilized not only as an intracellular elicitor in plant cell cultures but also as a permeabilizing agent (Lijavetzky et al., 2008). Fig. 37 shows the total RA production of the cultures. The results show that this phenolic compound was accumulated mainly inside the cells and only small amounts were released to the cell medium (Fig. 37). The CD treatment seemed to have slowly increased the release of RA from cells to the culture medium during the last part of the experiment, where a significant ($p<0.05$) amount of RA (279 and 0.247 mg L^{-1}) was detected only at days 18 and 21, respectively. The addition of MeJA alone did not affect the compound excretion, and only traces of RA were determined in the culture media of the cells treated with this elicitor. On the other hand, MeJA slowly increased the extracellular contents of RA in CD-treated cultures, especially at day 18 (376 g L^{-1}), probably due to its stimulatory effect on RA production.

At the end of the culture period, CD-treated cells remained a pale yellow color, while the culture medium became dark, whereas in control cultures, the cells were much darker and the culture medium remained a lighter color (Fig. 38). This effect could be due to the release of phenolic compounds from the cells to the culture medium and therefore could confirm the role of CD as a permeabilizing agent in our cultures.

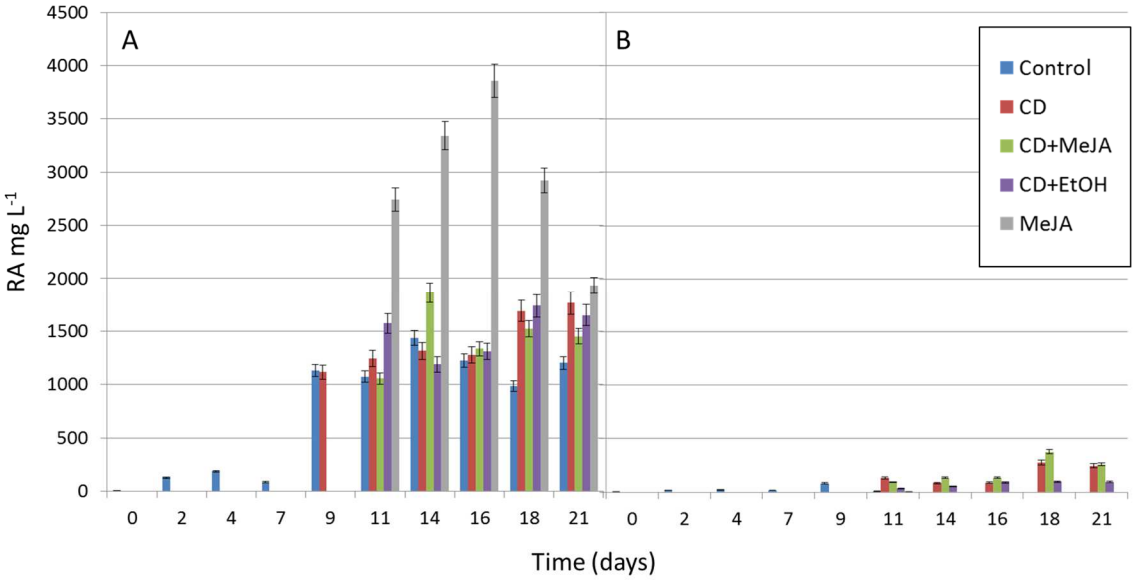

Figure 37. Time course of RA accumulation measured as mg L^{-1} in the cell biomass (A) and in the culture medium (B). Each result is the average of 3 replicates ± SD.

Table 7. Summary of the main results obtained in the elicitor experiments. Control, untreated cells, MeJa, methyl jasmonate, CD, cyclodextrins, EtOH, ethanol.

Treatment:	Control	MeJa	CD	CD+EtOH	CD+MeJa
Seed inoculum pcv (%)	10	10	10	10	10
Maximum biomass CFW (g L^{-1})	309.5	308.8	317.8	329.1	299.5
Maximum biomass CDW (L^{-1})	17.1	16.7	19	19.4	17.3
Growth rate μ_{max} (d^{-1})	0.12	0.11	0.12	0.12	0.12
Doubling time t_d (d)	5.9	6.4	5.7	5.5	5.8
Biomass productivity BMP_{CFW} (g d^{-1})	13.6	14.8	13.2	15.8	14.2
Maximum RA production (mg L^{-1}) (*day*)	1436 (*14*)	3858 (*16*)	1691 (*18*)	1738 (*18*)	1450 (**14**)
RA productivity (mg L^{-1} d^{-1})	69	241	94	97	103

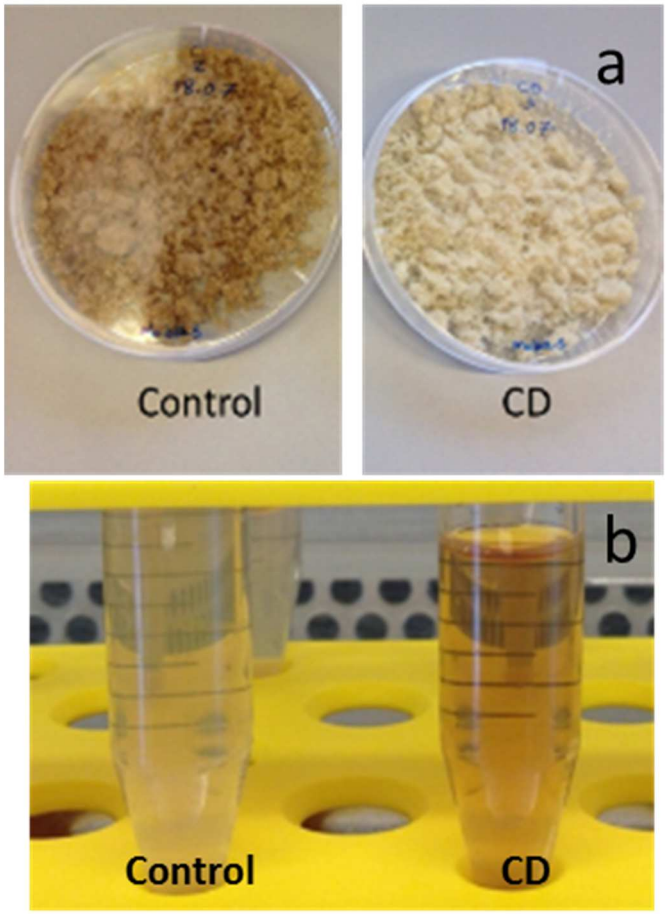

Figure 38. Comparative aspect of cell biomass (a) and culture media (b) of the control and CD-treated cultures.

7.1.3. Scale-up to benchtop bioreactor

In order to scale up the process to benchtop bioreactor-level in conditions optimized for RA production, a wave-mixed BIOSTAT CultiBag RM was utilized. As mentioned previously, the bioreactor was run in batch mode for a growth period of 21 days, with a culture volume of 1 L, inoculum density of 10%, rocking rate of 20 to 30 rpm, rocking angle of 6º, aeration of 0.1 (vvm) and a T of 25ºC. The pH was not controlled (Fig. 39). After 11 days, the cell culture was treated with 100 μM of MeJA. The obtained results were referred to biomass, and RA production was compared with levels obtained in control conditions (untreated cell cultures).

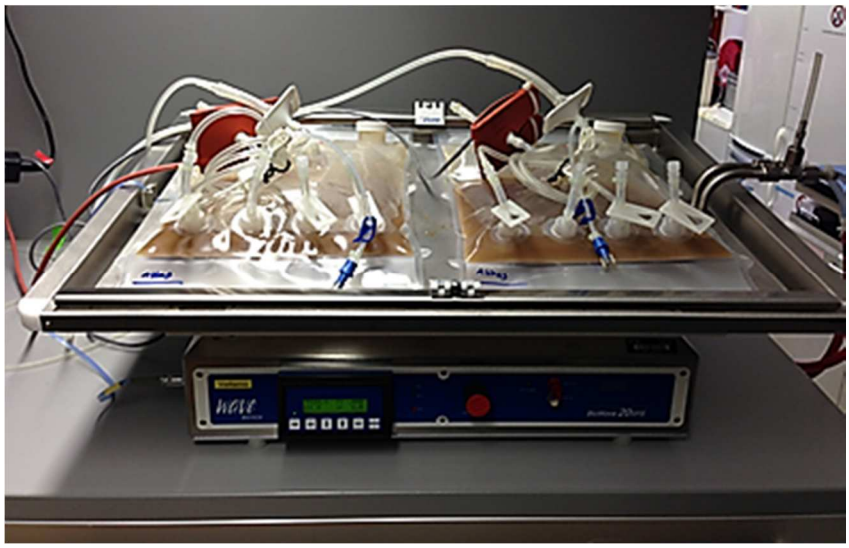

Figure 39. Aspect of the *S. khuzistanica* cell cultures in the wave-mixed BIOSTAT CultiBag RM.

7.1.3.1. Biomass production

An inoculum pcv of 10% (1-week-old) was prepared to optimize the growth capacity of the *S. khuzistanica* cell suspension in the wave-mixed bioreactor. Samples were taken every 2-3 days, and biomass accumulation was determined as pcv (ratio of the volume occupied by plant cells and aggregates to a volume of sample) and cell fresh and dry weight (CFW, CDW). Increase in CFW is due to cell growth and expansion, caused mainly by water uptake inside the cells, whereas CDW excludes errors caused by endogenous water contents and is normally considered to be a more precise measurement for biomass quantification (Eibl et al. 2009a).

The pcv time course showed that the *S. khuzistanica* cells began to grow as soon as they were transferred to bioreactor conditions, practically without a lag phase and with an exponential growth phase lasting until day 14. The cells then remained in a stationary phase until the end of the experiment at day 21 (Fig. 40). MeJA-treatment only decreased the cell growth capacity very slightly, measured as pcv at day 14, 5 days after the beginning of the elicitor treatment.

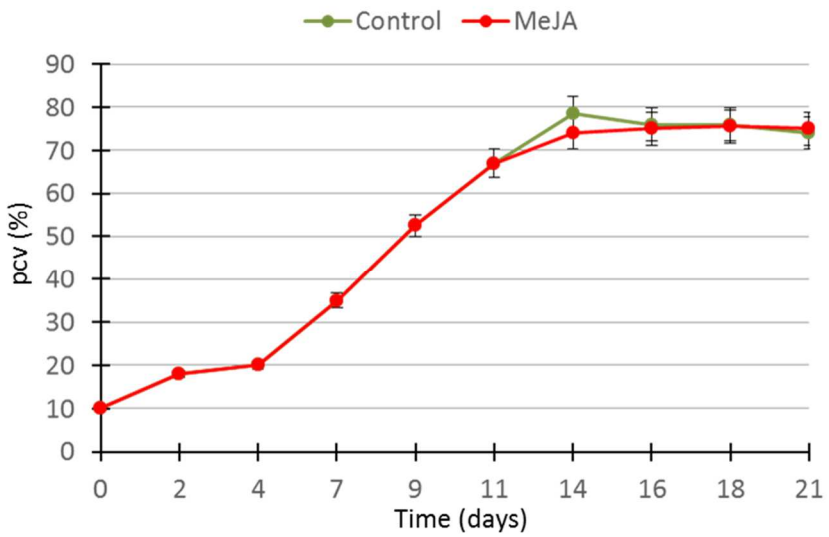

Figure 40. Time course of the pcv variations in the wave-mixed bioreactor, for a growth period of 21 days. Each result is the average of 2 replicates ± SD.

The time course of cell fresh biomass was similar to that of the pcv, although with some small differences. In this case, after a lag phase of 2 days, the CFW of the cells increased significantly, reaching a maximum at day 14, when the culture entered the stationary phase (Fig. 41). When the biomass was measured as CFW, the inhibitory effect of MeJA was more apparent than when comparing the pcv variations, and the CFW of the culture decreased from 363 g L^{-1} at day 11 to 316 g L^{-1} at day 14. This negative effect of MeJA on *S. khuzistanica* cultures was observed in the elicitor experiment in shake flasks, as reported previously. When comparing the CFW of both systems (wave-mixed bioreactor and shake flasks) in control conditions (Fig. 33), the suitability of the wave bioreactor for growing *S. khuzistanica* cell cultures was observed. Also, while the BMP_{CFW} of the cell cultures in shake flasks was 13.6 g L^{-1} day^{-1}, it increased 1.4-fold to 18.7 g L^{-1} day^{-1} in the bioreactors (Table 8). Similarly, in elicited cell cultures the BMP_{CFW} also increased from 14.2 g L^{-1} day^{-1} to 16.1 g L^{-1} day^{-1}, when comparing the growth of *S. khuzistanica* cell suspensions between shake flasks and the wave-mixed bag, respectively.

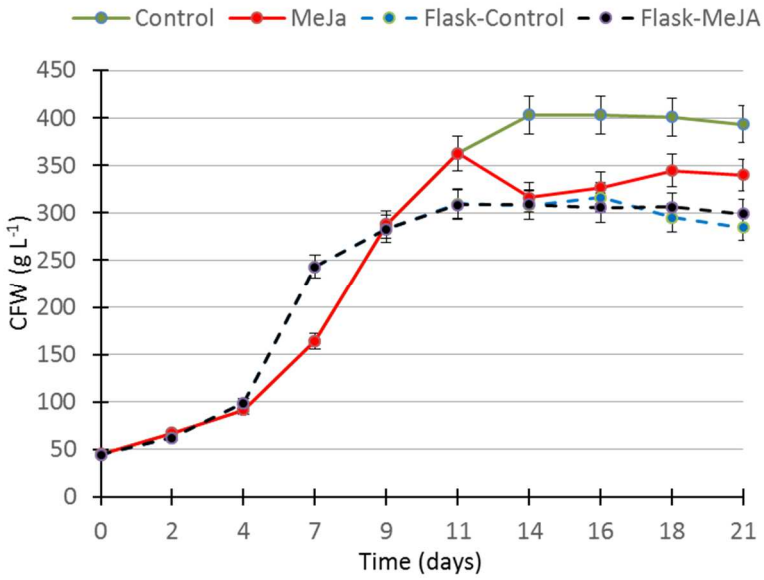

Figure 41. Time course of biomass production measured as CFW (g L^{-1}) in the bioreactor cultures, for a growth period of 21 days. Results are compared with those achieved in the shake flask cultures. Each result is the average of 2 replicates ± SD.

The CDW of the cultures showed a similar growth course, and according to these values, the stationary growth phase in the wave-mixed bioreactor began at day 14 (Fig. 42), similar to the results obtained on a fresh weight basis (Fig. 41). After day 14, the CDW decreased slightly until the end of the culture period, which means that the previously observed increase in CFW was only due to water uptake (Fig. 41). MeJA treatment provoked a dramatic decrease in the dry weight of the culture (Fig. 33), as was also reflected in the CFW time course. The growth course measured as CDW in shake flasks was very different: in this case the cultures showed a lag phase of 2 days followed by a short exponential growth phase until day 7. Thereafter, the cells remained in a stationary phase until the end of the culture period (day 21). This short exponential phase could explain why the final CDW in the bioreactor system was 1.4-times higher than in shake flasks. The maximum-specific growth rate (μ_{max}) and doubling time (t_d) using CDW were calculated by averaging the exponential growth phase, according to Werner et al. (2014). In the wave-mixed bag, the cell t_d was 4.4 d (μ_{max} of 0.16 d^{-1}), whereas in shake flasks the cell t_d was 5.9 d (μ_{max} of 0.11 d^{-1}) (Table 8), thus confirming the greater suitability of the wave-mixed BIOSTAT CultiBag RM bioreactor for the culture of *S. khuzistanica* cell suspensions (Table 8).

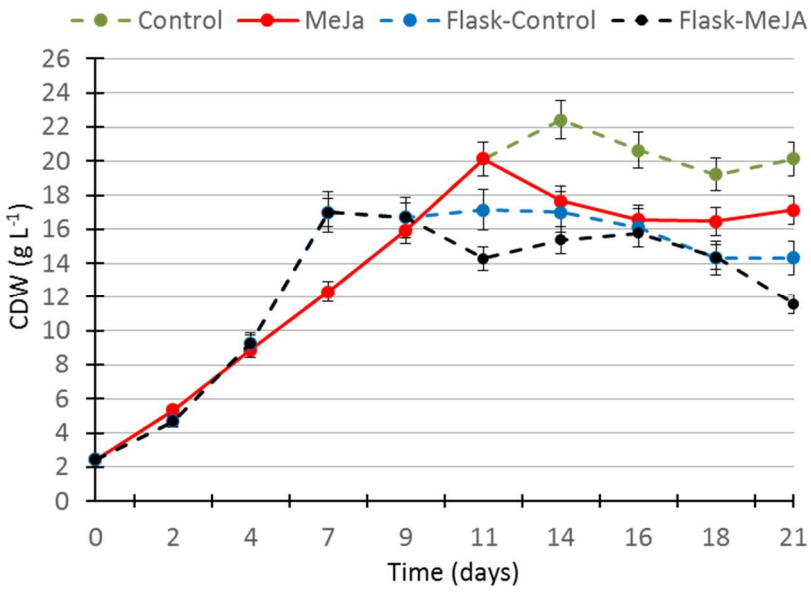

Figure 42. Time course of biomass production measured as CDW (g L^{-1}) in the bioreactor cultures, for a growth period of 21 days. Results are compared with those achieved in the shake flasks cultures. Each result is the average of 2 replicates ± SD.

7.1.3.2. pH and conductivity

The pH time course showed a considerable drop, from 5.7 to 4.7, during the first two days of culture, probably due to the initial massive consumption of ammonium present in Gamborg's B5 medium and the cell lysis that frequently occurs during the first 20-48 h after the cell subculture (Eibl et al., 2009b). After this period, a slight and steady increase in pH was observed until the end of the culture period at day 21 (Fig. 43). Fluctuations in pH in plant cell cultures have been attributed to an imbalance between anion and cation uptake (Chen et al., 2014).

The conductivity of the culture exhibited a decreasing trend during the exponential growth phase of *S. khuzistanica* cells (2–14 days). The reduction in medium conductivity was mainly due to the uptake of inorganic salts by the cells and the resulting biomass accumulation during the culture period. Our results showed an inverse relationship between biomass measured as CFW or CDW and medium conductivity during the active growth phase (days 2-14), suggesting that this parameter could be used as an indirect and non-invasive measure of the growth capacity of cell cultures, as reported previously (Chattopadhyay et al., 2002; Werner et al., 2014). The addition of the elicitor MeJA to the culture medium did not affect pH or conductivity (Fig. 43).

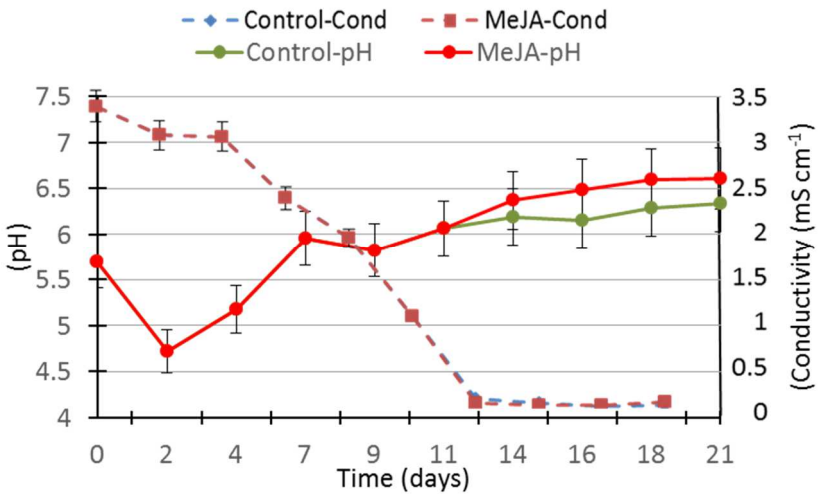

Figure 43. Time course of cell conductivity expressed as MS cm^{-1} and pH in the bioreactor cultures. Each result is the average of 2 replicates ± SD.

7.1.3.3. Rosmarinic acid production

Regarding RA production in *S. khuzistanica* cell cultures in the wave-mixed BIOSTAT CultiBag RM, our results showed that this biotechnological system could synthesize and accumulate RA mainly inside the cells and only traces of this compound were found in the culture media. The production of RA measured as g L^{-1} increased continuously over the culture period when the *S. khuzistanica* cell suspension was growing in control conditions, reaching a final production of 3102 mg L^{-1} (Fig. 44), which represents an RA productivity of 147 mg L^{-1} day^{-1} (Table 8). Normally, plant secondary compounds accumulate in low amounts in the producer cells in specific plant organs, and their accumulation is often lower in plant cell suspensions (Wilson and Roberts, 2012). In this context, the RA production of the *S. khuzistanica* cell cultures in control conditions could be considered as extremely high.

Elicitors like MeJA are frequently used to trigger the production of plant secondary metabolites in *in vitro* cultures (Wilson and Roberts, 2012). In this experiment, the addition of MeJA at day 11 to cell suspensions cultured in the wave-mixed bioreactor increased RA production 1.2-fold, which reached 3601 g L^{-1} at the end of the culture period (Fig. 44). When considering the RA production on a dry weight basis (specific production), the increased yield observed after MeJA-treatment was about 1.3-fold higher than in the control at the end of the culture period (Fig. 45), with a final production of 210 mg g DW^{-1}. The difference between the two measures (the volume of culture

medium or CDW) is because in the specific RA production, the decrease in growth provoked by MeJA is not taken into account.

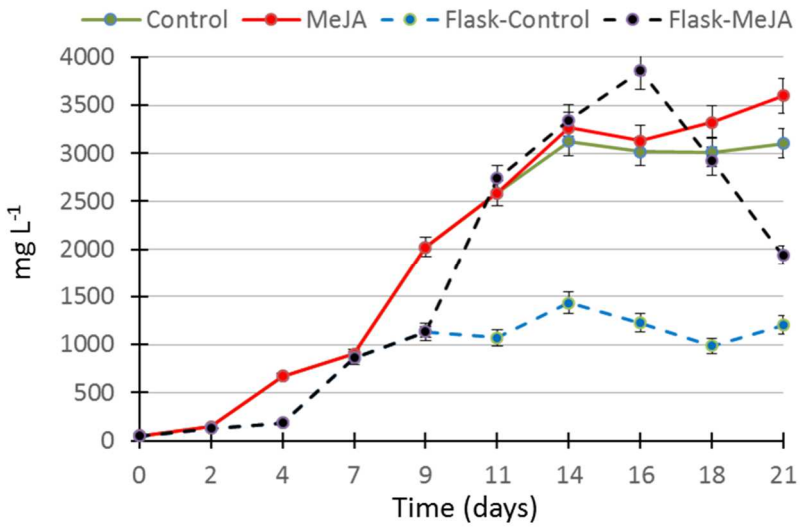

Figure 44. Time course of RA production expressed as mg L^{-1}. Each result is the average of 2 replicates ± SD.

When these results are compared with those obtained in shake flasks (Fig. 36-37), the suitability of the wave-mixed bioreactor for improving RA production in the scale-up process was clearly demonstrated. In control conditions, RA production in the bioreactor system (3102 mg L^{-1}) was 2.6-fold higher than in shake flasks on the same day (1201 mg L^{-1}), referring the production to the volume of culture medium, and about 1.8-fold higher if we consider the specific RA production (mg RA g DW^{-1}). Again, when the cultures were carried out in the bioreactor, a comparison of the two measurement systems reveals that the highly significant improvement in biomass accumulation is strongly related with the similarly improved RA production.

Also, regarding the time course of RA production in elicited *S. khuzistanica* cells (Table 8), in shake flasks the RA content peaked at day 16 (3858 mg L^{-1}), compared to day 21 (3601 mg L^{-1}) in the bioreactor. Thus, at the end of the experiment, the RA productivity was 171 mg L^{-1} day^{-1} in the wave-mixed bioreactor compared to 92 mg L^{-1} day^{-1} in the small-scale system. The high amount of RA in shake flasks at day 16, followed by a significant decrease in subsequent days, could be due to additional conditions of stress in this system absent in the bioreactor cultures, as plant secondary

metabolites are plant defense compounds and are associated with stressful situations (Mazid et al. 2011).

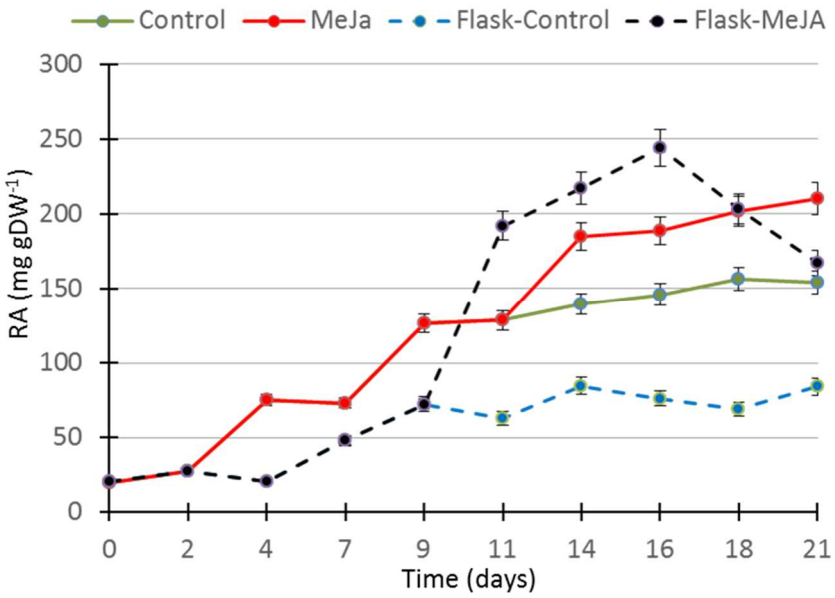

Figure 45. Time course of RA productivity expressed as mg g DW^{-1}. Each result is the average of 2 replicates ± SD.

Table 8. Summary of the main results obtained with the BIOSTAT CultiBag RM compared with those achieved in shake flasks.

Cultivation system:	**BIOSTAT**	**BIOSTAT**	**Shake flaks**	**Shake flaks**
Treatment	Control	MeJa	Control	MeJA
Seed inoculum pcv (%)	10	10	10	10
Maximum biomass CFW (g L^{-1})	403.24	344.75	316.4	314.6
Maximum biomass CDW (L^{-1})	22.42	20.14	16.7	16.7
Growth rate μ_{max} (d^{-1})	0.16	0.14	0.11	0.11
Doubling time t_d (d)	4.4	5	5.9	5.9
Biomass productivity BMP_{CFW} (g d^{-1})	18.7	16.1	13.6	14.2
Maximum RA production (mg L^{-1}) (*day*)	3102(*21*)	3601 (*21*)	1436 (*14*)	3858 (*16*)
RA productivity (mg L^{-1} d^{-1})	147	171	68	92

7.1.4. Coronatine assay

7.1.4.1. Effect of coronatine on a small scale

Elicitation with coronatine (COR) has been shown to produce a positive effect on intracellular taxane accumulation in *in vitro* cultures of *Taxus* spp. (Onrubia et al., 2013). However, the effects of COR elicitation on the accumulation of the target secondary metabolite, in this case RA, depend on the elicitor concentration and time of exposure, plant species and cell line.

To optimize the growth capacity of the suspension culture of *S. khuzistanica*, a starting PCV of 10% was prepared under the conditions previously established as optimal (see Section 7.1.1). As shown in Fig. 46, after more than a week in the lag phase (until day 11), growth measured as CFW shifted to the exponential phase, which lasted until day 16. Thereafter, a slight increase of CFW was observed and at day 18 the cultures entered the stationary growth phase, reaching a final biomass of 339.5 g L^{-1} in control conditions. The elicitor, which was added on day 14, had a significant ($p<0.05$) effect on the growth capacity of the suspension cultures, as the biomass of COR-treated cultures was significantly reduced. These results are similar to those described for MeJA (Fig. 32), confirming the negative effect of the elicitor-treatment on biomass production in *S. khuzistanica* cell cultures. However, the negative effects of COR on the growth, measured as maximum biomass CFW, were greater (nearly 10%) than the reduction provoked by MeJA (only 1%) (see Tables 8 and 10), suggesting that COR was more toxic than MeJA. These results were further confirmed when the average doubling time (t_d) values in both systems were compared, the value in the COR-treated cell line being 2-fold higher than in the MeJA-treated cell lines.

The time course of the growth measured as CDW was very similar to that of the CFW, as the lag phase also lasted until day 11 and a significant decrease of CDW was observed in the COR-treated cultures compared to the control during the exponential growth phase, the CDW being significantly lower at the end of the culture period (Fig. 47). This confirms that the elicitor significantly reduced the growth capacity of *S. khuzistanica* cell suspension cultures, when measured either as CFW or CDW. The lag phase at the beginning of the culture did not affect the growth capacity of the cell line in control conditions, because the growth rate (0.11) measured as μ_{max} (d^{-1}) was very similar in the same cell line in the previous experiment (0.12), where the lag phase only lasted 2 days (Table 10).

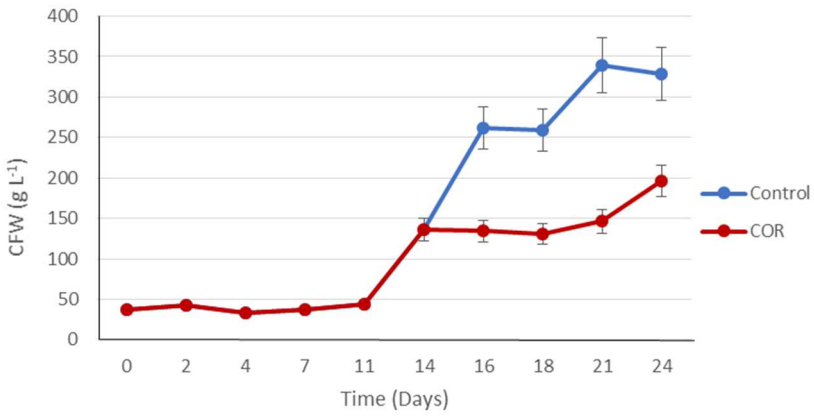

Figure 46. Time course of changes in cell fresh weight (CFW) expressed as g L^{-1}, over a growth period of 24 days. Each result is the average of 3 replicates ± SD.

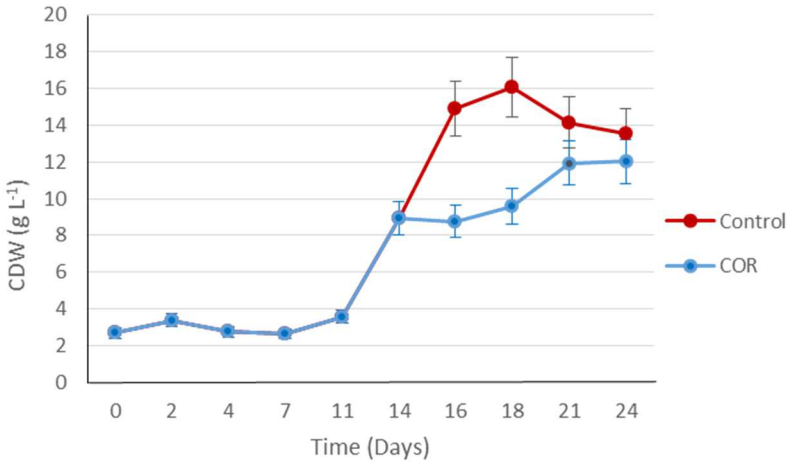

Figure 47. Time course of changes in cell dry weight (CDW). Each result is the average of 3 replicates ± SD.

Cells absorb nutrients from the medium, including the macro- and microelements that are added to the medium as ions/salts. As a result, when cells grow, the concentration of ions decreases, which in turn reduces the medium conductivity (Mustafa et al., 2011). Throughout the period of our experiment, the conductivity decreased continuously. Under the control conditions, it dropped significantly at day 16 (exponential growth phase), whereas in the COR-treated suspension cultures it continued to decrease only slightly after this day (Fig 48). The difference is probably due to the high consumption of medium salts in control cultures and the lower growth capacities of the treated cultures.

In other words, the increase in CFW and CDW in control cultures was inversely correlated with nitrate consumption and the decrease in conductivity.

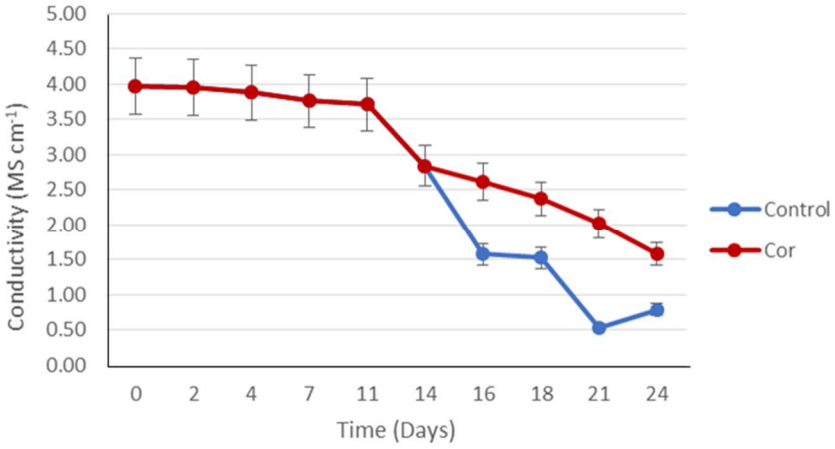

Figure 48. Time course of changes in conductivity. Each result is the average of 3 replicates ± SD.

The cell suspension cultures were initiated with a pH of 5.8, which dropped to 4.9 at day 7, probably due to the high uptake of ammonium. Subsequently, when the exponential growth phase began, the pH started to increase again, probably due to the nitrate uptake. The effect of the elicitor on the pH can be clearly observed, as it increased after the day of elicitation (day 14). The pH then started to decrease continuously until day 21, thereafter increasing again as the cells entered the death phase (Fig. 49).

The specific production of RA in suspension cultures of *S. khuzistanica* was calculated every two days throughout the experiment and expressed as mg g CDW^{-1}. In control conditions (untreated cells), the production of RA increased throughout the culture period, reaching a final content of 164 mg g CDW^{-1}. The addition of COR significantly enhanced the accumulation of RA in the culture (Fig. 50). In the treated cells, the RA production reached a maximum of nearly 221 mg g CDW^{-1} between days 4 and 10 after the elicitation. At day 18 (96 h after elicitation), the RA content was about 1.5-fold higher than in the control cells. These results show the positive effect of COR on RA production.

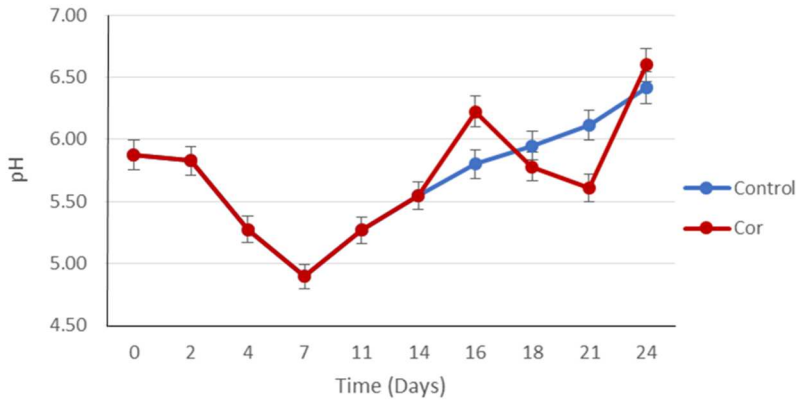

Figure 49. Time course of changes in pH levels. Each result is the average of 3 replicates ± SD.

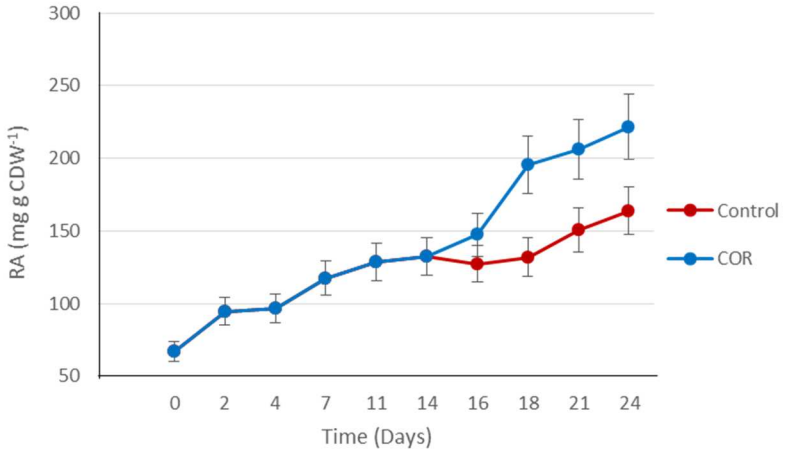

Figure 50. Time courses of RA production expressed as mg g CDW^{-1}, in *S. khuzistanica* elicited suspension cultures with pre-optimized COR (1 µM), up to 10 days after inoculation. Each result is the average of 3 replicates ± SD.

The RA production of the biotechnological system was also expressed as mg RA L^{-1} of culture volume (Fig. 51), which also takes into account the capacity of the system for producing biomass, unlike when expressed as mg g CDW^{-1}. As COR significantly reduced the CDW, RA productivity also decreased significantly after the elicitation treatment (Fig. 37). However, at day 21 (168 h after elicitation), the RA production levels of COR-treated cells started to overtake those of the control cells, reaching more than 2600 mg L^{-1} after 240 h, which was significantly ($p>0.05$) higher than the control.

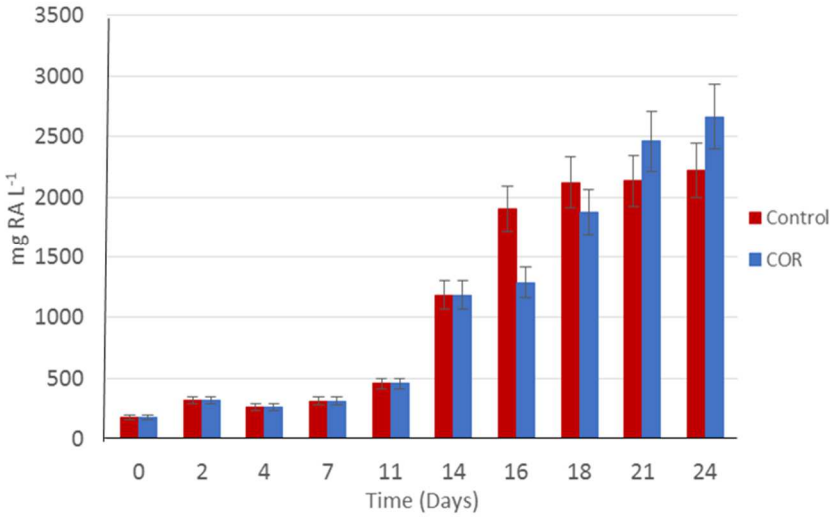

Figure 51. Time course of the RA production expressed as mg L^{-1}, in *S. khuzistanica* elicited suspension cultures (under the same conditions as specified in Fig. 50). Each result is the average of 3 replicates ± SD.

7.1.4.2. Effect of coronatine on enzymatic activity

RA biosynthesis in plants has been suggested to involve both the phenylpropanoid pathway and a tyrosine-derived pathway, as shown in Fig. 4. Eight enzymes involved in RA biosynthesis have been identified and characterized, and among them PAL, TAT and RAS were described as playing an important role in the regulation of RA biosynthesis in suspension cultures of *S. scutellarioides* and *C. blumei* (Szabo *et al.,* 1999*;* Petersen and Metzger, 1993). PAL is a key enzyme at the beginning of the phenylpropanoid pathway (Chevolot *et al*., 1997); TAT is the first enzyme in the tyrosine-derived pathway (Petersen and Metzger, 1993), and RAS catalyzes the adduct formation between 4-OH-phenyl-lactic acid and 4-OH-cinnamyl-CoA in RA biosynthesis (Berger *et al.,* 2006; Yan et al., 2006). In this study, we examined the effects of COR elicitation on the activities of PAL, TAT, and RAS using *S. khuzistanica* suspension cultures. These activities were measured and compared with the amount of intracellular RA accumulation after the COR treatment.

Our results showed that PAL activity was generally affected by COR elicitation. Although it increased sharply 48 h after the elicitor treatment, after 96 h the activity dropped and continued to decrease in the remaining period of culture (Fig 52A).

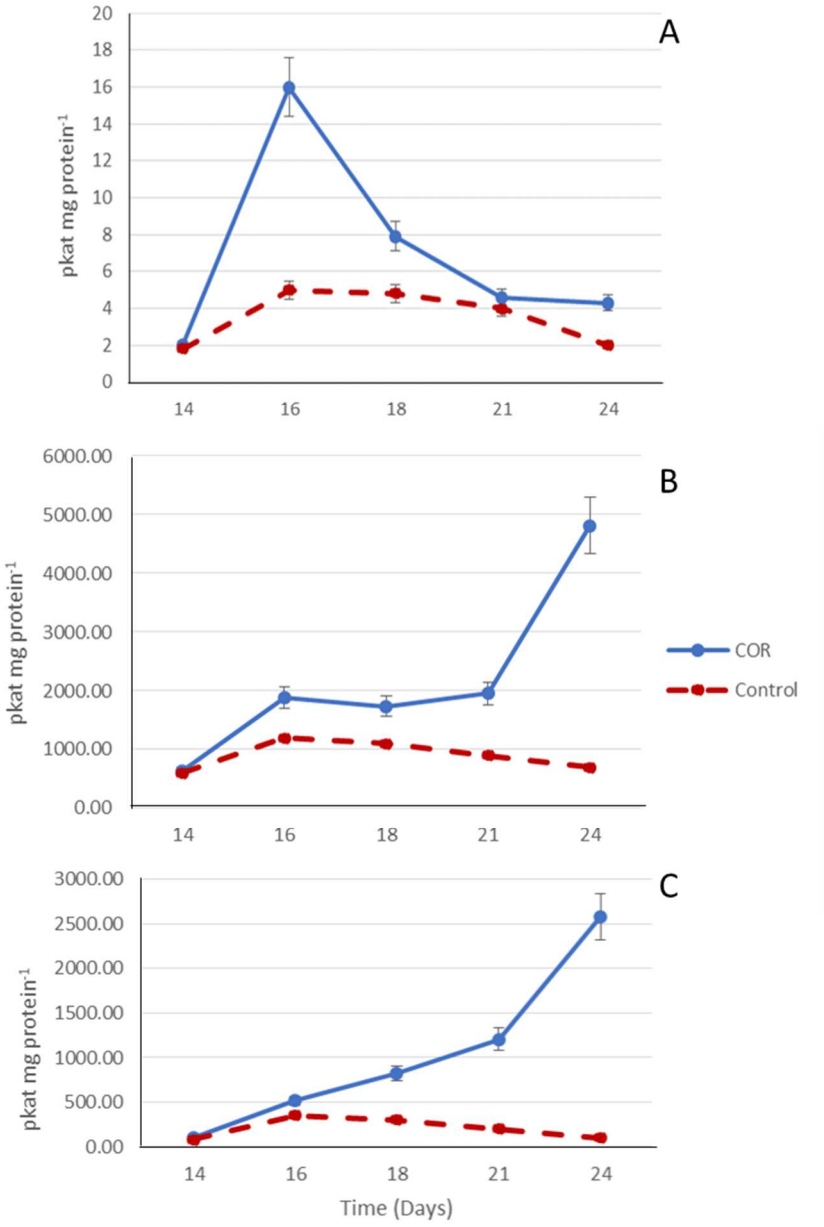

Figure 52. Time course of PAL (A), TAT (B) and RAS (C) activities after elicitation (under the same conditions as specified in Fig. 50). Each result is the average of 3 replicates ± SD.

As previously mentioned, TAT is the first enzyme in the tyrosine-derived pathway for the biosynthesis of RA (Ellis and Towers, 1970; Yan et al., 2006). The results obtained in this work show that TAT activity was enhanced significantly by COR elicitation, reaching up to 4813 pkat mg protein^{-1}, 240 h after the elicitation. In control conditions, TAT activity increased slightly in the first part of the culture, and then decreased slightly after day 18 (Fig. 52B). The RAS activity in COR-treated *S. khuzistanica* suspension

cultures rose to much higher levels within 48 h of elicitation and continued to increase significantly until the last day of the experiment (240 h after elicitation), reaching a maximum of 2577.7 pkat mg protein^{-1} (Fig. 52C). Taken as a whole, these results show that COR had a strong effect on TAT and RAS activity, especially at the end of the culture period, whereas its impact on PAL activity was far less and occurred only during the first days of treatment (Fig. 52).

7.1.4.3. Scaling up the process to a benchtop bioreactor

As mentioned, the COR assay was scaled up to a 2 L CellBag with a working volume of 1 L, shaken at 38 rpm in the dark at 25ºC. After a culture period of 11 days, the system was elicited with 1 µM COR. Samples were taken in triplicate at days 0, 4, 7, 9, 11, 14, 16, 18 and 21. The experiment was run in duplicate.

The time course of the biomass production measured as CFW shows a typical growth curve with a lag phase of 2-4 days, followed by an exponential growth phase until day 14, when the culture entered the stationary phase. Thereafter, the CFW did not increase for the remainder of the culture period until day 21 (Fig. 53). A similar curve was obtained when the growth was measured as CDW, although in this case the lag phase of the culture was not apparent (Fig. 53). In control conditions (untreated cells), when compared with the experiment in shake flasks, the lag phase in the bioreactor was clearly shorter, but this did not drive the culture to achieve a better growth rate μ_{max} (d^{-1}). In contrast, under elicited conditions, the negative effect of COR on growth capacity was lower in the bioreactor cultures than in the shake flasks (Table 9), reaching a BMP_{CFW} of 12.5 g d^{-1}, which was 1.7-fold higher than in the small-scale system (7.0 g d^{-1}). Unlike the previous experiment using MeJA as the elicitor, in this case the scaling up improved the biomass productivity only under elicited conditions.

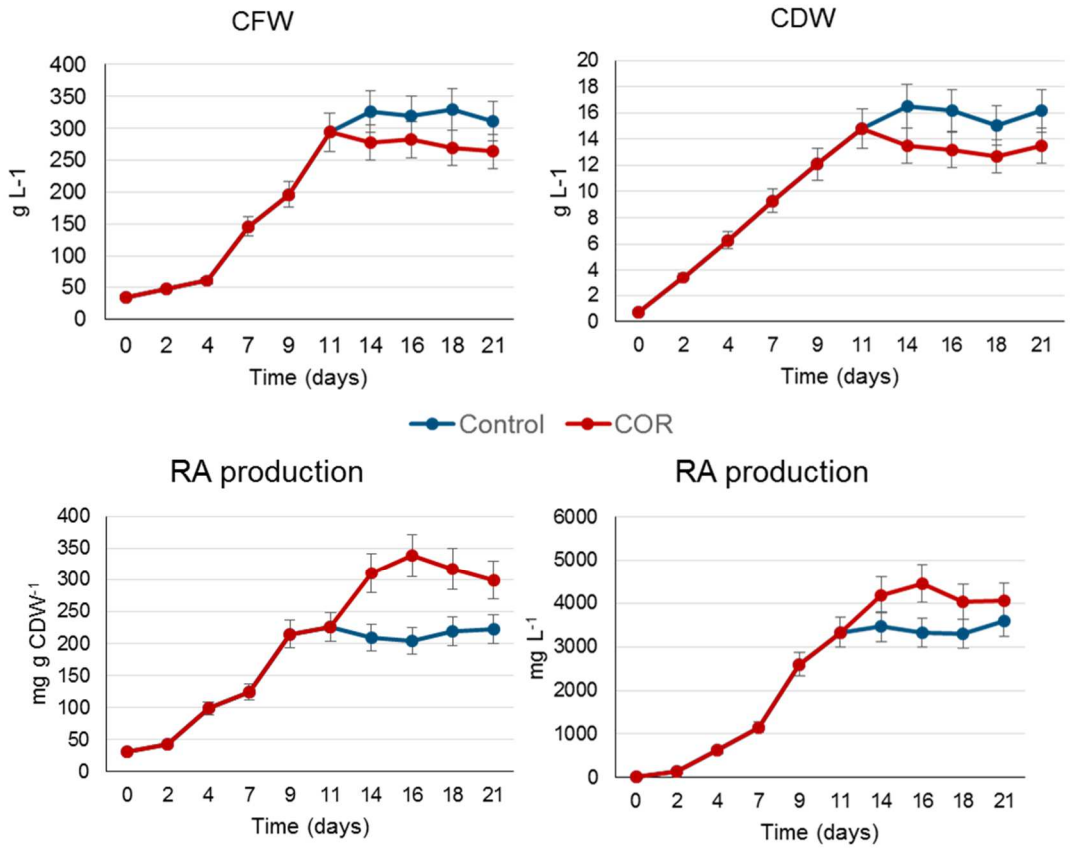

Figure 53. Biomass and RA production in *S. khuzistanica* control and COR-elicited cultures in a benchtop bioreactor during a growth period of 21 days. The data are the average of 3 replicates ± SD.

Similar to the experiment in shake flasks, COR significantly increased ($p>0.05$) the RA production capacity of the bioreactor system, achieving a specific production of 338.2 mg g CDW^{-1} at day 16, which was 1.7-fold higher than in control conditions (untreated cells). When measured as mg RA L^{-1} of culture medium, the production pattern was similar and from day 14 the yield was higher in the COR-treated cultures. It is noteworthy that the cultures in the bioreactor grew better than in shake flasks and the negative effects of COR on biomass production were much less apparent. This fact, together with a high specific RA production (g CDW^{-1}) in bioreactor conditions, resulted in yields that were 2.3-fold higher at day 16 than in the small-scale system. Such a high production also drove the scaled-up system to achieve the highest productivity (279 mg RA L^{-1} d^{-1}), 13% higher than in control conditions and 44% higher than in the MeJA-elicited cultures.

Table 9. Summary of the main results obtained with the shaken CellBag compared with those achieved in shake flasks.

Cultivation system:	**Shaken CellBag**		**Shake flasks**	
Treatment	Control	COR	Control	COR
Seed inoculum pcv (%)	10	10	10	10
Maximun biomass CFW (g L^{-1})	329.73	293.72	339.55	196.17
Maximum biomass CDW (L^{-1})	16.16	13.53	16.07	12.02
Growth rate μ_{max} (d^{-1})	0.11	0.10	0.11	0.06
Doubling time t_d (d)	6.3	6.9	6.3	11.6
Biomass productivity BMP_{CFW} (g d^{-1})	14.8	12.5	16.1	7.0
Maximum RA production (mg L^{-1}) (*day*)	3460(*14*)	4460 (*16*)	2221 (*24*)	2665 (*24*)
RA productivity (mg L^{-1} d^{-1})	247	279	92	111

Overall, the results confirm that *S. khuzistanica* cell cultures are suitable for the biotechnological production of RA and that the scale-up of the cultures to a wave-mixed bag or an orbitally shaken bag improves productivity even more. The main results of this experiment, summarized in Table 9, clearly show that the highest productivity as well as the highest specific RA production (day 16) were achieved in the COR-elicited bioreactor culture, without significantly affecting the growth capacity of the system, measured as CFW or doubling time. In contrast, in shake flasks COR induced a significant decrease in growth, reducing the doubling time by nearly half, which provoked a lower final production compared with bioreactor conditions.

7.1.5. Comparative study of the antiproliferative activity of rosmarinic acid and methanolic *S. khuzistanica* extracts.

As previously mentioned, the potential activity of RA against cancer cells prompted us to check its effect on the viability of MCF-7 and HepG2 cancer cell lines. We also assayed the effects of the *S. khuzistanica* extracts (SKE) on the caspase mechanism, a target for chemotherapeutics, as its activation enhances apoptosis.

7.1.5.1. Reduction of MCF-7 cell viability by S. khuzistanica extracts

The effect of SKE on cell viability was studied in MCF-7 and HepG2 cells, either non-treated or incubated for 48 h with different amounts of SKE, RA (positive control known to reduce cell viability) or vehicle (DMSO). As shown in Figure 54, the use of up to 0.4 % (v/v) of DMSO as a vehicle did not significantly affect MCF-7 and HepG2 cell viability. Therefore, DMSO was selected as the solvent for subsequent experiments at concentrations of up to 0.4 %. The highest concentration of SKE assayed (0.6 mg mL^{-1}) significantly reduced the viability of HepG2 cells up to about 25 % of the values observed in control cells, and decreased MCF-7 cell viability to barely detectable levels. A similar cytotoxic effect on MCF-7 and HepG2 cells was observed with the highest concentration of RA used (0.4 mM). Remarkably, even though RA content in 0.6 mg/ml SKE was estimated to be 0.064 mM, the biotechnologically produced extract had a stronger effect on MCF-7 and HepG2 cell viability than 0.2 mM RA.

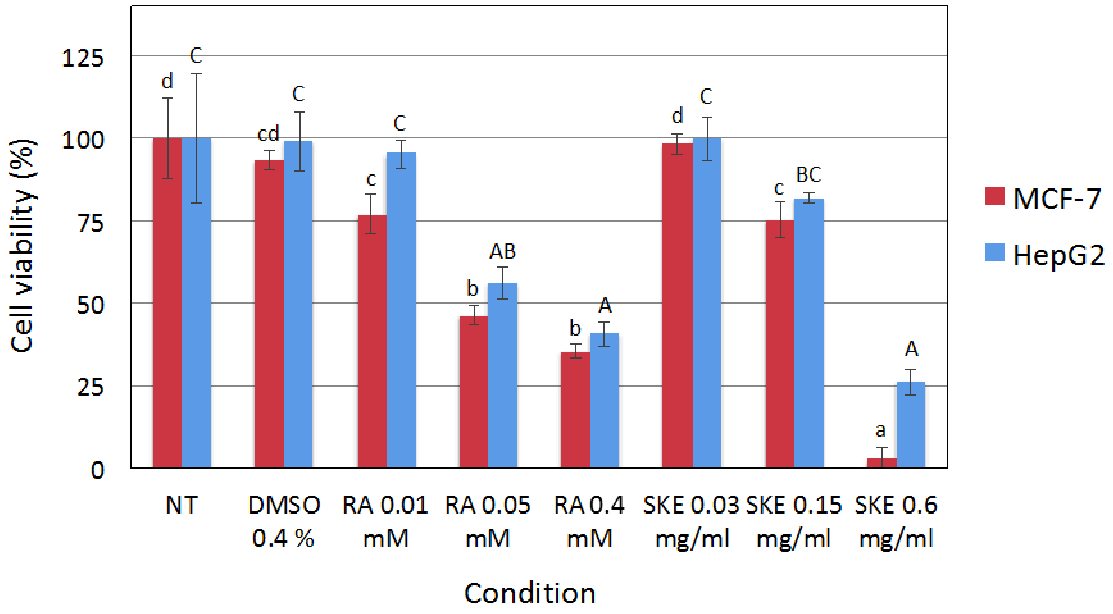

Figure 54. Effect of *S. khuzistanica* extracts (SKE) on viability of MCF-7 and HepG2 cells. Cell viability was assayed 48 h following treatment with 0.03 mg mL^{-1}, 0.15 mg mL^{-1} and 0.6 mg mL^{-1} SKE, 0.01 mM, 0.05 mM and 0.2 mM rosmarinic acid (RA) or 0.4 % DMSO (vehicle). Control cells were non-treated (NT). Each bar represents the mean ± SD of three replicates. Different letters (lower case for MCF-7 cells and upper case for HepG2 cells) indicate significant differences between treatments ($p<0.01$).

7.1.5.2. Induction of MCF-7 cell cycle arrest by S. khuzistanica extracts

Given that SKE had a stronger impact on the viability of MCF-7 cells than HepG2 cells and based on reports that RA induces cell cycle arrest in human-derived cell lines such as MCF-7 (Wu et al., 2015), the effect of SKE on the cell cycle was studied in the latter, using flow cytometry. To this end, MCF-7 cells were incubated for up to 48 h with SKE, RA or DMSO (vehicle). As previously reported, RA significantly increased the sub-G0/G1 cell population (apoptotic cells) at 48 h post-treatment. Flow cytometry analysis revealed that 0.6 mg mL^{-1} of SKE also significantly increased the percentage of sub-G0/G1 cells both at 24 h (Fig. 55A) and 48 h (Fig. 55B) post-treatment, and a tendency to reduce the cell fractions in the G0/G1 and G2/M phases was found. As in the cell viability, 0.6 mg ml^{-1} SKE had a stronger effect on the MCF-7 cell cycle than the maximum RA concentration used (0.4 mM).

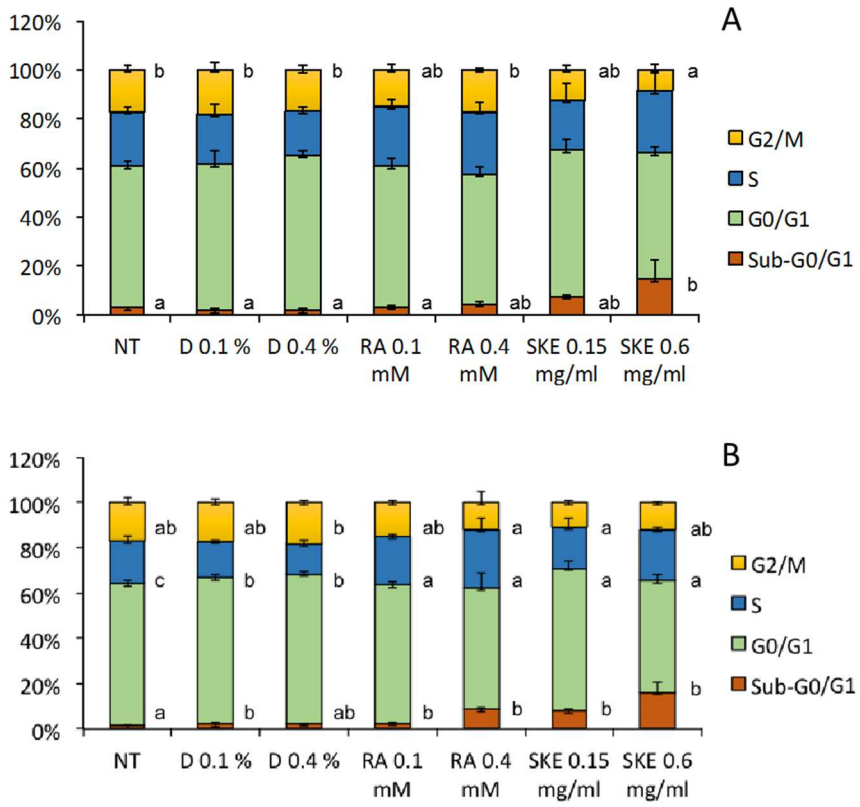

Figure 55. Effect of *S. khuzistanica* extracts (SKE) on the percentage of MCF-7 cells in each phase of the cell cycle. Cell cycle distribution of MCF-7 cells treated for 24 h (A) and 48 h (B) with 0.15 mg ml^{-1} and 0.6 mg mL^{-1} SKE, 0.1 mM and 0.4 mM rosmarinic acid (RA) or 0.1 % and 0.4 % DMSO (vehicle; D). Control cells were non-treated (NT). Each bar represents the mean ± SD of three replicates. For each phase of the cell cycle, different letters indicate significant differences between treatments ($p<0.05$).

7.1.5.3. Effect of S. khuzistanica extracts on caspase activity of MCF-7 cells

The fact that induction of the sub-G0/G1 cell population is associated with cell apoptosis and that RA is known to induce apoptosis in human cancer-derived cell lines (Jang et al., 2018; Huang et al., 2018) prompted us to analyze the impact of SKE on the activity of inflammatory caspase-1 and -5, initiator caspase-2, -8 and -9, as well as executioner caspase-6 (Fig. 56). No effect on the activity of inflammatory caspases was observed at 48 h after treatment. However, compared to non-treated cells, SKE significantly enhanced caspase-8 initiator activity, which is known to mediate activation of the extrinsic apoptosis pathway (MacIlwain et al., 2013). Although not significant, the same trend was observed in MCF-7 cells incubated with RA. No significant effects were found on the activity of caspase-2 and -9. Regarding executioner caspases, SKE, and to a lesser extent RA, showed a tendency to increase the activity of caspase-6. Previous studies have reported that involvement of caspases in apoptosis induction by RA is cell type-specific (Wu et al., 2015). Overall, our findings suggest that SKE and RA may induce apoptosis of MCF-7 cells by activating the extrinsic pathway. Conceivably, ligand binding to death receptors in the cell membrane would trigger caspase-8 dimerization and activation, which in turn may lead to direct cleavage and activation of executioner caspases. However, *S. khuzistanica* extracts and RA failed to significantly modify the activity of caspase-9, which is the initiator caspase responsible for the intrinsic apoptosis pathway. Given that 0.6 mg/ml SKE (containing 0.064 mM RA) was able to produce a stronger action on MCF-7 cells than 0.4 mM RA, our findings indicate that other compounds present in the cell plant extract may act synergistically with RA to promote anticancer effects.

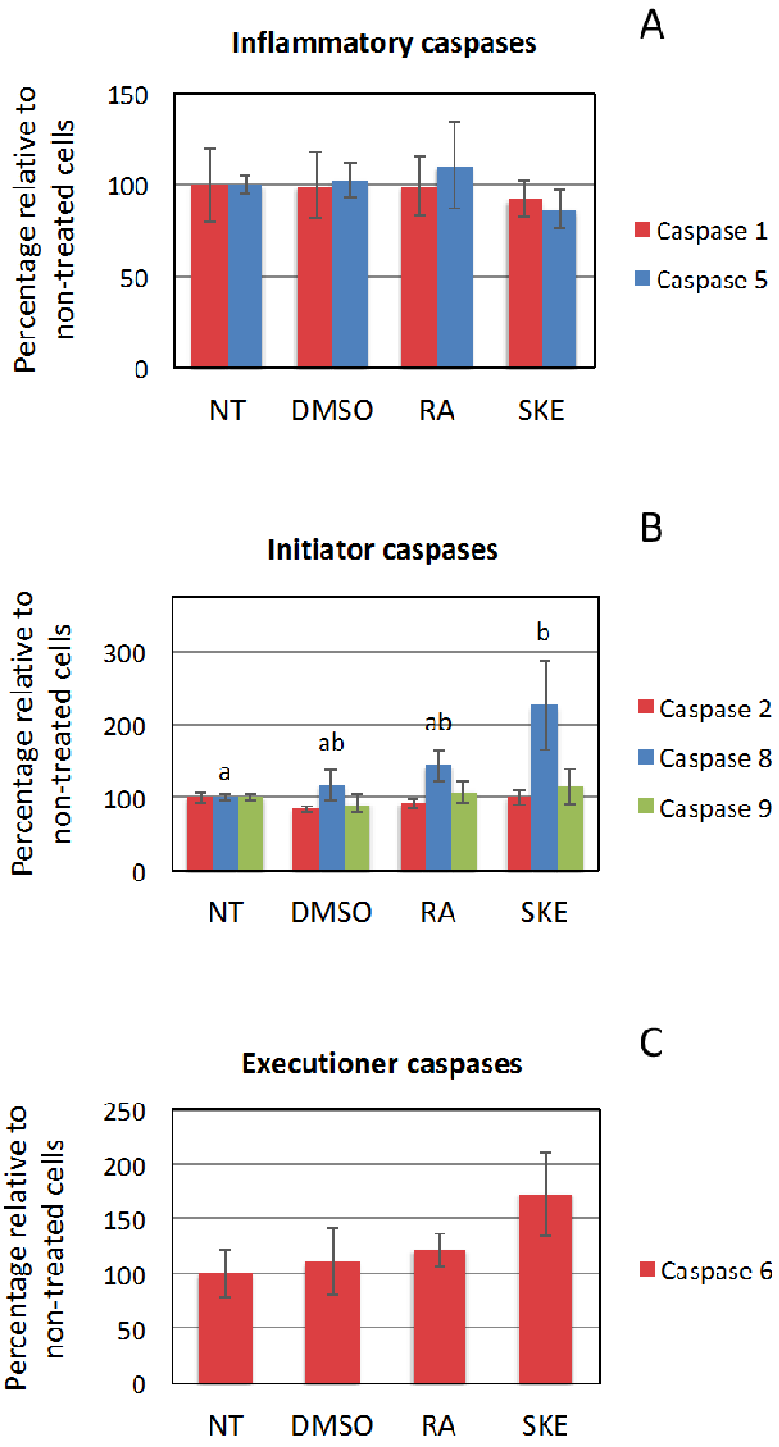

Figure 56. Assay of caspase activity in MCF-7 cells treated with *S. khuzistanica* extracts (SKE). The activity of inflammatory (A), initiator (B) and executioner (C) caspases was assayed in MCF-7 cells treated for 48 h with 0.6 mg mL^{-1} SKE, 0.4 mM rosmarinic acid (RA) or 0.4 % DMSO (vehicle). Control cells were non-treated (NT). Each bar represents the mean ± SD of three replicates. Different letters indicate significant differences between treatments ($p<0.05$).

7.2. Ruscogenin production

7.2.1. Optimization of callus induction

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 we have described (Palazon et al., 2006), callus induction from rhizomes, phylloclades and embryos of this species is very difficult, 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, as described in Material and Methods.

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. 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 25ºC in the dark. For each treatment, more than 50 explants were inoculated in the different media tested. The results are shown in Table 10.

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 tested, 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 the organogenesis of 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.

Table 10. 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}) + GA_3 (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.
* Medium tested previously by Palazon et al. (2006).

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 to obtain *R. aculeatus* calli. The media assayed for callus development are shown in Table 11.

Table 11. Callus induction from embryos after 8 weeks of culture.

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 medium is in gray.			

Of all the media tested, the MS medium supplemented with **Pic 4 mg L^{-1} and 4-PU-30 3 mg L^{-1}** was optimum for callus induction from embryos, as in the rhizome experiments, but in this case organogenesis from the calli was not completely inhibited. The medium supplemented with **2,4-D 0.5 mg L^{-1} and BA 0.05 mg L^{-1}** resulted in calli with less organogenesis, so was selected for further experiments. Comparing the results of callus induction from rhizomes and embryos showed that embryos were a better source of explants, generally producing calli in less time than rhizomes and with less development of roots and shoots.

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

As already mentioned, the biggest problem for the growth of *Ruscus* calli in all the previously assayed media was their 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. For this reason, the calli obtained from rhizomes or germinated embryos were cultured testing several media with the aim of completely blocking the organogenesis and developing enough friable callus biomass. The assayed media and the characteristics of the established calli are shown in Table 12.

Table 12. Growth capacity, friability and organogenesis of the *R. aculeatus* calli when cultured in different media for a period of 4-6 weeks.

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}) + AIA (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}) + GA_3 (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.					

Among the culture media assayed, those that resulted in better growth and friable calli were based on the MS mineral nutrition with the addition of picloram (at a concentration of 2-4 mg L^{-1}) and Kin (at a concentration of 0.1-0.5 mg L^{-1}). Also, the addition of GA_3 (0.5 mg L^{-1}) resulted in 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}) + GA_3 (0.5 mg L^{-1})**. However, after several subcultures in this medium we detected a darkening of the calli and a loss of their growth capacity, so for further assays we used the medium **B5 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.

7.2.3. Callus disintegration and establishment of cell suspensions

Small pieces of callus (≈ 2 g FW) were transferred to 175-mL flasks (Sigma VO633) with 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 assay the viability and growth capacity of the cells in the different culture media assayed.

Table 13. Assayed media and growth capacity of the *R. aculeatus* cell suspensions.

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.			

From the results shown in Table 13, 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. 58). As shown in the figure, pectinase attacked the middle lamella of the cell walls and destroyed most of the clusters, resulting in a finer cell suspension after filtration.

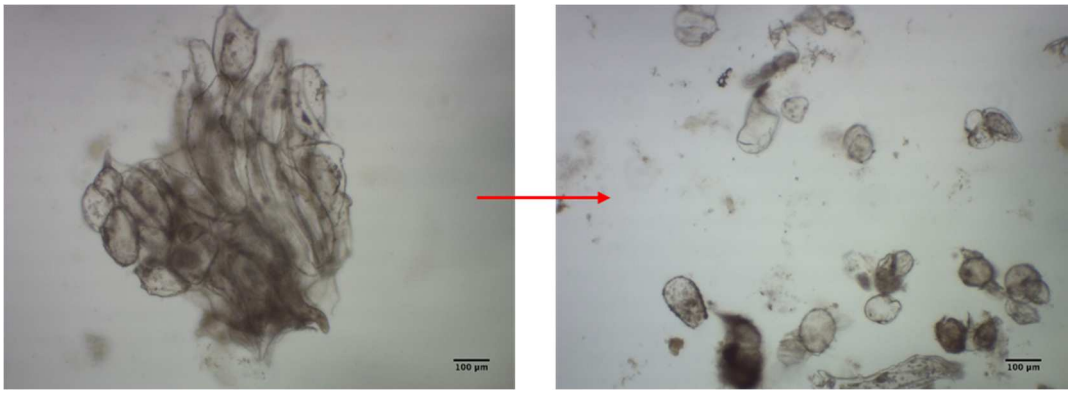

Figure 58. *R. aculeatus* cell disintegration effect of the pectinase treatment.

7.2.4. 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* plantlet cultures, callus cultures derived from embryos, plant cell suspensions and root-rhizome cultures. The latter two grew in both dark and light conditions, whereas plantlets were cultured only in light, and calli and cell suspensions only in darkness. All the biotechnological systems were treated after 2 weeks of culture with the elicitor COR 1 µM, 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. In Fig. 59, which shows the different plant biotechnological systems, a higher cell sedimentation can be seen in the cluster cultures (Fig. 59C) than in the fine cell suspensions; greening of the root-rhizome cultures when exposed to the light can also be observed (Fig. 59E).

Figure 60 shows the biomass production of the different systems expressed as a growth index (GI) [(DW harvested-DW inoculum)/DW inoculum]. In control conditions (without elicitation), *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 the root-rhizomes grown in darkness also reached a GI higher than 1.5. Biomass formation was reduced in root-rhizomes on transfer to light conditions 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. 3). In all cases, the elicitor treatment with COR 1 µM 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 had a good capacity to generate biomass, despite the recalcitrance of *R. aculeatus* to *in vitro* adaptation and the initial difficulties in obtaining the callus and cell suspension cultures.

Figure 59. External appearance of the different *R. aculeatus* culture systems after a 4-week growth period.

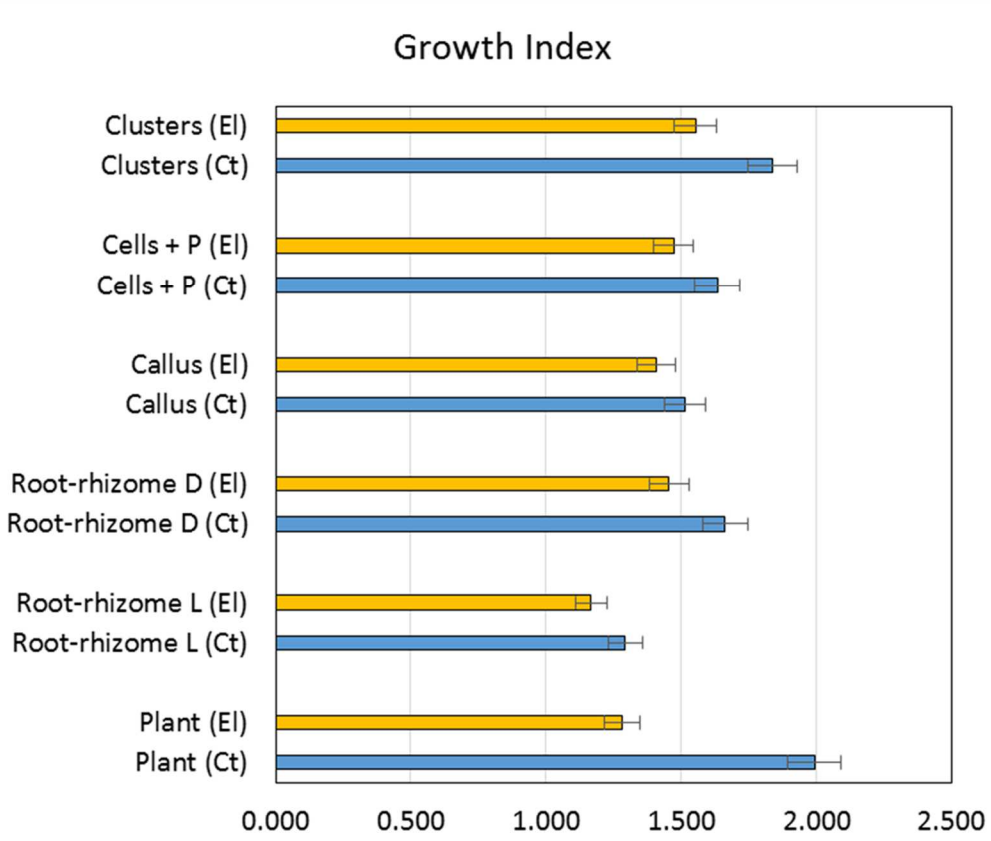

Figure 60. Biomass production of the biotechnological systems expressed as a growth index after a 4-week growth period. El: elicitor treatment, Ct: control conditions, L: light, D: darkness. Each value is the average of 5 biological replicates ± SE.

All the *in vitro* cultures developed the capacity to biosynthesize ruscogenin and neoruscogenin (Fig. 61). 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% of which was ruscogenin. The production was also high in the root-rhizomes, which reached a total saponin content of 1.014±0.087 mg g DW^{-1} when cultured in darkness, whereas in light conditions 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 callus cultures, where the saponin production was on average 0.369±0.063 mg g DW^{-1}. The results (see Fig. 61) also show that the disintegration of the calli and establishment of the cell suspension enhanced the capacity of the culture to produce ruscogenins, the cell suspension achieving a production of 0.851±0.098, which was 2.3-fold higher than that of the calli.

Treatment of the different *in vitro* cultures with COR 1 µM 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. 61). In general, the elicitor treatment was more effective in the undifferentiated cultures (calli and cells) than in the well-structured cultures (plantlets and root-rhizomes). In summary, the results demonstrate the effectiveness of the elicitor for increasing ruscogenin production. The % of ruscogenin in the total saponin contents (rucogenin + neoruscogenin) was quite variable, ranging from 44 % in the elicited root-rhizome cultures 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.

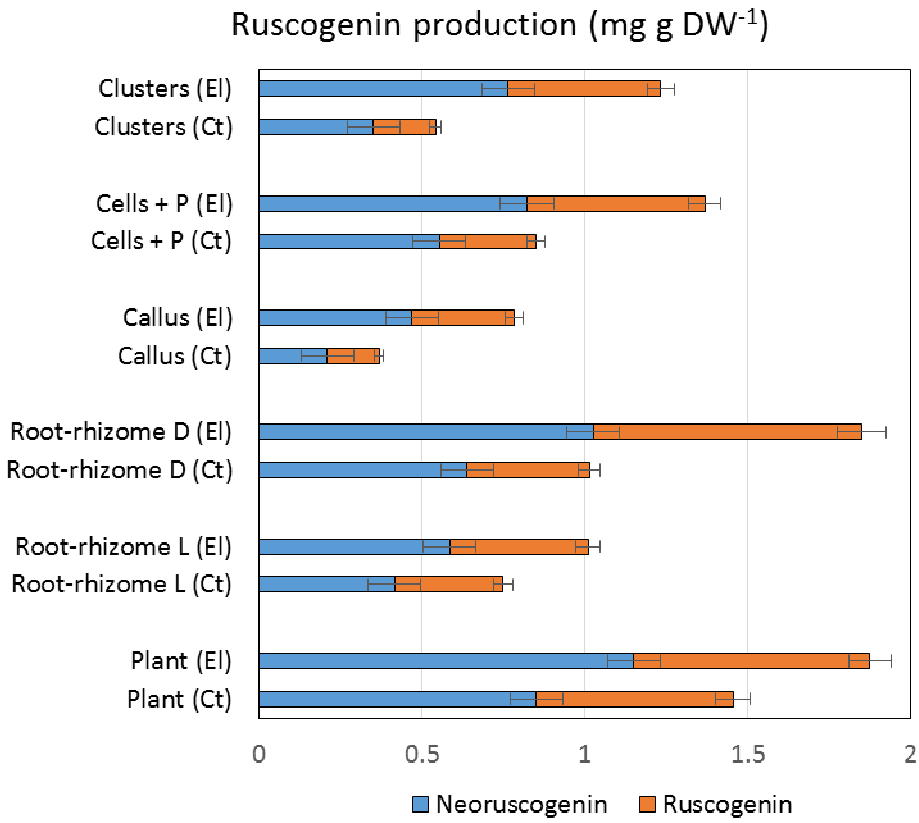

Figure 61. Ruscogenin production expressed as mg g DW^{-1}, after 4 weeks of culture. El: elicitor treatment (COR 1 µM), Ct: control conditions (untreated cultures), L: light, D: darkness. Each value is the average of 5 biological replicates ± SE.

7.2.5. Benchtop bioreactor scale.

As the most productive ruscogenin-producing system, root-rhizome cultures were selected for scaling up to a 2 L CellBag with a working volume of 500 mL, shaken in a Khuner orbital shaker in the dark at 25ºC and 35 rpm. After 2 weeks the culture was elicited with 1 µM COR and two weeks later the bioreactor was harvested to determine the growth capacity of the culture, measured as GI, and the content of the steroidal saponins ruscogenin and neoruscogenin. The results (Fig. 62) were compared with those obtained on a small scale in the same system (shake flasks). After a growth period of 4 weeks, the GI of the root-rhizome cultures in the orbitally shaken bag was nearly 1.5, a result very similar to that achieved on a small scale, and no significant differences were observed ($p \geq 0.05$) regarding the biomass production of both systems (Fig. 62A).

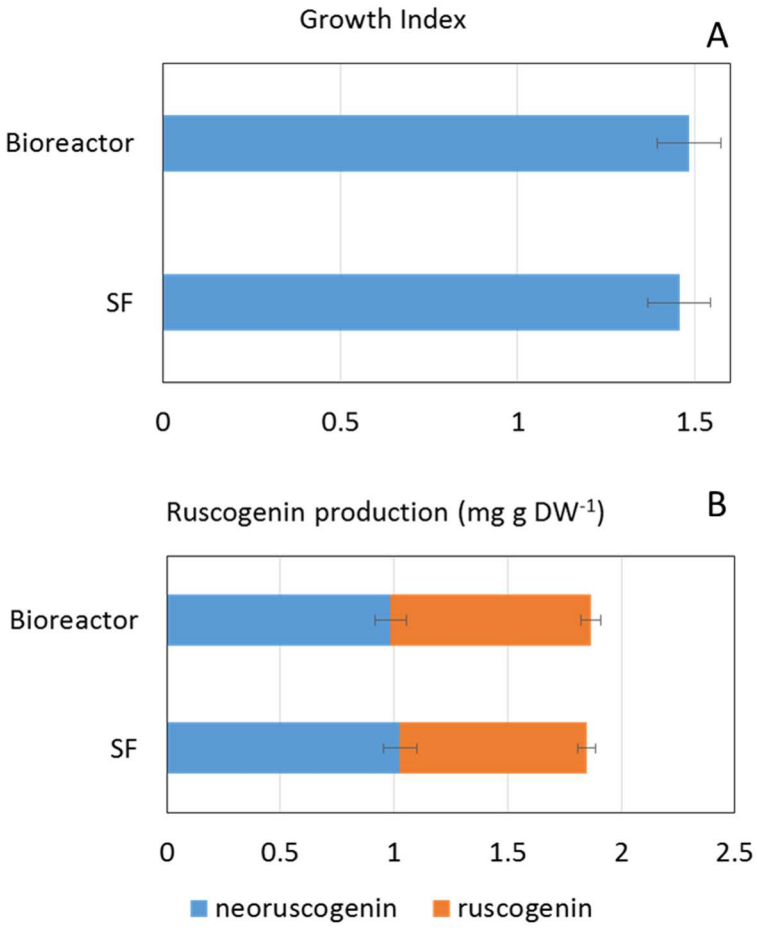

Figure 62. Comparative study of biomass production measured as a growth index and ruscogenin production of the root-rhizome system elicited with 1 µM COR in the bioreactor and shake flasks (SF) after a 4-week culture period. Each value is the average of 2 biological replicates ± SE.

The ruscogenin production (Fig. 62B) in the shake flasks and the orbitally shaken bag was also very similar, as was the ruscogenin/neoruscogenin ratio. Thus, we can confirm that the scale-up of the root-rhizome cultures to a benchtop bioreactor does not decrease the capacity of the system for the production of biomass and ruscogenins.

6. Discussion

Inoculum size has a strong influence on the growth curve in cell suspension cultures. In general, the smaller the inoculum, the longer the lag phase, and below a minimum amount the cell culture will not grow. On the contrary, a larger inoculum leads to an earlier stationary phase (Schripsema et al., 1990). Subculturing at the end of the exponential growth phase will achieve the greatest biomass production per time, and performing the subculture when the cells are in the stationary phase usually results in a longer lag phase due to lower cell viability. Consequently, the subculture regime of the cell suspensions, as well as the inoculum density and quality, are the key parameters to consider when developing the biotechnological process. The choice of parameters will depend on the ultimate goal or priority, for example, optimal cell viability, easy maintenance, usage in further experiments to optimize biomass or secondary metabolite production, etc. (Stafford et al., 1985).

In this work, in order to optimize biomass and RA production in *S. khuzistanica* cell lines, two inoculum densities were assayed: 10 and 20% of pcv. When the inoculum density was 20%, the growth curve of the cell suspension showed a long lag phase of approximately 1 week, followed by a short exponential growth phase until day 14, and practically no stationary phase, the biomass of the cell culture decreasing until day 21 (see Fig. 28). In contrast, with an inoculum density of 10%, the lag phase was considerably reduced, whereas the exponential growth phase was delayed from day 7 to 11, followed by a longer stationary phase from day 11 to the end of the culture period (day 21). In the exponential growth phase, most carbon/energy is used for cell division, and primary metabolism is very active in the cell culture. In contrast, in the stationary phase the energy in the cells is channeled to other metabolic pathways, for example, for cell enlargement/elongation, and often for the production of plant secondary metabolites (Mustafa et al., 2011). Consequently, a long stationary phase is desirable when a biotechnological process is designed for secondary metabolite production.

The productivity of a biotechnological process depends not only on the capacity of the plant cells to produce and accumulate the target compounds, but also on the capacity of the system to produce cell biomass (Murthy et al., 2014). According with our results, not only the density but also the quality of the inoculum are factors to consider in

the context of productivity. Thus, when we utilized a young inoculum (1-week old) formed by cells in the exponential growth phase, we achieved 2-fold more biomass than with an older inoculum (2-weeks old) (see Figs. 31 and 32). It should be pointed out that the importance of the inoculum age as a productivity factor is scarcely mentioned in the literature.

In optimized inoculum conditions (10% pcv, 1-week-old) the *S. khuzistanica* cell line showed a typical growth curve with a short lag phase of 1-2 days and an exponential phase until day 11, when the cells entered the stationary phase until the end of the culture period on day 21 (Fig. 32). During this period, biomass increased more than 5-fold, demonstrating the high capacity of the system to generate cell biomass and the effective optimization of the biomass production; the results were an improvement on those previously obtained with this cell line in non-optimized conditions (Sahraroo et al., 2014).

In the first set of experiments (elicitation with MeJA and CDs), elicitor treatments generally did not significantly affect the cell fresh and dry weight (CFW and CDW) of the *S. khuzistanica* cell cultures. Only treatment with 100 μM of MeJA significantly reduced the CDW of the culture at the end of the experiment. MeJA has been previously utilized as an elicitor in other RA-producing plant species (Mizukami et al., 1993; Szabo et al., 1999; Ogata et al., 2004; Tsuruga et al., 2006; Georgiev et al., 2007) and in most cases the growth capacity of the cell lines decreased. More recently, Krzyzanowska et al. (2012) demonstrated that the MeJA-induced growth inhibition in cell cultures of *Mentha x piperita* depends on the elicitor concentration and the duration of the treatment, reaching a maximum at the concentration of 200 μM. The effects of MeJA have not been tested previously in *S. khuzistanica* cell cultures, but our results are in agreement with studies of other RA-producing cell cultures that report a low inhibition of growth by this elicitor.

On the other hand, the negative effects of MeJA on growth measured as CDW (see Fig. 33) were reversed by the addition of CD, and the cells treated with MeJA and CD achieved a similar CDW at the end of the culture to the control (untreated cells). Although CD has not been tested in other RA-producing cell lines, Sabater-Jara et al. (2014) found a positive effect of CD on the growth capacity of *Taxus media* cell cultures.

The elicitor treatment increased RA production in the *S. khuzistanica* cell biomass, especially in the last stage of the cell culture. The effect of MeJA was significant after a week of treatment, when the cells accumulated up to 3.2-fold more RA than the control cells, achieving an RA concentration of approximately 25% of CDW and 3.86 g L^{-1} (see

Figs. 36 and 37), which is the highest production reported so far in plant *in vitro* cultures. Positive effects of MeJA elicitation on RA accumulation in cell cultures have been reported previously (see review by Bulgakov et al., 2012). Working with *Mentha x piperita* cell cultures, Krzyzanowska et al. (2012) reported an increase of RA production of up to 1.5-fold after MeJA treatment, the concentration of 100 μM being more effective than 200 μM. Recently, Kim et al. (2013) reported in MeJA-elicited cell cultures of *Agastache rugosa* a correlation between RA production and the expression level of the genes *ArPAL*, *Ar4CL* and *ArC4H,* which encode the enzymes phenylalanine ammonia lyase, cinnamate 4-hydroxylase and 4-coumarate:coenzyme-A (CoA) ligase, respectively, all of them involved in the RA biosynthetic pathway. This action of MeJA on the expression of the genes responsible for the biosynthesis of RA could explain the enhanced production of this plant secondary metabolite in our *S. khuzistanica* cultures, although this hypothesis requires further study. Similarly, Fatemi et al. (2019) recently demonstrated an increased expression of the RA biosynthetic genes *HPPR*, *RAS*, *PAL* and *TAT* in nodal segment cultures of *S. khuzistanica* when treated with MeJA.

CD are permeabilizing agents that can also act as efficient elicitors, as demonstrated in cell cultures of *Vitis vinifera* (Morales et al., 1998). In *S. khuzistanica* cell suspensions, the treatment with CD provoked only a moderate accumulation of RA inside the cells in the last stage of the culture (days 18-21) (see Fig. 37). This effect could be due to a real elicitor action of this compound when added to the culture media, as described previously for the biotechnological production of another phenolic compound, resveratrol (Lijavetzky et al., 2008). Alternatively, it could be the result of a reduction in the feed-back inhibition that frequently provokes the accumulation of plant secondary compounds inside the cells, as CD also increased the release of RA outside the cells to a certain degree (see Fig. 37). This type of effect has also been reported in *T. media* cell cultures treated with the same concentration of CD (Sabater-Jara et al., 2014). More effective was the combined CD + MeJA treatment, under which the cell cultures not only accumulated significantly higher amounts of RA than the control cells (untreated cultures) (see Fig. 36 and Table 7), but also reached the maximum production at day 14, one week before the end of the culture period. In any case, the RA production was higher than when the cells were treated only with MeJA.

Although the *S. khuzistanica* cell cultures were very productive the isolation of RA requires its extraction from the dry material, as it accumulates intracellularly. Microspectrophotometric evaluation as well as protoplast and vacuole isolation have clearly demonstrated that RA is stored in the vacuole (Chaprin and Ellis, 1984; Häusler et al., 1993). In order to facilitate extraction, Park and Martinez (1992, 1994)

permeabilized RA-producing *Coleus blumei* cell suspensions with 0.1 % of DMSO, resulting in a release of 65% of total RA production to the culture medium. Unfortunately, however, this system proved unsuitable for all *C. blumei* cultures, because some cell lines very actively excrete peroxidases to the culture medium, where they rapidly metabolize the RA released (Spencer, 1994).

Regarding the permeabilizing effect of CD-treatment, our results showed only small amounts of RA in the medium at the end of the culture period, although these were significantly higher in the case of the combined CD+MeJA treatment. The slow effect of CD in the *S. khuzistanica* cell suspensions contrasts with the results previously reported in cell cultures of *Vitis vinifera* or *Taxus* sp., where CDs acted simultaneously as strong elicitors and permeabilizing agents (Sabater-Jara et al., 2014). Nevertheless, after the CD treatment, our cells remained pale yellow, while the culture medium became dark, in contrast with the control cultures, where the cells were much darker, while the culture medium remained a lighter color (Fig. 38). This effect could be due to the release of phenolic compounds from the cells to the culture medium, which would confirm the role of CDs as permeabilizing agents and suggests that RA was degraded outside the cells. As mentioned above and also according to Szabo et al. (1999), RA is rapidly metabolized outside the cells by the high peroxidase activity of the apoplast, which could explain why we detected such small amounts, even if it was released in large quantities into the culture medium. Thus, CD probably had a stronger elicition effect than what was observable, which calls for the development of new approaches to protect RA from peroxidase-degradation in the culture medium.

Cell suspensions of several plant species have been used for the biotechnological production of RA but an industrial-scale production has not been achieved so far (Bulgakov et al., 2012). This is probably due to the inherent characteristics of the cell cultures, which impede their scale up to bioreactor level. In general, the growth of plant cell suspensions is characterized by an increased cell aggregation. This tendency, together with a high cell concentration and the specific morphology of the plant cells, results in greater broth viscosity, leading to a non-Newtonian behavior of the culture (Chattopadhyay et al., 2002 and references therein). The viscosity of the culture medium is also increased by the release of polysaccharides and other metabolites, making it difficult to carry out the plant cell cultures in bioreactors.

Production of RA in bioreactors has been reported previously (Ulrich et al., 1985; Su et al., 1993, 1995; Zhong et al., 2001). Ulrich et al. (1985) developed a two-stage cultivation process for the production of RA by a *Coleus blumei* cell suspension culture.

In the first stage, *C. blumei* cells were cultured in a 32 L-airlift bioreactor for 8 days and then 30-50% of the cell suspension was transferred to a 32 L-stirred bioreactor with 5% of sucrose. In these conditions, after 14 days the system achieved a CDW of 25.7 g L^{-1} with a RA production of 21.4% DW. Su et al. (1993) used a perfusion cultivation technique for RA production in a suspension culture of *Anchusa officinalis*, and also studied the influence of dissolved oxygen, finding that 30% of air saturation is optimum for RA production (Su et al., 1995). More recently, Pavlov et al. (2005a), working with a 3 L-stirred bioreactor, demonstrated that for *Lavandula vera* cell suspensions a dO_2 of 30%, 300 rpm and 28ºC are optimum settings for RA production. Zhong et al. (2001) investigated the kinetics of Ti-transformed *Salvia miltiorrhiza* cell cultures in both flasks and stirred bioreactors with different impeller size, showing that a large turbine impeller reactor was suitable for cell growth and RA production. Until now, wave-mixed bioreactors have not been used for RA-producing cell suspensions.

BIOSTAT® CultiBag RM has been successfully used for the culture of cell suspensions of several plant species including *Vitis vinifera*, *Malus domestica*, *Nicotiana tabacum* and *Hordeum vulgare* (Eibl et al., 2009b and references therein). Computational fluid dynamics (CFD) simulations showed a more homogeneous energy dissipation and shear stress pattern in this bioreactor than in stirred reusable bioreactors (Löffelholz et al., 2014). For these reasons, a BIOSTAT CultiBag RM was selected for scaling up the RA production in *S. khuzistanica* cell suspensions from shake flasks to a benchtop bioreactor.

In this work, the settings of the process were a rocking angle of 6º, rocker rate of 20 to 30 rpm, aeration rate of 0.1 vvm and 25ºC. The experiments lasted 21 days and were carried out in batch mode. In these conditions, the biomass productivity (BMP_{CFW}) of the system was 18.7 g d^{-1} with a RA productivity of 0.15 g L^{-1} d^{-1}, which were 1.7- and 3-fold higher, respectively, than that achieved in shake flasks (Table 8). In previous experiments, Bentebibel et al. (2005) demonstrated the suitability of the wave-mixed bioreactor for the production of the anticancer compound taxol in *Taxus baccata* cell cultures; after a culture period of 16 days the biomass productivity (BMP_{CFW}) was about 8.3 g d^{-1} and the taxol productivity 0.46 g L^{-1} d^{-1}. More recently, Lehmann *et al.* (2014), working with the BIOSTAT® CultiBag RM and a transgenic fast-growing tobacco cell line carrying the geraniol synthase gene of *Valeriana officinales* (VoGES), reported a BMP_{CFW} of 10 g L^{-1} d^{-1} and a production of up to 26 μg g^{-1} FW of geraniol, confirming the suitability of the wave-mixed bioreactors for the biotechnological production of plant secondary metabolites.

Under optimal elicitation conditions (100 μM MeJA), RA production in *S. khuzistanica* cell suspensions increased significantly when cultured in the BIOSTAT® CultiBag RM, reaching a value of 3601 mg L^{-1} and a CDW biomass of 20.14 g L^{-1}. It has previously been demonstrated that cell suspensions of *Lavandula vera*, another RA-producing plant species, when cultured in a stirred bioreactor in optimal conditions (50% air saturation, 400 rpm and 29.9ºC), achieved a maximum RA production of 3.5 g L^{-1} (Pavlov et al., 2005b), but the production decreased to 1.8 g L^{-1} when the agitation rate was 300 rpm and the DO 30% (Pavlov et al., 2005a), showing the importance of the culture conditions in reaching a high RA production. Taken as a whole, the results of this experiment clearly show the suitability of the biotechnological platform based on *S. khuzistanica* cell suspensions for the production of RA when cultured in the BIOSTAT® Cultivag RM bioreactor, and suggest that after a detailed study of the culture parameters (T, rocking angle, rocker rate, working volume, etc.) the biotechnological production of this bioactive natural compound could be increased even more.

In a second set of experiments, we tested the new elicitor COR (a jasmonate mimic) using a rational approach, aiming not only to reach a higher production of RA, but also to obtain insights into the regulation of the RA biosynthetic pathway. Thus, in the same *S. khuzistanica* cell line, we investigated the activity of three key enzymes in RA biosynthesis: phenylalanine ammonia lyase (PAL), tyrosine aminotransferase (TAT) and rosmarinic acid synthase (RAS), under control (unelicited) and COR-elicited conditions. The capacity of the system for producing biomass and RA was studied, and the process was scaled up to a benchtop bioreactor utilizing an orbitally shaken CellBag. This type of power input and bioreactor are suitable for the culture of mammalian and plant cells with a low oxygen demand (Klöckner et al., 2014).

In this approach, the cell line CFW followed a different time course compared to the MeJA experiment. In control conditions, the growth curve showed a longer lag phase (Fig. 46), but the doubling time (t_d) in both the MeJA and COR experiments was finally very similar (Tables 7 and 9). Differences in the growth capacity of a cell line between different subcultures could be due to small variations in the culture conditions and the inherent changes associated with culture aging (Deus-Neumann et al., 1984; Sierra et al., 1992; Kim et al., 2004). Correspondingly, our group has recently demonstrated that cell lines undergo epigenetic changes with successive subcultures, which can affect not only the growth capacity but also secondary metabolite production (Sanchez-Muñoz et al., 2018). This may well have occurred in our *S. khuzistanica* cell line, although to prove this hypothesis, an epigenetic study of the degree of DNA methylation of the cell line would have to be performed.

In this context, different levels of RA production were observed under MeJA and COR elicitation and in control conditions. In the MeJA experiment, a maximum RA production of about 1436 mg L^{-1} was achieved by the untreated cultures at day 14, and in the COR experiment more than 2200 mg L^{-1} at day 24, also in control conditions (Tables 8-9). These results indicate that the age of the cell line can affect not only the maximum yield but also the time course of production. Our results differ from those of previous studies (Kim et al., 2004; Fu et al., 2012), where the cell lines of other plant species lost the capacity to produce plant secondary metabolites with age.

When comparing the effects of the two elicitors, our results show that COR had a stronger negative effect on the growth capacity of the cell line than MeJA. The doubling time (t_d) of the MeJA-treated culture did not change significantly, whereas in the COR-treated cultures a 1.8-fold reduction of the t_d was observed, which provoked a drop in production during the first part of the experiment until day 18 (Fig. 51). The negative effect of COR on the cell growth, and a higher increase in the specific production of the MeJA-treated cell cultures, particularly at day 16, explain why the MeJA-treated cultures achieved a substantially greater productivity, with a peak of 241 mg RA L^{-1} d^{-1}. These results are different from those obtained with *Taxus* cell cultures where the induction of taxol production was higher with COR than with MeJA without significantly affecting the growth capacity of the cultures (Ramírez-Estrada et al., 2015).

To scale up the process, we utilized a 2 L CellBag (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) with a working volume of 1 L, shaken in a Kuhner orbital shaker in the dark at 25ºC and 35-38 rpm, with a shaking diameter of 50 mm. The experiments lasted 21 days and were carried out in batch mode. In these conditions, the BMP_{CFW} of the system was 14.8 g d^{-1} with a RA productivity of 247 g L^{-1} d^{-1} (Table 9). Although the BMP_{CFW} of the system, working in control conditions, did not improve in relation to the shake flasks, the RA productivity was 2.7-fold higher than in the small-scale system. These results demonstrate the suitability of the orbitally shaken CellBag for scaling up the suspension cultures of *S. khuzistanica*. Similar results have been achieved with *Centella asiatica* cell cultures, where the growth and centelloside production also increased at bioreactor level (Hidalgo et al., 2016).

When the biomass and RA production in both bioreactors, wave-mixed BIOSTAT® CultiBag RM and the orbitally shaken CellBag, are compared with those achieved in shake flasks, our results show the effectiveness of both bioreactor systems. In all the cases, the growth rate was higher at bioreactor level than in shake flasks, as was the maximum RA production (Tables 8-9). However, if we compare the productivity

achieved in control conditions (unelicited cells) in both types of bioreactor (Tables 8-9), our results show it was higher (247 mg L^{-1} d^{-1}) in the shaken CellBag than in the wave-mixed bioreactor (147 mg L^{-1} d^{-1}). The *S. khuzistanica* cell cultures were possibly better adapted to the latter system but, as mentioned, the two expriments were not developed simultaneously, and as reported in other studies, the age of a cell culture can have a dramatic effect on the productivity of the system (Kim et al., 2004; Fu et al., 2012).

It has been previously demonstrated that elictors are able to increase the expression of genes encoding key enzymes involved in plant secondary metabolism, and this ability has been harnessed to improve the production of target compounds in plant cell and organ cultures (Ramirez-Estrada et al., 2016). Accordingly, Ramirez-Estrada et al. (2017) recently reported that in cell cultures of *Taxus* sp. COR enhanced the expression of several genes involved in taxol biosynthesis, the elicited cell cultures reaching higher taxane contents than the untreated control. Also, Fatemi et al. (2019) reported an increase in the expression of the genes *HPPR*, *PAL*, *TAT* and *RAS* in MeJA-elicited nodal segment cultures of *S. khuzistanica*. One of the aims of this PhD thesis was therefore to evaluate the effect of COR on the activity of some key enzymes involved in RA biosynthesis. As mentioned, these enzymes are PAL, the first enzyme acting in the phenylalanine branch of the RA biosynthetic pathway, TAT, the first enzyme involved in the tyrosine branch, and RAS, which catalyzes the condensation of both precursors of RA, 4-hydroxyphenyllactic and 4-coumaroyl-CoA, synthesized by each of the two parallel branches (Fig. 4) (Hüchering and Petersen, 2013).

The time course of PAL activity in *S. khuzistanica* suspension cultures was affected by COR elicitation, reaching a maximum at day 16 of the culture, 28 h after the treatment, and then decreasing markedly to the levels of the control cultures (Fig. 52); however, it was lower than in a MeJA-elicited cell suspension of *Coleus blumei*, which was mostly in the range of 20–80 pkat mg $protein^{-1}$ (Szabo et al., 1999). A time-curve similar to our results was observed in *Solenostemon scutellarioides* cultures (Sahu et al., 2013), in which PAL activity increased sharply at 24 h after elicitation with MeJA, decreasing thereafter until the end of the culture period. As indicated by Yan et al. (2006), one possible cause of the reduced PAL activity in plant tissues in response to elicitation could be the activation of endogenous PAL inhibitors after an elicitor or stress challenge (Ritenour and Saltveit, 1996). This would suggest that PAL activity can differ according to the elicitor type and concentration, as well as the plant species. On the other hand, the lack of correlation between phenolic accumulation and PAL activity does not necessarily imply that enhanced phenolic biosynthesis is independent of PAL

expression, which may have previously reached sufficient levels to catalyze the metabolic reaction (Yan et al., 2006).

In contrast with PAL, the maximum activity of TAT (4813 pkat mg protein^{-1}) was achieved at the end of the culture period (Fig. 52), when the RA production also reached its maximum (Fig. 50), suggesting a strong correlation between the two parameters. These results are agreement with those reported for *Salvia miltiorrhiza* hairy root cultures elicited with Ag+ and yeast extract (Yan et al., 2006). Similarly, Dong et al. (2010) observed a high increment of TAT activity in S. *miltiorrhiza* cell cultures elicited with salicylic acid. In *S. miltiorrhiza* hairy root cultures elicited with MeJA, Zhang *et al.* (2014) demonstrated a higher correlation between RA biosynthesis and the enzyme activities of the tyrosine-derived pathway (TAT, among others) than the phenylpropanoid pathway (PAL, among others), corroborating the results obtained in our work.

Regarding the RAS activity, levels reported by Sahu *et al*. (2013) in control *S. miltiorrhiza* hairy root cultures (0-300 pkat mg protein^{-1}) are similar to the basal levels in *S. khuzistanica* control cell cultures (Fig. 52). However, in our case, when the cultures were elicited with COR the activity of RAS (2577.70 pkat mg protein^{-1}) increased dramatically at the end of the culture period, when the RA production was also at its maximum (Fig. 50), reaching levels that were even higher than those reported by Yan et al. (2006) in *S. miltiorrhiza* hairy root cultures elicited with Ag^{+} and yeast extract.

The different responses of the enzymes to elicitor treatments are likely due to their multiple roles in the secondary metabolic network. Enzymes of the phenylpropanoid pathway (PAL, C4H, 4CL) are ubiquitous in land plants and represent branch points in the formation of several widely distributed plant secondary metabolites, such as flavonoids, lignin, chlorogenic acid and anthocyanin. When the flux of metabolites through this pathway is increased, they are diverted to numerous products. In contrast, enzymes in the tyrosine-derived branch are involved in the biosynthesis of far fewer metabolites, including RA, and this specificity could be harnessed for a higher production of the target compound (Zang et al., 2014a).

According to the American Institute for Cancer Research, the age-standardised rate for all cancers (including non-melanoma skin cancer) in 2018 for men and women combined was 197.9 per 100,000. For this reason, cancer prevention and the search for new anticancer agents is one of the most significant public health challenges of the 21st century [https://www.wcrf.org/dietandcancer/cancer-trends]. Promising new anticancer therapies are based on plant-derived compounds that activate the apoptotic pathway leading to programmed cell death (Pfeffer and Sing, 2018).

In order to investigate the potential role of RA in apoptosis, we tested the effect of an *S. khuzistanica* RA-enriched extract on the viability of two human cancer cell lines: MCF-7 and HepG2. The *S. khuzistanica* extract exerted cytotoxic activity against both cancer cell lines, although the effects were greater against MCF-7 (Fig. 54). These results, together with recent evidence for the cytotoxic effects of RA, drove us to look for a possible mechanism of RA-induced apoptosis in human cancer cell lines. Our results showed that the action of the *S. khuzistanica* extract was higher compared to the equivalent concentration of pure RA, although far from reported effects of taxol on HeLa cell viability, which was tested in concentrations of nM and not mM, as here (Gallego et al., 2017).

Aiming to achieve a more in-depth understanding of the RA mechanisms of action, we also checked the effect of RA and the *S. khuzistanica* extract on cell cycle arrest in the MCF-7 cell line. We observed a significant increase of cells in the sub-G0/G1 phase (apoptotic cells), which indicates that the advance of the cell cycle to the G2/M phase had been partially stopped and apoptotic cell death had been induced (Fig. 55) (Jang et al., 2018; Huang et al., 2018).

Caspases are cysteine aspartate-specific proteases whose protein-cleaving activity is essential for the induction of apoptosis. The four known initiator caspases (caspase-2, -8, -9 and -10) are responsible for the hydrolysis and activation of the executioner caspases (caspase-3, -6, -7), which cleave the target proteins and eventually cause cell death (Li and Yuan, 2008). The induction of the sub-G0/G1 cell cycle arrest in the MCF-7 cells prompted us to investigate the effects of RA and *S. khuzistanica* on caspase activity. A significant increase in the activity of the caspase-8 initiator and executioner caspase-6 was observed, demonstrating the effectiveness of the *S. khuzistanica* extract in inducing apoptosis in cancer cell lines. In a similar experiment carried out on osteogenic sarcoma cells (U-2 OS), Lu et al. (2005) demonstrated that Taxol also induces sub-G0/G1 phase arrest in cancer cells and is able to increase caspase activity, mainly the executioner caspase-3, at lower concentrations (μM) than the RA concentrations (mM) used in this experiment. Taken as a whole, our results show that RA and to a greater extent the *S. khuzistanica* extract display apoptotic activity, although at a level far from that achieved with other anticancer agents currently utilized in chemotherapy.

As mentioned in the Introduction, 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 increased 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* micropropagation 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 in those that developed aerial shoots.

There are two types of approaches to developing a biotechnological platform for producing plant secondary metabolites, empirical and rational. Although the latter can give us new information about plant secondary metabolism, it needs some prior knowledge of biosynthetic pathways and their regulation, which is very limited in the case of ruscogenins. In contrast, empirical approaches are based on the effects on cell growth and bioactive compound production of in-put factors, such as variable 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 system.

Thus, in the current work, *R. aculeatus* callus induction and culture was carried out to obtain friable calli as an initial step in the development of a biotechnological platform for ruscogenin production based on plant cell cultures. 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 best 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 the 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 best medium for *R. aculeatus* cell growth was found to be the one optimized for callus cultures. Although very laborious and time-consuming, this phase is essential for establishing an optimized biotechnological platform for producing plant secondary metabolites (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 mainly by groups of cells (see Fig. 58). We therefore treated the cluster cultures with pectinase, an enzyme that degrades the cell middle lamella and 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,

pectinase treatment allowed single cell cultures to be obtained from the aggregates but the growth of the system was reduced (see Fig. 60) because of decreased cell viability (data not shown).

Compared with cell suspensions, organized cultures such as roots and shoots have more genetic stability and are able to produce the same spectrum of plant secondary products as the mother plant (Alvarez, 2014). Therefore, in a second phase of this work, we established root-rhizome cultures to investigate their capacity to produce ruscogenins. The obtained results were compared with those of the cell cultures and also with the production of the *in vitro* plantlets. It was found that the root-rhizome cultures had a high capacity to produce biomass in hormone-free medium as well as a range of ruscogenins, similar to the plantlets and the cell and cluster cultures.

Although the cell and cluster cultures 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 *in vitro*-cultured plants (Fig. 60). Palazon et al. (2006) demonstrated that the development of roots in *R. aculeatus* calli increased the level of ruscogenins but to a lesser extent than in calli developing shoots. Thus, the results obtained in this work confirm those previously reported by our research group.

The commercial source of ruscogenins is the root-rhizome, which is the part of the plant where they accumulate the most (Ivanova et al., 2015), but it is unknown where in the plant ruscogenins are biosynthesized. The fact that our *in vitro* plants accumulated higher ruscogenin contents than the root-rhizome cultures (Fig. 61) suggests the aerial part of the plant may play an active role in the biosynthesis of these compounds, which could later 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 relation to the total ruscogenins ranged from 35% in undifferentiated systems (clusters and isolated cells) to nearly 45% in root-rhizome cultures, a percentage similar to that achieved in the *in vitro* plants (around 41%).

The high ruscogenin contents of the *R. aculeatus in vitro* plants could not be attributed to a direct effect of light on saponin production, because the ruscogenin

production of the root-rhizome system was higher in conditions of darkness than light (Fig. 61). As mentioned previously, it is well accepted that the main source of the IpPP utilized in the biosynthesis of steroidal saponins is the cytoplasmic mevalonate pathway, although there is also a partial contribution from the chloroplastic MEP pathway, due to the crosstalk of both pathways at the IpPP level (Upadhyay et al., 2018). This suggests that in the whole plant, where the aerial part has a developed plastidial system, an active biosynthesis of IpPP can occur in these organelles. Consequently, the plant would dispose of 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 increases the production of ginsenosides in *Panax ginseng* roots because of a better developed plast system in the leaves.

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 for increasing the biotechnological production of saponins and other plant secondary metabolites in cell and organ cultures is 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 *R. aculeatus* in the biotechnological platforms developed, we tested the new elicitor COR. As mentioned previously, COR is a more powerful elicitor than MeJA and has been used to increase 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., 1018) and phytosterols (Kim et al., 2017), among others.

In this work, ruscogenin production increased dramatically when COR was added to the culture medium in all the biotechnological platforms established. The contents of ruscogenin and neoruscogenin were higher in the cultures treated with this elicitor than in the untreated cultures after 4 weeks of growth (Fig. 61). Also, COR had a greater stimulatory effect on the less productive unelicited systems. For example, the least productive system, the callus cultures, which achieved a total ruscogenin content of 0.369±0.070 mg g DW^{-1} in control conditions, increased their production more than 2-fold (up to 0.784±0.096 mg g DW^{-1}) when elicited. In contrast, in the plantlet *in vitro* cultures, which was the most productive system, the COR-induced increase was only 1.3-fold, and the elicited plantlets reached a total ruscogenin content of 1.872 mg g DW^{-1}. These results demonstrate the effectiveness of the COR treatment for inducing ruscogenin production in *in vitro* plant systems, confirming those of previous studies (Ramirez-Estrada el al., 2016).

In one last step, we scaled up the most productive biotechnogical system, the root-rhizome cultures elicited with 1 μM COR, to an orbitally shaken bag of 2 L with a working volume of 500 mL. In this case, scaling up the process from shake flasks to a bioreactor did not significantly improve the productivity of the system, and biomass and RA production were very similar (Fig. 62).

According to Georgiev et al. (2007), hairy root cultures have an advantage over undifferentiated systems such as callus and cell cultures in that they produce the same type of compounds as the root of the whole plant and in similar levels. The main drawback of this system (and generally all biotechnological systems based on organ cultures) is the difficulty of scaling up to bioreactor level, although there are some successful examples. Advances in bioreactor design have facilitated the industrial implementation of root cultures and currently the South Korean CBN Biotech Company is producing *Panax ginseng* roots in a bioreactor of 10 m^3 (Hidalgo et al., 2018). There is less information available about root-rhizome cultures, although Sivakumar et al. (2017) reported the production of the antimitotic compound colchicine in biorhizome cultures of *Gloriosa superba* in an airlift bioreactor. Our promising results indicate that root-rhizome cultures should be further explored for the production of ruscogenins, testing different culture conditions and bioreactor types.

7. Conclusions

The search for new anticancer agents is one of the greatest public health challenges of the 21st century. Nature, especially plants, offers us a plethora of organic molecules with potential anticancer activity. In this pipeline, rosmarinic acid (RA) and the steroidal saponins of *R. aculeatus*, ruscogenin and neoruscogenin, have recently been reported to show cytotoxic/anticancer activities and have future potential as chemotherapeutic agents in cancer treatment. On the other hand, both *Satureja khuzistania*, a rich source of RA, and *Ruscus acculeatus*, a ruscogenin producer, are now endangered plant species, making it vital to find new sources of these compounds to meet the current market demand. Thus, this PhD thesis has a dual objective: to develop plant biofactories based on cell and organ cultures optimized for the production of two target compounds, RA and ruscogenins. The results obtained led to the following conclusions:

1. *S. khuzistanica* cell cultures have considerable potential as a highly efficient biotechnological platform for the production of RA. Working in shake flasks and under control conditions (unelicited cells), our system was able to generate a high quantity of biomass and the target compound, which accumulated mainly inside the cells. When the cell suspensions were elicited with methyl jasmonate (MeJA) (100 μM), an RA yield of nearly 4 g L^{-1} was achieved, one of the highest reported to date, which demonstrates the effectiveness of this elicitor in improving the biotechnological production of RA.
2. In the same system, the cyclodextrin (CD) treatment increased the intracellular accumulation of RA without affecting the biomass production of the culture, but this elicitor did not significantly improve the extracellular RA content. Under combined MeJA and CD elicitation, RA production not only increased significantly, but its maximum accumulation was achieved 7 days earlier than in control conditions. However, the RA production was comparable with that reached in the cell cultures treated only with MeJA.
3. Considering the above results, the *S. khuzistanica* cell suspension culture was scaled up to a wave-mixed bioreactor, BioSTAT CultiBag RM, in optimized elicitation conditions (100 μM of MeJA). The scaling up of the process under these conditions improved both the biomass and RA production of the system, thus confirming the suitability of the wave-mixed bioreactor for the culture of *S.*

khuzistanica cells. In contrast with other processes, in which the scale up from shake flask to bioreactor level has led to a decrease in productivity, our system achieved a maximum fresh biomass of 403.24 g L^{-1} and an RA production of 3.6 g L^{-1}. This opens the possibility of implementing a biotechnological platform based on elicited *S. khuzistanica* cell cultures for the production of RA on a commercial scale.

4. In the same *S. khuzistanica* cell culture system, we applied a new elicitor, coronatine (COR), in a rational approach. Our results demonstrated that this elicitor was also suitable for improving RA production but compared to MeJA its effect on biomass production was more detrimental. The same experiment allowed us to demonstrate the capacity of COR to increase RA production by activating key enzymes in its biosynthesis. As in the previous study, the system was scaled up to an orbitally shaken bag, and it was confirmed that scaling up the *S. khuzistanica* cell cultures to a benchtop bioreactor improves their RA production. Orbitally shaken single-use bioreactors are available in a working volume of up to 2.5 m^3.

5. When an enriched RA extract obtained from the *S. khuzistanica* cell cultures and pure RA were investigated for potential anticancer activity, both showed a similar ability to decrease the viability of the human cancer MCF-7 cell line by inducing apoptosis via a mechanism involving the activation of the initiator caspase-8 and the executioner caspase-6.

6. After testing more than 70 *in vitro* culture media with different compositions of nutrient mineral salts, plant growth regulators and other organic components, we finally obtained *Ruscus aculeatus* calli and derived cell lines. From these results, we can conclude that *R. aculeatus* is recalcitrant to *in vitro* culture and that the B5 medium supplemented with 1 mg L^{-1} of IBA and 5 mg L^{-1} of BA is optimum for the biomass production of *R. aculeatus* plant cell cultures.

7. In order to develop an optimized biotechnological platform for the production of ruscogenins, we compared the *in vitro* production of the target compounds in four different systems (calli, cell suspensions, root-rhizomes and plantlets), all of them in control (untreated) or 1 μM CORO-elicited conditions. The results led us to conclude that the most productive system was the elicited root-rhizome cultures growing in darkness, although in all cases the production of the tested systems was lower than that achieved by the *in vitro* plants.

8. Regarding ruscogenin biosynthesis, the obtained results indicate it is not directly increased by light. Instead, a well-developed plastidial system, as found in plant

leaves, probably constitutes an additional source of precursors for the biosynthesis of the target compounds in the root-rhizomes.

9. Finally, our results showed the cultures could be easily scaled up without loss of productivity: scaling up the most productive *in vitro* system, COR-elicited root-rhizome cultures, to an orbitally shaken bag achieved a biomass and RA production similar to that of the shake flasks.

4. References

Abdollahi M., Salehnia A., Mortazavi S., Ebrahimi M., Shafiee A., F. Fouladian F., Keshavarz K., Kazemi A. (2003) Antioxidant, antidiabetic, antihyperlipidemic, reproduction stimulatory properties and safety of essential oil of *Satureja khuzestanica* in rat *in vivo*: an oxicopharmacological study. *Med. Sci. Monit.* 9, 331-335.

Almagro L, López-Pérez AJ, Pedreño MA. (2011) New method to enhance ajmalicine production in *Catharanthus roseus* cell cultures based on the use of cyclodextrins. *Biotechnol. Lett.* 33, 381-385.

Alvarez MA. (2014) *In Vitro* Plant Cultures as Biofactories. In: Plant Biotechnology for Health. Springer, Cham

Amanlou M, Fazeli M., Arvin A, Amin HG, Farsam H. (2004) Antimicrobial activity of crude methanolic extract of *Satueja khuzistanica*. *Fitoterapia* 75, 768-770.

Aranha H. (2004) Disposable systems. *Bioprocess Int.* 2, 6–16.

Arone G, Wilcock CC. (1994) Reproductive characteristics and breeding system on shrubs of the Mediterranean region. *Funct. Ecol*. 8, 69-76.

Aronne G, Wilcock CC. (1997) Reproductive phenology in Mediterranean macchia vegetaltion. *Lagascalia* 19, 445-454.

Bais HP, Walker TS, Schweizer HP, Vivanco JM. (2002) Root specific elicitation and antimicrobial activity of rosmarinic acid in hairy root cultures of sweet basil (*Ocimum basilicum* L.). *Plant Physiol. Biochem.* 40, 983–995.

Barbaroux M, Gueneron M. (2012) Single-use biopharmaceutical device for producing, storing, and transporting e.g. culture medium product, has tube with contact layer that is fused to contact layer of bag, with physical continuity existing between contact layers, Patent, WO2012022906-A1 2012.

Barberini S, Savona M, Raffi D, Leonardi M, Pistelli L, Stochmal A, Vainstein A, Pistelli L, Ruffoni B. (2013) Molecular cloning of SoHPPR encoding a hydroxyphenylpyruvate reductase, and its expression in cell suspension cultures of *Salvia officinalis*. *Plant Cell Tiss. Org. Cult.* 114, 131–138.

Bauer N, Kiseljak D, Jelaska S. (2009) The effect of yeast extract and methyl jasmonate on rosmarinic acid accumulation in *Coleus blumei* hairy roots. *Biol. Plant.* 53, 650–656.

Bauer N, Leljak-Levanic D, Jelaska S. (2004) Rosmarinic acid synthesis in transformed callus culture of *Coleus blumei* benth. *Z. Naturforsch. C. J. Biosci*. 59, 554–560.

Belchí-Navarro S, Pedreño MA, Corchete P. (2011) Methyl jasmonate increases silymarin production in *Silybum marianum* (L.) Gaernt cell cultures treated with β-cyclodextrins. *Biotechnol. Lett.* 3, 179-184.

Bentebibel S, Moyano E, Palazón J, Cusidó RM, Bonfill M, Eibl R, Piñol MT. (2005) Effects of immobilization by entrapment in alginate and scale-up on paclitaxel and baccatin III production in cell suspension cultures of *Taxus baccata*. *Biotechnol. Bioeng*. 89, 647-55.

Berger A, Meinhard J, Petersen M. (2006) Rosmarinic acid synthase is a new member of the superfamily of BAHD acyltransferases. *Planta* 224, 1503–1510.

Bertani W, Forni GP. (1984) HPLC determination of ruscogenin and neoruscogenin in raw material and pharmaceutical preperations. *Fitoterapia* 55, 101-104.

Bourgaud F, Gravot A, Milesi S, Gontier E. (2001) Production of plant secondary metabolites: A historical perspective. *Plant Sci.* 161, 839-851.

Bouskela E, Cyrino FZ, Marcelon GJ. (1993) Effects of *Ruscus* extract in the internal diameter of arteriales and venules of the hamster cheek pouch microcirculation. *Cardiovasc. Pharmacol.* 22, 221-222.

Bouskela E, Cyrino FZ, Marcelon GJ. (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.

Bouskela E, Cyrino FZ. (1994) Possible mechanism for the effects of *Ruscus* extract on microvascular permeability and diameter. *Clin. Hemorh*. 14, S23-S36.

Bouskela E, Cyrino FZ, Marcelon GJ. (1993) Effects of *Ruscus* extract in the internal diameter of arteriales and venules of the hamster cheek pouch microcirculation. *Cardiovasc. Pharmacol.* 22, 221-222.

Bradford MM. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72, 248–254.

Briceño Z, Almagro L, Sabater-Jara AB, Calderón AA, Pedreño MA, Ferrer MA. (2012) Enhancement of phytosterols, taraxasterol and induction of extracellular pathogenesis-related proteins in cell cultures of *Solanum lycopersicum* cv Micro-Tom elicited with cyclodextrins and methyl jasmonate. *J. Plant Physiol.* 169, 1050-1058.

Brodelius P, Deus B, Mosbach K, Zenk MH. (1979) Immobilized plant cells for the production of natural products. *FEBS. Lett.* 103, 93-97.

Bru R, Sellés S, Casado-Vela J, Belchí-Navarro S, Pedreño MA. (2006) Modified cyclodextrins are chemically defined glucan inducers of defense responses in grapevine cell cultures. *J. Agric. Food Chem.* 54, 65-71.

Bulgakov VP, Inyushkina YV, Fedoreyev SA. (2012) Rosmarinic acid and its derivatives: biotechnology and applications. *Crit. Rev. Biotechnol.* 32, 203-217.

Bulgakov VP, Veselova MV, Tchernoded GK, Kiselev KV, Fedoreyev SA, Zhuravlev YN. (2005) Inhibitory effect of the *Agrobacterium rhizogenes rolC* gene on rabdosiin and rosmarinic acid production in *Eritrichium sericeum* and *Lithospermum erythrorhizon* transformed cell cultures. *Planta* 221, 471–478.

Chaprin N, Ellis BE. (1984) Microspectrophotometric evaluation of rosmarinic acid accumulation in single cultured plant cells. *Can. J. Bot.* 62, 2278-2282.

Chattopadhyay S, Bisaria VS, Scheper T, Srivastava AK. (2002) Non-invasive methods for determination of cellular growth in *Podophyllum hexandrum* suspension cultures. *Biotechnol. Bioprocess Eng.* 7, 331-334.

Chaudhary S, Chikara SK, Sharma MC, Chaudhary A, Alam Syed B, Chaudhary PS, Mehta A, Patel M, Ghosh A, Iriti M. (2015) Elicitation of diosgenin production in *Trigonella foenum-graecum* (Fenugreek) seedlings by methyl jasmonate. *Int. J. Mol Sci*. 16, 29889-29899.

Chen CC, Bates R, Carlson J. (2014) Effect of environmental and cultural conditions in medium pH and plant growth performance of Douglas-fir (*Pseudotsuga menziesii*) shoot culture. *F1000Research* 3, 298.

Chen H, Chena F, Chiu FC, Lo CM. (2001) The effect of yeast elicitor on the growth and secondary metabolism of hairy root cultures of *Salvia miltiorrhiza*. *Enzyme Microb. Technol*. 28, 100–105.

Chevolot L, Dubois-Dauphin R, Yvin JC, and Liénart Y. (1997) High performance liquid chromatography and photodiode array detection of phenylpropanoids and benzoates in *Rubus* protoplasts elicited by kinetin. *Phytochem. Anal*. 8, 22–26.

Cusido RM, Onrubia M, Sabater-Jara AB, Moyano E, Bonfill M5, Goossens A, Pedreño MA, Palazon J. (2014) A rational approach to improving the biotechnological production of taxanes in plant cell cultures of *Taxus* spp. *Biotechnol. Adv.* 32, 1157-1167.

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.

De Vincenzi M, Stammati A, De Vincenzi A, Silano M. (2004) Constituents of aromatic plants: carvacrol. *Fitoterapia* 75, 801-804.

De-Eknamkul W, Ellis BE. (1987) Tyrosine aminotransferase: The entrypoint enzyme of the tyrosine-derived pathway in rosmarinic acid biosynthesis. *Phytochemistry* 26, 1941-1946

Deus-Neumann B; Zenk MH. (1984) Instability of indole alkaloid production in *Catharanthus roseus* cell suspension cultures. *Planta Med*. 50, 427-431.

Di Pasqua R, Betts G, Hoskins N, Edwards M, Ercolini D, Mauriello G. (2007) Membrane toxicity of antimicrobial compounds from essential oils. *J. Agric. Food Chem.* 13, 4863-4870.

Dong J, Wan G, Liang Z. (2010) Accumulation of salicylic acid-induced phenolic compounds and raised activities of secondary metabolic and antioxidative enzymes in *Salvia miltiorrhiza* cell culture. *J. Biotechnol.* 148, 99–104.

Douce R, Joyard J. (1996) Biosynthesis of thylakoid membrane lipids. In: Ort DR, Yocum CF. (Eds.), Advances in Photosynthesis, vol. 4. Kluwer, Dordrecht, pp: 69-101.

Dubos C, Stracke R, Grotewold E, Weisshaar B, Martin C, Lepiniec L. (2010) MYB transcription factors in *Arabidopsis*. *Trends Plant Sci.* 15, 573–581.

Eberle D, Ullmann P, Werck-Reichhart D, Petersen M. (2009) cDNA cloning and functional characterisation of CYP98A14 and NADPH:cytochrome P450 reductase from *Coleus blumei* involved in rosmarinic acid biosynthesis. *Plant Mol. Biol.* 69, 239–253.

Eibl R, Brändli J, Eibl D. (2012). Plant cell bioreactors, in biotechnology. Encyclopedia of life support systems (EOLSS), developed under the auspices of the UNESCO. Eolss Publishers, Oxford.

Eibl R, Werner S, Eibl D. (2009a) Bag bioreactor based on wave-induced motion: characteristics and applications. *Adv. Biochem. Engin/Biotechnol.* 115, 55-87.

Eibl R, Werner S, Eibl D. (2009b) Disposable bioreactors for plant liquid cultures at Litre-scale. *Eng. Life Sci.* 3, 156-164.

Ellis BE, Towers GH. (1970) Biogenesis of rosmarinic acid in Mentha. *Biochem. J.* 118, 291–297.

Expósito O, Syklowska-Baranek K, Moyano E, Onrubia M, Bonfill M, Palazón J, Cusidó RM. (2010) Metabolic responses of *Taxus media* transformed cell cultures to the addition of methyl jasmonate. *Biotechnol. Prog.* 26, 1145-53.

Facino RM, 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.

Fallarini S, Miglio G, Paoletti T, Minassi A, Amoruso A, Bardelli C, Brunelleschi S, Lombardi G. (2009) Clovamide and rosmarinic acid induce neuroprotective effects in *in vitro* models of neuronal death. *Br. J. Pharmacol.* 157, 1072–1084.

Farsam H, Amanlou M, Radpour MR, Salehinia AN, Shafiee A. (2004) Composition of the essential oils of wild and cultivated *Satureja khuzistanica* Jamzad from Iran. *Flavour Fragrance J.* 19, 308-310.

Fatemi F, Abdollahi MR, Mirzaie-asl A, Dastan D, Garagounis C. (2019) Identification and expression profiling of rosmarinic acid biosynthetic genes from *Satureja khuzistanica* under carbon nanotubes and methyl jasmonate elicitation. *Plant Cell Tiss. Organ Cult.* 136, 561-573.

Fattahi M, Nazerib V, Torras-Claveria L, Sefidkond F, Cusido RM, Zamani Z, Palazon J. (2013) A new biotechnological source of rosmarinic acid and surface flavonoids: Hairy root cultures of *Dracocephalum kotschyi* Boiss. *Ind. Crops Prod.* 50, 256–263.

Fedoreyev SA, Veselova MV, Krivoschekova OE, Mischenko NP, Denisenko VA, Dmitrenok PS, Glazunov VP, Bulgakov VP, Tchernoded GK, Zhuravlev YN. (2005) Caffeic acid metabolites from *Eritrichium sericeum* cell cultures. *Planta Med.* 71, 446-451.

Fei L, Weathers PJ. (2014) From cells to embryos to rooted plantlets in a mist bioreactor. *Plant Cell Tiss. Organ Cult.* 116, 37–46.

Françoise B, Hossein S, Halimeh H, Zahra NF. (2007) Growth optimization of *Zataria multiflora* Boiss. Tissue cultures and rosmarinic acid production improvement. *Pak. J. Biol. Sci.* 10, 3395-3399.

Fu C, Li L, Wu W, Li M, Yu X, Yu L. (2012) Assessment of genetic and epigenetic variation during long-term *Taxus* cell culture. *Plant Cell Rep.* 31, 1321–1331.

Furtado RA, de Araújo FR, Resende FA, Cunha WR, Tavares DC. (2010) Protective effect of rosmarinic acid on V79 cells evaluated by the micronucleus and comet assays. *J. Appl. Toxicol.* 30, 254–259.

Gallego A, Imseng N, Bonfill M, Cusido RM, Palazon J, Eibl R, Moyano E. (2015) Development of a hazel cell culture-based paclitaxel and baccatin III production process on a benchtop scale. *J. Biotechnol.* 195, 93-102.

Gallego A, Metón I, Baanante IV, Ouazzani J, Adelin E, Palazon J, Bonfill M, Moyano E. (2017) Viability-reducing activity of *Coryllus avellana* L. extracts against human cancer cell lines. *Biomed. Pharmacother.* 89, 565-572.

Gamborg OL, Miller RA, Ojima K. (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell. Res.* 50, 151-158.

Georgiev M, Kovacheva E, Marcheva N, Ileva M. (2006) Purification of rosmarinic acid extracts from *Lavandula vera* MM cell biomass. *Food Chem.* 94, 111-114.

Georgiev M, Kuzeva S, Pavlov A, Kovacheva E, Ilieva M. (2006) Enhanced rosmarinic acid production by *Lavandula vera* MM cell suspension culture through elicitation with vanadyl sulfate. *Z. Naturforsch. C* 61, 241-244.

Georgiev M, Pavlov A, Ilieva M. (2004) Rosmarinic acid production by *Lavandula vera* MM cell suspension: the effect of temperature. *Biotechnol. Lett.* 26, 855-856.

Georgiev MI, Eibl R, Zhong JJ. (2013) Hosting the plant cells *in vitro*: recent trends in bioreactors. *Appl. Microbiol. Biotechnol.* 97, 3787-3800.

Georgiev MI, Kuzeva SL, Pavlov AI, Kovacheva EG, Ilieva MP. (2007) Elicitation of rosmarinic acid by *Lavandula vera* MM cell suspension culture with abiotic elicitors. *World J. Microbiol. Biotechnol.* 23, 301–304.

Georgiev MI, Pavlov AI, Bley T. (2007) Hairy root type plant *in vitro* systems as sources of bioactive substances. *Appl. Microbiol. Biotechnol*. 74, 1175-1185.

Georgiev MI, Weber J, Maciuk A. (2009) Bioprocessing of plant cell cultures for mass production of targeted compounds. *Appl. Microbiol. Biotechnol.* 83, 809-823.

Georgiev MI. (2014) Design of Bioreactors for Plant Cell and Organ Cultures. In: Paek KY, Murthy HN, Zhong JJ (Eds) Production of Biomass and Bioactive Compounds Using Bioreactor Technology. Springer Science+Business Media Dordrecht, pp: 3-16.

Goossens A, Rischer H. (2009) Implementation of functional genomics in alkaloid producing plants. *Phytochem. Rev.* 6, 35-49.

Grzegorczyk I, Krolicka A, Wysokinska H. (2006) Establishment of *Salvia officinalis* L. hairy root cultures for the production of rosmarinic acid. *Z. Naturforsch. C* 61, 351-356.

Hadian J, Mirjalili MH, Kanani MR, Salehnia A,Ganjipoor P. (2011) Phytochemical and morphological characterization of *Satureja khuzistanica* Jamzad populations from Iran. *Chem. Biodiv.* 8, 902-914.

Hadian J, Ebrahimi SN, Salehi P. (2010) Variability of morphological and phytochemical characteristics among *Satureja hortensis* L. accessions of Iran. *Ind. Crop. Prod.* 32, 62-69.

Hadzifejzovic N, Kukic-Markovic J, Petrovic S, Sokovic M, Glamoclija J, Stojkovic D, Nahrstedt A. (2013) Bioactivity of the extracts and compounds of *Ruscus aculeatus* L. and *Ruscus hypoglossum* L. *Ind. Crops Prod*. 49, 407–411.

Haeri S, Minaie B, Amin G, Nikfar S, Khorasani R, Esmaily H, Salehnia A, Abdollahi M. (2006) Effect of *Satureja khuzestanica* essential oil on male rat fertility. *Fitoterapia* 77, 495-499.

Hakkim FL, Kalyani S, Essa M, S. Girija S, Song H. (2011) Production of rosmarinic in *Ocimum sanctum* cell cultures by the influence of sucrose, phenylalanine, yeast extract, and methyl jasmonate. *Int. J. Biol. Med. Res.* 2, 1070 -1074.

Hamaguchi T, Ono K, Murase A, Yamada M. (2009) Phenolic compounds prevent Alzheimer's pathology through different effects on the amyloid-beta aggregation pathway. *Am. J. Pathol.* 175, 2557–2565.

Häusler E, Petersen M, Alfermann AW. (1991) Hydroxyphenylpyruvate reductase from cell suspension cultures of *Coleus blumei* Benth. *Z. Naturforsch*. 46, 371–376.

Häusler E, Petersen M, Alfermann AW. (1993) Isolation of protoplasts and vacuoles from cell suspension cultures of *Coleus blumei* Benth. *Plant Cell Rep.* 12, 510-512.

Heftmann E. (1983) Biogenesis of steroids in Solanaceae. *Phytochemistry*. 22, 1843.

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.* 2018. 25, 3577-3596.

Hidalgo D, Steinmetz V, Brossat M, Tournier-Couturier L, Cusido RM, Corchete P, Palazon J. (2017) An optimized biotechnological system for the production of

centellosides based on elicitation and bioconversion of *Centella asiatica* cell cultures. *Eng. Life Sci.* 17, 413-419

Hill MO, Preston CD, Roy DB (2004) PLANTATT – Atributes of British and Irish Plants: Status, Size, History, Geography and Habitats. Centr for Ecology and Hydrology, Huntingdom, UK.

Hillman EM. (1979) A study of *Ruscus aculeatus* on Bookham Common. *London Naturalist* 58, 44-45.

Hippolyte I, Marin B, Baccou JC, Jonard R. (1992) Growth and rosmarinic acid production in cell suspension cultures of *Salvia officinalis* L. *Plant Cell Rep.* 11, 109-12.

Hirasuna TJ; Pestchanker LJ.; Srinivasan V; Shuler ML. (1996) Taxol production in suspension cultures of *Taxus baccata*. *Plant Cell Tiss. Org. Cult.* 44, 95-102.

Hirayama Y, Yamada T, Oya Y, Ito M, Kato M, Imaichi R. (2007) Expression patterns of class 1 KNOX and YABBY genes in *Ruscus aculeatus* (Asparagaceae) with implications for cladode homology. *Dev Genes Evol.* 217, 363-372.

Hraš AR, Hadolin M, Knez Z, Bauman D. (2000) Comparison of antioxidative and synergistic effects of rosemary extract with α-tocopherol, ascorbyl palmitate and citric acid in sunflower oil. *Food Chem.* 71, 229-233.

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

Huang LD, Van SJ. (2002) *Salvia chamelaeagnea* can be micropropagated and its callus induced to produce rosmarinic acid. *S. Afr. J. Bot.* 68, 177-180.

Huang TK, McDonald KA. (2012) Bioreactor systems for *in vitro* production of foreign proteins using plant cell cultures. *Biotechnol. Adv.* 30, 398-409.

Huang Y, Cai Y, Huang R, Zheng X. (2018) Rosmarinic acid combined with adriamycin induces apoptosis by triggering mitochondria-mediated signaling pathway in HepG2 and Bel-7402 Cells. *Med. Sci. Monit.* 24, 7898-7908.

Hücherig S, Petersen M. (2013) RNAi suppression and overexpression studies of hydroxyphenylpyruvate reductase (HPPR) and rosmarinic acid synthase (RAS) genes related to rosmarinic acid biosynthesis in hairy root cultures of *Coleus blumei*. *Plant Cell Tiss. Organ Cult.* 113, 375–85.

Hur YG, Suh CH, Kim S, Won J. (2007) Rosmarinic acid induces apoptosis of activated T cells from rheumatoid arthritis patients via mitochondrial pathway. *J. Clin. Immunol.* 27, 36–45.

Inyushkina YV, Kiselev KV, Bulgakov VP, Zhuravlev YN. (2009) Specific genes of cytochrome P450 monooxygenases are implicated in biosynthesis of caffeic acid metabolites in rolC-transgenic culture of *Eritrichium sericeum*. *Biochemistry Mosc.* 74, 917–924.

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.

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.

Jang IB, Lee DY, Yu J, Park HW, Mo HS, Park KC, Hyun DY, Lee EH, Kim KH, Oh CS (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, 345-53.

Jang YG, Hwang KA, Choi KC. (2018) Rosmarinic acid, a component of rosemary tea, induced the cell cycle arrest and apoptosis through modulation of HDAC2 expression in prostate cancer cell lines. *Nutrients* 10, pii: E1784.

Javanmardi J, Khalighi A, Kashi A, Bais HP, Vivanco JM. (2002) Chemical characterization of basil (*Ocimum basilicum* L.) found in local accessions and used in traditional medicines in Iran. *J. Agric. Food. Chem.* 50, 5878-83.

Kadarusman J, Bhatia R, McLaughlin J, Lin WLR. (2005) Growing cholesterol-dependent NS0 myeloma cell line in the wave bioreactor system: overcoming cholesterol-polymer interaction by using pretreated polymer or inert fluorinated ethylene propylene. *Biotechnol. Prog.* 21, 1341-1346.

Karam NS, Jawad FM, Arikat NA, Shibli RA. (2003) Growth and rosmarinic acid accumulation in callus, cell suspension, and root cultures of wild *Salvia fruticosa*. *Plant Cell Tiss. Organ Cult.* 73, 117-121.

Katsir L, Schilmiller AL, Staswick PE, He SY, Howe GA. (2008) COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. *Proc. Natl. Acad. Sci. U.S.A.* 105, 7100-7105.

Kay QON, Page J. (1985) Dioecism and pollination in *Ruscus aculeatus*. *Watsonia* 15, 261-264.

Kim BJ, Gibson DM, Shuler ML. (2004). Effect of subculture and elicitation on instability of taxol production in *Taxus* sp. suspension cultures. *Biotechnol. Prog*. 20, 1666–1673.

Kim HK, Oh SR, Lee HK, Huh H. (2001) Benzothiadiazole enhances the elicitation of rosmarinic acid production in a suspension culture of *Agastache rugosa* O. Kuntze. *Biotechnol. Lett.* 23, 55-60.

Kim JY, Kim HY, Jeon JY, Kim DM, Zhou Y, Lee JS, Lee H, Choi HK. (2017) Effects of coronatine elicitation on growth and metabolic profiles of *Lemna paucicostata* culture. *PLoS One.* 12, e0187622.

Kim JH, Lee BJ, Kim JH, Yu YS, Kim MY, Kim KW. (2009) Rosmarinic acid suppresses retinal neovascularization via cell cycle arrest with increase of p21(WAF1) expression. *Eur. J. Pharmacol.* 615, 150–154.

Kim KH, Janiak V, Petersen M. (2004) Purification, cloning and functional expression of hydroxyphenylpyruvate reductase involved in rosmarinic acid biosynthesis in cell cultures of *Coleus blumei*. *Plant Mol. Biol*. 54, 311–332.

Kim YB, Kim JK, Uddin MR, Xu H, Park WT, Tuan PA, Li X, Chung E, Lee JH, Park SU. (2013) Metabolomics analysis and biosynthesis of rosmarinic acid in *Agastache rugosa* Kuntze treated with methyl jasmonate. *PloS one*. 8, e64199.

Kintzios S, Kollias H, Straitouris E, Makri O. (2004) Scale-up micropropagation of sweet basil (*Ocimum basilicum* L.) in an airlift bioreactor and accumulation of rosmarinic acid. *Biotechnol. Lett.* 26, 521–523.

Klöckner W, Diederichs S, Büchs J. (2014) Orbitally shaken single-use bioreactors. *Adv. Biochem. Eng. Biotechnol.*138, 45-60.

Krzyzanowska J, Czubacka A, Pecio L, Przybys M, Doroszewska T, Stochmal A, Oleszek W. (2012) The effects of jasmonic acid and methyl jasmonate on rosmarinic acid production in *Mentha x piperita* cell suspension cultures. *Plant Cell Tiss. Organ Cult.* 108, 73–81.

Kumar V, Singh SK, Bandopadhyay R, Sharma MM, Chandra S. (2014) *In vitro* organogenesis secondary metabolite production and heavy metal analysis in *Swertia chirayita*. *Cent. Eur. J. Biol*. 9, 686-698.

Lee JK, Eom SH, Hyun TK. (2018) Enhanced biosynthesis of saponins by coronatine in cell suspension culture of *Kalopanax septemlobus*. *3 Biotech*. 8, 59.

Lee SY, Lee CY, Eom SH, Kim YK, Park NI, Park SU. (2010) Rosmarinic acid production from transformed root cultures of *Nepeta cataria* L. *Scient. Res. Assays* 5, 1122-1126.

Lee SY, Xu H, Kim YK, Park SU. (2008) Rosmarinic acid production in hairy root cultures of *Agastache rugosa* Kuntze. *World J. Microbiol. Biotechnol*. 24, 969-972.

Lee TL, Shultz RW, Hanley-Bowdoin L, Thompson WF. (2004) Establishment of rapidly proliferating rice cell suspension culture and its characterization by fluorescence-activated cell sorting analyses. *Plant Mol. Biol. Rep.* 22, 259.

Lee JK, Eom SH, Hyun TK. (2018) Enhanced biosynthesis of saponins by coronatine in cell suspension culture of *Kalopanax septemlobus*. *3 Biotech*. 8, 59.

Lehmann N, Dittler I, Lämsä M, Ritala A, Rischer H, Eibl D, Oksman-Caldentey K-M, Eibl R. (2014) Disposable Bioreactors for Cultivation of Plant Cell Cultures. In: Paek KY, Murthy HN, Zhong JJ (Eds.) Production of Biomass and Bioactive Compounds Using Bioreactor Technology. Springer Science+Business Media Dordrecht, pp: 17-46.

Li F, Fan XX, Chu C, Zhang Y, Kou JP, Yu BY. (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.

Li GS, Jiang WL, Tian JW, Qu GW, Zhu HB, Fu FH. (2010) *In vitro* and *in vivo* antifibrotic effects of rosmarinic acid on experimental liver fibrosis. *Phytomedicine* 17, 282-288.

Li X, Gao MJ, Pan HY, Cui DJ, Gruber MY. (2010) Purple canola: Arabidopsis PAP1 increases antioxidants and phenolics in *Brassica napus* leaves. *J. Agric. Food Chem*. 58, 1639-1645.

Li J, Yuan J. (2008) Caspases in apoptosis and beyond. *Oncogene* 27, 6149-6206.

Li W, Koike K, Asada Y, Yoshikawa T, Nikaido T. (2005) Rosmarinic acid production by *Coleus forskohlii* hairy root cultures. *Plant Cell Tiss. Organ Cult.* 80, 151-155.

Liang Z, Ma Y, Xu T, Cui B, Liu Y, Guo Z, Yang D. (2013) Effects of abscisic acid, gibberellin, ethylene and their interactions on production of phenolic acids in *Salvia miltiorrhiza* bunge hairy roots. *PloS one* 8, e72806.

Lijavetzky D, Almagro L, Belchí-Navarro S, Martínez-Zapater JM, Bru R, Pedreño MA. (2008) Synergistic effect of methyl jasmonate and cyclodextrin on stilbene biosynthesis pathway gene expression and resveratrol production in Monastrell grapevine cell cultures. *BMC Res. Notes* 1, 132–140.

Llieva M, Pavlov A. (1997) Rosmarinic acid production by *Lavandula vera* MM cell-suspension culture. *Appl. Microbiol. Biotechnol.* 47, 683-688.

Löffelholz C, Kaiser SC, Kraume M, Eibl D, Eibl R. (2014) Dynamic single-use bioreactors used in modern liter- and m^3- scale biotechnological processes: engineering characteristics and scaling up. In: Eibl D and Eibl R (Eds.) Disposable bioreactors II. *Adv. Biochem. Eng. Biotechnol.* 138, 1-44.

Lu KH, Lue KH, Chou MC, Chung JG. (2005) Paclitaxel induces apoptosis via caspase-3 activation in human osteogenic sarcoma cells (U-2 OS). *J Orthop. Res*. 23, 988-994.

Lu X, Hao L, Wang F, Huang C, Wu S. (2013) Molecular cloning and overexpression of the tyrosine aminotransferase (TAT) gene leads to increased rosmarinic acid yield in *Perilla frutescens*. *Plant Cell Tiss. Org. Cult.* 115, 69–83.

Ma AZ. (2014) Mechanism of Ruscogenin on Suppressing Pulmonary A549 and H460 Cells. Nanjing University of Chinese Medicine.

Mallol A, Cusidó RM, Palazón J, Bonfill M, Morales C, Piñol MT. (2001) Ginsenoside production in different phenotypes of *Panax ginseng* transformed roots. *Phytochemistry* 57, 365-371.

Mangas S, Bonfill M, Osuna L, Moyano E, Tortoriello J, Cusidó RM, Piñol MT, Palazon 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.

Martinez BC, Park CH. (1994) Immobilization of *Coleus blumei* cells in a column reactor using a spray feeding system. *Biotechnol. Techniq*. 8, 301-306.

Martinez-Pallé E, Aronne G. (2000) Pollination failure in mediterranean *Ruscus aculeatus* L. *Bot. J. Linn. Soc.* 134, 443-452.

Martinez-Pallé E, Aronne G. (1999) Flower development and reproductive continuity of *Ruscus* in Mediterranean *Ruscus aculeatus* L. (Liliaceae). *Protoplasma* 208, 58-64.

Masullo M, Pizza C, Piacente S. (2016) *Ruscus* genus: a rich source of bioactive steroidal saponind. *Planta Med.* 82, 1513-1524.

Mazid M, Khan TA, Mohammad F. (2011) Role of secondary metabolites in defense mechanisms of plants. *Biol. Med.* 3, 232-249.

McIlwain DR, Berger T, Mak TW. (2013) Caspase functions in cell death and disease. *Cold Spring Harb. Perspect. Biol*. 5, a008656.

Melotto M, Underwood W, He SY. (2008) Role of stomata in plant innate immunity and foliar bacterial diseases. *Annu Rev. Phytopathol.* 46, 101-22.

Milos M, Radoni A, Bezi N, V. Dunki V. (2001) Localities and seasonal variations in the chemical composition of essential oils of *Satureja montana* L. and *S. cuneifolia* Ten. *Flavour Fragrance J.* 16, 157-160.

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.

Mizukami H, Ogawa T, Ellis BE. (1992) Induction of rosmarinic acid biosynthesis in *Lithospermum-erythrorhizon* cell suspension cultures by yeast extract. *Plant Cell Rep*. 11, 480-483.

Mizukami H, Tabira Y, Ellis BE. (1993) Methyl-jasmonate-induced rosmarinic acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures. *Plant Cell Rep*. 12, 706–709.

Morales M, Bru R, García-Carmona F, Ros-Barceló A, Pedreño MA. (1998) Effect of dimethyl-ß-cyclodextrins on resveratrol metabolism in Gamay grapevine cell cultures before and after inoculation with *Xylophilus ampelinus*. *Plant Cell. Tiss. Org. Cult.* 53, 179-187.

Moyano E, Montero M, Bionfill M, Cusidó RM, Palazón J, Piñol MT. (2006) *In vitro* micropopagation of *Ruscus aculeatus*. *Biol. Plant.* 50, 441-443.

Murakami Y, Omoto T, Asai I, Shimomura K, Yoshihira K, Ishimaru K. (1998) Rosmarinic acid and related phenolics in transformed root cultures of *Hyssopus officinalis*. *Plant Cell Tiss. Organ Cult.* 53, 75–78.

Murthy HN, Lee EJ, Paek KY. (2014) Production of secondary metabolites from cell and organ cultures: strategies and approaches for biomass improvement and metabolite accumulation. *Plant Cell Tiss. Organ Cult.* 118, 1–16.

Mustafa NR, de Winter W, van Iren F, Verpoorte R. (2011) Initiation, growth and cryopreservation of plant cell suspension cultures. *Nat. Protoc*. 6, 715-742.

Naill MC, Roberts SC. (2004) Preparation of single cells from aggregated *Taxus suspension* cultures for population analysis. *Biotechnol. Bioeng.* 2004, 86, 817-826.

Namdeo AG. (2007) Plant cell elicitation for production of secondary metabolites: A review. *Phcog. Rev.* 1, 69-79.

Ogata A, Tsuruga A, Matsunob M, Mizukami H. (2004) Elicitor induced rosmarinic acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures: Activities of rosmarinic acid synthase and the final two cytochrome P450-catalyzed hydroxylations. *Plant Biotechnol.* 21, 393–396.

Onrubia M, E. Moyano E, M. Bonfill M, R. M. Cusidó RM, A. Goossens A, and J. Palazón J. (2013a) Coronatine, a more powerful elicitor for inducing taxane biosynthesis in *Taxus media* cell cultures than methyl jasmonate. *J. Plant Physiol.* 170, 211–219.

Onrubia M, Cusidó RM, Ramirez K, Hernández-Vázquez L, Moyano E, Bonfill M, Palazon J. (2013b) Bioprocessing of plant *in vitro* systems for the mass production of pharmaceutically important metabolites: paclitaxel and its derivatives. *Curr. Med. Chem.* 20, 880-891.

Onrubia M, Pollier J, Vanden Bossche R, Goethals M, Gevaert K, Moyano E, Vidal-Limon H, Cusidó RM, Palazón J, Goossens A. (2014) Taximin, a conserved plant-specific peptide is involved in the modulation of plant-specialized metabolism. *Plant Biotech. J.* 12, 971-983.

Palazon J, Moyano E, Bonfill M, Osuna L, Cusidó RM, Piñol MT. (2006) Effect of organogenesis on steroidal saponins biosynthesis in calli cultures of *Ruscus aculeatus*. *Fitoterapia* 77, 216-220.

Paluszczak J, Krajka-Kuzniak V, Baer-Dubowska W. (2010) The effect of dietary polyphenols on the epigenetic regulation of gene expression in MCF7 breast cancer cells. *Toxicol. Lett.* 192, 119–125.

Panya A, Laguerre M, Lecomte J, Villeneuve P, Weiss J, McClements DJ, Decker EA. (2010) Effects of chitosan and rosmarinate esters on the physical and oxidative stability of liposomes. *J. Agric. Food Chem.* 58, 5679–5684.

Park CH, Martinez B. (1994) Growth and production characteristics of permeabilized *Coleus blumei* cells in immobilized fed-batch culture. *Plant Cell Rep.* 13, 459-463.

Park SU, Uddin MR, Xu H, Kim YK, Lee SY. (2008) Biotechnological applications for rosmarinic acid production in plants. *African J. Biotechnol.* 7, 4959-4965.

Park, CH, Martinez BC. (1992) Enhanced release of rosmarinic acid from *Coleus blumei* permeabilized by dimethyl sulfoxide DMSO while preserving cell viability and growth. *Biotechnol. Bioeng.* 40, 459-464.

Pavlov AI, Georgiev MI, Ilieva MP. (2005a) Production of rosmarinic acid by *Lavandula vera* MM cell suspension in bioreactor: effect of dissolved oxygen concentration and agitation. *World J. Microb. Biot.* 21, 389–392.

Pavlov A, Georgiev M, Panchev I, Ileva M. (2005b) Optimization of rosmarinic acid production by *Lavandula vera* MM plant cell suspension in a laboratory bioreactor. *Biotechnol. Prog*. 21, 394-396.

Pérez-Fons L, Garzón MT, Micol V. (2010) Relationship between the antioxidant capacity and effect of rosemary (*Rosmarinus officinalis* L.) polyphenols on membrane phospholipid order. *J. Agric. Food Chem.* 58, 161–171.

Petersen M, Abdullah Y, Benner J, Eberle D, Gehlen K, Hücherig S, Janiak V, Kim KH, Sander M, Weitzel C, Wolters S. (2009) Evolution of rosmarinic acid biosynthesis. *Phytochemistry* 70, 1663-1679.

Petersen M, Alfermann AW. (1988) Two new enzymes of rosmarinic acid biosynthesis from cell cultures of *Coleus blumei*: hydroxyphenylpyruvate reductase and rosmarinic acid synthase. *Z. Naturforsch. C: Biosci.* 43, 501–504.

Petersen M, Haeusler E, Meinhard J, Karwatzki B, Gerlowski C. (1994) The biosynthesis of rosmarinic acid in suspension cultures of *Coleus blumei*. *Plant Cell Tiss. Organ Cult*. 38, 171-179.

Petersen M, Häusler E, Karwatzki B, Meinhard J. (1993) Proposed biosynthetic pathway for rosmarinic acid in cell cultures of *Coleus blumei*. *Planta*, 189, 10–14.

Petersen M, Simmonds MSJ. (2003) Molecules of interest: rosmarinic acid. *Phytochemistry* 62, 121-125.

Petersen M, Metzger JW (1993) Identification of the reaction products of rosmarinic acid synthase from cell cultures of *Coleus blumei* by ion spray mass spectrometry and tandem mass spectrometry. *Phytochem. Anal.* 4, 131–134

Petersen M. (1997) Cytochrome P-450-dependent hydroxylation in the biosynthesis of rosmarinic acid in *Coleus*. *Phytochemistry* 45, 1165–1172.

Petersen M. (2013) Rosmarinic acid: new aspects. *Phytochem. Rev*. 12, 207-227.

Pfeffer CM, Singh TK (2018) Apoptosis: a target for anticancer therapy. *Int. J. Mol. Sci.* 19, 448.

Pignatti S. (1982) Flora d'Italia, Vol. 3. Edagricole, Bologna.

Purwito A, Prima Muklisa P, Maharijaya DA. (2005) *In vitro* propagation of *Ruscus* (*Ruscus hypophyllum*). *Bul. Agron.* 33, 39-45.

Qiu J, Sun S, Luo S, Zhang J, Xiao X, Zhang L, Wang F, Liu S. (2014) *Arabidopsis* AtPAP1 transcription factor induces anthocyanin production in transgenic *Taraxacum brevicorniculatum*. *Plant Cell Rep.* 33, 669–80

Rader RA, Langer ES. (2011) Upstream single-use bioprocessing systems future market trends and growth assessment. *Bioprocess Int.* 10, 12–18.

Rao SR, Ravishankar GA. (2002) Plant cell cultures: Chemical factories of secondary metabolites. *Biotechnol. Adv.* 20, 101–153.

Ramirez-Estrada K, Osuma L, Moyano E, Bonfill M, Tapia N, Cusido RM, Palazon J. (2015) Changes in gene transcription and taxane production in elicited cell cultures of *Taxus x media* and *Taxus globosa*. *Phytochemistry* 117, 174-184.

Ramirez-Estrada K, Vidal-Limon H, Hidalgo D, Moyano E, Golenioswki M, Cusidó RM, Palazon J. (2016) Elicitation, an effective strategy for the biotechnological production of bioactive high-added value compounds in plant cell factories. *Molecules* 21, 182.

Redman DA. (2000) *Ruscus aculeatus* (Butcher's broom) as a potential treatment for pypotension, with a case report. *J. Altern. Complement. Med*. 6, 539-549.

Ritenour MA, Saltveit ME. (1996) Identification of α-phenylalanine ammonia-lyase inactivating factor in harvested head lettuce (*Lactuca sativa*). *Physiol. Plant*. 97, 327-331.

Rowan DD, Cao M, Lin-Wang K, Cooney JM, Jensen DJ, Austin PT, Hunt MB, Norling C, Hellens RP, Schaffer RJ, Allan AC. (2009) Environmental regulation of leaf colour in red 35S:PAP1 *Arabidopsis thaliana*. *New Phytol.* 182, 102–115.

Rudofsky G. (1991) Effect of *Ruscus* extracts on the capillary filtration rate. In. Retum Circulation and Norepinephine: An update. Vanhoute PM. (Ed). John Libbey Eurotext. pp: 219-224.

Sabater-Jarra, AB, Tudela LR, López-Pérez AJ. (2010a) *In vitro* culture of *Taxus* sp.: strategies to increase cell growth and taxoid production. *Phytochem. Rev.* 9, 343-56.

Sabater-Jara AB, Almagro L, Belchí-Navarro S, Ferrer MA, Ros Barceló A, Pedreño MA. (2010b) Induction of sesquiterpenes, phytoesterols and extracelular pathogenesis-related proteins in elicited cell cultures of *Capsicum annuum*. *J. Plant Physiol.* 167: 1273-81.

Sabater-Jara AB, Onrubia M, Moyano E, Bonfill M, Palazon J, Pedreño MA, Cusido RM. (2014) Synergistic effect of cyclodextrins and methyl jasmonate on taxane production in *Taxus x media* cell cultures. *Plant Biotechnol. J.* 12, 1075-1084

Sadeghi-Nejad B, Shiravi F, Ghanbari S, Alinejadi M, Zarrin M. (2010) Antifungal activity of *Satureja khuzestanica* (Jamzad) leaves extracts. *Jundishapur J. Microbiol.* 3, 36-40.

Saenger W. (1980) Cyclodextrin inclusion compounds in research and industry. *Angew. Chem. Int.* 19, 344–362.

Sahraroo A, Babalar M, Mirjalili MH, Fattahi Moghadam MR, Nejad Ebrahimi S. (2014) *In vitro* callus induction and rosmarinic acid quantification in callus culture of *Satureja khuzistanica* Jamzad (Lamiaceae). *Iran J. Pharm. Res.* 13, 1447-1456.

Sahu NP, Banerjee S, Mondal B, Mandal D. (2008) Steroidal saponins. In: Fortschritte der Chemie Organischer Naturstoffe. Progress in the Chemistry of Organic Natural Products. Kinghorn AD, Falk H, Kobayashi J (Eds). Springer, Wien, New York.

Sahu R, Gangopadhyay M, Dewanjee S. (2013) Elicitor-induced rosmarinic acid accumulation and secondary metabolism enzyme activities in *Solenostemon scutellarioides*. *Acta Physiol Plant.* 35, 1473–1481.

Sanchez-Muñoz R, Bonfill M, Cusidó RM, Palazon J, Moyano E. (2018) Advances in the regulation of *in vitro* paclitaxel production: methylation of a Y-Patch promoter region alters BAPT gene expression in *Taxus* cell cultures. *Plant Cell. Physiol*. 59, 2255-2267.

Sannie C, Lapin H. (1957) Sterolic sapogenins. VII. Neoruscogenin (3β,1-dihydroxy-22b,25L-5-spirostene), a new sapogenin from *Ruscus aculeatus*. *Bull. Soc. Chim. Fr.* 10, 1237-1241.

Scarpati ML, Oriente G. (1958) Isolamento e costituzione dell'acido rosmarinico (dal rosmarinus off.). *Ric. Sci.* 28, 2329–2333.

Schripsema J, Meijer AH, van Iren F, ten Hoopen HJG, Verpoorte R. (1990) Dissimilation curves as a simple method for the characterization of growth of plant cell suspension cultures. *Plant Cell Tiss. Org. Cult*. 22, 55–64.

Sefidkon F, Jamzad Z, Mirza M. (2004) Chemical variation in the essential oil of *Satureja sahendica* from Iran. *Food Chem*. 88, 325-328.

Shahsavari R, Ehsani-Zonouz A, Houshmand M, Salehnia A, Ahangari G, Firoozrai M. (2009) Plasma glucose lowering effect of the wild *Satureja khuzestanica* Jamzad essential oil in diabetic rats: role of decreased gluconeogenesis. *Pak. J. Biol. Sci.* 15, 140-145.

Sharma P, Sharma S, Yadau S, Srivastava A, Puroit I, Shirivastava N. (2014) Plant Derived Biomolecules: Culture Vessels to Bioreactors. In: Paek KY, Murthy HN, Zhong JJ. (Eds.) Production of Biomass and Bioactive Compounds Using Bioreactor Technology. Springer Science+Business Media Dordrecht, pp: 47-63.

Shimojo Y, Kosaka K, Noda Y, Shimizu T, Shirasawa T. (2010) Effect of rosmarinic acid in motor dysfunction and life span in a mouse model of familial amyotrophic lateral sclerosis. *J. Neurosci. Res.* 88, 896-904.

Sierra MI, van der Heijden R, van der Leer T, Verpoorte R. (1992) Stability of alkaloid production in cell suspension cultures of *Tabernaemontana divaricata* during long-term subculture. *Plant Cell Tiss. Org. Cult*. 28, 59- 68.

Singh P, Singh G, Bhandawat A, Singh G, Parmar R, Seth R, Sharma RK. (2017) Spatial transcriptome analysis provides insights of key gene(s) involved in steroidal saponin biosynthesis in medicinally important herb *Trillium govanianum*. *Sci. Rep.* 28, 45295.

Sivakumar G, Alba K, Phillips GC. (2017) Biorhizome: a biosynthetic platform for colchicine biomanufacturing. *Front. Plant Sci.* 8, 1137.

Sonawane PD, Pollier J, Panda S, Szymanski J, Massalha H, Yona M, Unger T, Malitsky S, Arendt P, Pauwels L, Almekias-Siegl E, Rogachev I, Meir S, Cárdenas PD, Masri A, Petrikov M, Schaller H, Schaffer AA, Kamble A, Giri AP, Goossens A, Aharoni A. (2016) Plant cholesterol biosynthetic pathway overlaps with phytosterol metabolism. *Nat. Plants* 22, 16205.

Spencer M. (1994) The production of novel peroxidases from plant cell cultures. Presented at the Symposium “Plant tissue and organ cultures in liquid media”. Prague; July 1994.

Srivastava S, Srivastava AK. (2007) Hairy root culture for mass-production of high-value secondary metabolites. *Crit. Rev. Biotechnol.* 27, 29-43.

Stafford A, Smith L, Fowler MW. (1985) Regulation of product synthesis in cell cultures of *Catharanthus roseus* (L) G. Don. *Plant Cell Tiss. Org. Cult.* 4, 83–94.

Su WW, Humphrey AE. (1991) Production of rosmarinic acid from perfusion culture of *Anchusa officinalis* in a membrane-aerated bioreactor. *Biotechnol. Lett.* 13, 889-892.

Su WW, Lei F, Kao NP. (1995) High density cultivation of *Anchusa officinalis* in a stirred-tank bioreactor with *in situ* filtration. *Appl. Microbiol. Biotechnol.* 44, 293-299.

Su WW, Lei F, Su LY. (1993) Perfusion strategy for rosmarinic acid production by *Anchusa officinalis*. *Biotechnol. Bioeng.* 42, 884-890.

Su WW, Lei F. (1993) Rosmarinic acid production in perfused *Anchusa officinalis* culture: Effect of inoculum size. *Biotechnol. Lett.* 15, 1035-1038.

Sumaryono W, Proksch P, Hartmann T, Nimtz M, Wray V. (1991) Induction of rosmarinic acid accumulation in cell suspension cultures of *Orthosiphon aristatus* after treatment with yeast extract. *Phytochemistry* 30, 3267-3272.

Svensjo E, Bouskela E, Cyrino FZ. Bougaret S. (1997) Antipermeabolity effects of Cyclo 3 Frt in hamster with moderate diabetes. *Clin. Hemorheol. Microcirc*. 17, 385-388.

Szabo E, Thelen A, Petersen M. (1999) Fungal elicitor preparations and methyl jasmonate enhance rosmarinic acid accumulation in suspension cultures of *Coleus blumei*. *Plant Cell Rep.*18, 485-489.

Tada H, Murakami Y, Omoto T, Shimomura K, Ishimaru K. (1996) Rosmarinic acid and related phenolics in hairy root cultures of *Ocimum basilicum*. *Phytochemistry* 42, 431–434.

Thomas PA, Mukassabi A. (2014) Biological flora of the British Isles: *Ruscus aculeatus*. *J. Ecol.* 102, 1083-1100.

Thomassen YE, Joyce E. van der Welle JE, van Eikenhorst G, Leo A. van der Pol LA, A.M. Bakker WAM. (2012) Transfer of an adherent Vero cell culture method between two different rocking motion type bioreactors with respect to cell growth and metabolic rates. *Process Biochem.* 47, 288–296

Timm S, Nunes-Nesi A, Pärnik T, Morgenthal K, Wienkoop S, Keerberg O, Weckwerth W, Kleczkowski L, Ferni, AR, Bauwe H. (2008) A cytosolic pathway for the conversion of hydroxypyruvate to glycerate during photorespiration in *Arabidopsis*. *Plant Cell* 20, 2848–2859.

Tsuruga A, Terasaka K, Kamiya K, Satake T, Mizukami H. (2006) Elicitor-induced activity of isorinic acid 3'-hydroxylase, an enzyme catalyzing the final step of rosmarinic

acid biosynthesis in *Lithospermum erythrorhizon* cell suspension cultures. *Plant Biotechnol.* 23, 297–301.

Ulrich, B., Wiesner, W. and Arens, H. (1985) Large-scale production of rosmarinic acid from plant cell cultures of *Coleus blumei* Benth. In: Neumann KH, Barza W, Reinhard E. (Eds) Primary and Secondary Metabolism of Plant Cell Cultures. Springer, Berlin, Heidelberg, New York, pp: 293-303.

Upadhayay S, Jeena GS, Shikha, Shukla RK. (2018) Recent advances in steroidal saponins biosynthesis and *in vitro* production. *Planta*, 248, 519-544.

Vanhamel S, Masy C. (2011) Production of disposable bags: a manufacturer's report. In: Eibl R, Eibl D. (eds.) Single-use technology in biopharmaceutical manufacture. Wiley, Hoboken, pp: 113–134.

Verpoorte R, Contin A, Memelink J. (2002) Biotechnology for the production of plant secondary metabolites. *Phytochem. Rev*. 1, 13-25.

Vidal-Limon H, Sanchez-Muñoz R, Khojasteh A, Moyano E, Cusido RM, 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

Vogelsang K., Schneider B., M. Petersen M. (2006) Production of rosmarinic acid and a new rosmarinic acid 3-O-beta-D-glucoside in suspension cultures of the hornwort *Anthoceros agrestis* Paton. *Planta*, 223, 369–373.

Vostálová J, Zdarilová A, Svobodová A. (2010) *Prunella vulgaris* extract and rosmarinic acid prevent UVB-induced DNA damage and oxidative stress in HaCaT keratinocytes. *Arch. Dermatol. Res.* 302, 171–181.

Walker K, Fujisaki S, Long R, Croteau R. (2002) Molecular cloning and heterologous expression of the C-13 phenylpropanoid side chain-CoA acyltransferase that functions in taxol biosynthesis. *Proc. Natl. Acad. Sci. U.S.A.* 99, 12715-12720.

Warren A. (1973) Survey of *Ruscus aculeatus* on Bookham Common: the first two years. *London Nat.* 52, 93-103.

Weathers PJ, Towler MJ, Xu J. (2010) Bench to batch: advances in plant cell culture for producing useful products. *Appl. Microbiol. Biotechnol.* 85, 1339–1351.

Webb DA. (2010) Volume 5: Alismataceae to Orchidaceae (Monocotyledones). In: Flora Europea. Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, Webb DA. (Eds). Cambridge University Press.

Weremczuk-Jezyna I, Grzegorczyk-Karolak I, Frydrych B, Królicka A, Wysokinska H. (2013) Hairy roots of *Dracocephalum moldavica*: rosmarinic acid content and antioxidant potential. *Acta Physiol. Plant.* 35, 2095–2103.

Werner S, Greulich J, Geipel K, Steingroewer J, Bley T, Eibl D. (2014) Mass propagation of *Helianthus annuus* suspension cell in orbital shaken bioreactors: Improved growth rate in single-use bioreactors. *Eng. Life Sci.* 14, 676-684.

Wilson SA, Roberts SC. (2012) Recent advances towards development and commercialization of plant cell culture processes for the synthesis of biomolecules. *Plant Biotechnol. J.* 10, 249-268.

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

Wu CF, Hong C, Klauck SM, Lin YL, Efferth T. (2015) Molecular mechanisms of rosmarinic acid from *Salvia miltiorrhiza* in acute lymphoblastic leukemia cells. *J. Ethnopharmacol.* 176, 55-68.

Xiao Y, Gao S, Di P, Chen J, Chen W, Zhang L. (2009) Methyl jasmonate dramatically enhances the accumulation of phenolic acids in *Salvia miltiorrhiza* hairy root cultures. *Physiol. Plant.* 137, 1–9.

Xiao Y, Gao S, Di P, Chen J, Chen W, Zhang L. (2010) Lithospermic acid B is more responsive to silver ions (Ag^+) than rosmarinic acid in *Salvia miltiorrhiza* hairy root cultures. *Biosci. Rep.* 30, 33–40.

Xiao Y, Zhang L, Gao S, Saechao S, Di P, Jungfen C, Wansheng C. (2011) The c4h, tat, hppr and hppd genes prompted engineering of rosmarinic acid biosynthetic pathway in *Salvia miltiorrhiza* hairy root cultures. *PLoS ONE*, 6, e29713.

Xing BY, Dang XL, Zhang JY, Wang B Chen ZY, Dong JE. (2013) Effects of methyl jasmonate on the biosynthesis of rosmarinic acid and related enzymes in *Salvia miltiorrhiza* suspension cultures. *Zhiwu Shengli Xuebao/Plant Physiol. J.* 49, 1326-1332.

Xu H, Kim YK, Jin XJ, Lee SY, Park SU. (2008) Rosmarinic acid biosynthesis in callus and cell cultures of *Agastache rugosa* Kuntze. *J. Med. Plants Res.* 2, 237-241.

Yamamoto H, Yazaki K, Inoue K. (2000a) Simultaneous analysis of shikimate-derived secondary metabolites in *Lithospermum erythrorhizon* cell suspension cultures by high-performance liquid chromatography. *J. Chromatogr. B* 738, 3–15.

Yamamoto H, Inoue K, Yazaki K. (2000b) Caffeic acid oligomers in *Lithospermum erythrorhizon* cell suspension cultures. *Phytochemistry* 53, 651–657.

Yan Q, Shi M, Ng J, Wu JY. (2006) Elicitor-induced rosmarinic acid accumulation and secondary metabolism enzyme activities in *Salvia miltiorrhiza* hairy roots. *Plant Sci*. 170, 853-858.

Yang R, Shetty K. (1998) Stimulation of rosmarinic acid in shoot cultures of oregano (*Origanum vulgare*) clonal line in response to proline, proline analogue, and proline precursors. *J. Agric. Food Chem*. 46, 2888–2893.

Yendo AC, de Costa F, Gosmann G, Fett-Neto AG. (2010) Production of plant bioactive triterpenoid saponins: elicitation strategies and target genes to improve yields. *Mol. Biotechnol*. 46, 94-104.

Yeo PF (1980) *Ruscus* L. In: Flora Europea, Tutin TG, Burges NA, Chater AO, Edmonson JR, Heywood VH, Moore DM. (Eds) Vol. 5. pp: 73-74. Cambridge University Press. Cambridge

Yeo PF. (1968) A contribution to the taxonomy of the genus *Ruscus*. *Notes Roy. Bot. Gard. Edinburgh*. 28, 237-264.

Zamboni A, Gatto P, Cestaro A, Pilati S, Viola R, Mattivi F. (2009) Grapevine cell early activation of specific responses to DIMEB, a resveratrol elicitor. *BMC Genomics* 10, 363-376.

Zhang Z, Perozziello G, Boccazzi P, Sinskey AJ, Geschke O, Jensen KF. (2007) Microbioreactors for Bioprocess Development. *J. Assoc. Lab. Autom.* 12, 143-152.

Zhang Y, Yan YP, Wang ZZ. (2010) The Arabidopsis PAP1 transcription factor plays an important role in the enrichment of phenolic acids in *Salvia miltiorrhiza*. *J. Agric. Food Chem.* 58, 12168–12175.

Zhang S, Yan Y, Wang B, Liang Z, Liu Y, Liu F, Qi Z. (2014a) Selective responses of enzymes in the two parallel pathways of rosmarinic acid biosynthetic pathway to elicitors in *Salvia miltiorrhiza* hairy root cultures. *J. Biosci. Bioeng.* 117, 645-651.

Zhang Y, Yan YP, Wu YC, Hua WP, Chen C, Ge Q, Wang ZZ. (2014b) Pathway engineering for phenolic acid accumulations in *Salvia miltiorrhiza* by combinational genetic manipulation. *Metab. Eng*. 21, 71-80.

Zhao J, Davis LC, Verpoorte R. (2005) Elicitor signal transduction leading to production of plant secondary metabolites. *Biotechnol. Adv.* 23, 283-333.

Zhong JJ, Chen H, Chen F. (2001) Production of rosmarinic acid, lithospermic acid B and tanshinones by suspension cultures of ti-transformed *Salvia miltiorrhiza* cells in bioreactors. *J. Plant Biotechnol.* 3, 107-112.

Zhou LL, Zeng HN, Shi MZ, Xie DY. (2008) Development of tobacco callus cultures over expressing *Arabidopsis* PAP1/MYB75 transcription factor and characterization of anthocyanin biosynthesis. *Planta* 229, 37–51.

Ziv M. (1983) The stimulatory effect of liquid induction medium on shoot proliferation of *Ruscus hypophyllum* L. *Sci Hort.* 19, 387-394.

Zucker M. (1965) Induction of phenylalanine deaminase by light and its relation to chlorogenic acid synthesis in potato tuber tissue. *Plant Physiol.* 40, 779-784.

Zuluaga DL, Gonzali S, Loreti E, Pucciariello C, Degl'Innocenti ED, Guidi L, Alpi A, Perata P. (2008) *Arabidopsis thaliana* MYB75/PAP1 transcription factor induces anthocyanin production in transgenic tomato plants. *Funct. Plant Biol.* 35, 606-618.