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The effect of methyl jasmonate on triterpene and sterol metabolisms of *Centella asiatica*, *Ruscus aculeatus* and *Galphimia glauca* cultured plants

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Abstract

Considering that exogenously applied methyl jasmonate can enhance secondary metabolite production in a variety of plant species and that 2,3-oxidosqualene is a common precursor of triterpenes and sterols in plants, we have studied *Centella asiatica* and *Galphimia glauca* (both synthesizing triterpenoid secondary compounds) and *Ruscus aculeatus* (which synthesizes steroidal secondary compounds) for their growth rate and content of free sterols and respective secondary compounds, after culturing with or without 100 μM methyl jasmonate. Our results show that elicited plantlets of *G. glauca* and to a higher degree *C. asiatica* (up to 152-times more) increased their content of triterpenoids directly synthesized from 2,3-oxidosqualene (ursane saponins and nor-seco-friedelane galphimines, respectively) at the same time as growth decreased. In contrast, the free sterol content of *C. asiatica* decreased notably, and remained practically unaltered in *G. glauca*. However, in the case of *R. aculeatus*, which synthesizes steroidal saponins (mainly spirostane type) indirectly from 2,3-oxidosqualene after the latter is converted to the plant phytosterol-precursor cycloartenol, while the growth rate and free sterol content clearly decreased, the spirostane saponine content was virtually unchanged (aerial part) or somewhat lower (roots) in presence of the same elicitor concentration. Our results suggest that while methyl jasmonate may be used as an inducer of enzymes involved in the triterpenoid synthesis downstream from 2,3-oxidosqualene in both *C. asiatica* and *G. glauca* plantlets, in those of *C. asiatica* and *R. aculeatus* it inhibited the enzymes involved in sterol synthesis downstream from cycloartenol.
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1. Introduction

Perhaps one of the most diverse groups of plant secondary metabolites are terpenoids, which are also found in microorganisms and animals. Included in this group are the triterpenes (C_{30}) and sterols (C_{18}–C_{29}), whose structurally diverse molecules proceed from a common precursor, the squalene. Metabolic pathways originating from squalene form an extensive net of compounds with defined branching points that diversify the end products, including compounds with primary roles in membrane architecture (sterols such as sitosterol, stigmasterol and campesterol) as well as a variety of secondary metabolites specific to each plant species (Grunwald, 1980; Seigler, 1998).

Centella asiatica (L.) Urban is a herbaceous plant with great medicinal value belonging to the Apiaceae family. Notable bioactive compounds of *C. asiatica* are the triterpene saponins madecassoside and asiaticoside, with their respective ursane type sapogenins madecassic and asiatic acid. As shown in Fig. 1, these compounds, referred to as centellosides, proceed from the cyclisation of 2,3-oxidosqualene by a specific oxidosqualene cyclase (OSC), β-amyrin synthase.

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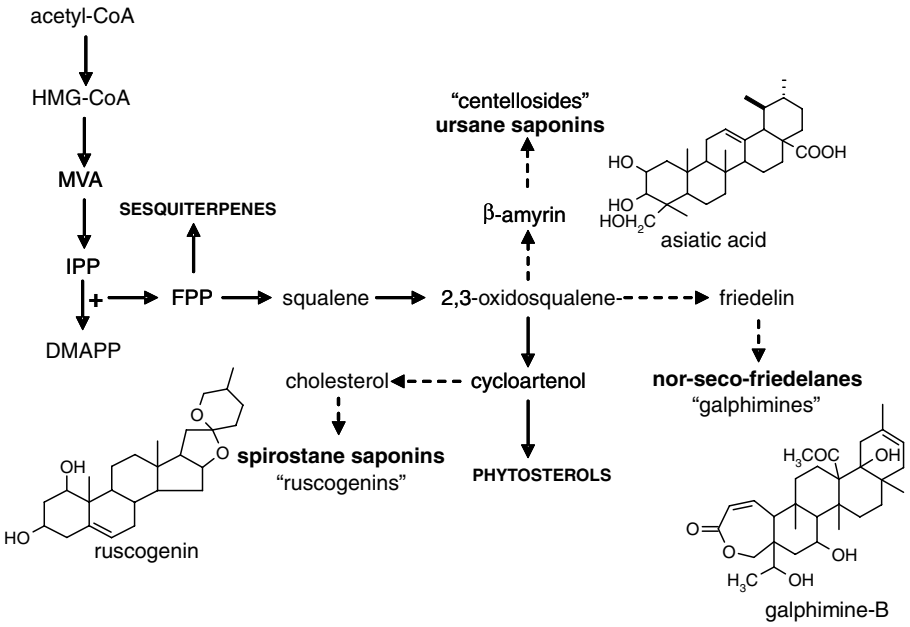

Fig. 1. Relationships between sterol and triterpene biosynthesis in *C. asiatica*, *R. aculeatus* and *G. glauca*.

Ruscus aculeatus L. (butcher's broom) of the Liliaceae family is a small evergreen shrub whose active components are steroidal saponins. Most of these are of the spirostane type, including the aglycones neoruscogenin and ruscogenin. Like triterpene saponins in *C. asiatica*, these compounds proceed from the cyclisation of 2,3-oxidosqualene, but their pathways differ in that steroidal saponins are formed via cycloartenol, a precursor they share with phytosterols (Fig. 1). Despite the interest of this medicinal plant, no studies on the biosynthesis of its active principles have been carried out until now.

Galphimia glauca Kav (Malpighiaceae) is a small evergreen tropical shrub extending from Mexico to Guatemala in Central America. Its main active component is galphimine-B, a nor-seco-triterpene of the friedelane type, with sedative and spasmolytic activities. Although the complete galphimine-B biosynthesis is not known, Corsino et al. (2000) have reported that the key branching point in the pathway to friedelane terpenoids is the cyclisation of 2,3-oxidosqualene by OSC. Similar to the biosynthesis of *C. asiatica* triterpenoids (Fig. 1), and differing from that of *R. aculeatus* steroidal saponins, the route to friedelanes is not through cycloartenol. Corsino et al. (2000) have observed that the conversion of 2,3-oxidosqualene to friedelin takes place in the leaves of *Maytenus aquifolium* and *Salacia campestris*, in the same way that galphimine-B is biosynthesised in the aerial parts of *G. glauca* (Lara-Ochoa et al., 2005).

Generally, when plant cells perceive environmental changes via specific receptors or perception mechanisms, they generate biological responses through specific signal transduction. Jasmonic acid and its methyl ester (methyl jasmonate, MeJA) have been reported to play an important role in a signal transduction process that regulates defense genes in plants (Farmer and Ryan, 1990). However, although exogenously applied MeJA is widely used in plant cell cultures to activate secondary metabolism, there are surprisingly few studies about its impact on plant growth, considering that jasmonates have a variety of biological activities, including inhibition of seed and pollen germination (Feys et al., 1994; McConn and Browse, 1996) or inhibition of root growth and photosynthetic apparatus (Staswick et al., 1992; Reinbothe et al., 1993a,b; Rossato et al., 2002).

Recently, Kim et al. (2004) have studied the accumulation of asiaticoside in whole plant cultures of *C. asiatica*, reporting an enhancement of its production by MeJA treatment. However, no elicitation studies with MeJA have been done with whole *R. aculeatus* and *G. glauca* plants, although it is of interest that Osuna et al. (1999) observed a higher production of galphimine-B in *G. glauca* calli after increasing the concentration of 2,4-dichlorophenoxyacetic acid (2,4-D) in the culture medium.

With respect to sterol metabolism it is known that the amount of sterols is generally constant among plant species (Benveniste, 2004). Triterpene and sterol biosynthesis begins with the conversion of farnesyl diphosphate into squalene (2,3-oxidosqualene), which determines the channelling of the isoprenoid pathway into the branches that produce phytosterols (Fig. 1). The sterol pathway involves a sequence of more than 30 enzyme-catalyzed reactions, all of which are membrane linked. Nothing is known about the catabolism of plant sterols. Upregulation of 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) in transgenic tobacco, corn and tomatoes results in the accumulation of only cycloartenol, rather than sterols. Potential strategies for sterol production are, however, likely to focus on overexpression of terminal enzymes in the pathway (Hartmann, 1998).

In this study we show that the production of potentially downstream 2,3-oxidosqualene compounds, such as sterols with a primary role and pharmacologically active triterpenoids and steroids, can be affected in the aerial part and roots of *C. asiatica*, *R. aculeatus* and *G. glauca* plantlets by the addition of 100 μM methyl jasmonate to the culture medium.

2. Results and discussion

2.1. Characterization of cultured plants

As shown in Table 1, in all cases the growth capacity of the treated plantlets declined in comparison with the controls. In the case of *Ruscus*, the reduction of growth mainly affected the roots. After 2 weeks of MeJA treatment, the fresh weight of elicited roots was 20% lower than those of the control, and after 4 weeks the reduction of root growth was more than 50%, due to a total inhibition of root growth during weeks 2–4. Furthermore, these roots also showed abnormal morphology traits, such as necrosis of the root apex (Fig. 2).

C. asiatica plantlets treated with MeJA also showed a considerable reduction of growth (Table 1), with the fresh weight of aerial parts reduced by over 50% and a decline in root growth of more than 70%. As in the *Ruscus* cultures, root growth was completely blocked during weeks 2–4. In addition, the elicited *Centella* plantlets showed necrosis symptoms in leaves and roots at the end of the culture period (Fig. 2).

The inhibitory effect of MeJA on the development of cultured *G. glauca* plantlets (compared to controls) was even more evident than in *Centella* and *Ruscus* cultures. After 2 weeks of culture, treated *Galphimia* plantlets not only showed a drastic reduction of growth (Table 1), but also accelerated symptoms of senescence and necrosis (Fig. 2), mainly in the roots, which led to the plants' death before the end of the culture period (4 weeks).

The effect of MeJA or of any elicitor (biotic or abiotic) is dependent on a number of factors which may interact. These include the elicitor's specificity and concentration, the duration of treatment and the growth stage of the culture (Holden et al., 1988). Researchers have frequently used MeJA at a concentration of 100 μM to increase secondary metabolism in in vitro cultures (Ketchum et al., 1999; Cusidó et al., 2002; Palazón et al., 2003; Kim et al., 2004), with the plant cells or organs coming into direct contact with the elicitor. In contrast, in our plant cultures only the roots were directly exposed to MeJA, which was probably why its effects on root development were more pronounced. In this context, it is worth noting that jasmonates inhibit the growth of seedlings, roots and cell division. As Koda et al. (1996) have reported, growth inhibition by MeJA appears to be caused mainly by the disruption of cortical microtubules, a phenomenon ubiquitous in plants.

2.2. Effects of the elicitor on specific secondary compound content

In *C. asiatica* the levels of the main active compounds, the triterpene saponins madecassoside and asiaticoside

Table 1
Effect of methyl jasmonate on plant growth measured as fresh (FW) and dry weight (DW) after 2 or 4 weeks of treatment

Growth	Week	Control		Elicited	
		AP	Roots	AP	Roots
Ruscus aculeatus					
FW	2	1.257 ± 0.093	4.818 ± 0.136	1.032 ± 0.098	4.231 ± 0.237
	4	1.915 ± 0.108	6.702 ± 0.288	1.448 ± 0.124	3.271 ± 0.338
DW	2	0.347 ± 0.013	0.735 ± 0.088	0.248 ± 0.023	0.595 ± 0.011
	4	0.435 ± 0.049	0.953 ± 0.078	0.357 ± 0.021	0.516 ± 0.057
Centella asiatica					
FW	2	4.506 ± 0.109	5.695 ± 0.366	3.391 ± 0.428	2.514 ± 0.398
	4	8.083 ± 0.437	7.116 ± 0.387	5.198 ± 0.511	2.381 ± 0.255
DW	2	0.612 ± 0.067	0.524 ± 0.044	0.412 ± 0.038	0.242 ± 0.022
	4	0.918 ± 0.088	0.551 ± 0.029	0.745 ± 0.066	0.191 ± 0.041

Growth	Week	Control	Elicited
		WP	WP
Galphimia glauca			
FW	2	0.277 ± 0.022	0.167 ± 0.024
	4	nd	nd
DW	2	0.057 ± 0.010	0.029 ± 0.011
	4	nd	nd

Each value is the mean of 8–10 determinations ± SE.
AP, aerial part; WP, whole plant.

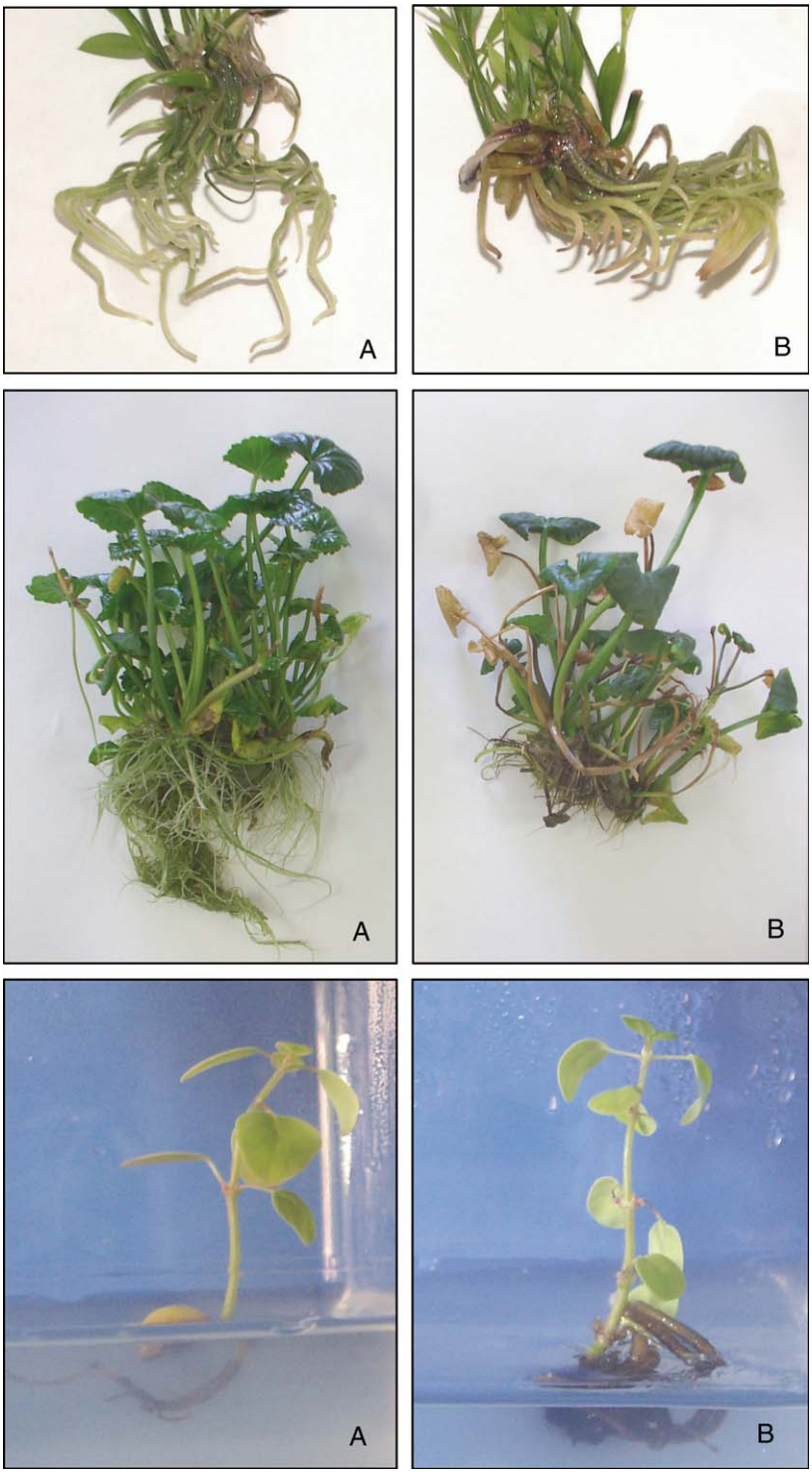

Fig. 2. Effects of methyl jasmonate on developing plantlets of *R. aculeatus* (up) and *C. asiatica* (center) and *G. glauca* (down). (A) Control plant, (B) elicited plant.

and their respective ursane sapogenins madecassic and asiatic acid, were determined in the aerial part and roots of both control and elicited plantlets after two and four weeks of culture. As shown in Fig. 3, in the aerial part of untreated plantlets the main compound was found to be asiaticoside followed by madecassoside and then asiatic acid and madecassic acid, while in the roots the asiaticoside content was very similar to or lower than that of madecassoside. The triterpene saponin content was always significantly ($p < 0.001$; *t*-test) higher in the aerial part than in the roots of plantlets, especially at 2 weeks of culture. The triterpenoid pattern in *Centella* differs according to

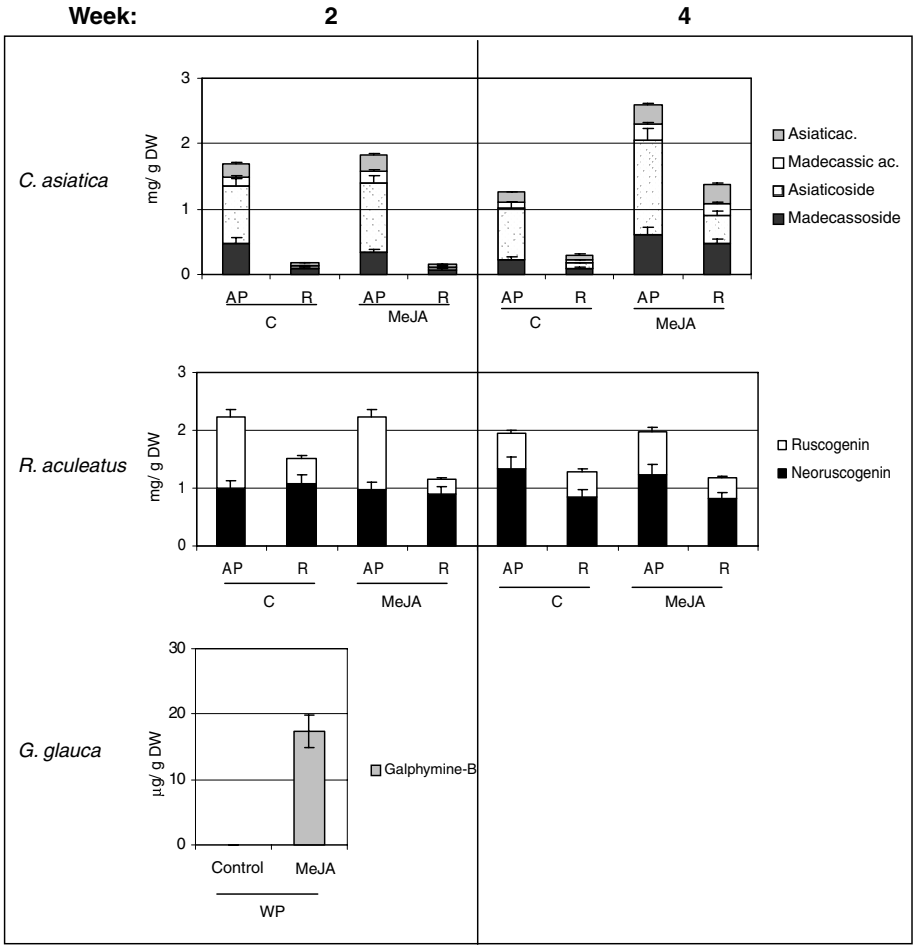

Fig. 3. Effects of methyl jasmonate on terpenoid secondary compound production of *C. asiatica*, *R. aculeatus* and *G. glauca* plantlets grown for 2 and 4 weeks with and without the elicitor (control). Each value is the mean of 3–6 determinations. Bars represent ±SE. AP, aerial part; R, roots; WP, whole plant; C, control; MeJA, methyl jasmonate.

the species and culture region (Rouillard-Guellec et al., 1997), but a survey of the bibliography shows both asiaticoside and madecassoside as the predominant compounds in whole plants, although very little is known about their content in roots. As shown in Fig. 4, in our *Centella* cultures a similar centelloside pattern was found in the aerial part and roots of plants. This pattern was not changed by elicitor treatment and in all samples the ratios of the different centellosides were asiaticoside > madecassoside > asiatic acid > madecassic acid.

Compared to the controls, the content of triterpene saponins and ursane sapogenins in elicited *C. asiatica* plantlets increased only slightly in the aerial part at week 2 and increased significantly ($p < 0.001$) in both the aerial part and roots at week 4. At the end of the culture period, as can be deduced from the values in Fig. 3, the level of

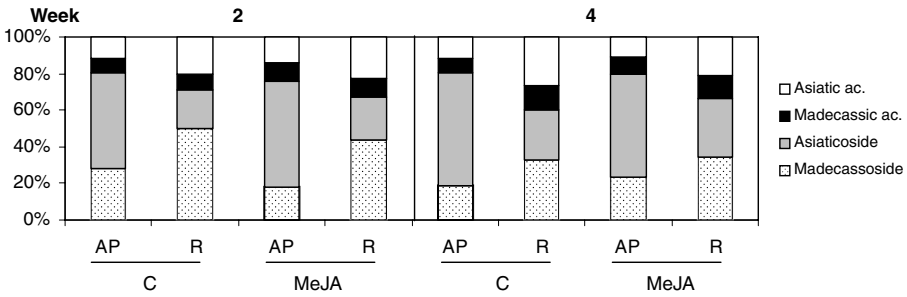

Fig. 4. Effects of methyl jasmonate on centelloside patterns (expressed as percentage of total centellosides) of *C. asiatica* plantlets grown for 2 and 4 weeks with and without the elicitor (control). Each value is the mean of 3–6 determinations. Bars represent ±SE. AP, aerial part; R, roots; C, control; MeJA, methyl jasmonate.

madecassoside, asiaticoside, madecassic acid and asiatic acid in the aerial part was 2–3-fold higher, and 4–6-fold higher in the roots. These data suggest that the longer the elicitor was in contact with the roots, the greater the increase in all these triterpenoid compounds in both the aerial part and roots of cultured plantlets. The relatively high asiaticoside content in our elicited roots differs from previous observations that have suggested its production in *C. asiatica* is tissue-specific, with synthesis occurring mainly in the leaves (9.56 mg/g DW) and very little in the roots (0.17 mg/g DW) of whole plants cultured in presence of MeJA 100 μM (Kim et al., 2004). This difference is probably due to the fact that in our study only the roots were in contact with the elicitor.

In contrast, the production of steroidal saponins ruscogenin and neoruscogenin in the aerial part and roots of elicited *R. aculeatus* plantlets (Fig. 3) after 2 and 4 weeks was similar have any specific effect on the different metabolic steps of the biosynthesis of both these steroidal saponins in our *Ruscus* cultures. MeJA concentrations higher than 100 μM produced several symptoms of necrosation in the roots and the cultured plantlets died before the end of the experiment (data not shown). Considering the lack of previous studies on the biosynthesis of these active compounds at the level of the whole plant, it was interesting to find that neoruscogenin was the main steroidal saponin to accumulate in both aerial parts and roots of the *R. aculeatus* plantlets. The only exception to this general observation was at week 2, when the aerial parts showed similar amounts of both steroidal saponins. Throughout the culture period, the content of steroidal saponins was higher in the aerial parts than in the roots. As reported by Palazón et al. (2006), *Ruscus* callus cultures show a limited capacity to biosynthesize steroidal saponins, but when the calli regenerate aerial shoots it increases drastically. This observation, together with our results, suggests that the synthesis of steroidal saponins ruscogenin and neoruscogenin occurs mainly in the leaves of *R. aculeatus*.

As previously mentioned, the inhibitory effect of MeJA on the development of cultured *G. glauca* plantlets was more evident than in both *Centella* and *Ruscus* cultures, and consequently, studies on the biosynthesis of their main active compound, the nor-seco-friedelane galphimine-B, had to be done using the whole plant. The lower MeJA concentration (50 μM) did not elicit the biosynthesis of the secondary compound of interest, since the nor-seco-friedelane galphimine-B content was not detectable, as in the control cultures (data not shown). In contrast, the presence of 100 μM MeJA in the culture medium of *G. glauca* plantlets caused a relatively considerable increase in their galphimine-B content (Fig. 3).

From the values depicted in Fig. 3, it can be deduced that the elicitor action specifically affected some metabolic steps of the ursane saponin (asiaticoside and madecassoside) and nor-seco-friedelane (galphimine-B) biosynthesis in the respective *C. asiatica* and *G. glauca* cultures, but not the steroidal saponins ruscogenin and neoruscogenin of *R. aculeatus*, which are not directly formed from 2,3-oxidosqualene but via its previous conversion to cycloartenol (Gross et al., 1985; Combarieu et al., 2002).

2.3. Effects of the elicitor on the content of free sterols

From the data shown in Fig. 5, it may be inferred that under the conditions of this work the level of free sterols with a primary role (sitosterol, stigmasterol and campesterol) and that of cholesterol was affected mainly by differences in biosynthetic activity among the cultured plantlets. Cholesterol, which appears not to have any primary role in plants, is found in small amounts in many plant species, where it serves as a precursor for other steroid derivatives (Heftman, 1984). When comparing sterol content in *C. asiatica* plantlets cultured for 2 and 4 weeks with and without MeJA, it can be deduced that the free sterol pattern in both aerial parts and roots was stigmasterol + campestrol > sitosterol while very small quantities of cholesterol were detected only in the roots of untreated plantlets (≈10 μg/g DW). Additionally, when considering the total content of free sterols (the sum of the sterols measured), our results show that in both control and elicited cultures it was higher in the aerial part than in the roots, and this was especially evident after 2 weeks of culture in plantlets grown without MeJA. It is also clear that the presence of MeJA in the culture medium reduced the free sterol levels, which were lower in the aerial parts and roots of all elicited plantlets than in the controls after 2 and 4 weeks of culture (2.4- and 1.3-fold at week 2, respectively, and 1.8- and 1.3-fold at week 4, respectively). The decrease in free sterols could be the result of elicited plantlets having a high capacity to synthesize triterpene saponins, and also might be explained by MeJA acting differently on the two metabolic pathways considered. This is supported by previous results reported by Kim et al. (2005a,b) which show an activation of β-amyrin synthase (*Cab*AS), a key enzyme in the biosynthesis of triterpene saponins, in *C. asiatica* plants elicited by MeJA but an inhibition of the expression of cycloartenol synthase (*Ca*CYS), the enzyme responsible for the first step in sterol biosynthesis. This also concurs with the fact that both sterols and triterpene saponins are synthesised from a common precursor, 2,3-oxidosqualene, via two different pathways (Fig. 1).

In the case of *R. aculeatus* (Fig. 5), the total free sterol content of plantlets grown without MeJA was always clearly higher in the roots than in the aerial part, and as observed in *C. asiatica* plantlets, the addition of the elicitor decreased this content in both the aerial part and roots throughout the experiment. This decrease could also be due to the aforementioned inhibition of the CYS enzyme expression by MeJA, although the elicitor did not affect the capacity of *Ruscus* to produce both ruscogenin and neoruscogenin in the aerial part and only slightly in the roots (Fig. 3). Moreover, considering the proposal that

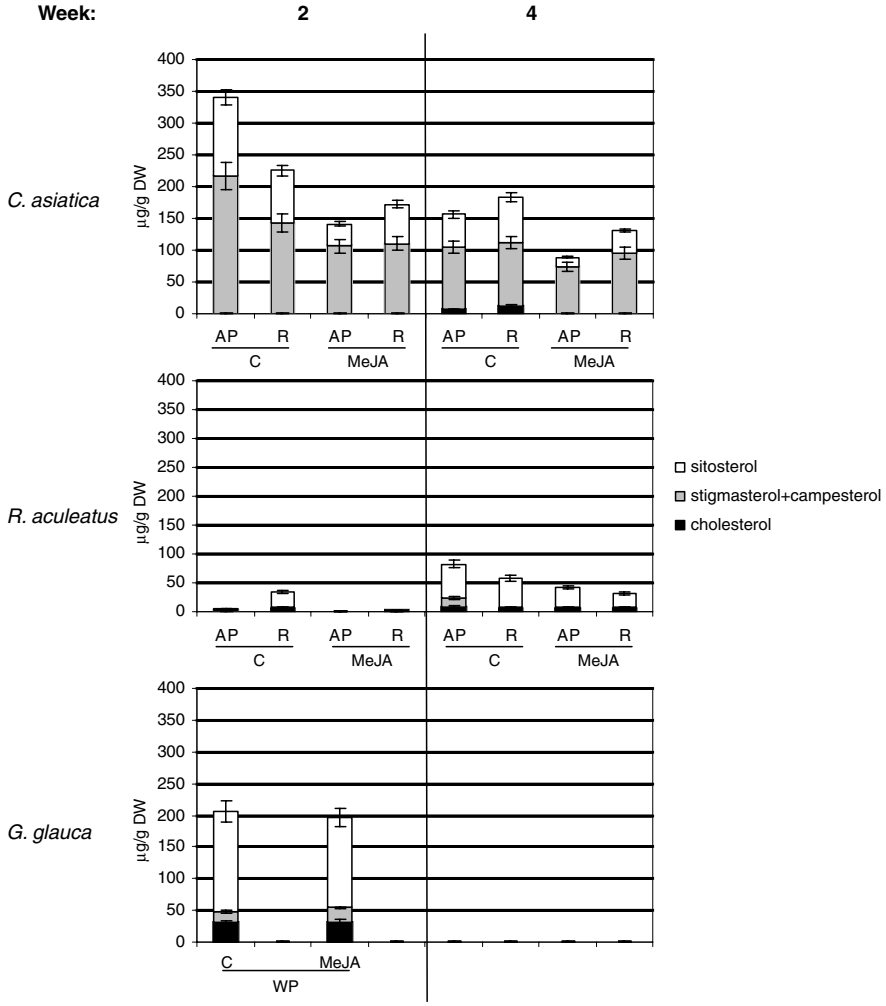

Fig. 5. Effects of methyl jasmonate on free sterol production of *C. asiatica*, *R. aculeatus* and *G. glauca* plantlets grown for 2 and 4 weeks with and without the elicitor (control). Each value is the mean of 3–4 determinations. Bars represent ±SE. AP, aerial part; R, roots; WP, whole plant; C, control; MeJA, methyl jasmonate.

Ruscus spirostane saponins (ruscogenins) are synthesized from the key precursor in plant sterol synthesis, cycloartenol, in a biosynthetic route including cholesterol (Gross et al., 1985; Combarieu et al., 2002), it was of interest that cholesterol, usually a minor sterol in most plant species, was present at relatively high levels in our *Ruscus* plantlets, comprising approximately 7–10% of total free sterol content.

The total free sterol content in *G. glauca* plants after 2 weeks of culture (Fig. 5) was very similar in plantlets grown with and without MeJA, always in the pattern of sitosterol > cholesterol > stigmasterol+campesterol. The level of cholesterol in our *Galphimia* plantlets was considerable, comprising approximately 15% of total free sterol content, which places *G. glauca* among the small number of plant species in which cholesterol is more than a minor sterol. In contrast with *R. aculeatus* plantlets, *G. glauca* cultures appeared to show an activation of the enzymes involved in the biosynthesis of galphimine-B, a nor-seco friedelane triterpenoid directly synthesized from 2,3-oxidosqualene, probably due to the presence of MeJA, since it was only detected in plantlets grown in presence of the elicitor (≈17 µg/g DW).

Our results demonstrate for the first time how in *R. aculeatus* and *G. glauca* cultured plants the production of both free sterols and specific secondary compounds (the steroidal saponins ruscogenin and neoruscogenin and the triterpenoid galphimine-B, respectively) can be affected by exogenous application of MeJA to the culture medium. We have also shown that the production of centellosides (the triterpene saponins madecassoside and asiaticoside, and their respective ursane type sapogenins madecassic and asiatic acids) clearly increased in both the aerial part and roots of *C. asiatica* plantlets in response to the presence of MeJA, although the triterpenoid pattern was not affected. Finally, considering that 2,3-oxidosqualene is a precursor of all the compounds studied in this experiment, another explanation for their contents in our *Centella*, *Ruscus* and *Galphimia* cultures could be that the reduced growth induced by MeJA feeds the precursors

for secondary metabolite production if they were endogenously limiting.

3. Experimental

3.1. General experimental procedures

Madecassoside, asiaticoside, asiatic acid, madecassic acid, ruscogenin, neoruscogenin and campesterol were obtained from CromaDex Inc. (USA). β-Sitosterol, stigmasterol and cholesterol were obtained from Sigma–Aldrich (USA). The standard compound galphimine-B was isolated from aerial parts of *G. glauca* as described in Osuna et al. (1999). Acetonitrile and MeOH (HPLC grade) and other chemicals used (analytical grade) were obtained from commercial sources without further purification. Methyl jasmonate was purchased from Sigma–Aldrich (USA).

The HPLC system consisted of a Pharmacia LKB-HPLC 2150 pump (Pharmacia-LKB, Uppsala, Sweden), an LC 2152 Controller (Pharmacia-LKB), an HPLC autosampler 465 (Kontron Instruments), a 2141 Variable Wavelength Monitor (Pharmacia-LKB) and a Biodacs integrator (Pharmacia-LKB).

3.2. Plant material

C. asiatica plants were obtained from seeds provided by the School of Pharmacy, Second Military Medical University (Shangai, China). *R. aculeatus* plants were grown in the greenhouse of the Faculty of Pharmacy of the University of Barcelona. A voucher specimen is kept in the Botanical Section of the Faculty of Health and Life Sciences of the University of Pompeu Fabra (Barcelona, Spain). Mature seeds of *G. glauca* were collected from wild plants in Guanajuato, Mexico, and adapted to the culture conditions of the Plant Physiology Laboratory of the University of Barcelona. Voucher specimens were deposited at the Instituto Mexicano del Seguro Social Herbarium (IMSSM) under the code numbers 8645 and 8646.

Nodes from plants of *C. asiatica*, embryos of *R. aculeatus* (Moyano et al., 2006) and seeds of *G. glauca* were cultured on MS medium (Murashige and Skoog, 1962) solidified with 0.27% of phytagel. When the plants were 4–7 cm high they were used as experimental material for controls and elicitor treatment.

3.3. Treatment with methyl jasmonate

Plantlets of *C. asiatica*, *R. aculeatus* and *G. glauca* obtained as described above were cultured for 4 weeks in MS solid medium (Murashige and Skoog, 1962) with and without methyl jasmonate (MeJA). Different concentrations of MeJA (50, 100 and 200 μM) were preliminarily assayed. Since 50 μM was not enough to produce elicitation in the three species, and 200 μM produced several symptoms of necrosation in the roots after 2 weeks of treatment, we chose to carry out our studies with 100 μM of MeJA. Control plants (grown without MeJA) and treated plants were sampled after 2 and 4 weeks of treatment. At each harvesting, 8–10 plants were separated into roots and aerial parts, except in the case of *G. glauca* cultures whose limited root development forced us to collect the whole plant. Each plant fraction was washed, weighed for fresh weight (FW) and lyophilised to obtain dry weight (DW) and analysed the triterpenes and free sterols.

3.4. Extraction and analysis of specific secondary compounds

To determine the quantity of madecassoside, asiaticoside, madecassic acid and asiatic acid, aerial parts and root, were taken separately from control and elicited *C. asiatica* plants at 2 and 4 weeks of culture. The samples were lyophilized and powdered, and 1 g was extracted as reported by Bonfill et al. (2006). The chromatographic analysis was performed at room temperature with a Spherisorb 5μ ODS2 (250 × 4 mm) column (Waters) using gradient elution, the eluents being acetonitrile (A) and water with ammonium dihydrogenphosphate 10 mM (pH 2.5 with ortophosphoric acid) (B) according to the following profile: 0–15 min, 80% A; 15–30 min, 62% A; 30–37 min, 30% A; 37–45 min, 80% A. The flow rate was 1 ml/min and the detector was set at 214 nm.

To determine the quantity of ruscogenin and neoruscogenin, aerial parts and roots were taken separately from control and elicited *R. aculeatus* plants at 2 and 4 weeks of culture. Lyophylized powdered samples (500 mg) were extracted as reported by Palazón et al. (2006) for HPLC analysis.

To determine the quantity of galphimine-B, whole plants were taken from control and elicited *G. glauca* plants at 4 weeks of culture. Lyophilized samples were extracted with MeOH (75 ml) during 72 h shaking and sonicated for 2 min. After filtration the solvent was evaporated to dryness and the residue was dissolved in 3 ml of MeOH and applied to HPLC. Quantification of galphimine-B was performed at room temperature using a Chromolit Performance RP_{18} column (10 cm length). The mobile phase, consisting of acetonitrile/water (35:65), was eluted isocratically at a constant flow rate of 1.7 ml/min. The detector was set at 232 nm. The identity and purity of the galphimine-B standard were confirmed by comparison with published spectral data.

3.5. Extraction and analysis of free sterols

The extraction method used was the same as that used for the extraction of ursane saponins from *C. asiatica*, as described above. The chromatographic analysis is based on the method of Manzi et al. (1996) but with some modifications. The separation was carried out isocratically at room temperature using a HYPERSIL 5μ ODS (250 × 4.6 mm) column (Thermo electron corporation)

with MeOH as the mobile phase. The flow rate was 0.9 ml/min and the detector was set at 210 nm.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.phytochem.2006.06.025.

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