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Coumarin Compounds in *Coronilla scorpioides* Callus Cultures

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Coronilla scorpioides (L.) W.D.J. Koch is known for producing several compounds with pharmaceutical interest, such as the hydroxycoumarins umbelliferone, scopoletin and daphnoretin, the dihydrofuranocoumarin marmesin, and the furocoumarin psoralen. *In vitro* callus cultures of *C. scorpioides* were established from hypocotyl, leaf, stem internode and root explants in order to evaluate the possibility of *in vitro* production of these active secondary metabolites. Calli were obtained with high frequency from all the explant types both in B5 and MS medium. However, after the third subculture, B5 medium, giving the best results, was selected for subsequent transfers. Homogeneous calli were kept either in darkness or in light. Chemical analyses showed that scopoletin and the intermediate products of the biogenetic pathway of psoralen, umbelliferone and marmesin, were always present in the calli and excreted into the media, while daphnoretin was never detected. Light seems to be a prerequisite for psoralen biosynthesis. Root-derived calli produced a significantly higher amount of psoralen (137.5 μg g^{-1} DW). Principal component analysis showed that umbelliferone, marmesin and psoralen contents are related to variables associated with different explant types.

Keywords: *Coronilla scorpioides*, *In vitro* cultures, Coumarins, Psoralen.

Coronilla scorpioides (L.) W.D.J. Koch (Fabaceae) is an annual species widely distributed in the Mediterranean basin and in other part of the world [1]. The seeds of several *Coronilla* species are known to contain, in addition to cardiac glycosides, several compounds with considerable pharmaceutical interest: the hydroxycoumarins umbelliferone, scopoletin and daphnoretin, and the furocoumarin psoralen [2]. Umbelliferone and scopoletin are active against *Mycobacterium tuberculosis* [3], while daphnoretin is a promising preventive and chemotherapeutic agent in the management of osteosarcoma [4]. Psoralen is used as a photosensitizing drug for the treatment of psoriasis and vitiligo [3]. Moreover, psoralen is known to cause cross-linking of DNA strands, leading to significant distortions in the DNA helix, creating sites that are recognized by DNA repair enzymes and is, therefore, a useful tool in DNA structural analyses [5].

In a previous work we investigated the coumarin contents of the seeds and vegetative organs of *C. scorpioides* [6]. The three hydroxycoumarins and the dihydrofuranocoumarin marmesin were detected, although not ubiquitously in the different plant organs. On the other hand, psoralen was detected in each part examined. The seeds are the accumulation organ and their content in *C. scorpioides* was 11.45 *vs.* 7.81 mg g^{-1} dry weight reported in *Psoralea corylifolia* seeds, a known source of psoralen [7]. Considering these results, it was considered to be of value to evaluate the *in vitro* production of these active secondary metabolites. Biotechnological approaches, specifically plant tissue cultures, represent a good alternative to traditional agriculture for industrial production of bioactive plant metabolites [8,9].

In this study, *in vitro* callus cultures of *C. scorpioides* were established from hypocotyl, leaf, stem internode and root explants and the production of coumarin compounds was investigated.

Calli were obtained with high frequency (about 90% of explants) from all the explant types (hypocotyl, leaf, stem internode, and root); no significant differences were revealed among Murashige and Skoog (MS) [10] and Gamborg *et al.* (B5) [11] media (Table 1).

Table 1: Effects of explant source and basal medium on callus induction and callus formation. The data are the percentage and the means ± SD of two set of experiments (100 explants per treatment).

explant source	callus induction (%)		callus formation	
	B5	MS	B5	MS
hypocotyl	94	95	3.9±0.1[b]	1.9±0.3[a]
leaf	95	98	4.0±0.0[b]	2.1±0.3[a]
stem internode	94	92	3.7±0.5[b]	2.0±0.3[a]
root	89	87	3.6±0.2[b]	1.7±0.5[a]

Mean values followed by the same letter are not significantly different at $P<0.05$ according to Tukey's test.

However, in the successive subcultures, B5 medium stimulated a significantly higher production of callus than MS medium, independently of the explant source, with a mean of 3.8 compared with 1.9, respectively (Table 1). After the third subculture, the medium B5, which gave the best results, was selected for subsequent transfers.

In order to investigate the influence of light on callus metabolism, homogeneous calli were kept either in darkness or in cool white fluorescent light (36 μmol m^{-2} s^{-1}) with a 16 h photoperiod. The calli grown in darkness appeared yellow and watery; under light conditions, the calli gradually became green and friable, independently of the organ explanted. From the third subculture onward, callus samples were evaluated for fresh weight. The calli showed a typical growth curve with a log phase from day 8 to day 21. The calli grown in light conditions showed a lower growth rate compared with the calli kept in darkness (Figure 1).

All the calli showed the characteristic coumarin fluorescence under UV light, clearly visible also in the surrounding growth medium, as a result of excretion of secondary products from the cells [12] (Figure 2).

Calli and the respective media were analyzed for their content of coumarin compounds. Extractions were carried out taking into account that in Fabaceae plants, coumarins may be present in both free and bound forms [2,13]. Moreover, preliminary research on wild *C. scorpioides* showed that considerable amounts of psoralen

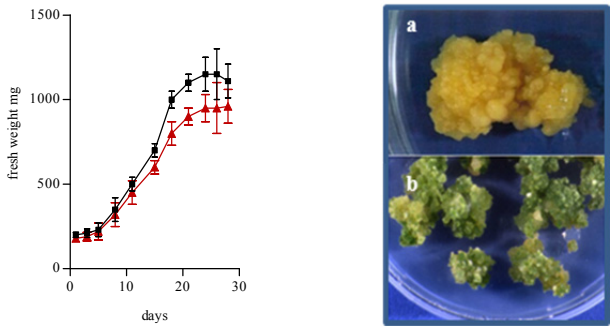

Figure 1: Calli of *C. scorpioides* grown in dark (a) and light (b) conditions and respective growth curves (black-dark, red-light). Values are expressed as mean ± SD of three replicates.

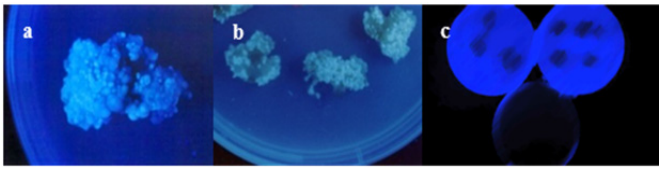

Figure 2: Calli of *C. scorpioides* grown in dark (a) and light (b) conditions, and subculture media (c) observed under ultraviolet light.

occur in bound form [6], and, therefore, an extraction procedure involving acid hydrolysis was followed for maximum coumarin recovery. Table 2 shows the coumarins identified in calli obtained from different explants, grown either in darkness or in light conditions, and in the respective subculture media. Scopoletin was always present in the calli and excreted in the media, while daphnoretin was never detected. Umbelliferone and marmesin, the intermediate products of the biogenetic pathway of the furocoumarin psoralen [14], were produced by all the callus types, both in dark and light conditions, while light seemed to promote their natural excretion from the cells. In regard to psoralen, light seems to be a prerequisite for psoralen biosynthesis. Indeed psoralen was present only in tissue cultures grown in light conditions. Formerly, it was hypothesized that light could affect psoralen biosynthesis, since key steps in furanocoumarin biosynthesis in *Ruta graveolens* involved chloroplast enzymes [14,15]. Recently, it has been reported that an UV-B light-induced overexpression of cyp98a22, a *p*-coumaroyl ester 3'-hydroxylase involved in the synthesis of coumarins, clearly produced an increase in the concentration of furanocoumarins, in support of the presumed role of light in furanocoumarin biosynthesis [16].

The psoralen contents are showed in Figure 3. As regards the total mean amount of psoralen, i.e. present in the calli and excreted in the media, calli derived from root explants showed a significantly higher (P<0.001) producing capability (137.5 µg g^{-1} DW). The calli obtained from hypocotyl, leaf and stem internode showed mean total psoralen levels in the range from 68.3 to 96.7 µg g^{-1} DW, and the differences were not statistically significant (P>0.05). Analysis of the media showed that the excretion rate of psoralen seemed to be correlated to the type of starting material used for callus induction, with the media of the leaf-derived calli being the richest for psoralen. Although it is well-known that the type of explant has an important influence on different processes, there are no data on its potential influence on the *in vitro* excretion process.

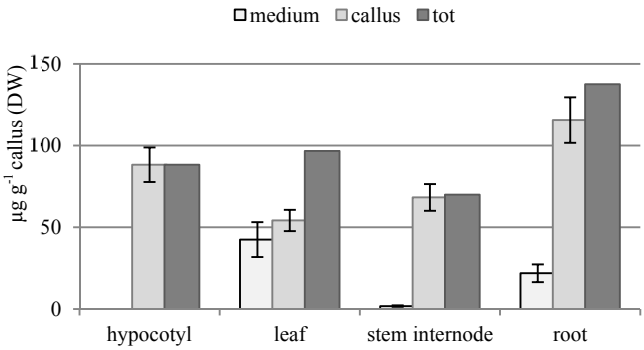

Figure 3: Psoralen contents (means ± SD) in calli from different explants and in their corresponding media: the data are respectively summed (tot) to represent the psoralen producing ability. Analyses were performed on three different samples of each callus type.

The umbelliferone, marmesin and psoralen contents in the calli were subjected to principal component analysis. Two principal components were extracted explaining up to 95.9 % of the total variance. Figure 4 shows the corresponding score plots.

At a glance, a clear separation between the calli derived from different explant types can be observed for both F1 and F2. Two clusterings of the scores of calli from hypocotyl and root explants occur along the F1 axis at positive values, while the scores of calli from leaf and stem internodes clustered along the F1 axis at negative values. It is well established that the source of explant is an important factor in determining *in vitro* responses, i.e. organogenesis, embryogenesis or callus induction [17-19]; on the contrary, the role of explant origin on *in vitro* secondary metabolite production has not yet been clearly defined. In two recent works it was proved that *in vitro* tissue cultures initiated from different explants exhibited different secondary metabolite production ability. It has been suggested that highly secondary metabolite producing explants could provide *in vitro* material with better biosynthetic potential [20,21]. Our results agree with this finding, as the roots are the vegetative organ with the highest psoralen level [6].

Table 2: Coumarins detected by HPLC UV analysis in calli obtained from different explants, grown either in darkness or in light conditions, and in the respective subculture media.

explant source		umbelliferone		scopoletin		daphnoretin		marmesin		psoralen	
		D	L	D	L	D	L	D	L	D	L
hypocotyl	*callus*	+	+	+	+	-	-	+	+	-	+
	medium	-	+	+	+	-	-	-	+	-	-
leaf	*callus*	+	+	+	+	-	-	+	+	-	+
	medium	+	+	+	+	-	-	+	+	-	+
stem internode	*callus*	+	+	+	+	-	-	+	+	-	+
	medium	+	+	+	+	-	-	+	+	-	+
root	*callus*	+	+	+	+	-	-	+	+	-	+
	medium	+	+	+	+	-	-	-	+	-	+

D = dark conditions; L = light conditions.

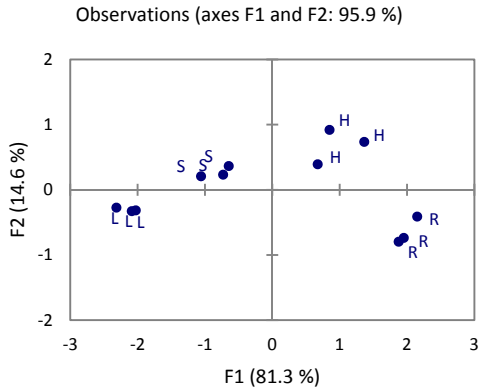

Figure 4: Principal component analysis of the different callus types: H from hypocotyl, L from leaf, S from stem internode and R from root. F1: principal component 1; F2: principal component 2.

The loading plot of F1 and F2 explains that calli from hypocotyl and root explants showed higher umbelliferone and psoralen contents (mean values 1.78 and 2.56 mg g^{-1} DW respectively) compared with calli from leaf and stem internode (mean values 0.15 and 0.65 mg g^{-1} DW respectively). It appears likely that umbelliferone level is critical for psoralen biosynthesis. This result seems to confirm that psoralen synthesis could be promoted by high concentrations of umbelliferone rather than marmesin, as previously reported in *in vitro* culture feeding studies [22,23].

Coumarin production by *in vitro* cultures has been extensively reported for different species known as coumarin producers [24,25] and it was seen that the amount of coumarin produced in unorganized cell cultures [26-28] was comparatively less than that obtained in organized cultures [29-32].

Psoralen production from calli was obtained only for *Bituminaria bituminosa* and *Ruta graveolens*, and the content was less than 60 µg g^{-1} DW [30,32]. *C. scorpiodes* calli produced 137 µg g^{-1} DW psoralen in derived-root calli. These data indicate that unorganized cell cultures of *C. scorpioides* could be a promising source of psoralen, also taking into account that psoralen is naturally excreted into the medium. Indeed it is recognized that, in order to design an optimal production process for secondary metabolites, the release of the metabolites into the extracellular medium is crucial [12]. However, it should be underlined that most of the useful metabolites biosynthesized by plant cells are stored within the cells [33], thus making their efficient and continuous production very difficult.

Experimental

Plant material: *Coronilla scorpioides* (L.) E.D.J. Koch seeds were collected and authenticated by Dr P. Palini and Dr S. Miotto (Botanical Garden of Padova) from native plants (San Dorligo della Valle, Trieste, North East-Italy). A voucher specimen (No. H0031421) was deposited in the Botanical Garden of Padua (Italy).

In vitro *germination:* Seeds were scarified by immersion in pure sulfuric acid for 15 min and surface sterilized with commercial bleach 15% (v/v) (5% of sodium hypochlorite) containing one drop of Tween-20 for 10 min. Seeds were then washed 3 times with sterile distilled water. The germination medium contained half-strength Murashige and Skoog (MS) macro- and microsalts [10] and vitamins, 2% (w/v) sucrose and 1% (w/v) agar. The pH of the medium was adjusted to 5.7 before autoclaving. After sterilization, the seeds were placed on the surface of the germination medium and cultivated in a tissue culture chamber at 25°C under cool white fluorescent lights (36 µmol m^{-2} s^{-1}) with a 16 h photoperiod.

Culture conditions: Hypocotyl, leaf, stem internode and root explants from 3 weeks old seedlings were used for callus induction. Explants (0.5–1 cm) were cultured on MS and Gamborg *et al.* (B5) [11] media containing 1.3 mg L^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid), 0.25 mg L^{-1} kin (kinetin) and 0.25 mg L^{-1} NAA (naphthaleneacetic acid). The media were supplemented with 30 g L^{-1} sucrose and solidified with agar (8 g L^{-1}); the pH of the media was adjusted to 5.7. Experiments were performed in 9 cm Petri dishes using 25 explants per dish. The explants were cultivated in the dark at 25°C and sub-cultured on fresh media every 4 weeks. Responses were recorded after 3 subcultures; the callogenesis was estimated by a '0–4 Scale' scoring system (Table 3) [18]. Each experiment was repeated twice with 2 replicates (100 explant type per treatment).

Table 3: A ''0–4 Scale'' scoring system developed for measuring callus formation.

Score	Description
0	no visible callus
1	5 mm callus at cut ends
2	5-10 mm callus
3	10-15 mm callus
4	> 15 mm callus

Callus samples (in triplicate) were evaluated for fresh weight.

Chemicals: Methanol was purchased from Merck, diethyl ether from Baker, and HPLC-grade acetonitrile from Fluka. Water was purified with a Milli-Q deionization unit (Millipore). Umbelliferone, scopoletin, daphnoretin, marmesin and psoralen were supplied by the Department of Pharmaceutical and Pharmacological Sciences of the University of Padova (Italy).

Extraction: Samples of calli (10^{th} subculture) were collected on the 20^{th} day of the growth cycle. Dried callus tissues (1 g) and the respective media were separately homogenized and refluxed for 2 h in 5% HCl. After cooling and filtration, a residue and an aqueous-acid phase were obtained. The residue was extracted with methanol; the aqueous-acid phase was extracted with diethyl ether and after distillation the residue was dissolved in methanol. The 2 methanolic solutions were combined and analyzed.

HPLC UV analysis: HPLC analysis was performed with a ChromQuest (Thermoseparation, San Josè, CA, USA) pump P4000 equipped with a photodiode array detector UV6000. The data were recorded and processed using a ChromQuest Chromatography Workstation. The separation was achieved with a Gemini RP-18 column (250 x 4.60 mm, 5 µm, Phenomenex, Torrance, CA, USA) and the injection volume was 6 µL. The mobile phase consisted of acetonitrile/water (32/68, v/v). The flow rate was kept at 1 mL min^{-1}. UV spectra were recorded in the 200–360 nm range; chromatograms were acquired at 227, 300 and 330 nm. The identification of the coumarins was carried out from their retention times and UV spectra, and by comparison with authentic samples. Umbelliferone, marmesin and psoralen contents were determined using the calibration curves constructed from standard solutions. Analyses were performed on 3 different samples of each callus type and the respective media.

Statistical analysis: Data were subjected to analysis of variance (ANOVA) and significance between the mean values was tested by Tukey's test at a confidence level of $P<0.05$. Principal component

analysis was used to establish statistical discrimination among callus samples. The statistical tests were generated with GraphPad Prism and XLSTAT.

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