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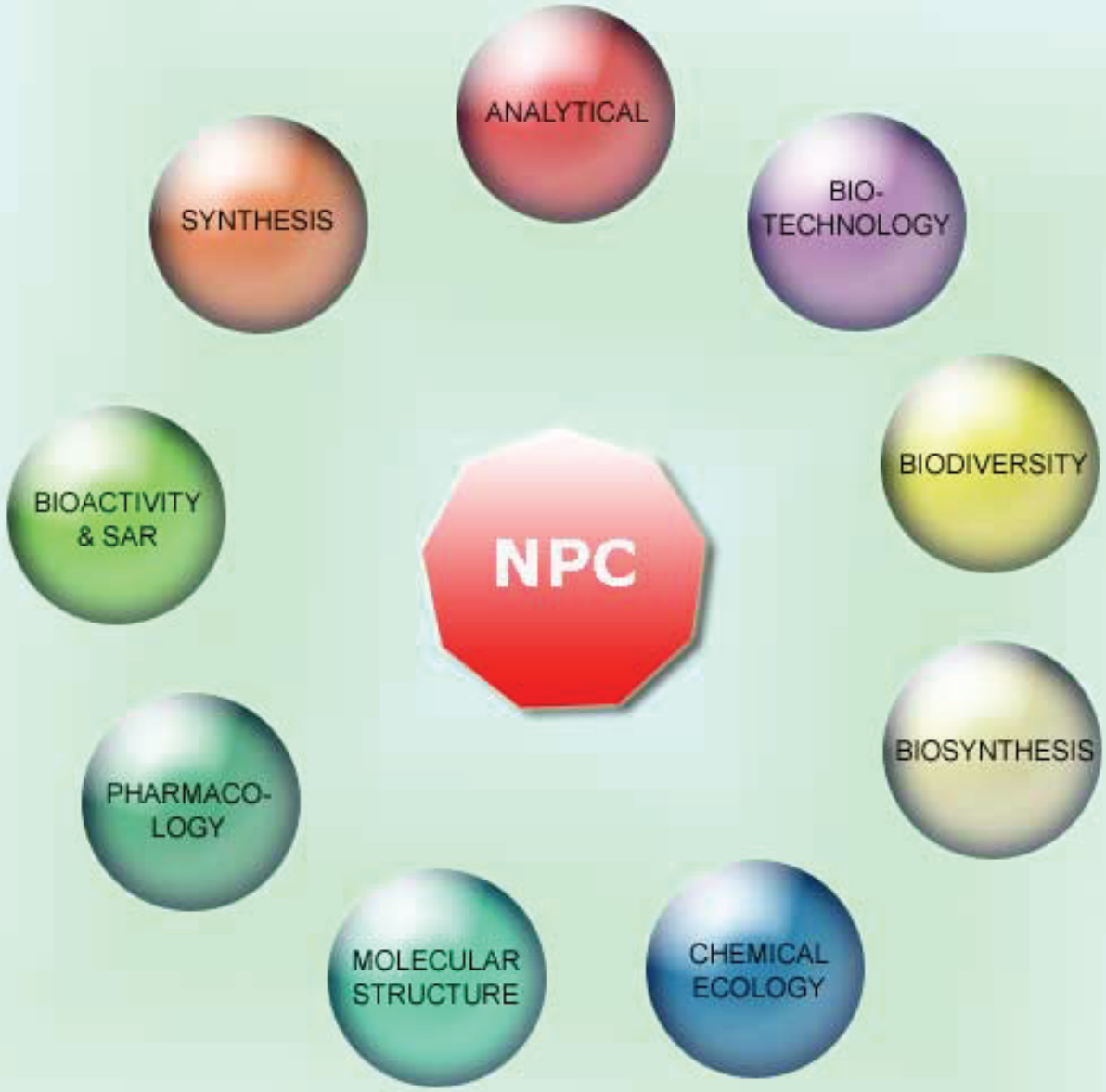

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Daidzein Production and HeLa Cytotoxicity of *Bituminaria bituminosa* Hairy Root Cultures

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Bituminaria bituminosa (L.) C.H. Stirt is a perennial species widely distributed in the Mediterranean basin and the Canary Islands. This species is used in folk medicine and currently has considerable pharmaceutical interest for its content in phenylpropanoids, furanocoumarins and pterocarpans. *In vitro* cultures (shoots and hairy roots) have been performed to obtain plant material useful for the production of these metabolites. Hairy root cultures were successfully established after inoculation of hypocotyls with the LBA 9402 *A. rhizogenes* strain. The HRPB3 line was selected for further analysis and elicited with chitosan and salicylic acid. All the HRPB3 cultures showed higher polyphenol content and greater DPPH-antioxidant activity than shoots cultured *in vitro*. The presence of isoflavone daidzein was detected in the hairy root extracts. The cytotoxic effect of HR extracts has been further tested on HeLa cells: the salicylic acid elicited HR exhibited good antiproliferative effects.

Keywords: *Agrobacterium rhizogenes*, HPLC-DAD, Polyphenol content, Elicitors, Daidzein, Cytotoxic effect, HeLa cells.

Bituminaria bituminosa (L.) C.H. Stirt (alias *Psoralea bituminosa* L., Fabaceae) is a perennial species of the Mediterranean area used to provide hay or forage for livestock [1] and in folk medicine as vulnerary, cicatrizing and disinfectant agent [2]. Its characteristic strong smell of bitumen is the result of a combination of several substances such as phenolics, sulphurated compounds, sesquiterpenes and probably short-chain hydrocarbons [3].

B. bituminosa, like other *Psoralea* spp. plants, is known for producing several compounds with considerable pharmaceutical interest, as isoflavones (daidzein and genistein) and furanocoumarins, represented by psoralen and its angular isomer angelicin [4,5]. The isoflavones are recognized as food compounds (nutraceuticals) providing protective effect against oxidative damage, and used in the animal diet for their phytoestrogenic properties [6,7]. A further application is the prevention of the cancer and cardiovascular diseases [8,9]. The furanocoumarin psoralen has photosensitizing, photobiological and phototherapeutic functions and is used for treatment of vitiligo and skin diseases, as psoriasis, mycosis fungoides and eczema [10,11]. Angelicin is known as sedative, anticonvulsant and photochemotherapeutic agent [12]. Moreover new pterocarpans erybraedin C and bitucarpin A were isolated and identified from *B. bituminosa* aerial parts [13] naturally grown in Elba island. These compounds showed cytotoxicity against different human cell lines [14] and erybraedin C exhibited a further inhibitory activity against the human topoisomerase I [15].

Spontaneous medicinal plants harvested for their metabolite content are often exposed to habitat destruction and biodiversity erosion. The production depends on several factors such as pedoclimatic conditions and genetic modification [16]. Thus their secondary metabolite profile is unpredictable in term of quality and quantity.

In vitro cultures represent a valid method to overcome the environmental factors and to improve the production of active compounds of pharmaceutical interest [17]. *In vitro* cultures of *B. bituminosa* have been performed to produce their characteristic secondary metabolites [18]. Callus and hairy root (HR) cultures have been successfully developed from *P. corylifolia* to produce furanocoumarins and isoflavones [19, 20] and their accumulation is influenced by the addition of elicitors [8-9].

The present study deals with the induction of HR from *in vitro* cultures of *B. bituminosa* and their production of known secondary metabolites. HeLa cytotoxic assay was further carried out to assess the inhibitory effect of HR methanolic extracts. After 25 days of *in vitro* culture, *B. bituminosa* seedlings exhibited a normal growth (Figure 1a) with good expanded green leaves and hypocotyls, and represent a good material for the further transformation.

Hairy roots (HR) were successfully produced by infection of *B. bituminosa* hypocotyls with *A. rhizogenes* strain LBA 9402. HR emerged after 45 days from hypocotyls. No HR were obtained from other organs (leaves and roots) in agreement with HR of *Psoralea corylifolia* [21]. The transformation efficiency of hypocotyls was 25%. Nguyen *et al*. [22] already reported a moderate formation and slow growth of HR in other *Psoralea* spp.

The different HR lines showed the characteristic behavior: good branching with the development of secondary hairs and negative geotropism (Figure 1b). Transformation was confirmed by identification on agarose gels of PCR after amplification of isolated DNA with primers for *A. rhizogenes rol*C gene (Figure 1d) [23]. The best growing line was selected (called HRPB3), then subcultured and stabilized in MS0 liquid medium. This line showed

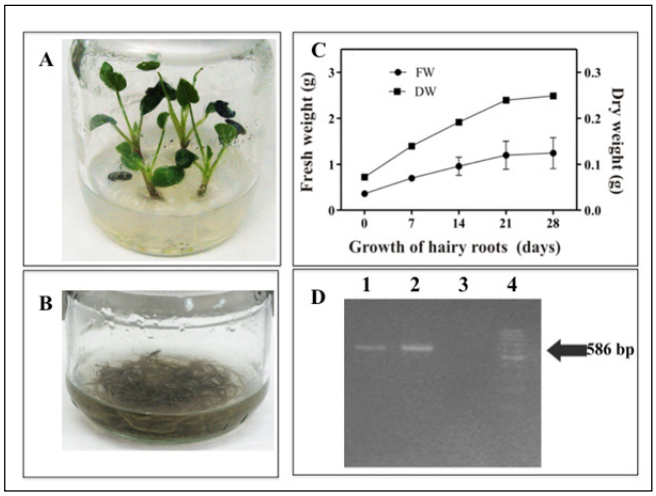

Figure 1: Production of hairy roots cultures of *B. bituminosa*. a) *In vitro* 25-days-old plantlets used for transformation b) detail of 28-days-old HRPB3 line grown in liquid MS0 medium. c) Fresh and dry weight growth curve of *B. bituminosa* HRPB3 in liquid MS medium (average ± SD of three replicates). d) Agarose gel electrophoresis of PCR products of roots transformed by *A. rhizogenes* LBA 9402. Arrow indicates 586 bp = *rolC* fragment. Lane 1= transformed hairy roots; lane 2 = *A. rhizogenes* LBA 9402; lane 3 = not-transformed hypocotyls lane 4= standard 100 pb DNA ladder.

a growth curve with a good increase of biomass both for fresh and dry weights until the end of log phase (21 day of culture) (Figure 1c). A similar growth was already observed in *P. corylifolia* [21] and in other HR cultures of medicinal plants [24].

Various factors influence the HR growth and their production of interesting secondary metabolites: e.g. medium composition, pH, temperature, light, biotic and abiotic elicitors [17, 25-26]. The 25day-old HRPB3 (control, HRc) was elicited by the addition of chitosan or salicylic acid for three days [8]. These elicitors were chosen because their addition has been shown to induce a defense mechanism associated with the production of secondary metabolites [8]. A slight increase of biomass in treated HR (HRchi and HRsa) was observed after three days of treatment without any morphological differences from the control line (data not shown).

Table 1: Content of total polyphenols and antioxidant capacity (DPPH-IC_{50}) of *in vitro* cultures of *B. bituminosa* (25day-shoots, HRc, HRchi, HRsa). Data are the mean values ± SD of three independent experiments with triplicates. Values with different letters are significantly different from each other ($p < 0.05$).

	Total Polyphenols, mg g^{-1} dry wt	DPPH-EC_{50}, µg mL^{-1}
25day-shoots	1.32±0.05[a]	152.92±24.86[b]
HRc	3.98±0.09[c]	34.82±4.41[a]
HRchi	3.73±0.32[b]	29.61±0.29[a]
HRsa	3.49±0.36[b]	32.65±1.90[a]

Methanolic extracts from various *in vitro* cultures (table 1) were used to assess the presence of typical *B. bituminosa* secondary metabolites and their antioxidant ability. The 25day-old *in vitro* shoots showed the lowest content of polyphenols (Table 1) whereas the highest amount was observed in HRc (3.98 mg g^{-1} dry wt). Both the elicited HR cultures showed very similar polyphenol content. The HR cultures exhibited the highest antioxidant activity if compared to *in vitro* plant organs. The EC_{50} of elicited HRchi (29.61 µg mL^{-1}) and HRsa (32.65 µg mL^{-1}) was similar to the control values (HRc, 34.82 µg mL^{-1}).

The phenolic content and antioxidant activity detected in all these *in vitro* cultures were at least three-fold lower than that reported in wild plants [27]. So it becomes interesting to further investigate the presence of the typical secondary metabolites in HR by HPLC analysis. For these analyses the extraction procedure excluded any

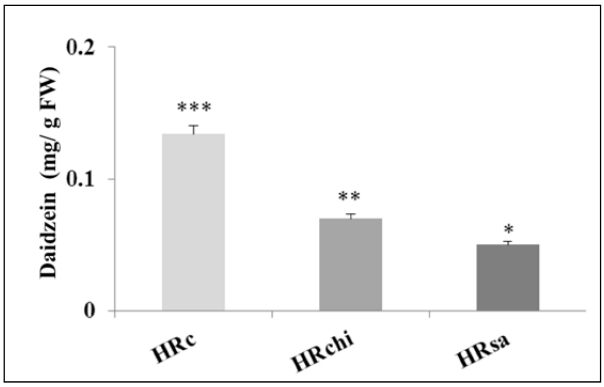

Figure 2: HPLC-DAD quantitative evaluation of the daidzein in different *HR* cultures of *B. bituminosa* (HRc, HRchi, HRsa). The mean content is expressed as mg g^{-1} fresh weight. Values are expressed as mean ± SD of three replicates ($p < 0.01$)

acidic treatment to avoid the degradation of active compounds [18]. Daidzein was identified in all the HR cultures (Figure 2), although the addition of elicitors decreased the production of this isoflavone. The daidzein amount was higher than the one detected in wild plants and in 25 days shoot cultures [18]. These results were in agreement with previous data obtained in HR from other *Psoralea* species [22, 28]. However, Shinde *et al*. [8] reported that the elicitation with 1mM salicylic acid or 10 mg l^{-1} chitosan optimized the production of the isoflavones daidzein and genistein in HR cultures of *P. corylifolia,* after few days of treatment (2 or 5 days). These different results can be correlated with the specific characteristics of the HRPB3 clone. In fact the accumulation of daidzein may depend on genotypic variation of *Psoralea* species and on the selection of HR clones [9,28].

The pterocarpans (erybraedin C and bitucarpin A) as well as the furanocoumarins (psoralen and angelicin) were not identified in these HR cultures, even though these compounds were produced either in wild plants or in *in vitro* 25-day shoots [13, 18, 29]. The absence of furanocoumarins in HR cultures could be justified by previous reports, where the synthesis and accumulation of furanocoumarins in *B. bituminosa* were influenced by environmental conditions, such as drought, extreme temperatures, and age of the plants [5, 29].

Table 2: Cytotoxicity assay in HeLa cells treated with extracts from different HR cultures of *B. bituminosa*. Values, expressed as relative percent of cell survival, are the mean ± SD of three replicates.

Type of extract	Extract dose (mg ml^{-1})		
	0.01	0.05	0.10
HRc	100.0±0.7	97.6±1.7	58.0±2.8[a]
HRchi	100.0±0.5	78.6±1.3[a]	52.0±1.7[b]
HRsa	94.6±2.9	41.8±1.5[b]	10.3±3.1[b]

[a]Significance vs. control value (100% cell survival), $p<0.01$ (Dunnett test).
[b]Significance vs. control value (100% cell survival), $p<0.001$ (Dunnett test).

The cytotoxicity assay of HR extracts was further assessed in HeLa cells (Table 2). The HR extracts showed a dose-dependent decrease in cell survival, even though the antiproliferative effects induced by HRc, HRchi did not reach 50% of the control value at the highest dose. Only the addition of salicylic acid in HR determined a higher degree of toxicity (10.3% of HeLa cells survival).

The present work confirmed that the HR cultures are an alternative system for production of secondary metabolites in *B. bituminosa*. However, these HR cultures produced only daidzein, and the use of elicitors did not improve its accumulation. The larger scale production of HR *B. bituminosa* culture could be a useful tool for the production of daidzein, an isoflavone characterized by important

pharmaceutical properties [6-7,9]. To this purpose further experiments are necessary to improve the daidzein production.

Experimental

Plant materials: shoots of *B. bituminosa* were *in vitro* cultured in Murashige and Skoog medium (MS0), containing sucrose 3% (w/v), agar 0.8% (w/v), Plant Preservative Mixture (PPM) 0.05% (v/v), as reported in previous paper [18], and used as starting plant material for hairy roots production.

Hairy root cultures establishment and confirmation of transformation: transformation was performed with wild-type hypervirulent *Agrobacterium rhizogenes* strain LBA 9402 (a gift from Dr. David Tepfer, INRA Versailles, France). Bacterial suspensions were grown for 48 h at 28°C on appropriate YMB medium [30]. Whole proliferated colonies were then suspended in an AAM liquid inoculation medium (A_{600} = 0.1) [31]. Ten mm-long segments of 25-day-old *in vitro B. bituminosa* organs (leaves, roots, hypocotyls) were soaked with the bacterial suspension for 15 min, while control explants were soaked in sterile distilled water for the same time. Infected and control fragments then transferred on MS0 medium at 23 ± 1°C for 48 h in darkness [22]. Afterwards the fragments were subcultured into MS medium added with cefotaxime (100 mg l^{-1}) to remove *A. rhizogenes* from the culture. Hairy roots emerged at the wounding sites after 45 days of culture. The formation of HR was monitored, recognized by their morphology and recorded as percentage.

Transformed nature of roots was confirmed by Polymerase chain reaction (PCR) amplification of *rol*C fragment (586 bp). Total genomic DNA was extracted from HR lines and control hypocotyls using a simple and rapid method [32]. Also DNA from the bacterial strain LBA 9402 was extracted [32]. DNA amplification was performed on a Biorad iCYCLER' termocycler. Each reaction mixture (15 µl) consisted in DNA template (maximum of 250 ng), 1 X Go Taq Green Master Mix (7.5 µl) (Promega, USA), and r*olC* primers (0.5 µM) (ROL F 5'-CTC CTG ACA TCA AAC TCG TC-3' and ROL R 5'-TGC TTC GAG TTA TGG GTA CA-3') [33]. The *rol*C fragment was amplified under the following conditions: 94°C for 4 min, 30 cycles of 94°C for 30 sec, 55°C for 30 sec, 72°C for 55 sec, and a final extension step of 72°C for 5 min. PCR products were examined by electrophoresis on agarose gels 2% (w/v) in TBE buffer 0.5% (v/v) under 260 nm UV light after staining with ethidium bromide. Among the transformed HR lines the called HRPB3 was selected for further experiments. Approximately 4-cm-long root segments of HRPB3 line were excised, and cultured in liquid MS medium (MS0, 50 mL) supplemented with PPM 0.05% (v/v). The liquid cultures were kept in the dark, on a rotary shaker (100 rpm) at 22 ± 1°C and subcultured every 4 weeks. For the determination of the growth curve, eighteen vessels containing 100 g of HR in 50 mL liquid medium were prepared and fresh and dry weights were recorded at 0, 7, 14, 21 and 28 days. The experiment was repeated three times.

Hairy roots treatments: HRPB3 line was cultured either in MS0 medium (control HRc) or MS0 supplemented with chitosan or elicitors. Stock solution of chitosan 2% (v/v) and salicylic acid (10 mM) were prepared as already known [8]. Approximately 150 mg (FW) of 25-day-old HR segments were inoculated on liquid MS0 medium added with chitosan (150 mg l^{-1}) (HRchi) or salicylic acid (final concentration 1 mM) (HRsa); flasks were kept on rotatory shaker at 100 rpm in darkness. The samples were harvested 3 days after elicitation treatment [8], frozen in liquid nitrogen and stored at -80 °C until use, or dried in oven for 48 h at 40°C.

Total soluble polyphenol compounds: were assayed in different sample extracts using the Folin-Ciocalteau's phenol protocol with minor modification [34]. Ten mg of dried different HRPB3 cultures and *in vitro* 25day-old shoots were pulverized and homogenized in a mortar with 1 mL of 70% (v/v) methanol to facilitate the extraction. The Absorbance was measured at 765 nm using the spectrophotometer Cintra 101(GBC scientific instrument, Australia). Results were expressed as mg of chlorogenic acid per g of dry weight (mg g^{-1} dry wt). All determinations were performed in triplicate.

Determination of antioxidant activity with 2,2-diphenyl- 1-picrylhydrazyl (DPPH) radical scavenging method: The antiradical activity of different HRPB3 cultures and *in vitro* 25day-old shoots was determined by using the stable 2,2-diphenyl-1-picrylhydrazyl radical (DPPH) [35]. The inhibition of the radical DPPH by the samples was calculated according to previous paper [36].

Phytochemical analysis: different 25day-old HRPB3 cultures (fresh materials) were extracted with chloroform and dried by rotatory evaporator. Each extract was dissolved in methanol (2.5 mg mL^{-1}) and filtered through a cartridge-type sample filtration, (25 mm) before HPLC unit with a polytetrafluoroethylene filter (PTFE, 0.45 µm, 25 mm) before HPLC injection. HPLC-DAD analyses were conducted as already reported [18]. The identification of each constituent was carried out by the comparison of the peaks obtained in the different chromatograms with the retention time and UV spectra of the authentic samples injected in the same chromatographic condition

Cytotoxicity assay: Cytotoxicity assay was performed in HeLa cells using the extracts obtained from HRPB3 cultures (HRc, HRchi, HRsa,) according to the method already published [18]. The results were expressed as percent of cell survival of the corresponding untreated extract, calculating the mean±SD of three independent experiments (replicates).

Statistical analysis: All the data are the mean values ± SD of three independent experiments with triplicates. The statistical differences between all extracts of *in vitro* cultures of *B. bituminosa* (HRc, HRchi, HRsa, 25d-shoots) were obtained by one-way ANOVA analysis with Student-Newmann-Keuls Multiple Comparison post-test ($p < 0.01$) and ($p < 0.05$). However, the cytotoxicity data were subsequently analyzed by the Dunnett test.

Chemicals: All standard compounds (bitucarpin A, erybraedin C, psoralen, daidzein, daidzein 7-O-glucoside) were included in a home-made data base of natural compounds isolated and characterized by NMR and MS techniques in our laboratory. HPLC-grade acetonitrile was provided by J. T. Baker (Baker Mallinckrodt, Phillisburg, NJ); HPLC grade water was prepared by a Milli-Ω^{50} purification system (Millipore Corp., Bedford, MA).

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