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# Phenolic compound production in relation to differentiation in cell and tissue cultures of *Larrea divaricata* (Cav.)

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ABSTRACT

The lignan nordihydroguaiaretic acid (NDGA) and its derivatives existing in *Larrea divaricata* species show a wide range of pharmacological activities which makes this genus an interesting target to consider the plant *in vitro* cultivation systems as a feasible alternative source for their production. These compounds are potentially useful in treating diseases related to heart condition, asthma, arteriosclerosis, viral and bacterial infections, inflammation and cancer. In the present study, calli, cell suspension cultures, and *in vitro* and wild plants of *L. divaricata* were investigated for their potential to synthesize phenolic compounds. Calli, both with and without organogenesis, produced NDGA and quercetin, as did plantlet and wild plants. NDGA was also produced by the cell suspension cultures, together with *p*-coumaric acid, ferulic acid and sinapyl alcohol. The capacity of undifferentiated tissues to form phenolic compounds is very limited, but when the calli underwent organogenesis, developing mainly adventitious shoots, the phenolic compound production increased significantly. Plantlets regenerated from adventitious shoots of *L. divaricata* calli did not show the same phenolic pattern as wild plants, with levels of NDGA and quercetin being 3.6- and 5.9-fold lower, respectively.



## 1. Introduction

*Larrea divaricata* Cav., also known as "Jarilla", is a perennial shrub widely documented among different communities of the "Monte" phytogeographical province (Salta to Chubut) [1,2].

Different ethnobotanical studies demonstrate that the medicinal value of this plant was discovered centuries ago by American Indian culture [3]. Various preparations are made from the plant, especially extractions from the leaves and steams. Several medicinal claims and uses have been made for the "Jarilla tea": treatment of stomach disorders, arthritis, rheumatism, bronchitis and other breathing problems, cancer and venereal diseases [3–5]. These activities have been attributed in part to the lignans, although the evidence suggests that other chemical substances (flavonoids) existing in the extracts could be acting synergically [3].

The most prevalent and biologically active compound present in the external surface of the leaves is the potent antioxidant nordihydroguaiaretic acid (NDGA), but this species has been previously shown to also contain a series of lignans, flavonoids, condensed tannins, triterpene saponins, essential oils and waxes [6–9].

To scientifically support the traditional use, several pharmacological studies have been carried out with "Jarilla" plant extracts and with isolated compounds. Many studies have shown that "Jarilla", NDGA and its derivatives are well known strong antioxidants as well as selective inhibitors of lipoxygenases [10–12]. It has also been reported that these compounds have been effective against several viruses (human immunodeficiency virus, herpes simplex virus, human papilloma virus, influenza virus, and Junin virus) [13–18] as well as that they inhibit the growth of tumors, both in cell cultures and in animal models [19–25]. The semi-synthetic natural product tetra-*O*-methyl NDGA ($M_4N$) can inhibit the growth of human xenograft tumors [26] and it is also currently undergoing Phase I/II clinical trials as a treatment for central nervous system and brain tumors [27].

Despite the growing potential of clinical uses for NDGA and its derivatives, there are few reports on the biosynthesis of these compounds [28], and studies on their production in *L. divaricata* plant cell cultures have only been carried out in the last two years [29]. Therefore, the need for the establishment of *in vitro* cultures from this plant is considered to be an important goal for plant biotechnology. The capacity for plant cells, tissue, and organ cultures to produce and accumulate many of the same valuable chemical compounds as the parent plant in nature has been recognized almost since the inception of *in vitro* technology. At the present time, many

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medicinal valuable compounds are produced by *in vitro* plant cell cultures; however, in other cases production requires more differentiated organ cultures such as shoot and root [30].

The aim of this study was to investigate the potential capacity of *L. divaricata in vitro* cultures to synthesize phenolic compounds and to evaluate the changes in phenolic compound content during the process of organogenesis. To secure a constant high quality supply two possible techniques might be followed. One of these is cultivation of a selected elite variety, for which plant biotechnology is important for the micropropagation, with the other method being *in vitro* plant cell or organ culture of the plant. In the present work, both micropropagation and the cell biotechnological production are addressed. By studying the various pathways connected with the production of the compounds of interest, more information concerning metabolic engineering strategy was provided.

## 2. Materials and methods

### 2.1. Chemicals

The different media were preformed using macro- and micronutrient salts, as well as vitamins of Murashige and Skoog [31]. These chemicals, plant growth regulators and Phytagel were purchased from Sigma–Aldrich (St. Louis, MO, USA).

All solvents for the HPLC analysis were of HPLC grade and purchased from Merck Co. (Darmstadt, Germany). NDGA, L-phenylalanine, *p*-coumaric acid, cinnamic acid, ferulic acid, caffeic acid, sinapic acid, sinapyl alcohol, coniferyl alcohol, quercetin and kaemferol aglycones were obtained from Sigma–Aldrich (St. Louis, MO, USA). The solvent used for the extraction of samples was of analytical grade and also obtained from Merck Co. (Darmstadt, Germany).

### 2.2. Callus induction and cell suspension cultures

Wild plants and seeds of *L. divaricata* Cav. were collected in Santa María de Punilla, Córdoba, Argentina. A voucher specimen has been deposited in the International Herbarium of the National University of Río Cuarto, Argentina, with the registration number RIOC 501.

*L. divaricata* seeds were surface-sterilized by immersion in 3% (w/v) sodium hypochlorite solution for 15 min. Subsequently, the seeds were washed with sterile distilled $H_2O$, germinated on half-strength MS medium without plant growth regulators at pH 5.8 and solidified with 0.8% (w/v) plant agar.

Leaf explants (50 explants) from aseptically obtained three-month-old seedlings were cultured in MS medium supplemented with 3% (w/v) sucrose and 0.1% (w/v) myo-inositol before being solidified with 0.27% (w/v) Phytagel.

The medium was supplemented with different types and combinations of auxins [Pi (Picloran):2,4-D (2,4-dichlorophenoxyacetic acid)] and cytokinins [KIN (kinetin):BAP ($N^6$-benzylaminopurine)] at different concentrations: 0.1, 1 and 2 mg/L. The pH of the media was adjusted to 5.6 prior to autoclaving. Cultures were kept in a growth chamber at 25 °C under a 16/8 h light/dark photoperiod using cool-white fluorescent light ($\mu mol\ m^{-2}\ s^{-1}$) in order to form calli, which were subcultured every 4 weeks.

For fresh weight determination (FW), twelve callus pieces were harvested from each treatment and weighed, with the dry (DW) being determined after lyophylization. These parameters were recorded after four months of culture.

Cell suspension cultures were established from the four-month-old calli to a liquid medium (MS, 2 mg/l of 2,4-D and 1 mg/l BAP, 3% (w/v) sucrose), and incubated under the same conditions as that for callus culture, but with continuous shaking (120 rpm).

The calli were incubated for 15 days after inoculation (1/10, p/v) and were harvested to determine the biomass and the phenolic compounds present by vacuum filtration at defined times.

### 2.3. Induction of adventitious shoots and roots

Half gram fresh mass of calli without organogenesis was isolated and cultivated in MS medium supplemented with 0.1 mg/l Pi in combination with three concentrations of BAP (2, 3 and 4 mg/l) or each phytohormone alone (0.1 mg/l of Pi or 4 mg/l of BAP). These calli were subcultured every 4 weeks. Adventitious shoots regenerated from these callus cultures were excised and cultivated in Magenta vessels (Sigma–Aldrich) with 150 ml MS medium supplemented with auxin indole-3-butyric acid (IBA) (2.5 μM) and 5% (w/v) sucrose. The plantlets were routinely subcultured every 4 weeks, and the cultures were kept in a growth chamber at 25 °C under a 16/8 h light/dark photoperiod using cool-white fluorescent light ($\mu mol\ m^{-2}\ s^{-1}$).

### 2.4. Extraction and analysis of phenolic compounds

Four-month-old calli without organogenesis; organogenic calli grown on medium supplemented with 4 mg/l BAP, cells cultured in suspension, three-month-old plantlets, and five-year-old wild plants were lyophilized overnight (Labconco Instrument, USA), before being ground to a powder with a pestle in a mortar, using liquid nitrogen to facilitate sample homogenization, and stored at −20 °C.

500 mg of each powered plant material was mixed with 10 ml of 99.5% ethanol in a 100 ml Erlenmeyer flask and covered with parafilm. Samples were shaken at 120 rpm for 24 h in the dark at room temperature, after which the resulting cell organic solvent extracts were filtered. The organic phase was centrifuged at 1500 rpm for 10 min and filtered under reduced pressure. The wild plant extract was also partitioned three times with water:diethyl ether (30 ml each time) and evaporated, since wild plants have a complex matrix.

The organic residues from each plant material were re-dissolved in methanol (1 ml), filtered through a membrane filter (0.45 μm pore size) and analyzed by HPLC, as described previously by Palacio et al. [29]. The phenolic compounds were detected at 265 nm for coniferyl alcohol, 272 nm for cinnamic acid, 275 nm for sinapyl alcohol, 281 nm for NDGA, 310 nm for *p*-coumaric acid, 325 nm for ferulic acid and caffeic acid, 328 nm for sinapic acid, and 370 nm for quercetin and kaemferol aglycones. The peak areas corresponding to each detected phenolic compound from the samples, and having the same retention time as authentic compounds, were integrated for comparison with an external standard calibration curve.

### 2.5. Statistical analysis

All experiments were repeated 3 times and the values are presented as means ± standard deviation (SD). The data from different *in vitro* culture tissues were statistically analyzed using a one-way analysis of variance and the means were compared by Duncan's multiple range tests at a 5% probability level.

## 3. Results and discussion

### 3.1. Callus induction and cultures

To evaluate the production of phenolic compounds in *L. divaricata* callus cultures, we initially tested more than 36 culture media using different hormonal treatments with the aim of obtaining friable calli of this plant species. Table 1 shows the concentrations of plant growth regulators tested in this study and

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**Table 1**
Callus induction and organogenesis from *L. divaricata* leaf explants.

| PGR[a] | | | | Callus induction | | Shoots (% explants) |
|---|---|---|---|---|---|---|
| Auxins | mg/l | Cytokinins | mg/l | % explants | FW (g)[b] | |
| Pi | 0.1 | BAP | 0.1 | – | – | – |
| | | | 1 | – | – | – |
| | | | 2 | 33.3 | 0.69 ± 0.11b[c] | 28.0 ± 4.0[c] |
| Pi | 1 | BAP | 0.1 | 58.3 | 0.77 ± 0.09b | – |
| | | | 1 | – | – | – |
| | | | 2 | – | – | – |
| Pi | 2 | BAP | 0.1 | 50.0 | 1.85 ± 0.12d | – |
| | | | 1 | 58.3 | 0.78 ± 0.08b | – |
| | | | 2 | – | – | – |
| 2,4-D | 0.1 | BAP | 0.1 | – | – | – |
| | | | 1 | 58.3 | 1.40 ± 0.08c | – |
| | | | 2 | – | – | – |
| 2,4-D | 1 | BAP | 0.1 | 58.3 | 0.32 ± 0.07a | – |
| | | | 1 | – | – | – |
| | | | 2 | 58.3 | 0.31 ± 0.09a | – |
| 2,4-D | 2 | BAP | 0.1 | 75.0 | 0.71 ± 0.01b | – |
| | | | 1 | 75.0 | 3.23 ± 0.14e | – |
| | | | 2 | – | – | – |
| 2,4-D | 0.1 | KIN | 0.1 | 48.3 | 0.39 ± 0.05a | – |
| | | | 1 | 48.3 | 0.38 ± 0.04a | – |
| | | | 2 | 58.3 | 0.31 ± 0.06a | – |
| 2,4-D | 1 | KIN | 0.1 | 50.0 | 0.86 ± 0.08b | – |
| | | | 1 | 58.3 | 1.29 ± 0.09c | – |
| | | | 2 | 58.3 | 1.82 ± 0.08d | – |
| 2,4-D | 2 | KIN | 0.1 | 58.3 | 1.66 ± 0.07d | – |
| | | | 1 | 41.6 | 1.69 ± 0.09d | – |
| | | | 2 | 58.3 | 1.38 ± 0.09c | – |
| Pi | 0.1 | KIN | 0.1 | – | – | – |
| | | | 1 | – | – | – |
| | | | 2 | – | – | – |
| Pi | 1 | KIN | 0.1 | – | – | – |
| | | | 1 | – | – | – |
| | | | 2 | – | – | – |
| Pi | 2 | KIN | 0.1 | – | – | – |
| | | | 1 | – | – | – |
| | | | 2 | – | – | – |

[a] PGR, plant growth regulator; BAP, ($N^6$-benzylaminopurine); KIN, kinetin; 2,4-D, 2,4-dichloroacetic acid; Pi, Picloran.
[b] FW, fresh weight.
[c] Organogenic calli.
Data of FW were collected after four months.
Values represent the mean ± S.D. Means following the same letter within columns are not significantly different, according to Duncan's multiple range test ($P < 0.05$).

the callus induction results after a four months growth period (subcultured every 4 weeks). Between 33.3 and 75.0% of callus induction and growth occurred. However, in nine treatments no formation of calli was observed. Different types and concentrations of growth regulators are known to have different effect on growth and developmental processes [32,33]. A particularly strong interaction was observed between 2 mg/l 2,4-D and 1 mg/l BAP. After 11–12 weeks, these calli became whitish-yellow and friable, with this indicating that different auxin and cytokinin types and ratios played important roles in callus induction and proliferation (Fig. 1B).

The medium with Pi/BAP produced compact, slow-growing calli with hard tissue. In the 0.1 mg/l Pi:2 mg/l BAP culture medium assayed, small calli appeared after 6 weeks of culture, but also simultaneously showed a certain degree of redifferentiation, and developed adventitious shoots (Table 1). The concentrations of Pi/KIN were toxic for the cultures, which proved to be the strongest inhibitor of callus growth (Table 1).

A cell suspension culture was also established in order to obtain plant material with the lowest degree of differentiation. The growth in suspension cultures of *L. divaricata* was studied in cells cultured in MS medium supplemented with 2 mg/l 2,4-D and 1 mg/l BAP, which was the medium that provided the strongest growth in callus cultures (Table 1). When yellow and friable calli were placed in liquid culture, they easily broke apart and dispersed into clumps. Further agitation fragmented these clumps into small cell aggregates. This cell suspension culture presented a normal growth cycle with a lag growth phase of approximately 5 days for fresh weight and 7 days for dry weight, an exponential phase until day 9 and a stationary phase until the end of the culture cycle (Fig. 2).

### 3.2. Study of callus organogenesis and plant regeneration

Small callus pieces (0.5 g FW) were cultured separately in MS medium supplemented with 0.1 mg/l Pi in combination with three concentrations of BAP (2, 3 and 4 mg/l) or each phytohormone alone (0.1 mg/l of Pi or 4 mg/l of BAP). Table 2 shows the callus

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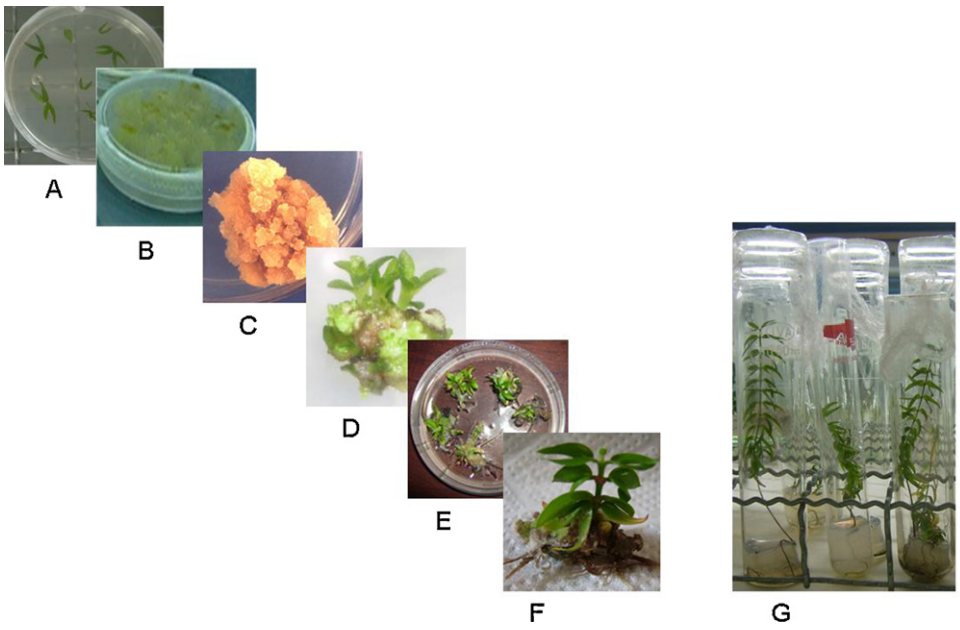


**Fig. 1.** Morphogenetic developments in tissue cultures of *L. divaricata* from leaf explants. (A) Leaf explants (0 day); (B and C) callus mass formation and growth; (D) adventitious shoot formation; (E and F) shoot elongation and growth and (G) full development.

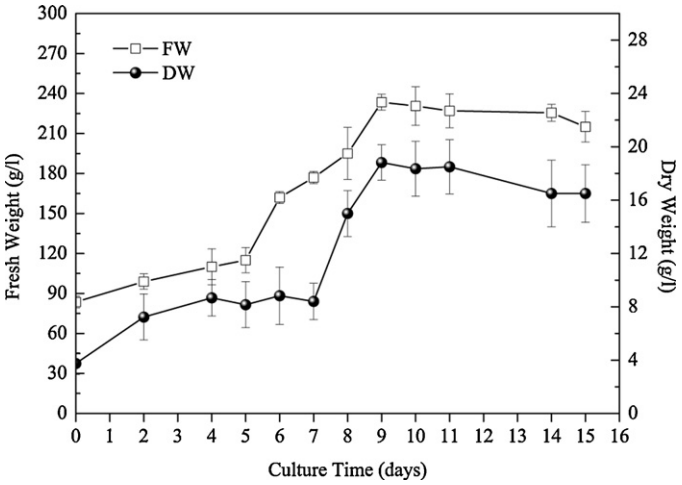


**Fig. 2.** Kinetics of biomass formation by *L. divaricata* cell cultures on MS medium containing 2,4-D and BA (2:1 mg/l). Data represent average values from 3 separate experiments ± SD. FW, fresh weight; DW, dry weight.

growth capacity and tendency to develop organogenesis. In this study, although the starting material was undifferentiated tissue, after six weeks of culture, the callus developed organogenesis. The presence of adventitious buds was apparent after 6 weeks, and adventitious shoot development was also observed after 16 weeks of culture (Fig. 1A–D). These results confirm the strong propensity of *L. divaricata* callus pieces grown on media with a high BAP concentration to develop organized structures and also demonstrate the low efficiency of the plant growth regulator treatments to completely inhibit this process.

BAP plays a key role in plant regeneration *in vitro* [34,35]. In the present experiment, BAP alone was able to induce adventitious shoot regeneration at a rate of 88.0%, whereas Pi alone appeared to have a suppressive effect on shoot differentiation (Table 2). Increasing the concentration of BAP in the culture media generally stimulated the development and regeneration of adventitious shoots, an observation previously found in several types of *in vitro* cultures for other plant species [36]. To the best of our knowledge, no successful method has been reported for adventitious shoots regeneration from the callus of *L. divaricata*.

When adventitious shoots regenerated from organogenic callus cultures were cultivated in MS together with 2.5 μM AIA and 5% (w/v) sucrose for rooting (as previously described [29]), 70% of adventitious shoots rooted after 6 weeks. The *in vitro* plants obtained showed a very similar morphology to those grown in greenhouse (data not shown). Consequently, this system is an easy protocol for the micropropagation of *L. divaricata* (Fig. 1A–G).

**Table 2**
Effects of different growth regulator combinations on adventitious shoot regeneration in *L. divaricata* calli.

| PGR[a] | | | | Percentage of explants forming shoots (%) | No. of shoots/explant |
|---|---|---|---|---|---|
| Auxin | | Cytokinin | | | |
| | mg/l | | mg/l | | |
| Pi | 0.1 | BAP | 2 | 27.3 ± 4.0a | 2.36 ± 0.95a |
| | 0.1 | | 3 | 34.6 ± 2.3a | 2.54 ± 1.11a |
| | 0.1 | | 4 | 53.3 ± 6.1b | 5.98 ± 0.82b |
| | – | | 4 | 88.0 ± 1.0c | 8.43 ± 0.81c |
| | 0.1 | | – | 0.0 | 0.0 |

[a] PGR, plant growth regulator; BAP, ($N^6$-benzylaminopurine); Pi, Picloran.
Data collected after four months of culture.
Values represent the mean ± S.D. Means following the same letter within columns are not significantly different, according to Duncan's multiple range test ($P < 0.05$).

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**Table 3**
Effects of different combinations of growth regulator on phenolic compound contents of *L. divaricata* calli.

| PGR[a] | | | | Growth | Phenolic compounds | |
|---|---|---|---|---|---|---|
| Auxins | mg/l | Cytokinins | mg/l | DW (mg)[b] | NDGA (μg/g DW) | Quercetin (μg/g DW) |
| Pi | 0.1 | BAP | 0.1 | – | – | – |
| | | | 1 | – | – | – |
| | | | 2 | 71.33 ± 9.12c[c] | 165.32 ± 10.54d[c] | 18.98 ± 1.98d[c] |
| Pi | 1 | BAP | 0.1 | 70.43 ± 8.23c | 2.87 ± 0.91a | – |
| | | | 1 | – | – | – |
| | | | 2 | – | – | – |
| Pi | 2 | BAP | 0.1 | 110.72 ± 12.21d | 14.89 ± 0.89b | 1.76 ± 0.87a |
| | | | 1 | 41.21 ± 4.42b | 25.32 ± 2.79[c] | 3.98 ± 0.99b |
| | | | 2 | – | – | – |
| 2,4-D | 0.1 | BAP | 0.1 | – | – | – |
| | | | 1 | 82.73 ± 6.36c | – | – |
| | | | 2 | – | – | – |
| 2,4-D | 1 | BAP | 0.1 | 36.76 ± 4.34b | 13.34 ± 2.01b | 4.78 ± 0.54b |
| | | | 1 | – | – | – |
| | | | 2 | 39.90 ± 9.31b | – | – |
| 2,4-D | 2 | BAP | 0.1 | 73.71 ± 8.91c | 15.52 ± 0.19b | 5.12 ± 0.45b |
| | | | 1 | 344.69 ± 9.3h | 29.21 ± 0.91c | 10.55 ± 0.71c |
| | | | 2 | – | – | – |
| 2,4-D | 0.1 | KIN | 0.1 | 21.3 ± 4.1a | – | – |
| | | | 1 | 38.8 ± 5.2b | – | – |
| | | | 2 | 43.9 ± 8.2b | – | – |
| 2,4-D | 1 | KIN | 0.1 | 210.3 ± 11.1f | – | – |
| | | | 1 | 84.7 ± 7.4c | – | – |
| | | | 2 | 104.9 ± 9.9d | – | – |
| 2,4-D | 2 | KIN | 0.1 | 291.3 ± 9.8g | – | – |
| | | | 1 | 156.5 ± 14.1e | – | – |
| | | | 2 | 70.5 ± 9.2c | – | – |

[a] PGR, plant growth regulator; BAP, ($N^6$-benzylaminopurine); KIN, kinetin; 2,4-D, 2,4 dichloroacetic acid; Pi, Picloran.
[b] DW, dry weight.
[c] Organogenic calli.
Data collected after four months.
Values represent the mean ± S.D. Means following the same letter within columns are not significantly different, according to Duncan's multiple range test ($P < 0.05$).

### 3.3. *In vitro* phenolic compound production

The capacity of *L. divaricata* calli, cell suspension cultures, plantlets and wild plants to produce the active metabolites: NDGA, *p*-coumaric acid, cinnamic acid, ferulic acid, caffeic acid, sinapic acid, sinapyl alcohol, coniferyl alcohol, quercetin and kaemferol aglycones was evaluated.

It has been previously established that manipulation of secondary product formation in medicinal plants is possible by varying the culture conditions, including the growth regulator type and concentration [32,37,38]. Table 3 shows the levels of the lignan NDGA and the flavanoid quercetin that were found in the calli. The type and concentration of auxin or cytokinin as well as the auxin/cytokinin ratio modified the pattern and concentration of secondary metabolites in plant cells and tissue cultures.

A high ratio of 2,4-D/BAP and Pi/BAP in the culture media promoted the production of phenolic compounds in non-organogenic calli. The highest mean quercetin level was produced with 2 mg/l 2,4-D:1 mg/l BAP medium, which was significantly greater than with the other media. The highest mean NDGA content was obtained with 2 mg/l 2,4-D:1 mg/l BAP and 2 mg/l Pi:1 mg/l BAP media. Our results also demonstrated that the substitution of KIN by BAP inhibited the synthesis of these phenolic compounds (Table 3), which callus grown at different concentrations of BAP and auxins (Pi or 2,4-D) having a selective ratio lignan (NDGA)/flavonoid (quercetin) accumulation response.

When the calli grown on 0.1 mg/l Pi and 2 mg/l BAP medium showed a certain degree of redifferentiation (adventitious shoot formation), the NDGA and quercetin accumulation increased more than in non-organogenic calli. Moreover NDGA and quercetin content depended on the state of callus differentiation (Table 3).

Plant cell suspension cultures presented a different phenolic compound pattern from the initial inoculum (calli grown on MS, 2,4-D 2 mg/l and BAP 1 mg/l). These cell cultures were able to produce four intracellular phenolic compounds: lignan NDGA and the phenylpropanoids *p*-coumaric acid, ferulic acid and sinapyl alcohol (Fig. 3). These phenylpropanoids are natural phenolic compounds that exhibit a wide range of biological effects against various diseases, including cancer, diabetes, cardiovascular and neurodegenerative illnesses. In addition, ferulic acid has been approved in Japan as a food additive which prevents oxidation [39].

As stated above, these simple-structured metabolites are found in several plant families, but no such phenolic compounds have yet been isolated from wild plants of *L. divaricata*. To date, ferulic acid has only been reported in the species of *Larrea nitida* [40]. In addition, secondary metabolite patterns have been observed to change in other plant species under *in vitro* conditions [36,41].

While the accumulation of secondary product synthesis may depend upon the state of differentiation of the cell or organ, the maximum levels of NDGA found in the present study in cell suspension cultures (day 4) were significantly greater (9.9-fold) than in non-organogenic callus cultures (Fig. 4). Similar findings were also reported for asiaticoside in *Centella asiatica* [42] and, for isoflavone in *Genista tinctoria* [41].

The calli showing the highest formation of fully developed adventitious shoots (grown on 4 mg/l BAP medium) were also

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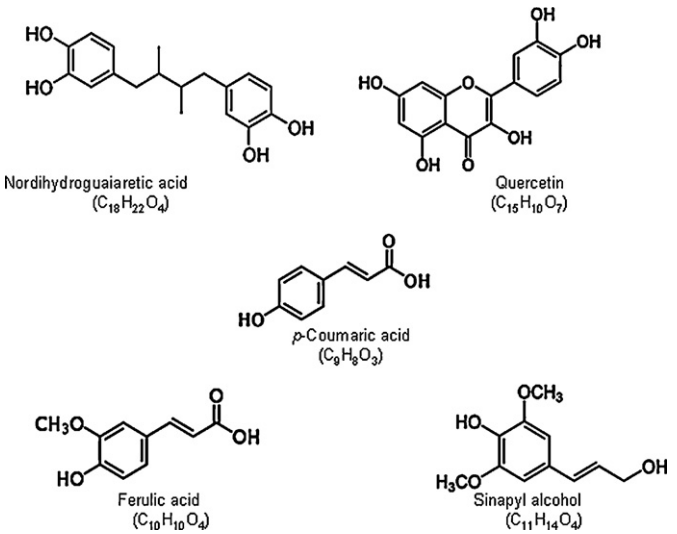


**Fig. 3.** Structural formulae of identified phenolic compounds in *Larrea divaricata* cell and tissue cultures.

assessed for phenolic compound accumulation, which revealed the three phenolic compounds NDGA, quercetin, and the phenylpropanoid ferulic acid. Organogenic calli cultures produced much higher levels of NDGA and quercetin than unorganized callus tissue, suggesting that tissue differentiation directly influences product accumulation (Fig. 4). Our findings are consistent with the results of Palazón et al. [43], who showed that a low amount of saponin was produced in undifferentiated tissues, but the production increased when the calli developed organogenesis. Sood and Chauhan [44] also reported that the production of Picroside-I in different types of *Picrorhiza kurroa* tissue cultures increased with the degree of morphological differentiation.

Plantlets of *L. divaricata* presented a greater capacity to synthesize and accumulate NDGA and quercetin (Fig. 4). Another metabolite detected in plantlets, although at a low level, was ferulic acid. On average, the NDGA and quercetin contents were 1.6-times and 1.2-times greater, respectively, in microplants than in calli, showing adventitious shoot formation (medium: 4 mg/l BAP). These results confirm the low capacity of the callus tissue and suspension-cultured cells to produce these phenolic compounds,

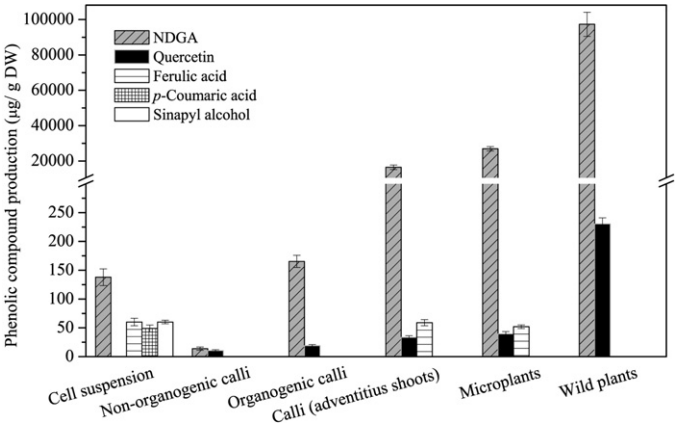


**Fig. 4.** Phenolic compound production in *L. divaricata* cultures with different degrees of differentiation. Cell suspension, MS medium supplemented with 2 mg/l 2,4-D and 1 mg/l BAP; non-organogenic calli, MS medium supplemented with 2 mg/l 2,4-D and 1 mg/l BAP; organogenic calli, MS medium supplemented with 0.1 mg/l Pi and 2 mg/l BAP; organogenic calli with adventitious shoots, MS medium supplemented with 4 mg/l BAP; microplants, MS medium supplemented with 2.5 μM AIA and 5% (w/v) sucrose. $^*N = 12 \pm$ S.D. DW, dry weight.

and that organized structures are necessary if these compounds are to be synthesized at high amounts. Another metabolite detected in plantlets, although at a low level, was ferulic acid. However, it was not possible to find a correlation between its level and the degree of differentiation. The low ferulic acid biosynthetic capacity observed in plantlets may be explained by a possible metabolization of this phenylpropanoid pathway intermediate to afford NDGA.

NDGA and quercetin were also accumulated in five-year-old wild plants, and their contents were found to be 3.6- and 5.9-fold higher, respectively, than in *in vitro* plants (Fig. 4). In addition, it was possible to produce adventitious shoots with a high content of NDGA in a short period of only three months, which could be useful for pharmacological studies. Regarding the phenylpropanoids, none of these metabolites were found in the evaluated *L. divaricata* wild plants.

Our data also indicated that the content of NDGA and quercetin increased with differentiation; with NDGA being the major product in all types of plant material. Thus, in *L. divaricata*, NDGA may be considered to be a marker for differentiation.

Taken as a whole, our results suggest that the aerial part of the *L. divaricata* plant is important for the biosynthesis of NDGA and quercetin, and that the undifferentiated tissues have only a limited capacity to synthesize these compounds. The analyses performed showed a clear increase of NDGA and quercetin levels in parallel with an increase in differentiation.

The two major classes of phenolic compounds found in *L. divaricata* tissue culture were lignan (NDGA) and flavonoid (quercetin). In the phenylpropanoid biosynthetic pathways, there were many branching points and the differential ratio of lignans/flavonoids could be controlled. The regulation of fluxes from cinnamic acid into lignan or flavonoid changed in response to the level of tissue differentiation. However, the cell suspension culture did not show the same phenolic compound pattern as callus cultures, *in vitro* or in wild plants, resulting in a lack of production of quercetin. In the callus cultures, a redistribution of the carbon flux between NDGA and quercetin production was observed, with the opening of a new pathway of quercetin apparently channeling the flux away from the NDGA branch, resulting in a considerable reduction of this lignan. In contrast, in the cultures with a higher degree of differentiation (organogenic calli and micropropagated plants) a higher level of the ratio NDGA/quercetin was found, as in the case of the wild plant.

Our studies showed that in cell culture systems, the production was lower than in intact plants. There also seemed to be a connection between the various phenolic pathways, suggesting the need for further studies which may reveal leads to future metabolic engineering of the pathways of interest. In addition, it might be possible to use external signals to activate certain pathways. At least for the present, however, biotechnology seems to be of great importance for breeding and growing *via* plant micropropagation.

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