COUMARIN PRODUCTION IN *Mikania glomerata* CALLUS

A.M.S. Pereira and S.C. França
Departamento de Biotecnologia Vegetal
Universidade de Ribeirão Preto – UNAERP
14096380 Ribeirão Preto, SP, Brazil

F.L.A Câmara
Departamento de Horticulutura
Faculdade de Agronomia UNESP
Botucatu, SP, Brazil

Keywords: guaco, *Mikania glomerata*, tissue culture, medicinal plant

Abstract

Cell cultures of *Mikania glomerata* Sprengel were established with leaf segments cultured on White medium supplemented with 1 mg/L BA and 3 mg/L NAA. Different types and concentrations of growth regulators were tested for callus maintenance. Determination of coumarin content was performed in HPLC using authentic coumarin standard. Growth regulator concentration affected biomass and coumarin accumulation. Cultures developed in semisolid medium containing both BA and NAA exhibited enhanced biomass production as well as coumarin accumulation. In the most favorable conditions tested, cells accumulated 25 μg/g of dry weight what is much inferior to the yield already reported in intact plants (5 mg/g of dry weight). However, results obtained so far suggest several alternatives for culture manipulation in order to optimize the productivity of coumarin by *M. glomerata* cultured cells.

1. Introduction

*Mikania glomerata* Sprengel, a medicinal species, popularly known in Brazil as guaco, belongs to the Asteraceae family, Eupatorieae tribe and Mikaniinae subtribe and has its monograph in the first edition of *Farmacopéia Brasileira* (Dias and Silva, 1929). The genus *Mikania* is composed of approximately 3,000 species distributed in tropical areas in America, Africa and Asia. Of these, about 150 are found in Brazil.

Biological assays carried out with *M. glomerata* showed that crude hydroalcoholic leaf extracts of this species have anti-inflammatory and expectorating activities, being the coumarin one of the substances associated to that effect (Ruppelt et al., 1991; Soares et al., 1990; Soares et al., 1996).

The aim of the present work was to establish favorable conditions for the production of cells of *M. glomerata* as a first step in the optimization of coumarin production in *in vitro* cultured cells.

2. Materials and methods

Axenic leaf segments (1 cm²) excised from in vitro cultured six-month old plantlets were inoculated on semisolid White (1951) medium supplemented with 3 mg/L naphthalene-acetic acid (NAA) and 1mg/L 6-benzylaminopurine (BAP) to induce callus formation.

After one year of culture, with monthly transfers, induced calli were inoculated on different culture media: a) White + 3mg/L NAA and 1mg/L BAP; b) MS (Murashige and Skoog, 1962) + 3mg/L NAA and 1mg/L BAP; c) B5 (Gamborg, 1984) + 3mg/L NAA and 1mg/L BAP; d) White + 3mg/L NAA; e) White + 1mg/L NAA; f) White + 1mg/L NAA + 0,1mg/L BAP; g) White +1mg/L NAA+ 0,1mg/L Kynetin (kin); h) White +3mg/L NAA and 1mg/L Kynetin; i) White 3mg/L 2,4D and 1mg/L BAP; j) White 3mg/L 2,4D and 1mg/L Kynetin.
In all experiments the explants were placed into glass flasks (8.5 x 5.5 cm) each containing 20 mL of medium supplemented with 30 g/L of sucrose, solidified with 2.0 g/L phytagel (Sigma), pH adjusted to 6.0. Culture flasks were closed with polypropylene closures (Bellco) and autoclaved at 121°C and 105 KPa for 15 minutes, sealed with plastic film and maintained at growth room at 25 ± 2°C, 55-60% of relative humidity under 16 h day photoperiod (40 μmol m⁻² s⁻¹, 85 W cool-white GE fluorescent lamp).

The experimental design adopted in the experiments was fully randomized with three replicates per treatment and ten explants per replicate. Data were analyzed statistically by ANOVA and means were compared by the Tukey test, with the level of significance set at 5%.

2.1. Establishment of the curve of biomass accumulation of cells of *M. glomerata* and coumarin production

One-year old callus obtained from a stock cell line subcultured every 30 days on White semi-solid medium, supplemented with 1 mg/L of BAP, 3 mg/L of NAA, 30 g/L sucrose and 0.2% of phytagel (Sigma) were transferred to glass flask (5.0 x 9.0 cm) containing 20 mL of MS semi-solid medium supplemented with 1mg/L of BAP, 3 mg/L of NAA, 30 g/L sucrose and 0.2% of phytagel. Callus were incubated at 27±2°C, under a 16 h day photoperiod (2000± 200 lux of luminous irradiation). The weight of the initial inoculum was standardized in 1 g and 22 samples of fresh calli biomass were harvested at regular intervals of 4 days to complete 32 days of culture. In parallel experiment, harvested samples were dried and coumarin contents were evaluated.

2.2. Analysis of coumarins

The quantitative analysis (determination of the content of coumarin in plant material) was based on an HPLC method preliminary reported (Celeghini *et al.*, 1996 a). Each sample of ground material (1.0 g) was extracted under sonication with 10 mL of EtOH : H₂O (1:1, v/v) for 20 min. The crude extract was filtered and directly analyzed by HPLC (high performance liquid chromatography) using a liquid chromatograph Shimadzu model LC-10 AD with manual injector model 7725i and photodioderearray UV-Vis detector SPD - M10A. The stationary phase column (Supelcosil LC-18) was 4.6 mm i.d. x 25 cm and 5 μm particle size. Samples were analyzed using as mobile phase CH₃CN : H₂O (40:60, v/v), isocratic mode, flow rate 1 mL/min. Sample volume was 20 μL. Quantitative analysis was done by external calibration method, using an authentic standard of coumarin (Sigma), with detector fixed at 274 nm. Every sample was analyzed at least in triplicate. For analytical validation of the HPLC method, see Celeghini *et al.* (1996 b).

3. Results and discussion

The initial growth of cells was extremely slow. It was necessary one year of culture to get of friable cells that were suitable to subculture. Initially the tissues of the leaves became rhizogenic and the production of cells was harmed due to the emerging of roots. Only leaf segments that did not produce roots were maintained in the experiment while the others were discarded. Investigation on the nutritional effects of different basal media on the proliferation of cells was conducted and it was also possible to observe morphologic aspects of the cells cultivated on B5, White and MS media. The concentration of salts in the White medium is reduced in comparison to B5, and this last one is enriched with nitrogen. Nutrients of White medium increased the production of
coumarin and reduced growth of *M. glomerata* cells and B5 medium caused opposite effects. It has been shown in several works that the increase on nitrogen and potassium levels reduces the production of most of the phenolic compounds (Khouri *et al.*, 1986). In general, when the culture medium favors cell proliferation, the nutrients and sources of carbon are absorbed by plant cells and the flux of primary metabolism is activated to the maximum. However, when the culture medium possesses low concentration of salts and the plant demands high level of nutrients, the cells undergo a stress process, the growth index is reduced and the secondary metabolism pathways are activated with the production and accumulation of special micromolecules (Khouri *et al.*, 1986; Yamamoto *et al.*, 1993; Meyer and Staden, 1995).

The study of the influence of growth regulators on cellular proliferation and coumarin production showed that the addition of NAA or 2,4 D (3 mg/L) associated to 1 mg/L of BAP promoted similar cellular proliferation. However, NAA favored coumarin production.

The association of NAA and cytokinin influenced positively both biomass accumulation and coumarin biosynthesis. The combination NAA and Kin increased cellular proliferation, while the association NAA and BAP favored coumarin production (Table 1). It is important to point out that the ratio NAA/BAP = 10 indeed was more favorable in terms of coumarin productivity (mg/g of dry biomass).

Literature is plenty of evidences to affirm that the concentration and type of auxin and cytokinin influence the synthesis and accumulation of secondary metabolites. Also, the response to growth regulators seems to be related to the plant cellular differentiation or organization. The accumulation of some types of coumarins and lignans have been stimulated by the presence of low levels of auxin (King, 1976; De-Eknamkul and Ellis, 1985). Hamdi *et al.* (1995) studied the effect of cytokinin on the production of coumarin (escopoline) in tissues of *Nicotiana tabacum* and they verified that the addition of BAP favored coumarin accumulation.

The amount of coumarin found in the aerial parts of *Mikania glomerata* is about 5 mg/g of dry weight (Pereira *et al.*, 1997), what means approximately 200 times more of what was produced in the callus.

Ellis (1984) stated that hundreds of structures of secondary metabolites have already been found in different species of plants cultured in vitro, but in general the production and accumulation of these substances are smaller than what is found in the intact plant, which grows under not controlled conditions. On the other hand, Scragg (1994) pointed out few examples of high producing plant cell cultures. There are strong evidences that the establishment of high producing cultures depends on the knowledge of the pathways, and how they can be manipulated to control the synthesis of many micromolecules.

Cell response to culture conditions was characterized by a long lag phase of sixteenth day while the log phase was observed from the twentieth, till the twenty-eighth day. During the lag phase the cells did not produce coumarin in detectable amounts, the accumulation occurred in the log phase of growth. This result seems to be related to the stage of maturation of the cell and also to alterations in the levels of some enzymes. Pereira, 1997, reported on the correlation of coumarin yields with the intense cellular division in young organs of *M. glomerata* intact plants. This is in agreement with our results in which cultured cells in the log phase of growth accumulated higher amounts of coumarin.

Overall results evidenced that culture conditions must be optimized to meet the cell requirements for growth. Minimum doubling time (T_D = 8.1 day) is still long, although within the limits generally accepted (Payne *et al.*, 1991).
4. References


<table>
<thead>
<tr>
<th>Basal medium</th>
<th>Growth regulator (mg/L)</th>
<th>Growth index</th>
<th>Coumarin Dry biomass (µg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>White</td>
<td>1.0 (NAA)</td>
<td>0.260 ± 0.07</td>
<td>N.D</td>
</tr>
<tr>
<td>White</td>
<td>3.0 (NAA)</td>
<td>0.356 ± 0.09</td>
<td>N.D</td>
</tr>
<tr>
<td>B5</td>
<td>3.0 (NAA) + 1.0 (BAP)</td>
<td>1.706 ± 0.23</td>
<td>1.6 ± 0.13</td>
</tr>
<tr>
<td>MS</td>
<td>3.0 (NAA) + 1.0 (BAP)</td>
<td>1.678 ± 0.32</td>
<td>2.0 ± 0.02</td>
</tr>
<tr>
<td>White</td>
<td>3.0 (NAA) + 1.0 (kynetin)</td>
<td>0.546 ± 0.04</td>
<td>2.8 ± 0.42</td>
</tr>
<tr>
<td>White</td>
<td>3.0 (2.4-D) + 1.0 (BAP)</td>
<td>1.306 ± 0.17</td>
<td>N.D</td>
</tr>
<tr>
<td>White</td>
<td>3.0 (2.4-D) + 1.0 (kinetin)</td>
<td>0.973 ± 0.12</td>
<td>1.6 ± 0.01</td>
</tr>
<tr>
<td>White</td>
<td>1.0 (NAA) + 0.1 (BAP)</td>
<td>0.680 ± 0.07</td>
<td>N.D</td>
</tr>
<tr>
<td>White</td>
<td>1.0 (NAA) + 0.1 (kinetin)</td>
<td>1.793 ± 0.12</td>
<td>2.0 ± 0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.543 ± 0.04</td>
<td>1.6 ± 0.01</td>
</tr>
</tbody>
</table>

Means values within rows, followed by the same letters are not significantly different (p < 0.05)