Production of Triterpenoids in Liquid-Cultivated Hairy Roots of *Galphimia glauca*

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Abstract

The production of three triterpenoids from *Galphimia glauca* hairy root cultures, the sedative principle galphimine E (**2**), the recently described glaucacetalin A (**3**), and maslinic acid (**6**), was quantified by HPLC in the biomass and the culture medium. Batch cultures of the hairy root line VYT, obtained through infecting cotyledons with *Agrobacterium rhizogenes* ATCC 15834, were grown for 41 days in shake flasks containing B5 medium without phytohormones. A maximum biomass of 11 g/L DW was obtained on day 33, while the doubling time was 6 days. Throughout the growth cycle fresh and dry weights as well as triterpene production were registered. Glaucacetalin A (**3**), excreted into the culture media, reached a maximum amount of 2.14 mg/L after 21 days while galphimine E (**2**) and maslinic acid (**6**) were recovered from the root biomasses reaching maximum concentrations of 0.11 and 0.43 mg/g, respectively, on day 39.

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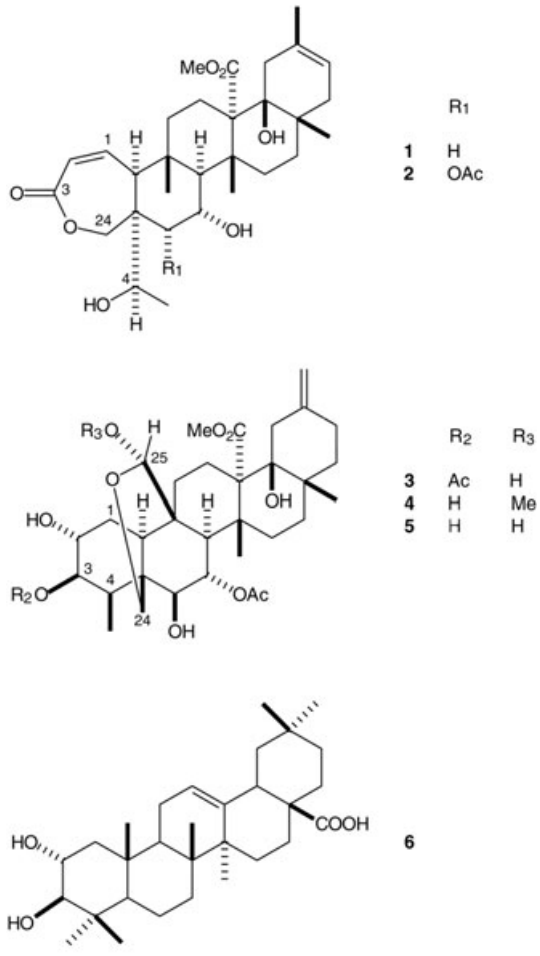

Of all the related nor-secofridelanes identified in the Mexican medicinal plant *Galphimia glauca* (Cav.) Kuntze (Malpighiaceae), collectively known as galphimines [1], only the sedative galphimine B (**1**) with its 6-acetoxy derivative, galphimine E (**2**), have so far been produced *in vitro*. Callus cultures were able to accumulate 0.15 mg/g DW of **1** and 0.56 mg/g DW of **2** in response to different hormonal treatments [2] whereas suspension cultures registered maximum accumulations of 0.21 mg/g DW of **1** and 0.11 mg/g DW of **2** [3]. Recently, the transformed hairy root line VYT was established by infecting cotyledons with *Agrobacterium rhizogenes* ATCC 15834 and the three novel nor-friedelanes then excreted into the culture media were isolated, their structures elucidated, and denominated as glaucacetalins A – C (**3–5**) [4]. All of the above-mentioned *in vitro* results, together with the purification of the complex mixture of galphimines from wild plants with comparable low triterpene production, indicate the need for new biotechnological investigations directed towards higher production of the sedative triterpenes as well as the characterization of their biosynthetic pathways. This paper is concerned with the production of triterpenoids in liquid-cultivated hairy roots of *G. glauca*.

The hairy root cell line VYT grew profusely in B5 medium without hormones. 10 g/L FW inocula in active growth of this cell line were transferred to fresh medium. The fresh and dry weight data of 41 days of culture were recorded (Fig. **1**). The transformed roots showed a high growth rate with a maximum biomass of 11 g/L DW, 14 times higher than the inocula. Root growth followed first order kinetics with a mean specific growth velocity (μ) of 0.12 d^{-1}. Triterpenes were identified at different growth phases in both the hairy root biomass and the culture media in order to establish the kinetics of their production (Fig. **2**). Galphimine E (**2**) and maslinic acid (**6**) obtained from the root biomass at day 39 exhibited maximum yields of 0.11 and 0.43 mg/g DW, respectively. This yield of compound **2** matched the previously obtained amount from cell suspension cultures (0.11 mg/g DW) [3] and was higher than that from callus cultures (0.06 mg/g DW) [2]. In advanced stages of the hairy root culture, the abovementioned active principle was detected as having a discontinuous peaking pattern of accumulation (Fig. **2**) similar to that observed both in cell suspensions [3] and in various plant cell cultures producing secondary metabolites [5]. Glaucacetalin A (**3**), the major recovered compound from the media, showed significant variations in production during the culture period. During

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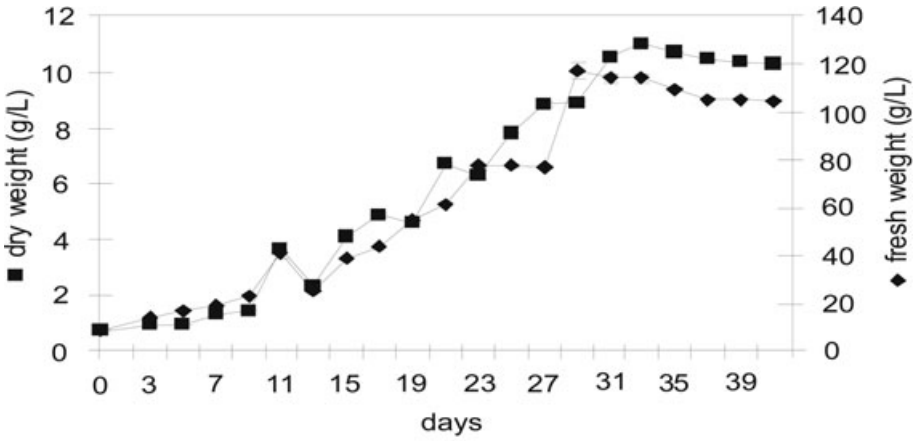

Fig. **1** Typical growth of the hairy root line VYT of *Galphimia glauca* culture grown in B5 medium (n = 3 ± SD).

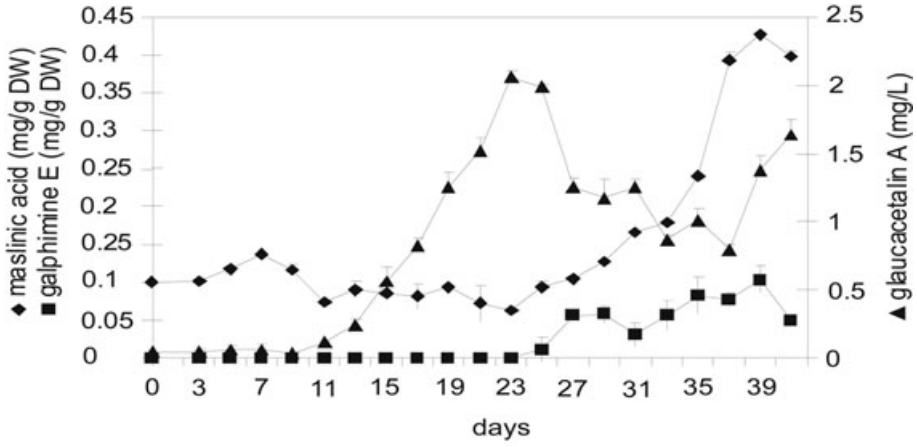

Fig. **2** Production of triterpenoids in hairy root culture of *Galphimia glauca*. Glaucacetalin A (**3**) recovered from culture medium and galphimine E (**2**) and maslinic acid (**6**) from biomass.

the active growth phase both the highest yield (2.14 mg/L) and maximum productivity (0.09 mg/L/d) of **3** were reached on day 21. Kinetic studies were not performed with glaucacetalins B (**4**) and C (**5**) because of their low production during the culture period, merely 0.41 and 0.16 mg/L, respectively.

In the wild plant, the oxidative cleavage of the C3/C4 bond is the predominant process leading to the formation of the ring A lactone although it is dependent on the participation of the primary alcohol in C24, a distinctive feature of all the isolated nor-secofriedelanes of the Malpighiaceae family [6], [7], [8], e. g., the galphimine series [1]. In the hairy roots, the oxidation does not involve cleavage but only generation of the cyclic acetal between the oxidized positions C24 and C25. The production of the glaucacetalines has been observed only in hairy root cultures.

Materials and Methods

The hairy root cell line VYT, cultivated for more than two years [4], was maintained in 250-mL Erlenmeyer flasks with 100 mL hormone-free B5 medium supplemented with sucrose (30 g/L), incubated at 26 – 27 °C, and kept under uniform light conditions (8 – 10 μmol s^{-1} m^{-2}) in a gyratory shaker (115 rpm). The roots were subcultured every three weeks by transferring 1 g FW into fresh medium. For kinetic studies on growth and triterpenoid production, roots were cultivated in a 41-day batch culture in three replicates. The following variables were measured every three days: dry weight, fresh weight, pH, viability, medium carbohydrates and triterpenoid production. Fresh weight was determined as filtered biomass, dry weight after lyophilization, and cell viability was measured using the fluorescein diacetate method [9].

Standard samples of galphimine E (**2**) and glaucacetalin A (**3**) for HPLC quantification were purified as previously described [1], [4]. To isolate maslinic acid (**6**), lyophilized samples of root biomass (ca. 50 g) were extracted by sonication at room temperature with EtOAc (150 mL, 3×) for 20 min and the resulting solution was dried. This extract (84 mg) was subjected to open column chromatography over silica gel (1.5 g), eluted isocratically with EtOAc-CH_2Cl_2 (2 : 1). A total of 30 fractions (2 mL each) was collected, and fractions 18 – 22 were combined yielding 3 mg of pure maslinic acid (**6**; Rf = 0.7, silica gel 60 F_{254} aluminium sheets using EtOAc as eluent). The identity of maslinic acid was confirmed through ^{1}H- and ^{13}C-NMR spectroscopic analysis and by comparison with reported data [10]. Copies of the original spectra are obtainable from the author of correspondence.

To analyze the kinetics of triterpenoid production, hairy root samples in triplicate were harvested every three days. The culture medium (100 mL) was extracted using 10 mL EtOAc (3×). The organic layers were dried over anhydrous Na_2SO_4, concentrated by heating under reduced pressure to a final volume of 500 μL, and then analyzed by HPLC. The biomass (1 g) was lyophilized, pulverized and extracted by sonication with EtOAc (10 mL, 3×)

for 10 min at room temperature. The extracts were filtered over Whatmann No. 1 paper after charcoal addition, the volume reduced to 500 μL and then filtered (0.5 μm). HPLC was used for triterpenoid quantification. Compounds **2** (t_R = 20.0 min) and **3** (t_R = 10.1 min) were analyzed on a Symmetry C_{18} column (Waters; 5 μm, 4.6 × 250 mm) with an isocratic elution of CH_3CN-H_2O (1 : 1), a flow rate of 0.7 mL/min, and detection at 232 and 216 nm for **2** and **3** (see Supporting Information), respectively. Maslinic acid (**6**; t_R = 15 min) was analyzed on a μporasil silica gel column (Waters; 10 mm, 3.9 × 300 mm) with an isocratic elution of hexane-EtOH (95 : 5), a flow rate of 0.7 mL/min, and detection at 212 nm. Suitable calibration curves for galphimine E (**2**), glaucacetalin A (**3**) and maslinic acid (**6**) were constructed from a series of injections of standard sample solutions ranging from 0.062 – 2 mg/mL. Determinations were carried out in triplicate and the peak areas for all tested concentrations were measured to obtain an average result for the construction of each calibration curve. Correlation coefficient values > 0.9997 indicated a linearity for the tested concentrations. Samples from hairy roots (10 μL) were injected and their peak areas were compared against the calibration curves.

Acknowledgments

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