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Lignan production by *Ipomoea cairica* callus cultures ☆

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Abstract

Callus cultures were established from the plant *Ipomoea cairica* (L.) Sweet, to verify whether they produce the same lignans, arctigenin and trachelogenin, as the intact plant. Medium components (auxin, pH, carbohydrates) were changed to influence the production level of the two compounds. Optimal conditions for culture growth and lignan production were determined, and the implications for in vitro lignan production are discussed. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Ipomoea cairica*; Convolvulaceae; Plant cell culture; Lignans; Arctigenin; Trachelogenin

1. Introduction

Lignans, such as arctigenin and trachelogenin, are interesting compounds due to their possible anti-tumour (Páska, Carrara, Innocenti, Cima & László, 1996; Trumm & Eich, 1989; Eich et al., 1990; Hirano, Gotoh & Oka, 1994), anti-HIV (Trumm & Eich, 1989; Eich et al., 1990; Schroder, Merz, Steffen & Muller, 1990; Eich et al., 1996), and Ca^{2+} antagonist activity (Ichikawa, Kinoshita, Nishibe & Sankawa, 1986). Arctigenin and trachelogenin are found in the tropical plant *Ipomoea cairica* (Trumm & Eich, 1989; Eich et al., 1990).

In order to produce these compounds in vitro (Arctigen, X = H; Trachelogenin, X = OH), the biosynthetic capacity of *I. cairica* cell cultures was examined. The establishment of callus cultures of *I. cairica* and identification of lignan compounds biosynthesized by them are reported here. Furthermore, in order to explore the possibility of higher growth rates and metabolite yields, experiments were carried out varying the pH, auxin (2,4-D) concentration, sucrose concentration, and carbon source of the medium. Although accelerated callus growth in general, did not favour secondary metabolite production, lignan production could be increased under certain conditions.

2. Results and discussion

Chemical analysis of *Ipomoea cairica* callus cultures showed the presence of two lignan compounds, arctigenin (Ozawa, Davin & Lewis, 1993; Rahman, Dewick, Jackson & Lucas, 1990) and trachelogenin (Nishibe et al., 1993; John & Tinto, 1992), the lignans occurring in the native plant. In the cultures, the two lignans were detected by TLC and HPLC in varying amounts depending on culture conditions.

The time course of growth and lignan contents are shown in Fig. 1a and b. Cultures initiated growth slowly, but accelerated from day 18 onwards. Fresh and dry weights changed in parallel, but water absorption increased with culture duration. Lignan content was highest at day 4 of culture. It may be hypothesized that trachelogenin appears here as a stress product and

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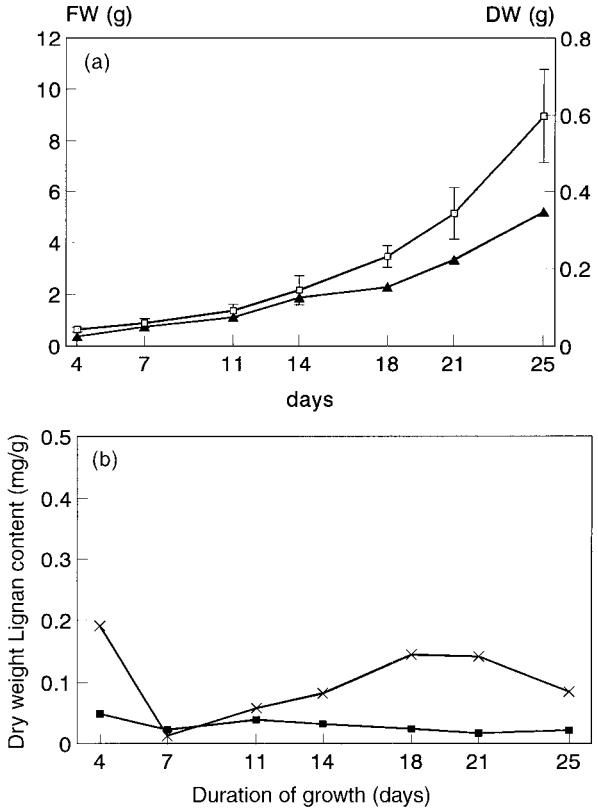


Fig. 1. (a) Time course of callus growth: □-fresh weight, g callus/plant box (±sd), ▲-dry weight, g callus/plant box; (b) time course of lignan content: ■-Arctigenin, ×-Trachelogenin, mg/g dry weight.

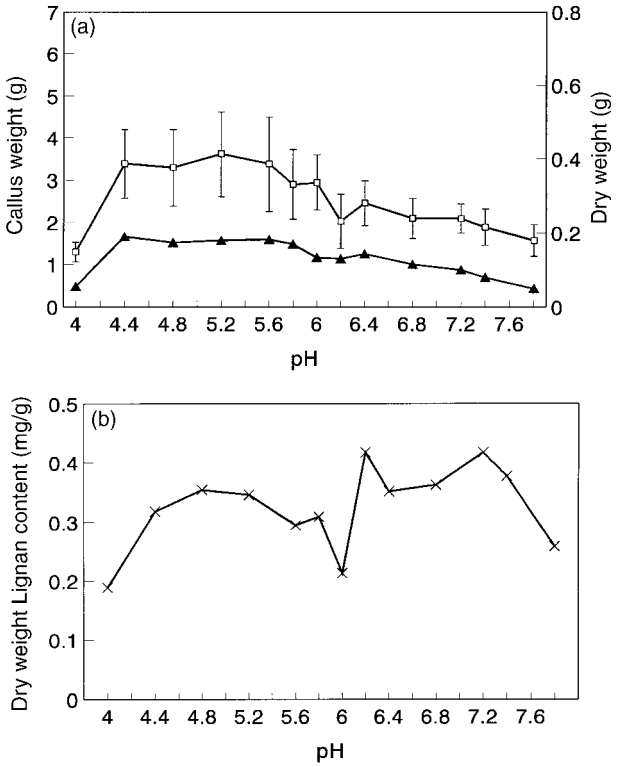


Fig. 2. (a) Callus growth at various pH values: □-fresh weight, g callus/plant box (±sd), ▲-dry weight, g callus/plant box; (b) lignan content at various pH values: ×-Trachelogenin mg/g dry weight, no Arctigenin.

is later metabolized by the cells. After day 7 normal lignan production started increasing gradually until day 18, when the medium was exhausted and lignan reserves were metabolized. Arctigenin content did not show apparent correlation with the growth curve, remaining low throughout the experiment. The best time for harvesting cultures appeared to be between days 18 and 21.

In the second experiment, the pH of the growth medium was varied from 4 to 7.8; the results of which are shown in Fig. 2a and b, in terms of fresh and dry weights and lignan contents. At the lowest pH, cell growth and lignan production were both inhibited, probably because of less efficient sucrose utilization (Martin & Rose, 1976). From pH 5.8 to 7.2, cell growth (fresh weight) and lignan production varied inversely, while between pH 4 and 5.6 growth and lignan production were similar and fairly constant. At the highest pH values, ion uptake was unfavourable (Martin & Rose, 1976), decreasing growth and metabolite production, arctigenin was not produced.

With varied auxin concentration, fresh and dry weights were somewhat parallel, and water absorption was high, with the cells becoming more compact as auxin was increased (Fig. 3a). Trachelogenin contents increased gradually with auxin concentration, sharply at 6 mg/l, and were independent of growth, at least at low auxin levels (Fig. 3b). The effect of stress (high auxin levels) acted as an inhibitor to both growth and trachelogenin production. Arctigenin content remained below the limit of detection throughout the experiment.

The effects of various sucrose concentrations on cell growth and metabolite production were examined. In this case, anomalies were found with respect to the other experiments, and fresh and dry weights did not correlate (Fig. 4a). Fresh weight was highest at 25 g/l sucrose level, with higher water content observed at low sucrose levels. With increasing sucrose, the cells converted more resources into dry weight, with a maximum at 35 g/l (Fig. 4a). At 25 g/l, arctigenin was at its maximum, and at 20 and 40 g/l (extreme values) trachelogenin content was high. Taken together, dry weight and trachelogenin production were optimal at 40 g/l sucrose.

To verify whether sucrose was the best carbon source for growth, various carbohydrates were tested for growth and lignan production (Fig. 5a and b). Glucose, fructose, and to an even greater extent maltose turned out also to be very favourable for the production of both lignans. This experiment was conducted in Petri dishes, and growth rates were correspondingly lower.

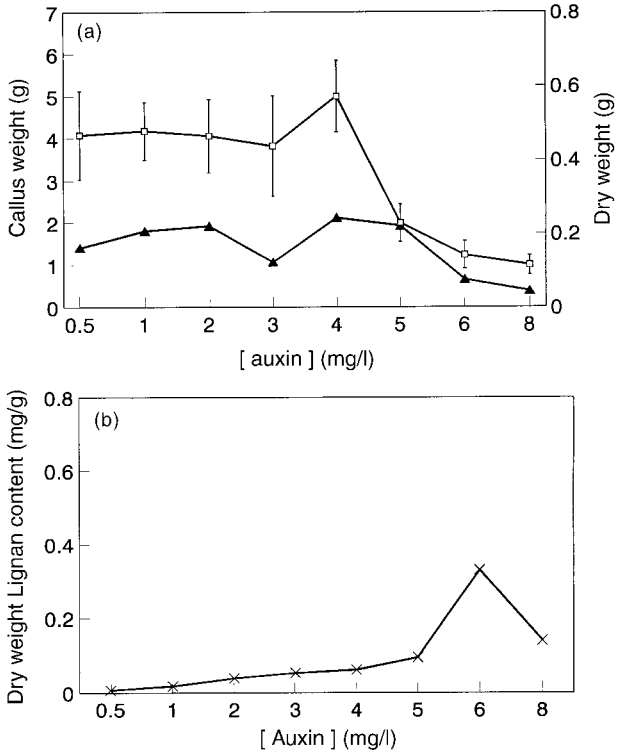
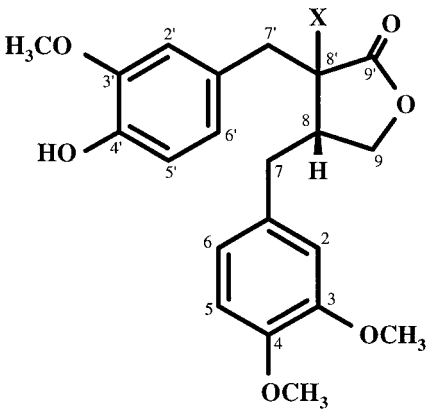


Fig. 3. (a) Callus growth at increasing auxin (2,4-D) levels: □-fresh weight, g callus/plant box (±sd), ▲-dry weight, g callus/plant box; (b) lignan content at increasing auxin (2,4-D) levels: ×-Trachelogenin mg/g dry weight, no Arctigenin.

In conclusion, cultures did produce lignans, but lignan production was subject to considerable oscillation due to callus growth changes and instability. Trachelogenin was always present, and was produced in greater quantities in conditions of stress, whereas arctigenin production fluctuated. In most cases high water absorption by cells seemed to be unfavourable for secondary metabolite production.

To optimize culture production, it will be possible to separate growth and secondary metabolite production in suspension cultures; culture may be initiated with a combination of pH and hormone (pH 5.2; auxin 4 mg/l) favouring growth, and pass to the production phase causing stress to the culture, changing to higher pH (6.2 or 7.2) and auxin level (6 mg/l) at the end of growth. Maltose seems to be suitable as a carbon source. The importance of selecting the right medium components is indicated by a 4-fold increase in yield in the best case (maltose 30 g/l, auxin 6 mg/l), with respect to controls in the single experiments (in favourable experimental conditions further increases are expected), while in the worst conditions the lignans may disappear entirely. For biomass production, an increase in fresh weight was not usually accompanied by any increase in dry weight. Rather, there was a greater risk of losing biomass in the different treatments.



3. Experimental

3.1. Cultures

Cell cultures were initiated from seeds of *I. cairica* (L.) Sweet supplied by CMI (Taipa, Macau) Serviços Agrarios, Index Seminum. After surface sterilization with 30% sodium hypochlorite, seeds were inoculated on hormone-free MS medium (Murashige & Skoog, 1962) containing 30 g/l sucrose and 10 g/l agar, pH 5.6.

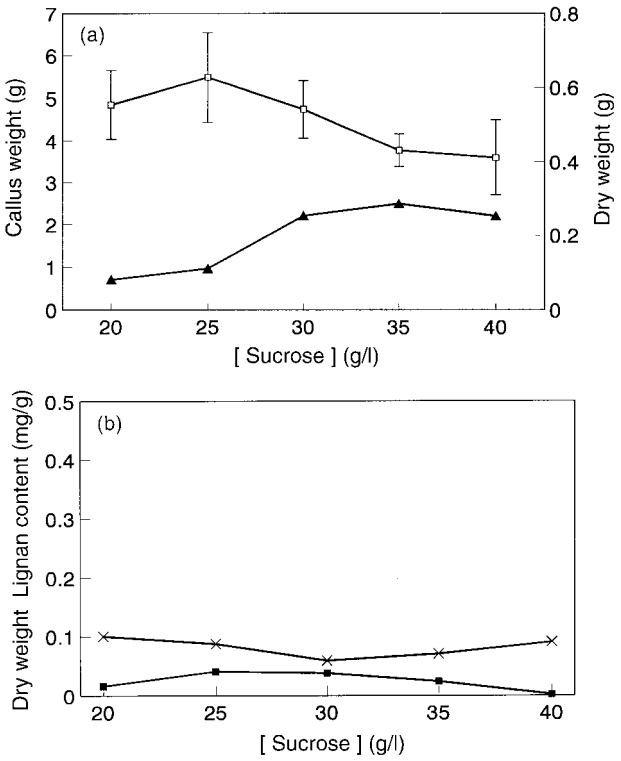


Fig. 4. (a) Callus growth at increasing sucrose levels: □-fresh weight, g callus/plant box (±sd), ▲-dry weight, g callus/plant box; (b) lignan content at increasing sucrose levels: ■-Arctigenin, ×-Trachelogenin, mg/g dry weight.

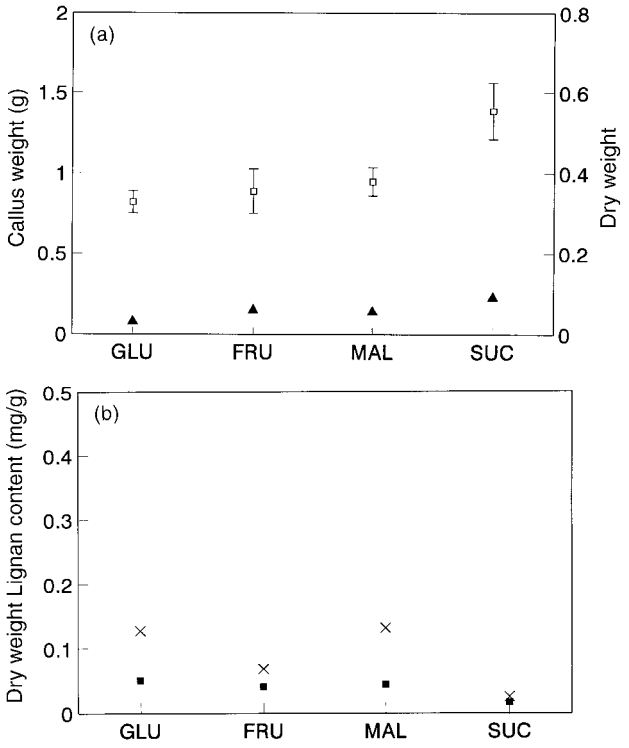


Fig. 5. (a) Callus growth with different carbon sources: GLU, glucose; FRU, fructose; MAL, maltose; SUC, sucrose; □-fresh weight, g callus/Petri dish (±sd), ▲-dry weight, g callus/Petri dish; (b) lignan content with different carbon sources: GLU, glucose; FRU, fructose; MAL, maltose; SUC, sucrose; ■-Arctigenin, ×-Trachelogenin, mg/g dry weight.

Stems and leaves of young sterile plantlets were cut into 2-cm pieces, and transferred to the same medium supplemented with 5 mg 2,4-dichlorophenoxyacetic acid and 0.1 mg 6-benzyl-aminopurine. Callus cultures were maintained on the same medium in plant boxes (80 m1/70 × 100 × 60 mm) and in Petri dishes, and transferred to fresh medium every 2 weeks, with a 12-h light period (Tungsram Warm White 36W F29, 45 μE/s/m^2) at 22°C.

Calli weighing 0.05 g were grown for 2 weeks (except for the growth time-course experiment) on several medium combinations, differing in only one component, such as pH, auxin level, sucrose level or carbon source. After harvest, callus fresh weight was measured, and dry weight measured after lyophilization. Parameters are given before autoclaving. Growth is given in grams callus/plant box, production in mg lignan/g dry weight. There were 3 replicates of every experiment with 110 calli each.

3.2. Chemical analysis

3.2.1. Extraction and isolation of lignans

20 grams of lyophilized, powdered tissue were extracted with methanol in Soxhlet extraction apparatus for 24 h, and extracts were then concentrated under vacuum, dissolved in $CHCl_3$/EtOAc (5:1) and fractionated by flash chromatography (Merck Kieselgel 60, size 0.015–0.040 μm) with the same solvent system. Fractions were further purified with TLC (Merck silica gel 60 F254), using $CHCl_3$/EtOAc (5:1). Purity was checked by HPLC. Arctigenin and trachelogenin were identified by UV and ^{1}H-NMR spectra, and data were consistent with authentic pure samples kindly supplied by Profs E. Eich, N. G. Lewis and S. Nishibe (Ozawa et al., 1993; Nishibe et al., 1993).

3.2.2. Determination of lignans

Samples (0.5 g) of lyophilized, powdered tissue from every experiment were extracted. Lignan contents were determined by HPLC under the following conditions: column LiChrosorb RP8 (Merck) 7 μm (250 × 4 mm) with precolumn (25 × 4 mm), mobile phase MeOH/H_20 (42:58), flow rate 0.9 ml min^{-1}, sample size 10 μl, UV detection at 230 nm. Retention times: arctigenin 14.1 min, trachelogenin 10.4 min.

Quantities were calculated on the basis of the relative peak areas of the lignans to a known amount of an internal standard (7-methoxy coumarin). Limits of detection were 6 μg (R = O.9991) and 3 μg (R = O.9986), for arctigenin and trachelogenin, respectively. Quoted data are the average values of quantitative determinations performed on three different callus samples. Standard deviation was less than 5%.

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References

Eich, E., Pertz, H., Kaloga, M., Schulz, J., Fesen, M. R., Mazumder, A., & Pommier, Y. (1996). *J. Med. Chem.*, *39*, 86.

Eich, E., Schulz, J., Trumm, S., Sarin, P. S., Maidhof, A., Merz, H., Schroder, H. C., & Muller, W. E. G. (1990). *Planta Medica*, *56*, 506.

Hirano, T., Gotoh, M., & Oka, K. (1994). *Life Sciences*, *55*(13), 1061.

Ichikawa, K., Kinoshita, T., Nishibe, S., & Sankawa, U. (1986). *Chem. Pharm. Bull.*, *34*(8), 3514.

John, L. M. D., & Tinto, W. F. (1992). *J. Nat. Prod.*, *55*(9), 1313.
Martin, S. M., & Rose, D. (1976). *Can. J. Bot.*, *54*, 1264.
Murashige, T., & Skoog, F. (1962). *Physiol. Plan.*, *15*, 473.
Nishibe, S., Fujimoto, T., Nose, M., Takeda, T., Ogihara, Y., & Xu, G. (1993). *Phytochemistry*, *32*(6), 1579.
Ozawa, S., Davin, L. B., & Lewis, N. G. (1993). *Phytochemistry*, *32*(3), 643.
Páska, C., Carrara, M., Innocenti, G., Cima, L., & László, M. (1996). *Toxicology letters*, *88*, 104 Suppl. 1.
Rahman, M. M. A., Dewick, P. M., Jackson, D. E., & Lucas, J. A. (1990). *Phytochemistry*, *29*(6), 1971.
Schroder, H. C., Merz, H., Steffen, R., & Muller, W. E. G. (1990). *Z. Naturforschung*, *45c*, 1215.
Trumm, S., & Eich, E. (1989). *Planta Medica*, *55*, 658.