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Phenolic profiles of untransformed and hairy root cultures of *Plantago lanceolata*

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Abstract — Untransformed root cultures and *Agrobacterium rhizogenes* induced root cultures (hairy roots) of *Plantago lanceolata* were investigated for caffeic acid glycoside esters, i.e. verbascoside (V) and plantamoside (P), by HPLC. Levels of V (6–12 mg·g^{-1} DW) and P (30–80 mg·g^{-1} DW) from untransformed and hairy root cultures were not modified by 0.1 mM (*E*)-cinnamic acid addition in Murashige and Skoog's culture medium. A part of the cinnamic acid was converted into (*E*)-*p*-coumaroyl-1-O-β-D-glucopyranoside, a phenolic derivative absent from control cultures without cinnamic acid. Maximum levels of this coumaroyl ester (6–8 mg·g^{-1} DW) were detected during 10 d and then slightly decreased from both root chemical profiles. © Elsevier, Paris

***Agrobacterium rhizogenes* / (*E*)-cinnamic acid / (*E*)-*p*-coumaroyl-1-O-β-D-glucopyranoside / *Plantago lanceolata* / plantamoside / root culture / verbascoside**

CGEs, caffeic acid glycoside esters / DW, dry weight / HPLC, high performance liquid chromatography / MS medium, Murashige and Skoog's medium / P, plantamoside / PCG, (*E*)-*p*-coumaroyl-1-O-β-D-glucopyranoside / SDS, sodium dodecyl sulfate / TLC, thin layer chromatography / V, verbascoside

1. INTRODUCTION

Chemical analyses of *Plantago lanceolata* (ribwort plantain) led to the isolation of iridoids [25], flavonoids [20], coumarins [19], volatile compounds [14] and caffeic acid glycoside esters (CGEs) [3, 29]. The two main CGEs of ribwort plantain (*figure 1*), i.e. verbascoside (V) and plantamoside (P) showed antimicrobial [1, 31], antioxidant [5, 24], anti-inflammatory [29] and antitumoural activities [21, 33]. In a previous paper [13], aerial and underground parts of in vitro cultured ribwort plantains were investigated for CGEs by thin layer chromatography (TLC) and high performance liquid chromatography (HPLC); V and P were highly concentrated in the roots with P levels double those of V.

The aim of the present study was to compare V and P accumulation in both untransformed and hairy root cultures produced by *P. lanceolata* tissues inoculated with *Agrobacterium rhizogenes*. This kind of tissue culture generally showed many advantages all along growth and production stages; transformed root clones can be maintained on medium without growth regulators. These clones are genetically more stable than cell cultures and show wide production field and high level of metabolite production over a long time course [35,

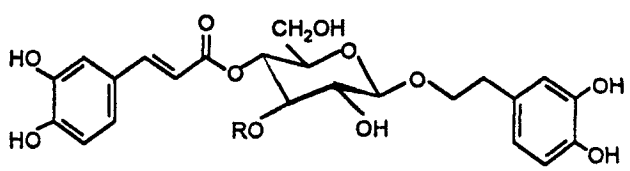

Figure 1. Structures of verbascoside (R, rhamnose) and plantamoside (R, glucose).

39]. On the other hand, untransformed and hairy root cultures of *P. lanceolata* fed with (*E*)-cinnamic acid were investigated for phenolic derivatives by HPLC. (*E*)-Cinnamic acid, which has been shown to be integrated into polyphenol pathway of different plant species [2, 9, 30, 32], was added in the culture medium in order to enhance P and V content and produce new phenolic metabolites.

2. RESULTS

2.1. Root production

Untransformed and hairy root cultures from *P. lanceolata* were induced from root fragments of young whole seedlings, 6 weeks after seed germination. Indeed, a previous study showed that whatever the age of seedlings (22 to 57-d-old), 67 % of root fragments and only 11 % of leaf fragments allowed untransformed or hairy root production [12]. Genetic transformation of T1 and T2 root clones (used for the cinnamic acid biotransformation experiment) was confirmed by Southern blotting using a labelled probe specific of T-DNA (*figure 2*). Hairy root cultures of *P. lanceolata* produced much more biomass than untransformed root cultures according to their tumoural origin.

2.2. V and P accumulation

Untransformed roots and hairy roots were analysed for caffeic acid glycoside esters (CGEs). In both root types of *P. lanceolata*, V and P were the two major CGEs detected by TLC and HPLC. On day 0 of the cinnamic acid biotransformation experiment, 42, 50 and 58-d-old untransformed and hairy root cultures (named A–B, C–D and E–F cultures, respectively) were transferred into either control MS medium or MS medium with added cinnamic acid. The twelve types of root cultures were investigated for V and P accumulation every 3–5 d from day 0 to day 19.

The average levels of V ranged from 6 to 12 $mg \cdot g^{-1}$ DW, whatever the age and the type of root, and the medium (*figure 3*), although V accumulation could reach 18 $mg \cdot g^{-1}$ DW at day 19 (*figure 3 F*) and

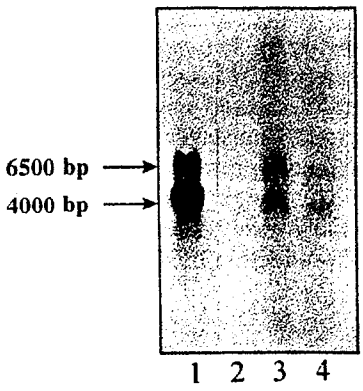

Figure 2. Southern blot analysis of hairy root cultures of *Plantago lanceolata*. 1, DNA from strain LBA 9402 of *A. rhizogenes*; 2, DNA from untransformed root clone; 3, 4, DNA from the two independent root clones induced by *A. rhizogenes* strain LBA 9402. DNA were digested with *Eco*RI. The probe was the EcoRI-15 fragment of R_i plasmid T_L-DNA of *A. rhizogenes* strain A_4. Lengths are indicated in bp.

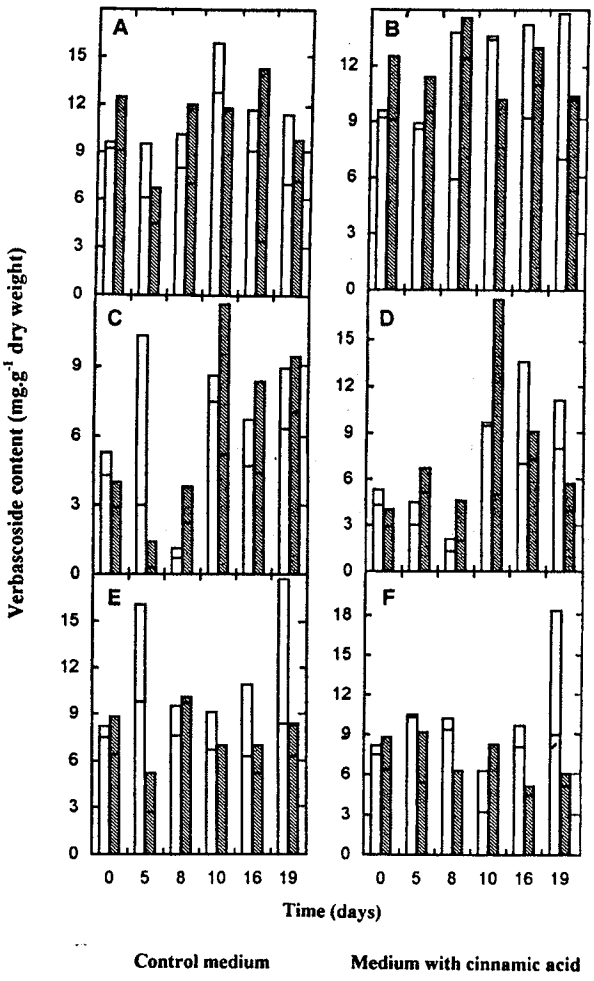

Figure 3. Verbascoside content in 42, 50 and 58-d-old cultures (A–B, C–D and E–F) of untransformed roots (■) and hairy roots (□) after their transfer (day 0) into either control MS medium (A, C, E) or MS medium with added cinnamic acid (B, D, F). Each experiment was carried out on two root clones and the two values were reported as superimposed bars.

decrease below 5 mg·g^{-1} DW for the first 8 d of the experiment (*figure 3 C, D*). V storage in root cultures was more stable and slightly lower than V storage in the roots of whole plants with average levels from 10 to 30 mg·g^{-1} DW [13].

P levels in all root cultures oscillated from 30 to 80 mg·g^{-1} DW (*figure 4*) but more extreme values (10–90 mg·g^{-1} DW) were found. P accumulation in root cultures matched those reported in the roots of whole seedlings [12, 13]. Cinnamic acid and culture age did not induce modifications of P levels.

P accumulation in root cultures was much higher and more variable than that of V as already found in whole seedling roots [12, 13].

2.3. Cinnamic acid biotransformation

Only traces of free cinnamic acid were detected in untransformed and hairy root cultures transferred into MS medium added with cinnamic acid. The phenolic profile of these two types of cultures was modified by the temporary appearance of a cinnamic derivative absent in both root types transferred into control MS medium. This cinnamic component (*figure 5*), previously identified as (*E*)-*p*-coumaroyl-1-O-β-D-glucopyranoside (PCG) [15], was detected by HPLC in 42, 50 and 58-d-old root cultures (*figure 6*). Maximum levels of PCG (6–8 mg·g^{-1} DW) were reached at day 8 for cultures A, day 10 for cultures B and day 5 for cultures C. In A, B and C untransformed and hairy root cultures, PCG accumulation progressively decreased after the tenth day of the experiment, except in B untransformed root cultures where high levels of PCG were still detected at day 19.

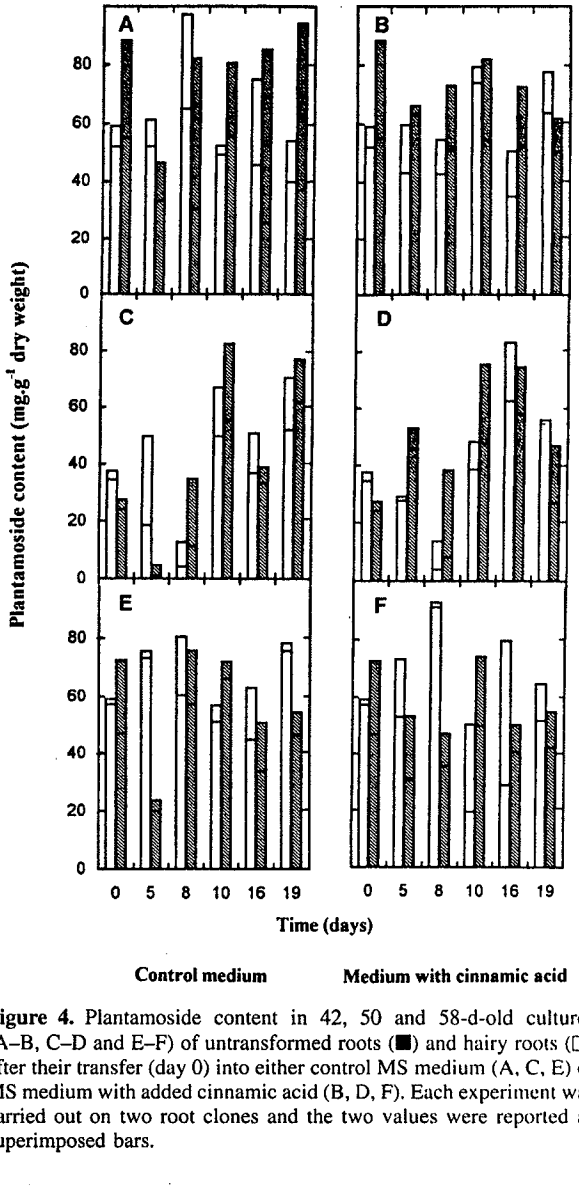

Figure 4. Plantamoside content in 42, 50 and 58-d-old cultures (A–B, C–D and E–F) of untransformed roots (■) and hairy roots (□) after their transfer (day 0) into either control MS medium (A, C, E) or MS medium with added cinnamic acid (B, D, F). Each experiment was carried out on two root clones and the two values were reported as superimposed bars.

3. DISCUSSION

Untransformed and hairy root cultures of *P. lanceolata* were investigated for their two major CGEs, i.e. verbascoside (V) and plantamoside (P). V accumulation in both root cultures was low and stable whereas P accumulation was much higher and more variable. Many studies have demonstrated that verbascoside (also called acteoside) was the most widespread CGE identified in the plant kingdom; other CGEs generally more specific to a plant genus were often detected with V [1, 4, 27]. Metabolically in the *P. lanceolata* CGEs pathway, V could be a parent compound of P which could explain its low and stable levels [1, 27]. According to this hypothesis, plantamoside (also called plantamajoside) [31], which was found particularly in *Plantago* sp., could be an end-derivative of the *P. lanceolata* CGEs pathway highly accumulated in both

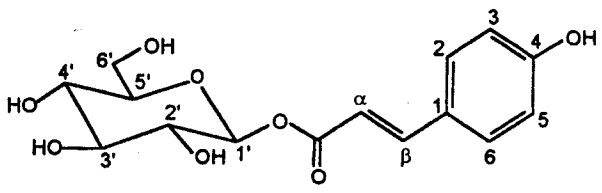

Figure 5. (*E*)-*p*-coumaroyl-1-O-β-D-glucopyranoside.

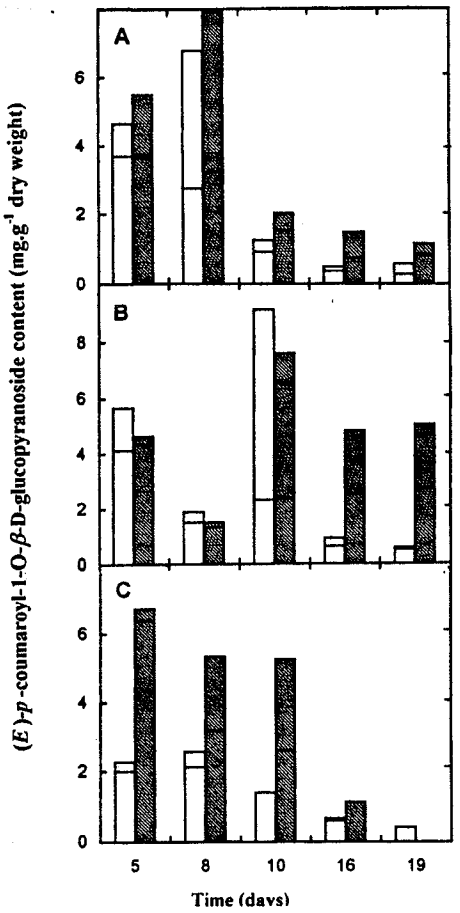

Figure 6. Time course of (*E*)-*p*-coumaroyl-1-O-β-D-glucopyranoside in 42, 50 and 58-d-old cultures (A–C) of untransformed roots (■) and hairy roots (□) after their transfer into MS medium with 0.1 mM cinnamic acid. Each experiment was carried out on two root clones and the two values were reported as superimposed bars.

root types. Hence, P accumulation could be influenced by various stresses, as are all polyphenol derivatives [27], that could explain the great variations in its levels.

V and P levels in both untransformed and hairy root cultures of *P. lanceolata* were globally similar to those found in roots of whole plants during our previous studies [12, 13, 15]. These results showed that V and P were biosynthesised in roots of whole plants and not only accumulated in underground tissues. This confirmed the importance of root cultures for CGEs bioproduction studies.

Genetic transformation by *A. rhizogenes* did not increase V and P accumulation in root cultures. However, hairy roots of *P. lanceolata* produced more biomass than untransformed roots that quickly degenerated. In literature, many secondary metabolites with broad spectrum of biological activities were bioproduced by various transformed root cultures at levels similar to those of entire plants, i.e. tropan alkaloids of *Solanaceae* [16], polyacetylenes and thiophenes of *Asteraceae* [11], and saponines and ginsenosides of *Araliaceae* [40]. On the other hand, metabolite production of some transformed root cultures could reach higher levels than those of the whole plant using optimised culture conditions, i.e. tropan alkaloids of *Atropa belladonna* [18], yohimbine alkaloids of *Catharanthus trichophyllus* [7], and indole alkaloids of *C. roseus* and *Amsonia elliptica* [23, 34]. Besides, root cultures may hemisynthesise various outer precursors, such as cinnamic acid, into expensive compounds used in different industries as has been reported for microorganisms [39]. Moreover, the high growth rate level of transformed root cultures are very attractive for such industrial production [39].

Cinnamic acid fed to culture cells [9, 32, 38] and tissue cultures [17, 26, 28] was usually biotransformed into *p*-coumaric acid and *p*-coumaroylglucose. According to our own experiments on *P. lanceolata*, the (*E*)-*p*-coumaroyl-1-O-β-D-glucopyranoside hemisynthesis capacity of untransformed and hairy root cultures was identical (from 6 to 8 $mg \cdot g^{-1}$ DW for 0.1 mM cinnamic acid added to the culture medium). Exogenous addition of phenolic compounds in plant culture medium could induce two types of glycosyl conjugation [36] and lead to either glycosides or esters [10, 30]. Few cell culture strains converted outer precursors into two conjugated derivative types, but in most cases, only one type of conjugation was described [37]. For cinnamic acid and analogues, glycosyl conjugation logically occurred on the acid function [9, 17, 26, 28, 38].

In conclusion, hairy root cultures of *P. lanceolata* are able to biotransform an outer precursor such as cinnamic acid into a phenolic derivative. Further investigations should be carried out in order to optimise the culture conditions of *P. lanceolata* roots and then, increase the bioproduction of biologically active metabolites.

4. METHODS

4.1. Plant material and growth conditions

Seeds of *Plantago lanceolata* were sterilised in a commercial solution of NaClO (3.6 %) for 20 min. The seeds were then cultured in Murashige and Skoog's (MS) medium with 10 $g \cdot L^{-1}$ agar in the dark.

4.2. Bacterial material

The strain of *Agrobacterium rhizogenes* (LBA 9402) was maintained on yeast mannitol agar (YMA) medium with added rifampicine. This rifampicine resistant strain contained the agropine plasmid pRi 1855 [22].

4.3. Root culture initiation

Roots of seedlings were cut into 15–20 mm length fragments, 6 weeks after germination. Inoculation was done using 10-d-old bacteria taken from YMA-grown colonies. Bacteria were applied on one tip of the fragments with a sterile scalpel [12, 22]. Control fragments and inoculated fragments were placed on solid MS medium for 2 d and then, both types of fragments were transferred on MS (N/5) medium containing 500 $mg \cdot L^{-1}$ filter-sterilised cefotaxim (Cef. 500 medium). Roots appearing 8 d later from both fragment types were excised and then transferred every 8 d on Cef. 500 medium.

4.4. Roots DNA analysis

Transformation of root clones was confirmed using Southern blotting with a labelled probe specific for T-DNA (EcoRI-15). Root DNA isolation was performed as described by Dellaporta et al. [8]. Two µg DNA were cleaved by restriction endonucleases; fragments were separated by electrophoresis and then transferred onto Hybond-N^+ filter (Amersham) in 0.4 N NaOH, 1 M NaCl. Hybridisation was performed at 42 °C during 24 h in 7 % SDS [6], using a [^{32}P]-labelled Prime-a-gene probe using the random Prime-a-Gene labelling system (Promega).

4.5. Feeding experiment

On day 0 of cinnamic acid biotransformation experiment, 42, 50 and 58-d-old untransformed and hairy root cultures were subcultured from Cef. 500 medium to both Cef. 300 medium (control medium) and Cef. 300 medium containing 0.1 mM (*E*)-cinnamic acid dissolved in 0.1 % dimethylsulfoxide, and then the experiment ran for a further 19 d. Each experiment was carried out on two root clones for both untransformed and hairy root cultures of *P. lanceolata*. The two values were reported as superimposed bars.

4.6. Phenolics compounds extraction

Freeze-dried roots were ground in a mortar and extracted with 70 % MeOH (150 $mL \cdot g^{-1}$ DW) for 30 min at room temperature.

4.7. Chemical analyses

Extracts were concentrated to dryness under vacuum, taken up in MeOH (1 $mL \cdot 100\ mg^{-1}$ DW) and then analysed by TLC and HPLC as previously described [12, 13].

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