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Flavonoid Accumulation Is Correlated with Adventitious Roots Formation in *Eucalyptus gunnii* Hook Micropropagated through Axillary Bud Stimulation[1]

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ABSTRACT

***Eucalyptus gunnii* Hook microcuttings, obtained *in vitro* through axillary bud stimulation, show different rooting responses on the same rooting medium depending on the physiological state induced by cytokinins used in the previous multiplication medium. 6-Furfurylamino purine and 6-(4-hydroxy-3-methylbut-2-enylamino)purine induced a physiological state characterized by high sensitivity of microcuttings to the rooting stimulus exerted by the auxin 3-indolebutyric acid, but N^6-benzyladenine did not produce the same effect. The former physiological state was characterized by an increased accumulation of two endogenous flavonoids (identified as quercetin glycosides) which may be markers of a well defined physiological state. They could have some direct influence on the rooting processes of the explants cultivated *in vitro*.**

A study has been carried out on physiological aspects of adventitious rooting in *Eucalyptus gunnii* Hook microcuttings, obtained *in vitro* through axillary bud stimulation. *E. gunnii* Hook, a plant cultivated in Italy and in other countries for cut green branches has, *in vivo*, a pronounced adventitious rooting ability only when the plants are very young. In fact, this capability which is typical for the cuttings taken from *Eucalyptus* seedlings disappears when the plants, still in juvenile phase, become 12 to 14 months old (6, 10, 13). Other physiological changes may occur (6, 24) that parallel the loss of the ability to form adventitious roots; for example, the accumulation of some phenolic substances was found to be correlated with the loss of rooting ability in *Eucalyptus grandis* cuttings (22). However, data concerning *in vitro* culture are not available.

On the other hand, a comparison of the metabolic substances extracted from the plant material in each of the different stages is not always straightforward, due to the wide genetic variations in *Eucalyptus* (15, 23). These genetic variations may well give rise to variable results. For these reasons, a standard *E. gunnii* clone (Iflos), which can be easily cultured *in vitro*, has been selected.

During our previous work (4), we found different rooting ability in *E. gunnii* microcuttings, depending on their endogenous flavonoids level. These experiments examine the correlation between the physiological state of the rooting ability in micropropagated material (microcuttings) and the level of endogenous flavonoids as affected by the hormone treatment during *in vitro* culture.

MATERIALS AND METHODS

Growth Conditions

During the multiplication phase, *Eucalyptus gunnii* Hook clone Iflos was grown *in vitro* on a modified de Fossard medium (4, 5) containing BA[5]; the Hartney medium (13) containing kinetin or zeatin was employed for the activation of the rhizogenetic potential in the explants. During the rooting phase a "medium O," containing IBA (4), was used (Table I). IBA was chosen as rooting hormone because it is much more stable than IAA in the culture medium (17).

Further details on the *in vitro* culture techniques and conditions are described elsewhere (4). The duration of each subculture was 1 month, and explants were grown at 26°C, 12 h photoperiod at 3000 lux. The *in vitro* culture trials had a duration of 3 years. Each year, 600 explants of the same clone, Iflos, (12 replications of 50 explants) were cultured for 5 months on a de Fossard modified medium (A medium). Half of them were then transferred (as microcuttings, 2 cm long) directly onto the O rooting medium and the remaining 300 explants were cultured for one additional month on Hartney medium containing different cytokinins before their transfer to the O rooting medium (Table II).

Extraction and Purification of Polyphenols

The polyphenols present in the tissues were extracted via an adapted Van Sumere procedure (29). Fresh plantlets (20 g

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[5] Abbreviations: BA, N^6-benzyladenine; kinetin, 6-furfurylamino purine; zeatin, 6-(4-hydroxy-3-methylbut-2-enylamino)purine; IBA, 3-indolebutyric acid; t-BuOH, *tert*-butyl alcohol; HOAc, acetic acid; *n*-BuOH, *n*-butyl alcohol; HMDS, hexamethyldisilazane.

Table I. *E. gunnii clone Iflos multiplication and rooting media*

mg/L	Medium		
	A	O	Hartney
Macroelements			
NH_4NO_3	400	50	
$MgSO_4 \cdot 7\ H_2O$	123	50	
$NaH_2PO_4 \cdot H_2O$	127.9	20	Murashige and Skoog half-strength[a]
KCl	141.5	6.6	
$CaCl_2 \cdot 2\ H_2O$	147		
$KH_2PO_4 \cdot H_2O$	150	43	
KNO_3	400	200	
$Ca(NO_3)_2 \cdot 4\ H_2O$		133.3	
Microelements			
H_3BO_3	9.27		
$MnSO_4 \cdot H_2O$	16.90		
$ZnSO_4 \cdot H_2O$	11.50	Murashige and Skoog quarter strength[a]	Murashige and Skoog half-strength[a]
$CuSO_4 \cdot 5\ H_2O$	0.37		
$Na_2MoO_4 \cdot 2\ H_2O$	0.24		
KI	0.83		
$CoCl_2 \cdot 6\ H_2O$	0.24		
Iron chelates	Murashige and Skoog[a]		
Organic compounds			
Myo-inositol	200.0		
Thiamine HCl	2.0	1.0	
Riboflavine	4.0		
Cysteine		2.0	
Ascorbic acid		100.0	
Citric acid		10.0	
α-Naphthaleneacetic acid			0.10
Saccharose (g/L)	41	20	20
Difco-Bacto agar (g/L)	8	8	8
pH	5.8	5.8	5.8

[a] Murashige and Skoog (21).

for each analysis) were homogenized in a Waring blender with 50 mL of boiling methanol/ethanol 1:1 (v/v), and the mixture was refluxed for 2 h under N_2 at 72°C. The extract was filtered, and the residue was extracted twice more as described above. The filtrates were pooled, centrifuged, and partitioned against petroleum ether (b.p. 100–140°C). The alcoholic phase containing the polyphenols was concentrated by evaporation of solvent *in vacuo* to 5 mL. Before chromatography, the latter solution was brought to 20 mL by addition of a water/methanol 1:1 (v/v) mixture.

Polyphenols were separated by paper chromatography (descending phase) using Whatman 3MM paper and the following solvents: TBA (*t*-BuOH-HOAc-H_2O, 3:1:1, v/v/v); 15% HOAc; and BAW (*n*-BuOH-H_2O-HOAc 4:5:1 v/v/v). Compounds were located by their color on paper and their fluorescence under UV light (280 and 366 nm) before and after treatment with ammonia vapor. The chromatographic spots were then eluted with methanol, and their UV spectra were recorded in methanol with a Hitachi double beam spectrophotometer (model 150-20). At the end of this step, only two eluted spots possessed two absorption peaks around 250 and 360 nm, and for this reason they were considered as likely flavonoids (11). Acid hydrolysis of the two possible flavonoidal substances was carried out with boiling 2 N HCl for 30 min (18). After hydrolysis, the products were separated and collected by column chromatography using a small glass column (1 cm diameter) packed with polyamide (Carlo Erba, Italy); the column was eluted first with 10 mL of double distilled water (to recover sugars) and then with 15 mL of methanol (to recover the aglycone).

Sugar Analysis

The sugars obtained after hydrolysis were separated and purified by TLC on silica gel 60 F-254 plates with ethyl acetate-pyridine-water 12:5:4 v/v/v according to Mabry *et al.* (18); R_F values were compared with those of authentic samples. The zones with sugars were scraped from the plates, and eluted with water (10 mL). After filtration through fiberglass, the eluates were dried in an oven (50°C), and then maintained under vacuum in the presence of P_2O_5 and NaOH overnight. The residues were dissolved in 100 μL of anhydrous pyridine, and the solution was allowed to stand for 15 h to obtain equilibration among the α, β, and γ forms. The trimethylsilyl

Table II. *In vitro E. gunnii clone Iflos rooting response to endogenous levels and exogenous supply of flavonoids*

1st Multiplication Medium	Flavonoid Content in Explants at the End of Culture	2nd Multiplication Medium	Flavonoid Content in Explants at the End of Culture	O Rooting Medium	Rooting Response
		Hartney + 0.5 mg/L BA	0.2 to 0.5 mg/100 g of fresh material	+ 0.5 mg/L IBA	No rooting
		Hartney + 0.5 mg/L Kinetin	8 mg/100 g of fresh material	+ 0.5 mg/L IBA	100% rooting
A medium (de Fossard modified) + 0.2 mg/L of BA	0.2 to 0.5 mg/100 g of fresh material	Hartney + 0.5 mg/L Zeatin	8 mg/100 g of fresh material	+ 0.5 mg/L IBA	100% rooting
		→	→	+ 0.5 mg/L IBA	No rooting
		→	→	+ 0.5 mg/L IBA + 1 mg/L quercetin	8–10% rooting
		→	→	+ 0.5 mg/L IBA + 1 mg/L isoquercitrin + 1 mg/L quercitrin	From 80–100% rooting

ethers were prepared by adding 100 μL of HMDS and 10 μL of TFA to the pyridine solution, followed by heating at 60°C for 10 min.

Sugars were quantified by GLC using a Hewlett Packard model HP 5730 A GC equipped with a hot wire detector and with a glass column (3.0 m × 2 mm) packed with Chromosorb W AW DMCS (80–100 mesh) coated with 1.5% SE-30 plus 1.5% SE-52. The carrier gas was helium with a flow rate of 30 mL/min. Injection port temperature was 240°C. The column initial temperature was 120°C and was increased to 260°C at 6.4°C min. The detector temperature was 350°C and the filament current 150 mA.

Flavonoid Analysis

The flavonoids were identified on the bases of UV absorption spectra in MeOH alone or in the presence of diagnostic reagents (MeOH + sodium methoxide; MeOH + $AlCl_3$; MeOH + $AlCl_3$ + HCl; MeOH + sodium acetate; and MeOH + NaOAc + H_3BO_3) (18) and chromatographic behavior on reverse phase HPLC.

The HPLC system consisted of a Liquid Chromatograph 1084 B Hewlett Packard, equipped with an HP-85 personal computer-operated photodiode array (HP 1040 A) high speed spectrophotometric detector. Analytical column: i.d. 4.6 mm, length 250 mm (Knauer); packing: Li-Chromosorb RP-18, 10 μm; eluent: solvent A = formic acid/water 5:95 (v/v), solvent B = methanol. Gradient profile: linear. Gradient range: 0 to 2 min = isocratic 7% B in A; 2 to 8 min = gradient 7% B in A–15% B in A; 8 to 25 min = gradient 15% B in A–75% B in A; 25 to 27 min = gradient 75% B in A–80% B in A; 27 to 29 min = isocratic 80% B in A. Flow rate: 2.5 mL/min; oven temperature: 35°C; eluent temperature, solvent A and B: 35°C; sample size: 100 μL loop valve. Attenuation: 2^5 absorbance × 10^{-4}/cm. Detection: 280 and 350 nm (optical bandwidth for both wavelengths 4 nm).

The concentration of the flavonoid glycosides was quantitatively determined by means of a Hitachi double beam spectrophotometer (model 150-20). For the latter purpose, the molar extinction coefficient of each substance (ϵ) in MeOH was employed (18). The molar extinction coefficient of the standards purchased from Sarsyntex (France), expressed as λ max (log ϵ) in MeOH (18) was used during the quantitative analysis of the plant flavonoids after identification; the values obtained were the following: for quercitrin, 349.2 (4.13) and for isoquercitrin, 357.6 (4.01).

RESULTS AND DISCUSSION

The *Eucalyptus gunnii* clone, Iflos (previously named clone 5), was chosen because of its excellent adaptability to *in vitro* culture. The Iflos clone shows a high multiplication rate on jelled media as well as some additional valuable characteristics, such as the production of healthy plants with attractive foliage. Previous *in vitro* studies have shown that cuttings of the Iflos clone grown on a medium containing BA do not easily root when transferred directly onto a rooting medium containing IBA. Therefore, a preliminary transfer of the explants for at least 1 month onto a culture medium containing cytokinins different from BA proved necessary (4). In fact, a high percentage of explants rooted when subcultured on a

medium containing kinetin or zeatin prior to culture on the rooting medium (Table II).

Moreover, the two different physiological states characterized in the microcuttings by either the absence or the presence of a sensitivity to the rooting stimulus were also shown to be biochemically different. Indeed, the explants grown on medium containing BA (and as such unable to root) showed an extremely low flavonoid concentration, around 0.2 to 0.5 mg/100 g of fresh weight (Table II); the same explants transferred to jelled media containing kinetin or zeatin acquired rooting ability, and increased their endogenous flavonoids level (8 mg/100 g of fresh weight, Table II).

The flavonoid glycosides found in the tissues were identified as isoquercitrin (quercetin 3-O-glucoside) and quercitrin (quercetin 3-O-rhamnoside) by HPLC (Figs. 1 and 2), and their ration in the tissues was 5:1. The two unknown flavonoids had the following retention times: for the first substance, 16.20 min; for the second substance, 15.19 min (Fig. 1). These two retention times, respectively, corresponded to the values obtained for authentic quercitrin and isoquercitrin (Fig. 2).

The two identified flavonoids were present in very low concentration (0.2–0.5 mg/100 g of fresh material) in the microcuttings unable to root, and in greater amount (8 mg/100 g of fresh material) in the microcuttings possessing rooting ability (Table II).

The sugar moieties obtained after acid hydrolysis from the two flavonoid glycosides proved to be glucose for the first compound, and rhamnose for the second one; in fact, the two unknown sugars gave rise, under our GLC analysis conditions, to α-glucose (retention time: 12.4 min), β-glucose (retention time: 13.7 min), γ-glucose (retention time: 11.7 min), and to α-rhamnose (retention time: 8.2 min) and β-rhamnose (retention time: 9.2 min) forms.

The presence of the aglycone quercetin in *E. gunnii* grown *in vivo* has already been reported (14), but the corresponding glycosides so far have not been identified. Furthermore, the aglycones kaempferol and myricetin, which have been detected in other *Eucalyptus* species (15), were not found in the Iflos clone. Addition of 1 mg/L of both quercetin glycosides to the rooting medium containing IBA enabled direct root formation in the microcuttings, after their transfer from the growth medium containing BA. The latter medium normally did not allow any root formation (Table II). Employment of the aglycone quercetin instead of quercetin glycosides resulted in a decreased rooting effect; in comparison with quercetin glycosides, only one-tenth of the rooting was obtained (Table II).

These results indicate a correlation between the chemical structure of cytokinins and their effect on flavonoid metabolism. In fact, only two cytokinins among the three tested were able to stimulate the accumulation of flavonoids in the explants. This stimulation of flavonoid accumulation *in vitro* in a *Eucalyptus* callus culture by cytokinins has been described by Samejima *et al.* (25). This effect also has been reported for tea cell culture (1). However, no correlation between the amounts of flavonoids produced and the physiological changes occurring in the tissues during growth were investigated. In the case of other genera grown *in vitro*, qualitative and quantitative modifications of flavonoids metabolism, in

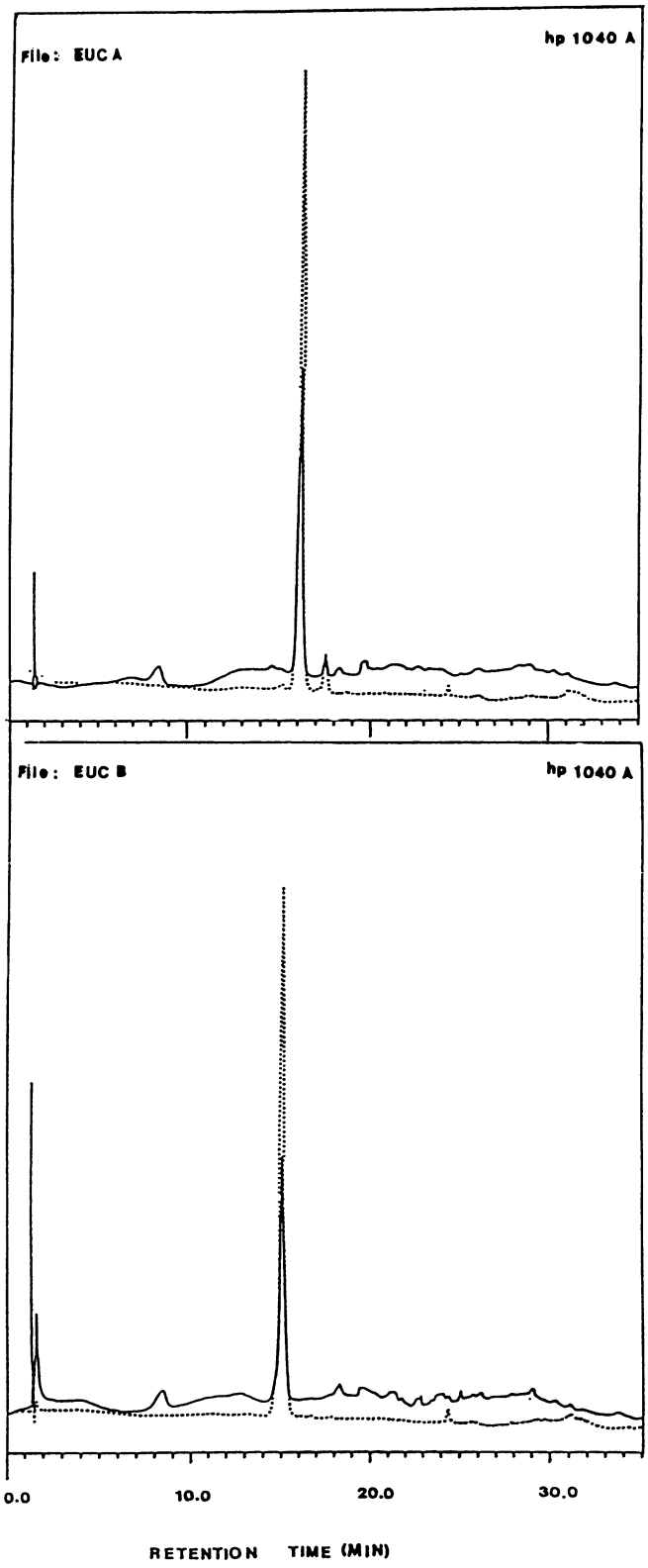

Figure 1. HPLC analysis of the two extracted flavonoids (named here Euc A and Euc B, respectively), obtained from *in vitro* cultivated *E. gunnii* plantlets. Retention time for Euc A = 16.203 min; retention time for Euc B = 15.189 min. Dotted line, absorbance at 350 nm; continuous line, absorbance at 280 nm.

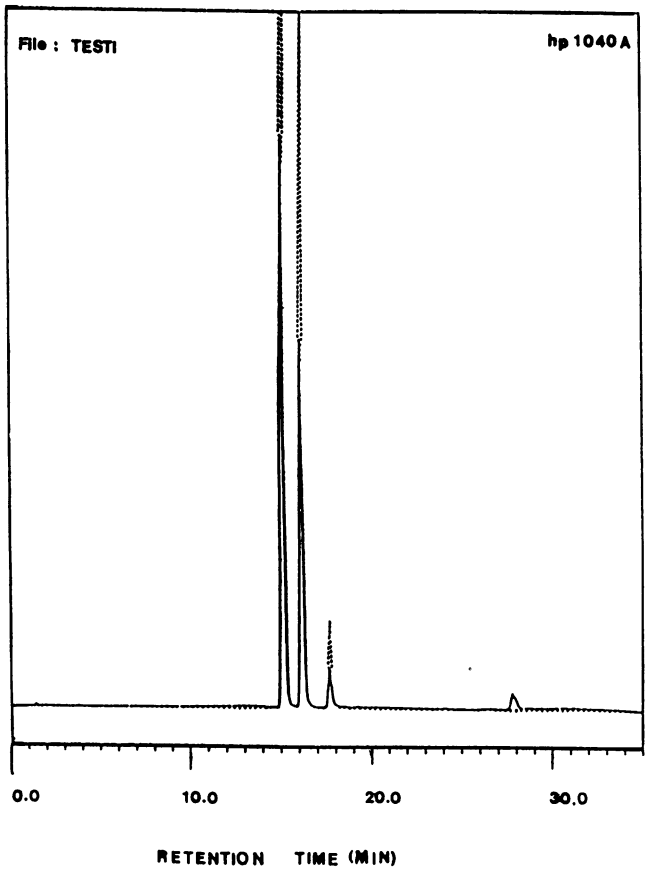

Figure 2. HPLC analysis of standard pure quercitrin (retention time = 16.204 min) and isoquercitrin (retention time = 15.144 min). Dotted line, absorbance at 350 nm; continuous line, absorbance at 280 nm.

comparison with the same plants cultivated *in vivo*, were found to occur (16, 25). Moreover, seasonal changes of flavonoid composition were reported for cultivated plants, depending on different physiological states (28).

Our study clearly indicates that flavonoids do not accumulate in the presence of BA, while kinetin and zeatin promote flavonoid accumulation (Table II). A hypothesis for the observed phenomenon could be formulated as follows: in cell cultures of many different species, flavonoid degradation occurs through oxidative pathways via peroxidative enzymes (2). BA has been demonstrated to promote peroxidase activity much more than kinetin and zeatin for some substrates (19). Thus, BA may promote flavonoid catabolism more strongly than either kinetin and zeatin. The promotion of flavonoid turnover by cytokinins has also been reported for *in vitro* cultured cells of *Cicer arietinum* (2).

Previous reports on *in vitro* cultures of *Eucalyptus* (3, 6) suggest an inhibitory role for the flavonoids with regard to plantlet development. However, perhaps under the growth conditions used by these authors endogenous flavonoids rapidly accumulated to toxic levels. In addition, the age of the explants used might have had an influence on rooting ability (22). In our work, the enhanced flavonoid accumulation appeared beneficial for the explants. Indeed, we found the increased accumulation of flavonoids was associated with the highest sensitivity of the microcuttings to the auxin present in the rooting medium. Thus, the two identified flavonoid glycosides can also be considered as markers of a well defined physiological state favorable to adventitious root formation.

The aglycone of these molecules has been described by many authors as an effective inhibitor of IAA oxidase (8, 9, 12). Moreover, recent research suggests that quercetin is not simply an inhibitor but rather a better substrate than IAA in the peroxidase and oxidase degradative enzymatic reactions (2). It is therefore quite possible that the aglycone quercetin plays an active role in adventitious root formation by maintenance of high levels of endogenous IAA (20, 26). For the same reason, the IAA derived from IBA transformation by plant tissues (7) may be available for a longer time before its oxidative degradation. However, in the latter connection, the weak rhizogenetic response of the explants to the addition of the free aglycone to the rooting medium must be stressed: the reason why glycosides were more active in promoting rhizogenesis may well reside in their high solubility and stability due to the presence of the sugar moieties.

Finally, it is worth mentioning that flavonol glucosides have been demonstrated to be energy rich compounds quickly available to the cells via glucosyl transferase reactions (27) although in our case, because of the low concentration detected, they are probably not a significant energy source.

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