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Restart of lignification in micropropagated walnut shoots coincides with rooting induction

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Abstract

The lignin content of walnut shoots did not change during *in vitro* shoot multiplication. Lignin content started to increase as soon as shoots were passed to a rooting medium with auxin. Exogenous auxin (applied for rooting) caused a transient elevation of the endogenous free indoleacetic acid (IAA) content with a simultaneous decrease of peroxidase activity. These events typically marked the completion of the rooting inductive phase (before any visible histological event, that is before the cell divisions beginning the rooting initiation phase). This meant that either the given exogenous auxin or the endogenous IAA has served as signal for the stimulation of lignification. Continued increase of lignification in the shoots required completion of root formation; this increase indeed was slown down when root emergence did not occur. It was further shown that lignification varied conversely to the content of the soluble phenol content, itself apparently being related to the activity of phenylalanine ammonia-lyase activity.

Additional key words: auxin, *Juglans regia* × *J. nigra*, *in vitro* culture, peroxidase.

Introduction

It is an evidence that the development of seminal and adventitious roots sustains and enhances growth and vigour of the aerial plant parts. The importance of root to shoot communication has been specially emphasized in the responses to environment stress (Davies and Jeffcoat 1990). Roots do not induce vascular differentiation by themselves nor must they be present in order to obtain vascular tissues in stems. The roots, however, have two major functions in vascular differentiation, namely: *1*) the roots orient the pattern of vascular differentiation towards the root tip by acting as a sink for the continuous flow of auxin deriving from young leaves, and *2*) the root apices are sources of inductive stimuli that promote vascular development (Aloni 1995). The major root developmental signal is cytokinin. It should be emphasized, however, that cytokinin alone, or root apices in the absence of an auxin source, do not induce vascular differentiation in stem tissues (Gaspar *et al.* 2002). The present paper poses the specific question of the restart of lignification in micropropagated shoot stems in coincidence with the process of adventitious rooting at their bases. The rooting process indeed, decomposed in successive interdependent physiological phases, involves changes in interrelated biochemical factors such as enzymes (peroxidase, phenylalanine ammonia-lyase, *etc.*), substrates or products (phenolic compounds) and hormones and related regulators (auxins, ethylene, polyamines) (Hausman *et al.* 1997, Gaspar *et al.* 1994, 1997, Kevers *et al.* 1997b); and most of these factors are also known to play a role in lignification (Ferrer *et al.* 1992, Whetten and Sederoff 1995, Bolwell 1997), as also exemplified in transgenic materials under- or over-expressing peroxidase activity, for instance in peroxidase overproducer tobacco plants (Lagrimini 1991). Such factors already have been

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Abbreviations: DKW - Driver and Kuniyki (1984) culture medium; f.m. - fresh mass; IAA - indoleacetic acid; IBA - indolebutyric acid; Mr - relative molecular mass; PO - soluble peroxidase; PVP - polyvinylpyrrolidone.
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investigated in the course of adventitious rooting by micropropagated walnut shoots (Ripetti *et al.* 1994, Heloir *et al.* 1996, Gatineau *et al.* 1997, Kevers *et al.* 1997a). Rooting of *in vitro* walnut shoots is now routinely achieved in a two-step process using two different culture media: a first medium with auxin for 7 d in the dark, covering the so-called rooting inductive phase (biochemical events preceding the cell divisions initiating root primordia), followed by a second medium without growth regulator in the light, covering the subsequent initiative (cell divisions and constitution of meristematic primordia) and expressive (growth of root primordia) rooting phases, according to a procedure designed by Jay-Allemand *et al.* 1992). We wanted to correlate these phases and their associated biochemical events with the restart of lignification in micropropagated walnut shoots, after their transfer from the multiplication medium to the rooting media.

Materials and methods

Plants and culture: *In vitro* shooting clusters of a hybrid walnut, *Juglans regia* × *Juglans nigra*, (F3 clone, NG23 × RA, AE 1996) were maintained through regular subcultures (every 3 weeks) by axillary proliferation on a DKW medium (Driver and Kuniyuki 1984) with 7.5 g dm^{-3} agar (*Roland*, Brussels, Belgium) and 1 mg dm^{-3} benzylaminopurine (BAP) under a photoperiod of 16-h with an irradiance (*Sylvania Gro-Lux* tubes, Danvers, USA) of 33 µmol m^{-2} s^{-1} and a temperature of 23 °C, as already described (Ripetti *et al.* 1994). At the end of a multiplication cycle, series of ten shoots were transferred, first to an induction rooting Murashige and Skoog (1962; MS) medium containing 3 mg dm^{-3} indole-3-butyric acid (IBA) and grown under darkness for one week, and subsequently subcultured for 4 weeks on a mixture of vermiculite and gelrite (0.25 % m/v) moistened with rooting DKW medium without auxin and grown under 16-h photoperiod as described above. All the analyses were performed during the multiplication phase (21 d), at the end of the rooting induction (28^{th} day) and initiation (35^{th} day) phases and during the rooting expression phase (from the 35^{th} to the 56^{th} day). Roots became well visible, emerging from the shoot bases, from the second week on the second rooting medium (that is on the 42^{nd} day) and growing well outside the shoots from the 3^{rd} week. The whole shoots (separated from the rooting zone, to avoid the eventual lignified vasculature of the forming roots) from the whole population were sampled for the biochemical analyses. About 20 % of rooting-recalcitrant shoots (at least with non-visible emerging roots) were separated from the rooting ones from 35^{th} day on to serve as control for the lignin analyses.

Determination of peroxidase and phenylalanine ammonia-lyase activities: Soluble peroxidase (PO) was extracted from whole shoots (random samples of 300 mg fresh mass, each representing *ca.* 4 shoots) ground in a 0.06 M phosphate buffer, pH 6.1, containing 1 % (m/v) insoluble polyvinylpyrrolidone (PVP, Mr 40 000). After centrifugation (10 000 *g*, 10 min), the supernatant was desalted on a *Sephadex G25* column. Cell wall bound peroxidase was extracted from the pellet with 1 M NaCl and 3 M $CaCl_2$ (Richardson and McDougall 1997); after 2 h incubation, the homogenate was centrifuged and the supernatant dialysed overnight against 50 mM Tris buffer at pH 7. PO activities were measured by a spectrophotometric method (*Uvikon 931 spectrophotometer, Kontron Instruments*, Milan, Italy) using guaiacol (0.1 % m/v) and H_2O_2 (0.006 % v/v) as substrates. PO activity was calculated using the coefficient of absorbance 26.6 $mmol^{-1}$ cm^{-1} at 470 nm for guaiacol and expressed in nkat mg^{-1}(protein).

Phenylalanine ammonia-lyase (PAL) was extracted in borate buffer (0.1 M, pH 8.8) in the presence of 1 % (m/v) insoluble PVP. PAL activity was evaluated by monitoring the formation of cinnamic acid at 290 nm in 0.1 M borate buffer at pH 8.8 as described by Letouzé (1975). Results were expressed as nmoles of cinnamic acid formed after the incubation at 40 °C for 1 h per 1 mg of proteins. A calibration curve using known concentrations of cinnamic acid was drawn.

Protein concentration was determined by the Coomassie blue method (Spector 1978). A calibration curve was drawn using known concentrations of bovine serum albumin.

Phenol and lignin content: Total soluble phenol content was estimated after acid extraction by the Folin reagent method (Druart *et al.* 1982). Lyophilised shoots were ground in 4 cm^3 0.1 M HCl, incubated for 3 h at 20 °C and then centrifuged at 10 000 *g* for 10 min. The supernatant was kept and the pellet was washed and centrifuged. The supernatants were mixed and adjusted to 10 cm^3 with 0.1 M HCl. The determination of phenol content was made at 750 nm after addition of the Folin reagent and 20 % (m/v) Na_2CO_3. A calibration curve was drawn using different concentrations of chlorogenic acid.

For lignin determination, the shoots were ground in water and centrifuged (10 min, 10 000 *g*); the resulting pellet was then resuspended in 2.5 cm^3 of 25 % (v/v) acetylbromide in glacial acetic acid and 0.1 cm^3 of 70 % perchloric acid. After heating at 70 °C for 30 min, the tubes were rapidly cooled, and supplemented with

2.5 cm^3 of 2 M NaOH and 12 cm^3 of glacial acetic acid. The mixture was filtered through a filter paper. The absorbance of the filtrate was measured at 280 nm and lignin content was estimated using the coefficient of absorbance 24 $mmol^{-1}$ cm^{-1} according to Kevers and Gaspar (1985).

Auxin extraction and determination: The extraction and analytical methods have been performed as described by Nordström and Eliasson (1991). About 500 mg (fresh mass) of whole shoots were homogenised in liquid nitrogen. The powder was extracted with 5 mM phosphate buffer (pH 6.5) containing containing 3-[5(n)-^{3}H]-indolylacetic acid as internal standard and butylated hydroxytoluene as antioxidant. After incubation in darkness for 1 h, the extract was filtered through a glass-fibre filter under vacuum. The filtrates were purified through *Bond-Elut C18* column conditioned at pH 6.5. The pH of the eluates was adjusted to 2.5 using 2.5 M phosphoric acid and then applied to *C18* columns (chromabond) pre-conditioned at pH 2.5. The columns were washed with distilled water, followed by acidic ethanol (ethanol/glacial acetic acid/water, 20/2/78 v/v). A second purification of the last eluates was performed on a second *C18* columns at pH 2.5. Auxins were eluted from the second *C18* columns with 2 × 0.3 cm^3 aliquots of 80 % methanol. 0.05 cm^3 of the methanolic extract were injected in an automated *Merck-Hitachi HPLC* (Darmstadt, Germany) system in the same conditions of elution pattern to those described by Nordström and Eliasson (1991): *Lichrospher 100-RP18 column*, 12.5 cm × 4 mm internal diameter, 5 μm particule size; column and solvent at 30 °C; flux 1 cm^3 min^{-1}; mobile phase acetonitrile:glacial acetic acid:water (10:2:88, v/v); detection by fluorescence detector (absorbance 292 nm, emission 360 nm).

Statistics: All results presented are means (± SD) of at least three independent batches from three separate experiments.

Table 1. Cell wall peroxidase activity [nkat mg^{-1}(protein)], phenol and lignin contents [mg g^{-1}(f.m.)] in rooted and non-rooted walnut shoots at the end of rooting process. Means ± SE.

	Rooted shoots	Non-rooted shoots
Peroxidase activity	9.41 ± 1.23	3.54 ± 0.26
Phenol content	0.22 ± 0.01	0.41 ± 0.05
Lignin content	8.74 ± 0.86	4.26 ± 0.27

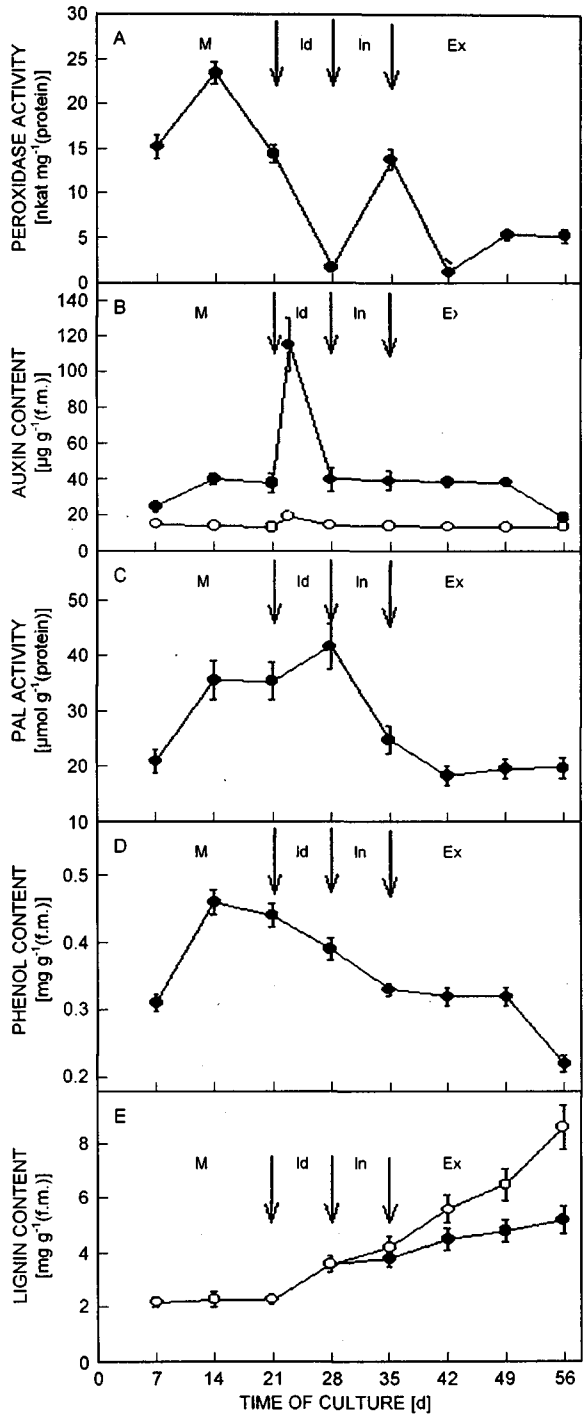

Fig. 1. Rooting process of walnut shoots: *A* - soluble peroxidase activity; *B* - contents in free IAA (*closed circles*) and conjugated IAA aspartate (*open circles*); *C* - phenylalanine ammonia-lyase activity; *D* - phenol content; *E* - lignin content of micropropagated walnut shoots with (*open circles*) or without (*closed circles*) root emergence during the rooting expression phase. Means ± SE, n = 3; M - preceding multiplication phase, Id, In, Ex - rooting induction, initiation, and expression phases, respectively.

Results and discussion

The duration of the successive inductive (Id), initiative (In) and expressive (Ex) rooting phases vary depending on the walnut clone and the culture conditions used (Scaltsoyiannes *et al.* 1998, Falasca *et al.* 2000). In our conditions, preceding studies (Ripetti *et al.* 1994, Heloir *et al.* 1996, Gatineau *et al.* 1997, Kevers *et al.* 1997a) have fixed and identified these three phases as beginning at the 21st, 28th and 35th days, respectively (see figures).

As reported previously, a dramatic decrease of peroxidase activity was observed from the time of application of the exogenous auxin at day 21 (after the multiplication cycle), followed by an increase starting at day 28, with the transfer on the auxin-free medium corresponding to the beginning of the rooting initiative phase (Fig. 1*A*). These changes marked the completion of the successive Id, In, and Ex phases (Gaspar *et al.* 1997). Achievement of the Id phase was further confirmed by the transient elevation of the endogenous free IAA content, the content of IAA-aspartate also varying to a lesser extent in this material (Fig. 1*B*). It is known from two preceding works on the same material that such a temporary elevation of content of endogenous auxins does not occur in control auxin-untreated (non-induced to rooting) shoots (Heloir *et al.* 1996, Gatineau *et al.* 1997). The rooting response was also correlated with the IAA amount (Caboni *et al.* 1997). Important to notice is the fact that the endogenous auxin increases during Id only concern the basal parts of the shoots where new roots will be formed (Moncousin *et al.* 1988, Gatineau *et al.* 1997, Hausman *et al.* 1997, Falasca *et al.* 2000). PAL activity of the whole shoots increased up to the end of the Id phase before decreasing with the beginning of the In phase (Fig. 1*C*)). The phenol content, which had reached a maximum at the end of the multiplication phase, decreased continuously during the successive Id, In, and Ex phases (Fig. 1*D*). This decrease coincided with a continuous increase of the lignin content, thus starting from the beginning of the Id phase (Fig. 1*E*). The accumulation of the phenolic compounds corresponded with the elevation of PAL activity and therefore suggests that lignification was at the expense of the phenolic compounds, some of them being or serving as precursors of monolignols. Interestingly, lignin accumulation was lower in the 20 % shoot population (Fig. 1*E*) which did not show visible signs (no swelling at the cutting base) of rooting. The inverse relationship between the phenol and lignin levels was further confirmed by analysis of rooted and non-rooted shoots. Rooting shoots showed a higher cell wall-bound peroxidase activity (Table 1). Cell wall bound peroxidase is considered to be involved in wall lignification (Ferrer *et al.* 1992, Pedreno *et al.* 1995).

These results thus indicate for the first time that increase of lignification in micropropagated walnut shoots restarted from the beginning of the rooting inductive phase, thus from the moment of the provision of the rooting auxin signal. The latter is either the exogenously supplied IBA, or the temporary elevated endogenous IAA (see Fig. 1*B*). Further, sustained lignification in the shoots required the successful running of the following rooting phases, that is the initiation and growth of root primordia (see Fig. 1*E*). This leads to conclude that, as supposed (see introduction), growing roots are source of signals for lignification, and presumably wood formation, in the stems. A recent study by Reverberi *et al.* (2001) shows, precisely in walnut microcuttings, that exogenous IAA induces the cambial cells of the stem to produce secondary xylem, thus enhance lignification. In *Picea* shoots, exogenously applied IAA also increased wood production (Denne and Wilson 1977). Furthermore, a positive correlation between net endogenous auxin and secondary xylem development was found in the stems of cottonwood (Degroote and Larson 1984). The question raised by the present work is thus how the locally (cutting base) increased level of endogenous auxins influence lignification in upper stem internodes.

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