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Role of phytohormones in organogenic ability of elm multiplicated shoots

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Abstract

The study presents the comparative analyses of endogenous contents of auxin (IAA), cytokinins (CKs), polyamines (PAs), and phenolic acids (PhAs) in apical and basal parts of elm multiplicated shoots with regard to the organogenic potential. The shoot-forming capacity was higher in the apical part than in the basal part. However, the timing of root formation was in the apical type of explant significantly delayed (compared with the organogenic potential of basal part). Significantly higher contents of free bases, ribosides and ribotides of isopentenyl adenine, zeatin and dihydrozeatin that were found in the apical segments, might be considered as the most important factor affecting *in vitro* shoot formation. The content of endogenous free IAA was approximately three times higher in the basal shoot parts than in the apical parts. The amounts of putrescine and spermidine were higher in the apical part which generally contains less differentiated tissues than the basal part of shoot. The predominant PhA in both types of explants was caffeic acid, and concentrations of other PhAs decreased in the following order: *p*-coumaric, ferulic, sinapic, vanillic, chlorogenic, *p*-hydroxybenzoic and gallic acids. The contents of all determined PhAs in their free forms and higher contents of glycoside-bound *p*-coumaric, ferulic and sinapic acids, precursors for lignin biosynthesis, were found in the basal parts.

Additional key words: auxin, cytokinins, multiplication of elm, phenolic substances, polyamines.

Introduction

Ulmus glabra Huds. is autochthonous elm species spread in mountain regions of the Czech Republic. However, Dutch elm disease has devastated the elm population in the last decades (Fenning *et al.* 1993). The elm trees, which have survived the attack of disease, are capable to maintain a high degree of resistance against the repeated infection. Various micropropagation systems have been reported for a range of elm species and hybrids (Fenning *et al.* 1993, Chalupa 1994, Gartland *et al.* 2000, Boroščíková *et al.* 2004).

The efficient *in vitro* propagation represents a process consisting of successive physiological phases with different exogenous requirements. The original hormonal content of explants is a very important factor directing *in vitro* responses (Centeno *et al.* 1997). However, the complex interactions among auxins, cytokinins (CKs), polyamines (PAs) and phenolic compounds are far from being elucidated. Beside the key roles of auxin and CKs, very important function in differentiation processes belongs to PAs. PAs have a wide spectrum of action with some similarities both with auxins and CKs and in co-operation with plant phytohormones modulate morphogenic processes (Altamura *et al.* 1993). Regeneration and organ differentiation in a number of plant species could be improved by application of PAs; however, PAs could not compensate CKs and auxin activity in micropropagation (Scholten 1998). PhAs, intermediates in phenylpropanoid metabolism, may also participate in maintaining "adequate" contents of phytohormones essential for efficient organogenesis (Cvikrová and Hrubcová 1999).

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Abbreviations: CaA - cafeic acid; *p*CA - *p*-coumaric acid; CKs - cytokinins; DHZ - dihydrozeatin; DHZR - dihydrozeatin riboside; DHZRP - dihydrozeatin riboside monophosphate; FA - ferulic acid; GA - gallic acid, *p*HBA - *p*-hydroxybenzoic acid; ChA - chlorogenic acid; IAA - auxin; iP - isopentenyl adenine; iPR - isopentenyl adenine riboside; iPRP - isopentenyl adenine riboside monophosphate; SiA - sinapic acid; PhAs - phenolic acids; PAs - polyamines; Put - putrescine; SAVO - 0.1% $NaClO_3$; Spd - spermidine; Spm - spermine; VA - vanillic acid; Z - zeatin; ZR - zeatin riboside; ZRP - zeatin riboside monophosphate.
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The *in vitro* induction and multiplication of shoots represent the first step in micropropagation. Early studies revealed that high ratio cytokinin/auxin promotes shoot formation (Skoog and Miller 1957) and hence the important role of CKs has been confirmed in many studies (D´Angeli *et al.* 2001, Caboni *et al.* 2002, Hiregoudar *et al.* 2005). It is generally accepted that endogenous auxin has a central role in the initiation of adventitious roots (Gaspar and Coumans 1987). Adventitious root formation proceeds apparently through three distinct phases and two of them are characterised by high endogenous auxin content (Nag *et al*. 2001). A key role of PAs in the process of adventitious rooting was shown in rooting of poplar cutting (Hausman *et al.* 1994, 1995). Root formation requires the high auxin/cytokinin ratio (Hausman *et al*. 1997). Beside the direct involvement of phenylpropanoids in lignin biosynthesis, which restarted with rooting process in micropropagated walnut shoots (Bisbis *et al.* 2003), phenolics play important role in the catabolism of IAA (Volpert *et al.* 1994).

In our previous work we described the efficient micropropagation system of elm (Malá 2000). The multiplicated shoots were cut to apical and basal parts and further cultured both on shoot and root inducing media in order to increase the shoot multiplication rate. Although both shoot parts produced shoots and roots depending on the composition of culture medium, the organogenic potential of apical and basal parts of shoots was different. The shoot forming capacity was higher in the apical part but the timing of root formation was in this type of explant significantly delayed (compared with the organogenic potential of basal part). In view of these findings, we examined the endogenous contents of IAA, CKs, PAs and PhAs in the apical and basal parts of elm shoots and we attempt to establish a relationship between the endogenous phytohormone contents and organogenic abilities of the explants.

Materials and methods

Plants and *in vitro* culture: For the establishment of primary elm cultures, up to 50 dormant buds were collected from 80-year-old donor plus elm trees (*Ulmus glabra* Hudson) during February 2001. The surface sterilization of buds was performed by their immersion in 0.1 % $NaClO_3$ for 10 min followed by immersion in 0.01 % $HgCl_2$ for 15 min. The buds were further rinsed three times with sterilised distilled water for 15 min and then placed onto the culture medium.

The excised apical meristems from dormant buds were placed onto agar - solidified Murashige and Skoog (1962; MS) medium with 0.2 mg dm^{-3} benzylamino-purine (BAP), 0.1 dm^{-3} β-indolebutyric acid (IBA), 10 mg dm^{-3} glutamine and 30 g dm^{-3} sucrose (pH media adjusted to 5.8). The buds were cultivated under white fluorescent light (*36W/33 Philips* tubes, Eindhoven, The Netherlands; irradiance of 30 μmol m^{-2} s^{-1} with 16-h photoperiod) and temperature of 24 °C for 4 - 6 weeks.

The shoots growing from the buds of 1 - 2 cm in the length were excised and placed on the fresh medium. For subsequent transfer we used MS medium modified by an increased concentration of cytokinins BAP (0.5 mg dm^{-3}), glutamine (200 mg dm^{-3}), and casein-hydrolysate (200 mg dm^{-3}). Explants were cultivated under the same growth conditions as during the organogenesis induction stage. The cultures were transferred onto fresh media every 3 - 4 weeks. Multiplicated shoots were cut to two parts, apical and basal, and both parts (each about 2 cm in length) were then cultivated separately on the shoot multiplication medium for 4 weeks. The efficiency of cut shoots in terms of shoot multiplication and root initiation was compared in 50 microcuttings each of both parts.

The apical and basal parts of 50 shoots (about 2 cm long) from multiplicating explant cultures were used for rooting. They were cultured in the dark on the agar medium with one-third strength MS enriched with 1 mg dm^{-3} α-naphthalenacetic acid (NAA) as the sole growth regulator. After 7 d the shoots were transferred onto the hormone-free medium (one-third strength MS) and exposed to 16-h photoperiod. The experiment was repeated twice.

Auxin and cytokinin analysis: IAA and CKs (1 g fresh mass of explant tissue) were extracted and purified simultaneously using *Si-C_{18}* columns (*SepPak Plus*, *Waters*, Milford, MC, USA) and *Oasis MCX* mixed mode (cation exchange reverse-phase) columns (150 mg, *Waters*) according to Dobrev and Kaminek (2002). Internal tritiated standards were added at the homogenisation step for recovery estimation of both auxin (3[5(n)-^{3}H] indolylacetic acid (*Amersham*, Prague, Czech Republic, specific activity 925 GBq $mmol^{-1}$, 2 pmol per sample) and cytokinins ([^{3}H] dehydrozeatin, synthesized by Dr. J. Hanuš, Isotopic Laboratory, Institute of Experimental Botany, Prague, 1.8 TBq $mmol^{-1}$, 2 pmol per sample).

IAA was determined using two-dimensional HPLC as developed by Dobrev and Kaminek (2002). The segment containing IAA obtained in the first dimension was collected in the loop of the fluid processor and redirected to the second HPLC dimension. IAA was quantified using fluorescence detection and external standardisation.

CKs were fractionated by HPLC on a reverse-phase C_{18} column (*Spherisorb 5 ODS*, 4 × 250 mm) by an increasing gradient (10 - 80 %) of methanol in water. Each sample was injected at least twice. The content of individual cytokinins in HPLC fractions was estimated by ELISA. Immunoassays were carried out using polyclonal antibodies and alkaline phosphate tracers (Strnad 1996). Three independent ELISA estimations were done.

Phenolic acid analysis: PhAs were extracted from the apical and basal parts of shoots as described in Cvikrová *et al.* (1991). Briefly, free (F_1), ester-bound (F_2, released after alkaline hydrolysis) and glycoside-bound (F_4, released after acid hydrolysis) PhAs were obtained from a methanol extract of tissue ground in liquid nitrogen. The fraction of cell wall-bound phenolic acids (F_3) was obtained after alkaline hydrolysis of the residual material following methanol extraction. The 2,6-diterc-butyl β-cresol was used as antioxidant and the samples were hydrolysed in nitrogen atmosphere. In spite of adding the antioxidant, the contents of caffeic and chlorogenic (3-*O*-(caffeoyl) quinate) acids in the fractions of ester-bound phenolics (F_2, F_3) were seriously lowered as indicated by the degradation of internal standard. For this reason the values of ester-bound fractions of these two acids are not shown in Table 1. PhAs were analysed by means of HPLC using a *Dionex* Liquid Chromatograph (*P660-HPLC* Pump, *ASI-100* automated sample injector, *TCC-100* termostated column compartment, *PDA-100* photodiode array detector, *Chromeleon Software 6.5*; *Dionex Sofron GmbH*, Germering, Germany) with C_{18} *Spherisorb 5 ODS* column (250 × 4.6 mm). For elution was used acetonitril and acetic acid gradient. Phenolic acids were detected in their absorption maximum. λ_{max} was detected from authentic compounds (*Sigma-Aldrich*, Prague, Czech Republic) that were used as references for quantitative analyses.

Polyamine analysis: The cells were ground in liquid nitrogen and extracted overnight at 4 °C with 5 % (v/v) perchloric acid (PCA) (1 cm^3 5 % PCA per 100 mg fresh mass). The extracts were centrifuged at 21 000 *g* for 15 min, and then PCA-soluble free PAs were determined. Standards (*Sigma-Aldrich*) and PCA-soluble free PAs were benzoylated as described by Flores and Galston (1982). HPLC analysis of benzoyl-amines was performed on HPLC *Beckmann System Gold* (*125S* solvent module pump, *168* detector; *Beckmann Instruments*, Fullerton, CA, USA), with C_{18} *Spherisorb 5 ODS2* column (250 × 4 mm) in methanol gradient according to Slocum *et al.* (1989). The column eluate was monitored at 254 nm.

Statistical analysis: One-way analysis of variance was used for the statistical evaluation of the results.

Results

The elm multiplicated shoots cut to apical and basal parts were cultured on the shoot and root inducing media. The shoot forming capacity was higher in the apical part than in the basal part. The new adventitive shoots arised from meristemoids that were formed in calluses on the bases of apical segments (Fig. 1*A*). The mean number of shoots per apical segment explant were 5.5 ± 1.6. The basal parts responded to culture on multiplication medium with the mean number 3.8 ± 1.8 of shoots per explant that originated from axillary buds (Fig. 1*B*). The differences in root development were scored as mean rooting time. The timing of root formation was significantly delayed in the apical part (13 d) in comparison with the basal part where the first roots appeared after 7 d. However, the percentage of rooted segments was similarly 89.8 ± 2.1 and 92.8 ± 2.1 % in the apical and basal segments, respectively.

The content of endogenous free IAA was approximately three times higher in the basal shoot parts when compared with the content determined in the apical parts (Fig. 2).

Significant difference was observed in the total

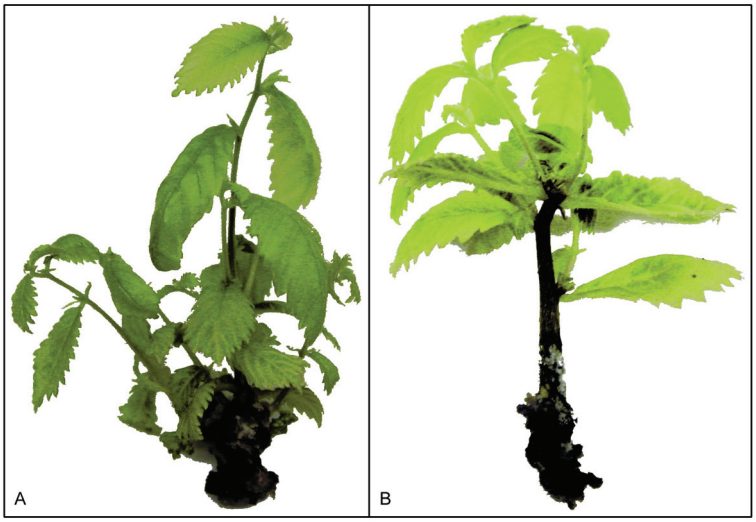

Fig.1. The shoot forming capacity of apical and basal parts of elm multiplicated shoots. *A* - The new adventitive shoots arose from meristemoids on the bases of apical segments. *B* - The new shoots in basal parts originated from axillary buds.

contents of active CKs represented by the sum of free bases and ribosides determined in the apical and basal shoot parts (Fig. 2). The apical shoot parts contained significantly higher amount of CK bases, isopentenyl adenine (iP), zeatin (Z) and dihydrozeatin (DHZ), and their ribosides (Fig. 3*A*,*B*). However, two times higher amount of cytokinin nucleotides was detected in the basal parts (Fig. 3*C*). The prevailing cytokinin forms both in the apical and basal shoot parts, were zeatin (Z), dihydrozeatin riboside (DHZR) and dihydrozeatin riboside monophosphate (DHZRP). Interestingly, the apical parts contained higher levels of Z and DHZR and lower level of DHZRP than the basal parts (Fig. 3*A*,*B*,*C*).

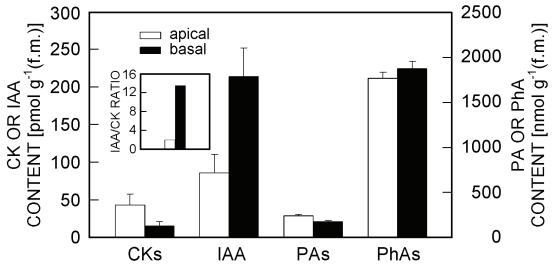

Fig. 2. Total contents of active forms of CKs, IAA, PAs and PhAs in apical and basal parts of elm shoots. Inset - IAA/CKs ratio in apical and basal parts of elm shoots. Means ± SE of two independent experiments with two replicates using material from 35 microcuttings in one experiment.

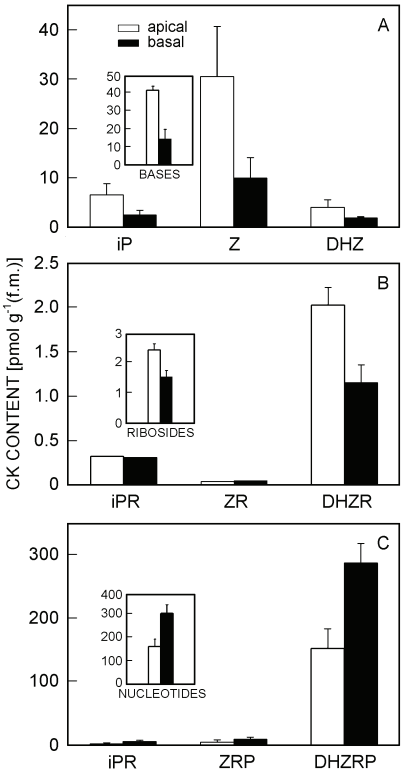

Fig. 3. Cytokinin contents in apical and basal parts of elm shoots. *A* - cytokinin free bases: isopentenyl adenine (iP), zeatin (Z) and dihydrozeatin (DHZ); inset - total content of free bases. *B* - cytokinin ribosides iPR, ZR and DHZR; inset - total content of ribosides. *C* - cytokinin nucleotides, iPRP, ZRP and DHZRP; inset - total content of nucleotides. Means ± SE of two independent experiments with two replicates using material from 35 microcuttings in one experiment.

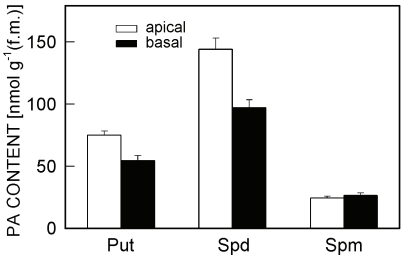

Fig. 4. Contents of free putrescine (Put), spermidine (Spd) and spermine (Spm) determined in apical and basal parts of elm shoots. Means ± SE of two independent experiments with two replicates using material from 35 microcuttings in one experiment.

The amounts of Put and Spd were higher in the apical part (Fig. 4), which generally contains less differentiated tissues than the basal parts of shoots. No significant difference was observed in the total contents of PhAs represented by the sum of free, soluble ester-, glycoside and insoluble cell wall-bound PhAs determined in the apical and basal shoot parts (Fig. 2). The predominant PhA in both types of explants was caffeic acid although only data of its free and glycoside-bound forms are presented in Table 1. Concentrations of other PhAs decreased in the following order: *p*-coumaric, ferulic, sinapic, vanillic, chlorogenic, *p*-hydroxybenzoic and gallic acids. The ester- and glycoside-bound PhAs from the methanolic extract (F_2 and F_4, respectively) represented the major phenolic fractions (Fig. 5). The contents of all determined phenolic acids in their free forms were higher in the basal parts than in the apical parts (Table 1). The higher total content of soluble ester-bound phenolics was found in the apical part than in

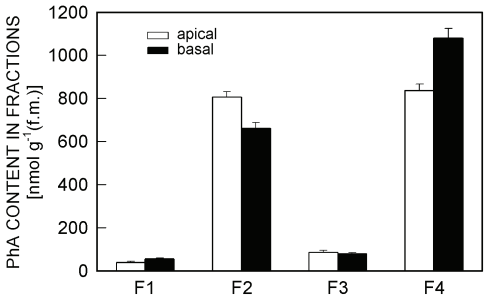

Fig. 5. Total contents of free PhAs (F_1), ester-bound methanol-soluble PhAs (F_2), ester-bound cell wall PhAs (F_3) and glycoside-bound methanol-soluble PhAs (F_4) determined in apical and basal parts of elm shoots. Means ± SE of two independent experiments with two replicates using material from 35 microcuttings in one experiment.

the basal one. On the contrary, the basal parts contained higher levels of phenolic glycosides, specifically of *p*-coumaric, ferulic and sinapic acids, which are precursors for lignin biosynthesis (Fig. 5, Table 1).

Table 1. Contents of individual PhAs [nmol g^{-1} (f.m.)] in the apical and basal parts of elm multiplicated shoots. F_1, free PhAs; F_2, ester-bound methanol-soluble PhAs; F_3, ester-bound cell wall PhAs; F_4, glycoside-bound methanol-soluble PhAs. CaA, cafeic acid; ChA, chlorogenic acid; FA, ferulic acid; GA, gallic acid; *p*CA, *p*-coumaric acid; *p*HBA, *p*-hydroxybenzoic acid; SiA, sinapic acid; VA, vanillic acid; n.d., not detectable. In spite of adding the antioxidant, the contents of caffeic and chlorogenic (3-*O*-(caffeoyl) quinate) acids in the fractions of ester-bound phenolics (F_2, F_3) were seriously lowered as indicated by the degradation of internal standard. For this reason the values of ester-bound fractions of these two acids are not shown. Means ± SE of two independent experiments with two replicates using material from 35 microcuttings in one experiment.

Explant	Fraction	GA	*p*HBA	VA	CaA	ChA	*p*CA	FA	SiA
Apical part	F_1	n.d.	2.31±0.25	3.75±0.37	3.24± 0.24	24.95±1.85	1.37± 0.19	2.64± 0.24	1.67±0.21
	F_2	n.d.	7.15±1.02	39.07±3.51	-	-	430.79±31.89	264.26±21.67	63.54±5.11
	F_3	n.d.	2.93±0.34	7.57±0.62	-	-	44.12± 4.03	32.12± 2.91	n.d.
	F_4	8.52±0.90	11.21±0.94	26.09±1.98	574.21±48.26	44.90±3.84	85.46± 6.86	38.81± 4.28	41.83±4.39
Basal part	F_1	n.d.	2.63±0.22	4.71±0.36	4.59± 0.36	34.42±3.26	1.73± 0.24	4.32± 0.35	1.96±0.33
	F_2	n.d.	7.89±1.17	68.26±5.43	-	-	328.64±31.67	175.56±15.26	83.25±6.61
	F_3	n.d.	2.83±0.25	4.03±0.34	-	-	33.79± 3.03	39.07± 2.13	n.d.
	F_4	13.03±1.24	19.32±1.74	45.41±3.77	738.86±46.23	55.67±4.92	102.86± 8.68	48.23± 3.19	67.65±5.42

Discussion

This work was focused on studying the possibility to increase the elm explant multiplication rate by cutting the multiplicated shoots to apical and basal parts. Our attempt was to establish a relationship between endogenous hormonal content and *in vitro* morphogenic responses of elm explants. The shoot forming capacity was higher in the apical part than in the basal one and the new adventitious shoots arose from the meristemoids that were formed in calluses on the bases of apical segments (Fig. 1*A*). Significantly higher contents of iP, Z and DHZ that were found in the elm apical segments (Fig. 3) might be considered as the most important factor affecting *in vitro* shoot formation in the apical segments. High contents of CKs were shown to have an important role in the initiation of proliferation centres in explants and in the subsequent bud primordia formation in rice (Zhu *et al*. 1995). The *in situ* localisation of CKs performed using antibodies with marked specificity against Z and iP pointed to a primary role of Z in cell differentiation during *in vitro* shoot formation in pear and apple (D´Angeli *et al*. 2001, Caboni *et al*. 2002). The importance of CKs for the initiation of organogenic centres was also reported earlier in *Nicotiana* by Heylen *et al*. (1991).

The removal of the apex released the axillar buds from apical dominance and the adventitious shoots formed on the elm shoot basal parts originated from axillary buds (Fig. 1*B*). It is generally accepted that axillary bud outgrowth is modulated through the antagonistic effects of CKs and IAA (Cline *et al.* 1997). The relationships between the endogenous phytohormone contents and axillary bud development were studied on decapitated nodal segments of pineapple. After 1-d culture on hormone free medium, marked decrease in free IAA content and progressive increase in ZR occurred, suggesting that Z-type CKs have an important role in organogenesis (Souza *et al*. 2003). Our results showed in the basal segments twice higher content of DZRP than in the apical parts (Fig. 3*C*). This compound may be considered as a "metabolic storage form" with rapid turnover. Comparison of the contents of Z and DHZ and their ribosides and ribotides (phosphates) points to the possibility that Z is metabolised to DHZ and which converts to DHZRP. DHZRP is inactive per se but may be quickly cleaved back to DHZR and DHZ. However, it was not shown that DHZ is active in this micro-propagation system. In the current work the IAA/CKs ratio was markedly higher in the basal parts of elm shoots than in the apical parts (Fig. 2). Throughout the axillary bud regrowth in decapitated pineapple explants was IAA/total CKs ratio lower when related to the value found in segments with the apex (Souza *et al*. 2003). However, we have to point out that we measured the endogenous phytohormone contents prior to the explant culture on shoot and/or root inducing culture media. During the culture the IAA/CKs ratio might have changed.

The induction of roots on explants from *in vitro* cultures is a crucial point in any micropropagation process. It is generally accepted that endogenous auxin has a central role in the initiation of adventitious roots (Gaspar and Coumans 1987). Although the percentage of rooted elm apical and basal shoot segments was similar, the timing of root formation was significantly delayed in

the apical part (13 d) in comparison to that in the basal part where the first roots appeared after 7 d. Higher IAA content (Fig. 2) and higher IAA/CKs ratio found in the basal shoot parts well correspond with the proposed role of IAA in the rooting process. The important role of endogenous IAA in root initiation in poplar shoots raised *in vitro* is also reported by Hausman *et al.* (1997). The possible role of CKs in rooting is still not clear. Rooting capacity of tree peony was favoured by a preliminary accumulation of endogenous IAA only when levels of the N^6-benzyladenine absorbed from the medium had decreased (Bouza *et al*. 1994). Cytokinins at high concentrations are known to inhibit adventitious rooting, however, their specific level is probably essential for the induction of cell division in the initial step in root formation (De Klerk *et al*. 2001).

Differentiation processes depend on endogenous factors, among which PAs are believed to play a very important role (Couee *et al*. 2004). The certain contents of free PAs are always associated with cell division and/or with re-programming of cells into new developmental pattern (Torrigiani *et al.* 1987). Higher contents of Put and Spd in the apical parts of elm multiplicated shoots might be together with high contents of active forms of Z and DHZ prerequisite for efficient adventitive shoot formation. On the other hand a positive correlation between PAs accumulation and the induction of adventitious rooting by auxin was observed in numerous woody species, suggesting that PAs could be used as markers of rooting process (Hausman *et al*. 1994, Neves *et al.* 2002). However, in our experimental system the levels of free PAs were probably not limiting factor for ongoing morphogenic processes during shoot and root formation.

There are still some unanswered questions about the precise role of phenolic substances in the processes of differentiation and morphogenesis. According to current knowledge, phenolics *1*) participate in some way in auxin catabolism (Legrand and Bouaza 1991) and *2*) they modulate the free PA levels by their conjugation (Cvikrova *et al.* 1999, Mader and Hanke 1997). Higher levels of free caffeic, chlorogenic and ferulic acids were found in the basal parts of elm shoots. These three phenolic acids exhibit *in vitro* IAA protection (Volpert *et al*. 1994) and might influence through the endogenous IAA content the appropriate hormonal balance required for root induction. Furthermore, the earlier appearance of adventitious roots in basal segments might be also linked to increased contents of *p*-coumaric, ferulic and sinapic acid glycosides, precursors of lignin biosynthesis, which were determined in this type of explant. Increase in the total phenolic content in the root regeneration zone in grape vine cultivars was earlier highlighted by Mouncousin (1986) and restart of lignification during the rooting inductive phase was described in micro-propagated walnut shoots (Bisbis *et al*. 2003). We may hypothesise that the lower IAA content and lower contents of lignin precursors in the apical shoot parts might be the cause of delayed root formation observed in this type of explant.

In conclusion, we have shown that the efficiency of our elm multiplication system in terms of shoot multiplication and rate of root initiation was significantly influenced by the endogenous levels of auxin, cytokinins (predominately zeatin and dihydrozeatin), polyamines and phenolic acids in the explants.

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