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Micropropagation of Wild Service Tree (*Sorbus torminalis* [L.] Crantz): The Regulative Role of Different Aromatic Cytokinins During Organogenesis

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Abstract The influences of three different aromatic cytokinin derivatives [6-benzylaminopurine, *meta*-topolin, and 6-(3-methoxybenzylamino)purine-9-ß-D-ribofuranoside (MeOBAPR)] on in vitro multiplication and rhizogenesis of the wild service tree (*Sorbus torminalis* [L.] Crantz) were compared. The highest micropropagation rate (24 new shoots per explant after 3 months of cultivation) was achieved on media containing BAP. On the other hand, the best rooting microcuttings were those multiplied on a medium containing MeoBAPR. To compare these results with the levels of endogenous cytokinins in multiplied explants, a newly developed UPLC-ESI(+)-MS/MS method was used to determine levels of 50 cytokinin metabolites in explants cultivated 12 weeks on media supplemented by BAP and of the two other aromatic cytokinin analogs used. Several significant differences among the levels of endogenous cytokinins, extracted from the explants, were found. The concentration of BAP9G, an important metabolite suspected to be responsible for inhibition of rooting and acclimatization problems of newly formed plantlets, was found to be the highest in microcuttings grown on media supplemented with BAP. This agrees well with the results of our rooting experiments; the lowest percentages of rooted plantlets 6 weeks after transferring shoots on rooting medium were present on explants multiplied on BAP. In contrast, BAP was still the most effective for the induction of bud formation on primary explants. Levels of the most active endogenous isoprenoid cytokinins, tZ, tZR, and iPR, as well as O-glucosides were also suppressed in explants grown on BAP compared with those of explants treated with other cytokinin derivatives. This may be the result of a very high BAP uptake into the explants grown on this cytokinin. On the other hand, endogenous concentrations of *cis*-zeatin derivatives as well as dihydrozeatin derivatives were not affected. Differences in the production of another plant hormone, ethylene, that plays an important role in controlling organogenesis in tissue culture, were also observed among *S. torminalis* plantlets grown in vitro on media containing different cytokinins tested. The highest ethylene levels were detected in the vessels containing media supplemented with mT. They were two to four times higher compared with the production by the *S. torminalis* explants cultivated on other media used. Finally, the levels of free IAA were also determined in the explants. *S. torminalis* plantlets grown on media containing BAP contained the lowest level of auxin, which is again in good agreement with their loss of rooting capacity. The results found in this study about optimal plant hormone concentrations may be used to improve in vitro rooting efficiency of the wild service tree and possibly also of other plant species.

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Abbreviations

BAP	6-Benzylaminopurine
MeOBAP	6-(3-Methoxybenzylamino) purine

*m*T	6-(3-Hydroxybenzylamino) purine
*o*T	6-(2-Hydroxybenzylamino) purine
iP	N^6-isopentenyladenine
cZ	*cis*-Zeatin
tZ	*trans*-Zeatin
DHZ	Dihydrozeatin
*R	9-β-D-ribofuranosyl derivative
*9G	9-β-D-glucopyranosyl derivative
*OG	O-β-D-glucopyranosyl derivative
*5′MP	5′-Monophosphate derivative
LC-MS	Liquid chromatography combined with mass spectrometry
UPLC-ESI(+)-MS/MS	Ultra-performance liquid chromatography combined with positive electrospray mass spectrometry
MS	Murashige-Skoog medium
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
NAA	α-Naphthylacetic acid

Introduction

The wild service tree (*Sorbus torminalis* [L.] Crantz) is one of a scarce species among forest trees and occurs in scattered populations at a low density in central Europe. It is a slow-growing tree that reaches a maximum height of 20–25 m at around 80–100 years. According to the latest data, the species is disappearing from forests of the Czech Republic and it is considered to be endangered. Nowadays, the wild service tree is rated as one of the most valuable hardwoods with a great potential for wider use in forestry and forest ecology; it is also important in the timber industry. Since the 1990s it has been among the highest-priced timber species in Europe (Demesure and others 2000).

The genetic diversity of this scattered species may be at risk, particularly in dense forests, which contain a high proportion of long-lived tree species. Because it grows in small fragmented populations, it is sensitive to high levels of interspecific competition. Such conditions could be a cause of a low gene exchange among populations (Hoebee and others 2006) and consequently of a strong decrease in genetic diversity in the species through reduction of population size. The regeneration of the wild service tree is gradually weakened, which could eventually lead to its local extinction. As for all forest trees, the wild service tree is particularly threatened by habitat fragmentation due to its low population density (Demesure and others 2000; Oddou-Muratorio and others 2001; Prat and Daniel 1993).

For long-term sustainability of wild service tree genetic resources, micropropagation technologies could prove to be useful (Chalupa 1992; Dujíčková and others 1992; Malá and others 2005). Clonal propagation could preserve the genetic diversity of the species over a long period of time, even if environmental conditions might worsen during the course of succession. At present there are 95 clones derived from mature elite wild service trees from various forest regions in the Czech Republic deposited in the Bank of Explants in the Forestry and Game Management Research Institute of the Czech Republic. Despite this, for standardized micropropagation procedures for the wild service tree and for its effective exploitation in forestry, some difficulties during the rooting stage of organogenesis remain to be solved.

Cytokinins, N^6-substituted purine derivatives, are an important class of plant hormones that regulate a large number of physiologic and developmental processes in plants (Letham and Palni 1983). They are classified as isoprenoid or aromatic, depending on the structure of the N^6-substituent (Strnad 1997). Endogenously, cytokinins can occur in different metabolic forms, including free bases, ribosides (R), N-glucosides (G), O-glucosides (OG), and nucleotides (5′MP) (Letham and Palni 1983).

In micropropagation technology, 6-benzylaminopurine (BAP) is widely used as one of the most effective and affordable cytokinins. Nevertheless, it often induces disproportional growth or inhibition of rooting in a number of plant species (Werbrouck and others 1995, 1996), including the wild service tree. Thus, research to find alternative phytohormones for micropropagation purposes remains important.

The high morphogenetic activity of *meta*-topolin [6-(3-hydroxybenzylamino)purine; mT] and its derivatives was previously described (Werbrouck and others 1996). Our LC-MS-based identification of naturally occurring aromatic cytokinins in plants and plant-pathogenic bacteria recently led to the discovery of several new phytohormones, including methoxytopolins (Tarkowská and others 2003). A large group of methoxytopolin analogs has been synthesized and characterized, and selected derivatives have been tested for use in micropropagation (Doležal and others 2006, 2007). 6-(3-Methoxybenzylamino)purine-9-ß-D-ribofuranoside (MeOBAPR), an endogenously occurring cytokinin and one of the most active derivatives from this large group discovered by us so far, was compared in this study with BAP and mT—standard and commercially available cytokinin free bases.

Ethylene is another plant hormone that plays an important role in controlling organogenesis in tissue culture, although its role in rooting has not been fully

elucidated (Kepczynski and others 2006). Increased ethylene levels caused reduction of root formation from pea cuttings (Nordstrom and Eliasson 1993). Moreover, exogenous application of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) inhibited root formation in apple shoot cultures by delaying root emergence and increasing callus formation at the bases of shoots (Ma and others 1998). The same authors also reported promotion of root formation by using various ethylene inhibitors (Ma and others 1998). On the other hand, ethylene was found to stimulate root formation from hazelnut cotyledons (Gonzalez and others 1991) as well as kiwi (*Actinidia deliciosa*) explants (Arigita and others 2003). Root formation in *Populus tremula* L. explants was either inhibited or stimulated by ethylene depending on its concentration within the culture vessels (Gonzalez and others 1997). This implies that the ethylene effect on rooting emergence and promotion is concentration dependent and knowledge about its optimal concentration may be used to improve in vitro rooting efficiency (Ma and others 1998).

Root formation has many practical implications in horticulture, agronomy, and forestry. There is a lot of commercial interest in root formation because of the many plant species that are difficult to root (Davies and others 1994). The auxins were the first plant hormone group to be used to stimulate rooting of cuttings. Their important role in root initialization and formation has been studied intensively for many years (although it is still not completely understood on a molecular level) (Ludwig-Müller and others 2005).

In this study we compared the influence of three different aromatic cytokinins (BAP, mT, and MeOBAPR) on the multiplication of explants and the rooting of microcuttings of the wild service tree. Subsequently, the endogenous cytokinin concentrations in the explants, with the focus on the level of potentially root-inhibiting cytokinin metabolites and their ethylene production and endogenous auxin (IAA) levels, were determined.

Material and Methods

Plant Material

Multiapex explant cultures derived from three clones of wild service tree from the Explant Bank of the Forestry and Game Management Research Institute and which had grown for 12 months on the multiplication medium:agar solidified MS (Murashige and Skoog 1962) medium with 0.2 mg dm^{-3} of BAP, 0.1 mg dm^{-3} of IBA, 100 mg dm^{-3} of glutamine, and 30 g dm^{-3} of sucrose (pH 5.8) were selected. The explants were cultivated under white fluorescent light (36 W/33 Philips tubes, Eindhoven, the Netherlands; irradiance of 30 μmol m^{-2} s^{-1} with 12-h photoperiods) and at 24°C.

Experimental Setup

Shoots derived from three clones were divided into three groups: control shoots cultivated on multiplication medium (see above) (30 cultures), shoots cultivated on a modified medium (multiplication medium with 0.2 mg dm^{-3} of mT instead of BAP) (30 cultures), and shoots cultivated on another modified medium (multiplication medium with 0.3 mg dm^{-3} of MeOBAPR instead of BAP) (30 cultures). All cultures were cultivated for 12 weeks (with transfer every 4 weeks into fresh multiplication medium of the same composition) under the same cultivation conditions. Ten shoot cultures were taken from each group for chemical analyses. For induction of rooting, 20 shoots (microcuttings derived from one clone) from each group were placed onto the agar rooting medium [one-third-strength MS medium enriched with NAA (14 mg dm^{-3}), that is, without cytokinins]. Transferred shoots were cultured in black boxes in an air-conditioned room at 25°C. After 1 week the shoots were transferred into fresh rooting medium (one-third-strength MS medium without NAA) and cultured under white fluorescent light (36 W/33 Philips tubes) with a 12-h photoperiod (irradiance = 30 μmol m^{-2} s^{-1}). The experiment was repeated three times.

Evaluation of Changes in Endogenous Cytokinin Levels

After 12 weeks of cultivation, the shoot cultures (100 mg per sample) of three different clones, grown on media containing different cytokinins, were taken for analyses. The endogenous concentrations of 50 cytokinin derivatives were determined by means of ultra-performance liquid chromatography combined with electrospray mass spectrometry [UPLC-ESI(+)-MS/MS] (Novák and others 2008). The cytokinins were analyzed using an Acquity UPLC™ (Waters, Milford, MA), equipped with a BEH C-18 (1.7 μm, 2.1 × 150 mm) column linked to a Quattro *micro*™ API detector (Waters MS Technologies, Manchester, UK) triple quadrupole mass spectrometer equipped with an electrospray interface.

The analytes were quantified by multiple-reaction monitoring of $[M + H]^+$ and the appropriate product ion (Novák and others 2008). The identity of all measured cytokinin metabolites was verified by comparing the mass spectra and chromatographic retention times with those of authentic standards. The measurements were done in triplicate.

Ethylene Measurement

Analysis was performed using the gas chromatography-flame ionization detector (GC-FID) method (Fišerová and others 2001). Cultivation vessels were ventilated 1 h before sampling. Subsequently, 1 ml of air was taken from each vessel and analyzed using a Finnigan Trace GC Ultra equipped with a FID detector and 50-m capillary column (HP-AL/S stationary phase, 15 μm, i.d. = 0.535). Injection temperature was set to 200°C, oven temperature to 40°C, and detector temperature to 220°C. The measurements were done in triplicate from three different vessels of each clone and cultivation medium used.

Determination of Endogenous IAA

Material was ground and homogenized with liquid nitrogen and weighed out into Eppendorf tubes. A cold solution of 0.05 M phosphate buffer (pH 7.0) containing 0.02% DEDTCA was used for extraction of material. To check the recovery during purification and to validate the quantification, a labeled internal standard of $13C_6$-IAA was added to each sample. Samples were stirred for 15 min at 4°C. After centrifugation (5 min at 12,000 rpm, 4°C), the supernatant was collected and acidified to pH 2.7 with 1 M HCl. Finally, samples were purified by solid-phase extraction on Varian BondElut C_8 (500 mg/3 ml) columns, evaporated to dryness, and store at -20°C before LC/MS analysis.

An Acquity UPLC System (Waters), including a binary solvent manager and a sample manager, and a Micromass Quattro *micro* API detector (Waters MS Technologies) were used for cytokinin analysis. All data were processed by MassLynx™ software with QuanLynx™ and QuantOptimize™ programs (ver. 4.0, Waters).

Samples were dissolved in 20 μl of ACN/water (10/90) and filtered (Micro-spin® filter tube; 0.2 μm; 3 min at 8000 rpm; Grace, USA); then 15 μl of sample was injected onto a reversed-phase column (BetaMax Neutral; 150 mm × 1 mm; particle size = 5 μm; Thermo Fisher Scientific, Waltham, MA) with UNIGUARD™ column protection (Hypurity advance; 10 mm × 1 mm; 5 μm; Thermo Fisher Scientific). The samples were eluted with a 20-min gradient as follows: 0–5 min 90/10 A/B, 5–10 min 80/20 A/B, 10–18 min 70/30 A/B, 18–19 min 50/50 A/B, 19–20 min 10/90 A/B (v/v), where A was 1% ACN with 0.1% formic acid and B was 95% ACN with 0.1% formic acid. At the end of the gradient the column was equilibrated to initial conditions for 5 min. The flow rate was 0.05 ml min^{-1} and the column was eluted at ambient temperature. Under these conditions, retention time for the monitored IAA was 15.36 min. The effluent was passed to the tandem mass spectrometer without post-column splitting. Indole-3-acetic acid was quantified by multiple-reaction monitoring of $[M + H]^+$ and the appropriate product ion.

For the selective multiple reaction monitoring mode (MRM) experiment, optimized conditions were as follows: capillary voltage, 3.0 kV; source/desolvation gas temperature, 100/250°C; cone/desolvation gas flow rates, 2.0/450 L h^{-1}; LM/HM resolution, 10.0; ion energy 1, 1.0 V; ion energy 2, 1.4 V; multiplier voltages, 700 eV. Argon was used as collision gas with an optimized pressure of 5×10^{-3} mbar. The dwell times (0.80 s), cone voltages (20 V), and collision energy (15 eV) for diagnostic transitions were optimized to maximize sensitivity.

Growth and Morphogenetic Evaluations

The newly developed shoots were counted in all shoot cultures after 3 months of cultivation. At the same time a qualitative evaluation of growth was carried out (numbers of new shoots per explant and average length of shoot).

Statistical Evaluation

The data were analyzed by analysis of variance (ANOVA) and Duncan's multiple-range test (Duncan 1955).

Evaluation of Rooting

The percentages of rooted plantlets were determined 6 weeks after transferring shoots onto rooting medium (see subsection "Experimental setup").

Results

Growth and Morphologic Evaluations

The highest multiplication rate (number of newly grown shoots per culture) was achieved using multiplication medium supplemented with BAP (24 ± 4 shoots). Only 55-60% of the multiplication rate observed with BAP was achieved in the media containing MeOBAPR and mT (Table 1). There were no significant differences in the number of new shoots among clones cultivated on the same media. The average numbers of new shoots in three clones is presented in Table 1. None of these cytokinins influenced either the morphologic formation or the length of shoots (3.2 ± 0.6 cm on average in all clones).

Rooting

Percentages of successfully rooted plantlets were determined after 6 weeks of cultivation in the rooting medium (see above). The highest percentages (15 ± 2.5%) of

Table 1 Comparison of the average numbers of new shoots influenced by different cytokinins (values combined from 3 experiments)

Cytokinin	No. of new shoots ± SD	mT	MeoBAPR	BAP
mT	14.2 ± 4.96	X	NS	*
MeoBAPR	13.3 ± 4.76	NS	X	*
BAP	24.3 ± 4.41	*	*	X

NS not significant

* Significant according to Duncan's multiple-range test at the 0.05 level (Duncan 1955)

Table 2 Comparison of the average percentages of rooted plantlets (values combined from 3 experiments)

Cytokinin	Percentage of rooted plantlets ± SD	MeoBAPR	mT	BAP
MeoBAPR	15.3 ± 2.5	X	*	*
mT	8.7 ± 3.5	*	X	NS
BAP	7.0 ± 3.0	*	NS	X

NS not significant

* Significant according to Duncan's multiple-range test at the 0.05 level (Duncan 1955)

rooted plantlets were achieved after transferring the microcuttings from medium with MeOBAPR into one-third MS medium without NAA (see subsection "Experimental setup"). This rate was significantly higher compared with the other two cytokinins tested (8.7 ± 3.5 for mT and 7.0 ± 3.0 for BAP) (Table 2).

Cytokinin Levels in the Explants

Fifty endogenous cytokinin metabolites were determined in the samples of *S. torminalis* plantlets grown in vitro on media containing three different cytokinin species. The levels of 27 cytokinin species were found to be present above the detection limit of the UPLC-MS/MS quantification method used (Novák and others 2008) and therefore could be quantified and compared in relation to cytokinins used in the cultivation media. The highest concentration (2.4 ± 0.8 pmol g^{-1} FW) of BAP9G was determined in the samples grown on BAP, whereas levels of this metabolite in the explants grown on MeoBAPR and/or mT were found to be significantly lower (0.41 ± 0.35 and 0.08 ± 0.06 pmol g^{-1} FW, respectively) (Table 3). Moreover, detectable levels of corresponding mT and MeoBAPR metabolites, mT9G and MeoBAP9G, were not found even in plantlets grown on mT and MeoBAPR, respectively. It is interesting to note that formation of BAP cytokinins was observed after application of mT to the culture medium (Table 3). Moreover, detectable levels of BAP as well as mT cytokinins were measured in the *S. torminalis* explants grown on media containing MeoBAPR (Table 3).

Among the isoprenoid cytokinins found, the highest concentrations detected were for the nucleotides, namely, iPR-5′MP, tZR-5′MP, and cZR-5′MP. On the other hand, levels of tZ, tZR, and iPR, which are considered the most active endogenous isoprenoid cytokinins, were suppressed in explants grown on BAP in comparison with the other two treatments (Table 3). Dihydrozeatin and *cis*-zeatin derivatives were almost unaffected by these cytokinin treatments.

Ethylene Induction

Levels of ethylene were measured in cultivation vessels during the incubation of *S. torminalis* plantlets in vitro on media containing three different cytokinins (Table 4). The highest concentration (272–392 nl L^{-1} depending on the clone used) of ethylene was determined in the vessels containing mT, whereas levels of this gaseous plant hormone produced by the explants grown on BAP and/or MeoBAPR were found to be significantly lower (Table 4).

IAA Levels in the Explants

Using UPLC-ESI(+)-MS/MS technology, endogenous IAA concentrations were measured in samples of *S. torminalis* plantlets (two different clones) grown in vitro on media containing three different cytokinins. A calibration curve ($y = 0.6167x - 0.0047$; $R^2 = 0.9972$) was created by plotting the known concentration of unlabeled/labeled analyte ratio against the calculated response area of the analyte/internal standard ratio. The indole-3-acetic acid limit of detection (LOD = 9.3 fmol) and quantification (LOQ = 30.8 fmol) were calculated from the signal-to-noise ratio of 3:1 and 10:1, respectively. The lowest concentration (29.4 ± 1.2 ng g^{-1} FW for clone 1 and 27.3 ± 0.7 ng g^{-1} FW for clone 3, respectively) of IAA was present in the plantlets cultivated on BAP. Levels of this compound in the explants grown on mT were found to be significantly higher in both clones (31.0 ± 0.2 ng g^{-1} FW in clone 1 and 51.2 ± 3.8 ng g^{-1} FW in clone 3, respectively) (Table 5). Whereas IAA concentration in the plantlets of clone 3, grown on another topolin derivative, MeoBAPR, were also significantly higher (33.8 ± 1.8 ng g^{-1} FW) compared with those grown on BAP, the IAA increase in clone 1 was not significant (Table 5).

Discussion

Plant micropropagation represents a complex process consisting of successive developmental stages controlled

Table 3 Cytokinin levels (pmol g^{-1} FW) in *S. torminalis* shoots determined by UPLC-ESI(+)-MS/MS

	tZ	tZR	tZR-5′MP	cZ	cZR	cZR-5′MP	DHZ	DHZR	iP
BAP	0.83 ± 0.34	0.69 ± 0.23	4.7 ± 1.3	0.30 ± 0.13	0.51 ± 0.14	4.8 ± 1.7	0.03 ± 0.02	0.12 ± 0.05	2.1 ± 1.3
MeOBAPR	3.4 ± 2.1	2.2 ± 0.9	3.7 ± 1.2	0.37 ± 0.21	0.64 ± 0.26	5.1 ± 2.3	0.11 ± 0.06	0.22 ± 0.12	1.2 ± 0.2
mT	5.7 ± 2.9	1.5 ± 0.3	2.7 ± 0.4	0.28 ± 0.07	0.52 ± 0.17	4.2 ± 1.0	0.24 ± 0.19	0.17 ± 0.05	0.8 ± 0.2
	iPR	iPR-5′MP	BAP	BAPR	BAP9G	BAPR-5′MP	mT	mTR	oT
BAP	0.95 ± 0.2	10.8 ± 2.8	2214 ± 611	261 ± 64	2.4 ± 0.8	753 ± 134	6.3 ± 4.3	0.60 ± 0.26	3.9 ± 1.5
MeOBAPR	2.1 ± 0.8	15.9 ± 3.6	69.3 ± 23	17 ± 12	0.41 ± 0.35	16.9 ± 6.4	37 ± 17.5	25.9 ± 12	1.6 ± 1.4
mT	1.4 ± 0.3	12.3 ± 2.3	24.1 ± 8.5	3.3 ± 1.8	0.08 ± 0.06	12.5 ± 7.6	167 ± 125	52 ± 41	3.9 ± 1.2
	oTR	MeOBAP	MeOBAPR	tZOG	tZROG	cZOG	cZROG	mTOG	mTROG
BAP	0.43 ± 0.22	<LOD	<LOD	3.15 ± 1.6	1.17 ± 0.54	0.53 ± 0.11	0.43 ± 0.11	5.7 ± 3.5	13.3 ± 3.6
MeOBAPR	0.46 ± 0.09	2177 ± 724	2255 ± 869	5.0 ± 2.5	1.96 ± 0.82	0.62 ± 0.17	0.58 ± 0.11	166 ± 41	160 ± 38
mT	0.44 ± 0.12	1.45 ± 0.3	<LOD	5.5 ± 0.5	1.90 ± 0.23	0.39 ± 0.14	0.43 ± 0.13	798 ± 428	523 ± 148

Amount ± SD represents mean values of three measurements of real samples. Only compounds above the detection limit are included. Twelve-week-old *S. torminalis* shoots (250 mg) were extracted. The extracts were purified by SPE followed by immunoaffinity chromatography, and then measured by UPLC-ESI(+)-MS/MS technique

Table 4 Ethylene production (nl L^{-1}) by *S. torminalis* explants determined by GC-FID 1 h after ventilation

Cytokinin	Clone number		
	1	2	3
MeoBAPR	33 ± 12	291 ± 28	175 ± 77
mT	392 ± 92	357 ± 68	272 ± 55
BAP	81 ± 16	119 ± 15	177 ± 18

The measurements were done in triplicate

Table 5 IAA levels (ng g^{-1} FW) in *S. torminalis* explants determined by UPLC-ESI(+)-MS/MS

Cytokinin	Clone number	
	1	3
MeoBAPR	30.9 ± 1.1	33.8 ± 1.8
mT	31.0 ± 0.2	51.2 ± 3.8
BAP	29.4 ± 1.2	27.3 ± 0.7

The measurements were done in triplicate

by various endogenous and exogenous stimuli which also regulate growth processes in vitro (Centeno and others 1996). However, cross-talk between auxins, cytokinins, and other active substances such as polyamines and phenolic acid derivatives that participate in morphogenetic processes during organ differentiation is far from being fully understood (Altamura and others 1993; Cvikrová and Hrubcová 1999; Scholten 1998). It has been shown that root formation proceeds through several growth stages that are characterized by a high level of endogenous auxins, particularly indole-3-acetic acid (Nag and others 2001). The presence of cytokinins is also essential for the induction of cell division at the beginning of root formation (De Klerk and others 2001), but in contrast to auxins, higher levels of cytokinins inhibit adventitious rooting (Bollmark and others 1988).

Problems with the rooting of microcuttings are why the wide utilization of micropropagation techniques are not used for many important tree species (Malá and others 2005) This is especially true for *S. torminalis*, plantlets of which root poorly, even when a protocol previously used successfully to micropropagate other poorly rooting broad-leaf tree species, for example, oak, is used (Malá and others 2000). Decreased ability to root adventitiously could be related to an increased level of BAP9G, mainly in the basal parts of explants. Accumulation of this metabolite at the plant base might be a reason for various acclimatization problems such as heterogeneity in growth and inhibition of rooting (Werbrouck and others 1995). The aim of the current work was to test other aromatic cytokinin derivatives, use of which could have strong organogenesis activity, comparable to that of BAP, but a minimal effect on subsequent rooting. Simultaneously, the levels of corresponding 9-glucosides, responsible for the decreased rooting capacity of newly formed explants, should be lowered. We confirmed the formation of this metabolite in *S. torminalis* explants, with the highest concentrations detected in the microcuttings grown on media containing BAP (Table 3). Accordingly, the lowest percentage of rooted plantlets 6 weeks after transferring shoots on rooting medium was among the explants multiplied on BAP (Table 2). On the other hand, this compound was still the most effective in the induction of adventitious buds as well as axillary bud

formation on primary explants. In this case the excised apical meristems were from dormant buds (Table 1).

To analyze this problem in more detail, we used our newly developed UPLC-ESI(+)-MS/MS method to determine levels of BAP, BAP9G, and 48 other endogenous cytokinin metabolites in the explants studied. Several significant differences were found between levels of the compounds extracted from the explants cultivated on media supplemented with BAP and those supplemented with cytokinin analogs. Levels of the most active endogenous isoprenoid cytokinins, tZ, tZR, their O-glucosides, and iPR, were suppressed in explants grown on BAP compared with other treatments. This suppression could probably be caused by very high concentrations of BAP cytokinins present in the explants grown on this cytokinin (Table 3). In contrast, mT exhibited massive formation of the corresponding O-glucosides (mTOG and mTROG) (Table 3), which are considered as storage forms and located in the vacuole (Fusseder and Ziegler 1988). Futhermore, MeoBAPR offers an even broader spectrum of possible metabolites (after alternative demethylation) (Table 3). On the other hand, endogenous concentrations of *cis*-zeatin and dihydrozeatin derivatives were comparable (Table 3). We describe here, also for the first time, limited formation of BAP derivatives after the application of *meta*-topolin as well as formation of mT and BAP cytokinins in MeOBAPR-treated explants (Table 3). Such metabolic conversions have not yet been described for isoprenoid cytokinins. However, the mechanism for such an in vivo transformation remains to be elucidated.

To analyze in more detail the different rooting abilities of explants multiplied on media supplemented with different cytokinins, the ethylene concentrations in cultivation vessels were also measured. In our experiment, the highest ethylene levels (272–392 nl L^{-1} depending on the clone) were detected in the vessels containing media supplemented with mT. They were several times higher compared with that produced by the *S. torminalis* explants cultivated on other media used (Table 4). This finding, that explants grown on media supplemented with mT have slightly elevated ethylene production, correlates well with the rooting ability of these explants (Table 2) as well as with earlier experiments performed on *Populus tremula* L. (Gonzalez and others 1991).

Although our work was focused on the role and practical use of another important plant hormone group, aromatic cytokinins, on the multiplication of explants, and on the rooting of microcuttings of the wild service tree, to get a complete picture of optimal endogenous plant hormone concentrations and their dependence on different exogenous cytokinins used in the cultivation media in relation to in vitro rooting efficiency of the wild service tree, we also analyzed their endogenous auxin (IAA) levels. As expected, the lowest level of IAA in the explant *S. torminalis* plantlets grown on media containing BAP was confirmed (Table 5). Because auxin is well known as the major factor in *de novo* root formation (Casson and Lindsey 2003), this finding is again in good agreement with the limited rooting capacity of the explants grown on BAP.

On the other hand, experiments on the CRE1/AHK4 cytokinin receptor mutant already showed that cytokinin is also required for correct vascular morphogenesis in the root (Casson and Lindsey 2003) and root formation (Higuchi and others 2004). However, the precise role of cytokinin in other aspects of root development is still to be determined. Moreover, we have shown that different cytokinin derivatives can exhibit different root-inducing capacities. It is possible that cytokinin, along with auxin (Sabatini and others 1999), provides positional cues required for meristem organization.

It is becoming clear that the optimal concentration of cytokinins and their metabolites and of the other plant hormones, including auxin and ethylene, is crucial for successful organ development including root emergence and formation from multiplicated explants. Subsequently, the results about optimal endogenous plant hormone concentrations and their dependence on different exogenous cytokinins used in the cultivation media may help to improve in vitro rooting efficiency of the wild service tree and possibly other plant species.

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