BIOLOGIA PLANTARUM 57 (1): 174-178, 2013 DOI:10.1007/s10535-012-0252-6

BRIEF COMMUNICATION

# The role of cytokinins during micropropagation of wych elm

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## Abstract

We have compared the influence of two aromatic cytokinin derivatives, $N^6$-benzyladenine (BA) and *meta*-topolin (mT), on the *in vitro* multiplication and senescence of wych elm (*Ulmus glabra* Huds.). After 3 months of cultivation, the micropropagation rate was higher (approx. six times more shoots developed) on Murashige and Skoog (MS) medium supplemented with mT than on MS supplemented with BA. Quantification of 50 endogenous cytokinins, using a recently developed UPLC-ESI(+)-MS/MS method, showed significant differences in the cytokinin metabolites (especially different glucosides) in explants cultivated on media supplemented with BA or mT. Differences in efficiency of photosystem II was also detected.

*Additional key words*: benzylaminopurine, chlorophyll fluorescence, meta-topolin, organogenesis, rooting, senescence, *Ulmus glabra*.

Elms (*Ulmus* sp.) are trees that are highly valued for their great strength, tightly twisted grain, durability, and tolerance of both cold stress and salinity (Heybroek *et al*. 1982). Wych elm (*Ulmus glabra* Huds.) is a native European species that is common in mountainous regions of the Czech Republic. However, during the 1970's, the disease epidemic destroyed most elm populations in Europe (Brasier 1990, Fenning *et al*. 1993). Hence, a program for conserving genetic resources of elms has been initiated, within the *EUFORGEN* framework, which includes establishment of collections of trees (clonal archives), presuming trees that survived the epidemic to have a high degree of resistance against repeated infection. Elite elm trees can be vegetatively propagated by grafting and cuttings but micropropagation is the most promising option (Gartland *et al.* 2004). However, although various micropropagation systems have been reported for numerous species and hybrids (Biondi *et al*. 1984, Fink *et al*. 1986, Chalupa 1994, Fenning *et al*. 1993, Corchete *et al.* 1993, Cheng and Shi 1995, Malá *et al*. 2005), some obstacles still remain during organogenesis, notably early senescence and low multiplication rates, both of which decrease the micropropagation efficiency.

Optimizing micropropagation is not straightforward since it is a complex process involving a sequence of developmental stages that are influenced by numerous endogenous and exogenous stimuli (Mohamed and Alsadon 2011). These stimuli include phytohormones, especially auxins and cytokinins (Pumisutapon *et al.*

*Received* 29 July 2011, *accepted* 5 April 2012.
*Abbreviations*: BA - $N^6$-benzyladenine; cZ - *cis*-zeatin; DHZ - dihydrozeatin; $F_v/F_m$ - variable to maximum chlorophyll fluorecence ratio; *9G - 9-β-D-glucopyranosyl derivative; IAC - immunoaffinity chromatography; IBA - indole-3-butyric acid; iP - $N^6$-isopentenyladenine; *MP - 5′-monophosphate derivative; LC-MS - liquid chromatography combined with mass spectrometry; MS - Murashige and Skoog medium; mT - *meta*-topolin [6-(3-hydroxybenzylamino)purine]; *OG - *O*-β-D-glucopyranosyl derivative; oT - *ortho*-topolin; PS II - photosystem II; *R - 9-β-D-ribofuranosyl derivative; tZ - *trans*-zeatin; UPLC-ESI(+)-MS/MS - ultra performance liquid chromatography combined with positive electrospray mass spectrometry.
*Acknowledgements:* This work was supported by grants from the Ministry of Agriculture of the Czech Republic (NAZV QI 92A247), Czech Ministry of Education (MSM 6198959216), and ED0007/01/01 Centre of the Region Haná for Biotechnological and Agricultural Research.
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2011). The focus here is on cytokinins which have been shown to play important regulative roles during organogenesis in numerous studies (*e.g.* D´Angeli *et al.* 2001, Caboni *et al.* 2002). The addition of exogenous cytokinins is essential, *inter alia*, for the induction of root formation (De Klerk *et al*. 2001) but (unlike auxins) high levels of cytokinins inhibit adventitious rooting (Bollmark *et al*. 1988). Thus, deeper understanding of the roles of endogenous cytokinins and effects of exogenous cytokinins could substantially facilitate the development of more effective micropropagation techniques. $N^6$-benzylaminopurine (BA) is an important aromatic cytokinin derivative that is routinely utilized to induce organogenesis in micropropagation but in some cases it may negatively influence growth, rooting, and acclimatization (Werbrouck *et al.* 1995). Hence, another aromatic cytokinin derivative *meta*-topolin (mT) appears to be more advantageous during the micropropagation and acclimatization of numerous plant species because differences in its metabolism ameliorate some of these adverse effects (Werbrouck *et al.* 1996, Bairu *et al.* 2009, Valero-Aracama *et al.* 2010, Wojtania 2010).

In the presented study, we compared the effects of these two cytokinin derivatives on the *in vitro* multiplication and senescence of wych elm explants. In addition, we determined concentrations of 50 cytokinin metabolites in explants cultivated for 12 weeks on media supplemented with BA or mT to compare their efficiency of photosystem II (PSII) and production of the senescence-related hormone ethylene (ET).

The explants were collected from donor trees, 40 to 80-year-old, in 2009 and were conserved in the Explant Bank of the Forestry and Game Management Research Institute, Czech Republic. For experiments, multiapex cultures derived from 15 clones of wych elm growing in the Explant Bank were used. The explants were cultivated at 24 °C for 18 months on multiplication medium consisting of agar-solidified Murashige and Skoog (1962; MS) medium supplemented with 0.5 mg $dm^{-3}$ BA, 0.1 mg $dm^{-3}$ indole-3-butyric acid (IBA), 100 mg $dm^{-3}$ glutamine, and 30 g $dm^{-3}$ sucrose, pH 5.8, under white fluorescent lamps (36W/33 *Philips* tubes, Eindhoven, the Netherlands) providing irradiance of 30 μmol $m^{-2}$ $s^{-1}$ in 12-h photoperiods. A pair of explant cultures derived from the multiapex cultures of each of the 15 clones was grown on MS multiplication medium with 0.5 mg $dm^{-3}$ (2.2 μM) BA, whereas another pair was grown on the same medium with 0.5 mg $dm^{-3}$ (2.1 μM) mT. All cultures were cultivated for 12 weeks (with transfer every 4 weeks to fresh MS multiplication medium) under the same cultivation conditions as in the Explant Bank. The new shoots that developed from each multiapex culture were then counted and their lengths were measured. In addition, four shoot cultures were randomly selected from the sets of 30 growing in the presence of each supplementary cytokinin derivative to analyze their endogenous cytokinin pools and levels of metabolites of the added cytokinins.

Triplicate portions (app. 200 mg) of the samples were extracted in 0.5 $cm^3$ of ice-cold 70 % (v/v) ethanol for 3 h, and a mixture of deuterium-labeled standards was added to each sample to check recovery during purification and to validate determinations (Novák *et al.* 2008). After extraction, the combined supernatants were purified using a *DEAE-Sephadex* (1.0 × 5.0 cm) column coupled to an octadecylsilica (0.5 × 1.5 cm) column followed by immunoaffinity chromatography (IAC) with a generic cytokinin monoclonal antibody (Faiss *et al.* 1997). This resulted in three fractions containing: *1*) the free cytokinin bases, ribosides, and *N*-glucosides, *2*) a ribotide fraction, and *3*) an *O*-glucoside fraction. Fractions 2 and 3 were further purified by IAC following treatment with alkaline phosphatase (*Roche Diagnostics*, Mannheim, Germany) and β-glucosidase (*G-0395*, *Sigma*, St. Louis, USA), respectively. The eluates from the IAC columns were evaporated to dryness and dissolved in 20 $mm^3$ of the initial mobile phase used for quantitative analysis. The concentrations of 50 endogenous cytokinins and metabolites of the added cytokinins were then determined by ultra-performance liquid chromatography combined with tandem mass spectrometry (UPLC-MS/MS) (Novák *et al*. 2008), using an *Acquity* UPLC ultra-performance liquid chromatograph (*Waters MS Technologies*, Manchester, UK) equipped with a *BEH C18* (1.7 μm; 2.1 × 150 mm) column linked to a *Quattro micro API* (*Waters MS Technologies*) triple quadrupole mass spectrometer equipped with an electrospray interface. The compounds were quantified by multiple reaction monitoring of $[M+H]^+$ ions and an appropriate product ion (Novák *et al*. 2008). The identities of all measured cytokinin metabolites were verified by comparing their mass spectra and chromatographic retention times with those of authentic standards. The measurements were done in triplicate.

Three clones, exhibiting signs of senescence (leaf yellowing and shedding) after long-term growing on the BA medium, were transferred to two multiplication media containing either 0.2 mg $dm^{-3}$ mT or 0.2 mg $dm^{-3}$ BA. The concentration of BA was chosen on the base of previous experience (Malá *et al*. 2005). The same concentration of mT was chosen for comparability of results. After 6 weeks of cultivation, their ethylene production was measured using the gas chromatography with the flame ionization detector (GC-FID) method described by Fišerová *et al.* (2001) and Malá *et al.* (2009). The measurements were done in triplicate. Finally, the leaf chlorophyll *a* fluorescence of the sets of 30 explants grown on both cultivation media was determined using an *Imaging-PAM* chlorophyll fluorometer (*Heinz Walz*, Effeltrich, Germany).

Between-treatment differences in mean numbers of shoots produced, the length of the shoots, levels of measured endogenous cytokinins, ethylene production, and photosystem activity were explored by two-way analysis of variance (*ANOVA*) and their significance was evaluated by the Tukey-Kramer test using *QC Expert* and *NCSS* software.

Number of growing shoots increased differently after

12 weeks in relation to the kind of cytokinin in nutrient medium. Explants cultivated on multiplication medium supplemented with mT produced six times more shoots than counterparts cultivated with BA (31.9 ± 28.1 *versus* 5.88 ± 4.03). High standard deviations are probably partly caused by the use of data obtained from all the shoots (also those with lower vitality) during subculturing to guarantee comprehensive results. However, despite great clonal variability in numbers of new shoots, the mT medium induced higher numbers of adventitious shoots in every case (Fig. 1). These findings are consistent with results obtained in experiments with other plant species, *e.g. Aloe ferox* (Bairu *et al.* 2009), *Pelargonium* (Wojtania 2010), *Pinus sylvestris* (De Diego *et al.* 2010), and *Prunus microcarpa* (Nas *et al*. 2010). Several authors have also reported that medium supplemented with mT induces stronger growth of various species (Werbrouck *et al.* 1996, Bairu *et al.* 2007, Valero-Aracama *et al*. 2010, Wojtania 2010). However, there was no significant difference between the average length of shoots produced by explants grown on mT (3.4 ± 1.4 cm) and those on BA (3.6 ± 1.2 cm).

Fig. 1. Wych elm regeneratnts on MS medium supplemented by 0.1 mg $dm^{-3}$ IBA, 100 mg $dm^{-3}$ glutamin, 30 g $dm^{-3}$ sucrose, and either 0.5 mg $dm^{-3}$ BA (*on the left*) or 0.5 mg $dm^{-3}$ mT (*on the right*).

The regenerants with signs of senescence were replanted into multiplication media with 0.2 mg $dm^{-3}$ mT or 0.2 mg $dm^{-3}$ BA. After 6 weeks of cultivation, the signs of senescence persisted only in cultures with BA. Accordingly, substantially higher ethylene content was detected in the vessels containing these cultures (99 ± 13 $mm^3$ $m^{-3}$, versus 41 ± 17 $mm^3$ $m^{-3}$ in vessels containing the cultures grown with mT). Similar results have also been obtained in experiments with cultured *Rosa hybrida* tissue (Doležal *et al*. 2003) and various senescence bioassays (Holub *et al.* 1998, Cag *et al.* 2003).

The maximum efficiency of PS II was determined as the variable to maximum fluorescence ratio ($F_v/F_m$) measured in the dark-adapted leaves (Papageorgiou and Govindjee 2004). The results showed that the explants growing on the mT medium had higher $F_v/F_m$ than those growing in the BA medium (Table 1) in accordance with their lack of visible signs of senescence (yellowing).

Table 1. Content of endogenous cytokinins [pmol $g^{-1}$(f.m.)] and $F_v/F_m$ in wych elm explants cultivated on medium supplemented with BA or mT. Endogenous cytokinins were determined by UPLC-ESI(+)-MS/MS. 12-week-old *U. glabra* shoots were extracted, the extracts were purified by SPE followed by immunoaffinity chromatography and content of cytokinins was then measured by UPLC-ESI(+)-MS/MS. Means ± SD represent from 3 independent technical replicates of 4 different clones. Only compounds above the detection limit (LOD) are included. The $F_v/F_m$ values were compared by two-way *ANOVA* and the Tukey-Kramer test. The difference is significant at $P \leq 0.01$, $n = 30$.

| | BA | mT |
|---|---|---|
| BA | 17798 ± 11595 | 2.61 ± 1.49 |
| BA9G | 4.71 ± 0.57 | <LOD |
| BAR | 92.5 ± 21.5 | 0.34 ± 0.29 |
| BARMP | 460 ± 148 | 0.75 ± 0.61 |
| DHZR | 0.09 ± 0.07 | 0.13 ± 0.08 |
| DHZROG | 0.07 ± 0.03 | 0.11 ± 0.06 |
| iP | 1.98 ± 0.5 | 1.07 ± 0.36 |
| iPR | 2.56 ± 0.59 | 1.48 ± 0.57 |
| iPRMP | 9.86 ± 3.19 | 4.31 ± 2.30 |
| mT | 7.10 ± 1.80 | 1141 ± 651 |
| mT9G | <LOD | 5.42 ± 3.37 |
| mTR | 1.66 ± 0.31 | 173 ± 116 |
| mTRMP | 3.16 ± 1.08 | 224 ± 84 |
| mTOG | 9.86 ± 3.09 | 1885 ± 434 |
| mTROG | 11.4 ± 2.74 | 1197 ± 349 |
| oT | 0.55 ± 0.25 | <LOD |
| oTR | 8.16 ± 1.55 | <LOD |
| tZ | 1.24 ± 0.60 | 1.30 ± 0.43 |
| tZ9G | 0.06 ± 0.01 | 0.44 ± 0.13 |
| tZR | 1.88 ± 1.63 | 4.32 ± 1.52 |
| tZRMP | 3.41 ± 3.02 | 6.27 ± 2.47 |
| tZOG | 2.00 ± 1.19 | 2.07 ± 0.56 |
| tZROG | 0.43 ± 0.32 | 0.67 ± 0.22 |
| cZ | 0.24 ± 0.11 | 0.28 ± 0.16 |
| cZR | 0.13 ± 0.05 | 0.50 ± 0.25 |
| cZRMP | <LOD | 1.59 ± 0.87 |
| cZOG | 0.13 ± 0.07 | 0.13 ± 0.06 |
| $F_v/F_m$ | 0.67 ± 0.14 | 0.80 ± 0.03 |

Fifty endogenous cytokinin metabolites were determined in the samples of wych elm plantlets grown *in vitro* on media containing the two cytokinins. The levels of 33 cytokinin species were found to be present at levels exceeding the detection limit of the UPLC-MS/MS quantification method used (Novák *et al*. 2008) and thus could be quantified and compared in relation to the cytokinins added to the cultivation media. We found much higher in-tissue concentrations of BA in the explants grown in the BA-containing medium than of mT in explants grown in the presence of mT (Table 1), in

accordance with results of previous experiments with *Sorbus torminalis* (Malá *et al.* 2009). In contrast, plants grown on both cytokinins generally contained very similar levels of their major metabolic forms (ribosides, nucleotides and 9-glucosides). However, much higher concentrations of *O*-glucosides were detected in the explants grown on mT as previously reported in *Spathiphyllum* (Werbrouck *et al*. 1996), whereas 9-glucosides were the major metabolites in explants grown with BA. These findings may be also related to the amelioration of acclimatization problems when mT is used (Werbrouck *et al.* 1996, Bairu *et al.* 2007). Hydroxylated aromatic cytokinins were also substantially more abundant in the tissue micropropagated on BA (Table 1).

In addition, we analyzed content of endogenously formed isoprenoid cytokinins in relation to the exogenously applied cytokinin in the media. In contrast to previously published results of experiments with *S. torminalis* (Malá *et al.* 2009), we did not find any significant differences in content of most isoprenoid cytokinin free bases – *trans*-zeatin (tZ), *cis*-zeatin (cZ), and dihydrozeatin (DHZ). However, content of their other metabolic forms – ribosides, nucleotides, and 9-glucosides – were slightly higher in plants grown on media with mT, whreas content of iP-type cytokinins were slightly higher in plants grown on BA (Table 1).

Clearly, optimal concentration of cytokinins and their metabolites, as well as other plant hormones, are crucial for successful and efficient plant *in vitro* micropropagation. Hence, determinations of optimal endogenous plant hormone concentrations and their dependence on exogenous cytokinins used in cultivation media may improve both the *in vitro* micropropagation efficiency and the quality of *ex vitro* acclimatized plants of wych elm. Although our current results have been obtained using wych elm explants only, they are in very good agreement with those of other authors obtained currently on other species (Bairu *et al*. 2007, Woitania *et al*. 2010). Therefore, possible exploitation of our results for micropropagation of other woody plant species is evident.

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