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In vitro propagation of *Tilia platyphyllos* by axillary shoot proliferation and somatic embryogenesis

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ABSTRACT: *In vitro* propagation of *Tilia platyphyllos* Scop. has been achieved by axillary shoot proliferation and somatic embryogenesis. The influence of tree age, explant source, genotype, and phytohormones on micropropagation of juvenile and mature trees of *Tilia platyphyllos* has been investigated. Nodal segments and shoot tips were used as initial explants for axillary shoot proliferation. Low concentration of cytokinin (BA, BPA, TDZ) plus auxin (IBA) stimulated fast shoot multiplication. Microshoots excised from proliferating cultures were rooted on low salt medium and produced trees were planted in the field. Embryogenic tissues were initiated from zygotic embryos cultured on MS medium supplemented with 2,4-D. After transfer of embryogenic tissues with developing embryoids on media lacking 2,4-D and supplemented with low concetration of IBA, the development of somatic embryos was enhanced. Secondary somatic embryogenesis led to the formation of new adventive somatic embryos. Trees produced from somatic embryos were planted in the field and exhibited normal growth and morphology.

Keywords: *in vitro* propagation; somatic embryogenesis; micropropagation; *Tilia platyphyllos*; field growth of micropropagated trees; organogenesis; somatic embryo; cytokinin; auxin; axillary shoot proliferation; embryogenic tissue; thidiazuron; phytohormone

Trees of *Tilia platyphyllos* (large-leaved linden) belong to important forest trees with a fast growth, great resistance to unfavourable external conditions and with important positive effects on soil fertility and forest stand stability. Linden trees are shade – tolerant and grow frequently in mixed forest stands with conifers and broadleaves. The root system of *Tilia* trees is deep and wide spreading and trees contribute to increased stability of forest stands. The decaying linden leaves are rich in nitrogen and minerals and have positive effects on soil fertility and formation of humus mold. Linden trees are also often used for ornamental planting. Flowers of *Tilia* trees are rich source of nectar and important source for honey production. For all these reasons *Tilia* trees are used in increasing number for reforestation and are often planted in Central European forests. Trees of *Tilia platyphyllos* grow frequently in hilly country and foothills at elevation of 300 to 700 meters. They grow to a height of 30 to 40 m. *Tilia platyphyllos* flowers 10 to 14 days before *Tilia cordata*. Both species hybridize and produce the hybrid *Tilia* × *europea (T. vulgaris*), a tall tree up to 45 m high.

Trees of *Tilia platyphyllos* are propagated mainly by seeds, however, the seed germination is often low, seeds are deeply dortmant and need a long stratification. Since *Tilia* trees are cross – pollinated, progress made in tree improvement is slow. Foresters are interested in linden vegetative propagation because of its potential to multiply economically important genotypes in large numbers. Forest yield can be enhanced significantly by large-scale multiplication of selected genotypes with improved growth rates, valuable quality of wood, high stress tolerance and disease resistance. Vegetative propagation is an important tool of preserving unique characterictics of the selected trees.

There are many advantages to use clonal *in vitro* propagation of elite *Tilia* trees. *In vitro* technologies offer the potential for production of great number of elite trees in a short time. The use of elite clones in forestry will improve possibilities to capture large genetic gains and to manage genetic diversity of forest stands. Clonal *in vitro* propagation technologies of elite genotypes could bring fast improvement of linden tree quality.

In our experiments with *in vitro* propagation of *Tilia* species, we have tested various methods for fast *in vitro* propagation of linden trees (CHALUPA 1981, 1983, 1984, 1987a,b,c, 1988, 1990a,b, 1991, 1999). Our experiments showed that micropropagation may be a practical solution for a rapid propagation of selected genotypes with desirable properties. In this study the results obtained in our recent experiments with *in vitro* propagation of *Tilia platyphyllos* are presented. The study was carried out to investigate the influence of tree age, explant source, genotype, medium composition and plant growth regulators on *in vitro* propagation of *Tilia platyphyllos* trees.

MATERIALS AND METHODS

Plant material

Nodal segments, shoot tips and zygotic embryos were used as initial explants for the establishment of tissue cultures of *Tilia platyphyllos*. Nodal segments and shoot tips were used as initial material for micropropagation of linden trees using method of axillary shoot proliferation. Zygotic embryos were used as initial explants for propagation of trees via somatic embryogenesis.

Nodal segments and shoot tips were used as initial explants for the establishment of tree cultures. For explant collection, 12 mature trees were selected (55 years old) and 25 seedlings. To study the effect of age on culture establishment and shoot multiplication, explants were taken from different parts of mature trees (from branches growing 2 m above ground, or from branches growing in top parts of trees), and from juvenile parts of trees (epicormic shoots growing in basal part of the trunk). For comparison, explants from 25 seedlings (2 years old) were used. Explants were collected in February and March. Nodal segments were surface sterilized in calcium hypochlorite solution (7.5% w/v) for 20 minutes and in mercury chloride solution (0.1–0.2%) for 20–40 minutes. After sterilization, nodal segments were thoroughly washed three times in sterile distilled water and placed on agar nutrient medium.

Immature and mature zygotic embryos collected from six open pollinated trees were used as explants for experiments with somatic embryogenesis. The fruits (spherical nuts) were collected weekly from the beginning of July to November. Fruits were surface sterilized in calcium hypochlorite solution (7.5% w/v) for 20 minutes and then washed twice in sterile distilled water. Following surface sterilization, zygotic embryos were excised from seeds and placed on agar nutrient media.

Culture media and conditions

Nodal segments were grown on modified MS medium (MURASHIGE, SKOOG 1962). The basal medium was supplemented with glutamine (100 mg/l) and casein hydrolysate (100 mg/l) and 30 g/1 sucrose. The media were solidified with 7g/1 Difco Bacto agar and adjusted to pH 5.7 before sterilization by autoclaving at 121ºC for 20 min. Growth regulators and glutamine were filter-sterilized. Growth regulators added to nutrient medium included 6-benzylaminopurine (BA), or 6-benzylamino-9-(2-tetrahydropyranyl)-9H-purine (BPA) or thidiazuron (TDZ) in combination with indole-3-butyric acid (IBA). BA and BPA were tested in concentrations 0.1–4.0 mg/l, TDZ in concentration 0.001–2.0 mg/l. Cultures were grown in cultivation rooms at 24º ± 1ºC with 16 h photoperiod under cool white fluorescent light with a photon flux density of 35 µmol/m^2/s. The explants were cultured in 100 ml flasks containing 30 ml of nutrient medium. In most cases explants were cultured for five week culture period and then transplanted to fresh medium. Shoot multiplication was stimulated by combinations of various concentrations of cytokinin (BA, BPA, TDZ) and auxin (IBA). The rooting medium for excised microshoots consisted of modified MS medium (half concentration of macro- and microelements, no aminoacids, sucrose 5 g/1). The medium was supplemented with auxin (IBA 0.5 mg/1, or IBA 0.5 mg/1 plus NAA 0.1 mg/l). Rooted plantlets were transplanted into containers and grown in potting mixture (peat and perlite 1:1, v/v) at high relative humidity under long photoperiod.

For induction of embryogenic cultures from excised embryos, modified MS medium was used. MS medium was supplemented with glutamine (200 mg/1) and casein hydrolysate (200 mg/1). The media contained varying concentration of auxin and cytokinin. Auxin 2,4-D was tested at concentrations 0.3, 0.5, 1.0, 1.5, 2.0 mg/l alone or in combination with BA (0.5, 1.0 mg/l). The embryogenic cultures with developing embryoids were transferred to MS medium lacking 2,4-D and supplemented with IBA (0.1, 0.2 mg/l).

RESULTS

In vitro shoot multiplication and rooting

Growth and development of shoots from axillary buds of nodal segments was stimulated on modified MS medium supplemented with a low concentration of cytokinin (BA, or BPA, or TDZ) and a low concentration of auxin (IBA 0.1 mg/1). Nodal segments cultured on nutrient medium produced new shoots within 5–6 weeks. The rate of shoot-forming buds was dependent on plant age, genotype

Table 1. Effect of explant source and cytokinin on shoot proliferation of *Tilia platyphyllos*

Explant source	BA		BPA		TDZ	
	Number of shoots	Shoot length (cm)	Number of shoots	Shoot length (cm)	Number of shoots	Shoot length (cm)
Seedlings	3.3 ± 1.5	2.2 ± 0.7	4.1 ± 1.8	2.3 ± 0.6	5.2 ± 2.3	1.4 ± 0.4
Mature trees (epicormic shoots)	2.5 ± 1.2	1.8 ± 0.6	2.8 ± 1.4	1.9 ± 0.5	3.4 ± 2.1	1.2 ± 0.4
Mature trees (low-growing branches)	2.3 ± 1.2	1.3 ± 0.4	2.5 ± 1.3	1.6 ± 0.5	2.8 ± 1.9	1.1 ± 0.3

Data based on *Tilia platyphyllos* nodal segments cultured on modified MS medium supplemented with auxin (IBA 0.1 mg/l) and cytokinin (BA 0.6 mg/l, BPA 0.6 mg/l, TDZ 0.005 mg/l)

Fig. 1. Shoot formation from axillary buds of *Tilia platyphyllos* nodal segment cultured on MS medium supplemented with cytokinin (BA) and auxin (IBA)

and bud position along shoot segment. The high multiplication rates were found in cultures derived from seedlings and from epicormic shoots growing in the basal parts of mature trees. Also explants from low-growing branches of mature trees exhibited good multiplication rates, while rates of cultures derived from branches growing in the top part of mature trees were significantly lower.

Shoot cultures derived from seedlings and from epicormic shoots of mature trees maintained a high multiplication coefficient during the experimental period (2 years), without a significant decrease in regeneration capacity. The results indicate that explants from epicormic shoots and from low-growing branches of mature trees can be used for shoot culture initiation and for *in vitro* propagation of mature trees. Some shoot cultures derived from epicormic shoots of mature trees were subcultured for 2 years without a decrease in multiplication capacity. Explants collected from seedlings (2 years old) were more responsive than those taken from branches of mature trees.

The development, elongation and number of shoots produced from axillary buds was significantly influenced by type and concentration of cytokinin and auxin in nutrient medium. All tested cytokinins (BA, BPA, TDZ) stimulated formation and growth of new shoots from axillary buds of nodal segments. Number of produced shoots and shoot length were significantly influenced by cytokinin concentration. Low concentration of cytokinin (BA or BPA 0.2–0.5 mg/1) stimulated shoot elongation more than higher concentrations. MS nutrient medium supplemented with a low concentration of BA or BPA (0.2–0.4 mg/l) plus IBA (0.1 mg/1) promoted formation of long shoots. Axillary buds started to grow in few days and produced new long shoots within 5–6 weeks. Multiplication ratio achieved on modified MS medium supplemented with low concentration of cytokinin (BA) and auxin (IBA) was sufficient for continuous shoot propagation.

Thidiazuron affected shoot proliferation significantly. Low concentration of TDZ (0.005–0.01 mg/1) together with IBA(0.1mg/1) stimulated formation and development of axillary buds. Shoots elongated slowly on media containing TDZ and were significantly shorter than those produced on media containing BA or BPA. Even leaves were smaller on media containing TDZ. Low concentration of TDZ (0.005–0.01mg/1) stimulated formation of axillary shoots, higher concentration inhibited shoot elongation. The number of shoots produced on MS medium supplemented with a low concentration of TDZ was higher than on medium supplemented with BA or BPA, however, the shoots were short. Higher concentrations of TDZ inhibited shoot elongation. To stimulate axillary bud formation and shoot elongation, nodal segments were grown on MS medium containing BA (0.3 mg/1) together with TDZ (0.005 mg/1) and IBA (0.1 mg/1). Nodal segments cultured on MS medium containing very low concentration of TDZ together with a low concentration of BA and IBA produced numerous longer shoots.

Microshoots excised from proliferating cultures were rooted on low salt media, containing low concentration of sucrose. Modified MS medium (half strength, aminoacids deleted) supplemented with a low concentration of auxin (IBA 0.3–0.5 mg/l or IBA 0.5 mg/l plus NAA 0.1 mg/l) stimulated root initiation. Within 2–3 weeks great part of microshoots (64–83%) formed roots. Rooted plants were grown under high humidity for 5–6 weeks and then were hardened off outside and later planted in the field.

Induction of somatic embryos and plant regeneration

The embryogenic tissue was initiated on modified MS medium supplemented with 2,4-D. Zygotic embryos of

Table 2. Effect of explant source and auxin on rooting of microshoots of *Tilia platyphyllos*

Explant source	IBA	IBA + NAA
	rooted microshoots (%)	
Seedlings	84	88
Mature trees (epicormic shoots)	72	74
Mature trees (low-growing branches)	58	62

Data based on 50 microshoots rooted on modified MS medium supplemented with IBA (0.5 mg/l) or IBA (0.5 mg/l) + NAA (0.1 mg/l)

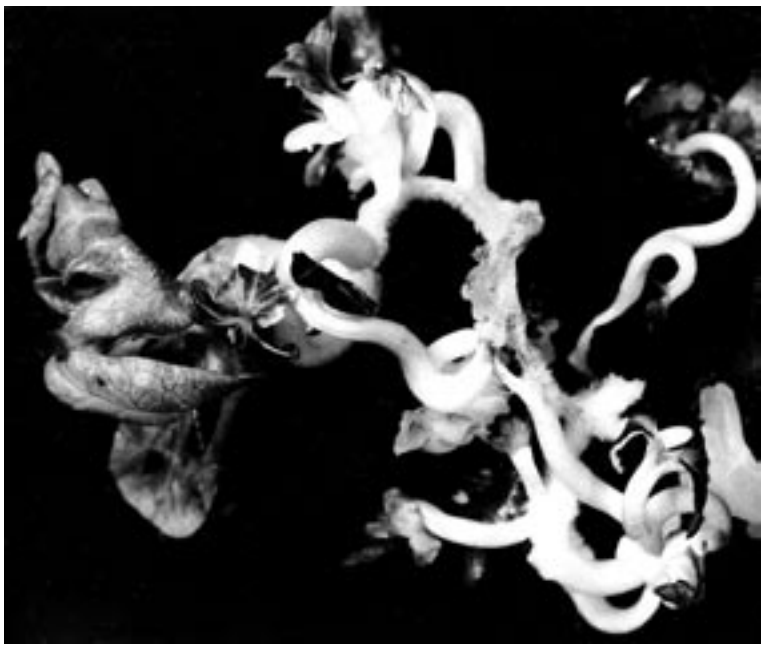

Fig. 2. Secondary somatic embryogenesis and development of new adventive somatic embryos in *Tilia platyphyllos* embryogenic culture

Tilia platyphyllos excised from seeds collected in July and August produced embryogenic cultures frequently. Immature zygotic embryos produced embryogenic tissues during the continuous exposure on medium containing 2,4-D. Embryogenic tissues developed within 6–10 weeks and white globular structures differentiated on MS medium containing 2,4-D. The most effective range of 2,4-D concentration was 0.3–1.5 mg/l. After 8–12 weeks in cultures, white globular structures developed, later giving rise to somatic embryos. Secondary somatic embryogenesis was frequent and led to the formation of new adventive somatic embryos.

The process of somatic embryo development from the initial globular stage to the bipolar embryo occurred on MS medium containing 2,4-D. However, 2,4-D generally arrested the development of somatic embryos, and only embryoids that were not in direct contact with the medium, exhibited good development. The development of somatic embryos was enhanced after transfer of embryogenic tissues with developing embryoids on MS medium lacking 2,4-D and supplemented with a low concentration of IBA (0.1–0.2 mg/l). Somatic embryos enlarged and bipolar embryos bearing both cotyledons and root primordia developed. Secondary somatic embryogenesis, the process when new somatic embryos are produced on the surface of previously differentiated somatic embryos, was frequent. Produced somatic embryos exhibited good capacity for secondary somatic embryogenesis. Secondary somatic embryos were produced on hypocotyl and root regions of the primary somatic embryos. Secondary somatic embryogenesis has been used for multiplication of somatic embryos. *Tilia* embryogenetic cultures have been maintained by subculturing of secondary somatic embryos.

The germination and conversion of *Tilia platyphyllos* somatic embryos to plants was stimulated on media supporting coordinated development of shoots and roots. The conversion of somatic embryos to plants was achieved on modified MS media containing a low concentration of IBA (0.1–0.2 mg/l) that supported the growth and development of shoots and roots. After the transfer of somatic embryos on germination medium, cotyledons and roots elongated and shoots started to grow. Within 5–7 weeks somatic embryos produced plantlets with roots and shoots. Plantlets with develop-

Fig. 3. Plantlet of *Tilia platyphyllos* regenerated from somatic embryo

ing shoots and roots were subcultured individually on fresh medium of the same composition. Well developed plantlets were transplanted into a potting mixture (peat and perlite 1:1, v/v) and were grown under high air humidity. Most transplanted plants survived transfer into soil and continued to grow. After acclimatization, the regenerated plants were moved to the greenhouse, where they continued in growth.

Acclimated plants of *Tilia platyphyllos*, regenerated from somatic embryos, were planted in the field where continued in growth. The height growth of *Tilia* trees produced from somatic embryos was comparable with the growth of trees produced from axillary bud cultures. Trees produced from somatic embryos exhibited normal growth and morphology.

DISCUSSION

Forest trees have a long generation period and fast reproduction of selected valuable genotypes is difficult. New biotechnological methods have a great significance for preservation and reproduction of valuable genotypes. The application of biotechnological methods will enable a shortening of time-consuming reproductive processes. Organogenesis and somatic embryogenesis in *Tilia* species was described in past years (CHALUPA 1981, 1983, 1984, 1987a,b,c, 1988, 1990a,b, 1991, 1999; KÄRKÖNEN 2000). Since that time, significant progress has been achieved. *In vitro* propagation of selected resistant and fast growing trees will bring a significant genetic gains in a short time. Trees with desirable genetic traits can be rapidly produced through *in vitro* multiplication of selected genotypes. The method of axillary shoot proliferation appears to be an appropriate method for *in vitro* fast reproduction of *Tilia* species. The main advantages of this method are that multiplication rates are high and cultures are genetically stable.

Somatic embryogenesis is considered to be a perspective method of producing large number of plants in a short time. Recent advances have increased the production of somatic embryos and the frequency of their conversion to plants. The induction of embryogenic cultures and the plant regeneration is highly affected by phytohormones. The application of exogenous phytohormones can often be crucial in the processes of somatic embryogenesis. Formation of *Tilia* embryogenic cultures was achieved in the presence of exogenous auxin. Deeper insight into factors influencing somatic embryogenesis can lead to their regulation and improvement of the somatic embryo initiation and conversion to plants. Since the initial reports of somatic embryogenesis in *Tilia* species (CHALUPA 1987b) significant progress has been achieved in *Tilia* propagation by somatic embryogenesis. Auxin was proved to be essential for the induction of somatic embryos in *Tilia* cultures. Somatic embryos exhibited a capacity for the secondary somatic embryogenesis. The secondary embryogenesis is used for the multiplication of somatic embryos and embryogenic cultures have been maintained by subculturing of secondary embryos.

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In vitro rozmnožování lípy velkolisté (*Tilia platyphyllos* Scop.) organogenezí a somatickou embryogenezí

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ABSTRAKT: *In vitro* reprodukce lípy velkolisté bylo dosaženo množením axilárních prýtů, pěstovaných na MS médiu, obsahujícím nízké koncentrace cytokininu (BA) a auxinu (IBA). U explantátů odebraných z juvenilních částí dospělých stromů a ze semenáčků bylo dosaženo rychlého množení prýtů při pěstování nodálních segmentů na agarových živných médiích. Prýty vypěstované *in vitro* byly zakořeněny na modifikovaném MS médiu obsahujícím nízké koncentrace auxinu (IBA, NAA). Po otužení byly stromky vypěstované *in vitro* vysazeny na venkovní pokusné plochy. Výškový a tloušťkový růst stromků vypěstovaných *in vitro* byl srovnatelný s růstem stromků, vypěstovaných ze semen. Embryogenní kultury byly iniciovány ze zygotických embryí, pěstovaných na MS médiu, obsahujícím nízkou koncentraci auxinu 2,4-D. Vytváření somatických embryí bylo dosaženo po přesazení embryogenních kultur na MS médium, obsahující nízkou koncentraci auxinu IBA. Sekundární somatická embryogeneze byla častá. Ze somatických embryí byly vypěstovány stromky, které byly vysazeny na venkovní pokusné plochy.

Klíčová slova: *in vitro* rozmnožování; somatická embryogeneze; mikropropagace; *Tilia platyphyllos;* venkovní růst *in vitro* rozmnožených stromů; organogeneze; somatické embryo; cytokinin; auxin; proliferace axilárních pupenů; embryogenní pletivo; thidiazuron; fytohormon

Reprodukce lesních stromů metodami *in vitro* má velký význam pro záchranu ohrožených populací lesních stromů a pro zachování odolných genotypů. Cílem *in vitro* rozmnožování stromů je zlepšení genetických vlastností pěstovaných porostů rozmnožením odolných a produktivních genotypů. Během relativně krátké doby lze pomocí *in vitro* metod rozmnožit vybrané genotypy na velký počet exemplářů. Rychlého klonového množení lesních stromů *in vitro* je možné dosáhnout pomocí orgánových kultur nebo embryogenních kultur.

Prováděné experimenty byly zaměřeny na vypracování vhodných metod pro rychlou *in vitro* reprodukci lípy velkolisté (*Tilia platyphyllos* Scop.). Byl zjišťován vliv fytohormonů ze skupiny cytokininů a auxinů na množení axilárních prýtů a na zakořeňování vypěstovaných prýtů. Zároveň se realizovaly pokusy s indukcí embryogenních kultur a s vytvářením somatických embryí a rostlin z embryogenních kultur. Pěstování a množení embryogenních buněk, z nichž každá je potenciálním zdrojem nového stromu, umožňuje velmi rychlé množení elitních genotypů. Experimentální práce s *in vitro* množením *Tilia platyphyllos* byly zaměřeny na zjištění vlivu stáří stromu, místa odběru explantátů, složení vhodného živného média a působení fytohormonů na růst a množení explantátových kultur a na produkci i růst vypěstovaných stromků lípy velkolisté.

Jako počáteční explantáty pro založení orgánových kultur byly použity jednak prýty odebrané z mladých rostlin (1–3leté semenáčky), jednak prýty z juvenilních částí dospělých stromů (kmenové výmladky rostoucí ve spodní části kmene a prýty z nízko rostoucích větví). Nodální segmenty s 1–2 axilárními pupeny byly po sterilizaci vysazeny na agarová živná média a byly pěstovány za kontrolovaných podmínek (24 °C, 16hodinová fotoperioda). Z fytohormonů přidaných do živných médií byl testován účinek cytokininu BA, BPA a TDZ, z auxinů vliv IBA a NAA. Z testovaných živných médií byl růst a množení axilárních pupenů nejvíce stimulován na modifikovaném MS médiu (Murashige a Skoog médium), které obsahovalo nízkou koncentraci cytokininu (BA, nebo BPA, nebo TDZ) spolu s nízkou koncentrací auxinu (IBA).

Po umístění nodálních segmentů na agarové živné médium se vytvořily z axilárních pupenů nové prýty během 3–5 týdnů. Vytváření a prodlužování prýtů z axilárních pupenů bylo značně ovlivňováno typem a koncentrací cytokininu v živném médiu. Se stoupající koncentrací cytokininu stoupal počet vytvořených prýtů, jejich délka však klesala. Nízké koncentrace BA a BPA (0,2–0,5 mg/1) při spolupůsobení auxinu (IBA 0,1–0,2 mg/l) stimulovaly vytváření a prodlužování prýtů z axilárních pupenů. Rovněž nízké koncentrace TDZ (0,005–0,01 mg/1) a IBA (0,1–0,2 mg/1) stimulovaly vytváření prýtů z pupenů, prýty však byly kratší než na médiu obsahujícím BA. Živná média, obsahující kombinaci cytokininů BA a TDZ a auxin IBA v nízké koncentraci, stimulovala růst a proliferaci prýtů. Z axilárních pupenů prýtů odebraných ze semenáčků a z juvenilních částí dospělých stromů (z kmenových výmladků) se vytvářely při jejich pěstování na MS médiu nové prýty, které byly dále množeny *in vitro*. Nejvyšší koeficient množení byl zjištěn u kultur vzniklých ze semenáčků a z prýtů odebraných z juvenilních částí dospělých stromů (kmenové výmladky v dolních částech kmene).

Prýty rozmnožené *in vitro* byly zakořeněny na modifikovaném MS médiu (poloviční koncentrace) obsahujícím nízké koncentrace auxinu (IBA nebo NAA 0,2–0,5 mg/1). Bylo dosaženo vysokého procenta zakořenění prýtů vy-

pěstovaných *in vitro* (tab. 2). Po otužení byly stromky vypěstované *in vitro* vysazeny na venkovní pokusné plochy, kde růst pokračoval. Výškový růst stromků vypěstovaných *in vitro* byl srovnatelný s růstem stromků vypěstovaných ze semen.

Embryogenní pletiva *Tilia platyphyllos* byla iniciována ze zygotických embryí pěstovaných na modifikovaném MS médiu obsahujícím nízkou koncentraci auxinu 2,4-D (0,5–2,0 mg/1). Nízké koncentrace 2,4-D v médiu stimulovaly vytváření embryogenních pletiv, avšak přítomnost 2,4-D v médiu zpomalovala vývin somatických embryí. Vytváření somatických embryí bylo stimulováno po přesazení embryogenních kultur s vyvíjejícími se embryi na modifikované MS médium obsahující nízkou koncentraci IBA (0,1–0,2 mg/1). Na těchto médiích se postupně vytvořila bipolární somatická embrya. Sekundární somatická embryogeneze byla častá a vedla k vytváření četných adventivních somatických embryí, zejména v oblasti hypokotylu a kořínku. Adventivní somatická embrya se postupně vyvinula ve zralá somatická embrya. Klíčení zralých somatických embryí bylo stimulováno na MS médiu, obsahujícím nízké koncentrace IBA (0,1 mg/1). Během 3–5 týdnů se vyvinuly ze somatických embryí rostliny, které byly přesazeny do nesterilního substrátu. Po otužení byly stromky vypěstované ze somatických embryí vysazeny na venkovní pokusné plochy, kde dále pokračovaly v růstu.

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