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# Micropropagation of Temperate Noble Hardwoods: An Overview

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## ABSTRACT

Temperate noble hardwoods are important economic resources of highly prized timber with excellent quality. Except for their economic value, they meet ecological functions for wildlife habitats or riparian buffers. This group of trees mostly does not form homogenous and continuous forest stands, they occur as an admixture associated with other main stand-forming species or may be found freely scattered in forests and semisteppes. The genepool of many noble hardwoods is subjected to extensive conservation efforts due to the rare occurrence, devastation of wild populations, and a demand for quality hardwood. Although the most extensive application of biotechnological tools in tree improvement has been made with some coniferous species and hardwoods of the genera *Populus* and *Eucalyptus*, tissue culture techniques may contribute to both genetic improvement and *ex situ* conservation strategies in noble hardwoods as well. In the past years the organogenic micropropagation of the juvenile planting stock dominated, recently *in vitro* regeneration of proven mature trees extends to a wider range of genotypes within the group. Somatic embryogenesis from leaves of mature trees opens a new route for micropropagation of superior genotypes with subsequent genetic transformation possibilities. Tree improvement and clonal propagation of noble hardwoods allows an increase in the availability and commercialization of selected genotypes carrying desired traits, mainly in rarely occurring species such as service trees or elms. Production of high quality trees and establishment in plantations may also ease the pressure of cutting elite genotypes from natural forests. For this reviewing purpose, the attention is focused on noble hardwood trees that belong to genera *Acer*, *Ulmus*, *Fraxinus*, *Prunus*, *Sorbus*, and *Juglans*.

**Keywords:** acclimatization, axillary shoot proliferation, cryopreservation, field performance, micropropagation, organogenesis, somatic embryogenesis

**Abbreviations: 2,4-D**, 2,4-dichlorophenoxyacetic acid; **2iPA**, 6-(γ-γ-dimethylallylamino)purine riboside; **4-CPPU**, *N*-(2-chloro-4-pyridyl)-*N′*-phenylurea; **ABA**, abscisic acid; **BAP**, 6-benzylaminopurine; **BPA**, 6-benzylamino-9-(2-tetrahydropyranyl)-9H-purine; **DED**, Dutch elm disease; **DKW**, Driver and Kuniyuki walnut medium; **$GA_3$**, gibberellic acid; **IAA**, indole-3-acetic acid; **IAAsp**, indole-3-acetylaspartic acid; **IBA**, indole-3-butyric acid; **KIN**, kinetin; **MS**, Murashige and Skoog medium; **NAA**, 1-naphthaleneacetic acid; **PAA**, phenylacetic acid; **PGR**, plant growth regulator; **TIBA**, 2,3,5-triiodobenzoic acid; **TDZ**, 1-phenyl-3-(1,2,3-thidiazol-5-yl)-urea or thidiazuron; **WPM**, woody plant medium

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## INTRODUCTION

Wood of many temperate noble hardwoods shows a special appearance in the texture, color, figure, and has excellent technical and machining properties. There is a high demand for this high quality wood. With regard to the occurrence in natural forests, the trees rarely form pure homogenous stands as the main stand-forming species [stands of narrow-leafed ash (*Fraxinus angustifolia*) in Pannonian lowland and flooded forests of northern Turkey are the exception]. As an admixture, noble hardwoods can often be found associated with other main forest stand constituents (beech, oak, hornbeam, alder, Norway spruce) or are freely scattered in forests, forest fringes, semisteppes, and extreme slopes.

Due to increasing human population and the increasing global demand for wood, consumption of forest products is exceeding the natural rate of regeneration in many areas of the world, resulting in forest loss and degradation. Therefore, the main goal of micropropagation of forest trees, including noble hardwoods as well, is aimed at the mass clonal production of selected elite genotypes for superior growth, form, and timber characteristics. Non-traditional uses of micropropagated woody plants include mainly the restoration of unsanitary landfills. Some noble hardwoods (*Acer platanoides*, *Fraxinus excelsior*, *Fraxinus pennsylvanica*, *Prunus avium*, *Prunus serotina*) are highly recommended trees for quick revegetating of waste landfills in many temperate countries (Kim and Lee 2005; Athy *et al*. 2006). Different basal media such as MS (Murashige and Skoog 1962), WPM (Lloyd and McCown 1980), DKW (Driver and Kuniyuki 1984), SH (Schenk and Hildebrandt 1972), LS (Linsmaier and Skoog 1965), QL (Quoirin and Lepoivre 1977), B5 (Gamborg *et al*. 1968), MCM (Bornman and Jansson 1981), Rugini medium (Rugini 1984), and their modifications are being used for induction of *in vitro* morphogenesis in noble hardwoods as well as in other broadleaves. In general, three morphogenic pathways leading to *in vitro* regeneration may be employed: Axillary shoot proliferation, adventitious shoot organogenesis, and somatic embryogenesis. Micropropagation without intermediate callus phase assures the genetic stability of regenerated plantlets that may be used for the establishment of clonal plantations.

A topic of the major role of biotechnology associated with plantation forests to achieve the global long-term sustainability, has been reviewed thoroughly by Fenning and Gershenzon (2002). Unfortunately, advances in the commercial micropropagation of noble hardwoods have not achieved the extent that may be seen in the genera *Populus* and *Eucalyptus* or in some coniferous species (*Pseudotsuga menziesii*, *Picea glauca*, *Pinus taeda*). Matters are not nearly so clear-cut. There are several reasons why the progress still lags behind those model fast-growing broadleaves or conifers. At first, the organogenic and embryogenic potentials of noble hardwoods are strongly affected by the maturity phase. As trees grow and attain maturity, the ability of vegetative tissues to differentiate plant organs declines. Experiments with micropropagation of kawakami maple (*Acer caudatifolium*) showed that explants sampled from the juvenile phase (2-year-old seedlings) were competent to morphogenesis and were responsive to culture conditions. Plantlets could be regenerated within several months (Ďurkovič 2003). In repeated experiments with explants sampled from the identical donor material in the transition phase (4- and 5-year-old plants) that were cultured under identical culture conditions, only recalcitrant responses were observed. Under the proceeded maturity, the explants showed no growth competence and no axillary shoot elongation and rooting could be achieved (**Fig. 1**). In case of mature genotypes and/or recalcitrant species that exhibit the strong influence of ontogenic aging, the effects of urea-based cytokinin-like compound TDZ are often superior to those of adenine-based cytokinins. Therefore, the application of TDZ instead of BAP seems to be more beneficial.

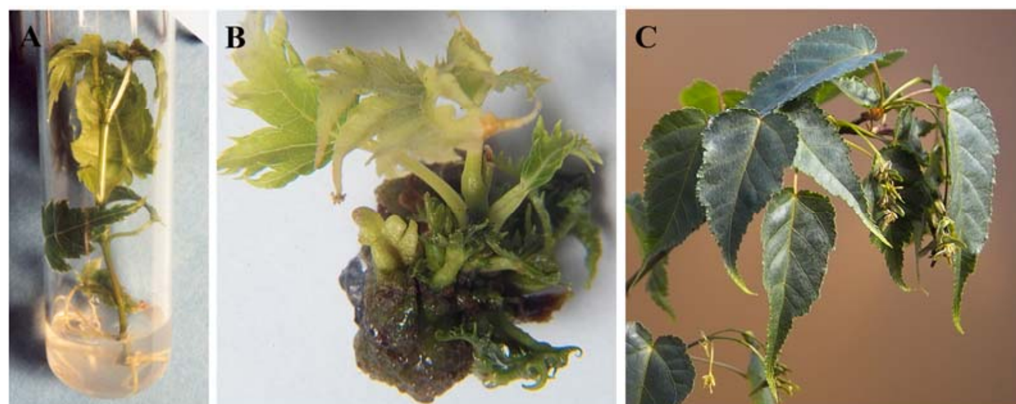


**Fig. 1 The effect of age on kawakami maple *in vitro* morphogenic competence.** (**A**) *In vitro* adventitious rooting from the basal part of elongated axillary shoot derived from the juvenile 2-year-old mother plant; (**B**) Halted shoot development from the axillary bud of the identical 5-year-old mother tree; (**C**) Rapid transition to the maturity phase documented by early flowering in the 5-year-old *in vitro* regenerant.

**Table 1** Summary of micropropagation via organogenesis and axillary shoot proliferation in genera of noble hardwoods.

| Species | Explant source | Age of donor trees | Multiplication medium | Rooting medium | Acclimatization (% of survival) | Field tests |
|---|---|---|---|---|---|---|
| *Acer caudatifolium* | Axillary buds | 2-yr-old seedlings | WPM 0.7 mg L$^{-1}$ BAP + 0.05 mg L$^{-1}$ NAA | ½ WPM 1.0 mg L$^{-1}$ IBA | 80% | NR |
| *A.* × *freemanii* 'Marmo' | Shoot tips; nodal segments | 4-yr-old rejuvenated trees | LS 1.0 µM BAP + 0.01 µM TDZ | LS PGR-free | 85% | NR |
| *A. grandidentatum* | Nodal segments | 2-yr-old seedlings | DKW 27.37 µM zeatin | DKW PGR-free | 100% | NR |
| *A. palmatum* 'Osakii' | Shoot tips; nodal segments | 4-yr-old trees | WPM 0.01 mg L$^{-1}$ TDZ | WPM 1.0 mg L$^{-1}$ IBA | 95% | NR |
| *A. platanoides* | Axillary buds | 2-yr-old seedlings; 10-yr-old trees | WPM 0.5 mg L$^{-1}$ KIN; WPM 0.1 µM TDZ | ½ WPM 1.0 mg L$^{-1}$ IBA | Successful | DP |
| *A. platanoides* 'Crimson King' | Shoot tips | Mature trees budded onto rootstock | LS 1.0 µM BAP + 0.005 µM TDZ | ½ LS 5.0 µM IBA + ½ LS PGR-free | Successful | NR |
| *A. pseudoplatanus* | Zygotic embryos | — | MS 1.0 µM BAP + 0.04 µM TDZ | MS 123.0 µM IBA + MS PGR-free | Successful | NR |
| | Stump sprouts; microcuttings | Germinated seedlings; 60- to 100-yr-old trees | MS 0.1 mg L$^{-1}$ KIN; MS PGR-free | MS 0.1 mg L$^{-1}$ KIN; MS PGR-free | Successful | NR |
| *A. rubrum* | Axillary buds | 20- to 40-yr-old trees | MS 0.1 mg L$^{-1}$ BAP + 0.01 mg L$^{-1}$ TDZ | ⅓ WPM 0.1 mg L$^{-1}$ IBA; MS PGR-free | 77% | After 5 GS |
| *A. rubrum* 'Red Sunset' | Nodal segments | NR | MS 0.01 mg L$^{-1}$ TDZ | WPM 1.0 mg L$^{-1}$ IBA; MS 1.0 mg L$^{-1}$ IBA | 95% | NR |
| *A. saccharinum* | Shoot tips; nodal segments | Juvenile seedlings; rejuvenated mature trees | DKW 0.01 µM TDZ | PGR-free *ex vitro* rooting; 1.0 mM IBA under *ex vitro* rooting | 90% | DP |
| *A. saccharinum* 'Pyramidale' | Shoot tips | Mature tree | LS 1.0 µM BAP + 0.005 µM TDZ | ½ LS 5.0 µM IBA + ½ LS PGR-free | Successful | NR |
| *A. saccharum* | Axillary buds | 2-yr-old seedlings | MS 2.0 mg L$^{-1}$ 2iPA + 0.01 mg L$^{-1}$ TDZ | MS 0.3 mg L$^{-1}$ PAA + 0.5 mg L$^{-1}$ IBA | NR | NR |
| *Fraxinus americana* | Zygotic embryos | — | MS 10.0 µM TDZ | 1.0 mM IBA under *ex vitro* rooting | Successful | After 6 GS |
| *F. angustifolia* | Zygotic embryos | — | ½ MS 4.4 µM BAP + 0.4 µM 2,4-D | WPM PGR-free | 80% | NR |
| *F. excelsior* | Shoot tips; nodal segments | Juvenile seedlings | MS 2.0 mg L$^{-1}$ BAP + 0.1 mg L$^{-1}$ IBA | ½ WPM 0.8 mg L$^{-1}$ NAA; ½ WPM 0.8 mg L$^{-1}$ IBA | 94% | DP |
| | Axillary buds | 16-yr-old trees | WPM 4.0 mg L$^{-1}$ BAP + 0.15 mg L$^{-1}$ IBA + 0.01 mg L$^{-1}$ TDZ | ½ MS 2.0 mg L$^{-1}$ IBA + 0.25 mg L$^{-1}$ BAP followed to *ex vitro* rooting | Successful | NR |
| *F. ornus* | Shoot tips; nodal segments | Germinated seedlings; 30-yr-old trees | Liquid RM 22.2 µM BAP + solidified RM PGR-free | HM 2.5 µM NAA + 0.4 µM BAP; HM 5.2 µM NAA + 0.4 µM BAP | 85% | NR |
| *F. pennsylvanica* | Axillary shoot tips | *In vitro* germinated seedlings | MS 40.0 µM BAP; MS 10.0 µM TDZ | NR | Successful | NR |
| *Juglans nigra* | Cotyledons of immature seeds | — | WPM 5.0 µM TDZ + 0.1 µM 2,4-D followed to a liquid DKW 10.0 µM BAP | 2.5 mM IBA + 1.25 mM NAA under *ex vitro* rooting | Successful | NR |
| *J. nigra* × *J. regia* | Embryonic axes | — | DKW 4.4 µM BAP + 0.005 µM IBA | ¼ DKW 32.0 µM IBA + ¼ DKW PGR-free with vermiculite | PBC | NR |
| *J. regia* cultivars | Embryonic axes | — | DKW 4.4 µM BAP + 0.005 µM IBA | ¼ DKW 24.6 µM IBA + ¼ DKW PGR-free with vermiculite | Successful | NR |
| | Shoot tips | Juvenile seedlings | DKW 4.4 µM BAP + 0.05 µM IBA | MS 15.0 µM IBA + ¼ DKW PGR-free with vermiculite | Successful | NR |
| *Prunus avium* | Shoot tips | Rejuvenated mature trees | MS 4.4 µM BAP + 0.49 µM IBA + 0.29 µM GA$_3$; MS 2.2 µM BAP | MS 14.7 µM IBA + 1.0 mM phloroglucinol | Successful | After 6 GS |
| | *In vitro* leaves | Rejuvenated mature trees | WPM 4.4 µM TDZ + 0.54 µM NAA | NR | NR | NR |
| | Axillary buds | Mature tree | WPM 0.5 mg L$^{-1}$ BAP + 0.05 mg L$^{-1}$ TDZ | ½ WPM 0.3 mg L$^{-1}$ IBA | Successful | DP |
| *P. serotina* | *In vitro* leaves | 1-yr-old seedlings; rejuvenated mature trees | WPM 2.27 µM TDZ + 0.54 µM NAA; WPM 4.4 µM TDZ + 0.54 µM NAA | MS 2.5 µM IBA | 86% | NR |
| | Axillary buds | Mature trees | MS 0.75 mg L$^{-1}$ BAP + 0.01 mg L$^{-1}$ IBA + 0.2 mg L$^{-1}$ GA$_3$ | MS 1.0 mg L$^{-1}$ IBA + 0.001 M rutin | 86% | After 6 GS |
| *Sorbus aucuparia* | Shoot tips; nodal segments | 45- to 50-yr-old trees | MS 1.0 mg L$^{-1}$ BAP + 0.1 mg L$^{-1}$ IBA; MS 0.01 mg L$^{-1}$ TDZ + 0.1 mg L$^{-1}$ IBA | ½ WPM 0.5 IBA; ½ WPM 0.4 mg L$^{-1}$ IBA + 0.4 mg L$^{-1}$ NAA | Successful | After 5 GS |
| *S. domestica* | Shoot tips; nodal segments | Juvenile seedlings; 30-yr-old trees | SH 22.2 µM BAP; SH 8.8 µM BAP | ½ MS 5.2 µM NAA + ½ MS PGR-free; HM 5.2 µM NAA + HM PGR-free | 70% | NR |

**Table 1** (Cont.)

| Species | Explant source | Age of donor trees | Multiplication medium | Rooting medium | Acclimatization (% of survival) | Field tests |
|---|---|---|---|---|---|---|
| *S. torminalis* | Shoot tips; nodal segments | Juvenile seedlings | MS 1.0 mg $L^{-1}$ BAP + 0.1 mg $L^{-1}$ IBA | ½ WPM 0.3 mg $L^{-1}$ IBA + 0.3 mg $L^{-1}$ NAA | Successful | NR |
| | Axillary buds | Mature trees | MS 1.0 mg $L^{-1}$ BAP + 1.0 mg $L^{-1}$ IBA + 0.1 mg $L^{-1}$ $GA_3$ | 2.0 g $L^{-1}$ IBA + MS PGR-free | 75% | NR |
| *Ulmus americana* | Leaves | 2-yr-old seedlings | DKW 22.5 µM TDZ; DKW 15.0 µM TDZ | *Ex vitro* rooting in a commercial Sunshine No.1 potting mix | Successful | NR |
| *U. glabra* | Axillary buds | 70- to 80-yr-old trees | MS 0.5 mg $L^{-1}$ BAP; WPM 0.4 mg $L^{-1}$ BAP + 0.05 mg $L^{-1}$ TDZ | ⅓ MS 1.0 mg $L^{-1}$ NAA; ½ WPM 0.4 mg $L^{-1}$ IBA | 88% | After 7 GS |
| *U.* × *hollandica* cultivars | Cambium strips; axillary buds | 5- to 15-yr-old trees | MS PGR-free followed to MS 14.0 µM $GA_3$; WPM 1.0 mg $L^{-1}$ BAP | MS 5.0 µM IBA; WPM 0.3 mg $L^{-1}$ IBA | 70% | DP |
| *U. parvifolia* 'A/Ross Central Park' | Nodal segments | Rejuvenated 20-yr-old trees | WPM 0.5 mg $L^{-1}$ BAP; WPM 2.0 mg $L^{-1}$ 4-CPPU + 0.5 mg $L^{-1}$ TDZ | WPM 1.0 mg $L^{-1}$ NAA | 100% | NR |
| *U. procera* | Shoot tips; *in vitro* leaves | Rejuvenated 2-yr-old trees | DKW 1.0 mg $L^{-1}$ BAP + 0.01 mg $L^{-1}$ IBA; DKW 4.0 mg $L^{-1}$ BAP | DKW 3.0 mg $L^{-1}$ NAA + DKW PGR-free | Successful | NR |
| *U. pumila* | Leaves; nodal segments | Juvenile seedlings; 20-yr-old trees | MS 15.0 µM BAP followed to DKW 2.0 µM BAP; MS 0.5 mg $L^{-1}$ BAP | *Ex vitro* rooting in a potting medium Sunshine Mix No.2; MS 0.1 mg $L^{-1}$ NAA | 70% | NR |
| *U. pumila* var. *pinnato-ramosa* | Axillary buds | 15-yr-old trees | WPM 1.3 mg $L^{-1}$ BAP | WPM 0.3 mg $L^{-1}$ IBA | Successful | DP |

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; 2iPA, 6-(γ-γ-dimethylallylamino)purine riboside; 4-CPPU, *N*-(2-chloro-4-pyridyl)-*N'*-phenylurea; BAP, 6-benzyl-aminopurine; DKW, Driver and Kuniyuki walnut medium; DP, demonstration planting; $GA_3$, gibberellic acid; GS, growing seasons; HM, Heller medium; IBA, indole-3-butyric acid; KIN, kinetin; LS, Linsmaier and Skoog medium; MS, Murashige and Skoog medium; NAA, 1-naphthaleneacetic acid; NR, not reported; PAA, phenylacetic acid; PBC, physiologically and biochemically characterized; PGR-free, plant growth regulator-free medium; RM, Rugini medium; SH, Schenk and Hildebrandt medium; TDZ, thidiazuron; WPM, woody plant medium.

Another option is the sampling of juvenile tissues that facilitate propagation of mature trees. The juvenile character may be preserved at the base of the tree. The degree of juvenility along trunk and branches is frequently inversely proportional to the distance between the root-shoot junction and meristems. In several of the hardwoods, the mature zone is characterized by the forked trunk and acute branch angles usually at the periphery of the tree in tissue that is ontogenetically older but young chronologically. The characteristic features of the juvenile zone are the single trunk, retention of leaves close to trunk untill winter and obtuse branch angles. When the juvenile material for the tissue culture establishment is not available, some manipulations for reversal of aging or partial rejuvenation are helpful. Explants sampled either from rejuvenated shoots following grafting or from rooted cuttings, are widely used for example in cherries. The second obstacle is pertinent to a narrow range of genotypes being propagated. Significant differences among various genotypes in culture initiation, multiplication, adventitious root formation, or acclimatization performances suggest that current protocols are not as efficient as they should be expected for a commercial practice that requires homogenous responses in a wider spectrum of proven genotypes. The effect of genotype still plays a crucial role in micropropagation of most noble hardwoods. With regard to somatic embryogenesis, there is a lack of reliable embryogenic systems capable of continuous production of somatic embryos without a loss of embryogenic competence, and with a high frequency of somatic embryo conversion into somatic seedlings that should be utilized on a commercial scale. Finally, information on the clonal performance of micropropagated noble hardwoods under field conditions is still insufficient. The most comprehensive results come from the field tests with micropropagated cherries, but the age of those plantations is relatively young in comparison with poplar plantings. Silvicultural applications of clonal forestry indicate that long-term field trials are required to fully evaluate the stability and performance of nonsomaclonal lines.

Cryopreservation in liquid nitrogen has become the important tool for the long-term storage of germplasm of noble hardwoods using minimum space and maintenance. It also allows propagation of selected genotypes throughout the year. The most frequently applied techniques are based mainly on the vitrification (pretreatment of explants with a highly concentrated cryoprotectant solution containing glycerol, ethylene glycol, sucrose and dimethylsulfoxide), encapsulation-dehydration (encapsulation of explants in calcium alginate beads, followed by the pregrowth treatment on medium containing a high level of sucrose and the subsequent desiccation of alginate beads), or simple desiccation (dehydration of the plant material before rapid freezing by direct immersion in liquid nitrogen). The explant material includes shoot tips, excised embryonic axes, somatic embryos, or cells in suspension cultures. Cryopreserved germplasm must of course be readily regenerated into whole plantlets for use, and the genetic stability needs to be checked.

This review highlights up to date progress made with micropropagation of temperate noble hardwoods, and gives an overview species by species and genus by genus. **Table 1** summarizes the achieved results on organogenesis and axillary shoot proliferation, **Table 2** on somatic embryogenesis. With regard to other temperate broadleaves (for example oak, beech, black locust, chestnut, linden, birch, sweetgum), coniferous species as well as other biotechnological topics such as gene transfer technologies, molecular marker assisted selection and mapping, wood formation and modification or deployment of improved transgenic trees, the readers are referred to the six volume set on somatic embryogenesis in woody plants edited by Jain *et al*. (1995) and other reviews (Merkle and Dean 2000; Wilhelm 2000; Giri *et al*. 2004; Leitch and Bossinger 2004; Merkle and Nairn 2005; Nehra *et al*. 2005; Merkle *et al*. 2007; Pijut *et al*. 2007).

## MAPLES

Several hard maples provide heavy, strong, and stiff wood of high commercial value that is used for the manufacturing of veneers for furniture, flooring, interior finishes, cabinets, crates, woodenware, novelties or even musical instruments. Wood is usually straight-grained, but occasionally has wavy or 'bird's-eye' grain. Sometimes it is distilled to make acetic acid and wood alcohol. In addition, Japanese shrubby species are extremely popular in horticultural plantings with

**Table 2** Summary of micropropagation via somatic embryogenesis in genera of noble hardwoods.

| Species | Donor tissue | Initiation medium | Cultivation | Proliferation medium | Maturation medium | Treatment | Conversion medium | Conversion rate (%) |
|---|---|---|---|---|---|---|---|---|
| *Acer palmatum* | Immature zygotic embryos | MS 10.0 μM BAP + 0.1 μM 2,4-D | Continuous light | MS 0.05 μM BAP + 0.5 μM NAA | MS 0.05 μM BAP + 0.5 μM NAA | — | ½ MS PGR-free, 1.5% sucrose | 44% conversion |
| *A. pseudoplatanus* | Immature zygotic embryos | MCM 0.025 mg $L^{-1}$ KIN + 0.25 mg $L^{-1}$ zeatin | Continuous light | MCM 0.1 mg $L^{-1}$ KIN + 1.0 mg $L^{-1}$ zeatin | MCM PGR-free | Chilling in darkness | MCM PGR-free | CNS |
| *Fraxinus americana* | Mature zygotic embryos | MS 1.0 μM TDZ + 10.0 μM 2,4-D | Continuous light | MS 0.05 μM BAP + 0.5 μM NAA | MS 0.05 μM BAP + 0.5 μM NAA | — | MS 0.05 μM BAP + 0.5 μM NAA | NR, CS |
| *F. angustifolia* | Immature zygotic embryos | ½ MS 4.4 μM BAP + 8.8 μM 2,4-D | Darkness 1 month | ½ MS 4.4 μM BAP + 0.44 μM 2,4-D | ½ MS PGR-free, 3% mannitol | Embryo encapsulation in alginate beads | MS PGR-free | 65% conversion |
| *F. excelsior* | Immature zygotic embryos | ½ MS 4.4 μM BAP + 8.8 μM 2,4-D | Darkness 2 months | MS 4.4 μM BAP + 0.44 μM 2,4-D | MS 0.44 μM BAP | 3-wk chilling in darkness | WPM 0.44 μM BAP | 40% conversion |
| *Juglans cinerea* | Immature zygotic embryos | DKW 1.0 mg $L^{-1}$ BAP + 2.0 mg $L^{-1}$ KIN + 0.01 mg $L^{-1}$ IBA; MS 0.25 mg $L^{-1}$ BAP + 2.0 mg $L^{-1}$ 2,4-D | Continuous darkness | DKW PGR-free; MS PGR-free | DKW PGR-free; MS PGR-free | 8-wk chilling in darkness | DKW PGR-free | CNS |
| *J. nigra* | Immature zygotic embryos | WPM 5.0 μM TDZ + 0.1 μM 2,4-D | Light 4 weeks | DKW PGR-free | LP PGR-free | — | NR | NR |
| *J. nigra × J. regia* | Immature zygotic embryos | DKW 1.0 mg $L^{-1}$ BAP + 2.0 mg $L^{-1}$ KIN + 0.01 mg $L^{-1}$ IBA | Continuous darkness | DKW PGR-free | DKW PGR-free | Desiccation 3-5 days; 8-wk chilling in darkness | Liquid DKW PGR-free with cotton compress | 46% germination |
| *J. regia cultivars* | Immature zygotic embryos; endosperm | DKW 1.0 mg $L^{-1}$ BAP + 2.0 mg $L^{-1}$ KIN + 0.01 mg $L^{-1}$ IBA | Continuous darkness | DKW PGR-free | DKW PGR-free | Desiccation 3-4 days; 2.9-25.7 μM $GA_3$; 4-12-wk chilling in darkness | DKW PGR-free; liquid DKW PGR-free with filter compress; ½ DKW PGR-free, 0.5% sucrose + 0.5% activated charcoal | 63% conversion |
| *Prunus avium* | Immature zygotic embryos | MS 9.3 μM KIN + 18.1 μM 2,4-D | Continuous darkness | MS 0.44 μM BAP + 0.46 μM KIN + 0.54 μM NAA | MS PGR-free, 88 and 263 mM maltose | 8-wk chilling in darkness | WPM PGR-free, 44 mM sucrose | 35% conversion |
| *Sorbus aucuparia* | Immature zygotic embryos | MS 1.0 μM BAP + 1.0 μM KIN + 0.5 μM NAA | Continuous darkness | MS 1.0 μM BAP + 1.0 μM KIN + 0.5 μM NAA | MS PGR-free | 2-wk chilling in darkness | MS PGR-free, 3% maltose | 20% germination |
| *S. domestica* | Anthers from 35-yr-old trees | MS 5.0 μM BAP + 25.0 μM IBA | Darkness 6 weeks | MS 2.5 μM BAP + 2.5 μM IAA | MS PGR-free | — | MS PGR-free | CNS |
| *Ulmus glabra* | Immature zygotic embryos | MS 0.9 μM 2,4-D | Darkness 6 weeks | MS 0.44 μM BAP | MS PGR-free | — | Liquid MS 0.22 μM BAP with filter paper support | 10% conversion |
| *U. minor* | *In vitro* leaves derived from a mature tree; immature zygotic embryos | MS 1.1 μM KIN + 2.3 μM 2,4-D; MS 0.2 mg $L^{-1}$ BAP + 0.2 mg $L^{-1}$ 2,4-D | Darkness 6-8 weeks | MS PGR-free; MS 0.44 μM BAP | MS PGR-free | — | ½ MS PGR-free, 1% sucrose | 36% conversion |

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BAP, 6-benzylaminopurine; CNS, conversion not successful; CS, conversion successful; DKW, Driver and Kuniyuki walnut medium; IAA, indole-3-acetic acid; IBA, indole-3-butyric acid; KIN, kinetin; LP, Long and Preece medium; MCM, Bornman and Jansson medium for conifer morphogenesis; MS, Murashige and Skoog medium; NAA, 1-naphthaleneacetic acid; NR, not reported; PGR-free, plant growth regulator-free medium; TDZ, thidiazuron; WPM, woody plant medium.

hundreds of selected cultivars available. Maples in tissue culture are amenable to propagation mainly via axillary shoot proliferation in the presence of TDZ that is applied in low concentration ranges (0.005-0.05 μM).

### *Acer platanoides* L.

Micropropagation of Norway maple was achieved by the single shoot proliferation from axillary buds sampled from juvenile 2-year-old seedlings (Ďurkovič 1996). Shoot growth was promoted by low concentrations of KIN, whereas BAP applied in both low and high concentration ranges was found to be too strong compound that induced a frequent basal callus formation. *In vitro* adventitious rooting was induced by IBA at a 70% frequency. Plantlets acclimatized readily to a greenhouse ambient environment. After overwintering, the regenerates were planted in the field (**Fig. 2**). In experiments with circa 10-year-old trees, Lindén and Riikonen (2006) achieved very low shoot multiplication rates from apical and axillary buds when using low concentrations of TDZ. However, shoot elongation failed and no adventitious rooting was reported. Strategy of apical shoot proliferation from mature trees was used in micropropagation of popular Norway maple cultivar 'Crimson King'

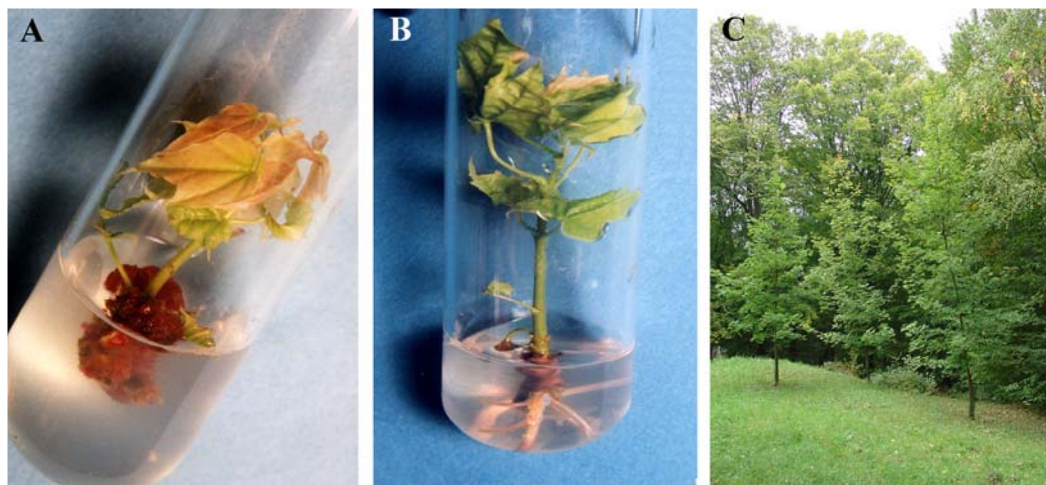


**Fig. 2 Micropropagation of Norway maple from juvenile seedlings.** (**A**) Axillary shoot elongation; (**B**) *In vitro* adventitious rooting; (**C**) Field growth of 13-year-old regenerates.

(Marks and Simpson 1994). Survival of axillary bud explants in the presence of BAP in combination with low concentrations of TDZ was limited to the first two subcultures only, whereas culture survival of apical explants was stabilized by the fifth subculture, and shoots subsequently elongated. After the formation of root primordia in the presence of IBA, the excised shoots rooted readily. Plantlets were successfully acclimatized under high humidity.

## *Acer pseudoplatanus* L.

Explants sampled from both juvenile and mature trees were used in several attempts to micropropagate sycamore maple. Wilhelm (1999) reported adventitious regeneration of plantlets from zygotic embryo explants. Adventitious shoots were formed on hypocotyl and plumule segments, whereas radicle segments showed only root elongation and callusing responses. Combination of BAP with a low concentration of TDZ promoted the best proliferation capacity. Twenty-four h applications of IBA followed by the transfer to PGR-free media resulted in moderate rooting frequency (65%). Interestingly, Hanus and Rohr (1987) achieved similar rooting frequencies when applying low concentrations of KIN. Successful regeneration of mature trees in age from 50 to more than 100 years, including specimens with wavy grain texture, was accomplished via *in vitro* rooting of stem microcuttings derived from apical and nodal explants (Hanus and Rohr 1987; Rohr and Hanus 1987). Adventitious roots formed on PGR-free media or media supplemented with a low concentration of KIN.

Naujoks (1995) initiated the formation of embryogenic structures from immature zygotic embryos that were collected from mature trees. However, the system suffered from a poor germination frequency, moreover, after several subcultures the embryogenic capacity was lost. Establishment of reliable embryogenic system for proven genotypes and adventitious shoot multiplication technique from mature explants still remain a challenge to date.

## *Acer rubrum* L.

Micropropagation of mature red maple trees in age from 20 to 40 years was achieved from axillary buds of both dormant and greenwood cuttings (Wann and Gates 1993). A combined application of a low concentration of TDZ with low concentrations of BAP induced the multiplication of shoots of both axillary and adventitious origin. Used alone, BAP only stimulated the formation of large amounts of basal callus. *In vitro* rooting of microshoots was remarkably easy on media containing either no PGRs or low concentrations of IBA. After acclimatization and overwintering, the authors established field demonstration plantings. Low concentrations of TDZ were also found efficient in axillary shoot proliferation from nodal segments in red maple cultivar 'Red Sunset' (Orlikowska and Gabryszewska 1995). However, the subsequent transfer of multiple shoot cultures to media supplemented with BAP instead of TDZ led to shoot necrosis. In the presence of IBA, 100% of shoots rooted easily *in vitro*. Rooted plantlets were successfully acclimatized in a greenhouse, and by the end of the first growing season they had reached up to 80 cm. McClelland *et al*. (1990) carried out interesting comparisons of anatomical and morphological properties in 'Red Sunset' microcuttings rooted under *in vitro* and *ex vitro* conditions. Roots formed *in vitro* had a greater diameter, enlarged cortical cells and underdeveloped vascular system, whereas *ex vitro* roots produced vascular cambium and advanced secondary growth. On the other hand, shoots from *in vitro* treatments continued to grow vigorously during adventitious root initiation and during acclimatization, so the plants were twice as tall as those from *ex vitro* treatments and shoot area was almost four times that of *ex vitro* rooted plants. During later stages of plant production, roots from both treatments had acquired similar *ex vitro*-type anatomical character, although *in vitro* rooted plants retained a prominent horizontal root system orientation.

In the field study, Sibley *et al*. (1995) compared annual growth rate, leaf morphology characteristics, fall colour patterns and gas exchange capacities for the two red maple cultivars 'Franksred' and 'October Glory' that were raised from both tissue culture and scions budded onto native rootstock. No significant differences between propagation methods for those cultivars were found in the above characteristics indicating that either method of propagation appears to be suitable for both cultivars.

## *Acer saccharinum* L.

Preece *et al*. (1991a) reported micropropagation of the juvenile silver maple. When using seedling single-node explants, TDZ in a lower range of concentrations was found again to be superior to BAP, KIN or zeatin to induce a massive axillary shoot proliferation (an average of 6.2. shoots per explant after 2 months, and 63.4 shoots per explant after 4 months). Single-node explants sampled from rejuvenated greenhouse-grown plants grown from rooted cuttings of mature trees, performed similarly to juvenile explants (an average of 4.5 shoots per explant after 2 months). Microshoots excised from proliferating cultures rooted well *ex vitro* without the addition of auxin. Micropropagated trees were planted in the field. The regenerates tall over 30 cm had circa 75% survival. Although in the following study with 90 genotypes (Preece *et al*. 1991b) the authors observed a significant effect of genotype within provenance on shoot multiplication rate and other *in vitro* growth characteristics, a micropropagation system based on the addition of 0.01 μM TDZ proved applicable to a wide variety of silver maple genotypes. Marks and Simpson (1994) established cultures of silver maple cultivar 'Pyramidale' from shoot tips of a mature tree. Used alone, BAP promoted

single shoot elongation accompanied by the production of large amounts of basal callus. The addition of TIBA in combination with BAP reduced callus development and increased the number of axillary shoots (an average of 3.9 shoots per explant). Similarly, combined use of BAP with a low concentration of TDZ enhanced axillary shoot growth up to 4.3 shoots per explant. In the presence of IBA, 100% of shoots rooted easily *in vitro*.

### *Acer × freemanii* Murr.

Kerns and Meyer (1987) published results on micropropagation of red-silver maple hybrid, cultivar 'Marmo'. Nodal segments were sampled from rejuvenated 4-year-old trees raised from both cuttings and scions budded onto silver maple rootstock. Low concentrations of TDZ in combination with BAP were found the most effective on shoot proliferation rates (an average of 5.3 shoots per explant). Microshoots rooted readily *in vitro* and plants were acclimatized under high humidity in a greenhouse.

### *Acer saccharum* Marsh.

The research of sugar maple propagation and improvement was thoroughly reviewed by Morselli (1989). For a longer period, *in vitro* techniques were only concerned with callus and shoot apex cultures. Hanus and Rohr (1985) described *in vitro* regeneration of sugar maple as well as box elder (*A. negundo*) from nodal segments explanted from germinated seedlings. Unexpectedly, the growth and development of rooted sugar maple plantlets halted after their transfer to soil. Brassard *et al*. (2003) reported the complete micropropagation of sugar maple from 2-year-old seedlings. The highest shoot multiplication rates were promoted by low concentrations of TDZ in combination with either 2iPA or PAA (an average of 4.3 shoots per explant or 3.3 shoots per explant, respectively). Adventitious roots formed on media supplemented with PAA in combination with IBA.

### *Acer grandidentatum* Nutt.

Bigtooth maple, another member in the section *Saccharina* Pax., belongs to the group of promising ornamental trees. Bowen-O'Connor *et al*. (2007) successfully micropropagated 2-year-old seedlings from nodal segments via axillary shoot proliferation. Expansion of axillary buds was promoted by low concentrations of TDZ, however, shoot proliferation increased with increasing concentration of zeatin without addition of TDZ. The authors recorded quite poor rooting frequencies in the presence of IAA, only up to 15%. Conversely, a spontaneous rooting without auxin was observed at 71% of explants. Rooted plantlets were potted in soilless media and acclimatized readily in an incubator.

### *Acer palmatum* Thunb.

Axillary shoot proliferation was also employed in micropropagation of frequently planted ornamental Japanese maple (Fernández-Lorenzo *et al*. 2000). Shoot tips and nodal segments sampled from the 4-year-old cultivar 'Osakii' were subjected to low concentration treatments of TDZ. Interestingly, the authors found that shoot multiplication rate was affected by the agar brand. Widely used Difco Bacto Agar was superior to local ABtE agar brand. Nevertheless, the best multiplication rates were quite low. High adventitious rooting performance (95%) was achieved on media containing IBA. Rooted plantlets acclimatized easily in a greenhouse.

Vlašínová and Havel (1999) reported somatic embryo formation in Japanese maple directly on immature zygotic embryos or on developed calluses. For induction of somatic embryogenesis the presence of 2,4-D was dispensable, whereas BAP proved essential. Secondary somatic embryos developed directly on cotyledons and hypocotyls of original somatic embryos as well as from a microcallus formed on the root tips of developing original somatic embryos. Selected cultures were able to produce somatic embryos continuosly without declining of this ability for more than 2 years. The authors succeeded in the maturation and germination of somatic embryos as well. Rooted plantlets were transferred to a greenhouse.

### Cryopreservation of maples

The potential of embryonic axes in the long-term cryopreservation of silver maple was outlined in the study of Beardmore and Whittle (2005). Tolerance of embryonic axes to desiccation and cryopreservation in liquid nitrogen was induced by the pretreatment with ABA and the compound tetcyclacis, which enhances endogenous ABA concentrations. Germination frequency of axes on PGR-free medium after 12 months of cryopreservation reached 54%, after 24 months it was 50%. Park *et al*. (2005) compared survival rates of cells in suspension culture of *A. pictum* (synonym of *A. mono*) derived from the callus when using different cryopreservation techniques. After one day of storage in liquid nitrogen, the highest survival rate of cryopreserved cells was observed with the vitrification (81%), whereas rates with techniques of the encapsulation-vitrification and simple freezing after sucrose pretreatment were lower (64% and 54%, respectively). Based on the identical random amplified polymorphic DNA patterns, no genetic changes were observed in cryopreserved cells.

## ELMS

During the 1920s–1940s, populations of elm trees in Eurasia and North America were strongly devastated by the fungus *Ophiostoma ulmi* causing DED. More recently, new aggressive strains of the fungus have emerged with sufficiently different cultural and molecular characters to warrant their designation as a new species, *Ophiostoma novo-ulmi*. Therefore, the existing germplasm is of a special interest in elm improvement (Smalley and Guries 1993; Merkle *et al*. 2007) and both *in situ* and *ex situ* conservation strategies. Wood of elm species has excellent bending qualities and is among the best in ease of gluing. Lumber is used for manufacture of veneers for furniture, containers, dairy and poultry supplies or for slack cooperage and kegs. Elms were among the first trees from which adventitious shoots were produced (Gautheret 1940). Elms in tissue culture are amenable to propagation by adventitious shoot multiplication as well as somatic embryogenesis from immature zygotic embryos or leaf explants.

### *Ulmus glabra* Huds.

In case of 80-year-old plus wych elm trees, Malá *et al*. (2005) reported adventitious shoot multiplication from apical parts that were excised from the established axillary shoots. Multiplication rate reached an average of 5.5 shoots per explant. Adventitious shoots arose from the meristemoids that were formed on the bases of apical segments. Basal parts gave rise to new axillary shoots from axillary buds at an average rate of 3.8 shoots per explant. Shoot multiplication ability was promoted by a moderate concentration of BAP. A significantly higher content of free bases, ribosides and ribotides of isopentenyl adenine, zeatin and dihydrozeatin that were found in the apical explants, might be considered as another important factor affecting the formation of new shoots. Up to 93% of shoots rooted readily in the presence of NAA. *Ex vitro* acclimatized and hardened regenerates were planted in the field (Malá *et al*. 2007). After 7 growing seasons, the plantation average was over 3.7 m in height with a growth rate over 0.5 m per year. No significant differences in growth rates, vitality, and morphology were found between micropropagated and seed-propagated trees derived from the same provenance. In experiments with over 70-year-old plus trees capable of natural regeneration and undamaged by DED, a continuous pro-

**Fig. 3 Micropropagation of wych elm from mature trees.** (**A**) Mother stand growing in the virgin forest, undamaged by DED; (**B**) Multiple shoot culture induced by combination of BAP and TDZ; (**C**) An autumn view of plantation with 8-year-old regenerates (photo courtesy P. Kapráľ, Forest Nursery Centre Šariš, Slovakia).

liferation of shoot cultures in the presence BAP or TDZ failed (Biroščíková *et al*. 2004). On the other hand, explants coming from the seed progeny in age of approximately 15 years were amenable to continuous micropropagation. The highest multiplication rate was reached in the presence of BAP in combination with a low concentration of TDZ (an average of 5.9 shoots per explant). Rooting frequencies were slightly lower than those reported by Malá *et al*. (2005), maximum 70% of rooted shoots in the presence of IBA. Successfully acclimatized and overwintered regenerates were planted in the experimental field plots and their growth is further monitored (**Fig. 3**).

Corredoira *et al*. (2002) described the induction of somatic embryogenesis from immature zygotic embryos. Addition of 2,4-D was necessary at early stages of zygotic development, whereas no PGRs were required at the mid-cotyledonary stage. The formation of the embryogenic callus was an essential step for the induction of proembryogenic cell masses as well as the maintenance of embryogenic competence during continuous subcultures on proliferation medium suplemeted with a low concentration of BAP. In the following experiments aimed at the improvement of somatic embryo proliferation and conversion, embryo production was higher with sucrose than with glucose or maltose (Corredoira *et al*. 2003). On the other hand, maltose improved the size and morphology of somatic embryos. The use of liquid medium with filter paper support and supplemented with a low concentration of BAP was essential for the survival of somatic embryos during germination and conversion into plantlets.

## *Ulmus minor* Mill.

A promising route for micropropagation of mature smooth elm via somatic embryogenesis has been opened by the use of leaf explants (Conde *et al*. 2004). The authors obtained somatic embryos from the embryogenic callus that formed on the surface of leaves after their exposure to a moderate concentration of 2,4-D in combination with KIN. The progress of globular somatic embryos to the cotyledonary stage occurred on PGR-free media. Longer and thinner embryos converted easily into somatic seedlings, whereas smaller and thicker ones proliferated and gave rise to new embryos. The acclimatization frequency of plants derived from somatic embryos reached up to 40% and was fully comparable to that of plants raised from axillary buds.

## *Ulmus procera* Salisb.

Fenning *et al*. (1993) successfully regenerated a 2-year-old clone of English elm from leaves excised from *in vitro* proliferating shoot cultures. In the presence of BAP, adventitious shoots grew through the layer of petiole callus and fully developed over a period of 5 weeks. Cutting the leaves across the midrib reduced the regeneration efficiency. When using axillary shoot tips as primary explants, the shoot multiplication rate reached 3.4 shoots per subculture, but had risen after one year in culture to 8.1 shoots per subculture. When using stem callus bases, over twenty elongated shoots could be produced over a 3-week subculture period per callus. Up to 83% of shoots rooted after exposure to a high concentration of NAA for 1 week followed by the transfer to PGR-free medium.

## *Ulmus americana* L.

Leaf explants were also used in the study of the shoot regenerative capacity from juvenile 2-year-old American elm seedlings (George and Tripepi 1994). TDZ was found clearly superior to BAP in shoot multiplication responses. After 15 subcultures of donor shoots, the authors observed the formation of 10 to 14 adventitious shoots per leaf explant. Those rates were promoted by a high concentration of TDZ. Unlike the average shoot multiplication rates, the highest multiplication ability was genotype-dependent. Regenerated shoots rooted *ex vitro* in a commercial potting mix in closed plastic containers with a success rate up to 94%. The similar study, based on the use of leaf sections sampled from greenhouse-grown seedlings of American elm that were exposed to a low concentration of TDZ, was published by Bolyard *et al*. (1991).

## *Ulmus parvifolia* Jacq.

Thakur and Karnosky (2007) reported a development of simple and efficient systems for micropropagation, germplasm conservation and distribution of historically important Central Park Splendor Chinese elm (*U. parvifolia* 'A/Ross Central Park') trees. This cultivar is larger than typical Chinese elms, unusually cold tolerant, and pest free. In the United States, the tree is being sold commercially as Central Park Splendor Chinese elm. Nodal segments were sampled from over 20-year-old rejuvenated trees. BAP was found to be the best PGR for axillary shoot proliferation, whereas 4-CPPU in combination with TDZ proved efficient for meristematic nodule formation (an average up to 20 meristematic nodules per explant). Further shoot elongation from meristematic nodules was significantly enhanced on media supplemented with zeatin. In the presence of NAA, *in vitro*-grown shoots gave higher rooting frequency (84%) than *ex vitro* microcuttings taken from greenhouse-grown plants (62%). Rooted plantlets were acclimatized readily to a greenhouse ambient environment and continued to grow well under greenhouse conditions.

## *Ulmus pumila* L.

Kapaun and Cheng (1997) observed a genotype-dependent potential of adventitious shoot regeneration from leaf tissues of greenhouse-grown Siberian elm seedlings. On average, 2.4 shoots per explant were produced in the presence of BAP. The authors recorded a frequency up to 90% of *ex vitro* rooting in potting soil. Corchete *et al*. (1993) successfully micropropagated a 20-year-old tree when using nodal segments as initial explants. Adventitious shoot multiplication (an average of 5.1 shoots per explant) was promoted by

a moderate concentration of BAP that proved an efficient PGR for the continuous culture proliferation over 2 years without declining of the multiplication ability. During *in vitro* rooting experiments, NAA was found superior to IBA when up to 70% of shoots rooted in the presence of NAA, whereas IBA induced extensive basal callusing responses only. Krajňáková and Longauer (1996) reported *in vitro* regeneration of the Siberian elm variety (*U. pumila* var. *pinnato-ramosa*) from axillary buds of circa 15-year-old trees. Significant effects of the genotype and year of sampling were observed in the stage of culture establishment and initiation of *in vitro* growth. Adventitious shoot multiplication performance was promoted by BAP that was applied at both moderate and high concentration ranges. Data from rooting experiments were not provided. A genetic fidelity of micropropagated plantlets was confirmed with isozyme markers.

### *Ulmus × hollandica* Mill.

Dutch elms belong to a group of interspecific hybrids that were commercially released as putatively 'resistant' to DED. Their parentage contains the complex of *U. glabra* and *U. minor* trees. One of those hybrids is 'Commelin'. Ben Jouira *et al*. (1998) reported rarely occurring plantlet regeneration from cambium strips sampled from 6-year-old 'Commelin' trees. Strips (constituted of part of the pholem, cambium, and a small chip of xylem) were placed horizontally onto media with a woody part above. On average, up to 15.2 adventitious buds per explant at a 100% frequency were induced on PGR-free media. In the presence of BAP, TDZ, and zeatin, the multiplication rates were either lower or even inhibited when the basal callus formation occurred. High concentrations of $GA_3$ improved the shoot elongation. The shoot-forming ability of explants was high from leaf fall in the autumn to the spring flush, but could be maintained by using cold storage of branches at 5-7°C. Elongated shoots rooted easily *in vitro* in the presence of a high concentration of IBA (96%). Under the identical rooting conditions, the activated charcoal had the inhibitory effect (46% of rooted shoots). Rooted plantlets were successfully acclimatized in a greenhouse. This method of micropropagation, based on cambium strip explants, was also efficient with another 'resistant' hybrid elm *U. pumila × U. japonica* but at a lower mutiplication rate, from 3 to 7 buds per cambium explant (Ben Jouira *et al*. 1997). Krajňáková and Longauer (1996) micropropagated mature trees of other Dutch elm cultivars such as 'Dodoens', 'Groeneveld', 'Lobel', and 'Plantijn'. The authors observed genotype-dependent responses in shoot elongation rates after *in vitro* culture initiation. Interestingly, the influence of donor explant site origin and medium composition on adventitious shoot multiplication of 'Dodoens' was negligible (a maximum average of 4.6 shoots per explant). The experimental field plots with micropropagated plantlets were established and their growth is further monitored.

### Cryopreservation of elms

The long-term preservation of threatened elm germplasm plays the important role in elm improvement programs. In the early study, Ulrich *et al*. (1984) achieved the successful regeneration of American elm plantlets from callus cultures after the short-term storage (4 min) in liquid nitrogen. Callus was treated with an aqueous cryoprotectant solution of polyethylene glycol, glucose and dimethylsulfoxide. Attempts for the long-term preservation of European elm genetic resources (wych elm, smooth elm, fluttering elm, and their hybrids) resulted in the establishment of the cryobank with a large collection of 444 clones (Harvengt *et al*. 2004). The authors used the technique based on the stepwise freezing of cryotubes containing dormant buds, and tested the potential of plant production from frozen buds after several years in liquid nitrogen. In a random sample, all clones except wych elm easily regenerated *in vitro*. Regrowth of cryopreserved wych elm buds was observed after micrografting on a micropropagated smooth elm rootstock. Growing plants were planted in the field to monitor their long-term growth behaviour. In addition, the authors provided the important operational costs comparison and found that the establishment of a cryobank including 200 bud explants per clone is about three times more costly than direct establishment of three ramets in the field. On the other hand, long-term maintenance costs of the cryobank are very low compared with the field management of trees.

## ASHES

Wood of ash species is heavy, hard, strong, stiff, and has excellent bending qualities. Much of the wood is used for the manufacturing of flooring, in furniture (especially in the bent part of chairs), slack cooperage, handles, and many types of sporting equipment including oars and bats. Ashes in tissue culture are amenable to propagation by adventitious shoot multiplication as well as somatic embryogenesis from immature zygotic embryos.

### *Fraxinus excelsior* L.

Micropropagation of common ash was achieved from both juvenile and mature trees. Axillary shoot proliferation, stimulated on shoot tips and nodal segments in the presence of high concentrations of BAP, is the common technique used for the formation of shoot cultures of the juvenile origin. Hammatt and Ridout (1992) used cotyledonary nodes from *in vitro* germinated seedlings as primary explants, whereas Chalupa (1990) sampled nodal segments from greenhouse-grown seedlings up to 6 months in age. Chalupa also used another adenine-type cytokinin BPA that effectively promoted axillary shoot proliferation to the extent similar to BAP (2-6 shoots per explant). Rooting frequencies were observed from 62% to 84% on media containing IBA and NAA. Acclimatized and hardened plants were planted in the field. During the first seasons in the field, the micropropagated trees grew uniformly and no signs of abnormal growth or morphology were observed (Chalupa 1990). Schoenweiss and Meier-Dinkel (2005) developed a protocol for micropropagation of grafted plants of 26 selected 16-year-old trees. Axillary buds and shoot tips were the most responsive explants for the culture establishment. For the improved axillary shoot proliferation, media were supplemented with a high concentration of BAP in combination with low concentrations of IBA and TDZ. The multiplication rate over the four tested clones increased on average from 1.6 to 2.0 shoots per explant. Unlike the explants of the juvenile origin, a high concentration of IBA combined with BAP induced none *in vitro* rooting in microshoots derived from explants of the mature origin. Interestingly, after the transfer to peat compost, the acclimatized microshoots rooted *ex vitro*. Rooting frequencies varied between 25% and 100%, with an average of 71%. This study clearly showed the significant influence of the clone responsiveness on the culture establishment and *in vitro* performance in mature trees. The impact of different periods of harvest on the ability of apical bud explants to initiate sprouting, was assessed by Silveira and Cottignies (1994). Explants harvested during shoot apical dormancy from September to March were able to sprout and slightly multiply on media containing high concentrations of BAP in combination with IBA. When the explants were sampled in May and June, which are the periods of cell proliferation in the apical bud and intense branch growth, no sprouting was obtained. After 2 months on multiplication medium, shoots formed scarious scales and dormant-like buds, and entered into a resting period (Silveira and Nougarède 1995; Nougarède *et al*. 1996). Following the subculture on a new multiplication medium, a dormant state reverted easily to a shoot growth recovery. Elongated shoots rooted readily on PGR-free media in frequencies from 87 to 100%. No significant differences in adventitious rooting among different dormancy periods of harvest were found.

Capuana *et al*. (2007) recently reported micropropagation via somatic embryogenesis. Immature zygotic embryos that showed the initial development of cotyledons were found the most ideal explants for the production of embryogenic tissues. Seeds sampled at more advanced stages of embryo development responded by the adventitious shoot organogenis. Somatic embryo formation was induced by 2,4-D applied with BAP in ratio 2:1. The progress of somatic embryos to the cotyledonary stage and maturation occurred on PGR-free media with the subsequent culture in the presence of a low concentration of BAP. Three-week storage in darkness at 4°C greatly improved embryo germination and conversion rates. Plantlets produced from somatic embryos exhibited normal growth and appearance, and were acclimatized in a greenhouse.

### *Fraxinus angustifolia* Vahl.

Tonon *et al*. micropropagated narrow-leafed ash plantlets from mature zygotic embryo explants via organogenesis (2001b), and from immature zygotic embryos via somatic embryogenesis (2001c). Embryo axes from mature seeds showed the greatest regenerative potential for organogenesis, whereas no adventitious shoots developed from the buds that formed on cotyledon segments. BAP in combination with 2,4-D promoted adventitious bud formation (an average of 5.4 buds per explant). Shoot multiplication ability was also affected by the gelling agent brand. Duchefa Gelrite was superior to Serva Gelrite and Difco Bacto Agar brands. Elongated shoots rooted readily *in vitro* in the presence of NAA as well as on PGR-free media. Rooted plantlets were acclimatized in a greenhouse.

With regard to somatic embryo formation, only embryo axes extracted from immature seeds containing the liquid endosperm and partially formed cotyledons were shown responsive to the combination of 2,4-D and BAP applied in a ratio of 2:1. Transfer of embryogenic structures to a medium with a decreased concentration of 2,4-D in combination with BAP made it possible to preserve the embryogenic potential and growth ability by the secondary embryogenesis for over three years. Mannitol and activated charcoal positively affected somatic embryo maturation. On the other hand, the encapsulation of somatic embryos in sodium alginate beads adversely affected plant conversion rate, regardless of nutrient additions to the alginate. Non-encapsulated embryos germinated at a frequency of 65%, whereas coated embryos showed normal germination at a frequency of 23%, respectively. To maintain a high production potential of somatic embryogenesis through automation, Tonon *et al.* (2001a) established a synchronous embryogenic system in the liquid culture. The fractionation of cells and cell clusters by the density gradient centrifugation in Ficoll solutions enabled the selection of the heaviest cell subpopulations with a synchronous development of somatic embryos at satisfactory rates.

### *Fraxinus ornus* L.

Arrillaga *et al*. (1992) reported micropropagation of flowering ash that is used mainly as an ornamental species in urban plantings. Shoot apices and nodal segments were sampled from juvenile seedlings and mature 30-year-old trees. Cultivation in a high salt concentration liquid medium supplemented with high concentrations of BAP, followed by the transfer to solidified PGR-free medium, promoted the highest shoot multiplication rates. For juvenile explants it was on average 6.5 shoots per explant, in case of mature explants it was on average 5.3 shoots per explant. *In vitro* rooting frequencies were significantly affected by the age of donor explants, and reached 71% for shoots of the juvenile explant origin and 53% for shoots derived from the mature explants, respectively. Effects of NAA in combination with BAP were superior to IAA/BAP or IBA/BAP treatments. When acclimatized and placed in a greenhouse, regenerated plantlets of both origins exhibited normal development compared with seedlings.

### *Fraxinus pennsylvanica* Marsh.

*In vitro* germinated seedlings were used in the study on green ash shoot proliferation (Kim *et al*. 1997). Axillary shoots tips formed on horizontally placed seedlings with removed radicles. High concentrations of BAP or TDZ promoted maximum shoot proliferation rates (14.3 shoots per culture vessel or 10.0 shoots per vessel, respectively). Significant differences in the axillary shoot proliferation rate and shoot biomass weight were apparent among clones. Excised shoots were rooted under *ex vitro* conditions and acclimatized in a greenhouse.

### *Fraxinus americana* L.

Bates *et al*. (1992) compared the organogenic potential of immature and mature nonstratified seeds of white ash. Mature seed explants showed a higher ability to adventitious shoot proliferation than explants from immature seeds. Adventitious shoots formed on the callus, cotyledons, and hypocotyls of the resulting seedlings in the presence of a high concentration of TDZ. In addition, somatic embryo formation from mature seed tissues was initiated on media containing 2,4-D and TDZ, and followed on media supplemented with BAP and NAA. Elongated adventitious shoots and germinated somatic embryos rooted *ex vitro* under an intermittent mist (rooting frequency of 80%) and were acclimatized in a greenhouse. Acclimatized and hardened regenerates were planted in several field studies with little transplant mortality (Van Sambeek and Preece 2007). After 6 growing seasons, survival in the study with 12 clones averaged between 70 and 100% except for a single clone where all regenerates had died. Variation among clones was twice that within clones. All of the surviving clones exhibited normal growth and morphology including the clone originating from organogenic callus.

### Cryopreservation of ashes

The vitrification proved to be the most suitable technique for cryopreservation of common ash shoot tips of both juvenile and mature origin (Schoenweiss *et al*. 2005). Juvenile clones were successfully cryopreserved with an average regrowth of 73%, in case of mature trees it was also a quite high frequency of 67%. The encapsulation-vitrification technique resulted in an average regrowth of only 16%. The encapsulation-dehydration technique was not successful because shoot tips were sensitive to osmotic dehydration. Brearley *et al*. (1995) subjected mature zygotic embryos of common ash to a simple desiccation treatment. When embryos were dried to the moisture content of 12-14% prior to rapid freezing in liquid nitrogen, the authors recorded the highest frequency of germination (63%).

## CHERRIES

Timber species of cherries are fast-growing trees with a relatively short crop rotation. Other members of this genus belong to the commercially important fruit species, rootstocks for proven cultivars or widely planted ornamentals. Wood of timber species (*Prunus avium* and *P. serotina*) is stiff and strong, but only moderately hard and heavy. Lumber is remanufactured into high quality furniture or flooring. Veneers utilize and display the exceptionally attractive reddish-brown colour and grain patterns. Wood is also used for woodenware, novelties, interior finishes for buildings, molding and trim. Cherries in tissue culture are amenable to propagation mainly by adventitious shoot multiplication, and in a limited extent also by somatic embryogenesis from immature zygotic embryos.

## *Prunus avium* L.

In most of the studies on micropropagation of selected mature wild cherry trees, the primary explants were provided either by rejuvenated trees that were previously grafted onto *P. avium* × *P. pseudocerasus* 'Colt' rootstock (Hammatt and Grant 1997, 1998; Grant and Hammatt 2000) or by rejuvenated shoots from root cuttings (Kitin *et al*. 2005). Adventitious shoots were induced from small furled leaves, extrafloral nectaries located at the base of leaf petioles, or directly from apical shoots. Shoot multiplication was promoted by high concentrations of BAP or TDZ. Hammatt and Grant (1997) made a screening of 10 woodland genotypes and 2 cultivars among which significant differences in organogenic potential were found. An average number of adventitious shoots per explant ranged from 1.2 to 6.6. Likewise, *in vitro* adventitious rooting on media supplemeted with IBA and phloroglucinol varied from 50 to 100%. This report documents how difficult is to find a protocol that should work optimally in a wide spectrum of genotypes. For the improved culture of less responsive cultivars, modifications in shoot culture medium composition were essential. Adventitious shoot multiplication rates reported by Kitin *et al*. (2005) varied from 3.6 to 8.9 shoots per explant depending on the concentration of BAP. On the other hand, high concentrations of BAP induced a higher frequency of fasciated shoots that are not a suitable material for adventitious rooting. Unlike the reports preferring explants sampled from rejuvenated trees, Ďurkovič (2006) used axillary buds sampled directly from a plus tree in a crop age. Combination of BAP with a low concentration of TDZ was found the most effective in adventitious shoot multiplication ability (6.8 shoots per explant). In the presence of IBA, rooting frequency reached 73%. Rooted plantlets were acclimatized readily to a greenhouse ambient environment and planted in the field (**Fig. 4**). Experiments with similar results were also done at the National Forest Centre in Zvolen, Slovakia (Jana Krajňáková, pers. comm.).

Micropropagation via somatic embryogenesis still encounters problems with a low conversion rate of embryos into plantlets. Early stages of somatic embryos were observed in protoplast-derived cell cultures (David *et al*. 1992). Later cotyledonary stages were obtained from immature zygotic embryos through direct somatic embryogenesis but in a low frequency. Moreover, most of the embryos were translucent and exhibited vacuolated cells without any storage reserves. Conversion into plantlets was also extremely low (de March *et al*. 1993; Garin *et al*. 1997). Improvement of somatic embryo maturation was achieved by the removal of auxin and cytokinin from the proliferation medium, and the addition of maltose and ABA. A higher concentration of maltose significantly increased a production of white somatic embryos that accumulated storage lipids and storage protein (Reidiboym-Talleux *et al*. 1999). Maturation and conversion of translucent somatic embryos was improved by the 2-month cold period in darkness at 4°C. This treatment increased the content of neutral glycerolipids and phosphatidylcholine to levels comparable to those observed in mature zygotic embryos. The content of phosphatidylethanolamine reached 10 times the level of that in mature zygotic embryos (Reidiboym-Talleux *et al*. 2000). The cold treatment was shown beneficial for translucent somatic embryos with the rate of conversion 35%. For white somatic embryos, this treatment was less effective due to precocious maturation with the rate of conversion only 8.5% (Reidiboym-Talleux *et al*. 1999).

Although considerable progress in organogenic micropropagation of proven mature trees was achieved, information on the field performance of *in vitro* regenerates is still poor. Hammatt (1999) compared the development of micropropagated trees, derived from the commercial cultivar F12/1, in the nursery and the field with cuttings and seedlings, over a total period of 6 years. In the season following propagation, micropropagated trees were more robust than cuttings. Increased uniformity, more rapid height gain and lighter branching were typical for micropropagated trees, in contrast to seedlings. In the first flowering season, a greater proportion of cuttings than micropropagated trees flowered. In the second flowering year, however, there were no differences in flowering habit between them. Propagation by cuttings or micropropagation did not consistently affect increments in stem diameters or heights. In addition, no evidence was found that long periods of exposure to cytokinin *in vitro* results in additional branch proliferation.

## *Prunus serotina* Ehrh.

For micropropagation of black cherry trees, both axillary shoot proliferation and adventitious shoot multiplication techniques were applied. Tricoli *et al*. (1985) reported BAP-induced multiple shoot culture formations from axillary buds of 50-year-old trees (a maximum average of 5.3 shoots per explant). IBA promoted a 94% rooting frequency under continuous darkness. Rooting percentage in the light was enhanced by rutin up to 70%. Leaf explants derived from shoot cultures of juvenile seedlings or rejuvenated trees proved useful for adventitious shoot production. In the work of Hammatt and Grant (1998), TDZ induced adventitious shoot regenerative responses to a greater extent than BAP. Shoot development was usually associated with sites of wounding, and in some cases shoots developed along the length of the adaxial surface of the midrib as well. The work of Espinosa *et al*. (2006) expanded upon the earlier study of Hammatt and Grant, both in the number of PGR concentration treatments and the lighting regimes. Clusters of shoot bud primordia regenerated from the margins of the leaf explant or at the leaf midrib. TDZ in combination with a low concentration of NAA was efficient to induce adventitious shoot multiplication (a maximum average of 5.1 shoots per explant). Interestingly, during subculturing of elongated shoots, the authors observed both complete and incomplete flowers on a small portion of adventitious

**Fig. 4 Micropropagation of wild cherry from mature trees.** (**A**) Multiple shoot culture induced by combination of BAP and TDZ; (**B**) *In vitro* adventitious rooting; (**C**) A view of plantation with 10-year-old regenerates (photo © Dr. J. Krajňáková, National Forest Centre, Zvolen, Slovakia).

shoots. The complete flowers remained alive for 2 months, then turned brown and died. In the presence of IBA, adventitious rooting performance of nodal explant-derived shoots reached up to a frequency of 70%, whereas rooting of adventitious shoots was rather poor, with only 27% of rooted shoots. Plantlets derived from leaf explants grew slower during the acclimatization process, had an 86% survival rate after acclimatization, and a 100% survival rate after overwintering. In rooting experiments with axillary shoots derived from a 95-year-old tree, Fuernkranz *et al.* (1990) assessed a spectral quality of light on rooting performance. Under low light intensity, the highest rooting percentage was provided by the yellow light (97%), whereas the blue light significantly reduced rooting (73%, and even zero rooting under moderate light intensity).

Information on the field performance of micropropagated regenerates over 6 growing seasons comes from Maynard (1994). In the first 3 years of growth in the plantation, micropropagated trees were smaller than control seedlings, but growth differences diminished in the following seasons. Pruning of side branches substantially increased the length of clear stems but adversely affected diameter growth. No plagiotropic growth was observed on any of the plantlets. After 6 growing seasons, the plantation average was over 4.5 m in height and 4.3 cm in diameter at breast height, with a height growth rate of over a meter per year.

### Other *Prunus* taxa

Besides studies devoted to micropropagation of important timber cherries *P. avium* and *P. serotina*, there is an impressive list of publications aimed at the biotechnological improvement of the fruit and ornamental species that belong to this genus. Both organogenesis and somatic embryogenesis are effectively employed in micropropagation of many *Prunus* taxons, including sweet cherry (*P. avium*) cultivars (Piagnani *et al.* 2002; Matt and Jehle 2005), hybrids of *P. avium* × *P. sargentii* (Hammatt and Grant 1998), *P. avium* × *P. pseudocerasus* ‘Colt’ (Mandegaran *et al.* 1999), *P. avium* ‘Mazzard’× *P. mahaleb* (Muna *et al.* 1999), sour cherry (*P. cerasus*) cultivars (Tang *et al.* 2000a, 2002), plum (*P. domestica* ‘Węgierka Zwykła’) cultivar (Nowak *et al.* 2004), peach (*P. persica*) taxons (Gentile *et al.* 2002; Fotopoulos and Sotiropoulos 2004), apricot (*P. armeniaca* ‘Bebecou’) cultivar (Koubouris and Vasilakakis 2006), almond (*P. dulcis*) cultivars (Channuntapipat *et al.* 2003), Mongolian (*P. fruticosa*) and Nanking (*P. tomentosa*) cherries (Pruski *et al.* 2005), *P. mume* cultivars (Ning *et al.* 2007a, 2007b), *P. incisa* cultivar ‘February Pink’ (Cheong and Pooler 2004), etc.

### Cryopreservation and minimal growth storage of cherry germplasm

Techniques applied routinely in cryopreservation of *Prunus* germplasm were reviewed thoroughly by de Boucaud *et al.* (2002). Following the vitrification technique, the highest regrowth rate of cryopreserved sweet cherry shoot tips (showing normal growth without necrosis) varied from 78% to 80% (Niino *et al.* 1997; Shatnawi *et al.* 2007). When using the encapsulation-dehydration technique, growth recovery from sweet cherry shoot tips reached up to 76% (Shatnawi *et al.* 2007). In case of wild cherry embryogenic tissues, cryoprotection was based on a pretreatment on solid medium with increased sucrose concentrations followed by the air desiccation to about 20% moisture content (Grenier-de March *et al.* 2005). When embryogenic tissues were treated with the vitrification solution, they turned necrotic. Regrowth rate of frozen embryogenic tissue was 89% with the most somatic embryos at the globular yellowish stage. On medium supplemented with maltose instead of sucrose, somatic embryos were able to develop to the cotyledonary stage. With regard to the genetic stability of *Prunus* plantlets recovered from cryopreserved apices, no genetic change was detected at the phenotypic, cytological and molecular level (Helliot *et al.* 2002).

The medium-term minimal growth storage of germplasm is another option. Slow growth cultures using low temperature regimes (0-4°C) with extended subculture intervals are available (Ashmore 1997; Pérez-Tornero *et al.* 1999).

## SERVICE TREES

Wood of service trees, due to their slow grown nature, is quite dense, fine-grained, and has good bending strength. It was used in the past to make screws for winepresses, musical instruments, inlays, billiard queue sticks, yardsticks, for turnery and joinery. Today it is usually used for decorative veneers. In the 1990’s, *Sorbus torminalis* was the highest priced timber species in Europe. Service trees in tissue culture are amenable to propagation by adventitious shoot multiplication.

### *Sorbus domestica* L.

Micropropagation of true service tree has been achieved from both juvenile seedlings and mature trees. Hypocotyl segments explanted from 15-day-old seedlings induced a formation of caulogenic callus (Arrillaga and Segura 1992). The highest shoot induction frequency was observed on media supplemented with moderate concentrations of BAP and IAA and with $NO_3$:$NH_4$ ratio of 4:1. Regrettably, an average number of adventitious shoots per caulogenic explant was rather low, ranging from 2 to 3 with no variation among $NO_3$:$NH_4$ treatments. On the other hand, in the previous experiments with shoot apices and nodal segments explanted from 30-day-old seedlings that were cultured on media with high concentrations of BAP, the average shoot multiplication rates were very high, ranging from 8.0 to 12.8 shoots per explant (Arrillaga *et al.* 1991). During subcultures, shoot multiplication rates of shoot tips increased up to 15 shoots per explant. Such high coefficients of multiplication may be attributed to, except the explant type, the improved basal medium modification and very high concentrations of BAP. High average multiplication rates were also observed for shoot tips sampled from 25- to 30-year-old trees, with up to 8.0 shoots per explant (Arrillaga *et al.* 1991). *In vitro* rooting capacity was significantly affected by the age of donor explants, and reached 87% of rooted shoots for the juvenile explants and 33% of rooted shoots for mature explants, respectively. Rooted plantlets from explants of both origins were acclimatized in a controlled environment room to reduced relative humidity by the progressive removal of a glass cover during a period of 3 weeks and then were transferred to a greenhouse. The survival rate of the plantlets was 70%.

In addition, Arrillaga *et al.* (1995) reported the induction of somatic embryogenesis from anthers of 35-year-old trees in low frequencies. Anthers containing tetrads or uninucleate microspores that were cultured on media supplemented with high concentrations of BAP and IBA or BAP and IAA, responded by the callus proliferation with a sporadic formation of somatic embryos. The stage of binucleate pollen as well as cold pretreatment of flower buds inhibited embryo formation. Although no conversion of embryos into somatic seedlings was achieved, the potential to obtain haploid plantlets under improved culture conditions still exists.

### *Sorbus torminalis* (L.) Crantz

To this date, only a few short reports deal with the micropropagation of wild service tree. Chalupa (1987) induced multiple shoot culture formation from juvenile shoot tips and nodal segments. Shoot multiplication was promoted by moderate and high concentrations of BAP in combination with IBA. Excised shoots rooted easily in the presence of low concentrations of NAA and IBA. Battut *et al.* (1993) reported micropropagation of mature trees on media supplemented with high concentrations of BAP and IBA in

combination with $GA_3$. Regenerated plantlets acclimatized readily to *ex vitro* environment.

### *Sorbus aucuparia* L.

Except for service trees, micropropagation efforts in the genus *Sorbus* are focused on rowan. The species is planted as an ornamental tree, and the fruits are important food components for birds and game in mountainous regions. Wood is used for furniture, veneer, and pulping. Chalupa (1992) stimulated adventitious shoot multiplication of mature 45- to 50-year-old trees by means of BAP or TDZ in combination with IBA. The highest multiplication rate of nodal segments reached an average of 8.2 shoots per explant. Explant source was shown as an important factor affecting rates of shoot multiplication, adventitious rooting, and field performance. Explants sampled from juvenile parts of mature trees (epicormic shoots and lower branches) exhibited higher multiplication rates, rooting frequencies, and height growth rates than explants from mature parts of trees (top branches). Up to 82% of shoots derived from epicormic shoot explants rooted in the presence of IBA combined with NAA. After acclimatization and hardening, micropropagated trees were planted in the field. At the end of the fifth growing season, the regenerates derived from juvenile parts of mature trees attained a height from 342 to 355 cm that was comparable to a height of control trees originated from seeds (368 cm).

Lall *et al*. (2006) reported somatic embryogenesis from cotyledons of immature zygotic embryos. Enhanced formation of proliferating masses of globular stage embryos was observed on medium supplemented with low concentrations of BAP, KIN, NAA, and with additives glutamine and casein hydrolysate. After transfer to basal medium without PGRs, globular embryos developed into cotyledonary stage embryos. Cold-treated embryos on medium containing maltose changed into green translucent embryos and underwent bipolar germination. However, conversion rate was rather low, at only 20%. Embryos without a cold pretreatment did not germinate but formed a callus and, occasionally, secondary embryos and adventitious shoots.

## WALNUTS

Wood of *Juglans regia* and *J. nigra* is heavy, hard, strong, stiff, stains exceptionally well, and is easy glued. The chocolate brown heartwood is one of the most durable of any temperate hardwood. In furniture, wood is used as veneer or as solid wood. It is popular for any interior finish due to its striking grain and color. Wood is also prized for gunstocks because of its stability after seasoning, its fine machining qualities, its uniformity of texture, and its beauty. Light and soft wood of *J. cinerea* is also used for interior finishes and furniture. Walnuts in tissue culture are amenable to propagation by both axillary shoot proliferation and adventitious shoot multiplication, preferrably on DKW medium supplemented with BAP (concentration of 4.44 μM seems optimal) or TDZ. Somatic embryogenesis from immature zygotic embryos or endosperm tissue is also a choice. A high salt DKW medium (Driver and Kuniyuki 1984) was developed just for *in vitro* culture of walnut trees.

### *Juglans regia* L.

The genotype plays a crucial role in micropropagation of Persian walnut trees. Scaltsoyiannes *et al*. (1997) studied multiplication rates of 12 juvenile clones. Responses were genotype dependent. The highest production of axillary shoots was promoted by a high concentration of BAP in combination with IBA (an average of 5.4 shoots per explant). Different clone reactions were also observed during culture establishment and shoot elongation. With regard to adventitious root formation, the average rooting ability was moderate (44%). However, some clones showed extremely poor rooting ability (5%), whereas others showed high rooting ability (95%). This study clearly shows that a thorough selection of the most responsive clones has to precede a large-scale propagation. Breton *et al*. (2004) selected trees with an early flowering phenotype that may produce flowers within months of germination. *In vitro* microshoot lines of those 'Early Mature' trees were established from elongated epicotyls of germinated somatic or zygotic embryonic axes corresponding to half-sib progenies. During the multiplication phase promoted by a moderate concentration of BAP in combination with IBA, apical flower buds were formed on 2-cm microshoots after 3 to 6 subcultures. The flowers appearing *in vitro* on apical inflorescences were mostly hermaphrodite. Adventitious rooting rates varied from 23% to 77% depending again on the genotype. Flowering was observed in 3 out of 4 genotypes of 3-year-old regenerates transferred to the greenhouse. On the other hand, the flower production clearly reduced a height growth of regenerated trees. For the embryo culture, Sánchez-Zamora *et al*. (2006) found WPM superior to DKW or MS media. On WPM, 81% of the embryos germinated and over 60% evolved into complete plantlets, whereas frequencies with DKW medium were significantly lower (54% and 45%, respectively). The highest cluster proliferation rate (6 shoots per explant) was obtained at the moderate concentration of BAP. Fernández *et al*. (2000) investigated the morphogenic potential of embryonic axes under different culture conditions. Cell suspensions, obtained from an embryo-derived callus induced with IBA, divided following embryogenic patterns and cell aggregates which were associated with earliest steps of embryoid formation. Cell suspensions from 2,4-D induced callus contained homogenous cell populations with no apparent differentiation. Organogenic shoot and root development was dependent on both the type and concentration of auxin in the culture medium. IBA induced long and thin roots, whereas with NAA they were shorter and thicker. Axillary bud proliferation was promoted by BAP and influenced by the physical nature of the culture medium. The use of liquid medium reduced the period of BAP addition into the culture medium. The subsequent culture of embryonic axes in the absence of BAP always allowed bud elongation. As Persian walnut belongs to difficult-to-root species, the ability of adventitious root formation is also one of the main factors limiting micropropagation. Two-phase rooting procedure is usually applied: root induction in the darkness on auxin-based rooting medium followed by the root development in the light in a mixture of diluted PGR-free medium and vermiculite. Experiments of Heloir *et al*. (1996) were focused on the induction of the rooting process. The authors determined changes in the concentrations of endogenous free IAA, IAAsp, and polyamines during the 7-day culture period in the darkness on IBA-based root-inducing medium. Levels of free IAA and IAAsp in the whole shoot extracts showed a transient peak at around 60 h and then remained at relatively low concentrations for the remainder of the culture period. Early increases in the auxin concentration favoured the polyamine accumulation as well. The concentrations of IAA and IAAsp remained stable when the rooted shoots were transferred to a mixture of vermiculite and gelrite, and grown in the light. Vahdati *et al*. (2004) determined the significant effect of genotype and increased sucrose level on the rooting ability. On the other hand, either increasing or decreasing the nitrogen level in the multiplication medium and increased temperature had a negative effect. Shoot length did not affect rooting success. Based on the genotype, rooting performance varied from 27% (cultivar 'Vina') to 94% (cultivar 'Sunland').

Tulecke and McGranahan (1985) and McGranahan *et al*. (1987) initiated somatic embryogenesis from cotyledons of immature zygotic embryos that were sampled from 5 cultivars 6-11 weeks after anthesis. Formation of embryogenic tissue was induced with high concentrations of KIN and BAP, in combination with IBA. Somatic embryos developed on the original cotyledon explants (the highest number was 26 embryos per explant), and also formed secon-

dary adventive somatic embryos on their root tips, cotyledons and hypocotyls. Brown tissue masses derived from hypertrofied somatic embryos also produced globular and other stages of somatic embryos. Both of these sources were used to maintain embryogenic lines by the continuous subculture on PGR-free DKW medium. Vigorous somatic embryos were then subjected to a cold treatment to break dormancy. Germination and root growth were improved by medium modifications that included lowering sucrose content and addition of charcoal. Cotyledon growth, greening and apical development took place in the next 2-4 weeks. Germinating somatic seedlings were transferred to peat plugs in vials and acclimatized to ambient laboratory environment. The study of Tang *et al*. (2000b) on desiccation treatments was aimed at the improvement of somatic embryo germination rates and conversion into plantlets. After 2 months of cold storage in combination with a desiccation treatment by using a supersaturated solution of either $CaCl_2$ or $Ca(NO_3)_2$ for 3 days, the germination frequency of somatic embryos reached up to 92%. Control somatic embryos germinated at only 28%. Moreover, the rate of the germinating embryos with both shoots and roots was high (70%). Up to 63% of the germinating embryos with both shoots and roots converted into plantlets due to further post-germination treatments. Somatic seedlings were transplanted to pots and acclimatized to greenhouse conditions. In similar experiments, San and Dumanoglu (2007) observed significant differences in germination frequencies between mother trees that provided open-pollinated seeds. The highest germination rate reached 69% when a desiccation treatment consisted of a saturated $MgCl_2$ solution for 4 days, and was followed with the addition of $GA_3$. On the other hand, germination rates of embryos originating from open-pollinated and non-pollinated seeds derived from the same mother tree were similar (21% and 24%, respectively). Interestingly, Tulecke *et al*. (1988) reported regeneration of triploid plants (3n = 48) derived from somatic embryos that were initiated from endosperm tissue cultures of open-pollinated seeds of cultivar ‘Manregian’. The main potential use of triploid walnut plants is as rootstock. A cluster of globular and cotyledonary somatic embryos was induced directly from the triploid endosperm on media supplemented with KIN, BAP and IBA. This embryogenic line was maintained by the continuous subculture on PGR-free DKW medium using secondary somatic embryogenesis from the roots and hypocotyl of somatic embryos or using hypertrofied callus masses from aged somatic embryos. Opaque somatic embryos were then subjected to a cold treatment to overcome dormancy and prevent rosetting. A triploid chromose number in root tips of potted somatic seedlings was confirmed.

The influence of mycorrhizal inoculation on post-acclimatization growth of micropropagated plantlets often belongs to the underrated area in micropropagation studies. Dolcet-Sanjuan *et al*. (1996) found that early inoculation with the arbuscular mycorrhizal fungi *Glomulus mosseae* or *Glomulus intraradices* confers protection against a stress situation and significantly improves plant survival when transferred to the field.

### *Juglans nigra* L.

In black walnut, adventitious shoot multiplication was reported from cotyledon tissues excised from immature seeds (Long *et al*. 1995). The most optimal period of explant sampling was 14 weeks after anthesis. Adventitious shoot formation was induced with a high concentration of TDZ in combination with 2,4-D, whereas high concentrations of BAP with KIN in combination with IBA were not effective at all. The authors observed a significant interaction between gelling agent and light treatment. The highest multiplication rate (on average 28.9 shoots per explant) was found when explants were cultured on medium gelled with agar and incubated for 4 weeks in light, and the lowest rate (on average 11.0 shoots per explant) when gelrite was used, regardless of whether the explants were in light or darkness. Fasciation of some shoots was observed due to the presence of a high concentration of TDZ. Following 1-week *in vitro* rooting pretreatment with IBA, a reduced amount of nitrogen, increased sucrose, and increased light intensity, the elongated shoots rooted *ex vitro* at a 40% frequency. Acclimatized plantlets were placed in a greenhouse.

Somatic embryogenesis follows the similar pattern like in Persian walnut. It may be initiated also from cotyledons of immature zygotic embryos but when using TDZ and 2,4-D instead of KIN, BAP and IBA. Long *et al*. (1995) found the highest embryogenic ability of cotyledon explants 12 weeks after anthesis. A high concentration of TDZ in combination with 2,4-D, WPM solidified with agar, and light conditions for the first 4 weeks were the most beneficial treatments for the induction of embryogenesis (on average 7.0 somatic embryos per explant). The authors developed a new LP medium (a combination of half-strength WPM and half-strength DKW salts and organics) for the secondary somatic embryogenesis and maturation of embryos beyond the cotyledonary stage. Data from embryo germination were not provided. However, Cornu (1988) pointed out the extremely low conversion rates, and only few somatic seedlings could be raised.

### *Juglans nigra* L. × *Juglans regia* L.

More attention and with the greater success has been devoted to hybrid walnut trees. The pattern of somatic embryo development was again very similar to that of Persian walnut, including experiments with desiccation and chilling treatments to promote embryo germination. Interestingly, when desiccated somatic embryos were placed on medical cotton compresses in liquid germination medium, the frequency of germination reached up to 46%, whereas on solid medium it was only up to 12% (Deng and Cornu 1992). Other experiments were focused on adventitious rooting and acclimatization processes. Dolcet-Sanjuan *et al*. (2004) tested *in vitro* rooting ability of 9 juvenile clones. The authors concluded that adventitious rooting performance and the quality of primary and secondary roots were significantly affected by several factors among which the most important were the genotype, auxin type (IBA and NAA) and concentration, presence of vermiculite in the root elongation medium, 2-week-long prerooting conditioning culture of microshoots in the presence of BAP, reduced sucrose content, and carbon dioxide enrichment during the root elongation phase. Modifications of the above factors resulted in the reduction of callus phase, increasing the percentage of rooted shoots (maximum 90%) and consequently the rate of acclimatization of plantlets to soil. Bisbis *et al*. (2003) brought a specific insight into adventitious rooting. The authors provided evidence that adventitious root formation from bases of micropropagated shoots coincides with lignification in the stems. It seems that growing roots are source of signals for increased lignification and wood formation in the stems.

Chenevard *et al*. (1997) studied carbohydrate reserves and carbon dioxide balance during acclimatization in growth chambers. During the first 7 days of acclimatization, respiration dominated. The content of metabolisable carbohydrates which accumulated mainly in roots during the root development stage, decreased and was used for growth and maintenance processes. From day 8, the daily carbon dioxide balance became positive, suggesting an autotrophic period. At the end of acclimatization after 28 days, the content of carbohydrates remained very low but steady. However, their distribution within a plant showed considerable changes. They decreased strongly in the root system and slightly in the aerial part of a plant. Fully acclimatized plantlets became dependent on their net assimilation capacity.

### *Juglans cinerea* L.

Pijut (1993) initiated early stages of somatic embryogenesis in endangered butternut. Globular to mature somatic embryos were initiated on cotyledonary explants excised from developing fruits that were collected 9 weeks after anthesis. Cotyledons were cultured on DKW medium supplemented with high concentrations of KIN and BAP in combination with IBA, and then transferred to PGR-free medium. Differentiation of the embryogenic callus on MS medium supplemented with 2,4-D in combination with BAP was induced on cotyledons excised 8-11 weeks after anthesis. Globular to torpedo-shaped somatic embryos were differentiated from this callus. Although the competence of the embryogenic callus was maintained for 1 year by regular subculturing on PGR-free media, unfortunately the conversion of somatic embryos into whole plantlets failed. Experiments aimed at the improvement of the frequency of somatic embryo production and germination of embryos into somatic seedlings remain the challenge.

## Cryopreservation of walnuts

In case of Persian walnut, embryonic axes with variable moisture content were treated with the cryoprotectant solution containing a high concentration of propanediol and sucrose, and were subjected to rapid freezing in liquid nitrogen (de Boucaud *et al*. 1991). The large survival (85%) and regrowth rate (75%) were found in axes with 20% moisture content. Thus, the regrowth rate and multiplication of shoots obtained did not pose any problem if embryonic axes were excised in the first 3 months after nut harvesting. For hybrid *J. nigra* × *J. regia* somatic embryos, the cryoprotectant treatment involving a progressive plasmolysis with an increasing sucrose concentration followed by dehydration under laminar air flow was adopted as a cryopreservation protocol. This treatment was preceded with the cold hardening of embryos and preculture of the hardened material on medium supplemented with dimethylsulfoxide and proline (de Boucaud *et al*. 1994; de Boucaud and Brison 1995). The authors used rapid freezing as well as slow freezing to –40°C prior to direct immersion in liquid nitrogen. Both methods gave relatively low rates of regrowth, 30% for slow freezing and 43% for rapid freezing, respectively. On the other hand, due to the very high tolerance of some embryos to desiccation, there is a chance to improve the cryopreservation method in different ways. The results depend on the potential for tissue to tolerate the water removal by dehydration. Beardmore and Vong (1998) reported that butternut embryonic axes, when excised with approximately 3 mm of cotyledonary tissue attached to the hypocotyl area, tolerated exposure to –196°C without use of cryoprotectants. Reducing the water content by slow desiccation to 4.8% was a threshold below which embryonic axes germinated after 24 h of cryopreservation (maximum 36% in dependence on the genotype). Germinated axes formed viable seedlings.

## FUTURE PROSPECTS AND CONCLUSIONS

Unlike agronomic crops, some coniferous species (*Pseudotsuga menziesii*, *Picea glauca*, *Pinus taeda*) and the genera *Populus* and *Eucalyptus*, micropropagation protocols employing both organogenesis and somatic embryogenesis are not yet sufficiently refined for a commercial application in many temperate noble hardwoods. There are several areas which are in need of further investigation. The main problem is associated with restrictions of somatic cell duplication by the maturity phase. Full understanding of regulation mechanisms that control the phase change from juvenile to mature growth remains a challenge for the future research. Application of TDZ instead of BAP proves helpful to overcome recalcitrant responses of mature genotypes in a still limited spectrum of assayed genotypes. Genotype-dependent responses to a regeneration protocol were observed

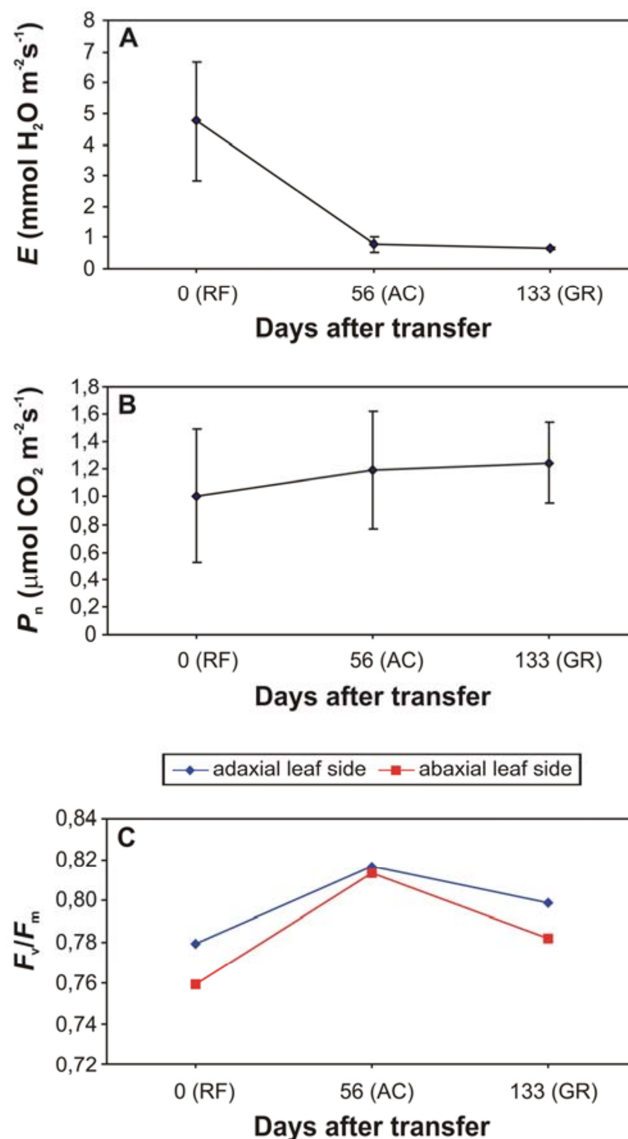


**Fig. 5 Changes in photosynthetic parameters during *ex vitro* acclimatization of black mulberry.** (**A**) Changes in mean transpiration rate ($E$); (**B**) Changes in mean net photosynthetic rate ($P_n$); (**C**) Changes in mean photosynthetic efficiency ($F_v/F_m$); RF, plantlets with *in vitro* formed roots transferred to *ex vitro* environmnent on day 0; AC, *ex vitro* acclimatized plantlets on day 56 after transfer; GR, vigorous greenhouse-grown regenerates on day 133 after transfer.

in many studies with noble hardwoods. In a commercial scale, the improved protocols should be working efficiently with several various genotypes, preferrably of the mature origin. Establishment of protocols with reduced steps of developmental pathways may significantly reduce time and costs. Conde *et al*. (2008) just developed the efficient organogenic protocol for smooth elm micropropagation that is based on two steps only: shoot proliferation, and direct acclimatization of thick stem shoots accompanied by *ex vitro* rooting. Development of such protocols would be extremely beneficial. Most reports on micropropagation either do not refer the acclimatization process or they refer very briefly. During the transfer to *ex vitro* conditions, a certain portion of regenerated plantlets usually dies. When transferred to soil, plantlets show an excess of water loss and rapid wilting if an appropriate care is not taken to maintain a high humidity in their new environment. The current research in our laboratory with micropropagated true service trees and black mulberry trees is aimed at the physiological assessment of acclimatization reponses in relation to changes in leaf anatomy, water loss, photosynthetic parameters, and wood formation. Identification the most beneficial environ-

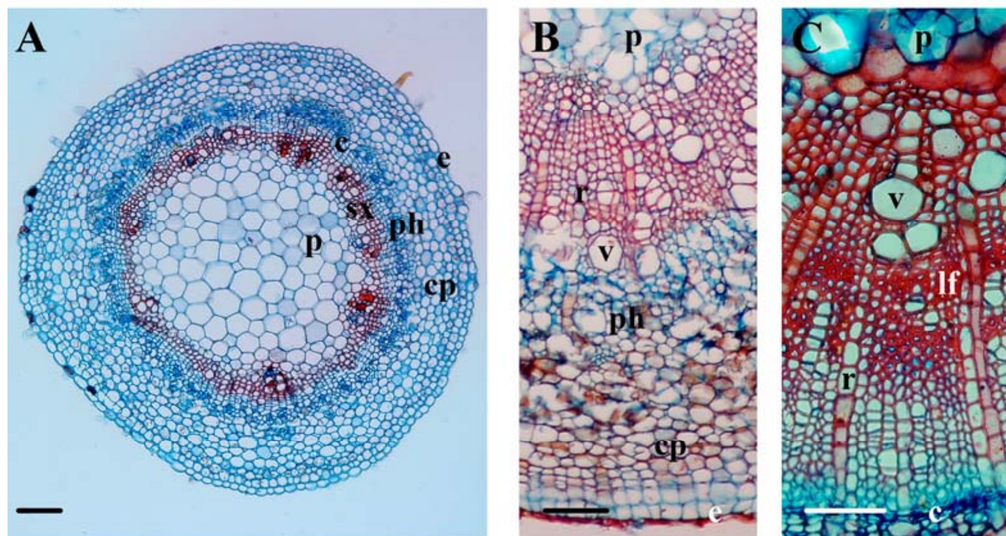


**Fig. 6 Xylogenesis during *ex vitro* acclimatization of black mulberry (safranin-Alcian blue double staining).** (**A**) Stem cross-section of plantlets with *in vitro* formed roots transferred to *ex vitro* environmnent on day 0, bar = 100 µm; (**B**) Stem cross-section of *ex vitro* acclimatized plantlets on day 56 after transfer, bar = 100 µm; (**C**) Stem cross-section of vigorous greenhouse-grown regenerates on day 133 after transfer, bar = 100 µm; c, cambium; cp, cortical parenchyma; e, epidermis; lf, libriform fibres; p, pith; ph, phloem; sx, secondary xylem; r, medullary ray; v, vessel.

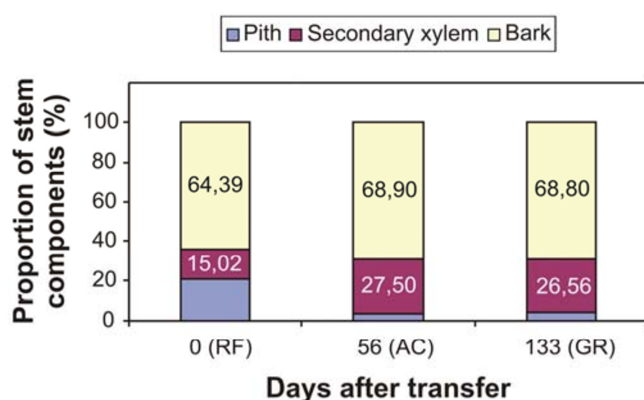


**Fig. 7 Changes in the proportion of stem tissues during *ex vitro* acclimatization of black mulberry.** RF, plantlets with *in vitro* formed roots transferred to *ex vitro* environmnent on day 0; AC, *ex vitro* acclimatized plantlets on day 56 after transfer; GR, vigorous greenhouse-grown regenerates on day 133 after transfer.

mental regimes with the physiological conditions of *in vitro* rooted plantlets may contribute to reduce a loss of the regenerates during acclimatization. Experiments with the regenerates derived from superior over 80-year-old black mulberry (*Morus nigra*) trees (high quality wood of black mulberry and white mulberry is used for manufacture of wine barrels in which world famous Tokaj wines are aged) show differences in photosynthetic parameters (**Fig. 5**) and wood anatomy (**Figs. 6** and **7**) mainly during the acclimatization phase. Experiments with the acclimatization responses of the regenerates derived from a superior over 90-year-old true service tree are just running. In addition, the early inoculation of *ex vitro* acclimatized plantlets with the mycorrhizal fungi may lead to a significant improvement of plant survival in the transplant process. Root colonization with the mycorrhizal fungi appears to be a protection against the stress situations and may reduce a loss of transplanted micropropagated plants. Furthermore, development of efficient regeneration systems that can be easily adapted is the prerequisite for the application of gene transfer technologies to facilitate a delivery of genetically modified noble hardwoods to the marketplace. An *Agrobacterium*-leaf disk transformation system has been used to produce transgenic American elm trees with an enhanced resistance to DED due to the expression of the synthetic antimicrobial peptide ESF39A (Newhouse *et al*. 2006, 2007). Transgenic English elm plantlets expressing the marker *uidA* gene encoding β-glucuronidase were regenerated from transformed internodal stem sections (Gartland *et al*. 2000). In Persian walnut, somatic embryos transformed with single inserts of the insecticidal crystal protein gene *cry*IA(c) from *Bacillus thuringiensis* exhibited host resistance to codling moth, *Cydia pomonella* (Dandekar *et al*. 1998). The somatic embryo-based transformation system using a green fluorescent protein selectable marker is also available for a rapid selection of non-chimeric transgenic somatic embryos (Escobar *et al*. 2000). Clonal reproduction of the planting stock together with transgenic technologies promise the improved establishment, preservation, and production of temperate noble hardwoods under the increasing demand for timber and paper around the world.

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