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BRIEF COMMUNICATION

In vitro regeneration of European linden

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Abstract

The regeneration of European linden (*Tilia* × *europaea* L.) *in vitro* was successful. When using axillary buds as primary explants it was possible to induce a new shoot growth. The highest number of shoots per explant (2.13 ± 1.09) was recorded at the presence of 0.2 mg dm^{-3} 6-benzylaminopurine. Up to 50 % of elongated shoots rooted in the presence of 2.0 mg dm^{-3} α-naphthaleneacetic acid.

Additional key words: callus formation, multiplication, organogenesis.

European linden (*Tilia* × *europaea* L.) is a hybrid between the bigleaf linden (*T. platyphyllos* Scop.) and the littleleaf linden (*T. cordata* Mill.), but is of uncertain origin (White 1995). Propagation of lindens is a challenge. Seed germinates with difficulty because of especially hard seedcoat and a frequent inability to set viable seed except in good summers. Vegetative propagation of interspecific hybrids or numerous cultivars is restricted to grafting. Plant tissue culture techniques enable to maintain the original genotypes of selected specimens through clonal propagation. Reports on organogenic tissue culturing of lindens include those on *T. cordata* (Chalupa 1983, 1990a, Youn *et al.* 1988, Pinker *et al.* 1995), and *T. amurensis* (Youn *et al.* 1989). Moreover, somatic embryo production and plantlet regeneration were described for *T. cordata* (Chalupa 1990b, Kärkönen and Simola 1999) and *T. platyphyllos* (Chalupa 1990c). All those works show on the possibility to *ex vitro* transfer. The aim of the present work was to optimize the culture conditions for European linden *in vitro* organogenesis and develop the procedure towards *ex vitro* transfer and subsequent tree growth.

Juvenile 2-year-old plants of European linden (*Tilia* × *europaea* L.) were selected as a source of explants. We used slightly unfolded axillary buds and greenwood cuttings before entering the stage of lignification. Explants were rinsed under tap water for 5 min, sterilized in 0.1 % mercury chloride solution with few drops of Tween 20 for 20 - 30 min, three times rinsed with sterile distilled water and placed onto WPM (Lloyd and McCown 1980) or MS (Murashige and Skoog 1962) culture media supplemented with phytohormones (BAP, kinetin, NAA, IBA) in various tested treatments. All nutrient media were solidified with agar (6 - 7 g dm^{-3}, *Sigma*, St. Louis, USA) and sucrose (20 g dm^{-3}). The pH was adjusted to 5.6 - 5.8 with 1 M KOH before autoclaving at 121 °C for 20 min. Cultures were maintained at day/night temperatures 25/19 °C, 16-h photoperiod with the irradiance of 62.5 μmol m^{-2} s^{-1} (cool white fluorescent lamps). Cultures were regularly sub-cultured after 4 weeks. Harvested microshoots of minimal length of 2 cm were transferred to rooting half strength WPM media (macro-, micro-elements, organic compounds) supplemented with auxins. Rooted plantlets were transplanted into pots containing perlite and were grown under high air humidity for 3 weeks. Significances of responses of axillary buds to phytohormones were tested by one-factor analysis of variance (*ANOVA*), frequencies in callus, shoot and root formation were tested by likelihood ratio χ^2-test (*G*-test), respectively.

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Abbreviations: BAP - 6-benzylaminopurine; IBA - indole-3-butyric acid; NAA - α-naphthaleneacetic acid.
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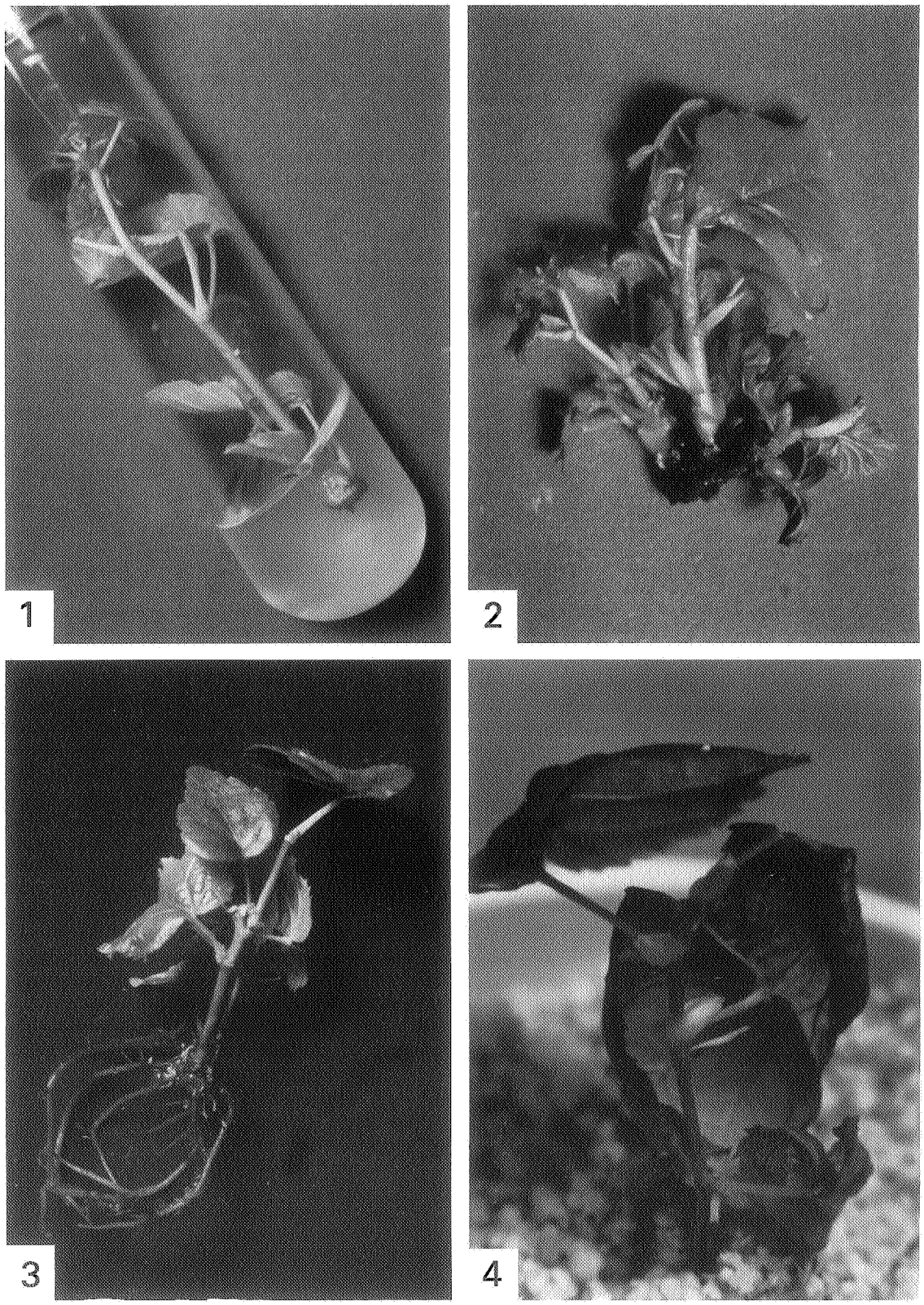

Fig. 1. Shoot proliferation from axillary bud.
Fig. 2. Multiple shoot culture.
Fig. 3. Root formation on excised shoot.
Fig. 4. Acclimatized regenerant transferred to *ex vitro*.

When considering the proliferation activity of explants there is no statistically significant difference between the application of BAP only and in combination with NAA. Established cultures proliferated to a various extent and differentiated growing shoots in all ten tested treatments (Table 1). However, during long-term tissue culture the number of shoots per explant varied from 1 up to 4 (the highest observed coefficient of multiplication, Figs. 1, 2). It was interesting that increasing the concentration of BAP only in media resulted in slowing down and decreasing the multiplication activity. On the other hand, at combined treatments of BAP with NAA the new shoots were formed at higher BAP concentrations. The application of 0.2 mg dm^{-3} BAP only was observed as the most effective treatment to achieve the maximal multiplication rate (*F*-test value 4.50, $P > 0.999$). In this case the mean number of shoots per explant equals 2.13 ± 1.09 (Table 1). Our results

Table 1. Effects of various concentrations of BAP and its combination with 0.1 mg dm^{-3} NAA on axillary bud culture on WPM medium. Means with the same letter are not significantly different according Duncan's test, $n = 30$, * - $n = 15$.

	BAP conc. [mg dm^{-3}]	Established cultures [%]	Shoot-forming cultures [%]	Number of shoots [explant^{-1}]
BAP	0.2	76.7	69.6	2.13 ± 1.09a
	0.5	70.0	52.4	1.73 ± 0.65ab
	1.0	66.7	60.0	1.75 ± 0.75a
	2.0	80.0	37.6	1.00 ± 0.00b
	3.0	73.3*	45.4	1.00 ± 0.00b
BAP+	0.2	60.0	38.9	1.00 ± 0.00b
NAA	0.5	76.7	60.9	1.00 ± 0.00b
	1.0	66.7	55.0	1.64 ± 0.67ab
	2.0	53.3	56.3	1.44 ± 0.53ab
	3.0	66.7*	40.0	1.50 ± 0.58ab

are comparable with those ones described for *T. cordata* explants (Youn *et al.* 1988) that were effectively multiplied also on WPM media. After reaching vigorous shoot development those authors transferred cultures onto basal media containing just low BAP dosage 0.2 mg dm^{-3} and within 4 weeks multiple shoots were formed. At those experiments an original shoot produced an average of 5.2 new shoots, the only different value.

The basal callus formation is the characteristic response of nodal segments. No clear and significant differences in dedifferentiation activity could be determined between media composition and tested phytohormone treatment. Redifferentiation processes towards the adventitious shoot formation and growth from dedifferentiated callus tissue seem very difficult to achieve in most hardwoods, and are limited mainly to herbaceous plants, crops or sub-tropical *Eucalyptus* (Subbaiah and Minocha 1990).

Table 2. Auxin effects on rooting of excised shoots, $n = 10$.

Auxin	Conc. [mg dm^{-3}]	Number of rooted shoots [%]
IBA	0.5	0
	1.0	30
	2.0	50
NAA	0.5	0
	1.0	20
	2.0	50
IBA+NAA	0.6+0.4	10

The effects of various concentrations of auxins IBA or NAA, and their combined application were tested to induce and promote rooting of excised shoots on half strength WPM medium (Table 2). As the best result, up to 50 % of rooted shoots was achieved using 2.0 mg dm^{-3} either IBA or NAA. Number of roots per rooted shoot (Fig. 3) varied from 2 to 6 depending on culturing duration in rooting media. Rooted regenerants were transferred to *ex vitro* conditions and were grown under high air humidity for 3 weeks (Fig. 4). All regenerated plantlets appeared to be phenotypically normal. Over 90 % rooted shoots were reported for *T. amurensis* (Youn *et al.* 1989), *T. cordata* (Chalupa 1990a) and *T. cordata* 'Wega' (Pinker *et al.* 1995). We observed up to 50 % rooted shoots after adding 2.0 mg dm^{-3} either IBA or NAA. These results may be compared to those ones for *T. cordata* (Youn *et al.* 1988). In that work the authors reported 48 % rooting on half strength WPM medium, and 64 % rooting on half strength IS medium (Saito and Ide 1985), respectively. Differences between our results and high rooting percentage of the above studies may be interpreted by a variation of interspecies responses, likewise different levels of endogenous auxins remain an open question. Our results show on the performance of *in vitro* regeneration of European linden though a special respect may be devoted to the rooting efficiency, not quite wishful in large-scale micropropagation and its further improvement may be searched yet for the rapid micropropagation.

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