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Possibilities for application of *in vitro* techniques in propagation of species of the genus *Tilia* sp.

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Abstract

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The propagation of the three types of linden used in the landscaping in Bulgaria is carried out mainly by seeds and cuttings, but both methods have a number of disadvantages. Alternative possibilities in this regard are provided by *in vitro* propagation, but studies on the types of explants, methods of surface disinfection, media composition, conditions for adaptation from *in vitro* to *ex vitro* growth are very limited. Having in mind that lindens are one of the most preferred and most widely used deciduous species in European and world ornamental horticulture, the clarification of these issues becomes particularly relevant. The aim of the present study was to develop an effective protocol for *in vitro* micropropagation of linden. Apical buds and stem cuttings from the top of mature cuttings or actively growing annual shoots of adult trees, apical and nodal segments of actively growing seedlings were used as initial explants. Two surface disinfection methods were studied – with 5% solution of calcium hypochlorite [$Ca(OCl)_2$] and 2% silver nitrate ($AgNO_3$). In the studies, nutrient media for multiplication based on MS (Murashige & Skoog, 1962), DKW (Driver & Kuniyuki, 1984) and WPM (McCown woody plant medium, 1980) were used. The effect of cytokinins 6-benzylaminopurine (BAP), Kinetin (6-furfurylaminopurine), *meta*-topolin (mT, [6- (3-hydroxybenzylamino) purine]), 2-iP (6-γ-γ- (dimethylalylamino) -purine) at an equimolar concentration of 5μM on the multiplication of large-leaved linden (*Tilia platyphyllos* Scop.) was studied. The best disinfection procedure was found by sequential application of $Ca(OCl_2)$ and $AgNO_3$ to explants from actively growing shoots, with the best results obtained with *Tilia cordata* Mill. In the multiplication of *Tilia platyphyllos* Scop. the maximum number of lateral shoots (2.93) was reported on the medium with *meta*-topolin, followed by those with BAP (1.73). The highest rooting rate (84.61%) of large-leaved linden (*Tilia platyphyllos* Scop.) was achieved on MS medium with half strength macronutrient content enriched with 0.3 mg l^{-1} indolyl-3- butyric acid (IBA).

Keywords: in vitro culture; *Tilia cordata* Mill.; *Tilia platyphyllos* Scop.; *Tilia tomentosa* Moench.; meta-topolin; auxins

Introduction

The genus *Tilia* includes over 40 species that originate mainly from Europe and North America. The most common in Europe are: *Tilia platyphyllos* Scop., *Tilia cordata* Mill., *Tilia tomentosa* Moench. In Bulgaria, lindens are found in natural habitats mainly in the low mountain belts with mixed deciduous vegetation. Linden is grown as a cultivated plant for landscaping and is widely used in parks and forest parks. It is one of the most preferred and most widely used deciduous species in European and world ornamental horticulture. That is why it is often the subject of scientific research, and the main directions are primarily related to the methods of propagation and methods of production of seedlings. The propagation of linden species is done mainly by seeds, but very often vegetative propagation by cuttings is applied. Re-

cently, methods for in vitro propagation have been developed, but all these methods are associated with many difficulties. They are due to the presence of a pronounced and prolonged dormancy of the seeds, their uneven maturation and poor formation, associated in some cases with adverse climatic conditions. The rooting of the cuttings is uncertain and the propagation coefficient is very low. Less widely used and generally not recommended for the production of seedlings from root shoots. Information on embryo culture has been published on embryo propagation (Chalupa, 1990, 2003; Kim et al., 2006; Hui & Ya-Juan, 2008); composition of the nutrient media, and in particular the concentration of cytokinins and sucrose (Chalupa, 1984; Ucler & Mollamehmetoúlu, 2001); rooting *in vitro* and *in vivo* (Peterson et al., 1960; Chalupa, 1984; Hansen, 1988; Pinker et al., 1995; Chalupa, 2003), etc. This information, however, concerns separated elements of the overall micropropagation process. Studies on the types of explants, methods of surface sterilization, composition of media for multiplication and rooting, conditions for adaptation from *in vitro* to *ex vitro* growth are very limited and no comprehensive effective protocol for *in vitro* micropropagation of this plant has been developed. Given that lindens are one of the most preferred and most widely used deciduous species in European and world ornamental horticulture, the clarification of these issues becomes particularly relevant. The aim of the present study was to develop an effective *in vitro* micropropagation protocol for linden.

Materials and Methods

The study was conducted at the Fruit Growing Institute, Plovdiv – Bulgaria, in the scientific laboratory of plant biotechnology. As plant material were used explants from the three types of linden, preferred in landscaping practice in Bulgaria – *Tilia platyphyllos* Scop. (large-leaved linden), *Tilia cordata* Mill. (small-leaved linden), *Tilia tomentosa* Moench. (silver linden). Three types of explants were studied – apical buds and stem cuttings from the top of mature cuttings or actively growing annual shoots of adult trees, as well as apical and nodal segments of actively growing seedlings.

The surface disinfection of the initial explants and establishment of *in vitro* culture

The standard laboratory procedure involves several steps and was used as a control. According to this procedure, the explants were washed with running tap water with detergent for one hour, treated with 70% (v/v) ethanol (2 min for explants from mature cuttings or 30 s for explants from actively growing parts), followed by 5 min of treatment with 5% calcium hypochlorite $Ca(ClO)_2$ and three rinses of 10 min with sterile distilled water. The explants remain in the last sterile distilled water until they were placed on the nutrient medium. All manipulations after rinsing with running water were performed under sterile laminar box conditions. The explants from mature cuttings were taken during the winter dormancy in February. After preliminary disinfection according to the standard procedure (described above), they were placed in sterile containers with sterile moist perlite and left in the growth chamber to stimulate their development (Figure 1a,b). After cracking, the buds were disinfected according to the standard procedure (control) or, after treatment with alcohol, they were soaked for 5 min with 2% silver nitrate ($AgNO_3$), followed by rinsing with sterile distilled water, remaining in the last water until the moment of inoculation on the nutrient medium. The explants from actively growing annual shoots and seedlings were taken in April-June. They have undergone a two-stage disinfection. After washing under running water for 1 hour, the explants were soaked for 30 seconds with 95% ethanol, treated with 2% $AgNO_3$ for 5 min, rinsed with sterile distilled water, treated for 5 minutes with 5% calcium hypochlorite $Ca(ClO)_2$ and three rinses for 10 minutes with sterile distilled water, the last water remaining until the moment of inoculation on the nutrient medium. The explants prepared in this way were placed on the respective nutrient media (Table 1). The studies used nutrient media based on MS (Murashige and Skoog, 1962), DKW (Driver and Kuniyuki, 1984) and WPM (McCown woody plant medium, 1980), enriched with 6-benzylaminopurine (BAP), indolyl-3- butyric acid (IBA), 30 g l^{-1} sucrose, 6.5 g l^{-1} agar (Duchefa), pH 5.6. The nutrient media were autoclaved for 20 minutes at 121°C and a pressure of 1 atm. Test tubes with a diameter of 25 mm were used, in which one explant was placed on 5 ml of nutrient medium. All explants were cultured at 22 ± 2°C and photoperiod 16 h day /8h night (OSRAM fluorescent lamps, 40 W; 40 µmol $m^{-2}s^{-1}$ PPFD). After a period of 21 days, the percentage of infected and uninfected (clean) explants was reported. The clean explants were transferred to fresh nutrient media by refreshing the sections at the base.

Multiplication

Experiments to optimize multiplication were performed with *in vitro* culture initiated by top explants from large-leaved linden seedlings (*Tilia platyphyllos* Scop.). In the experiments on establishement of *in vitro* culture, the best development of the explants was observed on a culture medium based on MS (V1) and therefore it was selected for further experiments in the multiplication stage. The effect of cytokinins 6-benzylaminopurine (BAP), Kinetin (6-furfurylaminopurine), meta-topolin (mT, [6- (3-hydroxybenzylami-

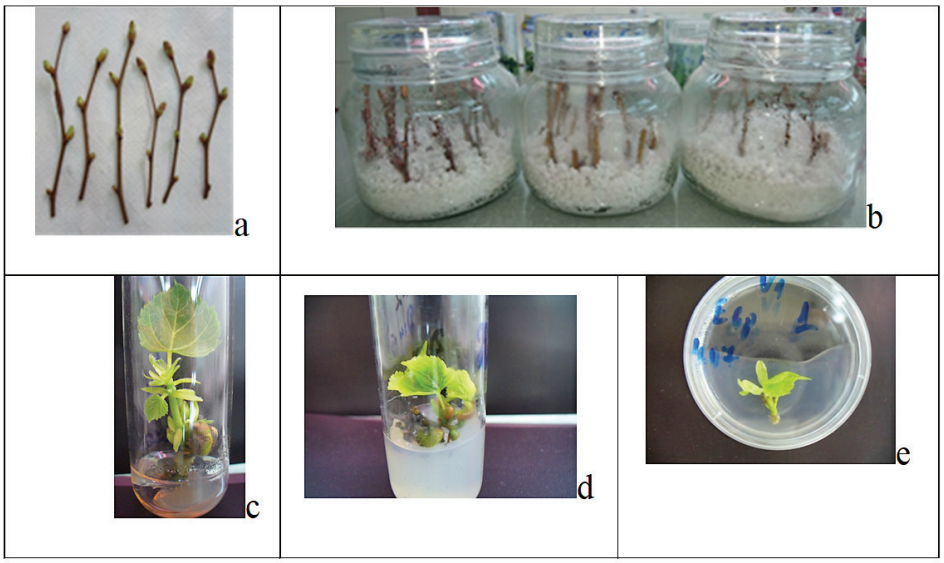

Fig. 1. Explants used in the establishment of linden *in vitro* culture: *a*. mature cuttings prepared for disinfection procedure; *b*. mature cuttings placed in sterile containers with sterile moist perlite and left in the growth chamber to stimulate their development; *c – e*. – initial development of explants from actively growing shoots; *c* – from silver-leaved linden; *d* – of small-leaved linden; *e* – large-leaved linden

no) purine]), 2-iP (6-γ-γ- (dimethylalylamino) -purine) at an equimolar concentration of 5μM were studied (Table 1). A nutrient medium without growth regulators was used as a control. In the multiplication stage, baby food glass jars (volume 190 ml) with 25 ml of nutrient medium were used, on which 4 tips (about 25-30 mm, with two pairs of leaves) were placed. The plants were grown at the temperature and light regime described above. After three consecutive passages (4 weeks each) of the respective nutrient medium, the following indicators were reported: fresh (FW) and dry (DW) mass of plantlet; number of newly obtained shoots from one explant (multiplication factor, K_M); length of central shoot; lateral shoot length; number of leaves.

Rooting of *in vitro* microcuttings

For the purpose of the experiment, large – leaf linden microcuttings with a length of about 20 mm, obtained in the multiplication stage, were used. Rooting nutrient media based on MS with half strength of macronutrients and enriched with auxins IAA, IBA or NAA (0.1 mg l^{-1} or 0.3 mg l^{-1}) (Table 1), 20 g l^{-1} sucrose, 6.5 g l^{-1} agar (Duchefa), pH 5.6 were applied. The same nutrient medium without growth regulators was used as a control. The micro-cuttings were transferred into polypropylene microboxes (600 ml) with white gas permeable filter (Sac O_2, Belgium) and in each container per 100 ml of nutrient medium 10 shoots were placed. For each variant of nutrient medium 5 containers were set. After 15 days, the following indicators were reported: rooting rate; average number of roots per plant; average length of roots; development of lateral roots; stem height and number of leaves.

Table 1. Nutrient media used at establishment, multiplication and rooting stages

Code of the medium	Basal medium	Growth regulators
Establishment of *in vitro* culture		
M D V1 V2 V3	MS	2.5 μM BAP + 0.005 μM IBA
	DKW	2.5 μM BAP + 0.005 μM IBA
	MS	10 μM BAP + 0.02 μM IBA
	WPM	10 μM BAP + 0.02 μM IBA
	DKW	10 μM BAP + 0.02 μM IBA
Multiplication		
V_{10} V_{12} V_{1k} V_{1t} V_{1p}	MS	Without growth regulators
	MS	5 μM BAP + 0.01 μM IBA
	MS	5 μM Kinetin + 0.01 μM IBA
	MS	5 μM mT + 0.01 μM IBA
	MS	5 μM 2iP + 0.01 μM IBA
Rooting		
M_0	50% Macro MS	Without growth regulators
MA_1	50% Macro MS	0.571 μM IAA (0.1 mg l^{-1})
MA_3	50% Macro MS	1.713 μM IAA (0.3 mg l^{-1})
MB_1	50% Macro MS	0.5 μM IBA (0.1 mg l^{-1})
MB_3	50% Макро MS	1.5 μM IBA (0.3 mg l^{-1})
MN_1	50% Macro MS	0.537 μM NAA (0.1 mg l^{-1})
MN_3	50% Macro MS	1.611 μM NAA (0.3 mg l^{-1})

Results and Discussion

The surface disinfection of the initial explants and establishment of *in vitro* culture

Mature cuttings

When disinfecting explants from mature cuttings according to the standard procedure, a high percentage of clean explants was obtained for all three types of lindens, ranging from 88 to 89.5% (Table 2). The highest percentage of uninfected explants was reported in *Tilia platyphyllos* in M_0 medium – 93%, followed by *Tilia tomentosa* with 91%. When sterilization with $AgNO_3$ was applied, the highest percentage of no infected explants was reported (97%) in *Tilia cordata*, which was higher than the control by 8.5%. In the other two linden species, the treatment with silver nitrate did not give better results (Table 2). It is known that woody plants grown outdoors have a rich microflora and this makes their sterilization difficult (Young et al., 1984). The high infection rate in our preliminary experiments necessitated the development of alternative protocols for explant disinfection and contamination control. In explants from actively growing shoots of adult trees and seedlings, a combination of the two sterilizing agents ($Ca(OCl)_2 + AgNO_3$) was used. With this method of disinfection a very high percentage of clean explants was achieved – over 89% in three studied types of linden (Table 3, Figure 1c, d, e), and no significant difference between the individual species was observed. In the apical buds, the clean, uninfected explants were in the narrow range from 94% (large-leaved linden) to 98% (small-leaved linden). The trend was similar for lateral buds. The least infected explants were reported for the plant material of *Tilia platyphyllos* – 98% of nutrient medium V_2.

In disinfection with a combination of calcium hypochlorite and silver nitrate of explants from actively grow-

Table 2. Influence of the sterilizing agent ($Ca(OCl)_2$ or $AgNO_3$)) on the success of disinfection of mature cuttings from *Tilia* (%)

Nutrient medium	*Tilia platyphyllos* Scop.		*Tilia cordata* Mill.		*Tilia tomentosa* Moench.	
	clean	infected	clean	infected	clean	infected
Sterilization by standard procedure (with 5% Ca $(OCl)_2$ – control)						
M	93	7	88	12	85	15
D_{10}	86	14	89	11	91	9
Average	89.5	10.5	88.5	11.5	88	12
Sterilization with $AgNO_3$						
M	78	22	96	4	77	23
D_{10}	81	19	98	2	72	28
Average	79.5	20.5	97	3	74.5	25.5

Table 3. Influence of sterilization with $Ca(OCl)_2 + AgNO_3$ on apical and lateral buds of actively growing shoots of adult *Tilia* trees (%)

Nutrient medium	*Tilia platyphyllos* Scop.		*Tilia cordata* Mill.		*Tilia tomentosa* Moench.	
	clean	infected	clean	infected	clean	infected
Apical buds						
V_1	95	5	98	2	96	4
V_2	97	3	96	4	97	3
V_3	96	4	96	4	97	3
D_{10}	94	6	95	5	95	5
Average	95.5	4.5	96.25	3.75	96.25	3.75
Lateral buds						
V_1	97	3	93	7	90	10
V_2	98	2	95	5	96	4
V_3	89	11	93	7	96	4
D_{10}	92	8	93	7	93	7
Average	94	6	93.5	6.5	93.75	6.25

Table 4. Effect of Ca $(OCl)_2$ + $AgNO_3$ sterilization on apical shoot tip explants from annual *Tilia* seedlings, %

Medium	*Tilia platyphyllos* Scop.		*Tilia tomentosa* Moench.	
	clean	infected	clean	infected
V_1	100	0	100	0

ing seedlings of large-leaved and silver-leaved linden, 100% efficiency was achieved (Table 4, Figure 1c). This method of surface disinfection of linden plant material was suitable for the establishment of explants *in vitro* from both actively growing shoots and mature cuttings. It confirmed our findings in apical explants from *Taxus baccata* L. that the combination of the two sterilizing agents led to a higher percentage of clean and vital explants (Ibrahim, 2011). These results were similar to the results of our previous study (Nacheva & Ivanova, 2017) with explants from *Ginkgo biloba* L. and plum (*Prunus domestica* x *Prunus cerasifera* 'Docera 6'), in which at the establishement of *in vitro* culture more than 80% and 70%, respectively, clean and vital explants were achieved by applying combined disinfection with $Ca(OCl)_2$ + $AgNO_3$.

Multiplication

On all tested nutrient media, the large-leaved linden explants developed successfully (Table 5, Figure 2). The plantlets cultivated on nutrient medium enriched with BAP (V_{12}) have the highest fresh mass – 1.2786 g, which is approximately 5 times more than those of the control variant (M_0). High results were also reported for nutrient medium with mT (V_1t) and 2iP (V_1p), 1.0091 g and 1.0027 g, respectively. Despite the high values of fresh mass in these variants, the highest percentage of dry mass is observed in the control – 17.95%, while the lowest is in V_{12} – 11.28%. Enrichment of the nutrient medium with the cytokinines BAP and mT stimulated the development of a larger central shoot, reaching 35.42 mm and 31.31 mm in length, respectively, which is almost two and a half times more than the control (12.16 mm). The other variants occupy an intermediate position. A similar trend was found with respect to the length of the lateral shoots – in nutrient medium with BAP and mT (V_{12} and V_1t) their length was the largest and statistically higher than the control. In these nutrient media the number of developed lateral shoots was also higher and with the inclusion of mT reaches 2.93 shoots, and BAP – up to 1.73 shoots. The number of explants obtained from one set explant (the so-called multiplication factor, K_M) is one of the most important indicators in *in vitro* propagation. Cytokinin mT is a hydroxylated analogue of BAP that improves the development and rooting of a number of species (Bairu et al., 2007; Aremu et al., 2012), including woody (Dimitrova et al., 2016). The better development of the explants on the medium with mT is also related to the development of a larger number of leaves (Table 5, Figure 2), as in this variant the largest number (8.73), significantly larger than control (2.07). The data on the morphological development of the microplants were also confirmed by the general outlook of the plantlets – the plants grown on nutrient medium with mT (V_1t) were distinguished with the best habitus, followed by those with medium with BAP (V_{12}) (Figure 2), which showed that these two nutrient media were most suitable for the multiplication of large-leaved linden.

Our results for the positive effect of cytokinin-enriched MS-based media are in consent with the reports of other authors. Chalupa (1987) also found that the nutrient medium MS enriched with BAP at low concentrations (0.2-0.6 mg l^{-1}) stimulated the growth and elongation of explants by *Tilia cordata* Mill. According to the same author (Chalupa, 2003) at a concentration of BAP 0.6 mg l^{-1}, a multiplication factor of 3 was achieved in *Til-*

Table 5. Morphological characteristics of *Tilia platyphyllos* Scop. plantlets at the multiplication stage

Medium	Fresh mass g	Dry mass %	Length of central shoot, mm	Length of lateral shoot, mm	Number of shoots, K_M	Number of leaves
M_0	0.2490	17.95	12.16	10.99	1.07	2.07
V_{1k}	0.6401	15.46	15.48	17.02	1.20	3.09
V_{12}	1.2786	11.28	35.42	18.74	1.73	5.53
V_{1p}	1.0027	14.59	20.05	13.90	1.33	3.60
V_{1t}	1.0091	12.80	31.31	18.76	2.93	8.73
GD p = 5.0%	3.08		10.45	6.17	1.52	2.11
GD p = 1.0%	4.47		15.14	8.94	2.21	3.06
GD p = 0.1%	6.70		22.71	13.41	3.31	4.60

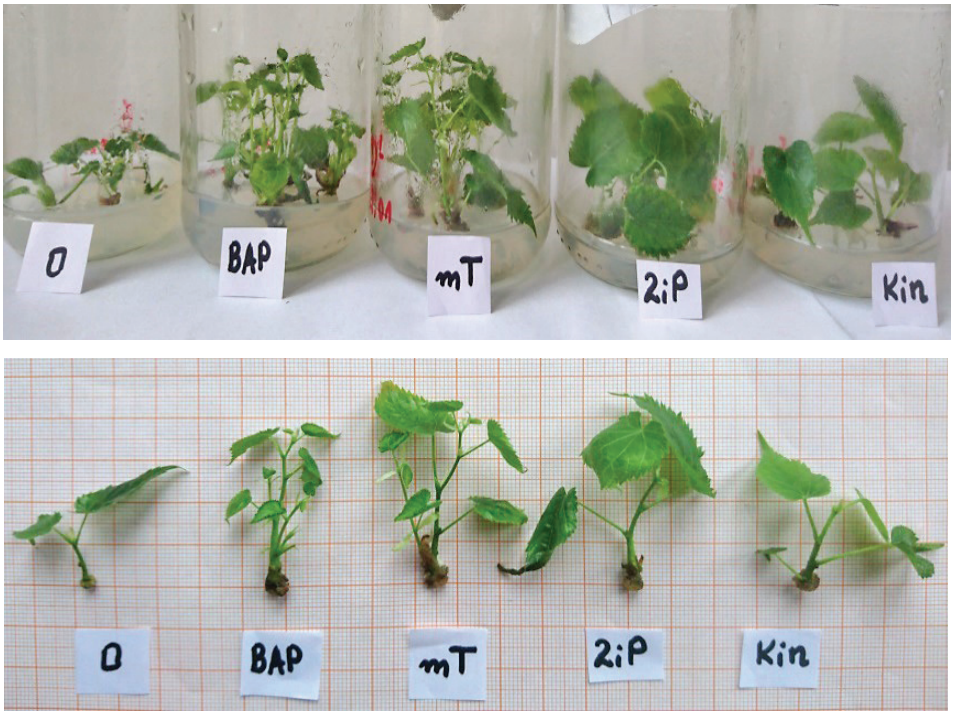

Fig. 2. Development of *Tilia pltyphyllos* microplants cultivated on multiplication media with different cytokinins (0 – control, without growth regulators, BAP – medium enriched with 5 µM BAP (V_{12}), mT – medium enriched with 5 µM mT (V_1t), 2iP – medium enriched with 5 µM 2iP (V_1p) and Kin – medium enriched with 5 µM Kinetin (V_1k)

ia platyphyllos. Sarvasova and Durkovic (2002) obtained between 2 and 3 shoots per explant in European linden (*Tilia x europeana* L.) when use for culturing the medium with 0.2 mg l^{-1} BAP. Similar results were achieved by Zurita-Valencia and co-authors (2014) of the nutrient medium MS enriched with 1 mg l^{-1} BAP and 0.25 mg l^{-1} NAA, which achieved a high K_M – 7.75 shoots per explant in *Tilia mexicana* Schlecht. According to Cvrčková, et al. (2018), the nutrient medium MS is most suitable for the growth and viability of explants from *Tilia cordata* Mill. Our results confirmed the findings of Ucler and Mollamehmetoglu (2001) that BAP is more effective than kinetin in multiplication of *Tilia platyphyllos*. Wei et al. (2013) reported that the largest number of shoots (2.5) and nodal segments (4.5) of *Tilia miqueliana* Maxim. were obtained on WPM medium with 2.22 µM BAP, 2.28 µM zeatin, and 0.1 µM IBA. Our results for the influence of mT on the multiplication of large-leaved linden, which achieved the maximum number of new lateral shoots (2.93) with optimal length (18.76 mm), suitable for transferring a new passage, as well as the maximum number of leaves, are the first in the scientific literature on the application of natural cytokinin mT in linden plants.

Rooting of the micro-shoots of linden

In all tested variants, as well as in the hormone free control, a relatively high percentage of rooted plants was reported – over 53% (Table 6, Figure 3). As expected, the lowest percentage of rooted plants was reported in control – 53.85%. Enrichment of the nutrient medium with auxins IAA, IBA, and NAA led to an increase in the percentage of rooted plants. At higher concentration of all three auxins, better rooting was obtained compared to lower concentrations (0.1 mg l^{-1}). The best results were achieved when enriching the nutrient medium with 0.3 mg l^{-1} IBA (84.61%). There were no significant differences in the number of roots in the studied variants, as well as in the number of leaves (Table 6). A larger number of plants grown at a higher concentration of the respective auxin developed lateral roots.

The plantlets from variants with 0.3 mg l^{-1} IBA and 0.1 mg l^{-1} IAA had the shortest stem length, but the differences with the control have not been statistically proven (Table 6). The differences of the other options compared to the control were not statistically proven. There was no significant difference in the number of leaves between the control and the auxin-treated microcuttings (Table 6). Sarvasova & Durkovic (2002) achieved 50% rooting on WPM medium with half-re-

Table 6. Growth parameters of *in vitro* rooted plants of large-leaved linden (*Tilia platyphyllos* Scop.)

Variants	Rooting (%)	Number of roots per plant	Lateral roots (%)	Length of root (mm)	Stem length (mm)	Number of leaves
M_0	53.85	1.85	23.07	48.96	18.91	2.46
MA_1	55.29	2.33	17.65	30.92	20.02	2.59
MA_3	68.75	1.64	25	35.53	14.43	1.93
MB_1	65.45	1.80	0	39.14	14.09	1.90
MB_3	84.61	1.73	30.71	35.31	19.34	2.46
MN_1	57.64	2.33	0	27.02	17.65	2.13
MN_3	70.58	1.36	35.29	48.39	17.84	2.35
GD p = 5.0%		1.56		5.71	4.86	2.12
GD p = 1.0%		2.19		8.02	6.82	2.97
GD p = 0.1%		3.10		11.32	9.63	4.2

Fig. 3. Influence of auxins on the rooting of microcuttings of *Tilia platyphyllos*. M0 – control, hormonal nutrient medium; MB1 and MB3 – nutrient media with IBA (0.1 and 0.3 mg l^{-1}, respectively); MN1 and MN3 – nutrient media with NAA (0.1 and 0.3 mg l^{-1}, respectively); MA1 and MA3 – nutrient media with IAA (0.1 and 0.3 mg l^{-1}, respectively)

duced macro- and micronutrients and enriched with 2 mg l^{-1} IBA or NAA. These results were close to those obtained by us on a hormone-free nutrient medium MS and one enriched with 0.1 mg l^{-1} IAA or NAA. When applying the three auxins (IAA, IBA or NAA) at a concentration of 0.3 mg l^{-1}, our results were significantly higher (65-84%). The beneficial effect of auxin IBA found by us is in confirmation of the results reported by Zurita-Valencia and co-authors (2014), which obtained 100% rooting of *Tilia mexicana* Schlecht. when applying also MS medium with IBA, but in significantly higher concentrations (5.0 mg l^{-1}). Similarly, Wei et al. (2013) reported that the highest rooting rate (91.7%) of *Tilia miqueliana* Maxim. obtained on MS medium with reduced by half and 3 mg l^{-1} IBA. These differences in rooting success are probably due to different genotypes, as it is known that the ability to form root, especially in woody species, is highly dependent on the genotype. Lindens are propagated mainly by seeds, but their germination is often low due to their deep dormancy and the need for a long period to overcome it. *Tilia* plants are cross-pollinated and the possibility of their improvement is very slow, so experts focus on the application of vegetative propagation of lindens, as the reproductive potential is much higher, which in economically important genotypes leads to their faster propagation and to obtain wood with more valuable qualities, high tolerance to stress and disease resistance. Our experiments showed that the micropropagation of linden could be a practical solution for rapid multiplication of selected genotypes with desired characteristics. *In vitro* propagation techniques of elite trees of the genus *Tilia* provide a number of advantages such as

the ability to produce a large number of trees with valuable economic qualities in a relatively short time. The use of elite genotypes could contribute to improving the quality of linden plantations.

Conclusions

The present study describe an effective protocol for *in vitro* micropropagation of linden.

- The most suitable explants for establishment of *in vitro* culture of linden are apical and stem cuttings of actively growing shoots;
- The most successful disinfection of plant material is a combination of 5% $Ca(OCl_2)$ and 2% $AgNO_3$;
- The nutrient media based on MS, enriched with 5 μM mT or 5 μM BAP and 0.01 μM IBA are the most effective for multiplication of large-leaved linden;
- Rooting of microcuttings is most successful when applying MS medium with reduced by half macronutrients, supplemented with 0.3mg l^{-1} IBA.

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