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In vitro propagation of *Fraxinus excelsior* L. by epicotyls

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Epicotyl segments from *in vitro* 45-day-old seedlings were used as explants. For inducing axillary shoot formation, WPM supplemented with IBA (0.01 mg l^{-1}), BAP (3.0 or 4.0 mg l^{-1}), or TDZ (0.1 or 0.5 mg l^{-1}) were applied. Multiplication rate was low after 8 weeks of cultivation. During this period, TDZ promoted a significantly higher rate of multiplication than BAP. After 16 weeks, the multiplication increased in all variants studied (mean number of shoots ranged from 4.80 ± 1.59 to 11.33 ± 2.33), but there were no significant differences between the cytokinins and their concentrations. Increasing of BAP concentration to 4.0 mg l^{-1} promoted multiple shoot formation, but shoots were short and often vitrified, and in some cases, formation of both fasciated and normal shoots was observed. Conventional and polarized light microscopy revealed anatomical differences between fasciated and normal shoots. For example, the vascular cylinder in fasciated stems was not circular but elliptical in cross section. Compared to normal stems, the cortex in fasciated stems appeared less differentiated on the 16th week of cultivation, and the stem thickness was considerably larger. Adventitious root formation was studied on half-strength WPM, supplemented with either IBA (0.5 mg l^{-1}), NAA (0.5 mg l^{-1}), or a combination of IBA and NAA (0.5 mg l^{-1} and 0.5 mg l^{-1}, respectively). After 7, 14, and 21 days on these inductive media, parts of the cultures were transferred on half-strength, hormone-free WPM (expressive medium). The highest percentage of rooting (96.67 ± 3.33%) and the most developed root system were achieved on an inductive medium with the combination of IBA and NAA for 14 days, and then transfer to a expressive medium.

Key words: common ash, fasciated shoots, *Fraxinus excelsior*, rhizogenesis, tissue culture.

Abbreviations: BAP: 6-benzylaminopurine; FAA: formalin acetic acid; IBA: indole-3-butyric acid; NAA: α-naphthalene acetic acid; TDZ: thidiazuron; WPM: woody plant medium.

INTRODUCTION

Common ash (*Fraxinus excelsior* L.) is an important forest tree in Europe. For large-scale nursery production, common ash is propagated by seeds that have to be subjected to long stratification (32 weeks) to overcome dormancy, which may otherwise last up to 6 years (Piotto, 1994; Suszka *et al.*, 1996; Milev *et al.*, 2004). *Fraxinus excelsior* cuttings are generally considered difficult to root, and rooting has been found to be possible only in juvenile material (Spethmann, 1982; Thompson *et al.*, 2001). Furthermore, the production of large quantities of grafts is limited by the season and the period of rootstock production.

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In vitro propagation allows large-scale production of plants and may provide rejuvenated plants with a high rooting capacity (Hackett & Murray, 1993). It has been reported that *in vitro* propagation of *F. excelsior* is possible by using (1) nodal and apical segments from 3-7 years old seedlings as explants (Chalupa, 1983, 1987a, b, 1990; Silveira & Cottignies, 1994), (2) buds from mature or grafted trees (Hammatt, 1994; Silveira & Nougarède, 1995; Nougarède *et al.,* 1996; Thompson *et al.,* 2001; Schoenweiss & Meier-Dinkel, 2005), and (3) adventitious shoot formation from embryo hypocotyls (Tabrett & Hammatt, 1992), cotyledons (Hammatt, 1996), leaves (Hammatt & Ridout, 1992), and petioles (Hammatt, 1996). Depending on the juvenility and rhizogenous activity of the clone used, different authors achieved 57 to 100% rooting (Chalupa, 1983, 1987a, b, 1990; Hammatt & Ridout, 1992; Hammatt, 1994, 1996; Silveira & Cottignies, 1994; Nougarède *et al.,* 1996; Thompson *et al.,* 2001).

The goal of this work was to find a method of large-scale production of *Fraxinus excelsior* plants by epicotyl segments. Our efforts were aimed at identifying successful types and concentrations of growth regulators for multiplication and adventitious root formation, and to compare the anatomical structure of fasciated shoots with that of normal shoots.

MATERIALS AND METHODS

Effects of the type and concentration of cytokinin on axillary shoot formation

Plant material

Epicotyl segments from *in vitro* 45-day-old seedlings were used as explants. They were with a length higher than 10 mm, having at least one internode and with their bottom leaves removed. Three replications, each containing ten explants were used per treatment.

Culture medium

For inducing axillary shoot formation, the WPM medium (Lloyd & McCown, 1980) was used in the following treatments: no cytokinin (control); 3.0 or 4.0 mg l^{-1} BAP plus 0.01 mg l^{-1} IBA (treatments WPM3 and WPM4, respectively); 0.1 or 0.5 mg l^{-1} TDZ plus 0.01 mg l^{-1} IBA (treatments WPM8 and WPM9, respectively).

Every two weeks, the explants were subcultured on the same fresh medium. Shoots that were longer than 20 mm and had one or two internodes were cut into shoot tips, their bottom leaves were removed, and then the tips were placed in the medium for the next multiplication. On the 8th and 16th week, the number of shoots and the length of each shoot were determined.

Anatomical study

Fasciated and normal shoots were harvested on the 16th week of cultivation and fixed in FAA solution. They were then transferred to an automatic tissue processor (Leica Histokinette 2000; Leica Microsystems, Wetzlar, Germany) and processed for embedding in paraffin, as described by Kitin *et al.* (2005). Histological sections were stained with Harris' hematoxylin and eosin for visualization of nuclei and mitotic activity of cells, or with alcian blue for visualization of polysaccharides. After dehydration, the sections were mounted in Entelan (Merck, Germany). Radial and cross sections were examined using conventional and a polarized light microscope (BX-50; Olympus, Tokyo, Japan), or a fluorescent microscope (BX-60, Olympus, Tokyo, Japan) under the following conditions: dichroic mirror (DM 505), excitation filter (BP 460-490), and long-pass emission filter (BA 515IF). Images were captured using a digital CCD camera (DP70; Olympus, Tokyo, Japan).

Effects of the type of auxin and inductive media on adventitious root formation

After the multiplication phase, shoots that were longer than 20 mm were transferred to different variants of an inductive, half-strength WPM rooting medium 1) free of auxin, 2) supplemented with 0.5 mg l^{-1} IBA, 3) supplemented with 0.5 mg l^{-1} NAA, and 4) a combination of 0.5 mg l^{-1} IBA and 0.5 mg l^{-1} NAA for 7, 14, and 21 days, and then on an expressive rooting medium (half-strength WPM without auxins). For comparison of the results, the shoots were cultivated on the same variants of half-strength WPM without transfer to an expressive medium.

Three replications, each containing ten explants, were cultured in each variant. After 42 days, the number of rooted plants, as well as the number and length of induced roots were determined.

Condition of the cultivation

Each medium and its variants contained 20 g l^{-1} sucrose and 7.1 g l^{-1} agar (Sigma). Shoot cultures were established in test tubes containing 12 ml of culture medium. The pH of all media was adjusted to 5.6 before autoclaving (under 118 kPa and 120°C for 20

min). The cultures were grown in a cultivation chamber at 25 ± 1 °C with 16 hrs of cool white fluorescent light at a photon flux density of 80 μmol m^{-2} s^{-1} per day.

The results were analyzed by ANOVA (post hoc LSD test) using SPSS 10.0 (SPSS for Windows, 1999).

RESULTS

Effects of the type and concentration of cytokinin on auxiliary shoot formation

All epicotyl segments died on cytokinin-free WPM medium after one week of cultivation (data not shown). The first signs of new shoot formation were detected in all other variants of the medium 10-14 days after culture establishment. Multiplication rates were low after 8 weeks of cultivation. During this period, higher concentrations of BAP did not influence multiplication rates, but TDZ promoted significantly higher levels of multiplication than BAP. The multiplication rates in all variants increased after 16 weeks of cultivation. However, there were no significant differences among multiplication rates obtained using BAP in different concentrations (Table 1). On the other hand, mortality was noticed in the TDZ-containing media after 8 weeks of cultivation. The highest concentration of BAP (4.0 mg l^{-1}) induced vitrification, whereas no vitrification was observed on the medium containing 3.0 mg l^{-1} BAP.

After 8 weeks of cultivation, shoot length was significantly higher in plants cultivated on medium containing 4 mg l^{-1} BAP and vice versa in plants cultivated on medium containing 0.5 mg l^{-1} TDZ. Also, it must be noted that there were no differences in the shoot length between variants containing lowest concentrations of BAP (3 mg l^{-1}) and TDZ (0.1 mg l^{-1}).

FIG. 1. Multiplication of *Fraxinus excelsior* by epicotyls after cultivation for 16 weeks on WPM supplemented with 3.0 mg l^{-1} BAP and 0.01 mg l^{-1} IBA (WPM3).

TABLE 1. Effects of the type and concentration of cytokinins on mean (± SE) shoot number

Growth regulators	8 weeks after culture establishment	16 weeks after culture establishment
3 mg l^{-1} BAP + 0.01 mg l^{-1} IBA	1.67 ± 0.33[b]	11.00 ± 1.82[a]
4 mg l^{-1} BAP + 0.01 mg l^{-1} IBA	1.40 ± 0.93[b]	11.33 ± 2.33[a]
0.1 mg l^{-1} TDZ + 0.01 mg l^{-1} IBA	5.00 ± 0.65[a]	11.17 ± 3.95[a]
0.5 mg l^{-1} TDZ + 0.01 mg l^{-1} IBA	5.40 ± 1.03[a]	4.80 ± 1.59[b]

Means followed by the same letters are not significantly different at $p \leq 0.05$

TABLE 2. Effects of the type and concentration of cytokinins on mean (± SE) shoot length (mm)

Growth regulators	8 weeks after culture establishment	16 weeks after culture establishment
3 mg l^{-1} BAP + 0.01 mg l^{-1} IBA	6.67 ± 1.15^{ab}	$12.05 \pm 1.28a$
4 mg l^{-1} BAP + 0.01 mg l^{-1} IBA	8.71 ± 1.30^{a}	10.18 ± 0.91^{a}
0.1 mg l^{-1} TDZ + 0.01 mg l^{-1} IBA	4.97 ± 0.44^{b}	3.63 ± 0.40^{b}
0.5 mg l^{-1} TDZ + 0.01 mg l^{-1} IBA	2.78 ± 0.34^{c}	2.54 ± 0.37^{b}

Means followed by the same letters are not significantly different at $p \leq 0.05$

After 16 weeks of cultivation, shoot length was significantly higher in plants cultivated on the BAP-containing medium than in those cultivated on the TDZ-containing medium. However, higher cytokinin concentrations did not influence shoot elongation rate (Table 2, Fig. 1).

Histological analysis of proliferated normal and fasciated shoots

The appearance of fasciated shoots was occasionally observed on the medium containing 4.0 mg l^{-1} BAP. The larger diameter of the flattened stems ranged from 3 to 5 mm and the smaller one was typically around 2 mm. Cross sections of normal and fasciated stems are shown in Figs 2-7. Fasciated shoots had irregular or elliptical shapes, whereas normally developed stems were more or less rounded. The vascular cylinder in normal stems was rounded, with small wedges of pith parenchyma and vascular cells projecting outwards into the cortex (Fig. 6). Mitotic activity of perimedullar parenchyma and cambial cells was observed in these pith wedges (Fig. 7). Division of cambial cells and differentiating vascular cells were seen in both normal and fasciated stems (Figs 8 and 9). The cortex width did not appear larger in fasciated stems than in normal stems. However, there was an increase in the cross-sectional area of pith in fasciated stems (Figs 2-5). This resulted in a considerable increase in the volume of fasciated stems compared to that of normal stems. In our sections, phloem fibers were frequently observed in normal stems (arrowhead in Figs 10 and 11) but were not seen in fasciated stems, which suggests that cortex differentiation occurs later in fasciated stems than in normal stems.

Effect of the type of auxin and inductive media on adventitious root formation

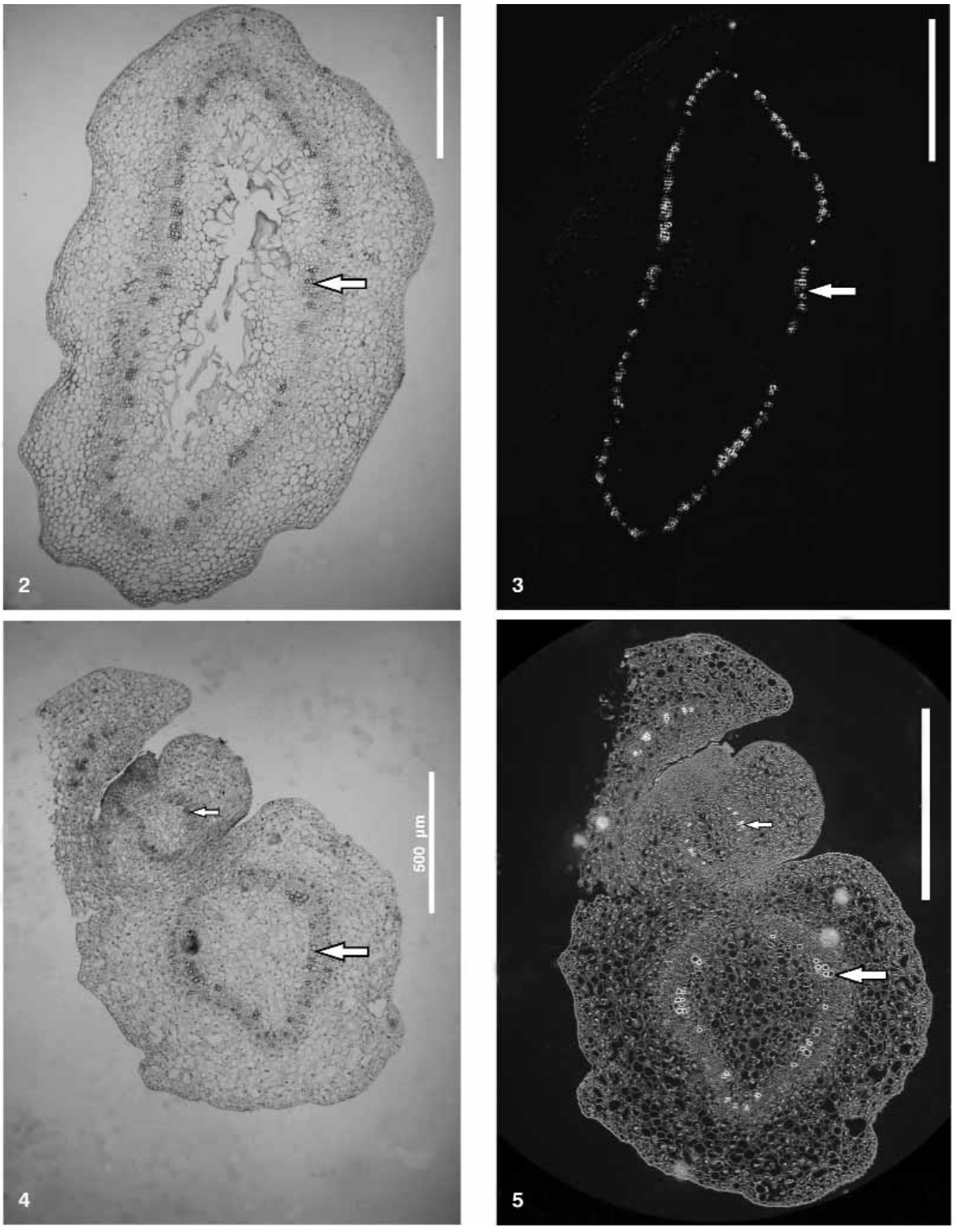
Rooting was observed in all rooting variants of the medium, but rooting percentage was significantly lower on auxin-free media. On the medium containing IBA or NAA alone, the percentage of rooted shoots was significantly higher when no transfer was performed (i.e., plants were cultivated on inductive medium only). When a combination of NAA and IBA was applied, the opposite tendency was observed. The rate of rooting was significantly increased after longer periods of cultivation (14 and 21 days) on inductive medium (Table 3).

The largest calluses were formed when NAA was applied to the medium. This trend was better expressed when no transfer was performed (inductive medium only). The adventitious roots were thick and with numerous second-order roots and fine hairs (Fig. 12). The opposite trend was noticed when IBA was used in the medium (Fig. 13). When NAA and IBA were applied in combination, the amount of callusing was small and numerous root hairs and lateral roots developed.

No callus formation was observed on the auxin-free medium (control) and the resulting roots were thin and without lateral branches.

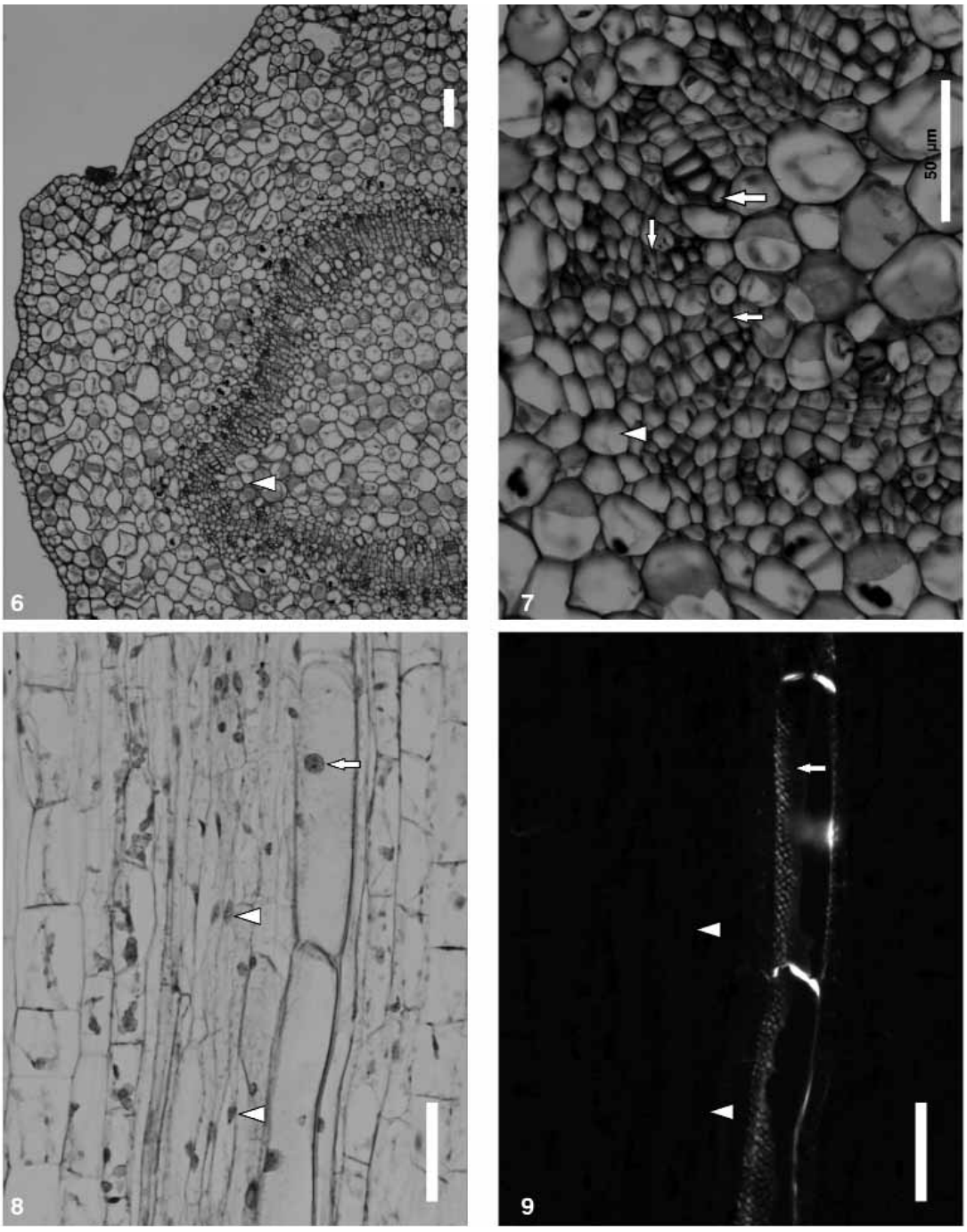
The highest root number (4.35 ± 0.51) was achieved on medium supplemented with NAA for 14 days (Table 4), and the highest root length was obtained when NAA was applied for 21 days (28.69 ± 1.85 mm) or when combination of IBA and NAA was used without transfer of plants on expressive rooting medium (28.46 ± 1.31 mm) (Table 5).

Rooting percentage depended on the type of auxin ($F = 16.57$, $p \leq 0.05$), the duration of cultivation on inductive medium ($F = 7.62$, $p \leq 0.05$) and the interaction between these two factors ($F = 3.37$, $p \leq 0.05$, Table 6). Root number depended on the duration of cultivation on inductive medium ($F = 3.84$, $p \leq 0.05$) and the interaction between type of auxin and duration of cultivation on inductive medium ($F = 4.08$, $p \leq 0.05$, Table 6). Root length depended only on the type of auxin ($F = 12.90$, $p \leq 0.05$, Table 6).



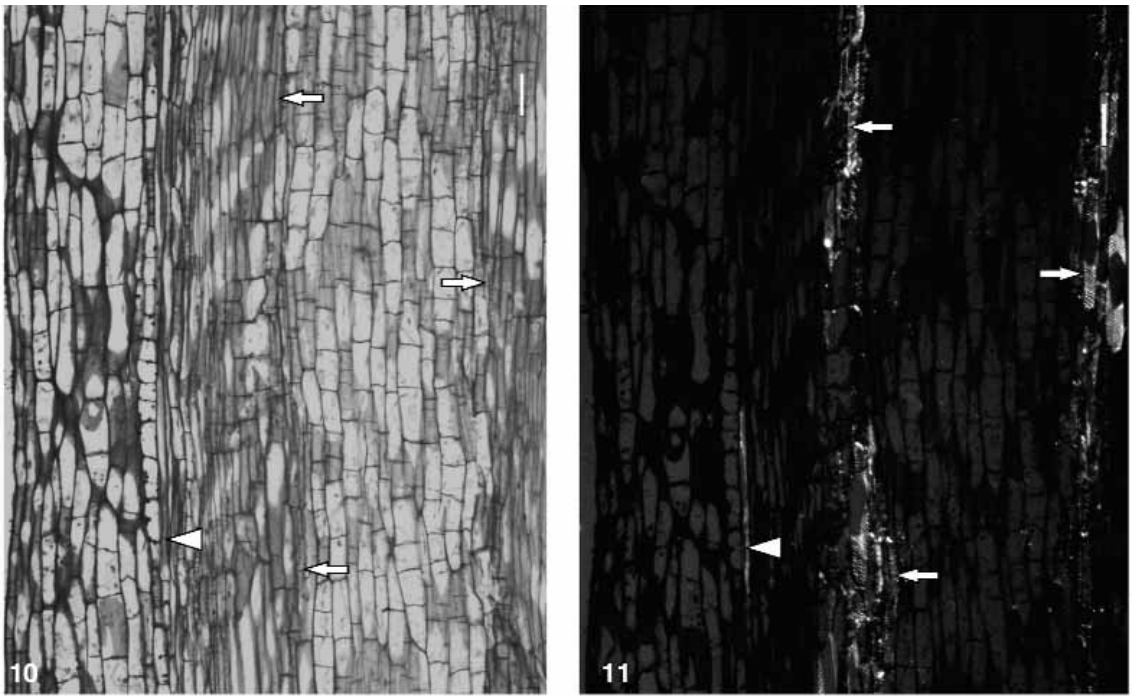
FIGS 2-5. Cross sections of fasciated and normal stems of *in vitro* proliferated *Fraxinus excelsior*.

Fig. 2 and Fig. 4. Bright-field images of a fasciated and a normal stem, respectively. Staining with hematoxylin-eozin.
Fig. 3. Corresponding image by polarized-light of the section in Fig. 2.
Fig. 5. Corresponding image of the section in Fig. 4 viewed by fluorescence microscopy. Large arrows point to xylem cells in the vascular cylinders which are birefringent under polarized light Fig. 3 and show brighter fluorescence Fig. 5. Small arrows in Fig. 4 and Fig. 5 point to the vascular tissue of a branch (another proliferating shoot). Note that the images in Figs 2, 3, 4 are viewed at the same magnification (Bars = 500 µm).



FIGS 6-9. Light microscopy images of normal stem of *in vitro* proliferated *Fraxinus excelsior*.

Fig. 6. Bright-field image showing a general view of the cortex, vascular cylinder, and pith. Staining with alcian blue. The shape of the vascular tissue in cross section is not circular but has wedges projecting outwards into the cortex (arrowhead).
Fig. 7. Image of the same section as in Fig. 6 showing an enlarged view of the wedged part of vascular cylinder. Large arrow points to xylem cells, small vertical arrow points to cambial cells producing xylem and phloem, small horizontal arrow points to dividing perimedullar cells, and arrowhead points to a cell of the inner layer of cortex.
Fig. 8. Bright-field image of a longitudinal section showing developing vascular tissue. Staining with hematoxylin-eozin. Arrow points to a nucleus of a differentiating vessel element. Cambium, phloem and cortical cells are at the left side of the differentiating vessel. Arrowheads point to nuclei in cambial cells.
Fig. 9. Corresponding image by polarized-light of the section in Fig. 8 showing birefringence of the walls of differentiating vessel elements (Bars = 50 µm).



FIGS 10, 11. Longitudinal section of a normal stem of *in vitro* proliferated *Fraxinus excelsior*. Staining with alcian blue.

Fig. 10. Bright-field image showing portions of cortex (to the left of the arrowhead), vascular tissue (between arrowhead and left arrows), and pith. Arrows point to xylem cells at the outer sides of the pith parenchyma. Arrowhead points to a phloem fiber at the inner side of the cortex.
Fig. 11. Corresponding image by polarized-light of the section in Fig. 10 showing birefringence of the walls of xylem cells (arrows), and phloem fibers (arrowhead) (Bars = 50 μm).

TABLE 3. Effects of the type of auxin and duration of cultivation on inductive medium on rooting of adventitious shoots (%)

Duration of cultivation on inductive medium (days)	Mean ± SE
1/2 WPM (control, auxin-free)	
Without transfer	26.67 ± 3.33
1/2 WPM + 0.5 mg l^{-1} NAA	
Without transfer	93.33 ± 3.33^{ab}
7 days	63.33 ± 3.33^{def}
14 days	66.67 ± 3.33^{def}
21 days	70.00 ± 10.00^{de}
1/2 WPM + 0.5 mg l^{-1} IBA	
Without transfer	96.67 ± 3.33^{a}
7 days	50.00 ± 5.77^{g}
14 days	56.67 ± 3.33^{fg}
21 days	73.33 ± 3.33^{cd}
1/2 WPM + 0.5 mg l^{-1} IBA + 0.5 mg l^{-1} NAA	
Without transfer	73.33 ± 3.33^{cd}
7 days	60.00 ± 0.00^{efg}
14 days	96.67 ± 3.33^{a}
21 days	83.33 ± 3.33^{bc}

Different letters indicate significant difference ($p \leq 0.05$)

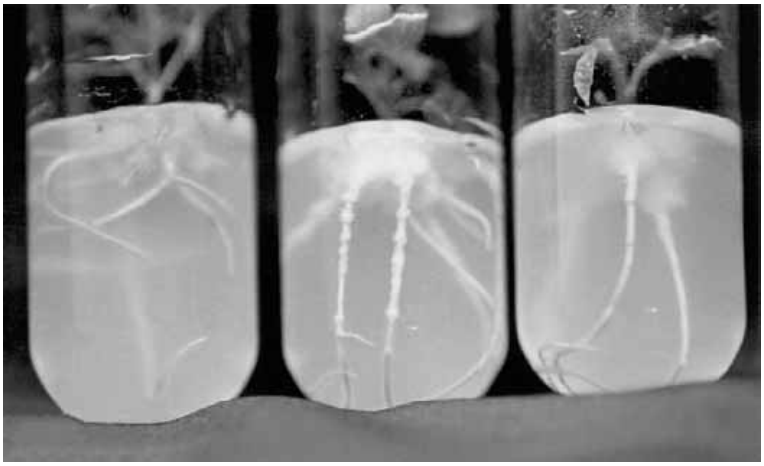


FIG. 12. Adventitious root induction after 42 days on half-strength WPM supplemented with 0.5 mg l^{-1} NAA.

FIG. 13. Adventitious root induction after 42 days on half-strength WPM supplemented with 0.5 mg l^{-1} IBA.

TABLE 4. Effects of the type of auxin and duration of cultivation on inductive medium on mean root number

Duration of cultivation on inductive medium (days)	Mean ± SE
1/2 WPM (control)	
Without transfer	3.72 ± 0.35^{abc}
1/2 WPM + 0.5 mg l^{-1} NAA	
Without transfer	$2.60 \pm 0.27de$
7 days	2.89 ± 0.22^{cde}
14 days	4.35 ± 0.51^{a}
21 days	2.26 ± 0.21^{e}
1/2 WPM + 0.5 mg l^{-1} IBA	
Without transfer	3.86 ± 0.53^{ab}
7 days	2.40 ± 0.21^{de}
14 days	2.59 ± 0.17^{de}
21 days	3.00 ± 0.21^{bcde}
1/2 WPM + 0.5 mg l^{-1} IBA + 0.5 mg l^{-1} NAA	
Without transfer	3.76 ± 0.38^{ab}
7 days	2.78 ± 0.17^{cde}
14 days	3.59 ± 0.24^{abc}
21 days	3.36 ± 0.26^{bcd}

Different letters indicate significant difference ($p \leq 0.05$)

TABLE 5. Effects of the type of auxins and duration of cultivation on inductive medium on mean root length (mm)

Duration of cultivation on inductive medium (days)	Mean ± SE
1/2 WPM (control)	
Without transfer	22.63 ± 0.94^{bcde}
1/2 WPM + 0.5 mg l^{-1} NAA	
Without transfer	23.52 ± 1.68^{bcde}
7 days	23.28 ± 1.30^{bcde}
14 days	25.88 ± 2.59^{abcd}
21 days	28.69 ± 1.85^{a}
1/2 WPM + 0.5 mg l^{-1} IBA	
Without transfer	21.15 ± 1.23^{ef}
7 days	17.83 ± 1.21^{f}
14 days	20.42 ± 1.88^{df}
21 days	20.55 ± 1.39^{df}
1/2 WPM +0.5 mg l^{-1} IBA + 0.5 mg l^{-1} NAA	
Without transfer	28.46 ± 1.31^{a}
7 days	26.85 ± 1.65^{abc}
14 days	27.37 ± 2.00^{ab}
21 days	25.87 ± 1.34^{abc}

Different letters indicate significant difference ($p \leq 0.05$) using LSD test

TABLE 6. Effects of different factors on the percent of rooted shoots, root number, and root length tested by ANOVA

Source of variation	Percent of rooted plants			Root number			Root length		
	d.f.	F-ratio	*p*-level	d.f	F-ratio	*p*-level	d.f.	F-ratio	*p*-level
A	3	16.57	0.001	3	1.31	0.27	3	12.90	0.001
T	3	7.62	0.001	3	3.86	0.01	3	1.11	0.34
A × T	6	3.37	0.001	6	4.08	0.001	6	1.49	0.18

A: Type of auxin used, T: Duration of cultivation on inductive media

DISCUSSION

In previous studies on *Fraxinus excelsior* it has been reported that TDZ is more effective than BAP in inducing shoot regeneration from embryo hypocotyls, cotyledons, whole leaves, and petioles (Tabrett & Hammatt, 1992; Hammatt, 1994, 1996; Thompson *et al.,* 2001). In the present study, a similar result was observed after 8 weeks of cultivation. After 16 weeks of cultivation, there was no significant difference between the influences of TDZ and BAP on shoot proliferation.

Although the presence of TDZ in the medium has been reported to allow shoot proliferation (Tabrett & Hammatt, 1992; Huetteman & Preece, 1993; Hammatt, 1994; 1996), our experiments showed that BAP is more effective than TDZ for shoot elongation.

Our results suggested that TDZ is an important agent for inducing shoot regeneration for a period of 8 weeks. Tabrett & Hammatt (1992) reported that the mortality in cultures increases with time on medium with TDZ. Our results agree with this finding and showed that TDZ causes mortality of the *in vitro*-obtained shoots and has a suppressive effect on the shoot elongation in comparison with BAP. BAP (3.0 mg l^{-1}) is suitable for inducing shoot regeneration and elongation for longer periods of cultivation.

In our experiments, fasciated shoots were occasionally observed in cultures containing 4.0 mg l^{-1} BAP in the medium. Formation of fasciated shoots of *in vitro* propagated *Fraxinus excelsior* has not been previously reported, although many experiments with this species have been performed (Chalupa, 1983, 1990; Hammatt & Ridout, 1992; Tabrett & Hammatt, 1992; Hammatt, 1994, 1996; Nougarède *et al.* 1996; Silveira & Nougarède, 1995; Schoenweiss & Meier-Dinkel, 2005). The appearance of fasciated shoots in this experiment was infrequent (only a few fasciated shoots were observed), but it should be noted that fasciated shoots have also been observed during *in vitro* propagation of *Betula pendula* (Iliev & Tomita, 2003; Iliev *et al*., 2003), and *Prunus avium* (Kitin *et al.*, 2005). The appearance of fasciation in *in vitro*-propagated silver birch may have been caused by p-fluorophenylalanine (FPA), because no fasciation was observed in the absence of FPA (Srivastava & Glock, 1987). Exogenously applied cytokinins induced fasciation in *Kalanchoe blossfeldiana* (Varga *et al.*, 1988), *Betula pendula* (Iliev & Tomita, 2003) *Prunus avium* (Kitin *et al.*, 2005) and *Mammillaria elongata* (Papafotiou *et al.*, 2001).

It has been suggested that in fasciated stems "a genetic mechanism might operate through a hormonal imbalance that is restricted to the meristem and its immediate vicinity" (Boke & Ross, 1978). Nilsson *et al*. (1996) reported that fasciated tissues in hybrid *Populus tremula* contained elevated levels of free cytokinins. It is not clear whether the fasciation observed in our experiments was caused by higher BAP concentrations. It was shown, however, that higher multiplication rates may result in the formation of fasciations.

It has been shown that the type of auxin has a central role in the initiation of adventitious roots (De Klerk, 2001; De Klerk *et al.*, 1997, 1999; Machakova *et al.*, 2008). Auxin is required to induce cell division, as well as for the organization of the primordium. After the inductive phase, once the cells have been determined to root formation, auxin is no longer required (Berthon *et al.* 1989, 1990; Hammatt & Ridout, 1992; Hammatt, 1996; Iliev & Iliev, 1997). Actually, at this stage, auxin becomes deleterious since it blocks the outgrowth of root primordia, the growth of roots, and the development of shoots (Gaspar & Coumans, 1987; De Klerk *et al*., 1997, 1999; De Klerk, 2001).

The quality of the root system is an important factor for the successful acclimatization of *in vitro*-propagated plants (McClelland *et al.*, 1990). Some researchers have observed that the *in vitro*-formed roots lack lateral roots, and either lack or have a reduced number of root hairs (Yde & Liaw, 1977). Similar effects were observed after using IBA for *in vitro* rooting of *Betula pendula* (Iliev *et al.*, 2001; Iliev & Tomita, 2003).

Our results showed that the percentage of rooting depends crucially on the type of auxin and duration of cultivation on inductive medium. These factors, however, had different effects on the quality of root systems formed. The highest percentage of rooted plants and the highest quality of root system were achieved when a combination of NAA and IBA was applied for 14 days.

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