IN VITRO PROPAGATION OF *Fraxinus excelsior* L.

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Abstract. Epicotyls, having at least one internode and with their bottom leaves removed and hypocotyls with cotyledons, isolated from *in vitro* 30-day-old seedlings were used as explants. For induction of axillary shoot formation, the explants were cultivated on WPM and MS media supplemented with 0.5 or 1.0 mg l^{-1} TDZ plus 0.1 mg l^{-1} IBA. The highest multiplication rate was found after cultivation for 8 weeks on MS medium supplemented with 1.0 mg l^{-1} TDZ plus 0.1 mg l^{-1} IBA. Also, in comparison with epicotyls, the multiplication rate of hypocotyls was lower (9.36 ± 1.94 and 4.12 ± 0.98, resp.). However, opposite tendency was found for the length of shoots. It was significantly higher in shoots, originated from hypocotyls on MS medium supplemented with 0.5 mg l^{-1} TDZ plus 0.1 mg l^{-1} IBA (15.04 ± 2.58 mm). Adventitious roots formation was studied on half-strength WPM, supplemented with 2.5 mg l^{-1} IBA plus 2.5 mg l NAA. After 24, 48 or 72 h on this inductive medium, the shoots were transferred on half-strength, auxin-free WPM (expressive medium). The highest rate of rooting (73.33 ± 3.33%) was achieved on an inductive medium applied for 24 h, and then transferred to an expressive medium.

Keywords: common ash, epicotyls, hypocotyls, rooting, thidiazuron.

Rezumat. Propagarea *in vitro* a *Fraxinus excelsior* L. Epicotilele, având cel puţin un internodul şi cu frunzele îndepărtate de la bază, şi hipocotilele cu cotiledoane, izolate din răsaduri de 30 de zile *in vitro* au fost folosite ca explante. Pentru inducerea formării lăstarilor auxiliari, explantele sunt cultivate pe medii WPM şi MS suplimentate cu 0,5 sau 1,0 mg l^{-1} TDZ plus 0,1 mg l^{-1} IBA. Cea mai mare rată de multiplicare a fost identificată după cultivarea timp de 8 săptămâni pe mediul MS suplimentat cu 1,0 mg L^{-1} TDZ plus 0,1 mg L^{-1} IBA. De asemenea, în comparaţie cu epicotilele, rata de multiplicare a hipocotilelor a fost mai mică (9,36 ± 1,94 şi respectiv 4,12 ± 0,98). Cu toate acestea, pentru lungimea lăstarilor tendinţa a fost opusă. Acesta a fost semnificativ mai mare în mugurii proveniţi din hipocotile pe mediul MS suplimentat cu 0,5 mg l^{-1} TDZ plus 0,1 mg l^{-1} IBA (15.04 ± 2,58 mm). Formarea de rădăcini adventive a fost studiată pe WPM redus la jumătate, suplimentat cu 2,5 mg L^{-1} IBA plus 2,5 mg l NAA. După 24, 48 sau 72 de ore, pe acest mediu inductiv, lăstarii au fost transferaţi pe un mediu WPM redus la jumătate şi fără auxină (mediu expresiv). Cea mai mare rată de înrădăcinare (73,33 ± 3,33%) a fost realizată pe un suport inductiv aplicat timp de 24 h şi apoi transferat pe un mediu expresiv.

Cuvinte cheie: frasin, epicotile, hipocotile, înrădăcinare, tidiazuron.

INTRODUCTION

Common ash (*Fraxinus excelsior* L.) is one of the most abundant and useful of the Bulgarian native ash species, providing both ecological and forest benefits. Also, it is famous for its diversity of ornamental cultivars (KRÜSMANN 1984; DIRR & HEUSER 1987; DIRR 1998), making it suitable for use in urban areas. It is known as a tree species with a rapid growth in the first decade after establishment (HEIN 2004), and there is some evidence that it is very productive when is mixed together with *Acer pseudoplatanus* (KERR & CAHALAN, 2004). Moreover, common ash was found to have positive effects on the soil, particularly on humus type and toposoil chemistry (WEBER et al., 1993; HEITZ 1998; HAGEN-THORN et al., 2004). The wood is economically important because of its hardness, beautiful texture and use in furniture. However, cloning of economically important genotypes and ornamental cultivars in a generative way is impossible because of the heterozygosity of this species. *F. excelsior* cuttings are generally considered difficult to root (GOOD et al. 1978; DIRR 1998). The rooting has been found to be possible only in juvenile material (CORNU et al., 1977; SPETHMANN 1982; JINKS 1995; DOUGLAS 2001; THOMPSON et al., 2001; DANCHEVA 2005). Limited success is reported after annual pruning of the adult trees (GOOD et al., 1978) or using of stump sprouts (STUTZ et al., 1983). Furthermore, the production of large quantities of grafts is limited by the season and the period of rootstock production and the success depends on the method of grafting (BODZAKOV 1962; KOHNERT 1991; KRÜSMANN 1964; DOUGLAS et al., 1996; THOMPSON et al., 2001; DANCHEVA 2009).

Several promising protocols for *in vitro* propagation of some economically important ash species were summarized by VAN SAMBEEK & PREECE (2007). The success of *in vitro* cloning of woody plants depends on the age of stock plant, explants used, and culture conditions. However, *in vitro* propagation could greatly increase the number of produced plants and may provide rejuvenated plants with high rooting capacity (BONGA & VON ADERKAS 1992; HACKETT & MURRAY 1993; HARTMANN et al., 2002).

It has been reported that *in vitro* propagation of several ash species is possible by somatic embryogenesis (PREECE et al., 1989; BATES et al., 1992; PREECE & BATES 1995), axillary shoot formation (PREECE et al., 1987; NAVARRETE et al., 1989), and adventitious shoots induction (NAVARRETE et al., 1989; BATES et al., 1992; TABRETT & HAMMAT 1992; TONON et al., 2001; VAN SAMBEEK et al., 2001; DU & PIJUT 2008; PALLA & PIJUT 2011; STEVENS & PIJUT 2012). In *F. excelsior* axillary shoots has been induced by using nodal and apical segments from 13 month to 16-year-old plants (SILVEIRA & COTTIGNIES 1994; NOUGARÈDE et al., 1996; SCHOENWEISS & MEIER-DINKEL 2005), buds from mature or grafted trees (HAMMATT 1994; SILVEIRA & NOUGARÈDE 1995; NOUGARÈDE et al., 1996; PIERIK & SPRENKELS 1997; THOMPSON et al., 2001; SCHOENWEISS & MEIER-DINKEL 2005), cotyledonary nodes (HAMMATT &

RIDOUT 1992), and epicotyls (MITRAS et al., 2009). However, some of the publications did not report for the age of stock plant, type of the induced shoots, and did not present data from experiments (CHALUPA 1983, 1987a,b, 1990).

It was reported that BAP support the axillary and adventitious shoot formation from hypocotyls and epicotyls from *F. americana* (NOUGARÈDE et al., 1996, PALLA & PIJUT, 2011) and *F. excelsior* (TABRETT & HAMMATT, 1992; HAMMATT & RIDOUT, 1992). However, it was found that TDZ is more effective than BAP for the induction of axillary shoots from epicotyls and hypocotyls from *F. americana* (BATES et al., 1992, NOUGARÈDE et al., 1996) and *F. excelsior* (TABRETT & HAMMATT, 1992; MITRAS et al., 2009).

Depending on the juvenility and auxin used and its concentration, different authors achieved 0 to 100% rooting in common ash (CHALUPA 1983, 1987a,b, 1990; HAMMATT & RIDOUT 1992; TABRETT & HAMMATT 1992; HAMMATT 1994, 1996; SILVEIRA & COTTIGNIES 1994; NOUGARÈDE et al. 1996; THOMPSON et al., 2001; SCHOENWEISS & MEIER-DINKEL 2005; MITRAS et al., 2009).

The goal of this work aimed at identifying successful explants, type of nutritive medium, type, and concentration of plant growth regulators (PGRs) for *in vitro* propagation of common ash.

MATERIALS AND METHODS

Plant material.

Fruits of *F. excelsior* were sampled from a single tree growing in a park in Sofia at altitude about 600 m. The samples were taken by the end of October 2011. They were preserved at 4°C till the end of August next year. Before the establishment of the cultures, the pericarp was removed from the seeds and they were soaked for 72 h in sterile distilled water. After the soaking, they were surface was disinfected for 8 min in 0.2% $HgCl_2$ followed by three times for 3 min rinses in sterile distilled water.

The embryos were isolated under sterile conditions and cultured on half-strength MS medium (MURASHIGE & SKOOG 1962).

Effect of the medium, concentration of TDZ and type of explant on axillary shoot induction.

Epicotyls, having at least one internode and with their bottom leaves removed and hypocotyls with cotyledons (Fig. 1), isolated from *in vitro* 30-day-old seedlings were used as explants. Their length was higher than 15 mm. Three replications, each containing seven explants were used per treatment.

For inducing adventitious shoot formation, the MS and WPM (LLOYD & MCCAWN 1980) media were used in the following treatments: 0.5 or 1.0 mg l^{-1} Thidiazuron (TDZ) plus 0.1 mg l^{-1} indole-3-butyric acid (IBA) (treatments MS1, MS2, WPM1, and WPM2, respectively). Every two weeks, the explants were subcultured on the same fresh medium.

Effect of the inductive medium on adventitious root formation.

After 8 weeks, shoots that were longer than 20 mm and having one or two internodes were transferred to an inductive, half-strength WPM rooting medium, supplemented with 2.5 mg l^{-1} IBA plus 2.5 mg l^{-1} α-naphthaleneacetic acid (NAA) for 24, 48 or 72 h., respectively and then on an expressive rooting medium (half-strength WPM without auxins). For comparison of the results, the shoots were cultivated on the same expressive medium (control). Three replications, each containing ten explants, were cultured in each variant. After 30 days, the percentage of rooted plants, as well as the number and length of induced roots were determined.

Conditions of the cultivation.

Each variant of the media contained 7 g l^{-1} agar (Sigma) and pH was adjusted to 5.6 - 5.7 before autoclaving (under pressure of 118 kPa and 120ºC for 20 min). The cultures were grown in a cultivation chamber at 25 ± 0.5ºC with 16 hrs of cool white fluorescent light at a photosynthetic photon flux density of 40 μmol m^{-2} s^{-1}, daily.

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

RESULTS

The first signs of callus formation were detected on the base of the explants in all variants of the media 14 days after culture establishment. One week later, the callus developed into nodule clusters that were often found in contact with the culture medium. These nodule clusters were globular in form, greenish in colour and compact in texture. The transferring of the explants to fresh medium gave rise to enlargement of the callus and also to new nodules formation. Simultaneously, axillary shoot formation was observed on the explants (Fig. 1). It was demonstrated that thidiazuron (TDZ) promoted significantly higher levels of multiplication than BAP. However, multiplication rates of the axillary shoots were low after 8 weeks of cultivation (MITRAS et al., 2009).

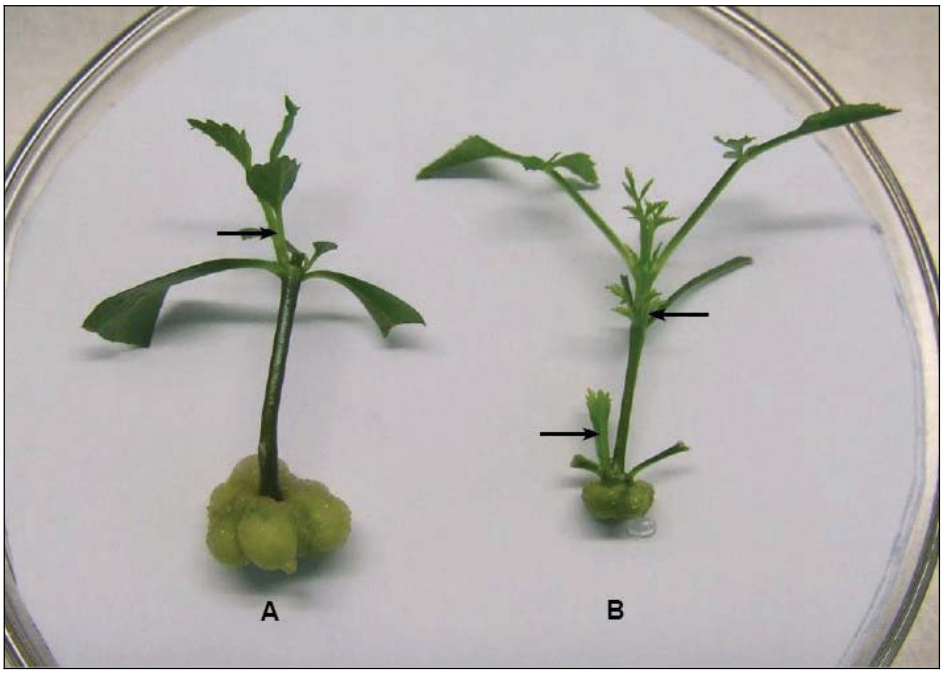

Figure 1. Callus formation on the base of hypocotyls (A) and epicotyl (B) and axillary shoots formation (arrows) on medium MS2 (original photograph).

After 4 weeks, regeneration of axillary shoots was observed. The highest multiplication rate (9.36 ± 1.94) was found on MS medium supplemented with 1.0 mg l^{-1} TDZ. The multiplication rate from the epicotyls was higher on MS medium in comparison with WPM. However, there were no significant differences among multiplication rates obtained using TZD in different concentrations on each medium. Similar tendency was not noticed for the axillary shoots formation from hypocotyls. The highest multiplication rate (4.12 ± 0.98) was achieved on MS medium supplemented with 1.0 mg l^{-1} TDZ but there was not found any statistical difference compared with other treatments. Also, in comparison with epicotyls, their multiplication rate was lower on the variants of MS medium, but there were not differences between the variants of WPM and MS medium (Table 1).

Table 1. Mean number of the induced axillary shoots, induced from epicotyls and hypocotyls.

Medium	Epicotyls	Hypocotyls
MS1	9.00 ± 1.52 a	2.55 ± 0.79 bc
MS2	9.36 ± 1.94 a	4.12 ± 0.98 b
WPM1	3.35 ± 0.40 bc	2.09 ± 0.28 bc
WPM2	2.95 ± 0.19 bc	1.75 ± 0.33 c

Legend: Values are mean (M) ± standard error (SE). Means followed by the same letter are not significantly different estimated by One-Way ANOVA followed by a post hoc LSD test at $p < 0.05$.

After 8 weeks of cultivation, the length was significantly higher in shoots, originated from hypocotyls on MS medium supplemented with 0.5 mg l^{-1} TDZ (15.04 ± 2.58 mm) (Fig. 2) and the increased concentration of TDZ induced depressive effect. However, similar reaction was not observed on the variants of WPM. The highest length of the shoots originated from epicotyls was observed on the variants of MS medium, but there were no differences in the shoot length between the used treatments (Table 2).

Table 2. Mean length (mm) of the axillary shoots, induced from epicotyls and hypocotyls.

Medium	Epicotyls	Hypocotyls
MS1	8.28 ± 0.74 bcd	15.04 ± 2.58 a
MS2	8.32 ± 0.69 bc	9.99 ± 1.40 b
WPM1	5.79 ± 0.49 d	9.57 ± 1.93 bc
WPM2	5.91 ± 0.30 cd	7.38 ± 1.15 bcd

Legend: Values are mean (M) ± standard error (SE). Means followed by the same letter are not significantly different estimated by One-Way ANOVA followed by a post hoc LSD test at $p < 0.05$.

Figure 2. Multiplication and elongation of axillary shoots on medium MS2 (Original photograph).

Figure 3. Rooted shoots after application of inductive medium for 24 h and transfer of the shoots to expressive medium (Original photograph).

The number of the formed shoots depended on the medium ($F = 36.003$, $p < 0.05$), the type of explant ($F = 32.643$, $p < 0.05$), and the interaction between these two factors ($F = 13.868$, $p < 0.05$). Root length depended on all investigated factors ($F = 14.217, 4.228, 15.754$, resp.; $p < 0.05$) and the interaction between concentration of TDZ and type of explant ($F = 4.605$, $p < 0.05$) (Table 3).

Table 3. Significance of the studied factors and their combinations on the axillary shoots formation estimated by a post hoc LSD test.

Factors	Number of the shoots		Length of the shoots (mm)	
	F	Level of significance	F	Level of significance
M	36.003	0.000	14.217	0.000
C	0.233	0.630	4.228	0.040
E	32.643	0.000	15.754	0.000
M × C	1.164	0.283	0.733	0.392
M × E	13.868	0.000	0.847	0.358
C × E	0.261	0.611	4.605	0.032
M × C × E	0.215	0.643	0.654	0.419

Legend: a R Squared = 0.078 (Adjusted R Squared = 0.063), M = Medium, C = Concentration of the TDZ, E = Explant, $p < 0.05$.

Effect of the inductive medium on adventitious root formation.

Rooting of the shoots was observed in all rooting variants of the medium, and very small amount of callus was observed on the base of the shoots. The roots were thin and some of them appeared from the stem and above the callus (Fig. 3, left). The rooting percentage was significantly higher on the inductive medium applied for 24 h. The rate of rooting decreased significantly after longer periods of cultivation (48 and 72 h) on inductive medium. Also, these results were lower in the comparison with the control (Table 4).

The root number was significantly lower when the shoots were cultured on control medium i.e. half-strength and auxin free WPM. When different durations of inductive phase were applied, the opposite tendency was observed but statistical differences were not observed between the applied treatments. However, the roots reached the highest length after the cultivation of the shoots on half-strength WPM without auxins i.e. on control medium (Table 4).

Table 4. Effect of the duration of inductive phase on the rooting of axillary shoots.

Duration of the inductive phase (h)	Rooted plants (%)	Mean number of the roots	Mean length of the roots (mm)
Control	60.74 ± 3.23 b	2.1 ± 0.2 b	32.4 ± 2.1 a
24	73.33 ± 3.33 a	3.9 ± 0.6 a	25.7 ± 1.9 b
48	42.73 ± 2.72 c	3.2 ± 0.5 ab	28.5 ± 2.9 ab
72	50.00 ± 2.62 c	3.8 ± 0.6 a	24.9 ± 2.0 b

Legend: Values are mean (M) ± standard error (SE). Means in the column followed by the same letter are not significantly different estimated by One-Way ANOVA followed by a post hoc LSD test at $p < 0.05$.

DISCUSSIONS

TDZ was found to be potential cytokinin for plant tissue cultures (HUETTEMANN & PREECE 1993; LU 1993; MURTHY et al., 1998; KHURANA et al., 2005). In previous studies on *Fraxinus excelsior* it has been reported that TDZ is effective in inducing shoot regeneration from embryo hypocotyls, cotyledons, whole leaves, and petioles (TABRETT & HAMMATT 1992; HAMMATT 1994, 1996; THOMPSON et al., 2001). To date, regeneration in common ash by adventitious shoots has only been reported from leaves (HAMMATT 1994) and embryo hypocotyls (TABRETT & HAMMATT, 1992). It was found that TDZ in concentrations 0.01-5.0 mg l^{-1} is the best for shoots induction (TABRETT & HAMMATT, 1992; HAMMATT 1994; SCHOENWEISS & MEIER-DINKEL, 2005). Our results (data not shown) are in agreement with the findings of TABRETT & HAMMATT (1992) that some of the initial explants died without producing ~~of~~ shoots. It was reported that WPM is more suitable than MS medium for the survival of the explants and mean number of the induced axillary shoots (HAMMATT & RIDOUT 1992; SILVEIRA & COTTIGNIES 1994; SILVEIRA & NOUGRÉDE 1995; NOUGARÉDE et al., 1996). However, we found that MS medium is more suitable for the formation of a greater mean number of axillary shoots from epicotyls but is not in significance for the induction of the axillary shoots from hypocotyls. It could be due to the different explants and TZD used instead of BAP in our experiments. Also, our results demonstrated that the used concentrations of TDZ did not affect the mean number of shoots induced from epicotyls and hypocotyls.

It was shown that the length of axillary shoots is higher on WPM in comparison with MS medium (HAMMATT & RIDOUT 1992) and depends on the used cytokinin and its concentration (SILVEIRA & COTTIGNIES 1994). Our results showed that the length of the axillary shoots was higher when they were induced from hypocotyls on MS medium. The high concentration of TDZ (1.0 mg l^{-1}) inhibited the elongation. However, the medium type and TDZ concentration did not affect the length of axillary shoots originated from epicotyls.

It has been shown that the type and concentration of auxin has a central role in the induction of adventitious roots (DE KLERK 2001; DE KLERK et al., 1997, 1999; KUREPIN et al., 2011). Also, it was demonstrated that auxin pulse treatment is critical for the rooting of plants *in vitro* and depends on the duration of inductive phase (DE KLERK 1996, 2001, 2002; KLERK et al. 1999; MITRAS et al., 2009). After the inductive phase, once the cells have been determined to root formation, auxin is no longer required for the rooting of *Fraxinus excelsior* shoots (HAMMATT 1996; HAMMATT & RIDOUT 1992; MITRAS et al., 2009). It was found that the rooting of common ash varies from 0 to 100% (HAMMAT & RIDOUT 1992; SILVEIRA & COTTIGNIES 1994; SCHOENWEISS & MEIER-DINKEL 2005; MITRAS et al., 2009) and depends on the age of the initial explants (SCHOENWEISS & MEIER-DINKEL 2005), genotype (TABRETT & HAMMATT 1992), type and concentration of the auxin used (HAMMAT & RIDOUT 1992; SCHOENWEISS & MEIER-DINKEL 2005; MITRAS et al., 2009). In comparison with our previous results (MITRAS et al., 2009), the pulse treatment of shoots with high concentration of auxin for shorter period lead to significantly smaller amount of callus formation. Our results showed that the percentage of rooting and number of roots depends on the duration of cultivation on inductive medium. The cultivation of shoots to a root induction medium for shorter period improved the rooting and number of the formed roots. These and our previous results (MITRAS et al., 2009) demonstrated that the balance between the concentration of the used auxin and the duration of inductive phase are critical for the rooting rate and quality of the root system.

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