ROOTING AND ACCLIMATIZATION OF MICROPROPAGATED SHOOTS OF *FRAXINUS EXCELSIOR* L.

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Abstract. Axillary shoot tips, originated *in vitro* were used as initial explants for the rooting experiments. They were cultured on an inductive, half-strength WPM, supplemented with a combination of 1.0 mg l^{-1} IBA and 1.0 mg l^{-1} NAA for 24, 48 or 72 h, respectively and then on half-strength, plant growth regulator-free (PGRs-free) expressive WPM. For comparison of the results, the shoots were cultivated on inductive or expressive medium without transfer. The rooted shoots were planted in 500 ml pots. In one part of the experiment, the posts contained peat and the plantlets were covered with transparent plastic vessels for 14 days to provide high air humidity. For the other experiment, the pots contained peat, perlite, peat and perlite (1 : 1, v/v), Dystric Alluvial Fluvisol and perlite (1 : 1, v/v) and the plantlets were covered with transparent plastic vessels. After 14 days the cover vessels were consecutively removed for 30 min, 60 min, 90 min, and 120 min, daily. All plantlets were grown in a cultivation chamber and after the removal of covers, were transferred in a greenhouse conditions. The highest rooting rate (90.00 ± 5.77%) was observed when the shoots were pulse treated with auxin for 24 h and decreasing of the mean number of roots was found with the increasing of the duration of inductive phase. High survival rate of the plantlets was manifested on different substrates (93-100%) but significant difference between them was not found. However, higher length was observed by using peat containing substrates (from 12.9 ± 1.0 to 19.4 ± 5.1 cm) but statistical difference between them was not found. By contrast, plants acclimatized on perlite containing substrates were smaller than these planted in peat containing substrates. These results demonstrated that the duration of inductive phase is critical for the rooting rate and quality of the root system and the type of the soil substrate is an important factor for the growth of the plants during the acclimatization.

Keywords: common ash, *in vitro*, tissue culture.

Rezumat. Înrădăcinarea şi aclimatizarea lăstarilor micropropagaţi de *Fraxinus excelsior* L. Vârfurile de lăstari axilari, de origine *in vitro*, au fost utilizate ca explante inițiale pentru experimente privind înrădăcinarea. Acestea au fost cultivate pe un WPM inductiv, redus la jumătate, suplimentat cu o combinație de 1,0 mg l^{-1} IBA și 1,0 mg l^{-1} NAA timp de 24, 48 sau 72 de ore și pe WPM expresiv, fără un regulator de creştere a plantelor (PGRs-free). Pentru a compara rezultatele, lăstarii au fost cultivaţi pe mediu inductiv sau expresiv, fără transfer. Lăstarii înrădăcinați au fost plantați în vase de 500 ml. Pentru o parte a experimentului, substratul a conţinut turbă și plantulele au fost acoperite cu vase din plastic transparent, timp de 14 zile pentru a menține umiditatea ridicată a aerului. Pentru celelălt experiment, vasele au conţinut turbă, perlit, turbă și perlit (1: 1, v / v), fluvisol distric aluvionar și perlit (1: 1, v / v) și plantulele au fost acoperite cu vase de plastic transparent. După 14 zile, vasele de acoperire au fost îndepărtate consecutiv timp de 30 min., 60 min., 90 min. și 120 min., zilnic. Toate plantule au fost păstrate într-o cameră de cultivare și după îndepărtarea capacelor, au fost transferate în condiții de seră. Cea mai mare rată de înrădăcinare (90,00 ± 5,77%) a fost observată atunci când lăstarii au fost tratati cu auxin pentru 24 de ore, în timp ce scăderea numărului mediu de rădăcini s-a constatat o dată cu creșterea duratei fazei inductive. S-a constatat o rată ridicată de supraviețuire a plantulelor pe diferite substraturi (93-100%) și nu s-a observat o diferenta semnificativă intre acestea. Cu toate acestea, s-a constatat o lungime mai mare în cazul substratului care conține turbă (de la 12,9 ± 1.0 - 19.4 ± 5,1 cm), dar nu s-a observat o diferență statistică semnificativă între acestea. Prin contrast, plantele aclimatizate pe substrat care conține perlit au fost mai mici decât cele cultivate pe substratul care conține turbă. Aceste rezultate au demonstrat că durata fazei inductive este critică pentru rata de înrădăcinare și calitatea sistemului radicular și că de tipul de sol este un factor important pentru creșterea plantelor pe perioada de aclimatizare.

Cuvinte cheie: frasin, *in vitro*, cultură de ţesut.

INTRODUCTION

Rooting of microshoots is critical for *in vitro* plant production systems. The induction of rooting depends on a series of interdependent phases (induction, initiation, and expression) (MONCOUSIN et al., 1988; DE KLERK et al., 1999; GASPAR et al., 1992, 1994).

Depending on the juvenility and auxin used and its concentration, different authors achieved 10 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 quality of the root system is one of the important factors for the successful acclimatization. It has been suggested that defects in root system functioning are one of the main reasons for the high mortality rate of plants derived from *in vitro* culture (YIE & LIAW, 1977; DAVID, 1982; PATEL et al., 1986). Furthermore, after the transfer from *in vitro* to *in vivo* conditions the plantlets should adapt to different environmental conditions. Usually the irradiance and air turbulence is much higher, air humidity much lower, there are fluctuating temperatures, the type of soil substrate and its humidity is different in comparison with *in vitro* conditions (ZIV 1986; KOZAI 1991; PREECE & SUTTER 1991; DONNELLY & TISDAL 1993; POSPÍŠILOVA et al., 1999; HAZARIKA 2003; ROHR et al., 2003; KOZAI & ZOBAYED, 2000). Depending on the biological peculiarities and genetic potential of the species, the methods which work for *in vivo* environment of one species are not necessary satisfactory to ensure the survival of another. However, there is only one report for high rate of *Fraxinus excelsior* acclimatization (LEBEDEV & SCHESTIBRATOV, 2013) but the effect of the soil substrate is not investigated.

The goal of this work was aimed at identifying suitable duration of inductive phase on rooting of axillary shoots and conditions of acclimatization of common ash plantlets.

MATERIAL AND METHODS

Effect of the inductive medium on the adventitious root formation.

In vitro originated axillary shoot tips (2 cm), having one or two internodes were cultured on an inductive, half-strength WPM (LLOYD & MCCOWN, 1980) rooting medium, supplemented with combination of 1.0 mg l^{-1} indole-3-butyric acid (IBA) and 1.0 mg l^{-1} α-naphthaleneacetic acid (NAA) for 24, 48 or 72 h, respectively and then on half-strength, plant growth regulator-free (PGRs-free) expressive WPM. For the comparison of the results, the shoots were cultivated on inductive or expressive medium without transfer.

Tree replications, each containing ten explants, were used for each variant. After 45 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 medium contained 20 g l^{-1} sucrose and 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.

Acclimatization

The rooted shoots were thoroughly washed to remove adhering gel and planted in 500 ml (∅ 95 mm, h = 110 mm) pots. In one part of the experiment the posts contained peat and the plantlets were covered with transparent plastic vessels (250 ml, ∅ 75 mm, h= 95 mm) for 14 days to provide high air humidity (Experiment 1). For the other experiment the pots contained peat, perlite, peat and perlite (1 : 1, v/v), Dystric Alluvial Fluvisol and perlite (1 : 1, v/v) were covered with transparent plastic vessels (Fig. 1). After 14 days the cover containers were consecutively removed for 30 min, 60 min, 90 min, and 120 min, daily (Experiment 2). All plantlets 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. After the removal of covers, all plantlets were transferred in a greenhouse with temperature of 20-25°C and regular irrigation.

Figure 1. Pre-acclimatization with covering of the plantlets in the cultivation chamber.

Ten plantlets were used per treatment and the experiment was repeated three times. After 60 days, the percentage of acclimatized plants, as well as the mean length of stem was determined.

The results were analysed by One-Way ANOVA followed by a post hoc LSD test at $p < 0.05$, using SPSS 10.0 for Windows (SPSS for Windows 1999). Percentage values were transformed using arcsine square root (√P) (COMPTON 1994) to normalize error distribution prior variance analysis.

RESULTS AND DISCUSSION

In many species, the application of endogenous auxin is required to achieve rooting (DE KLERK, 2001, 2002; DE KLERK et al., 1997, 1999; KUREPIN et al., 2011).

In the absence of auxin, the rooting rate in our experiment was low (Table 1), the emergence of new roots progressed slowly and callus formation was not observed on the base of the shoots in the end of the rooting period. On auxin enriched medium without transfer of the shoots, in the end of experiment, significant amount of callus and thicker roots were formed on the base of the shoots, in comparison with all other treatments (Fig. 2). However, statistical significant difference with the auxin-free medium was not found in the rooting rate (Table 1).

Figure 2. Significant amount of callus and formation of thick roots after cultivation on auxin enriched medium without transfer on expressive medium.

Figure 3. Rooted shoots, formed after the application of inductive medium for 24 h and evaluated after 45 days of cultivation.

Smaller amounts of callus and slenderer roots were formed on the base of shoots in the end of the experiment, after all pulse treatments. Our results demonstrated that auxin pulse treatments significantly improve the rooting of shoots *in vitro.* These results are in agreement with the findings of DE KLERK (1996, 2001, 2002), DE KLERK et al. (1999), MITRAS et al. (2009), LYUBOMIROVA & ILIEV (2013). The highest rooting rate (90.00 ± 5.77%) was observed when the shoots were pulse treated with auxin for 24 h (Table 1, Fig. 3). It was pointed that during the initial 24 h after the shoots have been taken, they are not yet very sensitive to auxin. It is considered that during this lag period, dedifferentiation occurs during which cells become competent to respond to the rhizogenic stimulus, auxin. Then up to 72 h, certain previously activated cells become committed to the formation of root primordia by the rhizogenic action of auxin in the induction phase (DE KLERK et al., 1999). However, statistical difference in the rooting rate between different pulse treatments was not found in our experiment. It was found that during this period, auxin pulses strongly increases the number of roots in pea (NORDSTRÖM et al., 1991) and *Helianthus annuus* (LIU & REID, 1992). Despite of that, decreasing of the mean number of roots was found with the increasing of the duration of inductive phase, but statistical difference between all treatments was not ascertained. The longest roots were observed after pulse treatment for 72 h and on auxin free medium without transfer to expressive medium (Table 1).

Our results showed that pulse treatment supports the rooting and the rate of rooting depends on the duration of inductive phase. After the cultivation of shoots to a root induction medium, auxin is no longer required for the rooting and improvement of the root system. These results are in agreement with the findings of other authors (BERTHON et al., 1989, 1990; HAMMATT & RIDOUT, 1992; HAMMATT, 1996; ILIEV & ILIEV, 1997, LYUBOMIROVA & ILIEV, 2013) but the duration of the inductive phase probably depends on the genotype peculiarities of the species, rate of the juvenility of the shoots, type and concentration of the used auxin.

Table 1. Effect of the duration of inductive phase on the rooting of shoots.

Duration of the inductive phase (h)	Rooted shoots (%)	Mean number of the roots	Mean length of the roots (mm)
Auxin enriched medium without transfer	30.0 ± 5.8 c	4.3 ± 1.4 ab	23.5 ± 4.1 b
PGRs-free medium without transfer	53.7 ± 6.7 bc	2.1 ± 0.2 b	32.4 ± 2.1 ab
24	90.0 ± 5.8 a	3.8 ± 0.5 a	29.1 ± 1.3 b
48	80.0 ± 5.8 a	3.1 ± 0.4 ab	29.4 ± 2.0 b
72	76.7 ± 3.3 ab	2.7 ± 0.4 ab	38.9 ± 4.6 a

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 $p < 0.05$.

Different systems for acclimatization are recommended (DEBERGH, 1991; CLAPA et al., 2013). It was reported that the effect of the type of soil substrate is an important factor for the acclimatization of plantlets to *in vivo* conditions (ILIEV et al., 2001; PINKER et al., 2007; JIMÉNEZ et al., 2011; RAGONEZI et al., 2012). High survival rate of the plantlets was manifested on different substrates (93-100%) but significant difference between them was not found (Fig. 4).

Our results indicated that significant elongation of the shoots on some substrates was noticed even in the cultivation room (data are not shown). The substrates studied can be divided into two groups according their effect on the elongation of micropropagated plants in the end of the investigated acclimatization period. Higher length was observed by using peat containing substrates (from 12.9 ± 1.0 to 19.4 ± 5.1 cm) but statistical difference between them was not found. By contrast, plants acclimatized on perlite containing substrates were smaller than these planted in peat containing substrates and statistical difference between them also was not found (Table 2, Fig. 4). The reason for this tendency could be not only the better retention of water and aeration of the peat, but also the higher concentration of nutritive substances in it.

Table 2. Effect of the substrate on acclimatization.

Substrate used	Acclimatized plants (%)	Mean length of the steam (cm)
Peat (Experiment 1)	93.3 ± 6.7 a	18.6 ± 1.9 a
Peat (Experiment 2)	93.3 ± 6.7 a	12.9 ± 1.0 ab
Peat and perlite (Experiment 2)	100.0 ± 0.0 a	19.4 ± 5.1 a
Perlite (Experiment 2)	93.3 ± 3.3 a	5.9 ± 0.5 b
Perlite and soil (Experiment 2)	93.3 ± 3.3 a	8.4 ± 0.6 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 $p < 0.05$.

Figure 4. Acclimatized plants in greenhouse conditions. Left: planted on perlite and Right: planted on mixture of peat and perlite.

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