EFFECT OF MEDIUM ON IN VITRO GERMINATION OF EMBRYOS OF *FRAXINUS EXCELSIOR* L.

DANCHEVA Desislava, ILIEV Ivan , SCALTSOIYANNES Apostolos, TSAKTSIRA Maria, TSOULPHA Parthena

Abstract. Dry seeds of common ash (*Fraxinus excelsior*) were sampled in October and stored at room temperature until July next year. After the soaking for 48h in sterile water they were surface disinfected for 8 min in 0.2% $HgCl_2$ (by volume) followed by three times for 3 min rinses in sterile water. The following 3 type of explants was used for in vitro cultivation: seeds with removed pericarp, seeds with excised one-four part of the opposite end of the embryo and embryos. All types of explants were placed on full and half strength variants of MS, DKW, WPM and Knop media. For the study of the pre-sowing the embryos were cultivated on half strength MS medium and subjected to a 4°C in darkness for 14 days. Only isolated embryos developed into viable seedlings. The germinative response of the embryos differed according the content of various culture media. Development of all organs is the best on half strength WPM medium (epicotyls - 8.29 ± 0.94 mm, hypocotyls - 26.32 ± 1.32, radicles - 71.77 ± 4.98 mm). Generally, the formation of normally developed seedlings was significantly better on half strength MS medium (65.00 ± 2.89 %) than the other media. For this reason this medium could be recommended as the most suitable for the germination of common ash embryos. Pre-sowing of the embryos for 14 days at 4ºC did not show stimulation effect upon seedling development. Organogenesis was poor and the rate of normally developed plantlets was low.

Keywords: culture medium, embryo, *Fraxinus excelsior*, in vitro germination.

Rezumat. Efectul mediului de cultură asupra germinaţiei in vitro a embrionilor de *Fraxinus excelsior* L. Seminţe uscate de frasin (*Fraxinus excelsior*), au fost recoltate în octombrie şi păstrate la temperatura camerei pâna în luna iulie a anului următor. După îmbibare cu apă sterilă timp de 48 ore, suprafaţa lor a fost dezinfectată timp de 8 minute cu o soluţie 0.2% $HgCl_2$, urmată de trei spălări timp de 3 minute în apa sterilă. Au fost cultivate in vitro următoarele trei tipuri de explante: seminţe cu pericarpul îndepărtat; seminţele având excizata ¼ din capătul opus embrionului şi embrioni. Toate cele trei tipuri de explante au fost plasate pe variante de mediu integral şi variante de mediu cu jumatate de MS, DKP, WPM şi mediu Knop. Studiul efectului unui pre-tratament, a fost realizat prin inocularea embrionilor pe mediu de cultură având jumătate din MS şi menţinuţi la 4ºC şi la întuneric, timp de 14 zile. Numai embrionii izolaţi au produs lăstari viabili. Raspunsul germinativ al embrionilor diferă după compoziţia variatelor medii de cultură. Dezvoltarea tuturor organelor este cea mai bună pe mediul cu jumatate de WPM (epicotil: 8.9 ± 0.94 mm, hipocotil: 26.32 ± 1.32, radicele: 71.77 ± 4.98 mm). In general, formarea unor puieţi dezvoltaţi normal, a fost semnificativ mai bun pe mediu cu jumatate din cantitatea de MS (65.00 ± 2.89%), decât pe alte medii. Pentru acest considerent, acest mediu este recomandat ca fiind cel mai potrivit pentru germinarea embrionilor de frasin. Pre-inocularea embrionilor timp de 14 zile la 4ºC şi la întuneric, nu a avut un efect stimulator asupra dezvoltării lăstarilor. Organogeneza şi rata dezvoltării normale a plantulelor au fost scăzute.

Cuvinte cheie: *Fraxinus excelsior*, embrion, germinatie în vitro, mediu de cultură.

INTRODUCTION

There appear to be up to three factors involved in ash seed dormancy, depending on the species: immature embryos, internal (chemical or hormonal) factors (VILLIERS & WAREING, 1964; BONNER, 1974; DIRR & HEUSER, 1987; STINEMETZ & ROBERTS, 1984; YOUNG & YOUNG, 1992), and oxygen-impermeable pericarps (VILLIERS & WAREING, 1964).

The seeds of the common ash generally present dormancy up to 6 years. During this period the embryos passes through different stages of morphological and physiological maturity, in the course of which the dormancy level changes characteristically (WAGNER, 1996; WAGNER & KAFKA, 1995): dormancy becomes apparent during embryogenesis, reaches a high level during the late phase of deposition of reserves, and declines at the time of seed maturation. A moderate increase in dormancy level can be observed in the first winter, but in the following spring the embryo is gradually released from dormancy and enlarges further. In forest tree nurseries, dormancy removal is classically obtained by a long time of stratification usually consisting in a warm treatment of 16 weeks followed by a cold treatment of 16 weeks (BONNER, 1974; YOUNG & YOUNG, 1992; MULLER et al., 1990; PIOTTO, 1994; SUSZKA et al., 1996). The causes of the dormancy were debated by VILLIERS & WAREING (1964). These authors obtained development of common ash embryos on moistened filter paper. The in vitro culture of seeds or embryos has been proposed to remove the dormancy (HO, 1987; PREECE et al., 1995; WAGNER & KAFKA, 1995; WAGNER, 1996). It was showed that in vitro germination of embryos after extraction was from 60% to 90% (WAGNER 1996, RAQUIN et al. 2002) and depended on the medium composition, pollination and reflects the functional stage of the embryo at a given time (WAGNER, 1996; RAQUIN et al., 2002).

The aim of this paper is to determine the influence of the endosperm on the in vitro dormancy of non stratified seeds, to overcome it by finding a medium for the germination of isolated embryos and to obtain normal seedlings.

MATERIAL AND METODS

Plant material

Fruits of *Fraxinus excelsior* L. were sampled from a single and isolated tree growing in a park in Sofia at about 600 m. It was shown that in July the final stage of embryo growth is completed and in the end of October the three major classes of dormancy (endo-, para- and ecodormancy) are typical for the common ash seeds (WAGNER, 1996). For this reason the samples were taken by the end of October 2002. They were preserved at room temperature (20-22°C) till the end of July next year. Before the establishment of the cultures, the pericarps were removed from the seeds (Fig. 1) and they were soaked for 48h in sterile water. Seeds damaged by insects, seeds without embryo or seeds with necrotic embryo were discarded.

After the soaking they were surface disinfected for 8 min in 0.2% $HgCl_2$ (by volume) followed by three times for 3 min rinses in sterile water.

The following 3 type of explants were used for *in vitro* cultivation: seeds with removed pericarp (type 1, Fig. 2), seeds with excised one-four part of the opposite end of the embryo (type 2, Fig. 3) and embryos (type 3, Fig. 4). All experiments were carried out in 3 replications, each with 20 embryos.

Figure 1. Seeds with pericarp and with removed pericarp.
Figura 1. Semințe cu pericarp și semințe cu pericarpul îndepărtat.

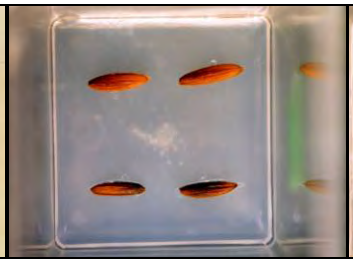

Figure 2. Seeds with removed pericarp (type 1).
Figura 2. Semințe cu pericarpul îndepărtat (tip 1).

Figure 3. Seeds with excised one four part of the opposite end of the embryo (type 2).
Figura 3. Semințe cu partea opusă a embrionului, secționată o pătrime (tipul 2).

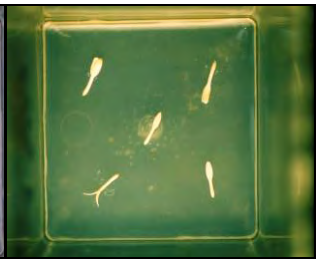

Figure 4. Isolated embryos (type 3).
Figura 4. Embrioni izolați (tipul 3).

Effect of culture medium on the germination and seedlings morphology

Treatment 1. Full strength and half strength variants of MS (MURASHIGE & SKOOG, 1962), WPM (LLOYD & MCCOWN, 1980), DKW (DRIVER & KUNIYUKI, 1984) and Knop (BOPP & BRANDES, 1985) media were used for the cultivation of explants from type 1, 2, and 3.

Treatment 2. For the study of the pre-sowing the embryos were cultivated on half strength MS medium and subjected to 4°C in darkness for 14 days.

Each of the above-mentioned media contained 20 g l^{-1} sucrose and 7.1 g l^{-1} agar. Knop medium contained 5 g l^{-1} sucrose.

The pH of all media was adjusted to 5.6 before autoclaving (under 1.2 atm. and temperature 120 ± 1°C for 20 min).

The cultures were grown in a cultivation room at a temperature 25 ± 1°C in a 16h-8h light-dark regime and cool white fluorescent light at photosynthetic photon flux density 40 µmol m^{-2} s^{-1}, daily.

The final status of the cultures was recorded at the end of a 5-week incubation period. The seedlings obtained, in response to the various culture media were separated in the following groups:

Figure 5. Seedlings type C.
Figura 5. Puiet tipul C.

A - any sign of growth
B - the embryo enlarged, but embryonic morphology maintained
C - cotyledons expanded and became green, the hypocotyl and the radicle remained white without elongation (Fig. 5)
D - cotyledons as in C, hypocotyl elongated,radicle had failed to emerge
E - full germination i.e. normal seedling (Fig. 6)

Figure 6. Normal seedlings (type E).
Figura 6. Puiet normal (tipul E).

Figure 7. Seedling (type F).
Figura 7. Puiet (tipul F).

Figure 8. Seedling (type G).
Figura 8. Puiet (tipul G).

F - normal hypocotyl and root, but the cotyledons with embryonic morphology (not enlarged) (Fig. 7)
G - normal hypocotyl, cotyledons and root, but the epycotyl does not appear (Fig. 8)

In this paper as in reference cited, the in vitro development of the embryo excised from mature seeds is called germination.

The results were analyzed by ANOVA (post hoc LSD test at level 0.05) using SPSS 10.0 (SPSS for Windows 1999).

RESULTS AND DISCUSSIONS

Germination of the embryos

The possible mechanisms imposing dormancy on the seeds of *F. excelsior* in the annual cycle were based on the data of VILLIERS & WAREING (1964). The essential factors maintaining dormancy in the seeds was considered to be the immaturity of the embryo, the restriction of gas exchanges by seed coast and the chilling requirement of the embryo.

A universally applicable terminology of dormancy, recently suggested by LANG et al. (1987) reduces the various dormancy phenomena to the major classes endo-, para-, and ecodormancy. Applying this terminology to the embryo dormancy of *F. excelsior*, it can be shown that all three mechanisms are involved in maintaining the embryo in a developmental but not germinative mode.

VILLIERS & WAREING (1965) reported that a growth inhibitor was produced in the embryo and endosperm of imbibed *Fraxinus excelsior* seeds. It was reported that an oxygen-impermeable pericarp is one of the reasons for the dormancy of common ash (VILLERS & WAREING, 1964).

It was reported that cutting one-third of the seed opposite the radicular end enhances germination of the seeds of *Fraxinus angustifolia* ssp. *oxycarpa* and could overcome the paradormancy (PREECE et al., 1995). Our experiments showed that the explants (seeds) from the types 1 and 2 do not germinated in all used media, after the applying of this technique. The fact could be due to the role of the inhibitors localized in the endosperm during the period of seed dormancy of common ash and they can not be overcome in vitro with scarification and without stratification.

Table 1. Germination of embryos (after 8 months preserving seeds) in different nutritive media in %.
Tabel 1. Germinaţia embrionilor (după păstrarea seminţelor timp de 8 luni) în diferite medii nutritive (%).

Type of the seedlings Medium	A M ± SE	B M ± SE	C M ± SE	D M ± SE	E M ± SE	F M ± SE	G M ± SE
½ MS	11.7 ± 1.7	0	8.3 ± 4.4	1.7 ± 1.7	65.0 ± 2.9 a[1]	6.7 ± 6.7	6.7 ± 3.3
MS	10.0 ± 0.0	3.3 ± 3.3	5.0 ± 0.0	1.7 ± 1.7	55.0 ± 2.9 b	6.7 ± 4.4	18.3 ± 6.7
½ WPM	6.7 ± 1.7	0	23.3 ± 1.7	3.3 ± 1.7	51.7 ± 3.3 b	0	15.0 ± 2.9
WPM	5.0 ± 0.0	1.7 ± 1.7	43.3 ± 1.7	0	36.7 ± 4.4 cd	0	13.3 ± 4.4
1/2 Knop	15.0 ± 2.9	10.0 ± 5.0	33.3 ± 4.4	0	25.0 ± 5.0 e	5.0 ± 2.9	11.7 ± 4.4
Knop	0	0	11.7 ± 4.4	0	31.7 ± 1.7 de	0	56.7 ± 6.0
1/2 DKW	8.3 ± 1.7	1.7 ± 1.7	11.7 ± 1.7	18.3 ± 1.7	50.0 ± 0.0 bc	0	8.3 ± 1.7
DKW	5.0 ± 2.9	18.3 ± 1.7	5.0 ± 2.9	8.3 ± 1.7	41.7 ± 1.7 c	8.3 ± 1.7	11.7 ± 1.7

[1]Different letters in the column indicate significant difference for the normal seedlings ($p \leq 0.05$), using ANOVA test. M - mean, SE - standard error of mean

Within a week of placement *in vitro*, cotyledons of the isolated embryos turned green and continued to expand with viable seedlings. However the germinative response of the embryos differed according to the content of various culture media. Some of them did not show any sing of growth (type A) or enlarged, but embryonic morphology was maintained (type B). Their mean percent in different nutritive studied media varied (Table 1) from 1.7 ± 1.7 % (type B, medium WPM) to 18.3 ± 1.7% (type B, medium DKW). The smallest percent of appearance of both types was observed on the medium WPM.

The mean percent of "seedlings" from type C in different media varied from 5.0 ± 2.9 and 5.0 ± 0.0 (media DKW and MS) to 43.3 ± 1.7 (medium WPM) (Table 1, Fig. 5). The expansions only of the cotyledons in present observation do not support the conclusion of BULARD & MONIN (1963) that in *F. excelsior* the inhibitory effect emanates from the cotyledons, this blocking the growth of the embryonic axis. Rather it appears that the embryo dormancy is largely regulated within the axis, as it has also been reported for maple embryos (PINFIELD et al. 1990). Moreover, the appearance of seedlings from the types D, E, F, and G was an indicator for the level of dormancy and showed that the embryonic organs of dormant ash embryos were heterogeneous. This confirms the opinion of COTTIGNES (1983) that the nuclear state within the embryogenic organs of dormant ash embryos was heterogeneous.

Generally, the formation of the seedlings type E were significantly better (Table 1, Fig. 6) on half strength MS medium (65.0 ± 2.9 %) than on other media. For this reason this medium could be recommended as the most suitable for the germination of common ash embryos.

Pre-sowing of the embryos for 14 days at 4ºC did not show stimulation effect upon seedling development. The rate of normally developed plantlets was low (26.7 ± 3.3 %). Embryos on this treatment developed mainly in seedlings from type G (without epicotyls) (40.0 ± 15.3) (Table 2).

Table 2. Influence of the pre-sowing (14 days at 4°C) on the embryos germination.
Tabel 2. Influența pre-însămânțării (14 zile la 4°C) asupra germinației embrionilor.

Type of the seedlings	A	B	C	D	E	F	G
M ± SE	6.7 ± 3.3	10.0 ± 5.0	6.7 ± 4.4	8.3 ± 4.4	26.7 ± 3.3	1.7 ± 1.7	40.0 ± 15.3

M - mean, SE - standard error of mean

The growth and survival of cultured embryos is greatly enhanced by addition of carbohydrates to the medium (HO, 1987). WAGNER & KAFKA (1995) reported that MURASHIGE and SKOOG (1962) medium without sucrose had not beneficial effect on ash plantlet morphogenesis. In our experiment, embryo cultures on Knop medium developed in seedlings mainly from type G and the percentage of normally developed seedlings was lower than in all other media (25.0 ± 5.0 on half strength; 31.7 ± 1.67 % on full Knop medium) (Table 1). The low percent of the normally developed seedlings (type E) on the variants of Knop medium can be due to poor nutrients or low concentration of sucrose (5%) in the medium.

Sucrose as the dominant nutrient compound suppresses the growth of embryos from the maturing seeds. When sucrose is the dominant nutrient, the cotyledons do not expand or form chlorophyll (WAGNER & KAFKA, 1995).

The possible reason for the best development of seedling on half strength MS medium can be due to the optimal concentration and form of calcium, potassium or nitrogen in the medium.

One of the most soluble forms of calcium is the chloride salt (BONGA & VON ADERKAS, 1992). On half strength MS media $CaCl_2 2H_2O$ is 220 mg l^{-1}. Increasing *Ca* in the medium such as $CaCl_2 2H_2O$ or $Ca(NO_3)_2 4H_2O$ significantly decreases percent of normally developed seedlings (from 25.00 ± 5.00 till 55.00 ± 2.89) (Table 1).

Potassium is the most abundant cation in the cell, playing an important role in osmotic control. In a properly balanced nutrient medium, tissues preferentially accumulate potassium than sodium. In an unbalanced medium, the preference shifts strongly towards sodium (BONGA & VON ADERKAS, 1992).

Nitrogenous compounds break dormancy and stimulate seed germination in many species (BONGA & VON ADERKAS, 1992). WAGNER & KAFKA (1995) reported that nitrates had no beneficial effect on the germination of isolated ash embryos, whether as components of a mineral mixture or sole nutrients. Our results confirm the conclusions of WAGNER & KAFKA (1995) that the response of ash embryos was similar if the medium contained nitrates or ammonium salts as the sole nutrients.

Development of the organs

The length of epicotyls reached 10.1 ± 1.6 mm after their cultivation on half strength MS medium, but it was not significantly different from the epicotyl length on half strength WPM (8.9 ± 0.9 mm) (Table 3). However, it should be noted that epicotyl growth was poor on all used media and the length depended on the medium. Development of all other organs was the best on half strength WPM.

The rich DKW and MS media did not have a good effect on the development of the organs. Because of a lack of microelements the poor Knop medium cannot provide the nutrients needed for normal development of the embryo, and on this medium the lowest lengths of epicotyl, hypocotyl and root were observed. These results did not differ significantly from the data on full DKW medium (Table 3).

Mean hypocotyl length is better on half strength WPM medium (26.3 ± 1.3 mm), there are not significant differences between full WPM and MS media. Lowest hypocotyl length is on DKW (full and half strength variants) and Knop media (Table 3).

Development of radicle is better on all half strength medium. The best results are achieved on half strength WPM medium (71.77 ± 4.98), but there is not significant difference with the results obtained after the using of full WPM medium (Table 3).

Table 3. Influence of the nutritive medium on the length (mm) of epicotyl, hypocotyl and root of the normally developed seedlings (type E).
Tabel 3. Influența mediului nutritiv asupra lungimii epicotilului, hipocotilului și rădăcinii (mm) în cazul puieților normal dezvoltați (tipul E).

Medium	Epicotyl M ± SE	Hypocotyl M ± SE	Root M ± SE
1/2 MS	10.1 ± 1.6 a[1]	21.2 ± 0.7 b	60.4 ± 4.3 b
MS	5.1 ± 0.6 ce	24.5 ± 0.9 a	48.3 ± 4.5 cde
1/2 WPM	8.3 ± 0.9 ab	26.3 ± 1.3 a	71.8 ± 5.0 a
WPM	7.2 ± 0.9 bc	23.9 ± 1.2 a	62.3 ± 6.7 ab
1/2 Knop	2.8 ± 0.6 de	19.1 ± 1.0 bc	56.3 ± 2.9 bc
Knop	2.6 ± 0.3 de	18.8 ± 0.9 bd	37.8 ± 3.0 ef
1/2 DKW	5.1 ± 0.6 cd	18.2 ± 0.6 cd	53.3 ± 2.7 bd
DKW	2.7 ± 0.4 de	18.3 ± 0.9 cd	31.4 ± 4.5 f

[1]Different letters in the column indicate significant difference for the normal seedlings ($p \leq 0.05$), using ANOVA test. M - mean, SE - standard error of mean

In the second treatment organogenesis was poor. Epicotyl and root length were lower (3.8 ± 1.1 mm and 34.9 ± 2.6 mm, respectively). Only hypocotyl length (28.0 ± 1.4 mm) was higher than according to the data received at first treatment (Table 4).

Table 4. Influence of the pre-sowing of the embryos (14 days at 4°C) on the length (mm) of the epicotyl, hipocotyl and root of the normally developed seedlings (type E).
Tabel 4. Influenţa pre-însămânţării embrionilor însămânţării (14 zile la 4°C) asupra lungimii epicotilului, hipocotilului şi rădăcinii (mm) în cazul puieţilor normal dezvoltaţi (tipul E).

	M ± SE
epicotyl	3.8 ± 1.1
hypocotyl	28.0 ± 1.4
root	34.9 ± 2.6

M - mean, SE - standard error of mean

CONCLUSIONS

The results from our investigation demonstrated that paradormancy could be overcomed by cultivation of isolated common ash embryos and using of suitable medium.

Half strength MS medium is the most suitable for embryo culture of common ash. Embryos on this medium showed good percent of vigorous developed seedlings. The using of dry seeds as initial explants can provide material for micropropagation during the whole year.

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Ivan Iliev, Desislava Dancheva
University of Forestry, 10 Kliment Ohridski blvd, Sofia 1756, Bulgaria
E-mail: iviliev@ltu.bg

Apostolos Scaltsoiyannes, Maria Tsaktsira, Parthena Tsoulpha
Department of Forestry and Natural Environment, Aristotle University, Thessaloniki 54006, Greece

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