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# Organogenesis of *Fraxinus excelsior* L. by isolated mature embryo culture

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## Abstract

The *in vitro* organogenesis of common ash *Fraxinus excelsior* L. on isolated mature embryo culture was investigated. Mature seeds of three genotypes (maternal trees) were collected from different ash populations of Lithuania: 'Kedainiai' (K-59), 'Nemencine' (N-5) and 'Telsiai' (T-3). The non-stratified seeds were disinfected with 70 % sulphur acid 1 min and with 80 % ethyl alcohol 3 min followed by four rinses in sterile distilled water. Mature zygotic embryos were aseptically removed from endosperm and placed on solified Murashige and Skoog medium supplemented with 1 mg $l^{-1}$ 6-benzylaminpurine, 1 mg $l^{-1}$ Ca pantetonate and 30 g $l^{-1}$ sucrose. Morphogenesis was observed every 10 days. The genotype of common ash had high effects on organogenesis. The T-3 maternal tree embryo showed the highest morphogenesis capacity under the same cultivation conditions, forming all organs within 20 days and at the highest frequency. Hypocotyls, embryonic leafs and embryonic stems could be used for direct organogenesis of *Fraxinus excelsior* L.

**Key words:** common ash, *Fraxinus excelsior* L., mature embryo, organogenesis, stratification.

## Introduction

Common ash (*Fraxinus excelsior* L.) is one of the most important forest tree species in many European countries. In Lithuania this hard wood tree occupies 50.8 thousand ha, attributing 2.89 % of the land area covered by all forest tree species. Breeding activities for hard wood species in Lithuania were initiated in 1998 (Pliura 2000).

Adventitious shoot organogenesis is a type of *in vitro* propagation that can be used for clonal reproduction of mass propagation of plants. Organogenesis involves *de novo* production of adventitious shoots on explants from many different sources, and this tissue culture technique is widely used in horticulture and forestry for production of ornamental plants and timber species (Tzfira et al. 1998). Although somatic embryogenesis and organogenesis has the potential to be a very efficient method for forest plant micropropagation *in vitro*, embryonic cultures produce only from seeds of zygotic embryos (Peña, Séguin 2001). There are few published studies on the somatic embryogenesis of common ash (Chalupa 1990; Hammat, Ridout, 1992; Hammat 1994). The isolated mature embryo culture could be helpful for tree research, as it does not involve seed stratification.

The objective of this study was to induce organogenesis and to obtain plant regeneration in isolated mature embryos from non-stratified seeds of *Fraxinus excelsior*.

## Materials and methods

Mature embryos, used as explants, were rescued from non-stratified seeds collected from common ash Lithuanian trees during autumn in 2000 and stored in the dark at 4 ± 2 ºC. Mature seeds from three genotypes (maternal trees) were collected from different ash populations of Lithuania: 'Kedainiai' (K-59), 'Nemencine' (N-5) and 'Telsiai' (T-3).

Pericarps were removed and seeds were sterilized with 70 % sulphur acid 1 min and with 80 % ethyl alcohol 3 min, followed by four rinses in sterile distilled water. The embryos were rescued by cutting the seeds along the edges and put them on solidified Murashige and Skoog (1962; MS) medium supplemented with 1 mg $l^{-1}$ 6-benzylaminpurine (BAP), 1 mg $l^{-1}$ Ca pantetonate and 30 g $l^{-1}$ sucrose. The pH was adjusted with KOH or HCl after dissolving gelling agents and before autoclaving (30 min at 120 ºC). Fifteen explants per replicate (four replicates) were initially placed into 85 mm diameter plastic Petri dishes, each containing 5 ml solidified medium. Culture tubes (20 × 145 mm) containing 10 ml medium were then used for the further plant regeneration. All cultures in first experiment were incubated under two light treatments: at 23 ± 2 ºC with a 16-h photoperiod under 80 W "cool-white" fluorescent lamps and in thermostat for darkness all day. The cultures for morphogenesis of different explants were cultivated at 23 ± 2 ºC with a 16-h photoperiod.

The research data was analysed using ANOVA.

## Results

The results demonstrate different morphogenesis frequencies for isolated mature embryo culture of common ash different maternal trees. Naturally, seeds germinate in darkness. Thus, we observed organogenesis in embryo culture of genotypes of common ash in the light and dark. Vitality of mature embryos was observed within ten days of culture. Fig. 1 presents morphogenesis success after 20 days cultivation under dark or light conditions. Leaves and callus were formed in the highest frequency under light, but dark conditions activated root and cotyledon formation in all genotypes. Embryos of the T-3 genotype formed callus and leaves in the highest frequency in comparison with the other two genotypes. In general, genotype T-3 seed embryos showed the highest organogenesis capacity in mature embryo culture.

The second experiment examined morphogenesis of isolated three types of explants (initial leaves, hypocotyls and embryonic stem) in tissue culture of the T-3genotype. Individual explants showed different response in tissue culture under the same conditions (Table 1). Hypocotyls formed roots in the highest frequency and they also formed callus. Initial leaves regenerated shoots, callus and roots. Embryonic stems formed shoots in the highest frequency.

## Discussion

Dormant seeds of ash acquire the ability to germinate after 6 to 7 months of stratification (Vorobyova 1981). For mass propagation it is important to find a technique that involves non-stratified seeds. Tissue technique, as isolated mature embryo culture, is promising for this purpose. The clonal propagation of ash by cuttings is frequently unsuccessful (Dirr,

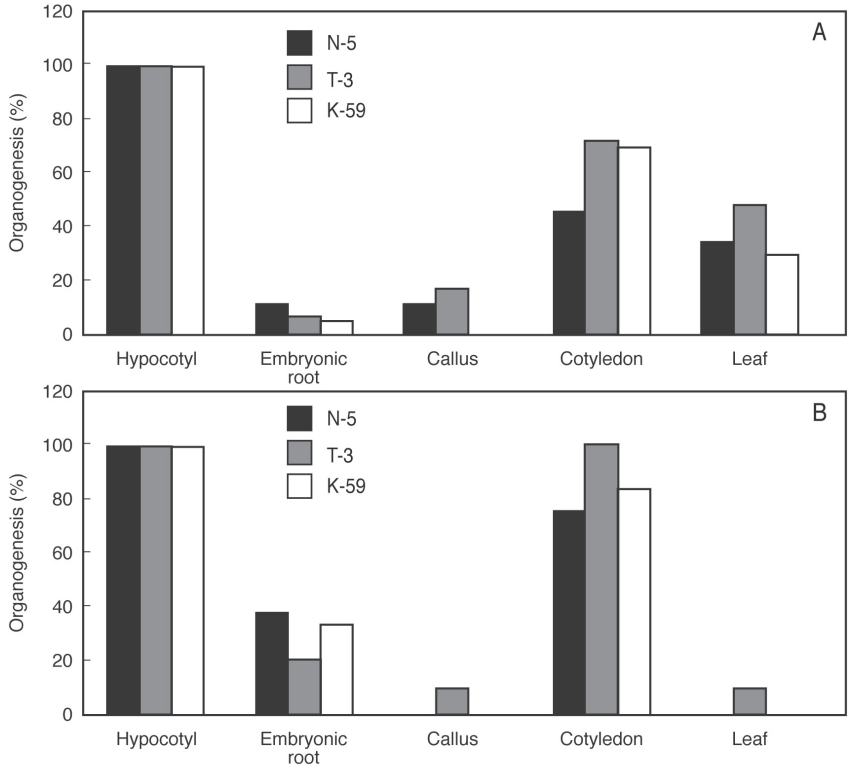


**Fig. 1.** Organogenesis in embryo culture of different genotypes of common ash in light (A) and darkness (B).

Heuser 1987), but there are some reports of *in vitro* regeneration of *Fraxinus* species. For example, both shoot organogenesis and somatic embryogenesis in white ash (*F. americana* L.) were achieved using cut mature seeds as primary explants (Bates et al. 1993; Bates, Preece 1995). In common ash (*F. excelsior*) micropropagation has been achieved from juvenile (Chalupa 1990; Hammat, Ridout 1992) and mature (Hammat 1994) plants. In our experiment, we attempted to induce *F. excelsior* organogenesis using by cutting sterile plantlets derived in mature embryo culture for receiving viable plants with roots.

In this work the medium was optimized for ash mature embryos culture initiation. After several days of cultivation, cotyledons, hypocotyls and embryonic stems developed.

**Table 1.** Effect of type of explant on morphogenesis of common ash genotype T-3

| Type of explant | Number of explants | Callus formation | | Root formation | | Shoot formation | |
|---|---|---|---|---|---|---|---|
| | | No. | % | No. | % | No. | % |
| Initial leaves | 53 | 17 | 32.08 | 2 | 3.77 | 10 | 18.87 |
| Hypocotyl | 61 | 3 | 4.92 | 33 | 54.09 | 10 | 16.39 |
| Embryonic stem | 16 | 0 | 0 | 0 | 0 | 2 | 12.5 |
| Sx % | | | 1.15 | | 1.49 | | 3.47 |

This observation is similar to that observed by Preece et al. (1989) in white ash (*F. americana*), where they achieved somatic embryogenesis and bud organogenesis on white ash cotyledons.

Morphogenetic processes, like rooting, somatic embryogenesis and bud organogenesis are often described as complex phenomena characterized by different phases, each with specific nutritional requirements (Gaspar et al. 1992). In the present study, MS medium proved to be effective for the induction and initiation phases. For common ash organogenesis, the medium was based on MS solution supplemented by additional growth regulators: 1 mg $l^{-1}$ BAP, 1 mg $l^{-1}$ Ca pantetonate.

Under the used conditions it was possible to achieve plant regeneration in mature embryos culture from non-stratified seeds of *F. excelsior*. Also, hypocotyls, initial leaves and stems as explants could be used for direct organogenesis of common ash.

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