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Note

Rapid seedling obtaining from European ash species *Fraxinus excelsior* (L.) and *Fraxinus angustifolia* (Vahl.)

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Abstract – Three different dissection treatments were applied to mature seeds of two European ash species: *Fraxinus excelsior* (L.) and *Fraxinus angustifolia* (Vahl.) in order to compare their relative efficiency for germination and further development. The in vitro embryo culture appeared to be the most efficient for both species (nearly 90% of viable plants). The two species expressed differences in germination rate without embryo culture.

***Fraxinus excelsior* / *Fraxinus angustifolia* / dormancy / embryo culture / germination**

Résumé – Une méthode d'obtention rapide de jeunes plants pour les frênes européens *Fraxinus excelsior* (L.) et *Fraxinus angustifolia* (Vahl.). L'efficacité de trois protocoles de dissection a été testée pour la germination de graines et le développement de deux espèces de frênes européens : *Fraxinus excelsior* (L.) et *Fraxinus angustifolia* (Vahl.). La culture in vitro d'embryons s'est avérée la plus efficace pour les deux espèces (près de 90 % de plantes viables). En l'absence de culture d'embryons, les taux de germination diffèrent pour les deux espèces.

***Fraxinus excelsior* / *Fraxinus angustifolia* / dormance / culture d'embryons / germination**

1. INTRODUCTION

In the forests of west Europe and especially in France the genus *Fraxinus* is essentially represented by two species, i.e. *Fraxinus excelsior* (L.) (common ash) and *Fraxinus angustifolia* (Vahl.) (narrow leaved ash). In the north part of France the common ash grows up to 30 m and is considered as a high valuable timber tree because of the toughness and elasticity of its wood. Therefore this species is largely used in reforestation programs in west Europe. On the opposite, the narrow leaved ash, which is appreciated in Mediterranean countries, exhibits under oceanic cool climate a poor development and a rather bad wood quality.

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Botanical traits of the two species generally allow to distinguish them but some individuals cannot be easily classified. In the sympatric areas, (e.g. Saône valley) well grown ash individuals exhibit intermediate characters. Therefore interspecific hybridisation was suspected for a long time but could not be ascertained up to now in natural conditions [8, 14]. Moreover we recently obtained ascertained hybrids between the two species under controlled conditions [9].

Seeds of both species generally present a dormancy, especially long in the common ash (up to 6 years). 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 [4, 6, 10]. The causes of the dormancy were debated for a long time by Villiers and Wareing ([11] and references therein). These authors obtained development of *F. excelsior* excised embryos on moistened filter paper. The in vitro culture of seeds or embryos has been proposed to remove the dormancy [7, 12, 13]. Wagner [12] showed that in vitro germination of embryos after extraction was possible for *F. excelsior* (germination rate from 60% to 90%), but her study was limited to the offspring of one single tree. On the other hand, Preece et al. [7] obtained very good results with partial cut seeds for *F. americana* (L.) and *F. pennsylvanica* (Marsh.). These authors mentioned a lower germination rate, less than 50%, for *F. angustifolia*; no data were given for *F. excelsior*. Embryo culture has been shown to remove embryo dormancy of *F. ornus* [1].

The aim of this paper is to compare different in vitro dormancy removal treatments, in order to find a procedure which can efficiently be applied to both species and their hybrids.

2. MATERIALS AND METHODS

2. 1. Plant material

Two populations typical of each species present in France were chosen for this experiment:

– common ash has been represented by the Saint-Gobain population (North of France);
– narrow leaved ash has been represented by the Cogolin/La Mole population (South-East of France).

According to the botanical traits and molecular markers [3], both populations were taxonomically pure.

Seeds were harvested from 20 trees per population, widely spaced one from each other (typically 50 m), in order to limit relatedness between individuals. Five sound seeds per tree per treatment were used, that is 100 seeds per species per treatment. Sound seeds were defined after sterilisation (see below).

2.2. Embryos and plants culture

2.2.1. Rehydration and sterilisation

Seeds were depericarped and soaked in a 0.3 M NaOH solution for 20 min. Rehydration and beginning of sterilisation were obtained in a 0.2% (w/v) calcium hypochlorite solution, $Ca(ClO)_2$ 70% active chlorine at 4 °C overnight. Sterilisation was achieved by soaking the seeds 2 hours at room temperature in a 2% (w/v) calcium hypochlorite solution. At the end of the sterilisation, seeds became white and translucent, so that the embryos were clearly visible (*figure 1a*). Seeds damaged by insects, seeds without embryo or seeds with necrotic embryo were discarded.

2.2.2. Culture medium and conditions

The different culture media contained the following common components per litre: macronutrients: 5 mM NH_4NO_3, 7.5 mM KNO_3, 1.5 mM $MgSO_4$, 1.5 mM $CaCl_2$, 2 mM KH_2PO_4 and 0.5 mM K_2HPO_4 so that the medium was directly buffered to pH 6.1. It was completed by 0.1 mM FeEDTA and half concentration of the mineral micronutrients of Murashige and Skoog [5]. 0.2% (w/v) Phytagel (Sigma) and 0.4% (w/v) agar were used as gelling agents before autoclaving 12 min at 120 °C. Culture medium named H_0 contained no sugar. 14 mM sucrose and 14 mM maltose (5 g per litre of each sugar) were added to H_0 giving H_{10} medium. The light regime of the growth chamber, provided by a mixture of fluorescent tubes (Philips TLD 36W33 4 000 K and Philips TLD 36W82 2 700 K) was 16 h d^{-1} (PAR: 25 µmol m^{-2} s^{-1}) and the temperature was 26 °C constant.

2.2.3. Treatments

• Treatment 1 (embryo culture):

After sterilisation, borders of seeds were carefully cut off and the remaining seeds were plated on H_0 medium for 24 hours, thus the embryos became free from the

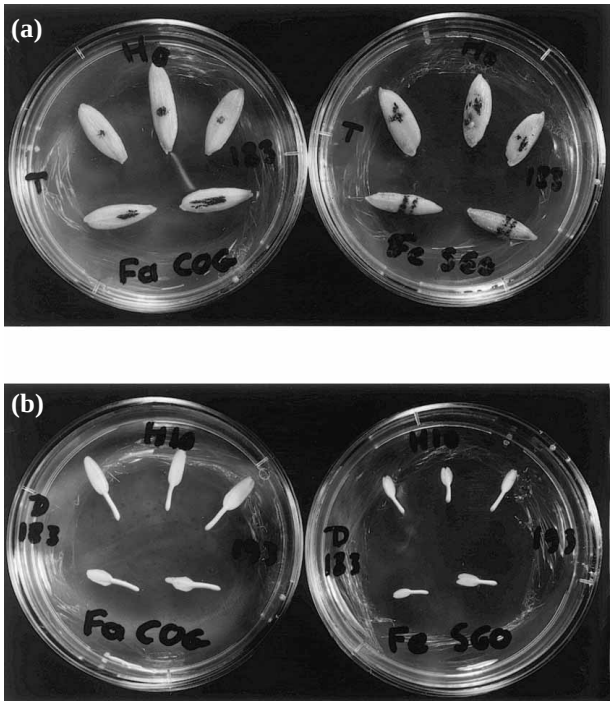


Figure 1. Rapid growth of European *Fraxinus* species with embryo culture. COG: *Fraxinus angustifolia* (Cogolin origin), on the left. SGO: *Fraxinus excelsior* (Saint-Gobain origin), on the right. (a) Seeds after sterilisation. (b) Embryos plated on H_{10} medium (24-h rehydration).

endosperm. Embryos were then first cultivated lying on H_{10} medium in Petri dishes (*figure 1b*) and transferred in culture tubes on the same H_{10} medium (1 embryo per tube), as soon as they started to grow, generally 2 to 3 days later. In this paper as in references cited, the in vitro development of the embryo excised from mature seeds is called germination.

- Treatment 2 (partial seed cutting):

Approximately one-third of the seed opposite to the radicle end was excised and discarded. Seeds were plated 24 hours on H_0 medium, then 48 hours on H_{10} medium and then transferred in culture tubes (as in treatment 1).

- Treatment 3 (intact seeds):

Same as treatment 2, without cutting (*figure 1a*).

2.2.4. Transfer in greenhouse and further growth

After 3 weeks, plants in tube, (*figure 2a*) had grown enough to be planted out in the greenhouse in mould. The light regime was 16 h d^{-1}. An additional lighting was given by lamps Philips SONT400W when necessary, one lamp per m^2. Only plants alive that produced at least 4 leaves (cotyledonary leaves excluded) were counted at 5 and 7 weeks after transfer in the greenhouse (*figure 2b*).

2.3. Statistical tests

Chi-square tests were performed in order to test both independence between development of plants and species (species effect) and independence between development and treatment (treatment effect). In addition, the presence of an interaction species × treatment effect was tested using interaction chi-square tests.

3. RESULTS

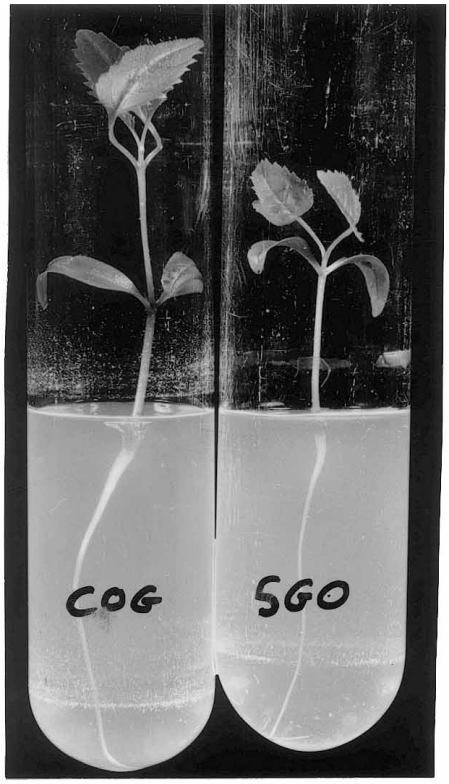
The different stages of germination and development are presented in *figures 1* and *2*. Embryos of *F. angustifolia* are generally larger than embryos of *F. excelsior* (*figure 1b*). They underwent root elongation as early as the first day of culture after excision.

Results for both species and for the three treatments are summarised in *table I*. As already mentioned, 100 safe seeds (i.e. 100 embryos) are involved per treatment per species. The chi-square values concerning the species effect were the following: at 5 weeks (respectively 7 weeks): $\chi^2 = 7.89$; df = 1; $p < 0.01$ ($\chi^2 = 8.33$; df = 1; $p < 0.01$). Concerning the treatment effect, the values were, at 5 weeks (resp. 7 weeks): $\chi^2 = 180.2$; df = 2; $p < 0.01$ ($\chi^2 = 165.3$; df = 2; $p < 0.01$). Thus, all chi-square tests concerning species or treatment effects were highly significant (at the 1% level), either 5 or 7 weeks after transfer to the greenhouse. An additional species × treatment interaction effect was observed at 5 weeks ($\chi^2 = 7.89$; df = 2; $p < 0.05$), but was no more

Table I. Percentage of developed plants 5 weeks (resp. 7) after transfer in the greenhouse.

	Treatment 1	Treatment 2	Treatment 3
F. angustifolia	88 (89)	39 (40)	17 (19)
F. excelsior	90 (88)	9 (14)	0 (0)

(a)



(b)

Figure 2. Rapid growth of European *Fraxinus* species with embryo culture corresponding to treatment 1. COG: *Fraxinus angustifolia* (Cogolin origin), SGO: *Fraxinus excelsior* (Saint-Gobain origin). (a) plantlets in culture tube (18 days of culture). (b) Seedlings in the greenhouse (10 weeks total culture).

significant at 7 weeks ($\chi^2 = 5.43$; df = 2; not significant at the 5% level).

Clearly, treatment 1 permits a good germination rate of the embryos and a good growth of seedlings of both species (almost 90% of plant normally developed after 10 weeks, 3 weeks in vitro then 7 weeks in the greenhouse). In contrast, treatment 3 gives poor results for *F. angustifolia* and no germination for *F. excelsior*. Treatment 2 leads to intermediate results. As a corollary, it appears that, without embryo extraction, seeds of *F. angustifolia* germinate better than seeds of *F. excelsior*.

4. DISCUSSION

Our results are quite in agreement with those of Wagner [12] (treatment 1 on *F. excelsior*) and Preece et al. [7] (treatment 2 on *F. angustifolia*) and extend them in 2 directions. In our experiment, treatment 2, as proposed by Preece et al. [7], gives fair results with *F. angustifolia* but poor results with *F. excelsior*. On the other hand, the embryo culture (treatment 1) gives us as good results with *F. excelsior* as with *F. angustifolia*. Moreover, the given percentages take into account all the stages from germination to growth in the greenhouse. In particular, we did not observe problems with the transfer to soil of the in vitro germinated seedlings as mentioned by Preece et al. [7].

Results observed without cutting or embryo culture (i.e. with treatment 3) indicate clear differences between common and narrow-leaved ash. Treatment 2 discriminates in the same way both species. These in vitro results are similar with those of Piotto [6] obtained in natural conditions. In both cases, *F. excelsior* exhibits a stronger dormancy than *F. angustifolia*.

Three possibilities may be proposed in order to explain the dormancy of ash seeds: embryo dormancy, tegument inhibition or endosperm inhibition. The latter were proposed by previous studies [1, 7, 11, 12]. The high rate (more than 90% of plated embryos) and high speed (less than 3 days after plating) of germination of the excised embryos show that there was no embryo dormancy in the mature seeds we used in this experiment. The strong (*F. angustifolia*) or total (*F. excelsior*) germination inhibition could be caused either by tegument or endosperm inhibition. The dormancy (or germination inhibition) is not affected by the sterilisation procedure. Experiments are currently undertaken in order to verify that the NaOH treatment followed by bleaching achieves to destroy the tegument. If true, the tegument inhibition could be excluded and therefore the endosperm inhibition ascertained.

The above developed technique allows to obtain easily and far quicker than the stratification a significant number of sterile ash seedlings. This can facilitate studies concerning ash mycorhization, because inoculation can be made with controlled fungus strains under aseptic conditions. An additional interest of our experiment is the possibility for foresters to use part of our sterilisation procedure in order to test the quality of ash seed lots. Ash seeds are very often damaged by fungi or insects [2]. For example, the seeds of some lots we used in this study were destroyed over 60% (data not shown). Moreover some sound seeds had a normal endosperm but no embryo. After the sterilisation process, about 90% of the seeds retained for treatment 1 (embryo culture) gave viable plants. So we properly evaluate the wholeness of the embryo in the seed. A good idea of the germination potential of a given lot can thus be obtained by this mean. As another practical issue of this experiment, the germination ability of *F. angustifolia* without any special treatment (vs. *F. excelsior*) could be used as a first test to detect contamination of seeds lots of *F. excelsior* by *F. angustifolia*.

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