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EFFECT OF DEUTERIUM-DEPLETED WATER ON *IN VIVO* AND *IN VITRO* GERMINATION IN BEECH AND BANATIAN BLACK PINE

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ABSTRACT*. Our study aimed to identify the effect of deuterium-depleted water (DDW), having 25 ppm deuterium, in the in vivo and in vitro germination process of two plant species of interest for biodiversity, from two natural protected sites in Romania, Fagus sylvatica L. and Pinus nigra var. banatica Georgescu & Ionescu. For this purpose, the seeds were watered, and the culture medium was prepared with distilled water (DW) (control) and DDW. At the end of the experiment, we concluded that, in the case of beech, in vivo, and in vitro, the germination rate and growth were higher in the version of seeds without pericarp treated with DDW. Also, the DKW culture medium, prepared with DDW, was better than the MS medium. The pine seeds did not germinate under the conditions of our experiment either in vivo or in vitro in the first year after sampling, but only in the second year after collection. Also, in the case of pine seeds, DDW optimized both the germination rate and growth indices.*

KEYWORDS: *deuterium, natural protected sites, beech nuts, rare plants, seeds*

Deuterium-depleted water is water with a low deuterium concentration below

150 ppm and has various physicochemical properties and different biological activities, with observable effects in plants (Petruș-Vancea 2018). Experiments carried out on two species of rice and maize grown on media with DDW revealed stimulation of seed germination and plant growth in different stages of ontogenetic development (Ștefănescu et al. 2002). The motivation for which we chose to replace DW with DDW, in the case of two species (beech and black pine), was based on the following aspects: beech seeds germinate easily in situ, they are plastic in response to environmental conditions (Muffler et al. 2021), but in vivo, under laboratory conditions, the germination is poor, especially due to infections that occur in the seed material (Kraj & Dolnicki 2003); black pine is a difficult species to regenerate (Oral 2019). In vitro plant cultures represent an alternative option for multiplication and conservation in the short-, medium-, or long-term of plants with recalcitrant seeds and plant species in danger of extinction (Rai 2022). Also, formulating the specific nutrient medium for the inoculum and species-specific is vital (Sudheer et al. 2022).

The European beech (*Fagus sylvatica* L.) grows under various climatic and environmental conditions (Bolte et al. 2007). Mass propagation by cloning is possible for this species, but it is not practiced due to the high costs involved (von Wuehlisch 2008). The banatian black pine (*Pinus nigra* var. banatica Georgescu & Ionescu) (Raab-Straube 2014+) is present in the Iron Gates Natural Park (IGNP) (Pătroescu et al. 2007), where it occupies small areas, on dolomitic substrate; however, its presence is discussed (Anastasiu et al. 2001).

Our study hypothesized that DDW will stimulate germination for both beech and pine. The objective was to increase the germination percentage and the survival rate in the first ontogenetic phases of the seedlings belonging to the two species.

The plant material consisted of beech seeds (*F. sylvatica*), taken from the spontaneous flora, from the Chitu Valley, at 400-450 m elevation (GPS: 45°16'16.0"N 23°22'54.0"E), from the Jiu Gorge National Park (JGNP) and Banatian black pine (*P. nigra* var. banatica), taken from the communities on Trescovăț Peak, 755 m elevation (GPS: 44°33'51.7"N 22°03'22.3" E), from the IGNP. Pine is a species protected by law, so the collection of plant material was carried out based on an agreement with the park administration. The seeds were collected from 5 trees, from each species, according to Bílek et al. (2009) protocol, in the second half of September 2022. All seeds were kept for 1 month at a temperature of 20 °C and then in a refrigerator at a low

temperature of 3 °C, as recommended by Kraj & Dolnicki (2003).

For in vivo germination, in February 2023, the beech seeds were washed with tap water, immersed for 30 seconds in 70% ethyl alcohol, rinsed with sterile water, and stratified by placing them in wet sand and kept cold. In April (according to the recommendations made by Standovár & Kenderes 2003), we put the seeds to germinate in a mixture of peat and sand, in a ratio of 1:1 (recommended by Elisovetcaia et al. 2022) or on filter paper. The culture substrate was treated with the fungicide Topsin 70 WDG in a dilution of 0.07%, 3 days after germination because infections of the seed material appeared. In the case of pine, after being stored in the refrigerator in April 2023, the seeds were washed under running water and germinated in vivo in a substrate consisting of peat and sand 1:1. In both species, the germination boxes were maintained outdoors at alternating day/night temperatures of 10–20 °C. The substrates were watered daily with distilled water (DW) in the control groups and deuterium-depleted water (DDW) with 25 ppm deuterium in the tested groups. After 90 days from in vivo germination and 30 days for in vitro, the measurements were made. We did not measure the pine roots to avoid destroying any seedlings.

For in vitro germination, in both species, we used two solid culture media, namely Murashige & Skook (1962) basal medium (MS) and Driver & Kuniyuki (1984) and McGranahan et al. (1987) (DKW), prepared with DW, for control lots, and with DDW, for test lots.

Germination in vitro was also done in April from the seeds kept in the refrigerator. Disinfection of nuts and pine seeds was carried out as follows: washing with a jet of water, immersion in 70% ethyl alcohol, immersion in sodium hypochlorite 5% with a few droplets of Tween 80 for 30 minutes, then cleared with sterile water 5 times and kept for 24 hours in sterile water, in a hood, after which the pericarp was removed from half of the nuts, respectively the coat from half of the pine seeds. They were again submerged for 3 minutes in 5% sodium hypochlorite and washed with sterile water, inoculated, and incubated at 21 °C, photoperiod 16/24h, lighting with fluorescent tubes with 2500 lx. We tested Qlarivia deuterium-depleted water produced with a patented installation (Ștefănescu & Țițescu, 2006). The data were mathematically processed, and the mean and standard deviation were calculated.

In *F. sylvatica*, in vivo germination was weak due to the infections that appeared, since the first days, after 30 days from germination, which were 100% on the filter paper substrate. Even if the nuts have a macroscopic

aspect of health, these being selected for germinating, after selection and removal of the endocarp, we identified that out of three lots of 100 seeds, 8% of them lacked seeds, being empty inside, which possibly was one of the causes of less germination in nuts, 20%, respectively 26%, compared to seeds, 34-39.5% (Table 1). The physical presence of the rest of the pericarp, which envelops the seed, does not physically affect germination, i.e., it does not prevent the emergence of the seedling. Still, it seems to have been a barrier to the disinfectant because, in nuts, a higher percentage, up to 53%, of infections was observed than in seeds (Table 1). A slight decrease in infections was observed in the lots watered with DDW, but it is not a question of considering DDW as a disinfecting agent. Also, regardless of the type of seed material, DDW led to the stimulation of the germination rate and the growth of the seedling in the first ontogenetic phases, especially rhizogenesis (Table 1 and Fig. 1).

Table 1. In vivo germination data at 90 days from germination (N – nuts; S – seeds).

Species	Type of substratum and seminiferous material	Germination rate (%)		Percent of infection (%)	Nuts without seeds (%)	Root length (cm)	Hypocotyl length (cm)	Epycotyl length (cm)	Plant length (cm)
F. sylvatica	V_{0N} - DW	20		53	8	1.0±0.1	2.1±0.3	2.0±0.5	5.1±0.1
	V_{0S} - DW	34		44		1.1±0.3	2.2±0.4	2.4±0.6	5.7±0.7
	V_{1N} - DDW	26		52.5		2.7±0.5	2.5±0.7	2.2±0.8	7.4±1.5
	V_{1S} - DDW	39.5		41		2.8±1.1	2.7±0.8	2.4±1.5	7.9±0.7
P. nigra var. banatica		1st year	2nd year						
	V_{0S} - DW	0	10	0	-	-	2.2±0.5	0.2±2.4	-
	V_{1S} - DDW	0	15	0	-	-	3.1±0.4	0.4±1.6	-

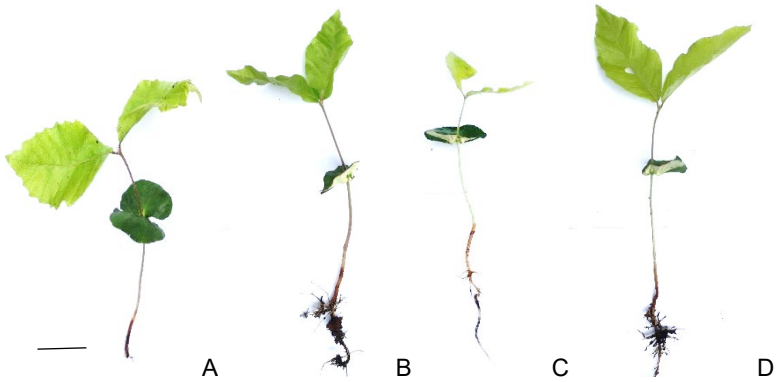

Figure 1. Beech seedling (*F. sylvatica* L.), at 90 days after germination, resulting from in vivo germination from nuts watered with DW(A), nuts watered with DDW (B), seeds with DW (C), and seeds with DDW (D) (bar means 1 cm).

Pine seeds germinated in vivo conditions but in the second year after collection. Thus, in the case of pine, we repeated the experiment in the next year, in the spring of 2024, after keeping the seeds in the dark, at 4°C, for one year. The seedlings had 7-8 cotyledons (Fig. 2). The lot treated with DDW showed a high germination rate and higher growth indices than the one treated with DW (Table 1). In vitro, these seeds did not become infected but did not germinate under the conditions we provided (Table 1).

Figure 2. *Pinus nigra* var. banatica seedling, at 90 days after germination, resulting from in vivo germination from seeds watered with DW(A), or with DDW (B) (bar means 1 cm).

In the case of in vitro germination of nuts or seeds, infections appeared, with a single exception from an inoculum point of view: seeds. The medium of this inoculum was MS-DDW, DKW-DW, and DKW-DDW (Table 2). Probably, the pericarp of nuts was a barrier in the action of the disinfectant.

In the case of *F. sylvatica*, we recorded a low germination rate (20-39.5%), compared to 68% reported by Ammer et al. (2002) in beech trees from Norway and 69.2% by Elisovetcaia et al. (2021) from Slovakia. Bressem (1998) reported that soil pH has a large influence on this process; thus, the best germination rate and subsequent development of beech seedlings occurred at pH (KCl) values of 4.3, while a pH of 3.0 inhibited plant development.

Generally, beech seeds are difficult to sterilize because they are usually heavily infested with microbes, and the ability to infiltrate the sterilizing agent into the cotyledons is limited (Nadel et al. 1991). In in vitro cultures, to reduce

crop infestation, antibiotics or fungicides can be introduced into the culture medium, but these substances are specific to certain microorganisms, can be easily decomposed in autoclaving, or can be toxic to cultivated plants (Guri & Kishor 1998). Optimization of micropropagation in beech was achieved with PPM (Plant Preserve Mixture), a substance with a very broad spectrum of microbes (Kraj & Dolnicki 2003).

We found that germination under laboratory conditions in beech contrasted with what can be observed in nature, where beech regenerated very well, even in locations with high abiotic stress, such as between railway track.

Table 2. In vitro germination data at 30 days from germination of *F. sylvatica* L. and *P. nigra* var. banatica (N – nuts; S – seeds; SC – seeds without coat).

Species	Type of medium	Type of inoculum	Germination rate (%)	Percent of infection (%)	Root length (cm)	Hypocotyl length (cm)
F. sylvatica	MS – DW	N	0	65	-	-
	MS – DW	S	0	70	-	0.0±0.0
	MS – DDW	N	0	58	-	-
	MS – DDW	S	5	45	0.4±0.6	0.0±0.0
	DKW – DW	N	0	60	-	-
	DKW – DW	S	11	43	0.3±1.5	0.0±0.0
	DKW - DDW	N	0	62	-	-
	DKW – DDW	S	16	50	1.1±0.8	0.9±1.2
P. nigra var. banatica	MS – DW	S	0	13	-	-
	MS – DW	SC	0	12	-	-
	MS – DDW	S	0	10	-	-
	MS – DDW	SC	0	9	-	-
	DKW – DW	S	0	11	-	-
	DKW – DW	SC	0	10	-	-
	DKW - DDW	S	0	12	-	-
	DKW – DDW	SC	0	10	-	-

On the other hand, in our studies, the Banatian black pine seeds did not germinate in vivo and in vitro in the first spring after sampling. The seeds germinated in similar conditions only in the second spring. In contrast to these results, Madosa et al. (2021) published a germination study, in vivo on moistened filter paper and in vitro on solid media, and they reported that germination starts on the second day, and after the sixth day, the intensity decreases. On the other hand, Oral (2019), in *Pinus nigra* subsp. pallasiana var. Yaltirikiana, reported a long time for germination and low germination

and seedling survival capacities, only 49% of the seeds were healthy, and 71% of them germinated in 28 days (Oral 2019). In *Pinus nigra* Arn. subsp. pallasiana (Lamb.) Holmboe, germination in Petri dishes on filter paper was over 80% without stress (Ulusan 2022). Šerý et al. (2020) significantly improved germination of *Pinus nigra* Arnold seeds with nonthermal plasma (NTP) treatment.

Even if some of the results of the experiments with *P. nigra* var. banatica were negative, we chose to present these results to highlight the difficulty of germination of this species and the importance of continuing studies in this regard. However, in the natural habitat on Trescovăț Peak, there are specimens of saplings aged 2-5 up to 10-15 years. The entire pine community is not very large, but the regeneration is better than in laboratory conditions.

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