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Some Problems Arising during the Initiation of Somatic Embryogenesis in *Pinus sylvestris* L. †

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Abstract: The use of biotechnological tools, in particular somatic embryogenesis (SE) for mass propagation of conifers, is relevant since this method allows to quickly replicate plant material with desired features. However, there are still a number of difficulties in obtaining an embryogenic cell culture for *Pinus sylvestris*. One of the important and unsolved problems is the search for SE-competent genotypes. We cultured 674 megagametophytes from 22 donor plants (16 genotypes) in vitro during the 2021 summer period. As a result of the experiment, callus formation was not recorded for the studied genotypes; however, 9.4 ± 1.0% of the explants formed plants. In addition to the genotype effect, unsuitable nutrient medium or late developmental stages of zygotic embryos could be the reasons for the lack of callus induction. To solve these problems, a number of studies were carried out: (1) the effect of the nutrient medium composition and density (MS, MSG, 1/2LV, DCR) on the callus initiation from mature seeds was analyzed, (2) the effect of various growth regulators concentrations on the initiation of callus formation was studied, (3) the analysis of the reproductive competence of donor plants was performed by the method of vegetative buds cultivation. As a result, several genotypes were found to have the ability for embryogenic callus formation, and the conditions for explants cultivation were selected.

Keywords: somatic embryogenesis; Scots pine; medium composition; mature seeds; vegetative buds; Karelia; forest seed plantation

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1. Introduction

Somatic embryogenesis (SE) is a promising and effective biotechnological method for obtaining a large amount of coniferous plant material throughout the year. Despite the availability of data on the successful initiation of this process in Scots pine (*Pinus sylvestris* L.), many researchers agree that this species is one of the most difficult to undergo SE [1–3]. It is considered that the successful initiation of SE in *P. sylvestris* depends on multiple factors [3–6]: the efficiency of surface sterilization protocol of plant material; the explant type; donor plant (genotype) capable of SE; cultivation conditions, in particular, the composition of the nutrient medium and the content of plant growth regulators; and the stage of the zygotic embryo development.

For several years, the team of our laboratory conducted research aimed at initiating SE in *P. sylvestris* using megagametophytes with immature zygotic embryos collected from plus trees' clones from the Petrozavodsk Seed Orchard (SO) of the 1st order as explants, and the protocol developed by M. Abrahamsson and co-authors [7]. Thus, in 2021, 674 megagametophytes were introduced into culture in vitro, which were collected from 22 clones of plus trees (16 genotypes). However, no embryonic-suspensor mass was obtained, and 9.4 ± 1.0% of the explants formed plants.

In this regard, we carried out a number of experiments aimed at finding out the possible reasons for the lack of SE initiation in *P. sylvestris* explants using vegetative buds and mature seeds.

2. Materials and methods

2.1. Determination of the Donor Plants' Reproductive Potential

Vegetative buds from plus trees clones (40 years old) growing on the Petrozavodsk SO of the 1st order (Karelia, Russia) [8] were collected during the period of forced dormancy in 2021, late February—early March (16 genotypes, 2 clones each) and in 2022, the end of March—beginning of April (6 genotypes, 2 clones each). Buds, without detaching from the shoot, were surface sterilized in a soap solution for 10 minutes, then washed under running water. Under aseptic conditions, buds were placed in 5% sodium hypochlorite solution for 10 minutes, with a three-fold treatment using sterile water, after which buds were placed in 20% hydrogen peroxide for 10 minutes with three thorough washings in sterile water. Buds were cleaned from integument layers in a laminar box, cut into 2–3 mm thick transverse disks, which were placed on Murashige-Skoog nutrient medium modified by A. Hohtola [9], 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BA) at concentrations of 2 and 1 mg/L, respectively, were used as growth regulators; 10 g/L sucrose served as a carbohydrate source. Five to six replicates were provided for each tree. Four explants were cultivated per jar (one replicate). The description of the ongoing processes was performed on the 30th day of the experiment. Parameters such as weight, initiation frequency, and proportion of light callus were analyzed.

The cytological analyses of the calluses obtained were conducted. The callus was placed on a glass slide, kept for 1–2 minutes in the dye (0.2% safranin water solution with the addition of a methylene blue drop) [6]. Squashed preparations were viewed under the light microscope (Carl Zeiss Primo Star) at 4× и10 × magnifications.

2.2. Study on the Effect of Plant Growth Regulators' Different Concentrations

The impact of phytohormones various concentrations on the megagametophytes reaction was performed on a DCR medium [10]. Twelve medium types were prepared, which differed in the content of plant growth regulators (PGR) and sucrose (Table 1). We used population mixtures of mature seeds collected from *P. sylvestris* trees located on the Petrozavodsk SO and in a park on the territory of Petrozavodsk (the age of the trees is 20 years) as explants. Explants were megagametophytes containing mature zygotic embryos. Sterilization of plant material was carried out in accordance with the protocol described above. Megagametophytes were extracted from mature seeds, peeled and placed horizontally on a medium, with 4 explants per jar (5 replicates). The formation of plants and/or calluses was registered on the 21st day of experiment.

Table 1. The content of growth regulators and sucrose in different types of the DCR medium.

Component	Nutrient Medium Number											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
2.4-D, μM	9.0	13.6	2.2	9.0	4.4	13.6	-	9.0	9.0	-	13.6	-
NAA, μM						-						2.7
BA, μM	4.4	2.2	2.2	9.0	4.4	13.6	4.4	-	2.2	-	9.0	9.0
Sucrose, g L^{-1}						30						10

Note. NAA—1-naphthylacetic acid.

2.3. Study of the Influence of Density and Composition of the Nutrient Medium

To study the effect of the composition and density of the nutrient medium on the reaction of *P. sylvestris* megagametophytes from mature seeds, we used explants from various habitats: the natural phytocoenosis of the Medvezhyegorsk region of the Republic

of Karelia (the age of the trees is 80–100 years) and the Petrozavodsk city park. During the study nutrient media MSG [5], MS [11], 1/2LV [6], DCR [7] with the same content of phytohormones 9.0 μM 2.4-D and 4.4 μM BA, which differ in the composition of micro- and macro-elements, sucrose content and have two density options (standard content and reduced content of gelling agent marked with "-") were used (Table 2). Sterilization, introduction and description of explants into culture in vitro was performed according to the protocol described above.

Table 2. The content of gelling agent and sucrose in different types of nutrient media.

Component g L^{-1}	Nutrient Medium							
	MSG	**MSG-**	**MS**	**MS-**	**1/2LV**	**1/2LV-**	**DCR**	**DCR-**
Sucrose	10		30		30		10	
Agar	7	3.5	6	3	7	3.5	-	3.5
Gelrite	-		-		-		3.5	-

2.4. Statistic Analysis

Data were statistically processed with Microsoft Excel 2007 and PAST (4.0). Spearman's rank correlation was used to measure the statistical dependence. All assays were performed at the Core Facility of the Karelian Research Centre RAS.

3. Results and Discussion

3.1. The Evaluation of the Donor-Plants Reproductive Potential

It was found that in 2021 the callus from the *P. sylvestris* vegetative buds was formed on the 5–11th day of cultivation. Data analysis showed that with an increase in the average mass of buds, callus (from 0 to 1 g), the proportion of light callus (from 4 to 61%) and the frequency of its initiation (from 38 to 90%) increases (Spearman correlation r = 0.52, $p = 0.002$ and r = 0.38, $p = 0.03$, respectively). Based on the data obtained, 6 genotypes were selected, which were capable on callus formation from buds with the highest mass (516, 856, 876, 1025, 1026). In 2022 explants were collected from these trees with further introduction into culture medium. On the 30th day of the study, the frequency of callus initiation in explants collected from different plus trees clones varied on average from 20 to 60%. Cytogenetic analysis showed that there are two types of cells forming the callus (Figure 1): meristematic (rounded) and parenchymal (elongated). Moreover, emerging single somatic embryos were registered in the genotype 1025-5 culture, which probably may indicate a predisposition of this genotype to SE. There is information in the literature about the formation of somatic embryoids in cell culture, where vegetative buds of *P. sylvestris* were used as explants [12].

3.2. Study of the Influence of Different Growth Regulators Concentrations

As a result of studying the effect of a substrate with different content of phytohormones, it was found that megagametophytes from plus trees clones located on the Petrozavodsk SO more often formed calluses on nutrient media № 3 и 5 (Table 2) while mature seeds from Petrozavodsk park formed a cell culture on substrate № 4 (Figure 2). It is important to note that explants collected from SO formed callus twice more often than seeds from the park. The auxin/cytokinin ratio (2:1) in the composition of nutrient medium is the most commonly used for SE initiation in conifers [6,7,13] et al. However, it was revealed in our study that the explants from mature seeds megagametophytes predominantly formed calluses on substrates with 1:1 auxin/cytokinin ratio. It should be noted that seed population mixture was used in this experiment, which contributed to a more effective assessment of nutrient media.

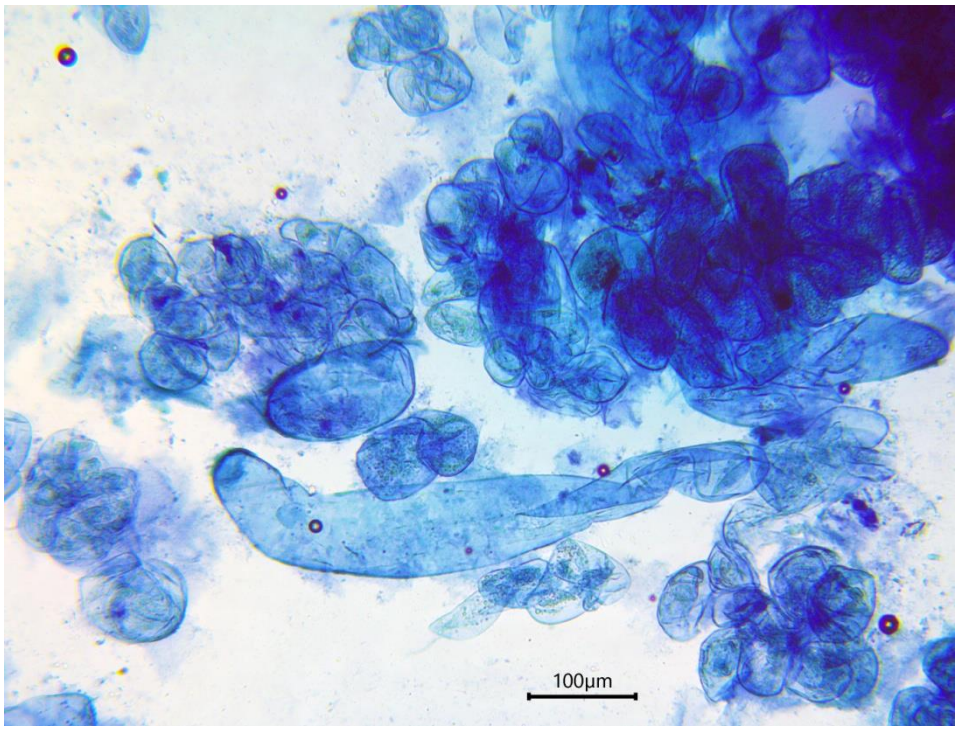

Figure 1. Meristematic and parenchymal types of somatic cells in *P. sylvestris* callus.

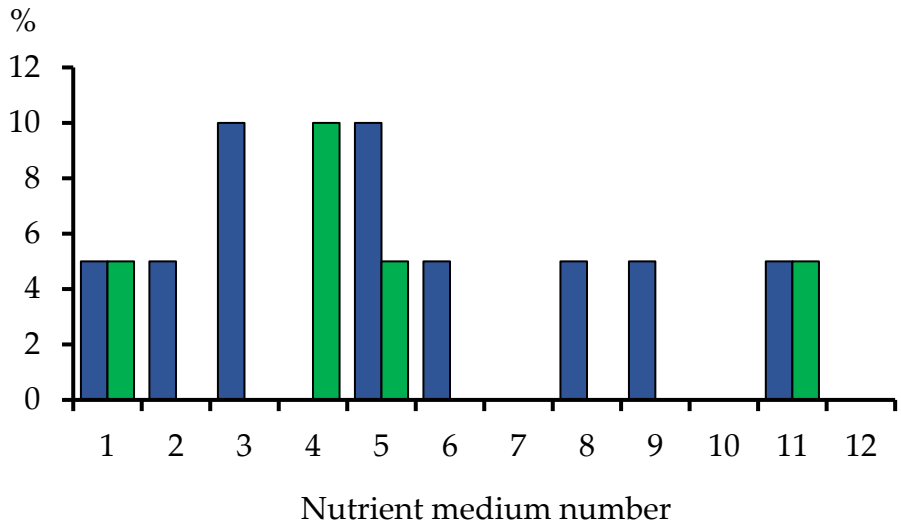

Figure 2. Frequency of callus formation on different nutrient media. Note: blue bars indicate Petrozavodsk SO, green bars—Petrozavodsk park.

3.3. Study of the Influence of Content and Density of the Nutrient Medium

It is known that the availability of water in the nutrient medium affects the development of the embryonic mass [13,14]. Several authors have shown that stress (including water deficiency) can trigger or improve embryogenesis in recalcitrant species [15,16]. It was found in our study that, in terms of the frequency of callus formation, the 1/2LV medium with the standard agar concentration turned out to be the most successful for *P. sylvestris* megagametophytes with mature embryos (Table 3). On the DCR- substrate, the proportion of explants (collected from trees in the natural phytocoenosis) formed callus averages 8.33 ± 3.3%, which is also a high value in this experiment. Analysis of the data obtained showed that the population mixture of seeds collected in the Medvezhye-

gorsk region of Karelia formed callus four times and plants 14 times more often than from megagametophytes of the Petrozavodsk park.

Table 3. Mean frequency of callus/plant formation from *Pinus sylvestris* mature seeds megagametophytes from different habitats on the nutrient media differed in composition and density.

Event/Medium, %	DCR	DCR-	MS	MS-	MSG	MSG-	1/2LV	1/2LV-
Medvezhyegorsk region								
Callus	1.67 ± 1.7	8.33 ± 3.3	3.33 ± 3.4	5.0 ± 2.8	6.67 ± 3.97	3.33 ± 2.4	9.67 ± 3.3	3.33 ± 2.4
Plant	8.33 ± 4.1	6.67 ± 3.1	0	0	11.67 ± 5.6	0	6.33 ± 2.9	0
Petrozavodsk park								
Callus	1.25 ± 1.3	0	-	-	1.25 ± 1.3	3.75 ± 2.1	5.0 ± 2.4	0
Plant	1.25 ± 1.3	0	-	-	0	1.25 ± 1.3	0	0

Note: Values in the table are the arithmetic mean of the frequency of callus/plant initiation ± standard error.

4. Conclusions

The data obtained indicate that the use of vegetative buds and mature seeds as explants can help identify *P. sylvestris* genotypes predisposed to SE, as well as select cultivation conditions throughout the year. As part of the experiments, it was found that there are genotypes on the Petrozavodsk SO that are probably capable of forming an embryonic-suspensor mass from immature embryos. The study showed that callus formed five times less frequently on the DCR nutrient medium than on the 1/2LV substrate. Perhaps, when *P. sylvestris* immature embryos collected in the middle taiga phytocoenoses of Karelia are introduced into culture in vitro, it is necessary to use this nutrient medium to initiate SE.

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