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INDUCTION AND DEVELOPMENT OF GRAND FIR (*ABIES GRANDIS* LINDL.) CALLUS IN TISSUE CULTURES

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ABSTRACT

The research is a methodological analysis of the first stage of micro-propagation of grand fir (*Abies grandis* Lindl.), which is included in the group of coniferous tree species introduced in Poland and characterised by a fast growth rate. The experiments were conducted on four types of grand fir explants (mature seeds, mega-gametophytes, mature zygotic embryos, buds collected in springtime from twenty year-old trees from the lower tree crown) with respect to initiation and further development of callus of this species. Nine experiments were conducted, where the following issues were analysed: efficiency of the disinfecting agent and the time of its operation on the percentage of grand fir explants of various origins not infected by bacteria or fungi, impact of media and growth regulators contained in the media on the initiation and development of callus and initiation of adventitious buds. Moreover, the research included the impact of the fungistatic and bacteriostatic agent – PPM and the conditions of conducting breeding (in light and in the dark) on callogenesis and organogenesis. The results of the research have shown that the best explants for *in-vitro* breeding of grand fir are zygotic embryos isolated from mature seeds (average frequency of regeneration 44.5%) and mega-gametophytes (average frequency of regeneration 10.3%).

Key words: *Abies grandis*; Embryonal suspensor mass (ESM); Plant Preservative Mixture (PPM); Somatic embryogenesis, Tissue culture

INTRODUCTION

The grand fir (*Abies grandis* Lindl.) is a coniferous species characterised by a fast growth rate. It is a Northern-American species characterised by high productivity, average requirements and good acclimatisation to the conditions of Western and Central Europe. Significant annual growth results in large timber mass and increases the abundance of the stand within a short time. Works on the introduction of grand fir in Europe have been pursued for several years. In Slovakia and the Czech Republic, there is an especially significant amount of planting of this species. In Poland, grand fir is cultivated in small areas in several forest inspectorates, mainly in the western part of the country.

Introduction of this species to plantation cultivation, in particular in North-Western Poland and in the Carpathians, may contribute to decreased devastation of the forests, e.g. by providing material for Christmas trees and Christmas decorations.

There are certain difficulties related with obtaining good seed material of *Abies grandis* in Poland, mainly due to the rare period of seed yielding (3 – 5 years in lowlands, 6 – 8 years in the mountains). Moreover, there are few trees in the reproductive stage, which results in frequent self-pollination. The imported seed material is usually characterised by low vitality. What is more, the selection of proper provenance is also important, because these specimens which winter well in our conditions have a higher propagation index. Therefore, in order to obtain planting material of high usefulness for forest economy and preservation of genotypes with economically valuable features, various solutions are looked for. One of them may be micro-propagation by means of *in vitro* cultures. This method allows for the obtaining of clones of selected trees in a relatively short period of time and in large amounts. One of the methods of clonal propagation is obtaining somatic embryos and, subsequently, seedlings through the stage of embryogenic callus. In the case of grand fir, it is difficult to obtain embryogenic callus; therefore, it is necessary to develop optimum cultivation conditions (selection of original explant, manner of disinfection, selection of medium and appropriate ratio of growth regulators contained therein).

MATERIAL AND METHODS

Research on obtaining grand fir (*Abies grandis* Lindl.) calluses was conducted. Nine experiments were carried out (Table 1 – 4) which analysed the efficiency of disinfecting agents and the time of their operation on the percentage of explants of various origin of grand fir not infected by bacteria or fungi (Table 1, 4) and the impact of medium and growth regulators contained in it (Table 2, 3) on initiation and development of grand fir callus (Table 2, 3) and on the initiation of adventitious buds (Table 4). Moreover, the research included the impact of PPM (Plant Preservative Mixture), a fungistatic and bacteriostatic medium developed by Plant Cell Technology Inc. (Table 2, 3), and conditions of conducting cultivation (in the dark and in light) on callogenesis and organogenesis.

Four types of explants were used in the research: mature seeds (Table 1), mega-gametophytes (Table 2), mature zygotic embryos (Table 3) and buds of grand fir collected in springtime from twenty year-old trees from the lower crown section (Table 4).

Table 1. Sterilisation efficiency, influence of media and PPM on initiation and development of the embryogenic callus of grand fir (*Abies grandis* Lindl.) on mature seeds derived from the state of Washington (provenance No. 12012, compartment 652)

Experiment No.	Sterilisation of plant material		Callus initiating medium (growth regulators $mg \cdot dm^{-3}$ PPM $cm^3 \cdot dm^{-3}$)
I	1.	Control *	MS BAP 1.0
	2.	Domestos 15% - 20 minutes	
	3.	Domestos 20% - 20 minutes	
	4.	NaOCl 5% - 15 minutes	
	5.	NaOCl 5% - 30 minutes	
	6.	NaOCl 10% - 30 minutes	
	7.	Chloramine 5% - 15 minutes	
	8.	Chloramine 5% - 30 minutes	
	9.	Chloramine 10% - 15 minutes	
II	1.	Control	MS BAP 1.0 PPM 2.0
	2.	Domestos 20% - 20 minutes	
	3.	Chloramine 10% - 15 minutes	
	4.	NaOCl 5% - 20 minutes	

Table 2. The influence of PPM on the percentage of non-infected explants and the influence of concentration of growth regulators in the medium and conditions of breeding on initiation and development of callus on mega-gametophytes of grand fir (*Abies grandis* Lindl.) disinfected with 10% hydrogen peroxide – H_2O_2 (3 minutes)

Experiment No.	Origin of plant material	Cone harvest date	SH callus initiating medium (growth regulators mg·dm^{-3}, PPM cm^3·dm^{-3})					
				BAP		2,4-D	NAA	PPM
III	Arboretum in Rogów – 5/28 (origin of tree - Cascade Range No. 5377)	14/09/1998	(breeding conditions: light, dark)	1.	1.0	0.5	0.5	-
				2.	1.0	0.5	0.5	0.5
				3.	0.5	0.5	1.0	0.5
				4.	0.5	1.0	2.0	0.5
				5.	1.0	0.5	1.0	0.5
				6.	1.0	2.0	0.5	0.5
				7.	2.0	1.0	1.0	0.5
				8.	2.0	4.0	2.0	0.5
				9.	4.0	2.0	2.0	0.5
				10.	1.0	0.5	0.5	2.0
				11.	0.5	0.5	1.0	2.0
				12.	0.5	1.0	2.0	2.0
				13.	1.0	0.5	1.0	2.0
				14.	1.0	2.0	0.5	2.0
				15.	2.0	1.0	1.0	2.0
				16.	2.0	4.0	2.0	2.0
				17.	4.0	2.0	2.0	2.0

Table 3. The impact of seed storage, concentration of growth regulators in the medium and conditions of breeding on the initiation and development of embryogenic callus on zygotic embryos of grand fir (*Abies grandis* Lindl) originating from the USA, Arboretum in Rogów and Kórnik.

Experiment No.	Origin of plant material	Starting date the experiment	Disinfection of plant material	Callus initiating medium (growth regulators mg·dm^{-3}; PPM cm^3·dm^{-3})			BAP	IBA	NAA	PPM
IV	Idaho - USA	25/10/1998	- no disinfection* - 5 % NaOCl–5 minutes	MS	In light, In the dark		2.0	0.5	1.25	-
							2.0			0.5
V	Arboretum in Rogów – 5/28 (origin of tree – Cascade Range No. 5377)	16/11/1998 (seeds stored for 2 months)	5 % NaOCl – 5 minutes		In the dark	1.	1.0			
						2.	2.0			
						3.	4.0			
						4.	8.0			
VI	Arboretum in Kórnik (origin of tree Nency – France)	21/01/1999 (seeds stored for 3 months)				1.	1.0			
						2.	2.0			
						3.	4.0			
						4.	8.0			
VII	Arboretum in Rogów – 5/28 (origin of tree – Cascade Range No. 5377)	12/02/1999 (seeds stored for 4 months)	5 % NaOCl – 5 minutes			1.	1.0	0.5	1.25	0.5
						2.	2.0			
						3.	3.0			
						4.	4.0			
		27/04/1999 (seeds stored for 6 months)				1.	1.0			
						2.	2.0			
						3.	3.0			
						4.	4.0			
		23/07/1999 (seeds stored for 9 months)				1.	1.0			
						2.	2.0			
						3.	3.0			
						4.	4.0			

Table 4. The efficiency of disinfection, heat treatment and the impact of soaking buds in PPM on the initiation of adventitious buds of grand fir (*Abies grandis* Lindl.) originating from Krynica (compartment 8c).

Experiment No.		Disinfection of plant material	Medium initiating adventitious buds (growth regulators $mg \cdot dm^{-3}$; PPM $cm^3 \cdot dm^{-3}$)
VIII	1.	Control	**MS** BAP 1.0 PPM 2.0
	2.	NaOCl 2% - 20 minutes	
	3.	NaOCl 5% - 10 minutes	
	4.	$Ca(OCl)_2$ 2% - 20 minutes	
	5.	$Ca(OCl)_2$ 5% - 10 minutes	
	6.	Chloramine 5% - 20 minutes	
	7.	Domestos 5% - 20 minutes	
	8.	Control + heat treatment	
	9.	NaOCl 2% - 20 minutes + heat treatment	
	10.	NaOCl 5% - 10 minutes + heat treatment	
	11.	$Ca(OCl)_2$ 2% - 20 minutes + heat treatment	
	12.	$Ca(OCl)_2$ 5% - 10 minutes + heat treatment	
	13.	Chloramine 5% - 20 minutes + heat treatment	
	14.	Domestos 5% - 20 minutes + heat treatment	
IX	1.	Control	**MS** BAP 1.0 PPM 2.0
	2.	NaOCl 10% - 20 minutes	
	3.	NaOCl 10% - 20 minutes + PPM 20 $cm^3 \cdot dm^{-3}$ in MS-24h	
	4.	NaOCl 10% - 20 minutes + PPM 20 $cm^3 \cdot dm^{-3}$ in MS-48h	
	5.	Domestos 10% - 20 minutes + PPM 20 $cm^3 \cdot dm^{-3}$ in MS-24h	
	6.	Domestos 10% - 20 minutes + PPM 20 $cm^3 \cdot dm^{-3}$ in MS-48h	

Five disinfecting agents were tested: Domestos (mature seeds, buds), sodium hypochlorite – NaOCl (mature seeds, zygotic embryos, buds), chloramine (mature seeds, buds), hydrogen peroxide - H_2O_2 (mega-gametophytes) and calcium hypochlorite $Ca(OCl)_2$ (buds). The type and the concentration of the disinfecting agent and the time of its operation on the plant material were adjusted to the type of explant (Table 1 – 4). Before disinfection, the plant material was soaked for 30 seconds in 70% ethanol (with the exception of mega-gametophytes), and after application of a proper disinfecting agent, the explants were rinsed three times in sterile distilled water. Only during disinfection of buds, additional combination with heat treatment of buds (2 – 3 seconds) was used (experiment VIII, Table 4) and soaking of buds in PPM for 24 and 48 hours according to the procedure recommended by the producer (http://www.ppm4plant-tc.com/references.htm). Moreover, the impact of PPM on the result of disinfection was analysed as well as its impact on initiation and further development of callus (experiments II – IX).

Two types of media were used: MS [24] and SH [28], containing macro and microelements, enriched with myo-inositol, glutamine, casein hydrolysate, vitamins and sucrose. Growth regulators were supplemented to the media in order to induce callus: cytokinins (BAP – 6-benzyloaminopurine, TDZ – thidiazuron, 2iP – 6-dimethylallyl-aminopurine) and auxins (2,4 D - 2,4-dichlorophenoxyacetic acid, NAA - N-Acetyl aspartate, IBA- Indole-3-butyric acid); they were applied in various combinations (Tables 2, 3). PPM was additionally introduced to the MS and SH media in experiments II – IX (0.5 or 2.0 $cm^3 \cdot dm^{-3}$) in order to eliminate bacteria and fungi, which were impossible to remove with the use of surface disinfection (Table 1 – 4). The pH of the media was adjusted to 5.6. Cultivation was carried out in the dark (experiments: I, II, V – IX), or both in the dark and in light (experiments: III, IV) at 24°C for 16 hours and at 20°C for 8 hours in an air-conditioned room. In all experiments, control combinations were used, i.e. without disinfection. Passages were made on every fourteenth day, and observation of non-infected and callus producing explants (type, development and mass of callus) were made every seven days. For experiments were used the components chiefly from the Sigma company [26].

RESULTS

The results of disinfection of grand fir explants used in experiments I – IX are presented in collective Table 5. Taking into account plant fragments from which the callus was obtained, 10% hydrogen peroxide provided the best results in disinfection of mega-gametophytes (3 minutes); whereas in the case of disinfection of seeds from which zygotic embryos were isolated, it was 5% NaOCl (5 minutes) (in both cases, 50% of non-infected explants).

The callus which developed on mature seeds was induced on the fourteenth day. Its very quick browning and dying out were recorded (Photo 1). This type of explant did not hold promise for further callogenetic development. PPM supplemented to the medium influenced an increase in the percentage of non-infected explants (from 9.2% to 60.5%); however, no callus formed after its application.

The highest frequency of callus formed on mega-gametophytes (18% - 33%) was achieved during application of cytokinins and auxins in a ratio of 1:3. In this case, PPM (2.0 $cm^3 \cdot dm^{-3}$) resulted in the increase of the percentage of non-infected explants up to 96.9%, at the same time not inhibiting the process of regeneration into callus (Table 7). Breeding in light or in the dark did not have any impact on regeneration in non-embryogenic callus.

Zygotic embryos of grand fir provided the highest percentage of callus, which depended on two factors: time of storing the seeds from which embryos were isolated and application of various combinations of cytokinin concentration – BAP (1.0, 2.0, 4.0, 8.0 $mg \cdot dm^{-3}$) (Table 3). 100% regeneration into callus of large mass (1400 mg on average) was recorded in the case of isolating zygotic embryos from seeds which were stored for a short time (2 months) on MS medium with 1.0 $mg \cdot dm^{-3}$ BAP. Use of BAP 2.0 $mg \cdot dm^{-3}$ concentration gave a relatively smaller percentage of regeneration frequency (72 %) (Table 8). Regeneration into callus in percentage terms was twice as high in the case of embryos bred in the dark (experiment IV, Table 3) (80%) than embryos bred in light (46%).

Buds collected in springtime from twenty year-old trees, from the lower section of the crown, did not produce adventitious buds, even though they were not infected in approx. 20%. Heat treatment of buds turned out to be ineffective, because both the heat treated buds and those buds which were not subject to heat treatment gave a similar percentage of non-infected buds (28 and 29%) (Table 4, 9). Additional soaking of buds in the PPM solution ($cm^3 \cdot dm^{-3}$) (Table 4) did not impact the increase of the percentage of non-infected buds and even contributed to its decrease to 7%.

The research has shown that the best explants for *in vitro* breeding of grand fir are zygotic embryos isolated from mature seeds (average frequency of regeneration is 44.5% after 42 days of breeding) and mega-gametophytes (average frequency of regeneration is 10.3% after 42 days of breeding) (Table 7, 8). However, despite applying numerous combinations of growth regulators (Table 2, 3), non-embryogenic callus developed on these explants; it was well-hydrated with a characteristic white-yellowish colour (Photo 2).

Table 5. Impact of five disinfecting agents and the time of their operation on the percentage of non-infected explants of *Abies grandis* (Lindl.) – comparison of results

No.	Disinfecting agent and the time of operation	Mature seeds	Mega-gametophytes	Zygotic embryos	Buds
1	Domestos 5 % - 20 minutes	*	*	*	–
2	Domestos 15 % - 20 minutes	–	*	*	*
3	Domestos 20 % - 20 minutes	+	*	*	*
4	Domestos 5% and heat treatment of buds	*	*	*	–
5	Domestos 10% and soaking of buds in PPM	*	*	*	–
6	NaOCl 2 % - 20 minutes	*	*	*	+
7	NaOCl 5 % - 5 minutes	*	*	+	*
8	NaOCl 5 % - 10 minutes	*	*	*	–
9	NaOCl 5 % - 15 minutes	–	*	*	*
10	NaOCl 5 % - 20 minutes	+	*	*	*
11	NaOCl 5 % - 30 minutes		*	*	*
12	NaOCl 10 % - 20 minutes	*	*	*	–
13	NaOCl 10 % - 30 minutes	–	*	*	*
14	NaOCl 2%, 5% and heat treatment of buds	*	*	*	+ –
15	NaOCl 10% and soaking of buds in PPM	*	*	*	–
16	$Ca(OCl)_2$ 2 % - 20 minutes	*	*	*	–
17	$Ca(OCl)_2$ 5 % - 10 minutes	*	*	*	–
18	$Ca(OCl)_2$ 2%, 5% and heat treatment of buds	*	*	*	–
19	Chloramine 5 % - 15 minutes	–	*	*	*
20	Chloramine 5 % - 20 minutes	*	*	*	–
21	Chloramine 5 % - 30 minutes	–	*	*	*
22	Chloramine 10 % -15 minutes	+	*	*	*
23	Chloramine 5% and heat treatment of buds	*	*	*	–
24	H_2O_2 10% - 3 minutes	*	+	*	*

\+ high percentage (above 50 %) of non-infected explants
– low percentage (below 50%) of non-infected explants
* no data (given agent not included in the research)

Table 6. Impact of disinfecting agent and PPM on the percentage of non-infected explants and the frequency of maturing of seeds of grand fir (*Abies grandis* Lindl.) regenerating in callus

Experiment No.	Explant	Combination*	Percentage of non-infected explants			Frequency of initiation in callus (%)			PPM in medium ($cm^3 \cdot dm^{-3}$)
			After days of breeding:			After days of breeding:			
			7	14	28	14	28	42	
I	Mature seeds	1- control	25	8	0	0	0	Dying out of breeding	0
		2	75	17	0	0	0		
		3	100	58	25	33	33		
		4	75	25	8	0	0		
		5	100	33	8	0	0		
		6	92	8	8	0	0		
		7	83	33	8	0	0		
		8	67	0	0	0	0		
		9	92	33	17	0	33		
		average	85.5	25.9	9.2	4.1	8.2		
II		1- control	0	0	0	0	0		2.0
		2	98	96	88	0	0		
		3	98	96	86	0	0		
		4	96	84	68	0	0		
		average	73.0	69.0	60.,5	0	0		

***** Type of combination specified in Table No. 1

Table 7. Impact of PPM on the percentage of non-infected explants and concentration of growth regulators and conditions of conducting culture on the frequency of mega-gametophytes of grand fir (*Abies grandis* Lindl.) regenerating in callus

Experiment No.	Explant	Origin of plant material	Combination*	Non-infected explants (%)		Frequency of initiation in callus (%)						PPM in medium ($cm^3 \cdot dm^{-3}$)
				After days of breeding:								
				14		14		28		42		
				Conditions of conducting breeding:								
				A	B	A	B	A	B	A	B	
III	Mega-gametophytes	Arboretum in Rogów – 5/28 (origin of tree – Cascade Range No. 5377)	1	5	50	0	20	0	20	0	20	0
			2	85	95	12	0	43	5	45	7	0.5
			3	60	95	0	0	0	16	0	18	
			4	75	55	0	0	0	0	0	0	
			5	85	65	0	0	0	0	0	0	
			6	65	30	0	0	0	17	0	17	
			7	25	25	0	0	0	0	0	0	
			8	35	15	0	0	14	33	14	33	
			9	60	50	0	0	25	0	25	0	
			average	55.0	53.3	1.5	0	10.2	10.1	9.3	10.5	
			10	55	95	9	0	33	0	33	0	2.0
			11	95	100	0	0	5	20	6	25	
			12	95	95	5	0	23	0	23	0	
			13	100	100	10	0	15	0	15	0	
			14	90	100	6	0	17	0	17	0	
			15	70	95	0	0	0	11	0	13	
			16	50	95	10	33	10	33	10	33	
			17	90	95	6	0	6	11	6	11	
			average	80.6	96.9	5.7	9.1	13.6	9.4	13.7	10.2	

***** Type of combination specified in Table No. 2
A – light; B – dark

Table 8. Impact of seed storage, concentration of growth regulators and conditions of conducting breeding on initiation and development of embryogenic callus on zygotic embryos of grand fir (*Abies grandis* Lindl.) originating from USA and arboretum in Rogów and Kórnik

Experiment No.	Explant	Combination*	Non-infected explants (%)		Frequency of initiation in callus (%)						PPM in medium ($cm^3 \cdot dm^{-3}$)
			After days of breeding:								
			14		21		28		42		
			Conditions of conducting breeding:								
			A	B	A	B	A	B	A	B	
IV	Zygotic embryos	1- control	0	0	0	0	0	0	0	0	0
		2	0	3	0	0	0	0	0	0	
		3	94	94	43	77	43	77	46	80	0.5
V		1	92		87		100		100		0.5
		2	58		62		72		72		
		3	82		78		78		80		
		4	48		8		13		14		
		average	70.0		58.7		65.7		66.5		
VI		1	92		92		46		46		
		2	94		92		46		46		
		3	80		80		33		33		
		4	88		84		40		40		
		average	88.5		87.0		41.2		41.2		
VII		1	90		33		33		33		
		2	90		10		15		15		
		3	84		4		4		4		
		4	98		0		0		0		
		average	90.5		11.7		13.0		13.0		
		1	56		24		32		32		
		2	76		24		24		24		
		3	94		18		21		21		
		4	94		11		11		11		
		average	80.0		21.2		22.0		22.0		
		1	0		0		0		0		
		2	0		0		0		0		
		3	0		0		0		0		
		4	0		0		0		0		
		average	0		0		0		0		

* Type of combination specified in Table No. 3
A – light; B – dark

Photo 1. Non-embryogenic callus of grand fir (*Abies grandis* Lindl.) developed on mature seeds

Photo 2. Non-embryogenic callus of grand fir (*Abies grandis* Lindl.) developed on mega-gametophyte

DISCUSSION

The research works on propagation of *Abies grandis* in *in vitro* conditions have been conducted since the mid 1980s. Among scientific centres dealing with this subject, a major role is played by the Slovak Academy of Sciences in Nitra, where research is conducted mainly on hybrid individuals of grand fir with other species from the *Abies* type [9,32,34].

This study tested the suitability of various types of explants of grand fir (mature seeds, embryos isolated from them, mega-gametophytes, buds) for the purposes of micro-propagation and determined the impact of disinfecting agents and the time of their operation and PPM (bactericidal and fungicidal agent) on the percentage of non-infected explants. Moreover, the impact of media and growth regulators contained in the media on the development and vitality of the induced callus of *Abies grandis* was examined.

According to literature, the type of explant constitutes one of the factors which impact the frequency of callus regeneration. Mainly immature zygotic embryos (mega-gametophytes) are used [29,34] as a source of fir explant as well as mature zygotic embryos isolated from seeds [3,13,14]. In this research, non-embryogenic callus was obtained both on zygotic embryos and on mega-gametophytes. In comparison with mega-gametophytes, zygotic embryos brought better results (on average 66.5% of regeneration frequency in callus). Hristoforoglu et al. [13,14] Jasik et al. [15], Szczygieł and Kowalczyk [31] arrived at similar conclusions. In these experiments (Table 1 – 4), non-embryogenic callus developed on mature, undamaged seeds, yet its quality was bad and it died out quickly. However, it has to be emphasised that mature seeds belong to the group of explants on which no research of this type has been conducted. Buds collected in springtime from twenty year-old trees from the lower section of the crown did not produce adventitious buds even though they were not infected in approx. 20%. Vookova et al. [32] obtained a small number of adventitious buds of mixtures of *Abies concolor* x *Abies grandis* forming on MS and SH mediums; the buds had visible morphological changes; therefore, the results obtained in this study regarding organogenic regeneration of buds do not differ significantly from previous studies on micro-propagation of grand fir by this method.

The review of publications and reports regarding disinfection of plant material indicates that authors most often do not specify the procedure and the efficiency of disinfection, only providing information about the chemical substance used and the time of its operation. This fact makes it impossible to compare the obtained results with other studies, even more so because a number of different agents are used for disinfection of explants of gymnospermae plants. The studies on disinfection of explants conducted by scientists from Slovakia [7,9] prove that application of mercuric chloride ($HgCl_2$) in a concentration of 0.1 – 0.2% (10-15 minutes) gives satisfactory results [5,18]. Also, a 10% solution of sodium hypochlorite (NaOCl) proved to be quite an efficient agent, especially with respect to firs, applied for 10 minutes [2]. In the case of *Abies alba,* soaking of seeds in 4% chloramine solution four or five times brings good results [19]. Calcium hypochlorite ($Ca(OCl)_2$ is a chemical compound frequently used for disinfection. However, in comparison with mercuric chloride, its efficiency of disinfection is lower [20,23]. Sometimes ready-made commercial preparations are used, such as Clorox [22] or Domestos [16,17] which, apart from active chemical agents, also contain additional patented disinfecting substances. The majority of highly concentrated chemical agents may even eliminate micro-organisms hidden deeply under the husk, yet eventually they do not have a positive impact on initiation, development and vitality of callus, which is shown by the above-mentioned studies. This study has shown that an effective disinfecting agent for mature seeds of *Abies grandis* is 20% Domestos (20 minutes), whose ingredients are NaOCl and 10% of chloramine (15 minutes) (Table 5). Apart from this, in the case of mature seeds of grand fir, introduction of PPM (2.0 $cm^3 \cdot dm^{-3}$) to the medium enhanced the sterility of the seeds and at the same time inhibited regeneration into callus. Compton and Compton and Koch [4] as well as Babaoglu and Yorgancilar [1] used PPM with good results, yet with respect to angiosperms. The experiments conducted on mega-gametophytes of *Abies grandis* have shown that a good way to sterilise them is with 10% hydrogen peroxide (H_2O_2) applied for 10 minutes after previous disinfection of cones in 5% H_2O_2 (10 minutes). In this manner, 71.4% of non-infected cultures were obtained on average (Table 7). According to literature, hydrogen peroxide is an effective agent for spruce and larch [30]; however, it is not very efficient with respect to mature seeds of fir, for which the optimum disinfecting agent is hypochlorite [11].

No adventitious buds were obtained on buds. It is assumed that this might have been caused by the use of disinfecting agents and additional procedures (heat-treatment of buds) in unsuitable (too drastic) concentration or time (Table 4).

In the literature on the subject, most studies are devoted to initiation and maturing of embryogenic callus obtained by means of somatic embryogenesis, which makes it impossible to compare these results regarding formation of non-embryogenic callus [5,10,11,13]. It is necessary to add that obtaining embryogenic cultures is very difficult in the case of trees, and the initiation of non-embryogenic callus, used most often for biochemical studies, is also not easy. After obtaining callus, in the course of its subsequent divisions and passages, some lines become brown and died out, which was observed in this study with respect to *Abies grandis* as well as in other studies with respect to *Picea abies* [31] and *Abies nordmanniana*, where after six months of maintaining cultures, only approx. 21% of callus lines continued to increase their mass (the rest died out) [25]. It is possible to induce initiation of embryogenic callus on formed non-embryogenic callus by changing the concentration of growth regulators in the medium or the concentration of caseine hydrolysate in the subsequent stages of *in vitro* cultivation. Therefore, the type of medium, enriched with optimum proportions of cytokinins, auxins and supplements of other ingredients such as phosphoglycerides, amino acids and vitamins, has significant impact on the callus initiation process. Well selected growth regulators in proper concentrations most often decide on the direction of morphogenetic transformations. According to various authors, only cytokinins should be used, e.g. BAP [8]; or cytokinins combined with a small concentration of auxins [6,27,33]. Therefore, in the conducted research, a number of combinations of media and growth regulators contained in them, as well as other compounds influencing initiation and development of callus, were used. BAP was used in a concentration of 0.5

– 8.0 $mg \cdot dm^{-3}$, 2,4D – 0.5 – 4.0 $mg \cdot dm^{-3}$, NAA – 0.5 – 2.0 $mg \cdot dm^{-3}$ (Table 2, 3). However, this did not bring the expected results due to the fact that embryogenic callus was not obtained on any of the explants. On the basis of studies conducted over a period of several years, it was recognised that the reason for the lack of embryogenic callus could have also been another factor, i.e. bad quality grand fir seeds or seeds with bad genotype. In the case of mega-gametophytes obtained from cones, the probable self-pollination of fir was the reason for absence of embryogenic callus. Non-embryogenic callus obtained in a frequency of approx. 28% formed on mega-gametophytes and zygotic embryos formed on SH and MS media with a supplement of cytokinins and auxins in a proportion of 1:3 (mega-gametophytes) and 1:2 (zygotic embryos).

Both the results of research presented in this study conducted on *Abies grandis*, as well as data from the literature on the subject, testify to the purposefulness of conducting research on obtaining callus in *in vitro* cultures due to the possibility of conducting various studies on it, e.g. regarding production of secondary metabolites or the use of callus for the needs of genetic engineering or phytopathologic *in vitro* studies on defence reactions of non-embryogenic and embryogenic callus with respect to pathogenic fungi [12,21,35].

CONCLUSIONS

1. The selection of explant is important in obtaining embryogenic callus. In the case of the grand fir, these are mega-gametophytes and embryos isolated from mature seeds.
2. The seeds have to be of high quality, stored not too long (up to 4 months) with an appropriate genotype and provenance (it is assumed that a higher propagation rate characterises these specimens which winter well in our conditions).
3. An appropriate disinfecting agent has to be selected for each type of explant; in the case of mega-gametophytes of grand fir, it is 10% hydrogen peroxide, and in the case of zygotic embryos, it is 5% sodium hypochlorite.
4. PPM applied in the medium in proper concentration (1.0–2.0 $cm^3 \cdot dm^{-3}$) contributes to achieving a higher percentage of non-infected explants; however, it does not result in inhibiting callogenesis.
5. The optimal proportion of cytokinin to auxin applied in the medium is 1:3 in the case of mega-gametophytes and 1:2 in the case of zygotic embryos isolated from mature seeds.

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