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PRESERVATION OF A RARE BOG PINE GENOTYPES USING MICROPROPAGATION TECHNIQUES

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Abstract

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Bog pine (*Pinus uncinata* DC. subsp. *uliginosa* (Neumann) Businský) is a subendemic species and appears to be one of the most endangered tree species of the Czech Republic. Its rare populations are at the present time greatly endangered namely by spontaneous hybridization with the Scots pine (*Pinus sylvestris* L.). Regarding the fact that its protection is insufficient even in national nature reserves (NNR) and the classical propagation by cuttings is problematic, modern methods were adopted for a long-term preservation of the taxon. Growth regulation conditions were investigated for the induction of organogenesis and somatic embryogenesis. Mature seeds were obtained from selected trees in the Žofinka NNR and from a locality near the village of Krajková in the Sokolov district. Cotyledon and hypocotyl segments from seedlings pregerminated in sterile conditions were used to induce the organogenesis. Bud proliferation was observed on the cotyledons after 4 weeks. The largest amount of buds was found on the medium with 1.5 mg.l^{-1} benzyladenine (BA) and 0.5 mg.l^{-1} 1-naphtylacetic acid (NAA) after 12 weeks. The hypocotyl segments showed only the formation of green callus. Isolated mature zygotic embryos were used for the induction of somatic embryogenesis. Development of mucilaginous callus was recorded after 3 weeks of cultivation on media with different combinations of BA, NAA and 2,4-dichlorophenoxy acetic acid (2,4-D) concentrations. When the callus induced on the medium with the combination of 0.56 (1.13) mg.l^{-1} BA and 1.86 mg.l^{-1} NAA was transferred on the medium with lower BA (0.113 mg.l^{-1}) and 2,4-D (0.44 mg.l^{-1}) concentrations, the first somatic embryos started to emerge after a period of other two weeks.

Pinus uncinata subsp. *uliginosa*, seeds, *in vitro* germination, organogenesis, somatic embryogenesis

Bog pine (*Pinus uncinata* DC. subsp. *uliginosa* (Neumann) Businský) is a subendemic species of the Czech Republic, a greater part of whose populations have a concentrated occurrence in localities of western Bohemia, in the Šumava Mts. in the valley of Křemelná and Vltava Rivers, in the Třeboňská pánev Basin and in one locality in the Žďárské vrchy Hills and one in the Hrubý Jeseník Mts. All localities of this taxon with the occurrence of clean-phenotype non-hybrid individuals behind the Czech borders (altogether less than ten) are either marginal parts of Czech populations or scanty residual populations. In Austria, Germany or elsewhere in Alpine countries (with the occurrence of allied taxa), no studies were published in several last decennia, dealing with the bog pine in its existing taxonomic demarcation, i.e. outside the Alps and/or the Schwarzwald. On the other hand, a number of works were published in Poland. Regional studies dealing directly with the bog pine (which is most often referred to by Polish authors as *P. uliginosa* Neumann) were focused on two residual populations growing not far from the Czech borders. More widely drafted works discussed a relevant allied group of pines (aggregate of *P. mugo* Turra) including the frequently occurring hybridization with *P. sylvestris* L. The mentioned works include morphometric studies (BORATYŃSKA et al., 2003), genetic studies (SIEDLEWSKA and PRUS-GLOWACKI, 1995; PRUS-GLOWACKI et al., 1998; LEWANDOWSKI et al., 2000, 2002) or phenological studies (BORATYŃSKI et al., 2003).

In addition to the geographic limitation and ecological distinctiveness, the bog pine is considerably endangered by long-term genetic erosion due to its spontaneous interspecific hybridization with the ecologically more flexible Scots pine (*P. sylvestris* L.). For many reasons, the bog pine is a much suitable model species (BUSINSKÝ, 1998) to be used in advanced research methods aiming at a long-term preservation of this taxon, which appears to be with the increasing knowledge one of the most endangered tree species of the Czech Republic. As to the long-term perspective of its survival, the mere passive protection of the species is insufficient even in national nature reserves.

Micropropagation techniques are the most convenient applicable method for a fast multiplication and preservation of rare genotypes of coniferous tree species. As reported by CHALUPA (1986) and MALÁ et al. (1999), organ cultures cultivated *in vitro* can be considered to be genetically stable because genetic changes do not occur. Regeneration using methods of organogenesis or somatic embryogenesis was studied in several pine species. Primary explants were in most cases axillary shoots from young seedlings or mature/immature embryos. Organogenesis was induced in *P. sylvestris* L. (CHALUPA, 1986; SUL and KORBAN, 1998; TORIBIO and PARDOS, 1989), *P. pinaster* Aiton (RANCILLAC, 1991; CALIXTO and PAIS, 1997) and *P. nigra* J. F. Arnold (SALAJOVÁ, 1992). Multiplication of individuals from adult trees continues to be still very difficult and it was successful only in some conifer species, e.g. in *P. lambertiana* Douglas (GUPTA and DURZAN, 1985) that belongs in another subgenus of pines. For the bog pine, only a method of organogenesis induction from isolated winter buds of adult individuals has been developed so far (VEJSADOVÁ and ŠEDIVÁ, 2002).

Explants derived from seeds after open pollination are not suitable for propagation due to the spontaneous hybridization of bog pine with *P. sylvestris*. In the *Pinus* genus, results of regeneration by the process of somatic embryogenesis suggest that although the success of this method depends on the genotype and on climatic conditions at the time of seed maturation, a sufficiently high amount of clones can be obtained through the modification of cultivation conditions for the establishment of viable populations (PARK et al., 2006). One of possibilities is the application of „saturated long-chain alcohol" triacontanol (TRIA) in the induction medium (MALABADI et al., 2005). Moreover, it was found out that the success in deriving the somatic embryogenesis can be enhanced by the selection of suitable parent couples in the *Pinus* genus (LELU et al., 1999; NISKANEN et al., 2004; MACKAY et al., 2006).

The objective of this work was to determine growth regulation conditions of organogenesis induction and somatic embryogenesis from the mature seeds of bog pine. To induce somatic embryogenesis the mature seeds were used to test the response of the zygotic embryos and megagametophyte to selected growth regulators.

MATERIALS AND METHODS

Organogenesis

Plant material

Mature seeds of bog pine (*P. uncinata* subsp. *uliginosa*) were used from the seed production of the Sofronka Arboretum in Bolevec. Donor trees were individuals from the Žofinka NNR – cadaster Dvory nad Lužnicí (seeds Z, Z1, harvested in 2007) and from the locality of Krajková in the Sokolov district (seeds K, K1, harvested in 2005). A portion of seeds was soaked in distilled water for 5 days at a temperature of 5 °C, another portion of seeds was stratified for 1–2 weeks without the application of water at 5 °C.

Surface sterilization of seeds

- Short washing with 70% ethanol.
- Application of 0.5–2.5% NaOCl (50% SAVO-commercial agent) or 7.2% of chlorinated lime filtrate with a few drops of Tween 80 for 30 minutes.
- Rinsing in sterile distilled water (3x).

Germination media

- 0.7% aqueous agar Sigma, pH 6.5.
- ¼ MS (MURASHIGE and SKOOG, 1962) concentration of macro- and microelements without vitamins, with 1.5% sucrose, 0.7% agar Sigma, pH 6.5.

Sowings was made into sterile plastic Petri dishes or into glass test tubes with slant agar. All media were sterilized at a temperature of 120 °C for 20 minutes. The seeds were germinated in a thermostat at 22 °C for 1–2 weeks.

Explant preparation

Cotyledons (primary leaves) and hypocotyl segments were isolated from the seedlings after 2–3 weeks. The explants were transferred onto the initiation media and cultivated at a photoperiod of 16 h, day/night temperature of 23/19 °C and light intensity 55 µmol m^{-2} s^{-1}. The percentage of explants with developing buds was established after 12 weeks. Subcultures were performed in 4-week intervals.

Induction media

The composition of induction media was a half-concentration of basic salts in the MS medium with vitamins, benzyladenine (BA) at a concentration of 1.5–2.5 mg.l^{-1}, combined with 1-naphtylacetic acid (NAA) at a concentration of 0.01–0.5 mg.l^{-1} or 0.5–1.0 mg.l^{-1} BA with indole-3-butyric acid (IBA) at a concentration of 0.1–0.5 mg.l^{-1}. The media contained 3% sucrose, 0.1% activated charcoal, 0.75% agar (Sigma), and the pH value of the media was adapted to 5.6 before autoclaving.

Statistical analysis

All data were analyzed using the one-way ANOVA analysis and the comparative Duncan's multiple range test at a significance level of $p = 0.05$.

Somatic embryogenesis

Plant material

Mature seeds of bog pine (*Pinus uncinata* DC. subsp. *uliginosa* (Neumann) Businský) were used from Tree No. 23, harvested in 2005, and not fully mature seeds from Borkovická blata, harvested on September 15, 2007.

Surface sterilization of old seeds (2005)

- Short washing with 70% ethanol.
- Application of the 20% SAVO commercial agent for 20 min.
- Rinsing in sterile distilled water (3x).
- The seeds were stored in the refrigerator at 4 °C immersed in sterile distilled water for 8 days.
- Before the preparation, the seeds were repeatedly rinsed in sterile distilled water.

Surface sterilization of seeds (2007)

- Short washing of the whole green cone with 70% ethanol.
- Rinsing in running water for about 15 min.
- 15 min. 20% SAVO.
- Rinsing in sterile distilled water (3x).
- Sterile withdrawal of the seeds.
- 10 min under the action of 10% hydrogen dioxide.
- Rinsing in sterile distilled water 3x.

Induction media

The total number of tested variants was 32 / (combinations of BA, 2,4-D or NAA) of DCR medium (GUPTA and DURZAN, 1985) with 50 mg.l^{-1} glutamine, 200 mg.l^{-1} inositol and 500 mg.l^{-1} casein hydrolyzate and/or 10 µg.l^{-1} triacontanole (TRIA), and either 3% sucrose or 3% maltose/. The media were solidified by Gelrit (Duchefa) or Phytagel (Sigma) (0.3%). The pH was adjusted to 5.8 before autoclaving.

Somatic embryogenesis was induced in sterile plastic Petri dishes (90 mm). Each treatment was implemented with a minimum number of 20 embryos and 20 megagametophytes.

Explant preparation

After removal of the testa, the embryos were transferred together with the isolated megagametophyte into the same Petri dish. The explants were cultivated in the dark at a temperature of 23 ± 1 °C. Morphogenetic response of the explants was observed gradually in one week intervals. Potentially embryogenic explants were transferred onto DCR multiplication medium with 0.113 mg.l^{-1} BA and 0.44 mg.l^{-1} 2,4-D and 2% sacrose. Subcultures were performed in two week intervals and documented using the Quick Photo Micro programme and the Deep focus module (Olympus).

RESULTS AND DISCUSSION

Organogenesis

It follows from the literary data (CHALUPA, 1986; SUL and KORBAN, 1998; MACKAY et al., 2006) that a successful organogenesis of conifers depends on various factors such as age and physiological condition of donor plants, date of collection, method and duration of initial material storage, surface sterilization, explant type and concentration of growth regulators in the cultivation medium. In our experiments, we used explants (shoot tips) from seedlings, in which the objective was an initiation of adventitious buds and shoots. The first step was to stimulate germination of long-term storaged bog pine seeds. The various variants of seed treatment were tested including the prolonged soaking in distilled water.

I: *Effect of pretreatment by water (5 days) and cold (5 °C) on the germination of bog pine seeds*

	Germination (%)		
Seeds[1]	Control[2]	Cold	Water + Cold
Z	0	0	60
Z1	0	0	5
K	0	0	75
K1	0	0	80

[1]Z, Z1 – the Žofinka locality; K, K1 – the Krajková locality, [2]Control – seeds without the pretreatment by water and cold

As shown above in Tab. I, the seeds germinated only after 5 days of soaking in water at 5 °C. Their germination rate ranged from 5 to 80% depending on the locality from which they originated. Tested were two sterilization agents, sodium hypochlorite and calcium hypochlorite (Tab. II). Successful was only the surface sterilization by 7.2% calcium hypochlorite, which stimulated most the germination of seeds from the Krajková locality.

II: *Effect of sterilization agent on the germination of bog pine seeds*

	Germination (%)		
Seeds[1]	0.5% NaOCl	2.5% NaOCl	7.2% $Ca(OCl)_2$
Z	0	0	60
Z1	0	0	5
K	0	0	75
K1	0	0	80

[1]Z, Z1 – the Žofinka locality; K, K1 – the Krajková locality; The seeds were given a 5-day pretreatement by water and cold.

Two media were tested for seed germination. However, no statistically significant variance was found

between the values of germination on the agar without mineral nutrients and sugar, and on the ¼ MS medium with 2% sucrose (Tab. III).

III: *Effect of medium on the germination of bog pine seeds*

	Germination (%)	
Seeds[1]	Aquaeous agar	¼ concentration of MS salts + saccharose
Z	60	60
Z1	5	5
K	75	75
K1	80	80

[1]Z, Z1 – the Žofinka locality; K, K1 – the Krajková locality

A difference in the response of explants was observed after 4 weeks with the cotyledons exhibiting bud proliferation (Fig. 1) and hypocotyl segments showing only green callus (Tab. IV).

Twelve weeks later a significantly highest bud formation occurred in the combination of 1.5 mg.l^{-1} BA with 0.5 mg.l^{-1} NAA (Tab. V), which was found in 23–85% of explants. Although the combination of 0.5 mg.l^{-1} BA with 0.2 mg.l^{-1} IBA was significantly lower in some treatments, it was observed to stimulate the development of adventitious buds (Fig. 2). Explants on the medium without growth regulators exhibited necrosis. Cotyledons from the pregerminated seeds collected from donor trees in the Žofinka NNR were more responsive than those from the Krajková locality although the germination of seeds showed a reversed effect (Tabs. I–III).

IV: *Effect of explant type on bud formation after 4 weeks*

Locality[1]/Explant	BA	BA/NAA	BA/IBA
	2.5 mg.l^{-1}	1.5/0.5 mg.l^{-1}	0.5/0.2 mg.l^{-1}
Z./cotyledons	Proliferation	Proliferation	Proliferation
Z./hypocotyl	Callus	Callus	Callus
Z1./cotyledons	Proliferation	Proliferation	Proliferation
Z1/hypocotyl	Callus	Callus	Callus
K/cotyledons	Proliferation	Proliferation	Proliferation
K./hypocotyl	Callus	Callus	Callus
K1/cotyledons	Proliferation	Proliferation	Proliferation
K1./hypocotyl	Callus	Callus	Callus

[1]Z, Z1 – the Žofinka locality; K, K1 – the Krajková locality

V: *Effect of growth regulators on in vitro regeneration after 12 weeks*

Explant[1]/regulator	mg.l^{-1}	Regenerating explants (%)			
		Z	Z1	K	K1
none[2]	-	-	-	-	-
BA	1.5	35.2a	31.3a	25.2a	23.7a
BA + NAA	1.5 + 0.5	85.1d	85.8c	60.3b	72.4c
BA	0.5	22.6ab	27.9a	21.5a	20.4a
BA + IBA	0.5 + 0.2	60.5[3]c	58.9[3]b	53.4b	51.2b

[1]Cotyledons of seedlings Z, Z1 – the Žofinka locality; K, K1 – the Krajková locality. [2]Necrosis. [3]Formation of adventitious buds. The values represent a mean of 10 replicates. Values followed by the same letter are not significantly different according to Duncan's test (p = 0.05).

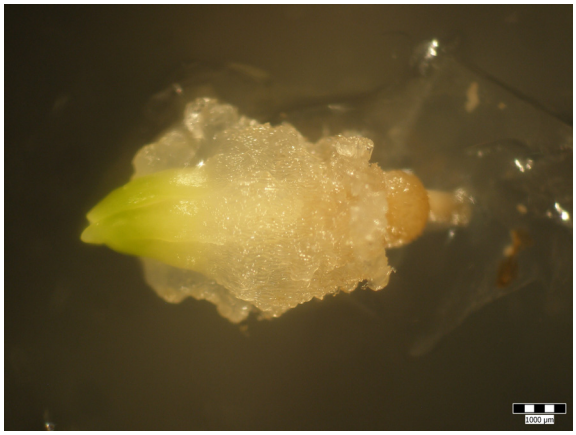
Somatic embryogenesis

Based on the preliminary experiments it was shown that if cytokinin BA is not present in the medium, the cotyledons exhibit a marked elongation. Among other things, the response apparently had to do with the presence of sucrose in the medium. When sucrose was replaced with maltose, the elongation of cotyledons was observed also on the medium with 1.13 mg.l^{-1} BA (Tab. VI). The elongation of cotyledons is usually connected with the termination of callus development, which is in other treatments observed namely on the hypocotyl (Fig. 3), and with the hypocotyl necrosis. Nevertheless, the deformed cotyledons exhibited a development of callus and tubular cells too (Fig. 4), and even roots under the influence of 0.93 mg.l^{-1} NAA (Fig. 5). Crumbly callus was developing on a whole scale of growth regu-

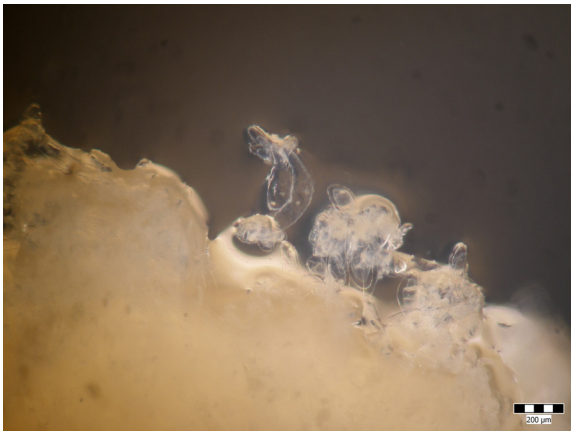
lator combinations (BA, NAA, 2,4-D) – see Tab. VI. However, after the transfer onto the proliferation medium, emerging somatic embryos were observed only with the use of lower BA concentrations (0.56 or 1.13 mg.l^{-1}) combined with 1.86 mg.l^{-1} NAA (Figs. 6 and 7), which corresponds well with the observations of LELU-WALTER et al. (2008) in *P. sylvestris* L. In both cases, the medium used our experiments contained triacontanol (10 µg.l^{-1}). This saturated long-chain alcohol is known to stimulate various physiological processes and proved well for the induction of somatic embryogenesis in *P. kesiya*, too (MALABADI et al., 2005). In contrast to the proliferation of axillary buds in the induction of organogenesis from seedlings where the optimum ratio of cytokinin and auxin was 3 : 1 (Tab. V), the development of buds on the cotyledons and apexes of zygotic embryos occurred at an opposite cytokinin/auxin ratio (1 : 1–4) – see Tab. VI. However, the developing buds were in that case largely adventitious (Fig. 8).

1: *Shoot tip proliferation*

2: *Formation of adventitious buds*



3: *Callus formation on the hypocotyl of zygotic embryos*

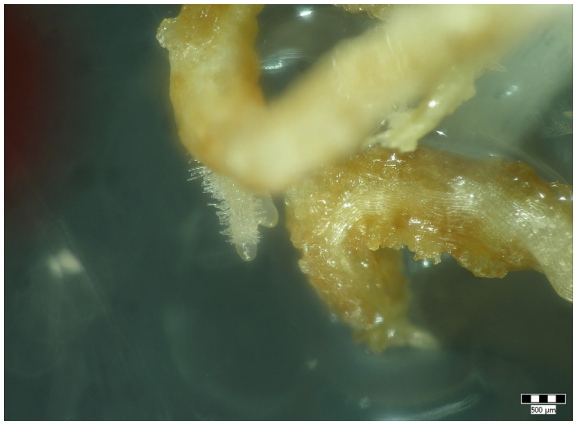


4: *Formation of tubullar cells on deformed cotyledons*

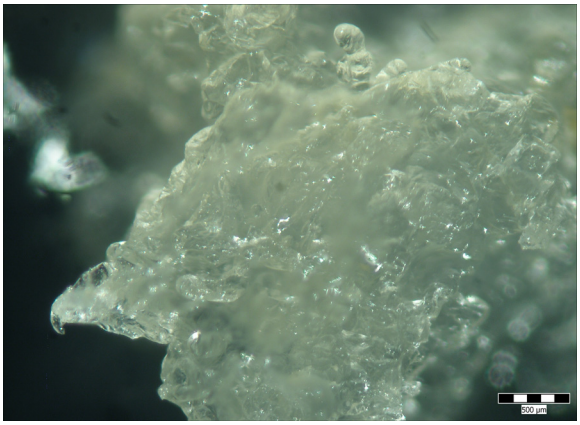
VI: *Morfological responses of zygotic embryos of bog pine after 3 weeks (C=cotyledons, H=hypocotyls, R=radicle)/% of response*

	components of induction medium								type of callus													other responses								
									consistent						mucilaginous							deformation		prolongation		organs		browning		
	BA	2,4-D	NAA	TRIA	Phyt	Gel	suc	malt	light			dark			light			dark												
	mg.l^{-1}	mg.l^{-1}	mg.l^{-1}	ug.l^{-1}	%	%	%	%	R	H	C	R	H	C	R	H	C	R	H	C	H	C	H	C	C	C	R	H	C
1	0	1.1	0	0	0.3		3																		80			100		100
2	0.56	2.2	0	0	0.3		3							70														100		100
3	2.25	0.55	0	0	0.3		3			50	50																			
4	2.25	2.2	0	0	0.3		3		50	50	50	80	80	80																
5	2.25	11	0	0	0.3		3				60					20							40				10			
6	0	1.1	0	0		0.3	3																					80		
7	0.56	2.2	0	0		0.3	3																		80					
8	2.25	0.55	0	0		0.3	3						50	50									10							
9	2.25	2.2	0	0		0.3	3									50	50		50	50			60		60			100		60
10	2.25	11	0	0		0.3	3					100	100	100														100	100	100
11	1.13	2.2	0	0		0.3	3				40	60	60	60									40		20		20	100	100	
12	0	0	0.93	0		0.3	3		20	20			80	80			20				60	100	100	100	30		80	80	80
13	0.56	0	1.86	0		0.3	3									80	80	20	20		40		50			50			
14	2.25	0	0.465	0		0.3	3									50	50		50	50	50		50						
15	2.25	0	1.86	0		0.3	3									40	40				60	60	80	100		30			
17	0	0	0.93	10		0.3	3		20	20											40	100	20	100		10			
18	0.56	0	1.86	10		0.3	3										80	80	80			60		60		40			
19	2.25	0	0.465	10		0.3	3			20			10						50	50	70		80			20			
20	2.25	0	1.86	10		0.3	3					40	40		10	10		60	60			20		20		40			
22	1.13	0	1.86	10		0.3	3		20	20		80	80								100	30	100	30		30	80	10	
23	0	2.2	0	10		0.3		3	90	90				80			10				90	100	100	100			10	10	80
24	1.13	2.2	0	10		0.3		3		10											10		30				10	10	
25	0	0		10		0.3		3									30		30		10		20						
26	1.13	0	1.86	10		0.3		3		40			60	60			10			20	100	100	100	100			80	80	
27	0.56	0.55	0	0		0.3	3									40				60		60							
28	0.56	0.55	0	10		0.3	3										40				70		70						
29	0.56	0	0.465	0		0.3	3										40				10								
33	0.56	0	0	0		0.3	3			10											20	100	60	90					
34	1.13	0	0	0		0.3	3			90											10	100	10	90					
35	2.25	0	0	0		0.3	3														50	100	50	100					

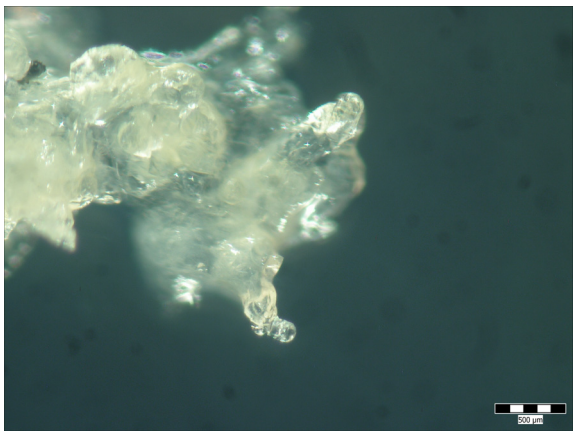
BAP – benzylaminopurine, 2,4-D – 2,4-dichlorophenoxyacetic acid, NAA – naphthylacetic acid, TRIA – triacontanol, Phyt – Phytagel (Sigma), Gel – Gelrite (Duchefa), suc – sucrose, malt – maltose



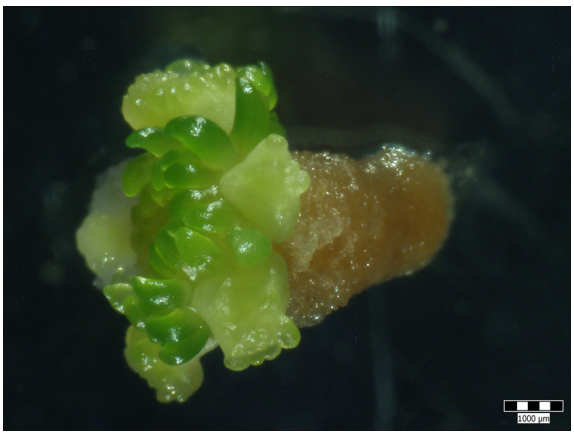
5: *Formation of roots on deformed cotyledons*



6: *Formation of somatic embryos after induction with 0.56 mg.l^{-1} BA and 1.86 mg.l^{-1} NAA*



7: *Formation of somatic embryos after induction with 1.13 mg.l^{-1} BA and 1.86 mg.l^{-1} NAA*



8: *Formation of buds on cotyledons and on the apex of zygotic embryos on the medium with 2.25 mg.l^{-1} BA and 1.86 mg.l^{-1} NAA*

SUMMARY

The objective of this work was to determine growth regulation conditions of organogenesis and somatic embryogenesis induction from the mature seeds of bog pine. In the *Pinus* genus, with respect to the induction of somatic embryogenesis occurs largely from retained embryos, which become extinct in the mature seeds, experiments with the mature seeds were used to test the response of the embryos to selected growth regulators. Bog pine (*Pinus uncinata* subsp. *uliginosa*) is a subendemic species of the Czech Republic, a greater part of whose populations occur in localities situated in western Bohemia, in the Šumava Mts. in the valley of Křemelná and Vltava Rivers, in the Třeboňská pánev Basin and by one locality in the Žďárské vrchy Hills and in the Hrubý Jeseník Mts. All localities of this taxon with the occurrence of clean-phenotype non-hybrid individuals behind the Czech borders (a total of less than ten) are either marginal parts of Czech populations or scanty residual populations. For many reasons, the bog pine is a much convenient model species (BUSINSKÝ, 1998) to be used in advanced research methods aiming at a long-term preservation of this taxon, which appears to be one of the most endangered tree species of the Czech Republic with the increasing knowledge. As to the long-term perspective of its survival, the merely passive protection of the species is insufficient even in the national nature reserves.

A great contribution of the developed technology for bog pine propagation consists in the harvesting of a sufficiently great amount of genetically pure clones that can be stored over a long time under both *in vitro* and *ex vitro* conditions. Results of regeneration by the process of somatic embryogenesis in the *Pinus* genus suggest that although the success of this method greatly depends on genotype and on climatic conditions at the time of seed maturation, a sufficient amount of clones can be obtained through modified cultivation conditions.

Mature seeds of bog pine (*Pinus uncinata* subsp. *uliginosa*) were used from seed production of the Sofronka Arboretum in Bolevec. Donor trees were individuals from the Žofinka NNR – cadaster Dvory nad Lužnicí (seeds Z, Z1, harvested in 2007) and from the locality of Krajková in the Sokolov district (seeds K, K1, harvested in 2005). A portion of seeds was soaked in distilled water for 5 days at a temperature of 5 °C, another portion of seeds was stratified for 1–2 weeks without water application at 5 °C. Cotyledons (primary leaves) and hypocotyl segments were cut from the seedlings after 2–3 weeks. The explants were transferred onto the induction media and cultivated at a photoperiod of 16 hours, day/night temperature of 23/19 °C and light intensity 55 µmol m^{-2} s^{-1}. The composition of induction media was based on a half-concentration of basic salts in the MS medium with a content of vitamins, benzyladenine (BA) at a concentration of 1.5–2.5 mg.l^{-1}, combined with 1-naphtylacetic acid (NAA) at a concentration of 0.01–0.5 mg.l^{-1} or 0.5–1.0 mg.l^{-1} BA with indole-3-butyric acid (IBA) at a concentration of 0.1–0.5 mg.l^{-1}. The media contained 3% sucrose, 0.1% charcoal activated, 0.75% agar (Sigma), and the pH value was 5.6.The percentage of explants with the developing buds was established after 12 weeks. Subcultures were implemented in 4-week intervals. After 4 weeks, the cotyledons were observed to exhibit bud proliferation and the hypocotyl segments showed formation of a green callus. After 12 weeks, the significantly highest amount of explants with the bud proliferation (23–85%) was established in the presence of 1.5 mg.l^{-1} BA combined with 0.5 mg.l^{-1} NAA. Although the combination of 0.5 mg.l^{-1} BA with 0.2 mg.l^{-1} IBA was significantly lower in some treatments, it was observed to stimulate the development of adventitious buds in the cultures. Experiments were performed by mature seeds to test the response of embryos on some growth regulators focused on the induction of somatic embryogenesis.

In a follow-up experiment, the response was tested of mature zygotic embryos from some individuals to growth regulators with respect to a possible induction of somatic embryogenesis. Surface sterilization of the seeds and soaking of the seeds in sterile distilled water at a temperature of 4 °C were followed by removal of the testa and by the extirpation of zygotic embryos. These were transferred together with the isolated megagametophytes onto induction medium DCR with different combinations of BA (0.56, 2.25 mg.l^{-1}), 2,4-D (0.55, 2.2 mg.l^{-1}), NAA (0.465, 0.93, 1.86 mg.l^{-1}) or with triacontanol (TRIA) at 10 µg.l^{-1}. In addition to growth regulators, the effects were tested of solidifying agents (Phytagel /Sigma/ and Gelrite /Duchefa/) (0.3%), sucrose or maltose (3%). After 3 weeks of cultivation, the morphological response of respective medium variants was evaluated. The comparison after 3 weeks of cultivation revealed a significant effect of BA. It was demonstrated that if the medium does not contain cytokinin, a pronounced elongation of cotyledons occurs. This response was modified by the presence of 3% maltose under the influence of which the elongation of cotyledons occurred on the medium with 1.13 mg.l^{-1} BA, too. The elongation of cotyledons was usually related to the terminated formation of callus, which developed in other treatments namely on the hypocotyl, and to hypocotyl necrosis. Nevertheless, these deformed cotyledons were observed to show the formation of callus and tubular cells, and even roots in the presence of 0.93 mg.l^{-1} NAA. The crumbly and potentially embryogenic tissue developed on a whole scale of growth regulator combinations (BA, NAA, 2,4-D) but after translation onto the proliferation medium with a lower concentration of growth regulators (0.113 mg.l^{-1} BA and 0.44 mg.l^{-1} 2,4-D), developing somatic embryos were detected only with the use of lower BA concentrations (0.56 or 1.13 mg.l^{-1}) combined with 1.86 mg.l^{-1} NAA for their initiation. In both cases, the media used in our experiments contained triacontanol at 10 µg.l^{-1}, which is known to stimulate various physiological processes. In contrast to previous experiments focused on the induction of organogenesis, in which the optimum cytokinin/auxin ratio was 3 : 1 (1.5 mg.l^{-1} BA and 0.5 mg.l^{-1} 2,4-D), the bud formation on the cotyledons and apexes of zygotic embryos occurred at a reversed cytokinin/auxin ratio of 1 : 1-4 (0.56 mg.l^{-1} BA, and 0.93 and 1.86 mg.l^{-1} 2,4-D). However, the developed buds were largely adventitious.

SOUHRN

Záchrana cenného genotypu borovice blatky s využitím mikropropagačních technik

Cílem práce bylo zjistit výživové a růstově regulační podmínky indukce *in vitro* regenerace ze semen borovice blatky. Vzhledem k tomu, že indukce somatické embryogeneze u rodu *Pinus* probíhá hlavně ze zadržených embryí, která u zralých semen zanikají, byly pokusy se zralými semeny využity k otestování reakce embryí na vybrané růstové regulátory.

Borovice blatka (*Pinus uncinata* DC. subsp. *uliginosa* (Neumann) Businský) je subendemitem České republiky, jehož převážná část populací je soustředěna na území České republiky (západní Čechy, Šumava v údolí Křemelné a Vltavy, Třeboňská pánev a po jedné lokalitě ve Žďárských vrších a Hrubém Jeseníku). Všechny zahraniční lokality tohoto taxonu s výskytem fenotypově čistých nehybridních jedinců (celkem méně než deset) jsou buď okrajové části českých populací nebo nepočetné zbytkové populace. Borovice blatka je z řady hledisek velmi vhodným modelovým druhem pro využi-

tí moderních výzkumných metod, směřujících k dlouhodobému zachování tohoto taxonu, který se s přibývajícím poznáním jeví jako jedna z nejvíce ohrožených dřevin České republiky. Z hlediska dlouhodobých perspektiv přežití blatky je její pasivní ochrana i v národních přírodních rezervacích nedostačující.

Velkým přínosem vypracované technologie množení borovice blatky je získání dostatečného množství geneticky čistých klonů, které mohou být dlouhodobě uchovány v *in*– a *ex vitro* podmínkách. Výsledky regenerace procesem somatické embryogeneze u rodu *Pinus* naznačují, že i když úspěšnost této metody je závislá na genotypu a na klimatických podmínkách v době zrání semen, je možno cestou modifikace kultivačních podmínek získat dostatečné množství klonů.

V pokuse byla použita zralá semena z osiva Arboreta Sofronka v Bolevci. Jako donorové stromy byly použiti jedinci z NPR Žofinka – katastrální území Dvory nad Lužnicí (semena Z, Z1, odběr v roce 2007) a z lokality u Krajkové, okr. Sokolov (semena K, K1, odběr v roce 2005). Část semen byla namočena do destilované vody po dobu 5 dní při teplotě 5 °C, část semen byla stratifikována bez aplikace vody 1–2 týdny při teplotě 5 °C. Po 2–3 týdnech byly odřezány ze semenáčů kotyledony (dělohy) a segmenty hypokotylu. Explantáty byly přeneseny na iniciační média a kultivovány za 16 h fotoperiody, teploty den/noc 23/19 °C a intenzity světla 55 µmol m^{-2} s^{-1}. Složení iniciačních médií vycházelo z poloviční koncentrace základních solí v MS živném médiu s obsahem vitaminů, benzyladeninu (BA) v koncentraci 1,5–2,5 mg.l^{-1} v kombinaci s kyselinou 1-naftyloctovou (NAA) v koncentraci 0,01–0,5 mg.l^{-1} nebo 0,5–1 mg.l^{-1} BA s kyselinou indolyl-3-máselnou (IBA) v koncentraci 0,1–0,5 mg.l^{-1}. Média obsahovala 3% sacharózu, 0,1% aktivní uhlí, 0,75% agar Sigma, pH médií bylo 5,6.

Po 12 týdnech bylo provedeno vyhodnocení. Subkultury byly prováděny ve čtyřtýdenních intervalech. Po čtyřech týdnech byla u kotyledonů pozorována proliferace pupenů, u segmentů hypokotylu se tvořil zelený kalus. Po 12 týdnech bylo zjištěno v přítomnosti 1,5 mg.l^{-1} BA v kombinaci s 0,5 mg.l^{-1} NAA průkazně nejvíce explantátů s proliferací pupenů (23–85 % explantátů). Kombinace 0,5 mg.l^{-1} BA s 0,2 mg.l^{-1} IBA byla sice u některých variant průkazně nižší, ale stimulovala u kultur tvorbu adventivních pupenů. Byly založeny pokusy se zralými semeny k otestování reakce embryí na vybrané růstové regulátory zaměřené na indukci somatické embryogeneze.

V návaznosti na tyto pokusy byla testována i reakce zralých zygotických embryí vybraných jedinců na růstové regulátory s ohledem na možnost indukce somatické embryogeneze. Po povrchové sterilizaci semen a jejich osmidenním máčení ve sterilní destilované vodě při teplotě 4 °C, byla odstraněna testa a extirpována zygotická embrya. Spolu s oddělenými megagametofyty byla přenesena na indukční médium DCR s různými kombinacemi BA (0,56; 2,25 mg.l^{-1}), 2,4-D (0,55; 2,2 mg.l^{-1}), NAA (0,465; 0,93; 1,86 mg.l^{-1}), eventuelně s triacontanolem (10 µg.l^{-1}) (TRIA). Kromě růstových regulátorů byl odzkoušen efekt ztužující substance (Phytagel (Sigma) a Gelrite (Duchefa) (0.3%)) a efekt sacharózy nebo maltózy (3%). Po třech týdnech kultivace byla sledována morfologická odezva jednotlivých variant média. Na základě srovnání po třech týdnech kultivace byl zjištěn významný efekt BA. Ukázalo, že pokud v médiu není přítomen cytokinin, dochází k výraznému prodlužování děloh. Tato reakce byla modifikována přítomností 3% maltózy, pod jejímž vlivem došlo k prodlužování děloh i na médiu s 1,13 mg.l^{-1} BA. Prodloužení děloh bylo většinou spojeno se zastavením tvorby kalusu, který se u jiných variant tvořil zejména na hypokotylu a k nekrotizaci hypokotylu, ale i na těchto deformovaných dělohách byl pozorován vznik kalusu, tubulárních buněk a za přítomnosti 0,93 mg.l^{-1} NAA, dokonce kořínků. Rozpadavý, potenciálně embryogenní kalus vznikal na celé škále kombinací růstových regulátorů (BA, NAA, 2,4-D), ale po přenesení na proliferační médium s nižší koncentrací růstových regulátorů (0,113 mg.l^{-1} BA a 0,44 mg.l^{-1} 2,4-D) byla pozorována vyvíjející se somatická embrya pouze při použití nižších koncentrací BAP (0,56 nebo 1,13 mg.l^{-1}) v kombinaci s 1,86 mg.l^{-1} NAA k jejich inicializaci. V obou těchto případech byl v našich experimentech v médiu přítomen triacontanol (10 µg.l^{-1}), o kterém je známo, že stimuluje různé fyziologické procesy. Na rozdíl od předcházejících experimentů, týkajících se indukce organogeneze, kde optimální poměr cytokininu a auxinu byl 3:1 (1,5 mg.l^{-1} BAP a 0,5 mg.l^{-1} 2,4-D), docházelo na dělohách a apexu zygotických embryí ke tvorbě pupenů při opačném poměru cytokininu a auxinu 1:1–4 (0,56 mg.l^{-1} BAP a 0,93 a 1,86 mg.l^{-1} 2,4-D. Zde se ale tvořily hlavně adventivní pupeny.

Pinus uncinata DC. subsp. *uliginosa* (Neumann) Businský, semena, organogeneze, somatická embryogeneze

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REFERENCES

BORATYŃSKA, K., BORATYŃSKI, A. AND LEWANDOWSKI, A., 2003: Morphology of *Pinus uliginosa* (*Pinaceae*) needles from populations exposed to and isolated from the direct influence of *Pinus sylvestris*. *Journal of the Linnean Society of London*, 142: 83–91.

BORATYŃSKI, A., BORATYŃSKA, K., LEWANDOWSKI, A., GOLAB, Z. and KICIŃSKI, P., 2003: Evidence of the possibility of natural reciprocal crosses between *Pinus sylvestris* and *P. uliginosa* based on the phenology of reproductive organs. *Flora*, 198: 377–388.

BUSINSKÝ, R., 1998: Agregát *Pinus mugo* v bývalém Československu – taxonomie, rozšíření, hybridní populace a ohrožení. *Zprávy Čes. Bot. Společ.*, Praha, 33: 29–52.

CALIXTO, F. and PAIS, M. S., 1997: Adventitious shoot formation and plant regeneration from *Pinus pinaster* Sol. ex Aiton. *In vitro Cellular and Developmental Biology*, 33: 119–124.

GUPTA, P. K. and DURZAN, D. J., 1985: Shoot multiplication from mature trees of Douglas-fir (*Pseudotsuga menziesii*) and sugar pine (*Pinus lambertiana*). – *Plant Cell Reports*, 4: 177–179.

CHALUPA, V., 1986: Rozmnožování jehličnatých stromů in vitro. *Lesnictví*, 32: 997–1010.

LELU, M. A., BASTIEN, C., DRUGEAULT, A., GOUEZ, M.L. and KLIMASZEWSKA, K., 1999: Somatic embryogenesis and plantlet development in *Pinus sylvestris* and *Pinus pinaster* on medium with and without growth regulators. *Physiologia Plantarum*, 105: 719–728.

LELU-WALTER, M.A., BERNIER-CARDOU, M. and KLIMASZEWSKA, K., 2008: Clonal plant production from self- and cross-pollinated seed families of *Pinus sylvestris* (L.) through somatic embryogenesis. *Plant Cell, Tissue and Organ Culture*, 92: 31–45.

LEWANDOWSKI, A., BORATYŃSKI, A. and MEJNARTOWICZ, L., 2000: Allozyme investigations on the genetic differentiation between closely related pines – *Pinus sylvestris*, *P. mugo*, *P. uncinata* and *P. uliginosa* (*Pinaceae*). *Plant Systematics and Evolution*, 221: 15–24.

LEWANDOWSKI, A., SAMOČKO, J., BORATYŃSKA, K. and BORATYŃSKI, A., 2002: Genetic differences between two Polish populations of *Pinus uliginosa*, compared to *P. sylvestris* and *P. mugo*. *Dendrobiology*, 48: 51–57.

MACKAY, J. J., BECWAR, M. R., PARK, Y. S., CORDERRO, J. P. and PULLMAN, G. S., 2006: Genetic control of somatic embryogenesis initiation in loblolly pine and implications for breeding. *Tree Genetics and Genomes*, 2: 1–9.

MALÁ, J., CVRČKOVÁ, H., MÁCHOVÁ, P. and ŠÍMA, P., 1999: Využití mikropropagace při záchraně cenných populací ušlechtilých listnatých lesních dřevin. *Zprávy lesnického výzkumu*, 44: 6–11.

MALABADI, R. B., MULGUND, G. S. and NATARAJA, K., 2005: Plant regeneration via somatic embryogenesis in *Pinus kesiya* (Royle ex. Gord.) influenced by triacontanol. *Acta Physiologiae Plantarum*, 27:531–537.

MURASHIGE, T. and SKOOG, F., 1962: A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiologia Plantarum*, 15: 473–497.

NISKANEN, A.M., LU, J., SEITZ, S., KEINONEN, K., VON WEISSENBERG, K. and PAPPINEN, A., 2004: Effect of parent genotype on somatic embryogenesis in Scots pine (*Pinus sylvestris*). *Tree Physiology*, 24: 1259–1265.

PARK, Y. S., LELU-WALTER, M. A., HARVENGT, L., TRONTIN, J. F., MACEACHERON, I., KLIMASZEWSKA, K. and BONGA, J. M., 2006: Initiation of somatic embryogenesis in *Pinus banksiana*, *P. strobus*, *P. pinaster*, and *P. sylvestris* at three laboratories in Canada and France. *Plant Cell, Tissue and Organ Culture*, 86: 87–101.

PRUS-GLOWACKI, W., BUJAS, E. and RATYNSKA, H., 1998: Taxonomic position of *Pinus uliginosa* Neumann as related to other taxa of *Pinus mugo* complex. *Acta Societatis Botanicorum Polonia*, 67: 269–274.

RANCILLAC, M., 1991: Maritime pine (*Pinus pinaster* Sol.). In: Bajaj Y.P.S. (ed.). *Biotechnology in Agriculture and Forestry*. Springer-Verlag, Berlin, 317–338.

SALAJOVÁ, T., 1992: Plantlet regeneration from axillary shoots of *Pinus nigra* ARN. *Biologia*, 47: 15–19.

SIEDLEWSKA, A. and PRUS-GLOWACKI, W., 1995: Genetic structure and taxonomic position of *Pinus uliginosa* Neumann population from Wielkie Torfowisko Batorowskie in Stolowe Mts. (locus classicus). *Acta Soc. Bot. Polonia*, 64: 51–58.

SUL, I. W. and KORBAN, S. S., 1998: Effects of media, carbon sources and cytokinins on shoot organogenesis in the Christmas tree Scots pine (*Pinus sylvestris* L.). *Journal of Horticultural Science and Biotechnology*, 73: 822–827.

TORIBIO, M. and PARDOS, J. A., 1989: Scots pine (*Pinus sylvestris*). In: Bajaj Y.P.S. (ed.). *Biotechnology in Agriculture and Forestry*, Springer-Verlag, Berlin, Vol. 5, 479–506.

VEJSADOVÁ, H. and ŠEDIVÁ, J., 2002: Mikropropagace ohroženého druhu borovice blatky (*Pinus rotundata* Link). *Acta Pruhoniciana*, 73: 37–47.

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