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Shoot organogenesis induction from genetically verified individuals of endangered bog pine (*Pinus uncinata* subsp. *uliginosa*)

H. Vejsadová, M. Lukášová

Department of Biodiversity, Silva Tarouca Institute for Landscape and Ornamental Gardening, Průhonice, Czech Republic

ABSTRACT: The objective of this work was to apply selected genetic markers to verify the genetic purity of bog pine (*Pinus uncinata* subsp. *uliginosa*) individuals from the Borkovická blata population in order to initiate *in vitro* cultures from them. Key factors of shoot organogenesis induction from seedling shoot apices were examined. The plastid DNA analysis was used as a molecular-genetic tool. The *trn*L-*trn*F region of chloroplast DNA was tested by PCR-RFLP method; furthermore, the chloroplast microsatellite region Pt41093 was also analysed. All the tested individuals from the studied population matched the *Pinus uncinata* subsp. *uliginosa* haplotype. In bog pine, regeneration and development of axillary shoots were significantly enhanced in the presence of 5 g·l^{-1} activated charcoal on the WV5 and WPM (Duchefa) media. The highest proliferation (28–30%) of short shoots (brachyblasts) was observed on the WV5 medium in the absence of benzyladenine (BA) or at its low concentration only (0.1 mg·l^{-1}).

Keywords: DNA extraction; *in vitro* regeneration; leaves; plastid DNA; seedling shoot apices

The bog pine, *Pinus uncinata* subsp. *uliginosa* (Neumann) Businský, a subendemic taxon in the Czech Republic, is found in two main distribution areas situated in South and West Bohemia. Of these, the populations in the South Bohemian Třeboň basin are the most endangered given their low altitude and correspondingly a higher anthropogenic pressure. The lowest situated and most isolated locality of the bog pine population is found in the Borkovická blata area near Soběslav in the Tábor district (altitude around 420 m a.s.l.). The surviving fragment of bog pine forest consists of old forest vegetation along the margin of exploited peatbog. A selected subpopulation has been fertile for over 20 years and given the height and accessibility of the individuals, the locality appears well suited to research aimed at preserving the bog pine and its genetic diversity. As there are evident threats to this population, it is appropriate to protect it from the inflow of introgressive genes and to sustain it by expansion of the number of individuals. It is advisable to conserve the genetic resources *ex situ*, in gene banks and *in vitro* collections, in order to provide a support for *in situ* conservation subsequently.

Being one of the most widely distributed genera of conifers, the genus *Pinus* exhibits high phenotype variability. It is the well-described introgressive hybridization between some pine species that complicates the taxonomic classification of individual taxa, including the bog pine. An earlier taxonomic approach to the aggregate of *Pinus mugo* according to Skalický (1988) incorporates three taxa in the rank of species: *Pinus mugo* Turra, *P. uncinata* Ramond ex DC and *P. rotundata* Link. According to taxonomic, biogeographical and nomenclatural reviews (Businský, Kirschner 2006; Businský 2008) the aggregate of *Pinus mugo* consists of the species *P. mugo* s. str. and *P. uncinata* with two

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subspecies: *P. uncinata* subsp. *uncinata* and *P. uncinata* subsp. *uliginosa*. As was presented by Lewandowski et al. (2002), the relatively high level of genetic differentiation between *P. uliginosa* populations may result from their isolation, small size and possibly different origin of these populations.

There is no unanimous agreement among specialists whether introgressive hybridization outside this aggregate, e.g. with surrounding species *P. sylvestris* L., exists. Some authors support this possibility (Businský, Weger 1995; Siedlewska, Prus-Glowacki 1995; Kormuťák et al. 2008), while others consider it not unlikely or refute it (Filppula et al. 1992; Neet-Sarqueda 1994; Christensen, Dar 1997). Businský (1998) concluded that the trait distinction between the taxa of the *P. mugo* aggregate is insignificant and that determination of the individuals without the knowledge of the whole population is unreliable. Genetic data can provide new facts and complement, detail or modify the current hybridization hypotheses. Therefore, morphological studies should preferably be completed with molecular analyses.

The variability of chloroplast DNA can serve as a useful diagnostic trait in hybridization studies. In conifers, cpDNA is inherited paternally so that it can help us define the direction and intensity of the hybridization process and its effect on the structure of sympatric populations. So far, several species-specific chloroplast DNA markers have been described to differentiate *P. sylvestris* and *P. mugo* (Vendramin et al. 1996; Wachowiak et al. 2000, 2004). These markers can help us to verify if the introgressive hybridization occurred in *P. uncinata* subsp. *uliginosa* population.

Clonal propagation by organogenesis is a tissue culture preparation method. It allows for *in vitro* regeneration of conifers, and is an efficient tool for the mass production of genetically pure material. As was shown by Malá et al. (1999), organ cultures of trees cultivated *in vitro* can be considered genetically stable as no chromosome changes take place. Clone material can be propagated by induction of organogenesis or by somatic embryogenesis on a primary explant. In coniferous trees, the organogenesis method is significantly more difficult as compared to deciduous trees. The success of the regeneration method depends on a number of factors, including the growth properties of the studied species. In *Pinus sylvestris* and *P. nigra*, organogenesis was induced via the induction of axillary buds and shoots (Chalupa 1986; Salajová 1992) or in cotyledons (Häggman et al. 1966; Sul, Korban 2004; Vejsadová et al. 2008).

The objective of this work was to apply selected genetic markers to verify the genetic purity of bog pine individuals in order to initiate *in vitro* cultures from them. Key factors of shoot organogenesis initiation were identified with the purpose of the conservation of genetically pure bog pine in an *in vitro* gene bank.

MATERIAL AND METHODS

DNA extraction

Young leaves of bog pine were collected from the population on the Borkovická blata peatbog (11 specimens). As referential material, sampling of Scots pine (*Pinus sylvestris*) young leaves was performed in a relict forest in the Hřebenec locality in the Třemšín massif (the Brdy Mts.) in the Příbram district (5 specimens).

DNA was extracted from lyophilised leaves using a commercial DNeasy Plant Mini Kit (Qiagen). This procedure is commonly used for deciduous trees and could be successfully applied to the leaves of evergreen conifers as well. The extracted DNA was of required quantity and quality for the following analyses.

Analysis of chloroplast DNA (cpDNA)

First, the single nucleotide polymorphism in the *trn*L-*trn*F region of chloroplast DNA was tested by PCR-RFLP method. Restriction sites are different in *P. sylvestris* and *P. mugo* and/or *P. uliginosa* (the basionym for the correct name of bog pine) if digested by the enzyme *Dra*I (Wachowiak et al. 2000). Universal primers were used (primer1: 5'-CGA AAT CGG TAG ACG CTA CG-3' and primer 2: 5'-ATT TGA ACT GGT GAC ACG AG-3') for PCR amplification of the non-coding region of cpDNA (Taberlet et al. 1991). The PCR mixture contained 10 ng of total DNA, 2.5mM $MgCl_2$, 100μM of each of dNTP, 0.2μM of each primer, 0.25 U *Taq* polymerase (Fermentas) and 1 × reaction buffer in the total volume of 15 μl (Wachowiak, Prus-Głowacki 2008). The PCR cycle profile comprised preliminary denaturation 5 min at 96°C, followed by 35 cycles of denaturation 30 s at 94°C, primer annealing 60 s at 53°C, DNA synthesis 90 s at 72°C, terminated with incubation 10 min at 72°C (Wachowiak et al. 2000). The volume of 10 μl of PCR products was subjected to restriction analysis at 37°C overnight using the restriction en-

zyme *Dra*I. Samples were separated in 2% agarose gel stained with ethidium bromide after digestion.

The second method employed was the analysis of the chloroplast microsatellite region Pt41093. This region differs in length in *P. sylvestris* and *P.mugo* and/or *P. uliginosa* (Wachowiak et al. 2006; Wachowiak, Prus-Głowacki 2008). Primers were used for PCR amplification (primer 1: 5'-TCC CGA AAA TAC TAA AAA AGC A-3' and primer 2: 5'-CTC ATT GTT GAA CTC ATC GAG A-3'), previously designed by Vendramin et al. (1996). The PCR mixture contained 10 ng of total DNA, 2.5mM $MgCl_2$, 100μM of each of dNTP, 0.2μM of each primer, 0.25 U *Taq* polymerase (Fermentas) and 1 × reaction buffer in the total volume of 10 μl (Wachowiak, Prus-Głowacki 2008). The PCR cycle profile comprised preliminary denaturation 5 min at 95°C, the enzyme addition 5 min at 80°C, followed by 25 cycles of denaturation 60 s at 94°C, primer annealing 60 s at 55°C, DNA synthesis 60 s at 72°C, terminated with final extension 8 min at 72°C (Vendramin et al. 1996). The PCR products were separated in 8% polyacrylamide gel, stained with silver.

In vitro plant material

We used mature seeds from genetically pure and non-hybrid (from the male line of descent) individuals of bog pine from the Borkovická blata locality. The seeds were sterilized using a 1.5% sodium hypochlorite solution for 25 min with a short pretreatment with 70% ethanol. The MS medium (Murashige, Skoog 1962) was used as the sowing medium, using 10times lower salt concentration without vitamins added and solidified with 7 $g \cdot l^{-1}$ agar (Sigma). The medium pH was adjusted to 6.5 before autoclaving. The seeds germinated in test tubes at a constant temperature in the thermostat of 22 ± 2°C.

Explant cultivation

Shoot apices (epicotyls) from 8–12 weeks old seedlings were used as the primary explants. The explants were transferred to three basic media: Westvaco WV5 (Duchefa), WPM (Lloyd, McCown 1980) (Duchefa) and the MS medium with a full concentration of salts. The media contained a mix of vitamins, cytokinin benzyladenine (BA) in the concentration of 5 and 10 $mg \cdot l^{-1}$, 3% sucrose and 7.5 $g \cdot l^{-1}$ Phytoagar (Duchefa). After 4 weeks of the explant cultivation on a growth regulator supplemented medium, shoots about 10 mm long were excised from explants and transferred onto a multiplication medium (WV5, WPM and MS) without regulators however with activated charcoal, 2.5% sucrose and 7.5 $g \cdot l^{-1}$ Phytoagar (Duchefa). The pH value of all media was adjusted to 5.6 before autoclaving.

Short shoots (brachyblasts) with the developed pairs of secondary leaves (needles) were excised from the cultures and further cultivated on the above media without growth regulators or at a low concentration of BA (0.1 and 0.5 $mg \cdot l^{-1}$). Subcultures were performed in 4–5 week intervals. The cultures were cultivated with a 16 h photoperiod, at a temperature of 23/19°C (day/night) and light intensity of 55 $\mu mol \cdot m^{-2} \cdot s^{-1}$ supplied from cool white fluorescent tubes.

Statistical analysis

The complete data were evaluated using the one-way analysis (ANOVA) and comparative Duncan's multiple range test at a significance level of $P = 0.05$. Each value represents the mean of two repeated experiments with 30 replications each.

RESULTS AND DISCUSSION

PCR-RFLP analysis of the *trn*L-*trn*F region

Wachowiak et al. (2000) described a single-nucleotide polymorphism in the non-coding region *trn*L-*trn*F of cpDNA. It provides a species-specific marker to distinguish between *P. sylvestris* and the *P. mugo* aggregate cpDNA. PCR product of the length around 1,030 bp remains undigested in the case of *P. sylvestris* (so-called S haplotype) if the restriction enzyme *Dra*I is applied. On the contrary, PCR product from *P. mugo* aggregate leads to two-band pattern (PCR product is digested to fragments about 800 bp and 250 bp – so-called M haplotype).

The amplification of the *trn*L-*trn*F region and digestion by restriction endonuclease *Dra*I were carried out on all 16 specimens. In 11 specimens from the Borkovická blata locality a specific restriction site was present and two obvious fragments were on the gel, sized approximately 800 bp and 250 bp. The specimens therefore matched the M haplotype. In contrast, the specimens of *P. sylvestris* from the relict pine forest in the Hřebenec locality remained undigested as expected – only 1 band of 1,030 bp was present.

SSR analysis of cpDNA – locus Pt41093

The length of the chloroplast microsatellite region Pt41093 varies in a range of 86 to 92 bp in *P. mugo* and *P. uliginosa* and in a range of 78 to 82 bp in *P. sylvestris* (Wachowiak et al. 2006; Wachowiak, Prus-Głowacki 2008). All 11 specimens from the Borkovická blata locality had the fragments of the length from 88 to 92 bp and, thus, they carry the cpDNA haplotype diagnostic for *P. uncinata* subsp. *uliginosa*.

In referential specimens from the Hřebenec locality we found fragments with an identical length of 79 bp. These comply with the cpDNA haplotype of *P. sylvestris*. It confirmed that the microsatellite locus Pt41093 can distinguish between these two species.

Induction and development of axillary buds and shoots

It follows from the literary data (Chalupa 1986; Sul, Korban 1998; Mackay et al. 2006) that 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 culture medium. In our experiments, we used explants (shoot apices) from genetically pure seedlings which are the objective of axillary bud and shoot initiation. The results about the bog pine germination were published in our previous work (Vejsadová et al. 2008).

Axillary buds induced on explants were observed after 4 weeks of cultivation on a BA supplemented medium (Table 1). The significantly highest regeneration of explants and formation of buds were observed on the Westvaco WV5 medium compared to the WPM and MS medium. At a concentration of 10 mg·l^{-1} BA the explant regeneration and bud development were significantly stimulated (by as much as 50%) compared to the tested lower concentration of cytokinin. The transfer of explants with developed buds to a regulator-free medium led to bud elongation.

A significantly higher regeneration of shoots occurred in explants cultivated in the presence of activated charcoal. On the WV5 and WPM media, markedly higher values of shoot regeneration and multiplication were found at a concentration of 5 g·l^{-1} activated charcoal (Table 2). No effect of charcoal concentrations was obtained on the MS medium.

Short shoot proliferation

Although recently success has been achieved in the micropropagation of pine species, in the bog pine, a standard *in vitro* protocol has been elaborated so far. Just like *Pinus nigra*, the bog pine is one of the coniferous trees that are difficult to propagate *in vitro*. So far, only incomplete results have been published (Vejsadová, Šedivá 2002; Vejsadová et al. 2008). In the present work we concentrated on the stimulation factors of shoot organogenesis initiation with the purpose of genetically pure individual conservation under *in vitro* conditions.

After 12 weeks of axillary shoot cultivation on a medium without growth regulators containing 5 g·l^{-1} activated charcoal, we observed proliferous axillary buds partly developed into primary leaf clusters and short shoots with the secondary leaf fascicles (Figs. 1 and 2). Significantly higher proliferation of short shoots (28–30%) was found on the WV5 medium without the presence of BA or at its low concentration (0.1 mg·l^{-1}) in the medium (Table 3). Low regen-

Table 1. The influence of initiation medium composition and benzyladenine (BA) on explant regeneration and axillary bud formation after 4 weeks of cultivation. Each treatment consisted of 30 explants

Basal medium	BA (mg·l^{-1})	Explant regeneration ± SE (%)	Mean No. buds/explant ± SE
WV5	5	50.3 ± 0.6^{b}	5.23 ± 0.21^{b}
	10	90.3 ± 0.6^{a}	8.14 ± 0.26^{a}
WPM	5	30.6 ± 2.5^{c}	1.13 ± 0.19^{c}
	10	40.6 ± 1.9^{b}	2.24 ± 0.17^{c}
MS	5	15.8 ± 4.2^{d}	1.08 ± 0.16^{c}
	10	20.1 ± 3.5^{d}	2.39 ± 0.29^{c}

Values followed by different letters are significantly different at the P = 0.05 level; SE – Standard error

Table 2. The influence of multiplication medium composition and activated charcoal on shoot regeneration and axillary shoot formation after 8 weeks of cultivation. The media were not supplemented with growth regulators. Each treatment consisted of 30 explants

Basal medium	Activated charcoal ($g \cdot l^{-1}$)	Shoot regeneration ± SE (%)	Mean No. shoots/explant ± SE
WV5	0	5.3 ± 4.6^{e}	2.18 ± 0.31^{d}
	2.5	45.3 ± 1.9^{b}	9.17 ± 0.84^{b}
	5.0	88.2 ± 0.4^{a}	17.20 ± 0.39^{a}
WPM	0	2.4 ± 4.8^{e}	1.25 ± 0.20^{d}
	2.5	10.6 ± 3.7^{d}	6.64 ± 0.71^{c}
	5.0	28.4 ± 2.6^{c}	10.42 ± 0.95^{b}
MS	0	1.2 ± 4.2^{e}	1.13 ± 0.22^{d}
	2.5	9.8 ± 3.4^{d}	5.87 ± 0.81^{c}
	5.0	11.3 ± 3.7^{d}	8.36 ± 0.93^{bc}

Values followed by different letters are significantly different at the $P = 0.05$ level; SE – Standard error

eration (6–8%) was observed on the WPM medium while there was no proliferation of short shoots on the MS medium at all.

Different *Pinus* species have specific nutritional requirements for the organogenesis induction. Sul and Korban (2004) used seven different media for shoot development in *Pinus pinea*. In their study, the half-strength MS medium induced the highest frequency of shoot regeneration in the presence of sucrose and 10μM BA. In our experiments, the Westvaco WV5 medium by Duchefa proved to produce the best induction and development of shoots in bog pine. Shoot formation and short shoot proliferation were significantly higher than on the other tested media with an identical supply of cytokinin BA (5 and 10 $mg \cdot l^{-1}$). On the full-strength MS medium we found the lowest values of shoot regeneration. These findings agree with results reported by Garcia-Ferriz et al. (1994) where the full-strength MS medium was significantly more inhibiting compared to the SH (Schenk, Hildebrandt 1972) medium. In *Pinus pinea*, the negative effect of the MS medium on shoot induction has been attributed to the high nitrogen content, likely to be due to the ammonium presence (Sul, Korban 2004). In *P. nigra*, the axillary shoot development was observed on the half-strength MS medium (Salajová 1992). Shoot organogenesis of pines is often facilitated using the BA cytokinin in combination with naphthyl acetic acid (NAA).

Table 3. The effect of benzyladenine (BA) on short shoot (brachyblast) proliferation after 12 weeks of cultivation. Each treatment consisted 30 explants

Basal medium	BA ($mg \cdot l^{-1}$)	Short shoot proliferation ± SE (%)
WV5	0	28 ± 3.1^{a}
	0.1	30 ± 2.9^{a}
	0.5	10 ± 1.8^{b}
WPM	0	6 ± 1.9^{a}
	0.1	8 ± 2.5^{a}
	0.5	1 ± 1.5^{c}
MS	0	–
	0.1	–
	0.5	–

Values followed by different letters are significantly different at the $P = 0.05$ level; SE – Standard error

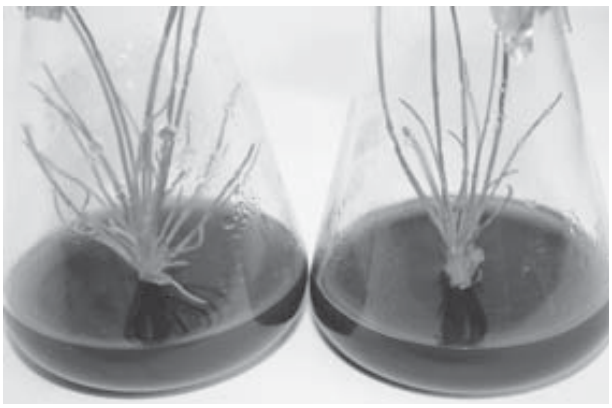

Fig. 1. Proliferous axillary buds partly developed into secondary leaf fascicles

Fig. 2. Short shoots with the secondary leaf fascicles

In *P. nigra* and *P. sylvestris*, the axillary buds were induced in the presence of 1 mg·l^{-1} BA in combination with 0.001 mg·l^{-1} NAA (Chalupa 1986; Salajová 1992). Our preliminary data (unpublished results) suggest that the low concentration of BA and NAA has no effect on the induction and development of axillary buds and shoots. It is obvious from the presented results that a higher concentration of BA (10 mg·l^{-1}) is stimulating for the induction of organogenesis in bog pine. In *P. pinea*, the shoot regeneration was significantly increased at a low concentration of 2 mg·l^{-1} BA in medium (Sul, Korban 2004).

A high concentration of activated charcoal and the absence of growth regulators were significant factors of the multiplication and elongation of shoots. In bog pine, the rooting of shoots is rather difficult and will require an additional study.

CONCLUSIONS

– All the tested specimens (11) from the studied population in the Borkovická blata locality matched the *P. uncinata* subsp. *uliginosa* haplotype.
– The regeneration of explants and the axillary bud formation were significantly increased (by as much as 50%) at a concentration of 10 mg·l^{-1} BA in the nutrient medium. Significantly higher regeneration and development of axillary shoots occurred in the presence of activated charcoal on the WV5 and WPM media. Significantly higher values of shoot multiplication were observed at a higher concentration (5 g·l^{-1}) of activated charcoal.
– The highest proliferation (28–30%) of short shoots (brachyblasts) was found on the WV5 medium without BA or at a low concentration (0.1 mg·l^{-1}) of this cytokinin in the medium.
– The genetic purity of *Pinus uncinata* subsp. *uliginosa* individuals was verified from the male line of descent using plastid DNA analysis and *in vitro* cultures were initiated from seedling shoot apices via shoot organogenesis.

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Corresponding author:

RNDr. Hana Vejsadová, CSc., Výzkumný ústav Silva Taroucy pro krajinu a okrasné zahradnictví, v.v.i.,
Oddělení biodiversity, Květnové náměstí 391, 252 43 Průhonice, Česká republika
tel.: + 420 296 528 345, fax: + 420 267 750 440, e-mail:vejsadova@vukoz.cz