ACTA BIOLOGICA CRACOVIENSIA Series Botanica 59/1: 93–103, 2017
DOI: 10.1515/abcsb-2017-0003

POLSKA AKADEMIA NAUK
ODDZIAŁ W KRAKOWIE

SOMACLONAL VARIATION DURING *PICEA ABIES* AND *P. OMORIKA* SOMATIC EMBRYOGENESIS AND CRYOPRESERVATION

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Received October 24, 2016; revision accepted March 9, 2017

Embryogenic cultures of plants are exposed to various stress factors both *in vitro* and during cryostorage. In order to safely include the plant material obtained by somatic embryogenesis in combination with cryopreservation for breeding programs, it is necessary to monitor its genetic stability. The aim of the present study was the assessment of somaclonal variation in plant material obtained from embryogenic cultures of *Picea abies* (L.) Karst. and *P. omorika* (Pančić) Purk. maintained *in vitro* or stored in liquid nitrogen by the pregrowth-dehydration method. The analysis of genetic conformity with using microsatellite markers was performed on cotyledonary somatic embryos (CSE), germinating somatic embryos (GSE) and somatic seedlings (SS), obtained from tissues maintained *in vitro* or from recovered embryogenic tissues (ETc) and CSE obtained after cryopreservation. The analysis revealed changes in the DNA of somatic embryogenesis-derived plant material of both *Picea* spp. They were found in plant material from 8 out of 10 tested embryogenic lines of *P. abies* and in 10 out of 19 embryogenic lines of *P. omorika* after *in vitro* culture. Changes were also detected in plant material obtained after cryopreservation. Somaclonal variation was observed in ETc and CSE of *P. omorika* and at ETv stage of *P. abies*. However, most of the changes were induced at the stage of somatic embryogenesis initiation. These results confirm the need for monitoring the genetic stability of plants obtained by somatic embryogenesis and after cryopreservation for both spruce species.

Keywords: microsatellites, somaclonal variations, somatic embryogenesis, spruce

Abbreviations: ET – Embryogenic tissue, ETc – Recovered ET after cryopreservation, ETv – Embryogenic tissue maintained *in vitro*, SE – Somatic embryo, CSE – Cotyledonary SE, GSE – Germinating SE, SS – Somatic seedlings, LED – Light-emitting diode, LM – Litvay's medium, LN – Liquid nitrogen, 2,4-D – 2,4-dichlorophenoxyacetic acid, NAA – 1-Naphtaleneacetic acid, Picloram – 4-Amino-3,5,6-trichloropicolinic acid, BA – Benzylaminopurine, ABA – Abscisic acid, IBA – Indolile-3-butyric acid, SE 3 ETc – 3-week old SE obtained from ETc, SE 5 ETc – 5-week old SE obtained from ETc, SSR – Simple sequence repeats

INTRODUCTION

Somatic embryogenesis (SE) is a propagation method used for accelerating the development and further mass production of high-value reproductive plant material. This technique is used successfully in micropropagation of various forest tree species of the genera *Abies*, *Picea*, *Pinus*, *Quercus* and *Eucalyptus* (Krajňáková et al., 2013; Mallón et al., 2012; Salaj et al., 1999; Pinto et al., 2002; von Arnold et al., 2005). A considerable amount of this research applies to various *Picea* spp., particularly *Picea abies*, *P. glauca*, *P. glauca* × *engelmannii*, *P. mariana* and *P. omorika* (Klimaszewska and Cyr, 2002; Mihaljević and Jelaska, 2005). In economic terms, most of these trees are important forest tree species for the forest industry (Klimaszewska and Cyr, 2002) which makes these species especially important objects for propagation *via* SE.

Both *Picea abies* (L.) Karst. and *P. omorika* (Pančić) Purk. have their natural range in Europe and play an important role in the natural forest

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PL ISSN 0001-5296

ecosystem and forest industry. Due to this, the development of efficient tools for SE has been of particular interest. Generally, conifers are regarded as very difficult material for micropropagation (Sarmast, 2016). Research into the micropropagation of *Picea abies via* SE was initiated by Chalupa (1985) and Hakman et al. (1985), and after decades of research these methods are still being continued and improved (von Arnold et al., 2005; Vondráková et al., 2015; Yakovlev et al., 2014). In the case of *P. omorika*, only a few studies on the SE method have been carried out as of yet (Hazubska-Przybył and Bojarczuk, 2008; Mihaljević and Jelaska, 2005). Currently, it is known that both spruce species can be propagated using this technique, and that their embryogenic cultures can also be successfully stored in liquid nitrogen (LN; Hazubska-Przybył et al., 2010, 2013; Find, 1998). These results will potentially allow for including *Picea abies* and *P. omorika* SE techniques into forest breeding programs and ornamental nurseries in the future.

The ultimate purpose of micropropagation of plants is to produce true-to-type individuals. Meanwhile, micropropagation of plants *via* organogenesis or SE is inextricably linked to the concept of somaclonal variation (Larkin and Scowcroft, 1981; Isah, 2015; Sarmast, 2016). Somaclonal variation may be induced by exposure of the propagated material to a high concentration of plant growth regulators and by its long-term maintenance under artificial conditions, i.e., *in vitro* culture (Marum et al., 2009; Sarmast, 2016). The risk of somaclonal changes to the genome of the propagated material is especially increased during SE, when the plant material is maintained in the form of callus (embryogenic tissue), which is particularly sensitive to such changes (Kunakh, 1999). Some results have shown that various changes at the morphological, biochemical, genetic and epigenetic levels appear not only during *in vitro* propagation (Bairu et al., 2011; Etienne and Bertrand, 2003; Peredo et al., 2006, Bradai et al., 2016), but also after cryopreservation, probably because of the routine use of factors with mutagenic effects, for example DMSO (Aronen et al., 1999). Genotypic instability during SE or storage in LN may have a significant influence on the quality of the material, i.e., clonal individuals obtained *via* tissue culture. Reduced quality of *in vitro*-derived plant material is often visible at the later stages of plant development. Therefore, for economic reasons, the genetic conformity of plant material should be assessed as early as possible, and is especially important for long-lived trees such as conifers, in order to minimize the costs of the process.

To detect somaclonal variation during somatic embryogenesis and after the cryopreservation of embryogenic cultures of trees, various genetic molecular markers have been used, for example AFLP (Hornero et al., 2001), RAPD (Krajňáková et al., 2011) and RFLP (DeVerno et al., 1994). Also, simple sequence repeats (SSR), also known as microsatellite markers, are used as the markers of choice to study the genetic conformity of plant material obtained *via* SE and/or stored in LN (Burg et al., 2007; Hazubska-Przybył et al., 2013; Helmersson et al., 2004; Lopes et al., 2009; Marum et al., 2009). Microsatellites are 1–6 nucleotide-long DNA sequences occurring extremely commonly in the genomes of *Eucaryota* in the form of perfect or near-perfect tandem iterations (Ellegren, 2004; Lopes et al., 2009). The number of these repeats may easily change due to various factors including those directly related to the mutagenic factors associated with *in vitro* techniques (Bairu et al., 2011). SSR markers demonstrate an advantage over other types of molecular markers mainly due to their characteristics, i.e., a high degree of polymorphism because of an intrinsically high mutation rate, codominant inheritance and a wide distribution over the genome. Additionally, the high reliability of this technique in comparison with other markers derived *via* random amplification of genome regions, such as RAPD, renders SSR a suitable tool for monitoring putative mutation events during SE (Bairu et al., 2011).

In our previous work, genetic analysis with SSR markers proved genetic fidelity of the *P. abies* material obtained *via* somatic embryogenesis and after cryopreservation (Hazubska-Przybył et al., 2013). However, those studies were focused mainly on optimizing of the protocol of cryostorage. Hence, we could not evaluate the influence of either the time of *in vitro* culturing or the time of cryopreservation on the genetic stability of the plant material. Since both these processes may induce somaclonal variation (Sarmast, 2016), their influence needs to be evaluated and the obtained results might guide the improvement of propagation protocols. Also, our previous investigations were limited only to a single genotype which precludes any general concluding with regard to the intrinsic factors of somaclonal variation, i.e., genotype-specific propensity to mutate which is also a highly discussed issue in terms of somaclonal variability (Sahijram et al., 2003; Marum et al., 2009, Salaj et al., 2011, Leva et al., 2012, Bradai et al., 2016). In this work we wanted to asses if the prolonged time of *in vitro* proliferation and storage in LN affects the genetic integrity of genomes of two spruce species, *P. abies* and *P. omorika*.

MATERIALS AND METHODS

PLANT MATERIAL

SE was initiated from mature zygotic embryos of *Picea abies* and *P. omorika* excised from seeds collected from open-pollinated trees. The seeds of *P. abies* originated from two trees (population Kolonowskie – K and Serwy – S) growing in the Institute of Dendrology Polish Academy of Sciences experimental forest 'Zwierzyniec'. The seeds of *P. omorika* were collected from two trees growing in the Kórnik Arboretum (AK1 and AK2) and from two trees in Dąbrówka Kościelna (DK1 and DK2) in Poland. The seeds were stored at +4°C. As the control for the genetic stability analysis, needles from the respective mother trees were used.

ESTABLISHEMENT OF EMBRYOGENIC CULTURES

Embryogenic tissues (ETs) of *P. abies* and *P. omorika* which originated from the Kórnik Arboretum were initiated at the end of 2009 and in 2010 respectively, whereas ETs of *P. omorika* which originated from Dąbrówka Kościelna were initiated in 2012. Modified LM medium (½ LM; with ½ of the full concentrations of media recommended by Litvay et al., 1985), supplemented with auxin (2,4-D, Picloram or NAA) at 9 μM and cytokinin (BA at 2.2, 4.5 or 8.8 μM) was used as the initiation medium. ETs were proliferated on the same medium but supplemented with Picloram (9 μM), BA (4.5 μM), sucrose (10 g/l) and L-glutamine (450 mg/l). The medium was solidified with Phytagel (5 g/l) and the cultures were kept in the dark at 22 ± 1°C.

To stimulate somatic embryo maturation, ETs of 11 lines of *P. abies* and 20 lines of *P. omorika* (Table 1) were spread over Whatman N° 2 filter paper and kept for one week on ½ LM medium, deprived of plant growth regulators but supplemented with activated charcoal (10 g/l). Next, the filter papers with ETs were placed on the medium with the addition of ABA (20 μM), IBA (1 μM) and sucrose (34 g/l). The cultures were incubated at a light intensity of 35 μmol m^{-2} s^{-1} (36 W fluorescent lamps) with a 16/8 h day/night photoperiod. After 5 weeks, a number of somatic embryos obtained per gram of FW tissue were estimated to evaluate the embryogenic potential of the individual ET lines.

To initiate germination, somatic embryos selected from 10 embryogenic lines of *P. abies* and 14 lines of *P. omorika* (Table 1) at the cotyledonary stage (CSE) were placed on Margara medium (1977), which was supplemented with sucrose (10 g/l) only. Next, Petri dishes with 20–60 embryos were transferred to darkness for two weeks, and then for the next two weeks to red and blue LED light, at an intensity of 35 μmol m^{-2} s^{-1}. After this, if possible, three to five properly germinating somatic embryos (GSE) with a clearly formed hypocotyl (about 10 mm long) and root (about 5 mm long) were transferred to Jiffy pots to obtain the somatic seedlings (SS) after a period of 6 months. For acclimatization to the *ex vitro* conditions, GSE of one line of *P. abies* and five lines of *P. omorika* were used (Table 1).

CRYOPRESERVATION PROCEDURE

Selected ET lines of *P. abies* (K4) and *P. omorika* (AK2.6 and DK1.3; Table 1) were frozen in liquid nitrogen by the pregrowth-dehydration method according to Hazubska-Przybył et al. (2013). Briefly, ETs were subjected to seven days of treatment on ½ LM medium supplemented with sucrose in increasing doses (0.25–1.00 M) and abscisic acid (ABA) at 10 μM. Next, ETs were air-dried over silica gel for 2 h at 25°C, to a water content of about 20% and frozen for 2 weeks in liquid nitrogen (LN). After this time, the samples were quickly thawed in a water bath at 42°C, rehydrated on medium containing decreasing doses of sucrose (1.00–0.25 M), and placed on $^1/_2$ LM proliferation medium, supplemented with sucrose at 10 g/l, Picloram at 9 μM and BA at 4.5 μM. After two to three months of proliferation, recovered ETs were placed on the standard maturation medium for three or five weeks to obtain somatic embryos of the tested spruce species.

SSR ANALYSIS OF GENETIC STABILITY

We designed two independent and distant in time experiments. The first one was dedicated to the genetic stability of plant material derived from *in vitro* culturing and the second one to the genetic stability of the ETs recovered from LN. Additionally, the first experiment was performed on the ETs lines, maintained under *in vitro* culture shorter than ETs analyzed in the second experiment. This allowed for assessing the impact of the culturing duration on the genetic stability. The control for both experiments was material obtained from the mother three. Each of the mother trees (K, S, DK, AK) was genotyped according to the procedure used for ET lines (see below). The similarity between the mother genotype and the derived lines and among the stages within the lines were calculated with Jaccard's similarity coefficient (1908).

TABLE 1. *Picea abies* and *P. omorika* embryogenic tissue (ET) lines of various origin from which plant material was obtained for genetic analysis.

Species	Origin	ET line	Embryogenic potential	Germination of SE	Acclimation of SS in Jiffy pots	Maintenance on proliferation medium (months)	Experiment number
Picea abies	Kolonowskie	K1^{v}	C	+	–	13	I
		K2^{v}	C	+	–	27	I
		K3^{v}	C	+	ns	27	I
		K4^{v+LN}	*B*	*ns**	*ns**	*42*	*II*
	Serwy	S1^{v}	C	+	+	10	I
		S2^{v}	C	+	–	10	I
		S3^{v}	C	+	–	10	I
		S4^{v}	A	+	–	27	I
		S5^{v}	C	+	ns	27	I
		S6^{v}	B	+	ns	27	I
		S7^{v}	B	+	ns	26	I
P. omorika	Kórnik Arboretum	AK1.1^{v}	B	+	+	12	I
		AK1.2^{v}	C	+	–	12	I
		AK1.3^{v}	B	+	+	12	I
		AK2.1^{v}	B	+	+	11	I
		AK2.2^{v}	B	+	+	11	I
		AK2.3^{v}	C	+	+	11	I
		AK2.4^{v}	C	+	ns	26	I
		AK2.5^{v}	B	–	ns	26	I
		AK2.6$^{v/v+LN}$	C	*+/ns**	ns/*ns**	*26/44*	*I/II*
		AK2.7^{v}	C	+	ns	26	I
		AK2.8^{v}	C	+	ns	26	I
		AK2.9^{v}	C	+	ns	26	I
		AK2.10^{v}	C	+	ns	26	I
	Dąbrówka Kościelna	DK1.1^{v}	C	+	ns	3	I
		DK1.2^{v}	B	+	ns	3	I
		DK1.3^{v+LN}	C	*ns**	*ns**	*18*	*II*
		DK2.1^{v}	C	–	ns	3	I
		DK2.2^{v}	C	–	ns	3	I
		DK2.3^{v}	C	–	ns	3	I
		DK2.4^{v}	C	–	ns	3	I

K, S, AK1, AK2, DK1, DK2 – origin of ET lines; successive numbers – line number of the specific origin; SE – somatic embryos; SS – somatic seedlings. v – plant material used for analysis after *in vitro* maintenance; v+LN – plant material used for analysis after cryopreservation (italic); ns – not studied; * – germination and acclimation after LN storage. A = ET lines produced more than 800 SEs per 1g of tissue; B = ET lines produced between 400 to 800 SEs per 1g of tissue; C = ET lines produced between 100 to 400 SEs per 1g of tissue

EXPERIMENT 1. GENETIC STABILITY DURING SOMATIC EMBRYO MATURATION AND GERMINATION

Plant material obtained from 10 ET lines of *P. abies* (origin Kolonowskie and Serwy) and 19 lines of *P. omorika* (origin the Kórnik Arboretum and Dąbrówka Kościelna) was used for SSR analysis (Table 1). ETs were maintained on the proliferation medium from 3–27 months, depending on the ET line. The following samples were used: ETv maintained *in vitro*, 5-week-old somatic embryos at the cotyledonary stage (CSE), 1-month-old germinating somatic embryos (GSE) or alternatively needles from 6-month-old somatic seedlings (SS) in some cases. Somatic seedlings were the final product of somatic embryogenesis but, due to high mortality, this stage was obtained only for some lines. Each sample of ETs consisted of approximately 100 mg of FW tissue, while each sample of CSE consisted of 50 mg FW of embryos. The GSE and SS samples consisted of three embryos with a properly developed hypocotyl and root or five needles collected from individual seedlings, respectively. All samples were stored at -80°C until DNA extraction.

DNA of the sampled material was analyzed at eight nuclear microsatellite loci designed for *Picea abies*. Five of the analyzed loci consisted of dinucleotide motifs (*SpAGC1*, *SpAGC2*, *SpAGG3*, *SpAC1F7* and *SpAC1H8*, Pfeiffer et al., 1997; Suppl. Table S1) and three were of three-nucleotide motifs (*EATC1E03*, *EATC1D02* and *EATC1B02* by Scotti et al., 2002; Suppl. Table S2).

EXPERIMENT 2. GENETIC STABILITY AFTER CRYOPRESERVATION OF EMBRYOGENIC CUTURES

ETs and plant material obtained from one ET line of *P. abies* (K4) and from two lines of *P. omorika* (AK2.6 and DK1.3) after storage in LN were used for genetic analysis (Table 1). *P. abies* ET was maintained on the proliferation medium for 42 months and two *P. omorika* ETs for 18 and 44 months before storage in LN. The following samples were analyzed: ETs maintained *in vitro* (ETv), ETs recovered and proliferated after cryopreservation (ETc) and 3- and 5-week-old somatic embryos obtained from ETc (SE 3 ETc and SE 5 ETc, respectively) incubated on maturation medium. Genetic analysis was performed with eight SSR loci as in the previous experiment. We added five additional loci that we wanted to test for their utility in analysis of genetic stability: *EATC2B02*, *WS0023.B03*, *WS0019.M09*, *WS0022.B15* and *WS00716.F13* (Suppl. Table S1) (Scotti et al., 2002; Rungis et al., 2004). For genetic analysis, the same type of samples was used as in Experiment 1.

DNA ISOLATION AND SSR ANALYSIS

Total genomic DNA isolation and analysis of genetic stability of the sampled plant material with five nuclear microsatellite loci: *SpAGC1*, *SpAGC2*, *SpAGG3*, *SpAC1H8*, *SpAC1F7* were performed according to the protocol applied in our earlier studies (Hazubska-Przybył et al., 2013). Three SSR loci, namely *EATC1E03*, *EATC1D02* and *EATC1B02*, were amplified in a PCR-Multiplex reaction using a Multiplex PCR Kit (Qiagen, Hilden, Germany) using fluorescently labeled primers with a "touch down" temperature profile as follows: pre-denaturation at 94°C for 15 min, followed by 10 cycles of denaturation at 94°C for 30 sec, then annealing at 60°C for 45 sec (with a 1°C reduction in the annealing temperature in each successive cycle) and extension of the amplification product at 72°C for 40 sec. This was followed by 25 cycles consisting of denaturation at 94°C for 30 sec, annealing at 50°C for 30 sec and extension at 72°C for 30 sec; the reaction ended with a final extension step of 7 min at 72°C. The amplified loci were separated on an automated ABI Prism 3130 genetic analyzer (Applied Biosystems) with GeneScan LIZ-500 as the internal size standard. Genotypes were scored with Gene Mapper v. 4.0 (Applied Biosystems). Each identified somaclonal variation event was verified by three independent amplification reactions and analysis on the 3130 Genetic Analyzer (Appilied Biosystems).

RESULTS

In this study, we detected 114 alleles in total across the 13 loci and two experiments conducted for *P. abies* and *P. omorika*. Non-fidelity in the amplification profiles was noted in eight loci. In total, 68 different mutations were recorded in the two spruce species and 31 lines investigated, which on average gives 2.19 mutation events per line (Suppl. Tables S2 and S3). However, in 20 cases of suspected mutations the total inconsistency between the homozygotic mother locus and homozygotic ET line's locus may suggest the presence of the 'null' allele in the mother locus. In such cases, only the father allele present in progeny (ET line) will be expressed leading to mismatch of genotypes at this locus/loci. Thus, after the correction of the mutation number by the presence of null alleles in mother genotypes, we obtained 52 mutations in total and 1.7 mutations per line.

During Experiment 1, variation in amplification profiles was detected in six ET lines of *P. abies* tested (60%) and in six loci out of the eight used (62.5%) (Suppl. Table S2). In total, 27 mutation events were detected of which 17 appeared at

the ETv stage, six at CSE stage and three at GSE stage. Most of the mutations from the ETv stage (15; 88.23%) were maintained at subsequent stages. Three out of six mutations noted at CSE stage were not detected at GSE stage at which again initial genotype from ETv stage was noted (Suppl. Table S2). In six lines more than one locus underwent mutation; in lines K2 and S4 four loci were mutated.

In the case of *P. omorika*, somaclonal variation was observed in ten out of 19 ET lines (52.6%) and in four loci out of eight used (50%) (Suppl. Table S2). Sixteen mutation events were recorded in total, of which seven were noted at ETv stage, six at CSE stage, two at GSE at single at SS stage. The mutations gained at ETv were generally detected at the subsequent stages (GSE/SS), and single reversed mutation into original genotype was also noted. The gain of another allele was noted also at the GSE stage (line AK2.8, AK2.9). Lines AK2.4 and AK2.9 were mutated in more than one locus – three and two, respectively.

In reference to Experiment 2 aiming at tracking the possible mutation events induced by cryopreservation, nine mutations were detected in total (Suppl. Table S3). Variation in allele size was recorded in all three lines tested, K4 (*P. abies*), AK2.6 and DK1.3 (*P. omorika*), and in five loci out of the 13 used (38.5%). Five mutations (55.5%) were recorded in embryogenic tissue (ETv), and two mutations (22.2%) in embryogenic tissue recovered after cryopreservation (ETc). Additionally, two mutations were detected in two loci in somatic embryos after 3 weeks (SE 3 ETc) of growth on the maturation medium (line AK2.6, *P. omorika*). However, in the case of *P. omorika* line AK2.6 mutation gained at ETc was not transferred to the subsequent stage and the initial genotype was recovered. An exceptional situation was noted in *P. omorika* line AK2.6 at locus SpAGC2, for which two successive mutation events were noted, one at the ETc stage and another during the development of SE on the maturation medium after three weeks (SE 3 ETc) (Suppl. Table S3). This change was maintained in SE that matured for further two weeks.

The estimators of genetic similarity for mother trees and derived ET lines and for stages within the respective ET line tested in two experiments are presented in Table 2. Generally, lower values of Jaccard's coefficient of similarity were noted for derived ET lines and their respective mother tree than among stages within the line originating from the single mother tree. For *P. omorika* lines DK.1-2 full fidelity was obtained and the lowest mean similarity was noted for lines K1-K3 and their mother tree (mean 0.676).

DISCUSSION

Plant material obtained from *in vitro* cultures and subjected to cryopreservation should be genetically stable if used in breeding programs. This implies the need for normalization of the procedures which seems to be especially important in the case of development of new micropropagation protocols (Thiem and Kikowska, 2008). The essential step in studies on SE and cryopreservation is the evaluation of the methods used with particular emphasis on their efficiency. However, the safety in terms of genetic stability should also be integrated into testing protocols. In this work, we demonstrated the presence of changes in the genome of both spruce species that were obtained by the SE method and stored in LN.

Our previous studies confirmed that *P. abies* and *P. omorika* are capable of micropropagation *via* the SE method and that the embryogenic cultures of these species may be successfully cryopreserved using the pregrowth-dehydration method (Hazubska-Przybył and Bojarczuk, 2008; Hazubska-Przybył et al., 2010, 2013). Using five SSR loci we also confirmed the genetic stability of the single line of *Picea abies* tested (Hazubska-Przybył et al., 2013). The present study, aiming at testing the genetic stability, was based on much wider material in order to be able to make more general conclusions about possible mutagenic effects of duration of *in vitro* culturing and storage in LN. Thus, we designed two experiments with two spruce species, a larger number of ETs lines that were maintained under *in vitro* conditions for a longer period in comparison to our previous study, in which *in vitro* culturing lasted 5 weeks and cryopreservation only 24 hours (Hazubska-Przybył et al., 2013). Moreover, in this study we validated additional SSR loci for their routine use in protocols of testing the genetic stability in these two spruce species.

Our results showed that all SSR loci were useful in the detection of mutations in plant material obtained *via* SE and after cryopreservation by pregrowth-dehydration because all loci were polymorphic. In a few cases, we did not obtain an amplification product (Suppl. Table S3). This suggests two possibilities: 1) some inhibitors of polymerase were present and stopped the reaction (probably the remains of chemical compounds used during the *in vitro* propagation procedure) or 2) some mutation might occur in the primer binding region and resulted in lack of amplification. Additionally, the fact that lack of amplification was noted exclusively in *P. omorika* suggests some problems with cross-amplification because the primers used in this study were originally generated for *P. abies*. Cross-amplification of SSR

TABLE 2. Jaccard's similarity coefficients for lines derived from *P. abies* and *P. omorika* seeds.

Experiment I	Jaccard's coefficient: Within the line	Jaccard's coefficient: Mother tree
P. abies		
K1	1.000	0.600
K2	0.733	0.502
K3	0.851	0.926
Mean	***0.861***	***0.676***
S1	1.000	1.000
S2	1.000	1.000
S3	1.000	0.600
S4	1.000	0.333
S5	0.921	0.454
S6	1.000	0.777
S7	0.851	0.718
Mean	***0.967***	***0.697***
P. omorika		
AK1.1	0.851	0.777
AK1.2	1.000	1.000
AK1.3	1.000	1.000
Mean	***0.950***	***0.926***
AK2.1	1.000	1.000
AK2.2	1.000	1.000
AK2.3	0.851	0.925
AK2.4	1.000	0.454
AK2.5	1.000	0.777
AK2.6	1.000	0.777
AK2.7	0.921	0.851
AK2.8	0.812	0.851
AK2.9	0.851	0.851
AK2.10	1.000	1.000
Mean	***0.945***	***0.866***
DK1.1	1.000	1.000
DK1.2	1.000	1.000
Mean	***1.000***	***1.000***
DK2.1	0.555	1.000
DK2.2	1.000	1.000
DK2.3	1.000	1.000
DK2.4	0.777	0.888
Mean	***0.833***	***0.972***
Experiment II		
P. abies		
K4	1.000	0.733
P. omorika		
AK2.6	0.862	0.652
DK1.3	1.000	0.857

loci is frequently applied in closely related species, but may result in low success rates and/or the presence of the null alleles (Boratyński et al., 2014; Sękiewicz et al., 2015).

Currently, SE together with cryopreservation are the biotechnological methods applied on a large scale in some countries for production of seedlings of some trees species both for forest management and commercial horticulture (Lelu-Walter et al., 2013). This method also seems to be a valuable tool in conservation activities concerning the protection and improvement of gene pools of an endangered species (Maruyama et al., 2007) or plants with medicinal properties (Karamian and Ghasemlou, 2014). Protocols for seedling reproduction *via* SE have been developed for many trees species so far (Corredoira et al., 2013) and ongoing work aiming at optimizing and improving SE process in many tree species is still being reported (Krajňáková et al., 2013; Pullman and Bucalo, 2014). However, these new procedures frequently involve new forms of stress, side effects such as induction of genetic changes in the SE-derived and cryopreserved plant material. The examples of these new stress-inducing factors can be Picloram or NAA added instead of 2,4-D to proliferation medium or strong drying of ETs after application of the pregrowth-dehydration as a cryopreservation method (Hazubska-Przybył and Bojarczuk, 2008; Hazubska-Przybył et al., 2010). The long-term proliferation of ETs of both spruce species on the medium supplemented with Picloram during their maintenance under *in vitro* cultures was

used in our present study. We hypothesized that both procedures used could negatively affect the genetic stability of plant material. Several studies have reported somaclonal variation in embryonic cultures both after *in vitro* cultivation and after cryopreservation (Burg et al., 2007; DeVerno et al., 1999; Helmersson et al., 2008; Nawrot-Chorabik, 2009; Isabel et al., 1996; Krajňáková et al., 2011; Dey et al., 2015). In this study we report substantial genetic instability in SSR genotypes of the studied material as 52 different mutation events in total were detected.

In our analysis, changes were detected in the SSR-assessed genotypes of plant material obtained from ETs of *P. abies* and *P. omorika* maintained *in vitro*. The mutation distribution was not random with regard to stage, as most of the changes were noted in DNA of embryogenic tissue maintained *in vitro* (ETv) (3–27 months in Experiment 1 and 18–44 months in Experiment 2) and somatic embryos exposed for the maturation medium (CSE) (Suppl. Table S2). In reference to the first stage (ETv), it seems that the initiation of the culture tissue is the most stressful procedure that generates the mutations. Most of the genetic changes gained at this very initial stage are stored at the subsequent stages. This resulted in a generally lower level of genetic similarity between the mother tree genotype and derived somatic lines but higher for within lines estimations (Table 2). The changes noted at CSE may result from a long-term proliferation/maintenance of ETs on the Picloram-containing medium (or from the stress caused by the conditions applied during maturation of somatic embryos, i.e., high osmoticum and the presence of ABA. The induction of somaclonal variation during SE is frequently ascribed to different physicochemical factors to which unprotected genetic material is exposed and reduced selection in the culture environment that enables the maintenance of mutated genomes (Bairu et al., 2011; Dey et al., 2015). Our results are consistent with the opinion that the cultivation period and conditions in which somatic embryos mature are likely the factors inducing somaclonal variations (Evans and Sharp, 1988; Nawrot-Chorabik, 2009; Dey et al., 2015). Long-term maintenance of embryogenic cultures *in vitro* was indicated as a possible factor of detected somaclonal variation in olive (Leva et al., 2012; Bradai et al., 2016).

Genotype-based propensity to mutation needs to be accounted also in discussion on the variability in the level of somaclonal variation. In this study, in eight lines of ET (K1, K2, S3-S5, S7 – *P. abies* and AK2.2, AK2.9 – *P. omorika*) and the ET line recovered after cryostorage (AK2.6 – *P. omorika*), mutations occurred at more than one locus which suggests generally lower stability of their genomes. Other studies also indicated genotype-dependent occurrence of somaclonal variation (Marum et al., 2009, Salaj et al., 2011, Leva et al., 2012). A recent study of Bradai et al. (2016) on somaclonal variation in olive plants revealed clear relationship between genotype and susceptibility to the morphological and physiological alternations of regenerated plants. It is known that the genetic make-up plays an important role in tolerance to different kinds of stress, including this induced with the SE procedure and so some genotypes may be less stable under conditions of tissue cultures (Karp, 1994). Frequently, the causal factors are transposable elements that are normally epigenetically silenced by DNA methylation (Zhang et al., 2014), and growth regulators present in regeneration medium are indicated as one of the factors affecting the methylation level and activation of the transposons (Sarmast, 2016).

In the case of the cryopreserved plant material, somaclonal variation was detected in recovered ETc in one out of the two tested embryogenic lines of *P. omorika* (AK2.6). In a single embryogenic line of *P. abies*, K4 mutation occurred before cryopreservation procedure at the stage of ETv, which corresponds to the results of Experiment 1 indicating that initiation of SE and prolonged cultivation are the main factors of somaclonal variation noted. Some genetic changes were not further transferred to subsequent stages, but others were. In our previous analysis of the genetic stability of *P. abies* embryogenic tissues after cryopreservation, we reported no changes in the genotypes assessed with the set of five SSR loci but this study was based on a single ET line (Hazubska-Przybył et al., 2013). The discrepancies between the current and previous studies may simply stem from including in this experiments a higher number of ET lines which increased the overall probability of revealing the mutation events. Additionally, as expressed previously, different genotypes may present different sensitivity to mutagenic factors (Marum et al., 2009). However, in our opinion, the most significant factor inducing the changes observed in ET lines is likely the long-term maintenance of embryogenic cultures on the proliferation medium – 42 months for *P. abies* and 44 months for *P. omorika* (Table 1). Also, the previously cryopreserved single *P. abies* ET line was cultured for 12 months (Hazubska-Przybył et al., 2013). In our opinion, long-term contact of ETs with plant growth regulators (Picloram and BA) in the proliferation medium could increase their sensitivity to the stress caused by the following procedure of cryopreservation. Additionally, the mutation discovered in the material after cryopreservation referred to the line AK2.6, which

was generally less genetically stable as it gained mutations in three loci and at more than one stage.

Somaclonal variation may entail the induction of genetic chimeras (Burg et al., 2007; López et al., 2004; Marum et al., 2009) when a subpopulation of cells avoids mutation and maintains non-mutated allele(s) along with mutated cells. In both experiments, we detected such chimeras. The inability to detect a mutation at later stages clearly suggests that these cells were not able to proliferate further in contrast to non-mutated cells. According to DeVerno et al. (1999), SE tissue may be composed of subpopulations of mutated and non-mutated cells, of which only those possessing genomes not affected by mutations survive and are capable of further development.

CONCLUSIONS

The results obtained in this work suggest that somaclonal variation may be quite frequent in SE and that cryopreservation may also have an impact on genetic stability, although to a lesser extent. Some of these changes were transferred to a subsequent stage and some were not, which implies that individuals with non-stable genomes may be disposed from the population of regenerated plants at later stages of the development. In fact, some of the mutated lines were not able to develop into seedlings (Suppl. Table S2). Hence, even if our results clearly indicate somaclonal variation during SE, we suggest that observations in later developmental stages should be included. Additionally, incorporating more than one system of genetic markers and other levels of genome organization are required for deep analysis of genome stability and potential consequences of changes during the process of SE. Our results call for more studies concerning the factors contributing to the occurrence of mutation events and the processes controlling their maintenance or loss. The non-random distribution of mutation suggests that individual propensity to mutations may play an important role. The detailed knowledge of the somaclonal variation process would contribute to more efficient and reliable SE and cryopreservation protocols.

AUTHORS' CONTRIBUTIONS

THP – designed and performed the ET experiments; THP and MD analyzed the data and wrote the manuscript. The authors declare that they have no conflicts of interest.

ACKNOWLEDGEMENTS

This article was supported by National Research Centre (NCN) in Krakow (project NN 309 130 837), and by the Institute of Dendrology Polish Academy of Sciences, Kórnik (statutory project), Poland. The authors thank A. Obarska and M. Łuczak for technical assistance.

REFERENCES

Aronen TS, Krajňáková J, Häggman H, and Ryynänen LA. 1999. Genetic fidelity of cryopreserved embryogenic cultures of open-pollinated *Abies cephalonica*. *Plant Science* 142: 163–172.

Bairu MW, Aremu A, and van Staden J. 2011. Somaclonal variation in plants: causes and detection methods. *Plant Growth Regulation* 63: 147–173.

Boratyński A, Wachowiak W, Dering M, Boratyńska K, Sękiewicz K, Sobierajska K, Jasińska AK, Klimko M, Montserrat JM, Romo A, Ok T, and Didukh Y. 2014. The biogeography and genetic relationships of *Juniperus oxycedrus* and related taxa from the Mediterranean and Macaronesian regions. *Botanical Journal of the Linnean Society* 174: 637–653.

Bradai F, Pliego-Alfaro F, Sánchez-Romero C. 2016. Long-term somatic embryogenesis in olive (*Olea europaea* L.): Influence on regeneration capability and quality of regenerated plants. *Scientia Horticuturae* 199: 23–31.

Burg K, Helmersson A, Bozhkov P, and von Arnold S. 2007. Developmental and genetic variation in nuclear microsatellite stability during somatic embryogenesis in pine. *Journal of Experimental Botany* 58: 687–698.

Chalupa V. 1985. Somatic embryogenesis and plantlet regeneration from cultured immature and mature embryos of *Picea abies* (L.) Karst. *Communications of the Institute for Forestry of the Czech Republic* 14: 57–63.

Corredoira E, Valladares S, Martinez MT, Vieitez AM, and San José MC. 2013. Somatic embryogenesis in *Alnus glutinosa* (L.) Gaertn. *Trees* 27: 1597–1608.

Deverno LL, Charest PJ, and Bonen L. 1994. Mitochondrial DNA variation in somatic embryogenic cultures of *Larix*. *Theoretical and Applied Genetics* 88: 727–732.

Deverno LL, Park YS, Bonga JM, and Barret JD. 1999. Somaclonal variation in cryopreserved clones of white spruce (*Picea glauca* (Moench) Voss.). *Plant Cell Reports* 18: 948–953.

Dey T, Saha S, and Ghosh PD. 2015. Somaclonal variation among somatic embryo derived plants – evaluation of agronomically important somaclones and detection of genetic changes by RAPD in *Cymbopogon winterianus*. *South African Journal of Botany* 96: 112–121.

Ellegren H. 2004. Microsatellites: simple sequences with complex evolution. *Nature Reviews Genetics* 5: 435–445.

Etienne H, and Bertrand B. 2003. Somaclonal variation in *Coffea arabica*: effects of genotype and embryogenic cell suspension age on frequency and phenotype of variants. *Tree Physiology* 23: 419–426.

Evans DA, and Sharp WR. 1988. Somaclonal and gametoclonal variation. In: Evans DA, Sharp WR, Ammirato PV [eds] *Handbook of Plant Cell Culture*, 97–132. Macmillan Publishing Company, New York 4.

Find JI, Kristersen MMH, Norgaard JV, and Krogstrup P. 1998. Effect of culture period and cell density on regrowth following cryopreservation of embryogenic suspension cultures of Norway spruce and Sitka spruce. *Plant Cell Tissue and Organ Culture* 53: 27–33.

Hakman I, Fowke LC, von Arnold S, and Eriksson T. 1985. The development of somatic embryos in tissue cultures initiated from immature embryos of *Picea abies* (Norway spruce). *Plant Science* 38: 53–59.

Hazubska-Przybył T, and Bojarczuk K. 2008. Somatic embryogenesis of selected spruce species (*Picea abies*, *P. omorika*, *P. pungens* 'Glauca' and *P. breweriana*). *Acta Societatis Botanicorum Poloniae* 77 (3): 189–199.

Hazubska-Przybył T, Chmielarz P, Michalak M, and Bojarczuk K. 2010. Cryopreservation of embryogenic tissues of *Picea omorika* (Serbian spruce). *Plant Cell Tissue and Organ Culture* 102 (1): 35–44.

Hazubska-Przybył T, Chmielarz P, Michalak M, Dering M, and Bojarczuk K. 2013. Survival and genetic stability of *Picea abies* embryogenic cultures after cryopreservation using a pregrowth-dehydration method. *Plant Cell Tissue and Organ Culture* 113 (2): 303–313.

Helmersson A, von Arnold S, Burg K, and Bozhkov PV. 2004. High stability of nuclear microsatellite loci during the early stages of somatic embryogenesis in Norway spruce. *Tree Physiology* 24: 1181–1186.

Helmersson A, Jansson G, Bozhkov PV, and von Arnold S. 2008. Genetic variation in microsatellite stability of somatic embryo plants of *Picea abies*: a case study using six unrelated full-sib families. *Scandinavian Journal of Forest Research* 23: 2–11.

Hornero J, Martinez I, Celestino C, Gallego FJ, Torres V, and Toribio M. 2001. Early checking of genetic stability of cork oak somatic embryos by AFLP analysis. *International Journal of Plant Science* 162: 827–833.

Isabel N, Boivin R, Levasseur C, Charest PM, Bousquet J, and Tremblay FM. 1996. Occurrence of somaclonal variation among somatic embryo-derived white spruces (*Picea glauca*, Pinaceae). *American Journal of Botany* 83: 1121–1130.

Isah T. 2015. Adjustments to in vitro culture conditions and associated anomalies in plants. *Acta Biologica Cracoviensia Series Botanica* 57(2): 9–28.

Jaccard P. 1908. Nouvelles recherches sur la distribution florale. *Bulletin of the Society of Natural Sciences* 44: 223–270 [In French].

Karamian R, and Ghasemlou F. 2014. Plant regeneration via organogenesis and somatic embryogenesis in *Verbascum sinuatum* L. *Acta Biologica Cracoviensia Series Botanica* 56(1): 97–103.

Karp A. 1994. Origins, causes and uses of variation in plant tissue cultures. In: Vasil IK, Thorpe TA [eds.] *Plant Cell and Tissue Culture*, 139–152. Kluwer Academic Publishers, Dordrecht.

Klimaszewska K, and Cyr DR. 2002. Conifer somatic embryogenesis: I. Development. *Dendrobiology* 48: 31–39.

Krajňáková J, Sutela S, Aronen TS, Gömöry D, Vianello A, and Häggman H. 2011. Long-term cryopreservation of Greek fir embryogenic cell lines: Recovery, maturation and genetic fidelity. *Cryobiology* 63: 17–25.

Krajňáková J, Bertolini A, Gömöry D, Vianello A, and Häggman H. 2013. Initiation, long-term cryopreservation, and recovery of *Abies alba* Mill. embryogenic cell lines. *In Vitro Cellular and Developmental Biology – Plant* 49: 560–571.

Kunakh VA. 1999. Variation of the plant genome upon dedifferentiation and callus formation in vitro. *Fiziologiya Rastenii* 46: 919–929.

Larkin P, and Scrowcroft W. 1981. Somaclonal variation—a novel source of variability from cell cultures for plant improvement. *Theoretical and Applied Genetics* 60: 197–214.

Lelu-Walter MA, Thompson D, Harvenght L, Sanchez L, Toribio M, and Pâques L. 2013. Somatic embryogenesis in forestry with a focus on Europe: state-of-the-art, benefits, challenges and future direction. *Tree Genetics and Genomes* 9: 883–899.

Leva AR, and Petruccelli R. 2012. Monitoring of cultivar identity in micropropagated olive plants using RAPD and ISSR markers. *Biologia Plantarum* 56: 373–376.

Leva AR, Petruccelli R, and Rinaldi LMR. 2012. Somaclonal variation in tissue cultures: a case study with olive. In: Leva A, Rinaldi [eds] *Recent Advances in Plant in vitro Culture*, 123–150. Intech.

Litvay JD, Verma DC, and Johnson MA. 1985. Influence of loblolly pine (*Pinus taeda* L.) culture medium and its components on growth and somatic embryogenesis of wild carrot (*Daucus carrota* L.). *Plant Cell Reports* 4: 325–328.

Lopes T, Capelo A, Brito G, Loureiro J, and Santos C. 2009. Genetic variability analyses of the somatic embryogenesis induction process in *Olea* spp. using nuclear microsatellites. *Trees* 23: 29–36.

López CMR, Wetten AC, and Wilkinson MJ. 2004. Detection and quantification of in vitro-culture induced chimerism using simple sequence repeat (SSR) analysis in *Theobroma cacao* (L.). *Theoretical and Applied Genetics* 110: 157–166.

Mallón R, Covelo P, and Vieitez AM. 2012. Improving secondary embryogenesis in *Quercus robur*: Application of temporary immersion for mass propagation. *Trees – Structure and Function* 26: 731–741.

Margara J. 1977. La multiplication vegetative de la betterave (*Beta vulgaris* L.) en culture in vitro. *Comptes rendus de l'Académie des Sciences Paris* 285: 1041–1044.

Marum L, Rocheta M, Maroco J, Oliveira MM, and Miguel C. 2009. Analysis of genetic stability at SSR loci during somatic embryogenesis in maritime pine (*Pinus pinaster*). *Plant Cell Reports* 28: 673–682.

Maruyama E, Hosoi Y, and Ishii K. 2007. Somatic embryogenesis and plant regeneration in yakutanegoyou, *Pinus armandii* Franch. var. *amamiana* (Koidz.) Hatusima, an endemic and endangered species in Japan. *In Vitro Cellular and Developmental Biology – Plant* 43(1): 28–34.

Mihaljević S, and Jelaska S. 2005. Omorica Spruce (*Picea omorika*). In: Jain SM, Gupta PK [eds] *Protocol for Somatic Embryogenesis in Woody Plants*, 35–45. Forestry Sciences, Springer-Verlag 77.

Nawrot-Chorabik K. 2009. Somaclonal variation in embryogenic cultures of silver fir (*Abies alba* Mill.). *Plant Biosystems* 143 (2): 377–385.

Peredo EL, Ángeles RM, and Arroyo-García R. 2006. Assessment of genetic and epigenetic variation in hop plants regenerated from sequential subcultures of organogenic calli. *Journal of Plant Physiology* 163: 1071–1079.

Pinto G, Santos C, Neves L, and Araújo C. 2002. Somatic embryogenesis and plant regeneration in *Eucalyptus globulus* Labill. *Plant Cell Reports* 21: 208–213.

Pfeiffer A, Olivieri AM, and Morgante M. 1997. Identification and characterization of microsatellites in Norway spruce (*Picea abies* K.). *Genome* 40: 411–419.

Pullman GS, and Bucalo K, Maruyama K. 2014. Pine somatic embryogenesis: analyses of seed tissue and medium to improve protocol development. *New Forests* 25: 353–377.

Rungis D, Bérubé Y, Zhang J, Ralph S, Ritland CE, Ellis BE, Douglas C, Bohlmann J, and Ritland K. 2004. Robust simple sequence repeat markers for spruce (*Picea* spp.) from expressed sequence tags. *Theoretical and Applied Genetics* 109: 1283–1294.

Sahijram L, Soneji JR, and Bollamma KT. 2003. Analyzing somaclonal variation in micropropagated bananas (*Musa* spp.). *In Vitro Cellular and Developmental Biology – Plant* 39: 551–556.

Salaj T, Salaj J, and Kormutak A. 1999. Initiation of embryogenic tissues and plantlet regeneration from somatic embryos of *Pinus nigra* Arn. *Plant Science* 145: 33–40.

Salaj T, Matušiková I, Fráterová L, Piršelová B, and Salaj J. 2011. Regrowth of embryogenic tissues of *Pinus nigra* following cryopreservation. *Plant Cell Tissue and Organ Culture* 106: 55–61.

Sarmast MK. 2016. Genetic transformation and somaclonal variation in conifers. *Plant Biotechnology Reports* 10: 309–325.

Scotti I, Magni F, Paglia GP, and Morgante M. 2002. Trinucleotide microsatellites in Norway spruce (*Picea abies*): their features and the development of molecular markers. *Theoretical and Applied Genetics* 106: 40–50.

Sękiewicz K, Dering M, Sękiewicz M, Boratyńska K, Iszkuło G, Litkowiec M, Ok T, Dagher-Kharrat MB, and Boratyński A. 2015. Effect of geographic range discontinuity on species differentiation – East-Mediterranean *Abies cilicica*: a case study. *Tree Genetics and Genomes* 11: 1–10.

Thiem B, and Kikowska M. 2008. Zapewnienie jakości roślin leczniczych rozmnażanych w kulturach *in vitro*. *Herba polonica* 54: 168-178.

Yakovlev IA, Lee KY, Rotter B, Olsen JE, Skroppa T, Johnsen Ø, and Fossdal CG. 2014. Temperature-dependent differential transcriptomes during formation of an epigenetic memory in Norway spruce embryogenesis. *Tree Genetics and Genomes* 10: 355–366.

Von Arnold S, Bozhkov P, Clapham D, Dyachok J, Filonova L, Högberg KA, Ingouff M, and Wiweger M. 2005. Propagation of Norway spruce via somatic embryogenesis. *Plant Cell Tissue and Organ Culture* 81: 323–329.

Vondráková Z, Eliašová K, Vágner M, Martincová O, and Cvikrová M. 2015. Exogenous putrescine affects endogenous polyamine levels and the development of *Picea abies* somatic embryos. *Plant Growth Regulation* 75: 405–41.

Zhang D, Wang Z, Wang N, Gao Y, Liu Y, Bai Y, Zhang Z, Lin X, Dong Y, Ou X, Xu CH, and Liu B. 2014. Tissue culture-induced heritable genomic variation in rice, and their phenotypic implications. *PlosOne* 9(5): e96879.