Cytogenetic Stability of Young and Long-Term Embryogenic Cultures of *Larix sibirica*

Olga V. Goryachkina^{1*}, Maria E. Park¹, Iraida N. Tretyakova¹,
Ekaterina D. Badaeva² and Elena N. Muratova¹

¹Sukachev Institute of Forest, Russian Academy of Sciences, Siberian Branch, Federal Research Center
Krasnoyarsk Science Center SB RAS, Akademgorodok 50/28, Krasnoyarsk 660036, Russia

²Vavilov Institute of General Genetics, Russian Academy of Sciences, Gubkin str., 3, Moscow 119333, Russia

Received April 10, 2018; accepted May 20, 2018

Summary We present the results of cytogenetic analysis of seven young and long-term cultured embryogenic cell lines (CLs) of *Larix sibirica* obtained by somatic embryogenesis. All CLs were found to be mixoploid. Three CLs (1, 2 and 6 years of cultivation) were the most stable cytogenetically with 91–96% cells having diploid chromosome number $2n=24$, typical for the species. These CLs can be successfully used for the development of planting material and in clonal plantation forestry. At the same time, some embryogenic CLs were shown to possess various chromosome and genome mutations. We found two aneuploidy proliferating CLs ($2n=25$ and 28) capable of somatic embryo formation. One of the CLs analyzed in a current study was found to be cytogenetically unstable with chromosome numbers ranging from 24 to 35 and containing one-two micronuclei in the interphase cells. Fluorescent *in situ* hybridization (FISH) and morphometric analyses of karyotypes of these embryogenic CLs allow an assumption that the trisomy for one or several chromosomes is the most widespread type of genomic disturbances in embryogenic CLs of *L. sibirica*.

Key words *Larix sibirica*, Somatic embryogenesis, Embryogenic cell line, Chromosome, Karyotype, Aneuploidy, FISH.

Somatic embryogenesis is a vegetative approach for mass multiplication of plants that can significantly accelerate genetic and breeding researches and broaden the scale of production of planting material of important conifer species carrying desired traits. Successful propagation of conifers *via* somatic embryogenesis requires the assessment of the genetic stability of the obtained embryogenic CLs. Such factors as conditions and duration of cultivation, application of growth regulators can cause various alterations in plant karyotype, in particular, increase mutation (Bairu *et al.* 2011, Sarmast 2016).

Data on the cytogenetic stability of embryogenic cultures of conifer plants available from the literature are rather controversial. It is well known that plants that have passed through *in vitro* propagation can display somaclonal variation (Larkin and Snowcroft 1981). Some authors described changes of chromosome number in embryogenic cultures of some conifer species, in particular, *Abies alba* (Roth *et al.* 1997), *Pinus nigra* (Salajova and Salaj 1992), *Larix×eurolepis* (Nkongolo and Klimaszevska 1995), *Pin. radiata* (O'Brien *et al.* 1996). That is more, cytogenetic instability of cell cultures and the derived clones has also been found in *Picea mariana*, *Pic. glauca*, *Pic. abies* (Fourré *et al.* 1997, Tremblay *et al.* 1999). By contrast, other authors demon-

strated the genetic stability of *in vitro* cultures of some *Picea* species (Mo *et al.* 1989, Eastman *et al.* 1991, Isabel *et al.* 1993, Nkongolo and Klimaszevska 1994, Harvenget *et al.* 2001, Helmersson *et al.* 2004).

Thus, cytogenetic peculiarities of embryogenic cultures of conifer plants were not sufficiently studied. The larch trees which prove to be perspective for mass multiplication *via* somatic embryogenesis are virtually not studied. In a current paper, we analyzed embryogenic cultures of the Siberian larch (*L. sibirica* Ledeb.), obtained by somatic embryogenesis with the aid to assess the level of cytogenetic stability of embryogenic CLs with different cultivation time.

Materials and methods

Plant materials

Seven continuously proliferating embryogenic CLs of *L. sibirica* (Table 1), which were characterized by different cultivation time, viability, proliferation activity, the number and size of somatic embryos (SEs) and their ability to mature, germinate, and form viable plantlets were selected for cytogenetic analysis from the Collection Bank of Embryogenic Cultures of the Laboratory of Forest Genetics and Breeding, Sukachev Institute of Forest SB RAS, Krasnoyarsk, Russia (Park *et al.* 2016).

These embryogenic CLs were developed from zygotic embryos of the donor tree *L. sibirica* A4 obtained by

* Corresponding author, e-mail: kvitko_olga@mail.ru
DOI: 10.1508/cytologia.83.323

Table 1. Characteristics of *L. sibirica* CLs.

Cell line	Year of initiation and plant material	Chromosome number, <i>2n</i> (N*)	Aberration (%)
17.7	2017, controlled pollination (<i>L. sibirica</i> × <i>L. sibirica</i>)	24 (106)	4.5
16.28	2016, open pollination	48 (5) 10 (1) 20 (1) 24 (107) 48 (4)	5.31
107	2013, open pollination	20 (3) 24 (1) 25 (1) 26 (3) 27 (6) 28 (85) 56 (1)	99.0
12	2015, controlled pollination (<i>L. sibirica</i> × <i>L. sibirica</i>)	23+1 dL** (91) 39+1 dL (3) 46+2 dL (6)	100.0
6	2011, open pollination	24 (91) 48 (9)	9.0
4	2009, open pollination	12 (1) 25 (85) 26 (1) 50 (12)	100.0
5	2009, controlled pollination (<i>L. sibirica</i> × <i>L. sukaczewii</i>)	24 (1) 25 (7) 26 (11) 27 (18) 28 (20) 29 (37) 30 (24) 31 (4) 35 (1) more than 50 (7)	99.2

*N=number of cell analyzed; ** deletion of chromosome segment; % aberration=% of cells with abnormal chromosome number. Chromosome numbers observed with the highest frequency are shown in bold.

self-pollination and by controlled pollination with pollen of *L. sibirica* and *L. sukaczewii*. The age of experimental trees was estimated to be about 50–70 years old; all trees were grown in the Arboretum of the Sukachev Institute of Forest SB RAS (Krasnoyarsk, Russia). Controlled pollination of *L. sibirica* aimed in obtaining zygotic embryos for subsequent *in vitro* cultivation was carried out at the end of the third decade of April, at the stage of microstrobile ripening and the beginning of pollen release.

In vitro induction of zygotic embryos, initiation and proliferation embryogenic cultures were carried out on a nutrient medium AI according to the protocol described earlier (Tretyakova and Barsukova 2012; patent No 456344; <http://www.freepatent.ru/images/patents/5/2456344/patent-2456344.pdf>; Park *et al.* 2016). Proliferating embryogenic CLs were sub-cultured on a fresh AI medium every two weeks and incubated in dark at 24±1°C. The productivity of embryogenic CLs was estimated by counting the SE number g⁻¹ of fresh embryonal-suspensor masses (ESM) a week after subculturing.

Cytogenetic analysis was carried out on globular SEs at the stage of proliferation. Different methods of treatment were used for metaphase arrest and chromosome

contraction, pre-treatment of the material with 0.2% colchicine for 18–20 h at room temperature provided the best metaphase yield for most lines. When working with CLs 12 and 107, the concentration of colchicine was raised up to 0.5%. Materials were fixed in ethanol–acetic acid (3 : 1) for 24 h.

Conventional karyotype analysis and silver staining of nucleoli

The material was stained in 1% aceto-hematoxylin solution at 50°C for 10–15 min. Then few SEs were dissected using a fine needle and forceps and placed in a drop of saturated chloral hydrate solution. Embryos were macerated with a razor blade for 2–3 min, until homogeneous suspension is obtained. The preparation was covered with a coverslip, drained with a filter paper and squashed. Chromosome number was assessed on 100 metaphase cells per each CL.

Nucleoli were visualized in interphase nuclei using silver staining according to standard protocols (Hizume *et al.* 1980, Muratova 1995). A small piece of fixed ESM not treated with colchicine was washed in distilled water for 1 h and stained in 50% silver nitrate at 60°C for 5 h.

The material was thoroughly washed with distilled water and placed in a drop of 2% acetocarmine to enhance contrast and then squashed. The number of nucleoli was assessed on 300–400 nuclei for each CL. The slides were examined using a MIKMED-6 (LOMO, Russia) microscope equipped with a digital camera MC-12 (LOMO, Russia).

FISH procedure

Materials were washed in distilled water for 1 h, macerated in 0.6% Cellulysine (Calbiochem, Switzerland) for 14–16 h at 24°C. The root tips were squashed in a drop of 45% acetic acid, and the coverslips were removed after freezing in liquid nitrogen. The slides were dehydrated in ethanol series and stored at 4°C before use.

Wheat 45S rDNA probe (pTa71; Gerlach and Bedbrook 1979) and 5S rDNA amplified by PCR (Trontin *et al.* 1999) from genomic DNA of *L. sibirica* were used as probes. The probes were labeled with biotin or digoxigenin by a nick translation according to manufacturer's protocol (Roche, Germany). FISH was conducted according to the protocol (Goryachkina *et al.* 2013) with minor modifications. The slides were counterstained with $1 \mu\text{g mL}^{-1}$ 4',6-diamidino-2-phenylindole (DAPI, Sigma, U.S.A.) in the Vectashield mount media (Vector Laboratory, U.S.A.) The slides were analyzed on an Imager D1 microscope (Carl Zeiss, Germany) coupled with

an AxioCam HRm camera (Carl Zeiss, Germany). The selected metaphase cells were captured using AxioVision software, release 4.6 (Carl Zeiss, Germany). Images were processed using Photoshop CS4 (Adobe Systems, U.S.A.).

Results

Proliferating embryogenic culture of conifers can exist as the semi-transparent mass of immature ESM, or filamentous embryos (Stasolla and Yeung 2003). The ESM of *L. sibirica* possesses polyembryonic complexes consisting of several embryos and well as independent single embryos. The continuous process of embryo multiplication takes place in embryogenic culture, which can explain the fact that the observed embryos represented by globules consisting of two to some tenth of cells are at the different stage of development.

Normally, the karyotype of larch consists of 24 chromosomes ($2n=2x=24$). CLs differ in cytogenetic stability and chromosome number; they all are mixoploids (Table 1). Three CLs differing in cultivation time (CLs 6, 16.28 and 17.7) contain cells with predominantly euploid chromosome sets $2n=24$ (Fig. 1). Single cells have the reduced chromosome number ($2n=10, 20$), which is likely to be due to spontaneous chromosome loss during abnormal cell divisions. We also recorded polyploid cells; their frequency ranged from 4.5 to 9.0%, whereas at the

Fig. 1. Metaphase cells of *L. sibirica* CLs with different number of chromosomes stained in aceto-hematoxylin. A: CL 6 ($2n=24$), B: CL 16.28 ($2n=24$), C: CL 17.7 ($2n=24$), D: CL 12 ($2n=23+1$), E: CL 4 ($2n=25$), F: CL 107 ($2n=28$), G: CL 5 ($2n=29$), H: CL 5 ($2n=30$), I: CL 5 ($2n=31$). Bar=10 μm .

Fig. 2. Aceto-hematoxylin stained metaphase chromosomes of *L. sibirica* CLs arranged according to the length. Chromosome pairs are identified by the morphology and distribution of secondary constrictions (shown by arrows). Bar=10 μ m.

natural level of mutagenesis somatic tissues of conifer trees usually contain less than 1% of the polyploid cells (Muratova 1991, Muratova *et al.* 2007, 2009, 2010, Goryachkina and Sizikh 2012). CLs 4, 12, and 107 are of the most interest because most cells show chromosome number different from that in *Larix* species. Thus, 85.0% cells in CLs 4 and 107 have 25 and 28 chromosomes, respectively. In the CL 12, 91.0% metaphase cells contain 24 chromosomes; however, the length of one chromosome was significantly reduced, approaching the length and shape of B chromosome (Fig. 1e). The chromosome number in cells of CLs 4, 12, and 107 varied within the ranges indicated in Table 1.

Hybrid line CL 5, obtained in 2009, was characterized by high cytogenetic instability. Metaphase cell containing 29 chromosomes are the most common, although their frequency is only 28.5%. Other variants of chromosome numbers have also been found (Table 1). Interphase nuclei of this line often possess micronuclei; their frequency reaches 10.5%. Micronuclei are the structures that derive from whole chromosomes or chromosome fragments which fail to bind to the spindle fibers and therefore are not included in the nuclei of daughter cells. Instead, they remain in the cytoplasm of the interphase cells, appearing as nucleus-like corpuscles, amounting for 1/3 to 1/5 of the nucleus size. Micronuclei are also recorded in interphase nuclei of CL 107; however, they occur with much lower frequency (2.7%).

Metaphase cells with a chromosomal set typical for each CL were selected for karyological analysis (Fig. 2). Normal karyotype of *L. sibirica* (CLs 6, 17.7, 16.28) consists of six pairs (I–VI) of long metacentric chromo-

somes and six pairs (VII–XII) of short submetacentric chromosomes (Krukliis 1974, Budaragin 1980, Hizume 1988, Muratova 1991, Muratova *et al.* 2007, 2009, Goryachkina *et al.* 2013). Two of the metacentric chromosome pairs (III and IV) carry secondary constrictions in the intercalary region of one of the arms. Chromosome pair VI is distinguishable because it is the smallest and most asymmetric among metacentrics. Chromosomes VII and VIII are close to subacrocentric, which allows for their discrimination. Chromosome pairs IX and X are submetacentrics and characterized by the larger short arm. Chromosomes XI and XII are defined as smallest submetacentrics. Despite morphological differences of submetacentric chromosomes VIII–XII, identification of individual *L. sibirica* chromosomes and their assignment to the pairs of homologs is possible only when the metaphase cell has good chromosome spreading and the minimal number of overlaps.

Chromosome measurements and identification of homologous pairs allow evaluation of most frequent types of genomic aberrations in CLs. Thus, in the CL 12, we deal with a deletion of the long arm of one of the submetacentric chromosomes, whereas the remaining 23 chromosomes correspond to normal chromosome complement of the species (Fig. 2). The CL 4 cells with $2n=25$, are trisomic for probably the submetacentric chromosomes VII (Fig. 2). All metaphase plates of CL 4 available for analysis have the same karyotype. In the metaphase cells of CL 107, we suppose trisomy for several chromosome pairs. These are two long metacentric chromosomes lacking secondary constrictions (I, II) and two long submetacentrics (VII, VIII).

Fig. 3. FISH image on metaphase chromosomes (A) and FISH karyotype (B) of *L. sibirica* CL 5 ($2n=29$). Signal color codes: 45S rDNA (red), 5S rDNA (green). DAPI staining (blue). Minor 45S rDNA signals are shown by arrows. Bar=10 μ m.

Table 2. The number of nucleoli per nucleus in *L. sibirica* CLs.

CLs	Total of cells examined	Average number of nucleoli	The frequency of cells with each number of nucleoli (%)				
			1	2	3	4	5
4	322	2.07 $\pm$ 0.05	32.3	36.3	23.6	7.8	—
5	319	2.91 $\pm$ 0.05	7.8	25.7	37.6	25.4	3.4
6	307	1.97 $\pm$ 0.05	36.2	34.2	26.4	3.3	—
12	366	1.63 $\pm$ 0.04	53.8	30.3	14.7	1.1	—
16.28	314	2.09 $\pm$ 0.05	27.7	43.3	21.0	8.0	—
17.7	307	2.19 $\pm$ 0.05	24.1	38.8	30.6	6.5	—
107	328	2.21 $\pm$ 0.05	24.1	36.9	32.6	6.4	—

In metaphase plates of CL 5 additions of various metacentric as well as subacrocentric chromosomes were observed. Cells with $2n=29$, which occur with the highest frequency show the following karyotype variants: 15M+14SM, 17M+12SM (Fig. 2). In addition to conventional staining, FISH with two rDNA probes for the analysis of CL 5 (Fig. 3A) confirmed that the cells of CL 5 were trisomic for several chromosomes. In normal karyotype of *L. sibirica* four major signals of 45S rDNA, or major nucleolus organizing regions (NORs), are observed on two pairs of large metacentric chromosomes (III, IV); they are located intercalary within the region of secondary constrictions. Two 5S rDNA sites are located in the subterminal region of the short arm of chromosome III, which also carries major NORs in an opposite arm. In the CL 5, we detect three chromosomes carrying signals of both the 45S and 5S rDNA probes, which imply that we are dealing with the trisomy for the chromosome III (Fig. 3B). Minor 45S rDNA loci are observed in the pericentromeric regions of chromosome pairs I, II, VI, and XII, and these minor NORs significantly simplify picking up homologous chromosomes for karyotype construction of *L. sibirica*.

All major 45S rDNA loci detected on *L. sibirica* chromosomes by FISH are functionally active, which is evidenced by silver staining of the interphase nuclei. The number of nucleoli per nucleus in *L. sibirica* varied from 1 to 4, whereas in the CL 5 some nuclei had five nucleoli

(Table 2). As a rule, the maximal number of nucleoli is detected at the stage of mitotic late telophase when the cell septum is not completely built. Senescent nuclei at the interphase stage usually possess just one large nucleolus. Thus, the number of nucleoli in the nucleus can reflect the level of mitotic activity.

Discussion

CLs of *L. sibirica* analyzed in a current study had different cultivation time. Long-term CLs (4, 5, and 6) retained their embryogenic potential during 6–8 years and were characterized by the mass formation of SEs. Such proliferating activity of embryogenic CLs has been already described for megagametophyte culture of *L. decidua* and in the embryogenic culture of hybrid larch *L. $\times$ eurolepis* и *L. $\times$ marschlinsii* (Von Aderkas *et al.* 2003, Lelu-Walter and Pâques 2009) that were capable to form ESM for 9–17 years. Noteworthy, under the same cultivation conditions the process of formation SEs in some *L. sibirica* CLs was more active than in other lines.

Only three out of seven *L. sibirica* CLs analyzed here (CLs 6, 17.7, and 16.28) had somatic chromosome number typical for the species ($2n=24$). The CL 17.7 was *in vitro* cultivated for less than a year; however, the CL 6 remained cytogenetic stability during all six years of cultivation. Genetic stability of this line has earlier been shown using microsatellite analysis: only one of the nine

loci showed non-conformity with the maternal genotype for two alleles (Tretyakova *et al.* 2016).

Interestingly, the CL 6 was characterized by low productivity (2040 ± 189 SE g⁻¹ ESM fresh weight) and by the lowest number of maturing SEs (0.6%). Nevertheless, from this CL we obtained the cloned plantlets of *L. sibirica*, which successfully grow in the greenhouse of Experimental station Pogorelskyi bor of the Sukachev Institute of Forest SB RAS (Park *et al.* 2016). According to microsatellite analysis, these clones are almost identical to the CL 6, from which they were obtained.

The results of analysis of CLs 4, 12 and 107 were of the special interest, as these lines possessed predominantly cells with a constant chromosome number different from normal. As most cells displayed one and the same type of abnormalities one may suggest that it has already been present in the material taken for cultivation, or it emerged *de novo* when the material was introduced to *in vitro* cultivation as cell response to the new environment. These CLs showed rather high productivity ranging from 3000 to 4000 SE g⁻¹ ESM fresh weight, and the CL 4 showed the highest number of maturing embryos (33.0%).

The CL 5 proved to be cytogenetically unstable, which is evidenced by the variation of chromosome number over metaphase cells and by the presence of micronuclei in interphase nuclei. Interestingly, this CL has been actively proliferating and forming SEs for eight years. Cytogenetic analysis revealed that this CL forms a large number of the globular SEs (3750 ± 163 SE g⁻¹ ESM fresh weight), which actively multiply, but remain at the globular stage of development. When SEs are transferred onto the medium containing abscisic acid they do not mature. Cytogenetic instability can be caused by the hybrid origin of the line or by long cultivation time.

Cytogenetic instability of embryogenic CLs of conifer plants has been reported in the literature. Thus, the emergence of polyploid cells during somatic embryogenesis was recorded in megagametophyte culture of *L. decidua*, however, variation of chromosome number in one of the lines did not lead to the loss of maturation ability by embryos (Von Aderkas *et al.* 2003). A high number of aneuploid cells (up to 70%) has been observed in the embryogenic culture of hybrid species *L. × eurolepis* (Nkongolo and Klimaszewska 1995). One of the *Pin. radiata* CLs carried one additional chromosome in the karyotype, which was confirmed by the assessment of DNA content (O'Brien *et al.* 1996). Somaclonal variation of the chromosome number was found in plants of *Pic. glauca*, *Pic. mariana* and *Pic. abies*, obtained using somatic embryogenesis (Fourré *et al.* 1997, Tremblay *et al.* 1999). The authors described both aneuploidy ($2n=38$) and several variants of chromosomal mosaicism ($2n=24$, 27, 36; $2n=30$, 39, 40, 55) in these species. The plant of *Pic. abies*, obtained *via* somatic embryogenesis, was a chimera, which possessed $2n=25$ in buds and normal

$2n=24$ in a root meristem (Fourré *et al.* 1997).

Our results have shown that CLs of *L. sibirica* can retain cytogenetic stability over years. These CLs can be successfully used in plantation forestry, however cytogenetic monitoring of CLs from the collection bank of embryogenic CLs is an essential step for their selection. Our data and the results published in the literature indicate that cytogenetic analysis is the most sensitive method of evaluating genetic stability of long-term proliferating CLs, and also in qualitative assessment of regenerated plants. Besides, cytogenetic studies of embryogenic CLs can contribute to the development of theoretical aspects of genetics, reproductive biology, and biotechnology of conifer plants.

Acknowledgements

Authors express sincerely thanks to Dr. Oreshkova N.V., V. N. Sukachev Institute of Forest SB RAS, for supplying of *L. sibirica* genomic DNA, and Dr. Shcherban A.B., The Federal Research Center Institute of Cytology and Genetics SB RAS for preparation of 5S rDNA probe. The study was funded by Russian Foundation for Basic Research and Government of Krasnoyarsk Territory, Krasnoyarsk Region Science and Technology Support (project N° 16-44-243068), Russian Foundation for Basic Research and Belarus (project 18-54-00010), Investment Foundation FASIE (project 0040522).

References

- Bairu, M. W., Aremu, A. O. and van Staden, J. 2011. Somaclonal variation in plants: causes and detection methods. *Plant Growth Regul.* **63**: 147–173.
- Budaragin, V. A. 1980. Karyotypes of the Main Forest-Forming Species of Kazakhstan. *Trudy of Kazakhstan Scientific and Research Institute of Silviculture. Protective Forestation and Problems of Selection in the North Kazakhstan, Alma-Ata.* pp. 116–122. (*in Russian*)
- Eastman, P., Webster, F. B., Pitel, J. A. and Roberts, D. R. 1991. Evaluation of somaclonal variation during somatic embryogenesis of interior spruce (*Picea glauca engelmannii* complex) using culture morphology and isozyme analysis. *Plant Cell Rep.* **10**: 425–430.
- Fourré, J. L., Berger, P., Niquet, L. and Andre, P. 1997. Somatic embryogenesis and somaclonal variation in Norway spruce: morphogenetic, cytogenetic and molecular approaches. *Theor. Appl. Genet.* **94**: 159–169.
- Gerlach, W. L. and Bedbrook, J. R. 1979. Cloning and characterization of ribosomal RNA genes from wheat and barley. *Nucleic Acids Res.* **7**: 1869–1885.
- Goryachkina, O. V., Badaeva, E. D., Muratova, E. N. and Zelenin, A. V. 2013. Molecular cytogenetic analysis of Siberian *Larix* species by fluorescence *in situ* hybridization. *Plant Syst. Evol.* **299**: 471–479.
- Goryachkina, O. V. and Sizikh, O. A. 2012. Cytogenetical reactions of conifer plants in anthropogenically disturbed areas of Krasnoyarsk and its environs. *Khvoinee Borealnoi Zoni* **30**: 46–51. (*in Russian*)
- Harvengt, L., Trontin, J. F., Reymond, I., Canlet, F. and Pâques, M. 2001. Molecular evidence of true-to-type propagation of a 3-year

- old Norway spruce through somatic embryogenesis. *Planta* **213**: 828–832.
- Helmersson, A., Von Arnold, S., Burg, K. and Bozhkov, P. V. 2004. High stability of nuclear microsatellite loci during the early stages of somatic embryogenesis in Norway spruce. *Tree Physiol.* **24**: 1181–1186.
- Hizume, M. 1988. Karyomorphological studies in the family Pinaceae. Mem. Fac. Educ. Ehime Univ. Ser III. Nat. Sci **8**: 1–108.
- Hizume, M., Sato, S. and Tanaka, A. 1980. A highly reproducible method of nucleolus organizing regions staining in plants. *Stain Technol.* **55**: 87–90.
- Isabel, N., Tremblay, L., Michaud, M., Tremblay, F. M. and Bousquet, J. 1993. RAPDs as an aid to evaluate the genetic integrity of somatic embryogenesis-derived populations of *Picea mariana* (Mill). *BSP. Theor. Appl. Genet.* **86**: 81–87.
- Krukalis, M. V. 1974. Karyological Features of *Larix czekanowskii* Sz. In: Minina, E. G. and Iroshnikov, A. I. (eds.). Variation in Woody Plants in Siberia. Krasnoyarsk. pp. 11–19. (in Russian).
- Larkin, P. J. and Scowcroft, W. R. 1981. Somaclonal variation—a novel source of variability from cell cultures for plant improvement. *Theor. Appl. Genet.* **60**: 197–214.
- Lelu-Walter, M.-A. and Pâques, L. E. 2009. Simplified and improved somatic embryogenesis of hybrid larches (*Larix×eurolepis* and *Larix×marschliensis*). Perspectives for breeding. *Ann. For. Sci.* **66**: 1–10.
- Mo, L. M., von Arnold, S. and Lagererantz, U. 1989. Morphogenic and genetic stability in long term embryogenic cultures and somatic embryos of Norway spruce (*Picea abies* [L.] Karst.). *Plant Cell Rep.* **8**: 375–378.
- Muratova, E. N. 1991. Karyological studies in *Larix sibirica* (Pinaceae) from different parts of its area. *Bot. Z.* **76**: 1586–1595. (in Russian)
- Muratova, E. N. 1995. Nucleolus staining methods for karyotype analysis of conifers. *Bot. Z.* **80**: 82–86. (in Russian)
- Muratova, E. N., Karpyuk, T. V., Vladimirova, O. S., Sizykh, O. A. and Kvitko, O. V. 2009. A cytological study of Siberian larch in anthropogenically disturbed areas of the city of Krasnoyarsk and its vicinity. *Theor. Appl. Ecol.* **9**: 99–108. (in Russian)
- Muratova, E. N., Sedelnikova, T. S., Pimenov, A. V., Karpyuk, T. V., Kvitko, O. V. and Sizykh, O. A. 2010. Karyological Polymorphism of Larch Species. In: Efremov, S. P. and Milyutin, L. I. (eds.). Larch Biodiversity of the Asian Russia. Academic Publ. House GEO, Novosibirsk. pp. 34–49. (in Russian).
- Muratova, E. N., Sedelnikova, T. S., Pimenov, A. V., Karpiuk, T. V., Sizikh, O. A. and Kvitko, O. V. 2007. Karyological analysis of larch species from Siberia and the Far East of Russia. *For. Sci. Tech.* **3**: 89–94.
- Nkongolo, K. K. and Klimasewska, K. 1994. Karyotype analysis and optimization of mitotic index in *Picea mariana* (Black spruce) preparations from seedling root tips and embryogenic cultures. *Heredity* **73**: 11–17.
- Nkongolo, K. K. and Klimasewska, K. 1995. Cytological and molecular relationships between *Larix decidua*, *L. leptolepis* and *Larix×eurolepis*: identification of species-specific chromosomes and synchronization of mitotic cell. *Theor. Appl. Genet.* **90**: 827–834.
- O'Brien, I. E. W., Smith, D. R., Gardner, R. C. and Murray, B. C. 1996. Flow cytometric determination of genome size in *Pinus*. *Plant Sci.* **115**: 91–99.
- Park, M. E., Ivanitskaya, A. S., Dvoynina, L. M. and Tretyakova, I. N. 2016. The embryogenic potential of long-term proliferation cell lines of *Larix sibirica* in vitro. *Siberian J. For. Sci.* **1**: 27–38. (in Russian)
- Roth, R., Ebert, I. and Schmidt, J. 1997. Trisomy associated with loss of maturation capacity in a long-term embryogenic culture of *Abies alba*. *Theor. Appl. Genet.* **95**: 353–358.
- Salajova, T. and Salaj, J. 1992. Somatic embryogenesis in European black pine (*Pinus nigra* Arn.). *Biol. Plant.* **34**: 213–218.
- Sarmast, M. K. 2016. Genetic transformation and somaclonal variation in conifers. *Plant Biotech. Rep.* **10**: 309–325.
- Stasolla, C. and Yeung, E. C. 2003. Recent advances in conifer somatic embryogenesis: improving somatic embryo quality. *Plant Cell Tissue Organ Cult.* **74**: 15–35.
- Tremblay, L., Levasseur, C. and Tremblay, F. M. 1999. Frequency of somaclonal variation in plants of Black spruce (*Picea mariana*, Pinaceae) and White spruce (*P. glauca*, Pinaceae) derived from somatic embryogenesis and identification of some factors involved in genetic instability. *Am. J. Bot.* **86**: 1373–1381.
- Tretyakova, I. N. and Barsukova, A. V. 2012. Somatic embryogenesis in in vitro culture of three larch species. *Russ. J. Dev. Biol.* **43**: 353–361.
- Tretyakova, I. N., Park, M. T., Ivanitskaya, A. S. and Oreshkova, N. V. 2016. Peculiarities of somatic embryogenesis of long-term proliferating embryogenic cell lines of *Larix sibirica* in vitro. *Russ. J. Plant Physiol.* **63**: 800–810.
- Trontin, J. F., Grandemange, C. and Favre, J. M. 1999. Two highly divergent 5S rDNA unit size classes occur in composite tandem array in European larch (*Larix decidua* Mill.) and Japanese larch (*Larix kaempferi* (Lamb.) Carr.). *Genome* **42**: 837–848.
- Von Aderkas, P., Pattanavibool, R., Hristoforoglu, K. and Ma, Y. 2003. Embryogenesis and genetic stability in long term megagametophyte-derived cultures of larch. *Plant Cell Tissue Organ Cult.* **74**: 27–34.