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RESEARCH PAPERS

Fatty Acid Composition of Total Lipids in Embryogenic and Nonembryogenic Callus Lines of Larch

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Abstract—Fatty acid (FA) composition of total lipids in embryogenic and nonembryogenic calli of Siberian larch (*Larix sibirica* Ledeb.) was investigated by the method of GC-MS. We detected a high content of oleic acid in total lipids of embryogenic cell culture (32–56% by weight of total FA), which apparently depends on a high activity of acyl carrier protein (stearoyl-ACP-Δ9-desaturase). At the same time, activity of Δ12-desaturase in the cells of embryogenic calli was considerably (2–3 times) lower than in nonembryogenic calli. We discuss a possibility that concentration of FA (oleic and linoleic acids) may be used as a marker of embryogenic potential when promising cell lines of Siberian larch are screened in the stage of early embryogenesis.

Keywords: Larix sibirica, calli, embryogenesis, lipids, fatty acids, desaturases

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INTRODUCTION

Larch forests in East Siberia, as some in other geographic zones, are a source of genetic diversity of such an important representative of the genus *Larix* as *Larix sibirica* [1]. However, because of rapid industrial development of Siberia, the areas occupied with by woods are swiftly reduced, which irretrievably deteriorates the gene pool of valuable forest species, including larch [1, 2]. Such a situation negatively affects wood ecosystems and results in their loss. It is obvious that an important role in preservation of larch genetic diversity belongs to approaches based on the methods of biotechnology and cell biology aiming at production of cell culture and investigation of feasibility of vegetative propagation of this valuable tree species.

Clonal propagation by means of somatic embryogenesis and organogenesis may be considered the most promising approach to preservation of rare genotypes with unique characteristics [3, 4]. However, production of somatic embryos is a difficult biotechnological problem for the majority of conifers, including those growing in Russia [4–6]. These difficulties depend on a lack of data about conditions and mechanisms of induction of somatic embryogenesis in gymnosperms. Genetically determined embryogenic potential is realized through cell competence [5, 7]. Such a competence may be characterized by biochemical composition of plant cells, including variation of qualitative and quantitative composition of cell lipids and their component fatty acids (FA) associated with changes in external (environmental) and internal (developmental) factors. This is especially characteristic of woody plants that live long, are attached to ground, and are incapable of avoiding adverse environmental factors.

The results of investigation of FA composition and the content of total lipids in certain stages of somatic embryogenesis are presented in few reports [8–11]. In our previous work [11], we compared FA composition of total lipids in the calli of two species of larch: *L. sibirica* and *L. gmelinii*; we found a high degree of FA unsaturation and the presence of Δ5 acids characteristic of conifers. In several reports [8, 9], it was shown that the content of total lipids rose and the content of FA changed in the course of maturation of somatic embryos of common spruce, which points to an important role of lipids (including FA) in embryonic development. The researchers came to a conclusion that the content and composition of callus total lipids necessary for embryonic development may be of use in estimation of the competence of somatic embryos for progression through the following stages

Abbreviations: ACR—acyl carrier protein; EM—embryonal mass; FAME—fatty acid methyl esters; PUFA—polyunsaturated fatty acids; UI—unsaturation index

of development. In this relation, we made an assumption that absolute content and FA composition of total lipids of Siberian larch calli and their level in embryogenic and nonembryogenic calli may provide important information about lipid metabolism in the tissues of these types.

The aim of this work was to compare the content and fatty acid composition of total lipids in embryogenic and nonembryogenic calli of Siberian larch.

MATERIALS AND METHODS

Experimental material. Somatic embryogenesis was induced in isolated zygotic embryos in the stage of cotyledon initiation taken from the seeds picked from 34 40–60-year-old trees of Siberian larch (*Larix sibirica* Ledeb.) growing in the territory of the arboretum of the Institute of Forestry, Siberian Branch, Russian Academy of Sciences (Krasnoyarsk) and in Khakassia. Geographic coordinates of the place of collection were 54°38′58″ North and 89°26′28″ East; the seeds were gathered in July 2007–2011.

The seeds were freed from cover scales and surface-sterilized with 5% alcoholic iodine for 3 min. After triple washing in sterile distilled water, megagametophytes were treated with 3% hydrogen peroxide for 5–10 min. Embryos were removed from megagametophytes under aseptic conditions, placed on wet filter paper in Petri dishes and then transferred to AI agar nutrient medium [12]. As growth regulators, we used 2.4-D (2 mg/L) and BAP (1 mg/L). In order to produce embryonal mass (EM), cytokinin concentration in base medium was reduced to 0.5 mg/L. EM was incubated in darkness at 24 ± 1°C with subculture interval of 28 d. Cell lines of larch obtained as a result of induction differed by proliferative activity, by the number of immature somatic embryos within embryonal mass, and by the opportunity to produce regenerant plants [4, 12].

FA lipid composition was determined in embryogenic (Cl2 and Cl6 producing mature embryos and regenerant plants); (Cl4, Cl5, and Cl10 producing immature embryos) and nonembryogenic (Cl23 and ClL) cell lines. In order to extract lipids, a sample of plant material (0.5 g) was fixed in liquid nitrogen; then, we added 0.001% ionol and ground to obtain a homogenous mass [13]. We next added 10 mL of chloroform : methanol mixture (1 : 2, v/v), mixed, and left for 30 min up to complete lipid diffusion into the solvent. The solution was quantitatively transferred through a filter to a separatory funnel; the mortar and filter were three times washed with the same solvent mixture. To improve separation, some water was added.

Total lipids were determined in lower chloroform fraction. Chloroform (special grade, stabilized with 0.005% amylene) was removed from lipid extract in a vacuum using an RVO-64 rotary evaporator (Czech Republic). In order to check lipid extraction (%), we added a known amount of nonadecanoic acid (C19:0) in the course of homogenization. FA methyl esters (FAME) were produced according to [14]. FAME were additionally purified by means of TLC on glass plates covered with KSK silica gel (Russia) with benzene as mobile phase (R_f = 0.71–0.73). In order to visualize FAME zone, the plates were sprayed with 10% H_2SO_4 in MeOH and heated in a drying oven at 100°C. FAME zone was removed from the plate with a spatula and eluated from silica gel with (*n*)-hexane. FAME were analyzed by means of GC-MS using a 5973/6890N MSD/DS chromato-mass spectrometer (Agilent Technologies, United States); detector—quadrupole mass spectrometer; ionization—electron impact; ionization energy—70 eV; analysis was made with recording of total ion current.

The mixture of FAME was separated on an HP-INNOWAX (30 m × 250 μm × 0.50 μm) capillary column with PEG as a stationary phase. Carrier gas was helium, and gas flow rate was 1 mL/min. Temperature of evaporator was 250°C, that of ion source was 230°C, and that of detector was 150°C; temperature of the line connecting chromatograph and mass spectrometer was 280°C. Range of scanning was 41–450 u. Volume of applied sample was 1 μL, and flow divider was 5 : 1. FAME were separated isothermally at 200°C. In order to identify FA, we used NIST 08 mass spectrum library and Christie's archive of FAME mass spectra [15]. Relative content of FA was determined by means of internal normalization in percent by weight (weight %) of their total content in the sample under investigation with due account of FA response coefficient. Absolute content of total lipids and FAME was determined by weighing on a GR-120 electron scale (A&N, Japan); the sample was dried to attain a constant weight.

Absolute content of oleic acid ($P_{18:1\Delta9}$) was calculated from the total mass of FAME (mg/g dry wt) in the sample and a percentage of this acid according to the formula (1):

$$P_{18:1\Delta9} = P_{FAME}C_{18:1\Delta9\%\ relative}/100. \quad (1)$$

Activity of Δ9- and Δ12-desaturases was assessed using stearoyl-desaturase (SDR) and oleoyl-desaturase (ODR) ratios calculated according to the modified formulas (2) and (3) [16], taking into consideration the fact that oleic acid is a precursor in biosynthesis of taxoleic (C18:Δ5,9) and pinolenic (C18:3Δ5,9,12) acids.

$$SDR = \%18{:}1\Delta9/(\%18{:}0 + \%18{:}1\Delta9 + \%18{:}2\omega6 + \%18{:}2\Delta5,9), \quad (2)$$

$$ODR = (\%18{:}2\omega6 + \%18{:}2\Delta5,9 + \%18{:}3\omega3 + \%18{:}3\Delta5,9,12)/(\%18{:}1\Delta9 + \%18{:}2\omega6 + \%18{:}2\Delta5,9 + \%18{:}3\omega3 + \%18{:}3\Delta5,9,12). \quad (3)$$

Table 1. Content of total lipids in embryogenic (e) and nonembryogenic (ne) calli of Siberian larch

Cell lines	Content of total lipids, mg/g dry wt
Cl2 (e)	51.2 ± 2.5
Cl6 (e)	69.9 ± 6.3
Cl4 (e)	45.2 ± 1.1
Cl5 (e)	78.0 ± 3.2
Cl10 (e)	74.6 ± 5.8
ClL (ne)	40.1 ± 1.4
Cl23 (ne)	35.8 ± 2.6

The table shows the means of six replications and their standard deviations. Significance of differences was assessed by means of *t*-test ($P < 0.05$).

In order to estimate unsaturation index of membrane lipid FA, we used unsaturation index (UI) [17] calculated according to the formula:

$$UI = \sum Pjnj/100, \quad (4)$$

where P_j is content of FA (weight %) and n_j is number of double bonds in each acid.

Statistical treatment. The tables show the means of 3–6 replications and their standard deviations. Experimental data were statistically treated using a Microsoft Office Excel 2010 package of statistical analysis and Statistica V-10.0 software (StatSoft Inc., United States). We used the methods of cluster analysis (hierarchical clustering based on the methods of furthest and nearest neighbor, Euclidean distances, and probability approach on the basis of *k*-means algorithm) and correlation analysis. Reliability of correlation coefficient was estimated by means of the *t*-test.

RESULTS AND DISCUSSION

The content of total lipids in embryogenic and nonembryogenic calli of Siberian larch is given in Table 1. The table shows that the weight of lipid fraction in individual cell lines considerably differed (from 35.8 ± 2.6 mg/g dry wt for Cl23 to 78.0 ± 3.2 mg/g dry wt for Cl5), with absolute content of total lipids in embryogenic calli being reliably higher than in nonembryogenic. The obtained results well agree with the data reported in [8, 9]. Grigova et al. [9] showed that the content of total lipids in proliferating embryonal-suspensor mass of *Picea abies* amounted to 24% of dry weight. This parameter for embryonal mass produced from zygotic embryos of *Acca sellowiana* was 20–21% of dry weight [8]. Apparently, such a high absolute content of lipids is characteristic of callus cultures of woody plants.

FA composition of in vitro calli of Siberian larch is shown in Table 2. In lipids of embryogenic and nonembryogenic calli, the main saturated acids were palmitic (16 : 0), stearic (18 : 0), arachic (20 : 0), and behenic (22 : 0). Minor saturated FA with the length of carbon chain of C14–C17 and their isomers accounted for less than 1% of total FA. The main unsaturated FA comprised oleic (C18:1Δ9), linoleic (C18:2Δ9,12), and linolenic (C18:Δ9,12,15) acids. In addition, we detected uncommon FA of Δ5 series: taxoleic, pinolenic, and sciadonic (C20:3Δ5,11,14) acids characteristic of conifers and some other taxons [18].

We revealed significant differences between embryogenic and nonembryogenic lines in respect to FA composition of total lipids. For instance, relative content of saturated FA was the lowest in lines Cl2 and Cl6 (18.7 and 18.2%, respectively). In other cell lines, this parameter was much greater (up to 33.4% in ClL). The most pronounced differences were detected in relative and absolute content of monounsaturated acids, and first of all of oleic acid. As to unsaturated FA in nonembryogenic calli, relative content of oleic acid was 12.0–14.8%. Lipids of embryogenic calli Cl2 and Cl6 with the content of immature embryos of up to 210 pieces/100 mg EM showed relative content of oleic acid at the level of 55.2 and 56.5%, respectively, whereas that in calli Cl4 and Cl5 contained more immature somatic embryos (370 pieces/100 mg EM) and the level of oleic acid was 32.1–36.5% in cell line Cl10 (data about the number of embryos in calli are given in [4, 11]). The greatest absolute content of oleic acid was also observed in callus lipids of embryogenic lines Cl2 and Cl6 (Fig. 1): 22.7 and 30.9 mg/g dry wt, respectively. Much lower (4–8 times) content of oleic acid was detected in lipids of nonembryogenic lines ClL and Cl23. In this respect, embryogenic lines producing more immature embryos (Cl4, Cl5, and Cl10) were intermediate (12.6, 20.6, and 18.5 mg/g dry wt, respectively). Such a high content of oleic acid in embryogenic calli as compared with nonembryogenic was associated with a high activity of Δ9-desaturase. Its values calculated for all the investigated lines are shown in Fig. 2a. The greatest activity of Δ9-desaturase was observed in lines Cl2 and Cl6 that were potentially capable of producing sound regenerant plants.

Among polyunsaturated FA (PUFA) of lipids from nonembryogenic larch calli, we observed a high relative content of acid C18:2Δ9,12 (40.1% for line Cl23 and 34.2% for line ClL), whereas its level was four times lower among lipids of embryogenic calli (Cl2 and Cl6) amounting to 10.5–10.8% of total FA. For cell lines Cl4, Cl5, and Cl10, the content of linoleic acid was approximately 1.5 times lower than in nonembryogenic lines and amounted to 16.3, 23.1, and 25.1%, respectively. Lower content of acid C18:2Δ9,12 in embryogenic lines apparently depended on a difference between two groups of calli in activity of acyl-lipid Δ12-desaturase, whose calculated value was much higher for the tissues of nonembryogenic lines (Fig. 2b), which is corroborated by correlation analysis ($r = -0.915$; estimated parameters: absolute content of oleic acid and oleoyl-desatu-

Table 2. FA composition (% of total FA) of total lipids in calli of embryogenic and nonembryogenic cell lines of Siberian larch

FA	Calli						
	embryogenic lines					nonembryogenic lines	
	Cl2	Cl6	Cl4	Cl5	Cl10	ClL	Cl23
C16:0	9.9 ± 1.0	9.1 ± 0.7	13.1 ± 0.5	16.0 ± 0.7	18.1 ± 0.6	21.7 ± 0.6	20.9 ± 1.3
iso-C17:0	1.8 ± 0.2	1.2 ± 0.1	3.2 ± 0.1	2.6 ± 0.2	1.7 ± 0.1	4.8 ± 0.3	3.1 ± 0.8
C18:0	2.2 ± 0.2	2.0 ± 0.3	5.0 ± 0.2	2.2 ± 0.2	3.8 ± 0.1	2.6 ± 0.2	3.2 ± 0.3
C18:1Δ9	55.2 ± 1.8	56.5 ± 0.9	36.5 ± 0.5	32.1 ± 0.7	32.5 ± 0.9	14.8 ± 0.3	12.0 ± 1.5
C18:2Δ5,9	6.6 ± 0.1	7.1 ± 0.2	7.2 ± 0.2	3.8 ± 0.2	4.6 ± 0.2	1.6 ± 0.2	1.7 ± 0.2
C18:2Δ9,12	10.8 ± 1.5	10.5 ± 1.3	16.3 ± 0.5	23.1 ± 0.9	25.1 ± 1.0	34.2 ± 0.8	40.1 ± 3.2
C18:3Δ5,9,12	0.4 ± 0.1	0.4 ± 0.2	2.9 ± 0.1	2.1 ± 0.2	2.7 ± 0.3	6.1 ± 0.1	6.7 ± 1.0
C18:3Δ9,12,15	4.7 ± 0.3	2.7 ± 0.3	3.4 ± 0.1	4.9 ± 0.3	4.5 ± 0.1	3.9 ± 0.1	6.2 ± 0.5
C20:0	1.6 ± 0.1	1.1 ± 0.1	2.5 ± 0.3	0.9 ± 0.1	0.6 ± 0.1	1.0 ± 0.1	0.6 ± 0.1
C20:1Δ11	1.1 ± 0.1	0.5 ± 0.1	0.9 ± 0.1	2.5 ± 0.1	n/d	0.3 ± 0.1	n/d
C20:2Δ9,12	1.1 ± 0.2	1.2 ± 0.2	0.8 ± 0.1	3.6 ± 0.2	0.9 ± 0.1	1.0 ± 0.1	0.4 ± 0.1
C20:3Δ5,11,14	0.2 ± 0.1	1.7 ± 0.2	3.0 ± 0.1	2.4 ± 0.1	1.6 ± 0.2	2.2 ± 0.2	1.6 ± 0.2
C22:0	1.6 ± 0.1	2.6 ± 0.1	3.7 ± 0.6	2.0 ± 0.1	2.2 ± 0.2	1.4 ± 0.2	1.4 ± 0.2
FA < 1%	2.8 ± 0.2	3.4 ± 0.2	1.5 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	4.4 ± 0.3	2.1 ± 0.2
Σ_{SFA}	18.7 ± 1.1	18.2 ± 0.9	28.7 ± 0.6	24.9 ± 0.6	27.9 ± 0.9	33.4 ± 0.8	30.3 ± 1.4
Σ_{MUFA}	57.5 ± 2.6	57.7 ± 1.1	37.7 ± 0.8	35.2 ± 0.8	32.7 ± 1.1	16.4 ± 0.9	13.0 ± 1.7
Σ_{PUFA}	23.8 ± 1.9	24.1 ± 0.6	33.6 ± 0.4	39.9 ± 0.54	39.4 ± 0.9	50.2 ± 0.9	56.7 ± 3.4
UI	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	1.3 ± 0.1	1.4 ± 0.1
Content of FAME, mg/g dry wt	41.1 ± 0.5	54.7 ± 5.1	34.4 ± 3.3	63.6 ± 1.4	56.9 ± 3.9	32.1 ± 1.7	27.4 ± 3.1

n/d—not detected; FA < 1%—total quantity of FA accounting for less than 1% of total FAME; Σ_{SFA}—total saturated FA; Σ_{MUFA}—total monounsaturated FA; Σ_{PUFA}—total PUFA; UI—unsaturation index calculated according to the formula (4). The table shows the means of six replicates and their standard deviations.

rase ratio). As to the acids of Δ5-series, lipids of embryogenic calli (Cl2 and Cl6), apart from a high content of oleic acid (up to 55–56%), also contained rather much monounsaturated taxoleic acid (up to 6.6–7.1%), whereas its level in nonembryogenic calli was only 1.6–1.7%. The content of pinolenic acid in callus lipids of lines Cl2 and Cl6 was 0.4% of total FA, whereas it was much greater in the lipids of nonem-

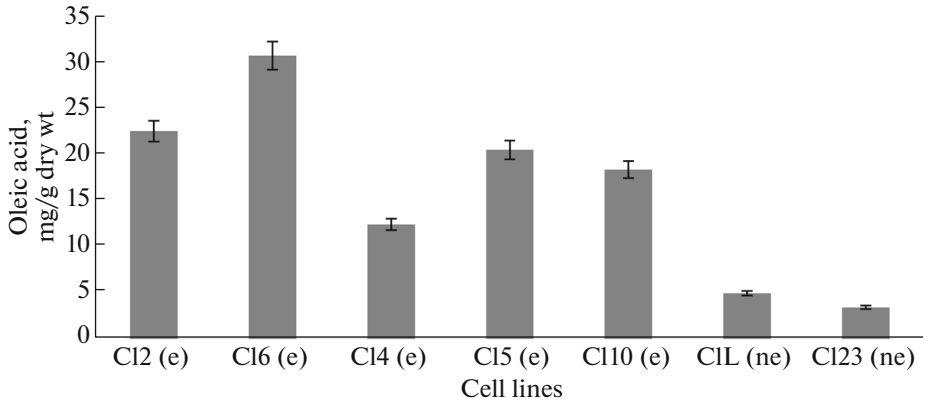

Fig. 1. Absolute content of oleic acid in total lipids of embryogenic (e) and nonembryogenic (ne) calli of Siberian larch.

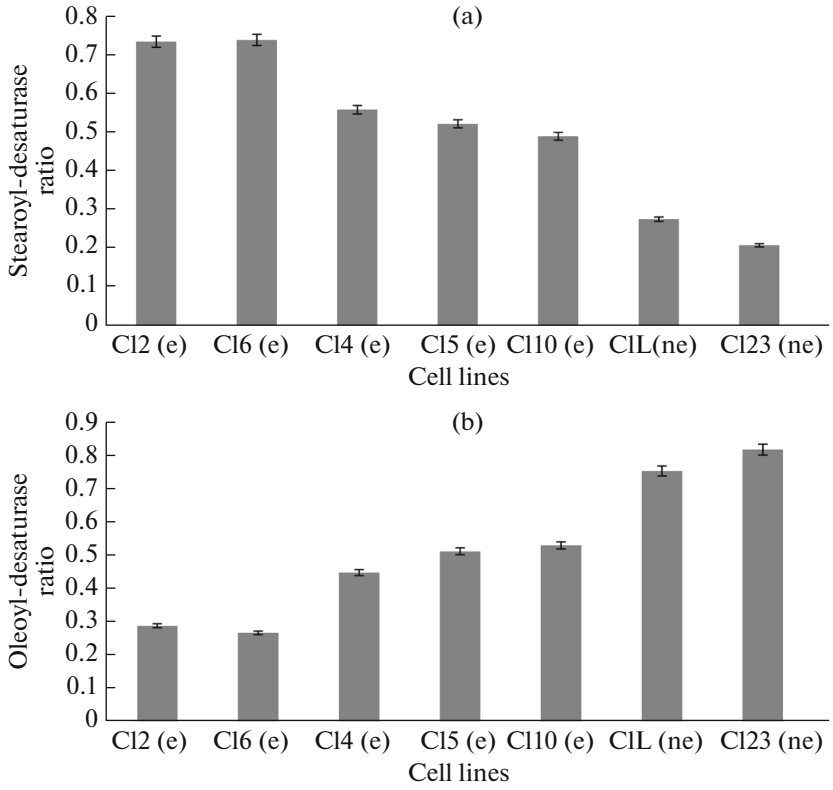

Fig. 2. Calculated activity of (a) Δ9-desaturase and (b) Δ12-desaturase in callus tissue of cell lines of Siberian larch. (e) embryogenic and (ne) nonembryogenic lines.

bryogenic calli and reached 6.1–6.7%. Relative content of sciadonic acid in callus lipids ranged from 0.4 to 3.6%. In respect to this parameter, there were no differences between the lines related to callus embryogeny. Unsaturation index (UI) did not reliably differ in the lines with different embryogenic potential. In all probability, differences stipulated by a high content of monounsaturated acids in the lipids of embryogenic calli were negated due to a high level of PUFA in the lipids of nonembryogenic calli, primarily of linoleic acid (C18:2Δ9,12).

In order to corroborate detected differences in FA composition in investigated callus lines of larch, we made cluster analysis of the data shown in Table 2 (Fig. 3). It was found that, by the tested parameters, nonembryogenic callus lines ClL and Cl23 are identical and form a homogenous cluster. Analysis of lipid FA composition has shown that embryogenic callus lines formed a separate cluster that comprised two subclusters: the first comprised lines Cl2 and Cl6 and the second comprised lines Cl4, Cl5, and Cl10. As was noted above, the lines included in these subclusters differed by productivity of immature somatic embryos (in the lines of subcluster 2, it was higher) and by the ability to produce regenerant plants (it was only observed in the calli of subcluster 1). A test for steadiness of clusterization based on comparison of the results obtained by means of application of so dissimilar algorithms as the methods of nearest neighbor and furthest neighbor has shown that classification is adequate. Analysis of clusters formed by means of calculation of *k*-means has shown that differences between clusters predominantly depend on the following parameters: content of C18:3Δ5,9,12 at significance level $P < 0.01$ and content of C18:1Δ9, C18:2Δ5,9 as well as Σ_{MUFA} and Σ_{PUFA} significance level $P < 0.05$ (Fig. 3).

It is known that stearoyl-CoA-desaturase (Δ9-desaturase) plays a key role in lipid metabolism [19]. It induces formation of the first *cis*-double bond between 9 and 10 carbon atoms in palmitic and stearic acids that are transformed into palmitoleic (C16:1Δ9) and oleic (C18:1Δ9) acids, respectively. These FA are the most widespread monounsaturated FA of phospholipids and triglycerides. These FA are not only lipid components; they act as intermediates in signal transduction, including processes of cell differentiation [20]. In this relation, the higher content of oleic acid we detected in the lipids of calli with emerging embryogenic zones seems quite predictable and probably plays an important metabolic and/or regulatory role in somatic embryogenesis in the course of production of embryo-like structures (embryoids) in cell and tissue cultures. One should

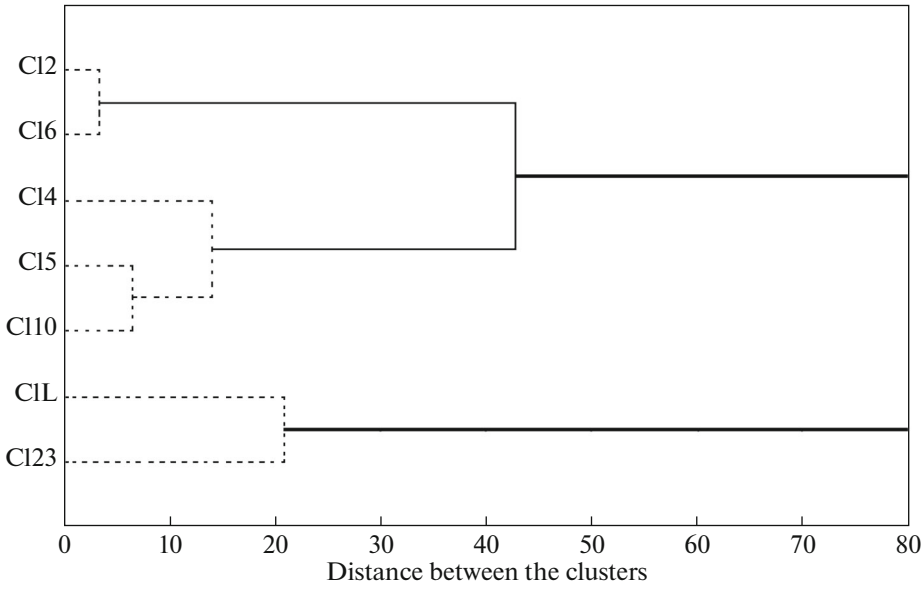

Fig. 3. Dendrogram of cluster analysis of FA composition of total lipids in embryogenic lines producing regenerant plants (Cl2 and Cl6), embryogenic lines not producing regenerant plants (Cl4, Cl5, and Cl10), and nonembryogenic (ClL and Cl6) cell lines in callus tissue of Siberian larch.

specially note that the greatest content of oleic acid (above 50%) we observed was characteristic of embryogenic lines (Cl2 and Cl6) that, as was shown earlier [3, 4, 12], produce mature somatic embryos and regenerant plants.

Use of relative content of oleic acid for diagnostics of the state of cultured tissue is shown in [9], where the ratio between oleic and linoleic acids served as a diagnostic marker measuring maturity of somatic embryos in the calli of common spruce. In our experiments with embryogenic calli of larch, this ratio was especially high in lines Cl2 and Cl6 where it came to 5.1–5.4. For lipids of embryogenic lines Cl4, Cl5, and Cl10, this ratio was 1.3–1.4 and 0.2 for lipids of nonembryogenic calli (lines Cl23 and ClL). Such a low ratio between the shares of oleic and linoleic acids is also observed in the membrane lipids of plant chloroplasts [9].

Investigation of FA composition in lipids from embryogenic and nonembryogenic cell lines of callus culture of Siberian larch has shown that this parameter within these two groups was identical. At the same time, the groups of callus lines with different embryogenic potential reliably differed in FA composition, especially in the content of oleic and linoleic acids. For instance, nonembryogenic lines contained 12–15 and 34–40% of oleic and linoleic acids, respectively; embryogenic lines not producing regenerant plants accumulated 32–36 and 16–25%; embryogenic lines producing regenerant plants contained 55–56 and 10–11%, respectively. Changes in relative content of unsaturated FA and, first of all, of monounsaturated acids in callus lipids in the course of somatic embryogenesis of Siberian larch culture are apparently associated with changes in enzyme activity of stearoyl-ACP-Δ9- and acyl-lipid-Δ12-desaturases. Further investigation of the mechanisms of changes in lipid FA composition in the course of morphogenesis in the culture of Siberian larch will contribute to elucidation of the biological role of changes in lipid metabolism in different stages of formation of somatic embryos. We believe that a high (55–56% of total FA) content of oleic acid in callus lipids may serve as a marker of embryogenic potential in selection of promising cell lines of Siberian larch in the stage of early embryogenesis. The results of these investigations may be subsequently used for working out a technique of clonal propagation of conifers.

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