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Induction of Androgenic Cultures of Siberian Larch (*Larix sibirica* Ledeb.)

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Abstract

The male buds of Siberian larch do not have an organic dormancy in the fall-winter period and, under favorable conditions, they are able to complete the development of the male generative structures. Cultivation of microsporophylls on MS medium containing 0.2-0.5 mg/l of hormone 2,4-D, during a month, resulted in the induction of androgenesis in vitro of Siberian larch. The increase of hormone concentration caused the development of necrotic processes. Data on androgenesis in vitro of Siberian larch, from representative gymnosperms, was obtained for the first time. Two development type embryos were obtained by cultivating microsporophylls of Siberian larch on MS medium.

Key Words: androgenesis, culture in vitro, embryoids, microsporocytes, Siberian larch

Introduction

At present, androgenesis *in vitro* occupies an important place in plant biology. It has been induced in many angiospermous plants. With the help of androgenesis *in vitro,* the selection process is accelerated and new types and lines of grasses can be created. The existence of conifer androgenesis *in vitro* was not known before. However, experiments on microstrobile culture were carried out using *Ginkgo bilobo* (Tulecke, 1953), *Picea abies*, *Pinus resinosa* (Bonga, Fowler, 1970; Bonga, Molnnis, 1975), *Pinus sibirica* (Skripachenko, 1982), and *Ephedra foliata* (Konar, 1963).

Trees of the genus *Larix* are ideal subjects for obtaining androgenous cultures of conifers as all the needed conditions for androgenesis initiation used for grass cultures *in vitro* are appropriate for them as well. These conditions include: effects of cold treatment, stages of explant development, and nutrition media with needed hormones.

The aim of the work was to initiate the *Larix sibirica* androgenesis *in vitro* and to make a cytological analysis of the process.

The main Tasks:

- to obtain androgenic cultures of *Larix sibirica*;
- to make a comparative cytological analysis of the processes occurring in androgenic cultures *in vitro* and in microsporocytes of *Larix sibirica in vivo*;
- to choose conditions for growing *Larix sibirica* pollen *in vitro*;

Material and methods

The subjects of this study were 40-year-old Siberian larch clone trees growing at the experimental base "Pogorelsky bor" and Arboretum of the Institute of Forest (Krasnoyarsk). The male buds from these trees were collected from October to May. Male buds were sterilized using 3% peroxide with a drop of Tween 20 (3 min.). After sterilization, microstrobiles were washed three times in sterile distilled water (15 min in each).

To study microspore development, callus tissue and embryonic microsporophylls were cultured on MS medium (Murashige, Skoog, 1962) + 30g/l of sucrose and 6 g/l of agar, without hormones and with the 2,4-D in concentration 0.2 - 10 mg/l. Explants were grown under light (16-h photoperiod) and at 24°C.

The explants were fixed with the Karnua solution (96% alcohol and acetic acid, 3:1) for 1-60 days of cultivation. For cytological analysis, preparations were stained with hematoxylin using the Heidenhein's procedure (Prozina, 1960). Before staining, the explants were treated with 1 N HCl at 60°C for 5 min. The preparations were examined under a KS-300 imaging system microscope (Germany). Morphological changes were fixed with a Nikon digital camera (Japan).

Results

Culture of microsporophylls; Autumn dates of cultivation

Microstrobiles collected in October-November consisted of microsporocytes at prophase I of meiosis (leptonema-pahynema) (Fig.1).

In 7 days of cultivation on MS media, with 0.2-0.5 mg/l 2,4-D, microsporocytes finished their meiosis (Fig. 2), which was accompanied by the decomposition of tetrads. One-nuclear microspores were formed (Fig. 3), and they reached the mature pollen grain size of 68,5 μm. At this period in the one-nuclear microsporocytes, cytoplasmic evagination proceeded. This process can be compared with the initiation of mature pollen germination. After 16 days of cultivation, the formation of coenocytes and 2-3-nuclear structures were observed. After 21 days of the inoculation of microsporophylls in culture *in vitro,* coenocytes and callus tissue developed. After 60 days of cultivation, the globules formation occurred in the callus (Fig. 4).

Microsporophylls of the same collection which were

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inoculated on MS medium with 2,4-D concentrations 1-2 mg/l, increased in size. After 14 days of cultivation, the meiosis finished and one-nuclear microspores were obtained. Cytoplasmic evagination occurred in these microspores and multi-cellular structures were formed. After 30 days of cultivation, the organogenic callus formed.

Cultivation of microsporophylls on MS medium with 5-10 mg/l 2,4-D resulted in formation of necrosis after 7 days and death after 14 days.

Cultivation of microsporophylls on MS medium without hormones has shown that meiosis finished in 7 days and microspores formed after 14 days. The microspores increased their size up to the size of pollen grain, but mitotic divisions were not observed.

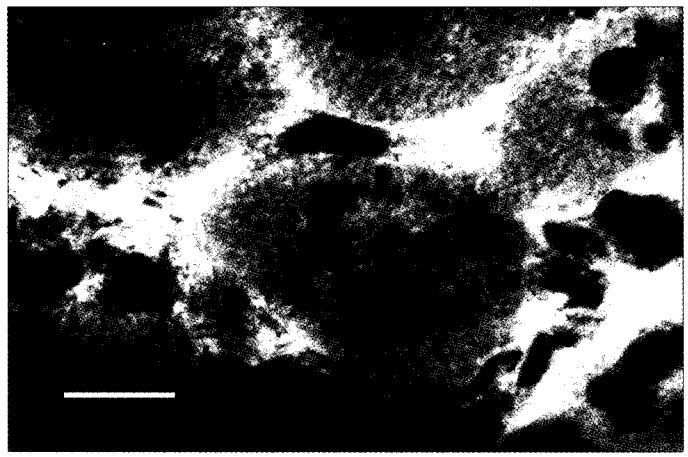

Fig. 1. Microsporocytes of Siberian larch in prophase I of meiosis. Scale is 10 μm.

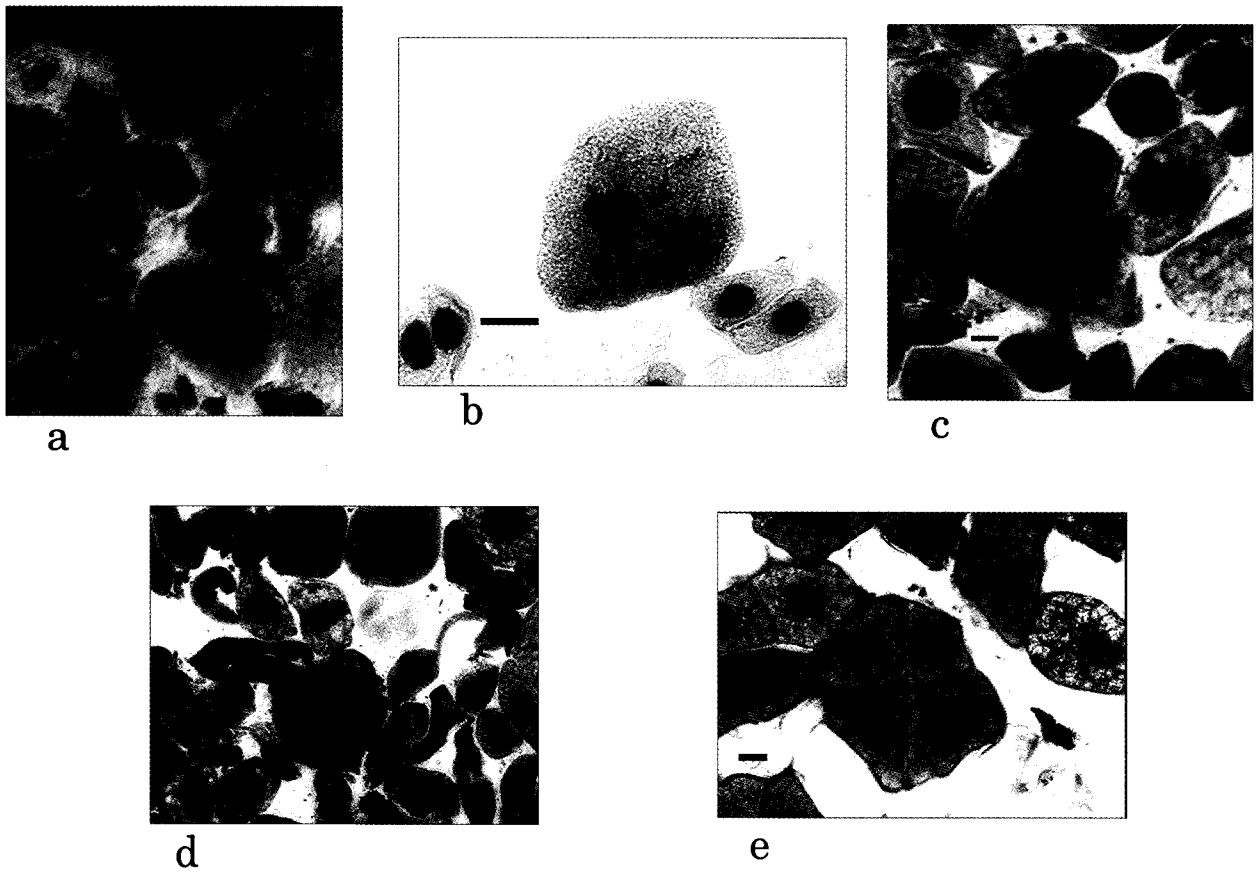

Fig. 2. Meiosis of Siberian larch in culture *in vitro* (September – November): a- prophase; b- metaphase II; c- the formation of diad; d- the formation of tetrad; e- tetrad. Scale is 10 μm.

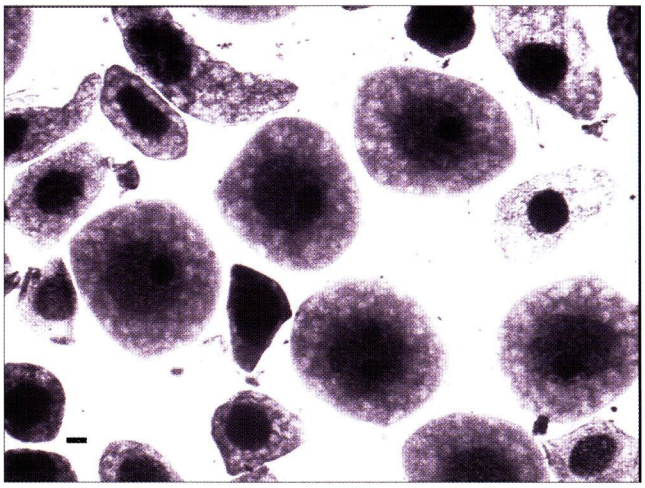

Fig. 3. The formation of microspores of Siberian larch in culture *in vitro*. Scale is 10 μm.

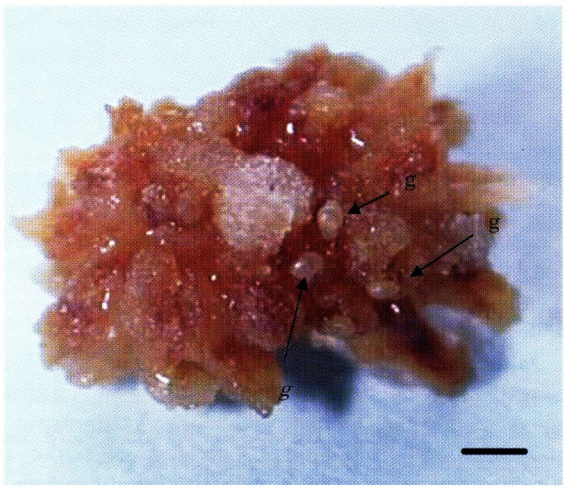

Fig. 4. Siberian larch organogenic callus with globular embryoids (g-globules). Scale is 2 mm.

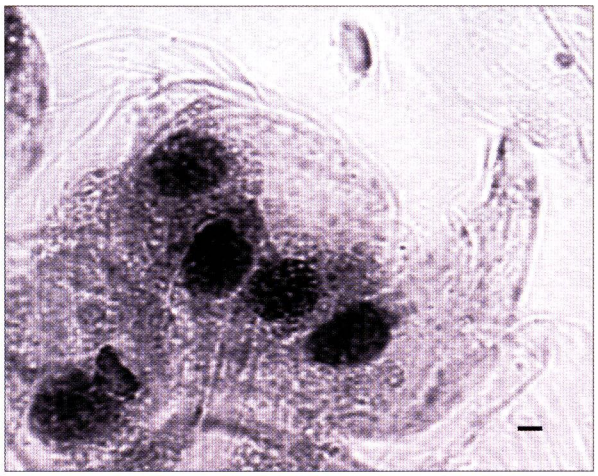

Fig. 5. The formation of multinuclear structures of Siberian larch *in vitro*. Scale is 10 μm.

Culture of microsporophylls; Winter dates of cultivation

In December-February on MS medium with 0.2-0.5 mg/l 2,4-D the meiosis ran for 7 days, as it did in autumn. After 14 days, formation and growth of microspores occurred. Cytoplasmic evagination was observed after 16 days of cultivation. Cultivation after 30-60 days resulted in the formation of organogenic callus, as it did in autumn.

On the medium with 1-2 mg/l 2,4-D, the microsporocytes finished their meiosis in 7 days. After 14 days, the formation of two-cell coenocytes occurred. Further cultivation resulted in multinuclear structures (Fig. 5). It looked like a yellowish, mellow callus of non-organogenic type.

The formation of necrotic zones was observed on the medium with 5-10 mg/l 2,4-D after 7 days, and after 14 days of cultivation, the microsporophylls died.

The microsporophylls cultivated on the media without hormones consisted of the microsporocytes. Reduction divisions were not observed and the formation of callus tissue did not occur. Further cultivation over the next 1-2 months resulted in the death of explants.

Spring dates of cultivation; Cultivation of microsporophylls in the reduction division period

In nature Larch reduction division begins in the end of March - beginning of April. Microstrobiles collected at this time were at different stages of development and there was a large asynchrony in the reduction division. When the microsporophylls were inoculated on MS medium with 0.2-0.5 mg/l 2,4-D, the decomposition of tetrads occurred on the second day of cultivation. After 9 days, the microspore increased in size up to 63.8 μm, and cytoplasmic evagination was observed. After 13 days, the mitosis resulted in the formation of two-nuclear cells. One of the nuclei went to a "pollen tube" and a cell coat was observed between forming cells. Instead of prothallial divisions resulting in the transformation of two unequal cells in pollen grain (gametophyte development), the division of the microspore into two equal cells resulted in a change of the microspore to anomaly (sporophytic) development in culture *in vitro*. From 14 to 21 days, active cell division was observed and the formation of two types of embryoids occurred. The division of the first cell

resulted in the formation of a globular structure "head" and the division of the second cell resulted in the formation of an elongated "leg" (Fig. 6). The length of multicellular embryoids of this development type is 160 μm; the width of the "head" is 110 μm.

The embryoids which developed in the second way grew on the surface of the organogenic callus. These types of multicell embryos had elongated, torpedo-like forms and consisted of meristematic cells (Fig. 7). The length of these embryoids was 282.8 μm, and their width was 73.6 μm.

Multicell embryoids can be formed in two ways: directly from microspores, or through the formation of an organogenic callus and the development of embryoids on its surface.

When the microsporophylls were inoculated on the medium with 1-2 mg/l 2,4-D, the development occurred similar to its cultivation with the lesser hormone concentration. After 13 days, the formation of two-cell structures occurred. Cultivation from 30 to 60 days resulted in the formation of coenocytes and an organogenic callus inside the microspore coat.

On the medium with 5-10 mg/l 2,4-D, the formation of necrotic zones was observed after 7 days; after 14 days of cultivation, the microsporophylls died.

On the media without hormones, microsporophylls passed meiosis after 3 days, and the decomposition of tetrads was observed. The microspore's size was 30.7 μm. After 14 days, the microspore's size increased to 46.5 μm; after 21 days, their size reached 52.5 μm. However, after further cultivation the microsporophylls died.

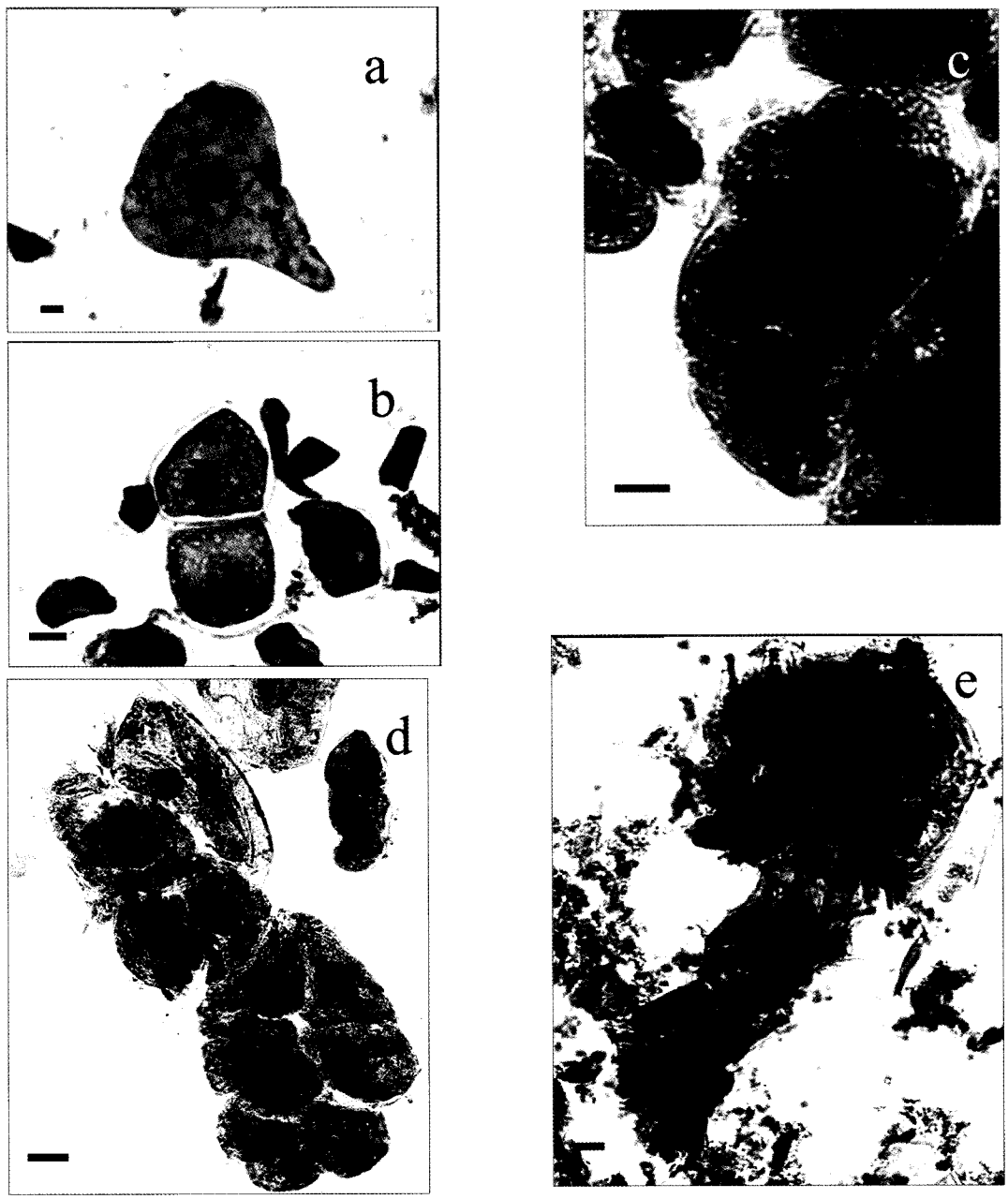

Fig. 6. Siberian larch embryoid formation in culture *in vitro* (first way of development): a- cytoplasmic evagination; b- division of nucleus and formation of two equal cells; c- four-cell embryoid formation; d- multicell embryoid formation; e- globular embryoid. Scale is 10 μm.

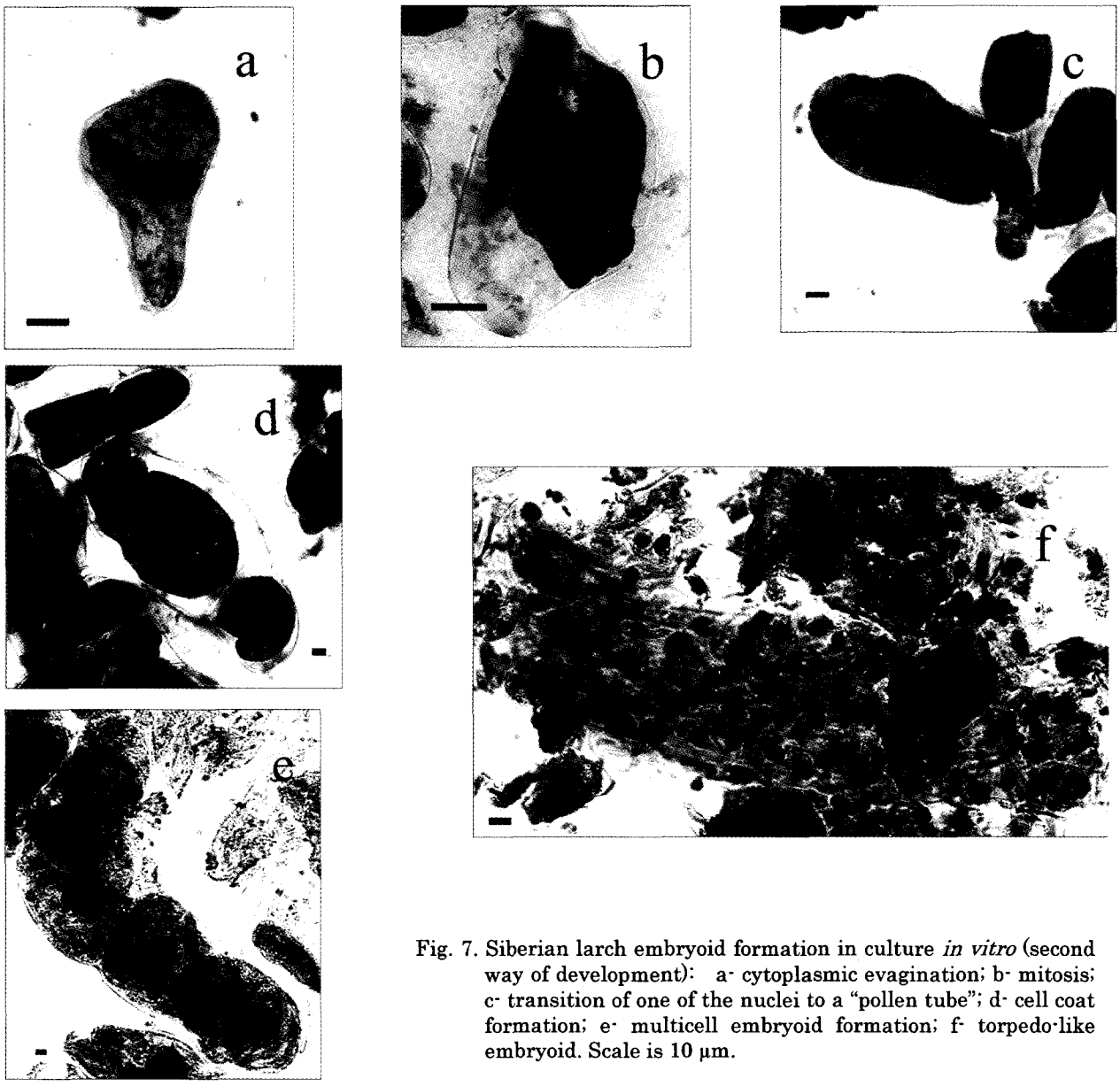

Fig. 7. Siberian larch embryoid formation in culture *in vitro* (second way of development): a- cytoplasmic evagination; b- mitosis; c- transition of one of the nuclei to a "pollen tube"; d- cell coat formation; e- multicell embryoid formation; f- torpedo-like embryoid. Scale is 10 μm.

Spring dates of cultivation; Cultivation of mononuclear microspores

In the second half of April, the reduction division of Siberian larch finished and the formation of the mononuclear microspores was observed. After 7 days, microspores inoculated on MS medium with 0.2-0.5 mg/l 2,4-D increased in size from 50.3 μm to 73 μm. After 14 days, the formation of "pollen tube" was observed and the size of pollen grains was 92 μm. After 21 days, the "pollen tube" grew and pollen grain sizes reached 124.1 μm. Further cultivation resulted in the formation of coenocytes.

After 7 and 14 days of cultivation, explants on the medium with 1-2 mg/l 2,4-D showed an increase in the size of microspores to 54.6 μm and 60.3 μm respectively. Further cultivation resulted in the formation of the organogenic callus.

On the medium with 5-10 mg/l 2,4-D, the formation of necrotic zones was observed after 7 days. After 14 days of cultivation, the microsporophylls were dead.

On the hormone-free medium the microspores increased in size to 53.8 μm and 57.4 μm after 7 and 14 days respectively. Further cultivation resulted in the formation of necrotic zones.

Spring dates of cultivation; Cultivation of mature pollen

In nature, the first divisions in the developing male gametophyte of Siberian larch begin 4-20 days after finishing meiosis. In meiosis, every microspore mother cell forms a tetrad of microspores. One to two days after tetrad formation, the destruction of the tetrad on single microspores occurs. After 3-4 days, the cell coat begins to form, and within a week the microspore is covered with a thick exine. There are four mitotic divisions in the developing male gametophyte before pollination. At the beginning of gametophyte formation, two prothallial cells are successively separated and later the generative and siphonogenic cells are formed. The generative cell is divided and produces spermiogenic and sterile cells. During pollination, the pollen grain is represented by a tree-cell structure, consisting of

siphonogenic, spermiogenic, and sterile cells, as well as two residual prothallial cells. This pollen grain is covered by thick exine and never germinates on nutrient media.

A series of experiments on germination *in vitro* of Siberian larch pollen grains was carried out. The experiments showed peroxide treatment of pollen promoted the loosening of exine, hydration of pollen and its active germination on MS medium with 0.2-0.5 mg/l 2,4-D (Fig. 8). Pollen germination occurred on the second day in light. Pollen tubes were 32 μm and more. Cultivation of pollen on nutrient medium resulted in the growth of pollen tubes and the formation of coenocytes within them. However, change from the gametophytic way of development to sporophytic was not observed.

Discussion

The presented results of our work have shown the ability of isolated microsporophylls of Siberian larch to form an androgenic callus and embryoids. Microsporocytes of Siberian larch collected at the meiotic stage of development (prophase I) finished their meiosis in culture *in vitro* during a few days and changed over to the sporophytic way of development during the whole autumn-winter period. Microsporocytes collected in the early spring were more responsive for cultivating.

It is known that different stresses can induce androgenesis *in vitro* in angiosperm plants. These stresses include heat treatment, osmotic shock, treatment by ultraviolet rays, low atmospheric pressure, etc. (Dunwell, 1982; Tukeeva, *et al.*, 1994). Some researchers believe treatment of isolated male generative organs by low positive temperature (+3^{0}C - +7^{0}C) is an important aspect of cultivation for male generative organs, when the highest number of embryos occur (Kruglova, Gorbunova, 1997; Abidovskaya *et al.*, 2003). It is believed that this change from gametophytic to sporophytic development occurs on a genetic level, i.e. with the influence of low positive temperature either switching off a gene or inhibiting a gene's functions, microspores change from gametophytic to sporophytic development (Chen *et al.*, 1986; Zou, 1994).

The outside temperature of Siberian larch microstrobile collecting in the autumn-winter period varies from 0 to – 30^0 C, and is stressful for microsporocytes. Inoculation of microsporocytes in culture *in vitro* at +24 - +26^{0}C triggers the process of microsporogenesis and microspore formation. Then the change of development from gametophytic to sporophytic occurs.

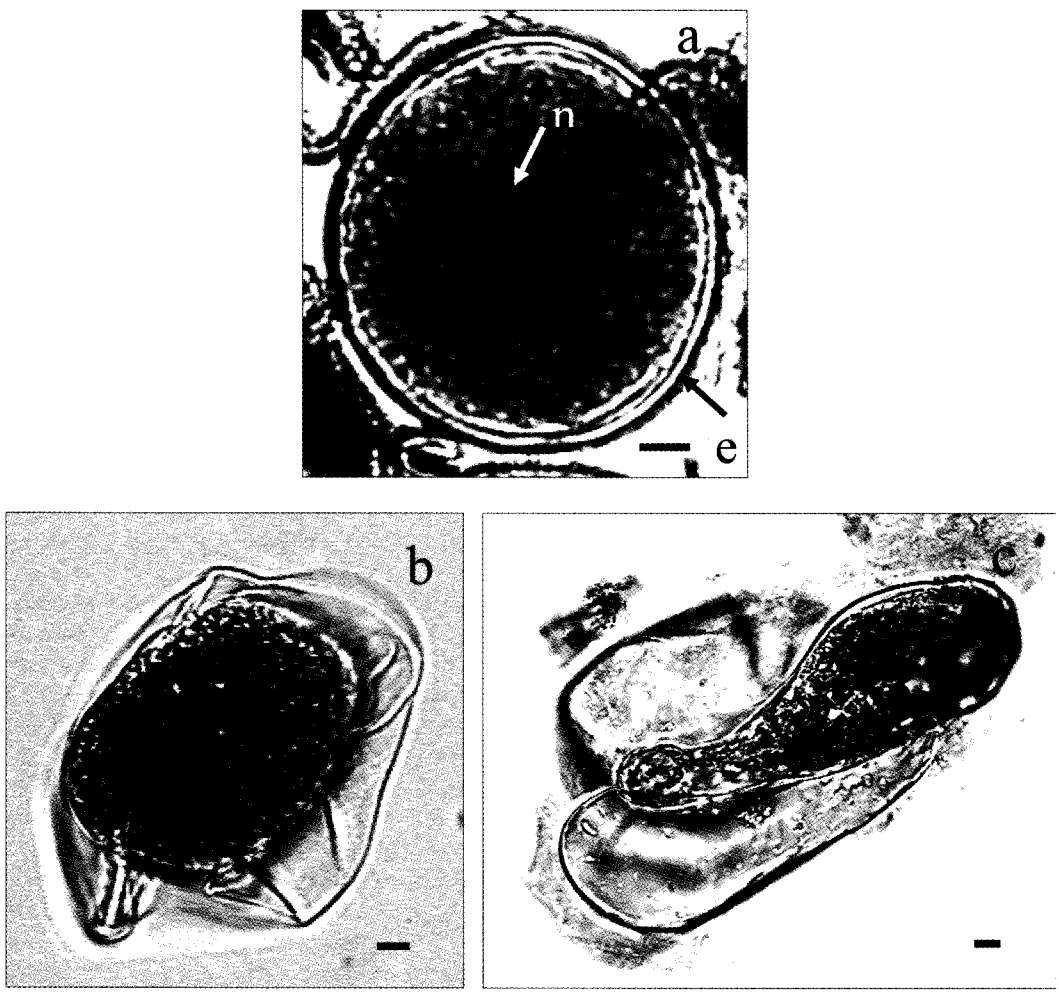

Fig. 8. Pollen grain of Siberian larch *in vivo* and their germination *in vitro*: a- pollen grain (n- nucleus, e- exine); b- loosening of exine; c- pollen tube formation. Scale is 10 μm.

It should be noted that the microstrobile development phase plays a special role in passing embryological transformation. Morphogenesis tendencies greatly depend on the development phase. According to Kruglova's data (Kruglova *et al.*, 1995), the wheat morphogenic microspore at the moment of inoculation on nutrient medium is at the great-vacuolized development phase which is optimal for inducing sporophytic morphogenesis *in vitro*. An androgenic callus was obtained from the anthers containing microsporocytes at meiosis stage in *Zycoparsisum* (Gresshoff, Dou, 1972), from one-nuclear pollen in *Lolium* and *Hordeum* (Clapham, 1971), and from mature pollen in *Brassica* (Kameya, Hinata, 1970). Formation of Siberian larch embryonic structures was most prominent at the meiosis period during winter, and especially in the early spring when reduction divisions became more active. At the end of April the change from gametophytic to sporophytic development, in male gametophytes (pollen grains) did not occur.

The presented results show that dependence of embryos and organic callus formation of Siberian larch are determined by cultivation conditions, nutrient medium composition, and 2,4-D concentration. It was determined that an increase in the 2,4-D concentration resulted in callus tissue formation. Further increase in the 2,4-D concentration resulted in embryogenesis when morphogenesis of barley and wheat anthers was studied (Tivary, 1989; Gorbunova *et al*, 1993; Gorbunova, Kruglova, 1994, 1997; Anapiyaev, 2000). In our experiments the best results were obtained on the medium with low 2,4-D concentration, 0.2-0.5 mg/l. The increased induction of embryogenesis from immature microspores of Siberian larch in culture *in vitro* was observed. High hormone concentrations have a negative influence on the larch microsporophyll embryonic process in culture *in vitro*. The development stopped and the tissue underwent necrosis.

Induction of Siberian larch androgenic cultures is influenced by auxins (2,4-D). Opinions about the influence of auxins on the process of plant sexualization are held by the Russian scientists E.G. Minina (1952) and D.A. Sabinin (1963) and English scientist J. Heslop-Harrison (1963). It was found that the optimal conditions for male sex determination are created at low auxin content, and that increased content of auxins resulted in female sexualization. It was shown that high auxin concentration provided feminization in herbaceous plants (Minina, 1952), woody species (Minina, 1965), and, in particular, in coniferous trees (Minina, Larionova, 1979). Perhaps the increased auxin concentration in the Siberian larch microsporophyll tissue, through 2,4-D injections, resulted in sex reversion of male generative organs. Development of male gametophyte did not occur; the formed microspore developed as an ovule. The newly-formed structures looked like embryos.

According to Batygina's reproductive theory (1992, 1993, 1994, 1999), the embryo is an asexually formed bipolar structure, a rudiment of a new plant organism, a structural unit of an asexually reproducing flowering plant *in vivo* and *in vitro*.

It is known that plant zygotic embryogenesis is a united process when the embryo is formed from a zygote, one initial cell. The embryo passes a range of discrete phases, which differ in their morphophysiological characteristics and duration (Batygina, 1993, 1994, 1999). The morphology of embryos of herbaceous plants is a "white, lusterless, compact globular structure, or, sometimes, clavate form" (Kruglova, 2001).

Development of larch embryos, as in the other conifer species, consists of three phases: proembryo, early embryo and late embryo (Tretyakova *et al.*, 2003). The first phase of proembryo formation occurs inside the archegonium. Divisions pass and a 16-cell proembryo which consists of 4 "floors" (four cells in each one) is formed (Tretyakova, 1990). Further development of the embryo occurs in the corrosium cavity; the embryogenic mass of cells appears from the lower floor. Embryo differentiation starts from the "torpedo" stage.

According to our data, development of larch embryoids in culture *in vitro* is original; it goes directly from morphogenic microspore or through the callus. In the first case, division of a microspore results in the formation of two equal cells, each of which is divided and the group of homogeneous cells develops inside the microspore coat. The formed multicellular structure of meristematic type looks like a globule, sometimes with a leg. The embryoids developed in the second way grow on the surface of the organogenic callus. Multicell embryoids of such type have an elongated torpedo-like form and consist of meristematic cells.

It is likely that the process of cultivating *in vitro* creates favorable conditions for realizing the potential of larch morphogenic microspores. Totipotency of plant cells (Butenko, 1964, 1984, 1994) or a multivariance of their development (Sabinin, 1963) is brightly displayed in larch specialized cells. Microsporocytes of larch realize their totipotency in culture *in vitro* through the development of androgenic callus or directly through embryoid formation.

For the first time, we have shown the possibility of androgenic culture development of gymnosperm plants in the sample of Siberian larch. Androgenic neo-formations from Siberian larch microsporocytes and microspores can develop to sporophytic embryogenesis and form embryoids.

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