ISSN 1062-3604, Russian Journal of Developmental Biology, 2018, Vol. 49, No. 4, pp. 222–233. © Pleiades Publishing, Inc., 2018.
Original Russian Text © I.N. Tret'yakova, M.E. Park, 2018, published in Ontogenez, 2018, Vol. 49, No. 4, pp. 251–263.

EXPERIMENTAL EMBRYOLOGY

Somatic Polyembriogenesis of *Larix sibirica* in Embryogenic in vitro Culture

I. N. Tret'yakova[a], * and M. E. Park[a]

[a]*Sukachev Institute of the Forest, Siberian Branch, Russian Academy of Sciences, Division of Krasnoyarsk Scientific Center Federal Research Center, Siberian Branch, Russian Academy of Sciences, Krasnoyarsk Russia*

**e-mail: culture@ksc.krasn.ru*

Received January 11, 2017

Abstract—The initiation of somatic embryos and their propagation in the long-term proliferating embryonal suspensor mass of *Larix sibirica* were studied. It was found that the increase in the number of somatic embryos in the embryogenic culture occurred as a result of cleavage of the globules of the somatic embryo and the suspensor; it less often occurred as the result of budding of the suspensor and the separation of the embryonal tubes of the suspensor. In the case of long-term proliferating cell lines (more than 8 years), the rate of cleavage did not weaken. A conclusion on the identity of morphogenic processes underlying the development of zygotic and somatic embryos of conifers is made, which is confirmed by the concept of T.B. Batygina (1999) on the parallelism of their development in vivo and in vitro.

Keywords: Larix sibirica, embryonal-suspensor mass, cleavage of globular embryos, budding, in vitro and in vivo embryonal tubes

DOI: 10.1134/S1062360418040069

INTRODUCTION

The reproduction of most eukaryotes, including coniferous species, is based on sexual and vegetative reproduction. The processes of development of embryonal structures in conifers are synchronized by the interaction of micro- and megametophytes, from the moment of germination of pollen to fertilization. The course of embryological processes is determined by the specifics of the species and intraspecific properties of the organism (physiological, biochemical, and molecular-genetic features), and is also controlled by the environment (Nekrasova, 1972; Tret'yakova, 1990; Singh, 1978).

At the same time, representatives of coniferous plants have multiple ways of realizing their reproductive potential: the possibility of asexual emergence of the embryo and polyembryony, including cleavage. The realization of this reproductive potential is widely manifested in the experimental conditions of in vitro culture, and, first of all, through somatic embryogenesis.

Cell regeneration in plants through somatic embryogenesis is a multistep process. This process in conifers is more complex and includes several stages: initiation (induction), proliferation, maturation of somatic embryos, germination of somatic embryos, regeneration, and acclimatization of somatic seedlings.

The proliferating embryogenic culture of conifers is characterized as translucent masses of immature somatic (globular) embryos—embryonal suspensor masses (ESM) (Stasolla et al., 2003). Up to the present, there has been no common view on the mechanism of initiation and the increase in the number of globular embryos in ESM.

The following mechanisms for the formation of somatic embryos were proposed:

(1) the formation of somatic embryos as a result of asynchronous division of individual cells of the explant (von Arnold and Hakman, 1988; Belorussova and Tret'yakova, 2008);

(2) cleavage polyembryony (von Aderkas et al., 1991);

(3) formation of somatic embryos as a result of division of cells located in the suspensor (Stasolla and Yeung, 2003).

The embryonal structures of angiosperms in vivo and in vitro have been repeatedly described (Haccius, 1955; Erdelska and Vidovensova, 1992, 1994; Batygina 1999, 2007). These formations were classified as a phenomenon of "a vegetative polyembryony," which occurs parthenogenetically under the effect of growth substances (Yakovlev and Snegirev, 1954), X-ray irradiation or inhibitors of the polar auxin transport (Titova et al., 2016).

In gymnosperms, especially in the Pinaceae family, the phenomenon of in vivo cleavage polyembryony is pronounced, as a result of which the embryonal initials of a zygotic embryo obtained from one fertilized ovum cleave into four identical embryonal units, each giving rise to four twin embryos (Tret'yakova, 1990).

In addition, the presence of several archegonies (usually 2–4) in the megagametophyte and the development of several zygotes, including zygotes from different male gametes, lead to the additional formation of embryos, which are also actively cleaved (Buchholz and Blakeslee, 1930). In *Pinus sibirica*, 12–16 embryos can develop in the embryonal canal of one megametophyte (one ovary) (Tret'yakova, 1990). It is not excluded that the presence of cleavage is archived in the plant cells of Pinaceae species, which can be realized in in vitro culture under the action of hormones.

Cleavage polyembryony is the most mysterious phenomenon of somatic embryos. This process takes place throughout the life of the embryogenic culture. For example, for 8 years, there is an active multiplication of somatic embryos through cleavage in the proliferating embryogenic cultures of *Larix sibirica* (Tret'yakova et al., 2015; Pak et al., 2016), which mature and form regenerants, when transferred to the medium supplemented with ABA.

The study was aimed at studying the propagation of somatic embryos in the proliferative 8-year-old embryogenic culture of *Larix sibirica* in vitro and comparing the patterns of somatic polyembryony in vitro and in vivo.

MATERIALS AND METHODS

Fifteen proliferating cell lines (Cl) obtained from three trees of the Siberian larch *Larix sibirica*, growing in the arboretum of Sukachev Institute of the Forest (Siberian Branch, Russian Academy of Sciences, Krasnoyarsk) (genotypes A4 and 10) and larch forests of the Republic of Khakassia (Chernoe ozero settlement) (genotype Ch2), served as the material for the study. Age of cultures was from 5 to 8 years.

Introduction to culture, initiation (induction) of somatic embryogenesis and proliferation of embryonal suspensor masses (ESM) in Siberian larch were carried out on AI medium (patent no. 2456344; http://www.freepatent.ru/images/patents/5/2456344/patent-2456344.pdf) and have been described earlier (Tret'yakova and Barsukova, 2012; Tret'yakova et al. 2015; Pak et al., 2016). Proliferating embryogenic cultures were subcultured on fresh AI medium every 2 weeks. The cultures were incubated in the dark at 24 ± 1°C.

Methods of light microscopy, modified by the authors (Tret'yakova, 1990; Belorussova and Tret'yakova, 2008), were applied to study the embryogenic cultures and somatic embryos. A cytoembryological and histological study of the ESM of somatic embryos was performed on pressurized preparations stained with safranin and hematoxylin (Kruglova et al., 2013) and on permanent preparations stained with prozionic dyes (Ivanov, 1982).

RESULTS

Initiation of Embryogenic Cultures

After the introduction of the zygotic embryos of larch at the stage of initiation of the cotyledon into the in vitro culture, the somatic cells, the length of which was 62.5 ± 6.5 μm, began to intensively enlarge along the entire length of the hypocotyl and reached a length of 191.7 ± 6.9 μm on the fifth day of cultivation. Figure 1 shows the process of formation of Siberian larch somatic embryos. The elongated cells were polarized (Figs. 1a, 1b). Two free nuclei were seen in them (Figs. 1b, 1c). Further, one of the nuclei was displaced to one of the ends of the elongated cell (Figs. 1b, 1c), and an asymmetric division with the formation of a small cell (embryonal initial of 39.2 ± 1.2 μm in diameter) and a long cell (200 ± 12.7 μm in length) occurred (Fig. 1d). Next, the cells of the embryonal initial divided and formed cellular conglomerates (globules); embryonal tubes were formed from the cells of the basal part of the globule (Figs. 1d, 2e, 2f, 2h). Thus, on the 12th–15th days of cultivation, many somatic embryos were observed, from the basal part of which embryonal tubes appeared periodically (Figs. 1f, 1g, 1i, 1j et_1, et_2,..., et_3, etc.). These tubes elongated and formed a suspensor. The globules consisted of isodiametric initial cells forming the embryonal mass (Figs. 1k, 2). Cells of embryonal globules had small dimensions (39.4 ± 0.5 μm), a large nucleus located in the center, and were able to actively divide. It should be noted that the embryonal globules contained a large amount of auxin; the embryonal tubes and the whole suspensor remained slightly stained for auxins and cytokinins (data of immunohistochemical analysis, in the press, Tret'yakova et al.).

Proliferation of Embryogenic Cultures (EC)

After 30–45 days, the cultures were transferred onto AI medium with reduced content of cytokinins and sucrose, and this transfer stimulated the multiplication of somatic embryos, consisting of embryonal globules and a suspensor (embryonal pipe system). There was an intensive increase in the embryonal suspensor mass (ESM).

Proliferating cell lines of Siberian larch were obtained by us in different years (2008–2016) from the A4, 10, and Ch2 genotypes by free and controlled pollination: 11 Cl from genotype A4, 3 Cl from genotype 10, and 1 Cl from genotype Ch2. The age of the cultures was 5–8 years. Externally, proliferating cultures looked like loose, easily scattering structures of white or cream color (Fig. 2).

The intensity of growth of ESM varied significantly in the Cl investigated. The mass of proliferative ESM for the period from transplantation to transplantation (14 days) increased by six times in Cl5 and Cl6 and by 23 times in the most actively growing Cl4. This cell line is interesting not only for the intensity of ESM

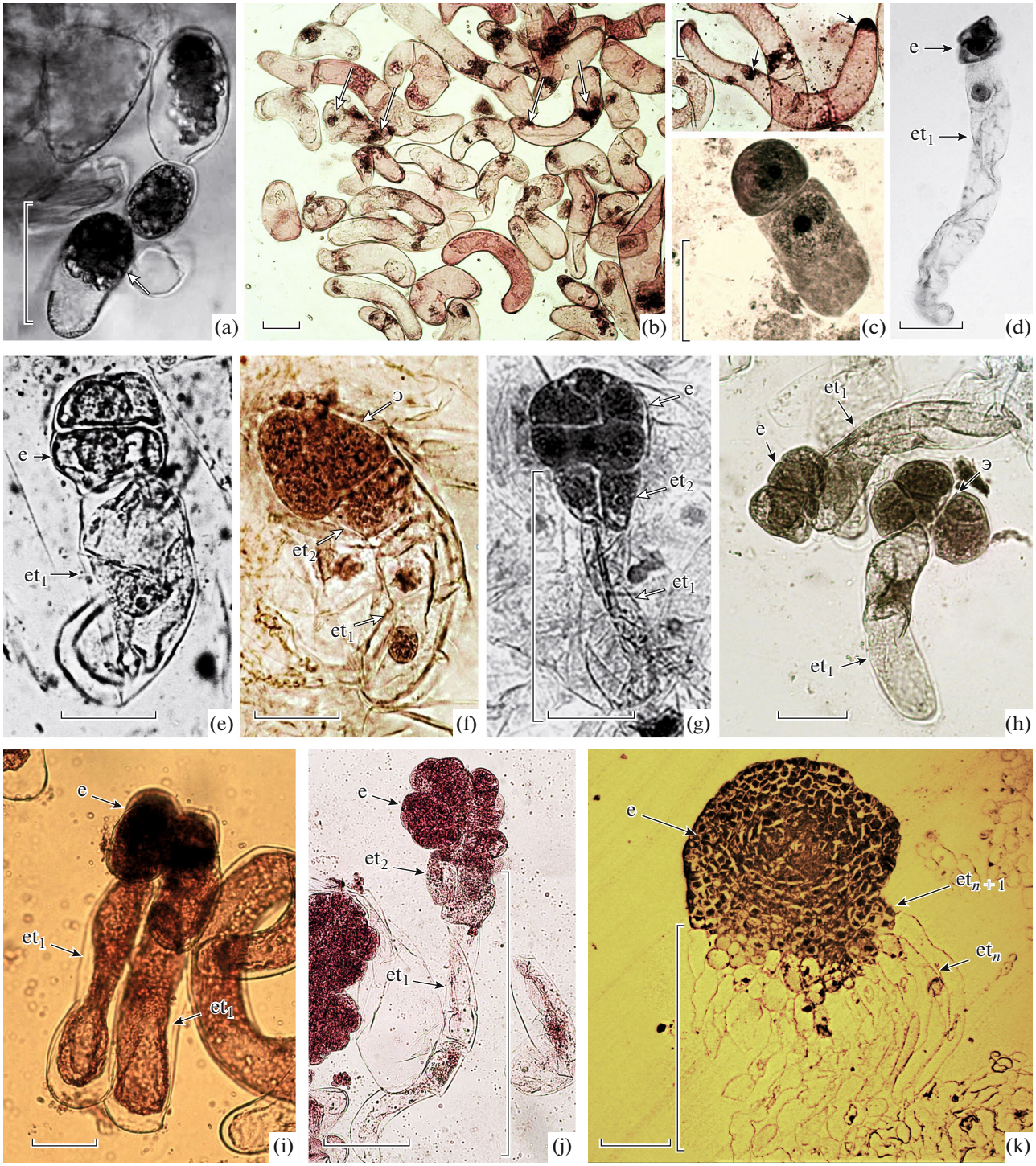

Fig. 1. Process of formation of somatic embryos in Siberian larch: (a, b) elongation and polarization of somatic cells (arrows denote the cellular nuclei), (c) displacement of the nucleus to one side of the cell, (d) first division of the initial cell of the embryo, (e) four-celled embryo with a single-celled suspensor, 50 μm scale, (f–k) globular embryo, scale for (j, k): 100 μm. Designations: e—embryo (actual embryo); et_1—first order embryonal tube; et_2–et_n—embryonal tubes of various orders formed later then the et_1; S—embryonal suspensor.

growth but also for the rate of maturation of somatic embryos: from 14–18 days, in contrast to the average duration of 45 days in the other investigated cells. The number of globular somatic embryos from different Cl of larch ranged from 2040 ± 189 (Cl6) to 11103 ± 259 (Cl10) per 1 g of ESM. The globules of somatic embryos in these Cl had a size ranging from 112 to 282 μm, the size of suspensors ranged from 592 to 2969 μm. The globules of the somatic embryos were often located on the surface of the cultures and formed clus-

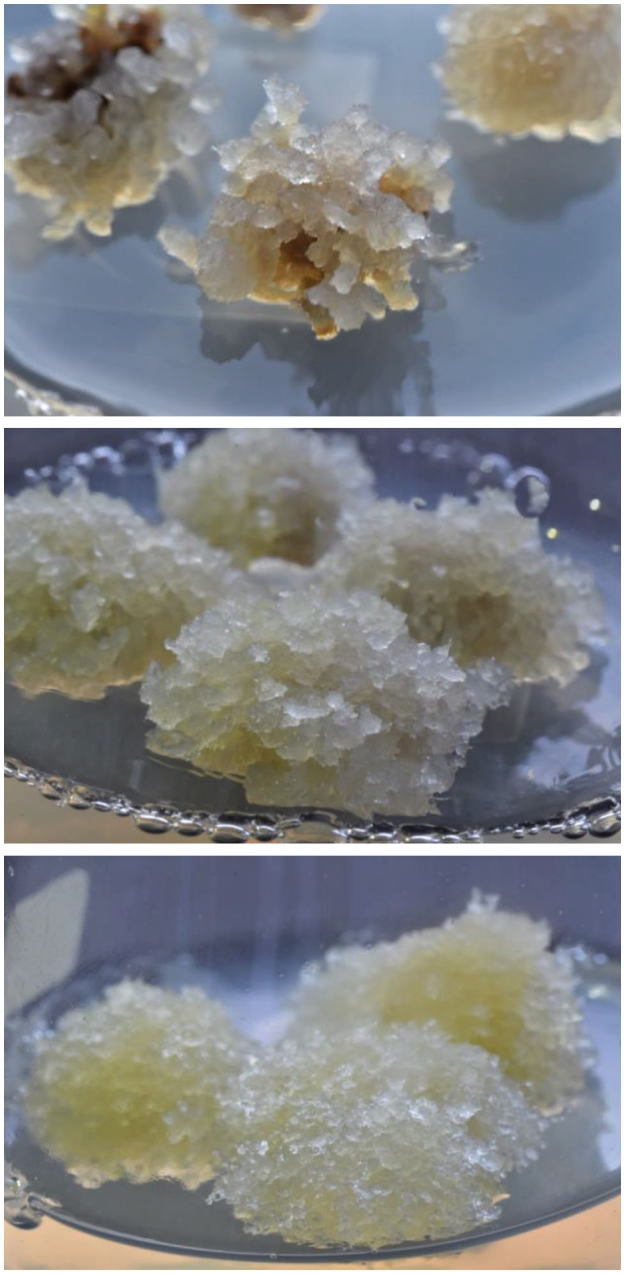

Fig. 2. Collection of long-proliferating embryogenic cell lines of *Larix sibirica*.

ters, and their suspensions closely adjoined each other (Fig. 3). Polyembryonal complexes consisting of globules and suspensions were formed. Further, there was a cleavage. In this case, the globules of somatic embryos began to separate from each other (Figs. 3a–3e). Cleavage could occur in small globules of 90 μm (Fig. 3a) and in larger globules (282 μm), where there was an active initiation of embryonal mass (Fig. 3b–3e). Then there was a cleavage of suspensors adjacent to the head of the somatic embryo (Figs. 3f, 3g, 3h). Somatic embryos began to disintegrate until they were completely separated from each other. Independent somatic embryos appeared (Fig. 3i). Cleavage did not weaken under prolonged cultivation (up to 8 years or more). There were somatic embryos that cleaved and disintegrated again and again.

In addition, the formation of somatic embryos from the cells of the suspenor as a result of lateral protrusion or budding was observed (Fig. 4).

However, the process of adventitious budding of the suspensor occurred much less frequently than the cleavage of the embryonal globule of the somatic embryo. At the same time, a phenomenon was observed: the embryonal tubes from which the suspensor consisted were cleaved (Fig. 5). Asynchronous division followed by the formation of a globule and a somatic embryo suspensor proceeded in the disjointed embryonal tubes. The picture resembled the initial stage of the initiation of somatic embryogenesis (Fig. 1). Such a phenomenon has not been described in the literature.

Thus, the formation of ESM actively proceeded as a result of cleavage of somatic embryos at the globular stage of development; it happened less often as a result of formation of adventitious buds of the suspensor and cleavage of embryonal tubes with their subsequent proliferation.

Maturation of Somatic Embryos

Inhibition of polyembryogenesis occurred under the influence of ABA. When the proliferating ESM was transferred to the AI nutrient medium supplemented with ABA (Tret'yakova et al., 2016; Pak et al., 2016), cleavage, budding, and disjoinment of the suspensor embryonal tubes stopped for several weeks. After 2 week of cultivation on the medium, each embryo became an independent structure, the embryo axis was formed and the programmed cell death of the suspensor started. Sometimes several globular embryos did not diverge in the basal part and still had a common degrading suspensor (Fig. 6). During the second week of cultivation on the medium supplemented with ABA, there were an active histodifferentiation and the development of apical meristems and cotyledons. At the beginning of the third week, the suspensor completely died out. The maturation of somatic embryos completed within 20–60 days. When the embryos were transferred to the 1/2AI medium (without hormones), the germination and the formation of regenerants occurred.

DISCUSSION

Sexual embryogenesis of angiosperms is based on polarity and asymmetric division, which occurs already at the first division of the zygote and leads to the formation of two unequal cells: a small terminal cell, giving rise to the embryo, and a large basal cell that forms the pituitary body and the suspensor (Batygina, 1999). In this case, the polarity inherent in the

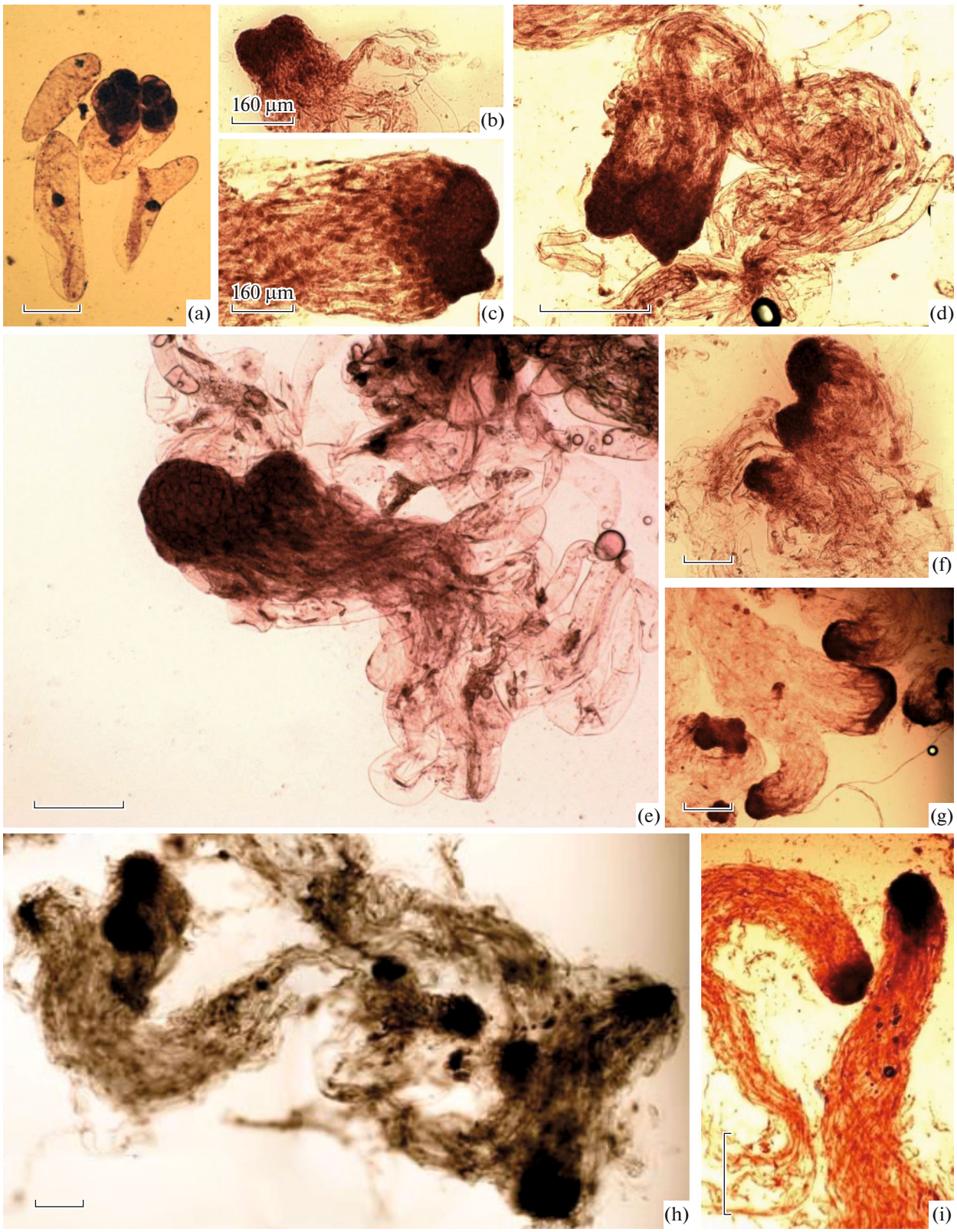

Fig. 3. Multiplication of globular somatic embryos of *Larix sibirica* through the cleavage: (a (50 μm scale), b, c, d, e) cleavage of the globules of somatic embryos from each other, (f, g) cleavage of suspensors adjacent to the head of the somatic embryo, (h) somatic embryos joined by a common suspensor, (i) independent globular somatic embryos. 160 μm scale.

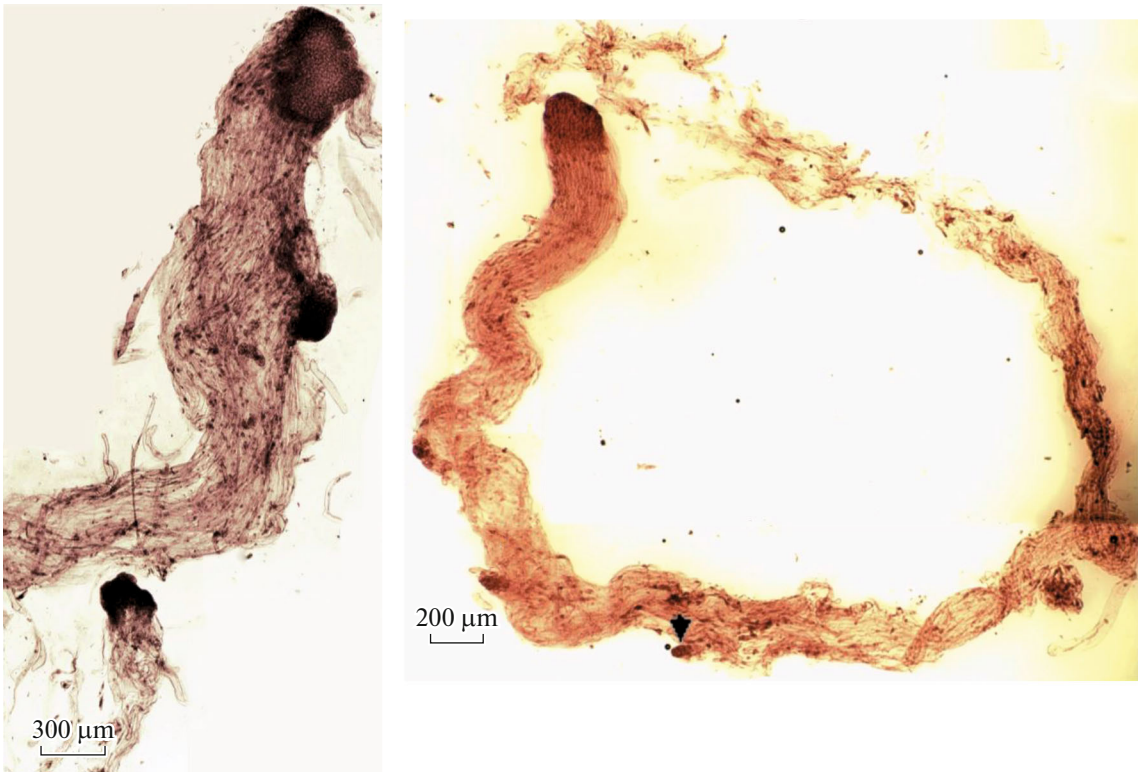

Fig. 4. Formation of somatic embryos from the cells of the suspensor as a result of lateral protrusion or budding.

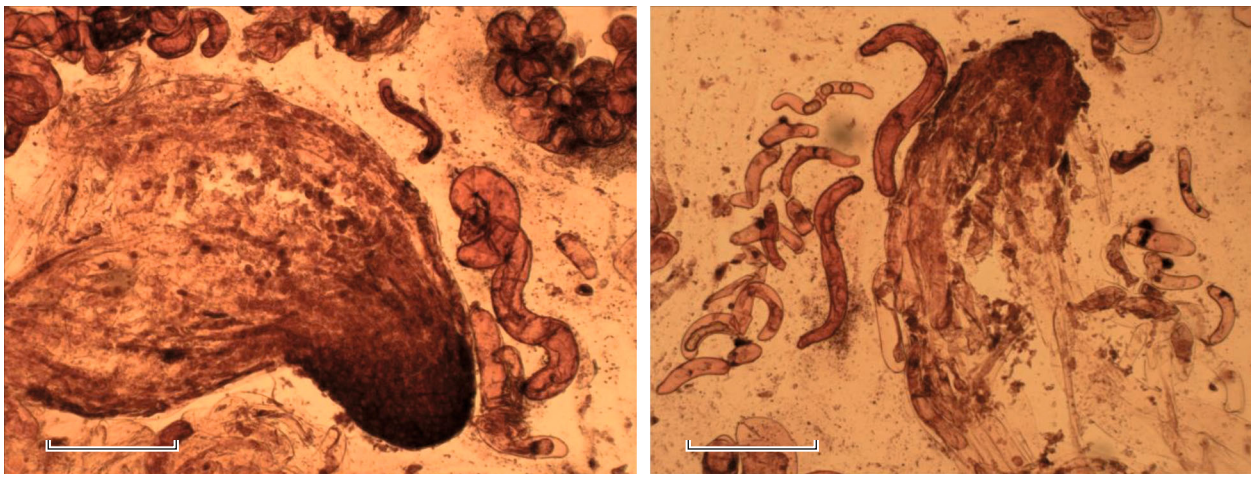

Fig. 5. Multiplication of globular somatic embryos from the cells of the suspensor. (Separation of embryonal tubes of a somatic embryo). 160 μm scale.

zygote is maintained and transmitted to the daughter cells as a result of its division (Lutova et al. 2010).

The polarity of the zygote is pronounced in both conifers and angiosperms. However, the two nuclei of the proembryo formed in the center of the zygote as a result of fertilization divide, and the four free nuclei of the proembryo, due to the zygote's polarity, move to the base of archegonia, where a sixteen-cell proembryo consisting of identical cells arranged in four tiers (four cells in each tier) of USE type forms (Singh, 1978; Dogra, 1978). Four embryonal initials (e), four embryonal cells of the suspensor (eS), derivatives of e-tier, four cells of the disfunctional suspensor (dS), and four cells of the open tier (upper tier of proembryo, U) are formed (Fig. 7). Describing the early embryogenesis in conifers, Singh (1978) named the cells of the embryonal suspension eS as et (embryonal tubes).

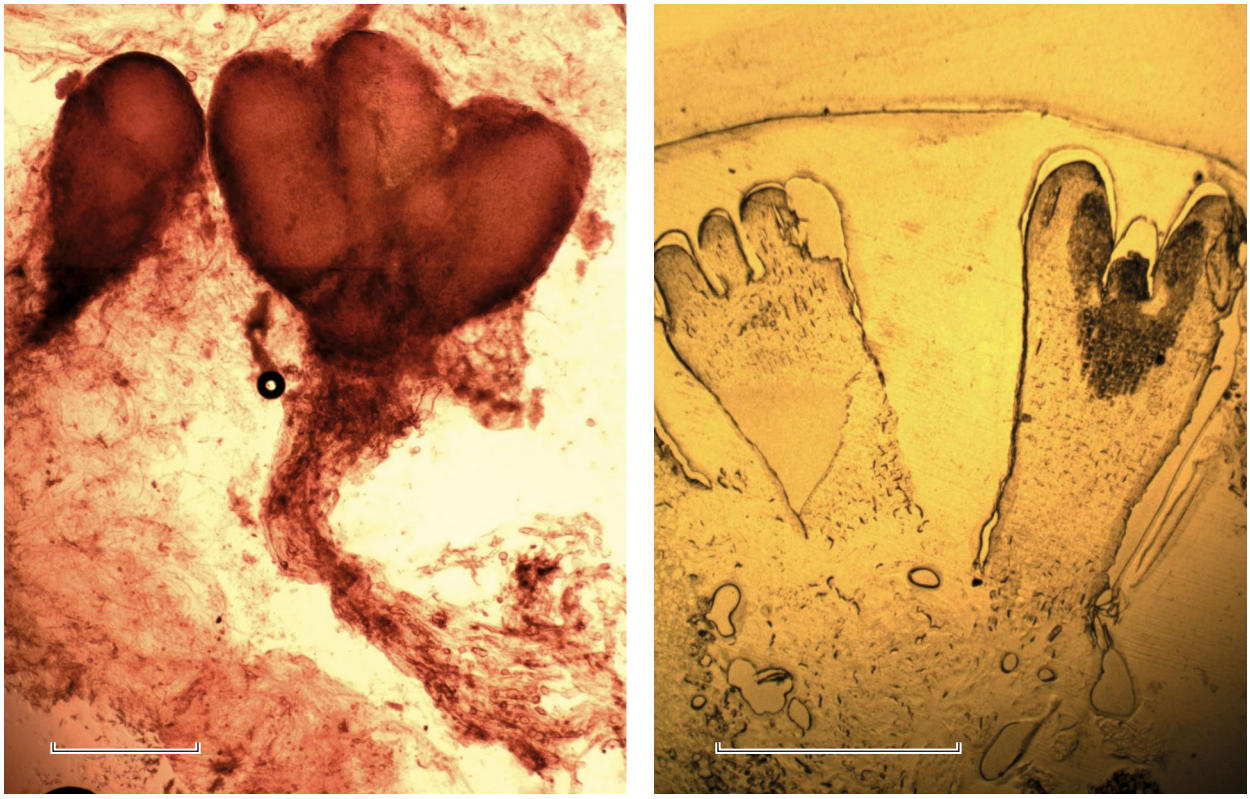

Fig. 6. Multiplication of globular somatic embryos from the cells of the suspensor: (a) pressurized preparation stained with safarin, (b) after 45 days on a medium supplemented with ABA, permanent preparation. 500 μm scale.

Therefore, in the further presentation of the material, we will call these cells the embryonal tubes (et).

After 7–10 days from the fertilization, the cells of the embryonal suspensor (eS or et) of *Pinaceae* species begin to enlarge rapidly up to 200–300 μm and push the cells of the lower tier (e) into a corrosion cavity of the female gametophyte. The enlargement of the embryonal tubes (et) occurs at an unequal rate, and the initial cells outrun one another (Fig. 8a). Cleavage, resulting in the formation of four embryo initials, each of which divides and forms four embryonal globules in a corrosive cavity of a megagametophyte, occurs. Then, the embryonal tubes are formed from the basal cells of each globule (Figs. 8b, 8c) (Tret'yakova, 1990).

According to Singh, the cleavage polyembryony in gymnosperms with in vivo zygotic embryogenesis consists of the independent behavior of embryonal units and the development of a suspensor apparatus (Singh, 1978). Cleavage can occur (a) during the formation of the walls of a young proembryo when different cells develop independently and form an embryonal mass and a suspensor. Such cleavage is found in *Erhedra*, *Sequoia*, and *Gnetum* (Singh, 1978); (b) cleavage may be caused by the enlargement of the suspensor tier S. In this case, the cells of the suspensor are separated from each other at the tip and each cell forms one or more embryonal units (Dogra, 1966). Cleavage of the suspensor is typical for the representatives of *Cryptomeria, Taxodium, Glyplastrobus,* and *Taxus*; (c) cleavage may result from the enlargement of the embryonal suspension (eS) typical for the representatives of *Coniferales* (Singh, 1978), which is formed from the embryonal tier (e). In this case, the cells of the embryonal suspensor eS are separated from each other and each cell functions as an independent embryonal unit, produc-

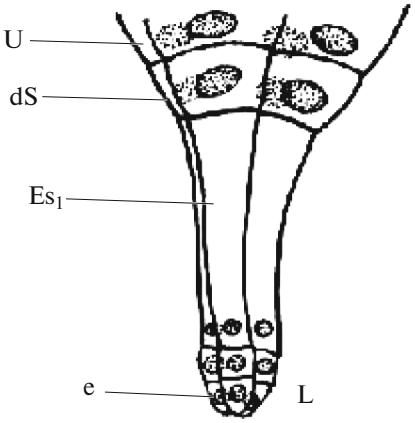

Fig. 7. Diagram of a USE-type proembryo (Singh 1987).

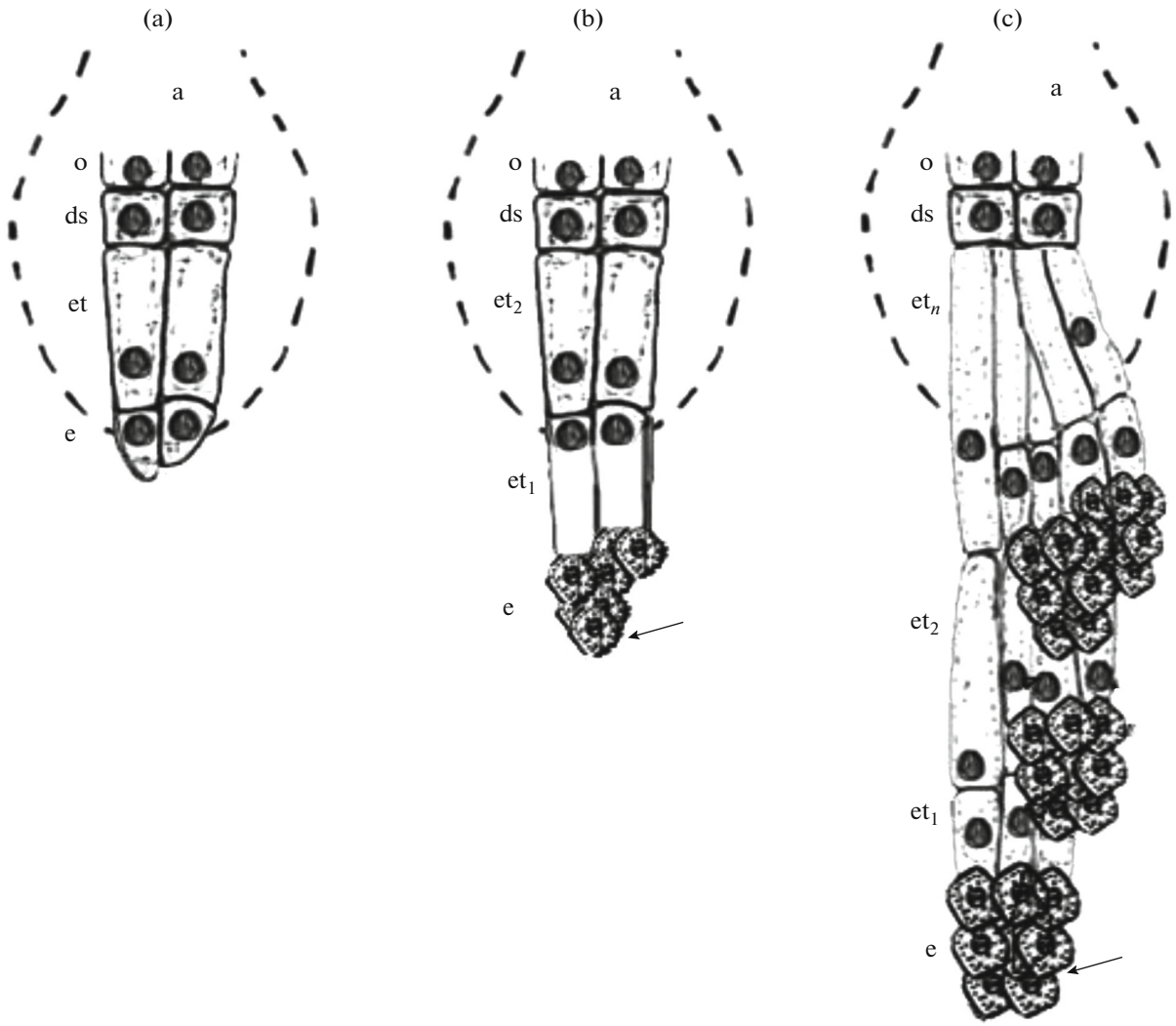

Fig. 8. Cleavage and formation of zygotic embryos (Tret'yakova 1990): (a) four embryo initials (e), four embryonal suspensor cells (et), tier derivatives (e), and then four cells of a disfunctional suspensor (ds) and four cells of the open tier (o) are formed in archegonia (a); one initial cell is nearer to the corrosive cavity of the megametophyte due to the uneven rate of enlargement of the cells of the embryonal suspensor (et); (b) the formation of four embryos; (c) four embryos in the corrosive cavity of the megametophyte. The leading embryo is visible (indicated by an arrow). Embryonal tubes (et) are formed from the cells of the basal part of the embryo.

ing an embryonal mass (embryonal clams—e), from the basal part of which the cells of the tube et_1, et_2, etc. develop, forming the suspensor. This type of cleavage is found in *Cupressaceae*, *Pinaceae*, *Sequoidendron*, *Cunningyamie*, and *Metasequaja* (Doyle and Brennan, 1971, 1972; Singh, 1978); (d) cleavage between the embryonal units occurs when the embryonal initials (e) are completely separated from each other and form embryonal tubes (et). This type of cleavage is described in *Podocarpaceae*, *Taxodiaceae*, *Surressus*, and *Cedrus sdeodara* (Singh, 1978); (e) lobing or budding of the embryonal mass is described in many gymnosperms (Singh, 1978). However, this type of regeneration is not a regular phenomenon and is considered to be a false cleavage (Dogra 1967); (f) determinate cleavage polyembryony is a condition when it becomes evident at an early stage which of the many embryos in the seed will be successful (Buchholz, 1933), as in *Dacrydium* (Quinn, 1966a, 1966b); (g) Indeterminate Cleavage Polyembryony is a condition when it is not clear at the beginning which of the embryos will become the leader, as in *Pinus* (Singh, 1978).

Thus, cleavage polyembryony is a regular and organized phenomenon caused by the independent growth of embryonal tubes and the splitting of the globule (embryonal mass) (Buchhols, 1918; Dogra, 1967).

Summarizing the cleavage in *Coniferales*, Singh (1978) considers the following types:

(1) Unitary Cleavage; in *Coniferales*, caused by the independent formation of embryos by pe cells and their derivatives (Dogra, 1967).

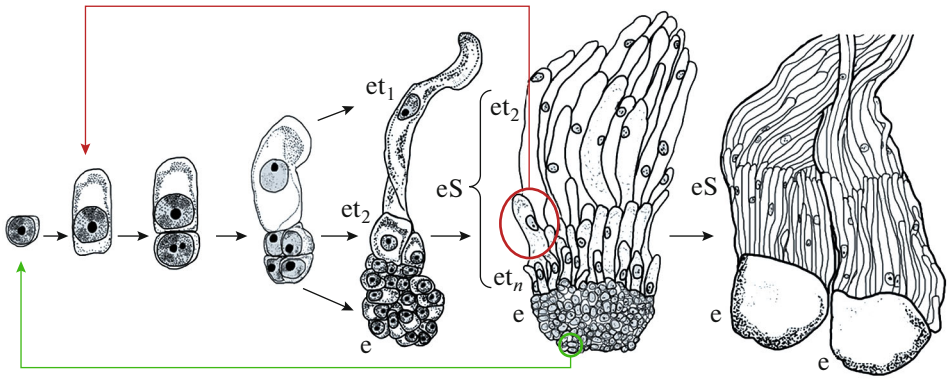

Fig. 9. Development and cleavage of somatic embryos of *Larix sibirica* in vitro (see Discussion).

(2) Partial Cleavage; occurring when two or three embryonal units remain joined together, while the other units are separated from each other.

(3) Total Cleavage; occurring when all embryonal units are completely separated from one another (Dogra, 1967).

(4) Inhibitory Cleavage; caused by the destruction of eS of one or more embryonal units and the enlargement of other units (Dogra, 1967).

(5) Unitary Lobing; occurring when the early embryonal mass is divided into lobes with the formation of embryonal tubes. Embryonal tubes sometimes pass through the free-nuclear stage before an early ontogenesis. It is assumed to be an accidental phenomenon caused by different growth rates of embryonal units in the embryonal mass (Dogra, 1967).

According to Williams and Maheswaran (1986), the formation of embryoids in conifers in an in vitro culture can occur through callus and in a direct way: (1) the direct formation of embryoids from the mass of embryonal cells of the explant, occurring as a result of the formation of long cells from cell aggregates with dense cytoplasm; (2) direct formation of embryoids from the peripheral cells of the explant or free-floating cells; (3) multiple formation of embryoids through the cleavage. The formation of embryoids through the coenocytes was observed in megametophytes of *Larix decidua* (von Aderkas and Bonga, 1988), *Pinus lambertiana, Pinus taeda* (Gupta and Dursan, 1986a, 1987), and *Pinus sibirica* (Voroshilova and Tret'yakova, 2014). This type of formation of embryoids has been described earlier in the pollen cultures of *Pinus resinosa* (Bonga, 1982), *Zamia taxus* (La Rue, 1954).

The enlargement of the somatic cells of the zygotic embryo, the appearance of the polarity probably under the action of 2,4-D and their asymmetric division (direct pathway) is a condition that is necessary for the initiation of somatic embryogenesis in in vitro culture in *Larix* species as well as in other species of conifers. Such a type of embryo formation has been described earlier in *Picea abies* (von Arnold and Nakman, 1988; Stasolla et al., 2003) and *Larix sibirica* (Tret'yakova et al., 2016) and does not differ in angiosperms.

Consequently, cell lengthening, polarity, and asymmetric division are the main criteria that determine the transition of larch cells to the path of somatic embryogenesis. The further development of zygotic and somatic embryos in *Pinaceae* proceeds in a similar way (Tret'yakova et al., 2015; Pak et al., 2016). Both form two groups of cells: embryonal initial and an elongated cell (possibly, the first embryonal tube). Embryonal globules are formed from the initial embryonal cells; embryonal tubes (et_1...et_n), forming the suspensor, are formed from the basal cells of the embryonal globules (Fig. 9). Thus, two structural units, one of which is the globular embryo and the other is the embryonal tubes, constituting the suspensor, are pronounced in both zygotic and somatic embryos of larch. Embryogenic cultures of *Larix* are an embryonal suspensor mass.

Somatic embryos are often combined into polyembryonal complexes. Somatic polyembryogenesis has been described in *Pinus taeda* and *Picea abies* (Gupta and Dursan, 1987; Jokinen and Dursan, 1994) and was later called a "repetitive cleavage process" (Becwar et al., 1990). A polycleavage process of somatic embryogenesis was detected in *Juniperus communis* (Holmersson and von Arnold, 2004) and in *Abies* (Vondrakova et al., 2011). The scheme for ESM proliferation was shown in *Pinus taeda* (Gupta and Dursan 1991).

We noted polyembryonal complexes in *Pinus pumila* (Tret'yakova and Shuvaev, 2015) and in *Larix sibirica* (Pak et al., 2016). In vitro cleavage in Siberian larch can be attributed to the unitary cleavage type (c), according to Singh (1978), due to the independent formation of the embryo through the embryonal cells of the globule and embryonal tubes (et), arising from the distal cells of the globule. This type of cleavage begins at the stage of the globule and does not depend on its size. Suspensors are initially closely pressed to each other and form a single complex, which separates as the embryonal tubes and globules of the somatic

embryo separate, and independent somatic embryos are formed (Fig. 9).

It is known that the cleavage is typical for the gymnosperms and is much less common in angiosperms. According to Titova et al. (2016), wheat forms the polyembryoids of two "multiple shoot meristems" classes, from which the phenotypes "siamese twins back to back" and "multiple meristems" are formed. Cleavage in "multiple meristems" phenotypes occurs at the early globular stage. In this case, polyembryons are not independent and represent polymeric structures with multiple meristems, shoots, cotyledons, and other organs usually fused to a common root pole. It is assumed that this phenomenon cannot be attributed to cleavage polyembryony (Fischer et al., 1997). However, according to Titova et al. (2016), the formation of polymeric structures with numerous meristems and a common root can be considered as a manifestation of incomplete cleavage polyembryony. At the same time, the constituent units of polyembryoids are not independent and form polymeric structures with multiple shoot meristems and fascicles fused to varying degrees.

The active cleavage polyembryony in *Larix* at the stage of development of the globular embryo can probably be related to the competence of its cells to different effects, in particular, to auxins. The impulse in the form of an inflow of auxins from the nutrient medium into the embryo leads to the reprogramming of the cells of the globule and to acquiring the ability to perceive and transport auxins. The possible accumulation of auxins in the globule and their lower content in the basal part creates a gradient of polarity resulting in the formation of the embryonal tubes (a suspensor), in which, according to the data of our immunohistochemical investigation (Tret'yakova et al., 2017, in press), auxins are not detected. Probably, the cleavage of the globule leads to complete cleavage of the suspensor and the isolation of the embryo.

The somatic polyembryogenesis in *Larix sibirica*, as well as in other conifers, can be considered as a repeated cleavage process established in a zygote (Gupta and Dursan, 1987). We suppose that it is an independent process. This process is actively realized in the in vitro culture of Siberian larch at the globule stage, when the conditions that are optimal for cleavage are created.

Reproduction of somatic embryos in the in vitro culture occurs not only as a result of cleavage polyembryony but much less often through the formation of adventitious buds in the suspensor, which was observed in *Picea abies* (von Aderkas et al., 1991) and in *Larix sibirica* (Tret'yakova et al., 2016). In addition, mass propagation of somatic embryos can also occur as a result of dissociation of embryonal tubes of the suspensor (Stasolla and Yeung, 2003). Dissociation of embryonal tubes of suspensors in the proliferating embryonal suspensor mass of Siberian larch leads to the multiple formation of globular somatic embryos through the asymmetric division. Moreover, the process of development of new somatic embryos does not differ from the described process of initiation of somatic embryogenesis: the same elongated polarized cells (embryonal tubes) asymmetrically divide and form embryonal globules and a suspensor.

The process of propagation of somatic embryos in the proliferating embryonal suspensor mass remains a mysterious phenomenon. This process does not weaken in long-cultivated embryogenic cultures (Pak et al., 2016). At regular transplantations, the proliferating embryogenic cultures of *Larix sibirica* intensively grow for 8 years, and somatic embryos in them are actively cleaved and formed from the embryonal tubes of the suspensor. The rate of formation of somatic embryos does not decrease. Such proliferative activity of embryogenic cultures was described earlier in the culture of megametophytes of *L. decudua* and the culture of embryos of larch hybrids (*Larix* × *eurolepis* and *Larix* × *marschlinsii*) (Lelu-Walter and Pâques, 2009), where the formation of ESM continued for 9 years and more. However, the mechanism of multiplication of somatic embryos in such cultures has not been disclosed. For the first time, we showed the process of in vitro somatic polyembryogenesis in conifers using *Larix sibirica* as the example. This process can be caused by cleavage, budding of the suspensor and mass cleavage of the embryonal suspensor tubes, followed by their transition to the embryogenesis.

The generality of morphogenetic processes underlying the development of zygotic and somatic embryos of conifers once again confirms the concept of the parallelism of their development in vivo and in vitro.

ACKNOWLEDGMENTS

The study was carried out with the financial support of the Russian Foundation for Basic Research, the Government of Krasnoyarsk krai, and the Krasnoyarsk Territory Fund for Support of Scientific and Technical Activity within the framework of scientific projects nos. 16-44-240509 and 16-44-243068.

REFERENCES

Von Aderkas, P. and Bonga, J.M., Formation of haploid embryoids of *Larix decidua*: early embryogenesis, *Am. J. Bot.*, 1988, vol. 75, pp. 690–700.

Von Aderkas, P., Bonga, J., Klimaszewska, K., and Owens, J., Comparison of larch embryogeny in vivo and in vitro, in *Woody Plant Biotechnology*, New York: Plenum Press, 1991, pp. 139–155.

Von Arnold, S. and Hakman, I., Regulation of somatic embryo development in *Picea abies* by abscisic acid (ABA), *J. Plant Physiol.*, 1988, vol. 132, pp. 164–169.

Batygina, T.B., Embryogenesis and morphogenesis of zygotic and somatic embryos, *Russ. J. Plant Physiol.*, 1999, vol. 46, no. 6, pp. 774–788.

Becwar, M.R., Nagmani, R., and Wann, S.R., Initiation of embryogenic cultures and somatic embryo development in loblolly pine (*Pinus taeda*), *Can. J. For. Res.*, 1990, vol. 20, pp. 810–817.

Belorussova, A.S. and Tret'yakova, I.N., Patterns of somatic embryo formation in Siberian larch: embryological aspects, *Russ. J. Dev. Biol.*, 2008, vol. 39, no. 2, pp. 83–91.

Bonga, J.M. Haploid tissue culture and cytology of conifers. Collogue international sur la culture 'in vitro' des essences forestieres, Association Foret-Cellulose, Nangis, France, 1982, pp. 283–293.

Buchholz, J.T. and Blakeslee, A.F., Pollen tube growth and control of gametophytic selection in cocklebur, a 25-chromosome datura, *Bot. Gaz.*, 1930, pp. 366–383.

Buchholz, J.T., Determinate cleavage polyembryony, with special reference to *Dacrydium, Bot. Gaz.*, 1933, vol. 94, pp. 579–588.

Dogra, P.D., Observations on *Abies* pindrow with a discussion on the question of occurrence of apomixis in gymnosperms, *Silvae Genet.,* 1966, vol. 15, pp. 11–20.

Dogra, P.D., Seed sterility and disturbances in embryogeny in conifers with particular reference to seed testing and tree breeding in Pinaceae, *Stud. Forst. Suecica*, 1967, no. 45, pp. 1–97.

Dogra, P.D., Morphology, development and nomenclature of conifer embryo, *Phytomorphology*, 1978, vol. 28, no. 3, pp. 307–322.

Doyle, J. and Brennan, M., Cleavage polyembryony in conifers and taxads: a survey. I. Podocarps, taxads and taxodioids, *Roy. Dublin Soc. Sci. Proc. Ser. A*, 1971, pp. 57–88.

Doyle, J. and Brennan, M., Cleavage polyembryony in conifers and taxads: a survey. II. Cupressaceae, Pinaceae and conclusions, *Sci. Proc. Roy. Dublin Soc., A*, 1972, vol. 4, no. 10, pp. 137–158.

Erdelska, O. and Vidovencova, Z., Cleavage polyembryony in maize, *Sex. Plant Reprod.*, 1992, vol. 5, no. 3, pp. 224–226.

Erdelska, O. and Vidovencova, Z., Cleavage polyembryony in vivo and in vitro, *Biol. Plant.*, 1994, vol. 36, no. 3, pp. 329–334.

Fischer, C., Speth, V., Fleig-Eberenz, S., and Neuhaus, G., Induction of zygotic polyembryos in wheat: influence of auxin polar transport, *Plant Cell*, 1997, vol. 9, no. 10, pp. 1767–1780.

Gupta, P.K. and Durzan, D.J., Somatic polyembryogenesis from callus of mature sugar pine embryos, *Biotechnology*, 1986, vol. 4, pp. 643–645.

Gupta, P.K. and Durzan, D.J., Somatic embryos from protoplasts of loblolly pine proembryonal cells, *Nat. Biotechnol.*, 1987, vol. 5, no. 7, pp. 710–712.

Gupta, P.K. and Durzan, D.J., Loblolly pine (*Pinus taeda* L.), in *Trees*, Berlin: Springer, 1991, vol. 3, pp. 383–407.

Gupta, P.K., Durzan, D.J., and Finkle, B.J., Somatic polyembryogenesis in embryogenic cell masses of *Picea abies* (Norway spruce) and *Pinus taeda* (loblolly pine) after thawing from liquid nitrogen, *Can. J. For. Res.*, 1987, vol. 17, no. 9, pp. 1130–1134.

Haccius, B., Experimentally induced twinning in plants, *Nature*, 1955, vol. 176, no. 4477, pp. 355–356.

Holmersson, A., von Arnold, S., Burg, K., and Bozhkov, P.V., High stability of nuclear microsatellite loci during the early stages of somatic embryogenesis in Norway spruce, *Tree Physiol.*, 2004, vol. 24, pp. 1181–1186.

Ivanov, V.B., *Aktivnye krasiteli v biologii* (Reactive Dyes in Biology), Moscow: Nauka, 1982.

Jokinen, K.J. and Durzan, D.J., Properties of rescued embryonal suspensor masses of Norway spruce determined by the genotype and the environment in vitro, *Silva Fennica*, 1994, vol. 28, pp. 95–106.

Kruglova, N.N., Egorova, O.V., Sel'dimirova, O.A., Zaitsev, D.Yu., and Zinatullina, A.E., *Svetovoi mikroskop kak instrument v biotekhnologii rastenii* (Light Microscope as a Tool in Plant Biotechnology), Ufa: Gilem, Bashk. Entsikl., 2013.

Lelu-Walter, M.-A. and Pâques, L.E., Simplified and improved somatic embryogenesis of hybrid larches (*Larix* × *eurolepis* and *Larix* × *marschlinsii*). Perspectives for breeding, *Ann. For. Sci.*, 2009, vol. 66, p. 104.

La Rue, C.D. Studies on growth and regeneration in gametophytes and sporophytes of gymnosperms. *Brookhaven Symp. Biol.*, 1954, vol. 6, pp. 187–208.

Lutova, L.A., Ezhova, T.A., Dodueva, I.E., and Osipova, M.A., *Genetika razvitiya rastenii* (Genetics of Plant Development), St. Petersburg: Izd. N-L, 2010.

Nekrasova, T.P., *Biologicheskie osnovy semenosheniya kedra sibirskogo* (Biological Basics of Seed Production by Siberian Cedar), Novosibirsk: Nauka, 1972.

Pak, M.E., Ivanitskaya, A.S., Dvoinina, L.M., and Tret'yakova, I.N., Embryogenic potential of long-term proliferating *Larix sibirica* cell lines in vitro, *Sib. Lesn. Zh.*, 2016, no. 1, pp. 27–38.

Quinn, C.J., Gametophyte development and embryogeny in Podocarpaceae. III. *Dacrydium bidwilii, Phytomorphology*, 1966a, vol. 16, pp. 81–91.

Quinn, C.J., Gametophyte development and embryogeny in the Podocarpaceae. IV. *Dacrydium colensoi*: general conclusion, *Phytomorphology*, 1966b, vol. 16, pp. 199–211.

Rober-Kleber, N., Albrechtová, J.T.P., Fleig, S., Huck, N., Michalke, W., Wagner, E., Speth, V., Neuhaus, G., and Fischer-Iglesias, C., Plasma membrane H^+-ATPase is involved in auxin-mediated cell elongation during wheat embryo development, *Plant Physiol.*, 2003, vol. 131, no. 3, pp. 1302–1312.

Singh, H., *Embryology of Gymnosperms*, 1978.

Stasolla, C. and Yeung, E.C., Recent advances in conifer somatic embryogenesis: improving somatic embryo quality, *Plant Cell Tissue Organ Culture*, 2003, vol. 74, no. 1, pp. 15–35.

Titova, G.E., Sel'dimirova, O.A., Kruglova, N.N., Galin, I.R., and Batygina, T.B., Phenomenon of "Siamese embryos" in cereals in vivo and in vitro: cleavage polyembryony and fasciations, *Russ. J. Dev. Biol.*, 2016, vol. 47, no. 3, pp. 122–137.

Tret'yakova, I.N., *Embriologiya khvoinykh: fiziologicheskie aspekty* (Embryology of Conifers: Physiological Aspects), Novosibirsk: Nauka, 1990.

Tret'yakova, I.N. and Barsukova, A.S., Somatic embryogenesis in in vitro culture of three larch species, *Russ. J. Dev. Biol.*, 2012, vol. 43, no. 6, pp. 353–361.

Tret'yakova, I.N. and Shuvaev, D.N., Somatic embryogenesis in *Pinus pumila* and productivity of embryogenic lines during long-term cultivation in vitro, *Russ. J. Dev. Biol.*, 2015, vol. 46, no. 5, pp. 276–285.

Tret'yakova, I.N. and Voroshilova, E.V., Embryo initiation from *Pinus sibirica* megagametophytes in in vitro culture, *Russ. J. Dev. Biol.*, 2014, vol. 45, no. 2, pp. 93–100.

Tret'yakova, I.N., Ivanitskaya, A.S., and Pak, M.E., The productivity of embryogenic cell lines and their somaclonal variability in Siberian larch in vitro, *Lesovedenie*, 2015, no. 1, pp. 27–35.

Tret'yakova, I.N., Pak, M.E., Ivanitskaya, A.S., and Oreshkova, N.V., Peculiarities of somatic embryogenesis of long-term proliferating embryogenic cell lines of *Larix sibirica* in vitro, *Russ. J. Plant Physiol.*, 2016, vol. 63, no. 6, pp. 800–810.

Vondráková, Z., Eliášová, K., Fischerová, L., and Vágner, M., The role of auxins in somatic embryogenesis of *Abies alba, Centr. Eur. J. Biol.*, 2011, vol. 6, no. 4, pp. 587–596.

Williams, E.G. and Maheswaran, G., Somatic embryogenesis: factors influencing coordinated behaviour of cells as an embryogenic group, *Ann. Bot.*, 1986, vol. 57, no. 4, pp. 443–462.

Yakovlev, M.S. and Snegirev, D.P., Effect of growth substances on the formation of polyembryonic grains in wheat, *Bot. Zh.*, 1954, vol. 39, no. 2, pp. 187–194.

Translated by M. Shulskaya