SOMATIC EMBRYOGENESIS IN *LOLIUM MULTIFLORUM* SUSPENSION CULTURE

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ABSTRACT

The embryogenic cell suspension was obtained from immature embryos of *Lolium multiflorum* through a callus culture. Somatic embryogenesis was induced by addition of 2,4-D, dicamba and picloram in 0,5 mg/l concentrations in MS liquid nutrient medium. It was shown that somatic embryos arised from single cells. In globular embryoids, the meristematic cells are characterized by the presence of phytoferritin inclusions in the leucoplasts.

KEY WORDS: immature embryo, suspension culture, ryegrass, somatic embryo, phytoferritin.

INTRODUCTION

Application of cell-engineering technologies in selection of fodder grasses is limited by low regenerative capacity *in vitro*. Therefore, it is necessary to improve the biotechnological methods for induction and supporting the long term embryogenic state in both callus and suspension cultures.

Induced morphogenesis and somatic embryogenesis are known to be the common regeneration ways *in vitro* for obtaining plants in all cereal grasses (Vasil, Vasil, 1984). Somatic embryogenesis is considered a most effective method of clonal propagation of plants. Although there are numerous investigations of a somatic embryogenesis phenomenon in different species, only restricted studies have been made with ryegrass, the main fodder culture in temperate climate. Conditions for obtaining embryonic suspension cultures of ryegrass and the somatic embryo ultrastructure were the goal of this paper.

MATERIAL AND METHODS

Callus was obtained from immature embryos of *Lolium multiflorum* Lam, cv. Volodar plants grown in the field. Caryopses were collected at 12-14 days after artificial pollination, sterilized in diluted (1:4 v/v) aqueous solution of commercial Chlorox for 20 min and rinsed twice in sterile distilled water. The isolated embryos were placed with the scutellum upward in Petri dishes with agar nutrient medium of Murashige and Skoog (1962) supplemented with 5 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D), 1 g/l casein hydrolisate, 100 ml/l (v/v) coconut water and 7 g/l agar. Pieces of formed callus were transferred to the liquid medium with the same concentration of growth substances and were shaken (100 rpm/min) at 24°C under slight illumination (100 luxes). After

3-4 weeks the primary dense suspension consisted of large cell clumps settled in the bottom and supernatant containing single cells and small cell clusters. After removing the cell aggregates the remaining suspension contained separate cells. One part of them was of meristematic type; such cells were 20-30 µm of size, single or assembled in small aggregates from 6-10 ones; they had an izodiametric shape, a dense cytoplasm and a large nucleus. The other part was represented by cells of 120-150 µm size with large vacuoles. These cells, growing by elongation, were removed from the suspension using a selective method of increasing osmotic pressure of the medium. For this 5 ml aliquots were transferred into 50 ml flasks with 5 ml MS medium with 10 % saccharose (MS1). The large vacuolated cells were destroyed in the conditions of such a medium, and the suspension was enriched with a population of small cells of meristematic type (Fig. 1). After three days the supernatant was removed from the flasks and fresh liquid medium containig 2,4-D, dicamba and picloram in concentration of 0,5 mg/l accordingly, 3% saccharose, 15% coconut water and 20 mg/l AgNO₃ (MS2) for induction of somatic embryogenesis was added into the flasks.

For electron-microscopic study the samples from the sediment in the flasks were fixed for 2 h in 1% glutaraldehyde in 0,1 M phosphate buffer pH 7,2, then in 2% OsO₄ in the same buffer for 12 h. Standard dehydration was performed in ascending concentrations of ethanol and acetone followed by embedding in a mixture of Epon and Araldite. The ultra-thin sections were stained with lead citrate and examined in the transmission electron microscope JEM-1200 EX.

RESULTS AND DISCUSSION

Callus began to form on the scutellum of isolated immature embryos at 5th or 6th day of cultivation on the MS agar me-

Fig. 1. Embryogenic cells from the selected suspension in MS1 media.

Fig. 2. Portion of a callus surface with somatic embryos.

Fig. 3. Transversal section of a somatic globular embryo from the MS2 medium.

Fig. 4. Leucoplast with the phytoferritin inclusion. Bar = 200 nm
x 40000

Figs 5-7. Highly organized "crystalline" inclusions of phytoferritin.

Fig. 5. Bar = 100 nm
x 120000

Fig. 6. Bar = 100 nm
x 120000

Fig. 7. Bar = 100 nm
x 230000

dium. After a month of cultivation two types of calli have been formed: compact, nodulated of white colour and 2) friable, light-yellow. Sometimes, a formation of somatic embryo was observed directly at the callus surface (Fig. 2).

The selection cell suspension after two-week growth in MS2 contained numerous globular somatic embryos. The next differentiation of somatic embryos occurred after its transferring into hormone-free medium. Globular embryoids of different size had a similar internal structure. The central part of embryoids consisted of differentiating, small meristematic cells with large nuclei occupying 2/3 of the cell volume; the nuclei contained a considerable amount of condensed chromatin and 1-2 nucleoli. There were small vacuoles, mitochondria with well developed cristae, plastids with starch grains and numerous ribosomes. These ultrastructural characteristics indicate that the cells are in an active metabolic state. The rows of 3-5 cells dividing synchronously and cells at different phases of mitosis were observed at transversal sections of the embryoids. The peripheral part of the embryoids consisted of large cells with a central vacuole and a narrow layer of the cytoplasm (Fig. 3).

As a rule, 3-4 meristematic cells in the section of the globular embryoid had plastids with crystalline structures (Fig. 4). The number of these inclusions in the leucoplast varied from 1 to 4. The inclusions consisted of electron-dense particles 100-120 Å in diameter. particles were arranged in a regular 2 "crystalline" order (Figs 5-7).

The inclusions in leucoplasts of globular embryoids were structurally identical with deposits of phytoferritin, described in chloroplasts and leucoplasts in dicotyledons and monocotyledons (Hyde et al., 1963; Wildman, Hunt., 1976). Phytoferritin is assumed to be a reserve, which is utilized by developing plastids. The first phytoferritin deposits occur in leucoplasts of globular embryoids. We can assume that phytoferritin appearance is a sign of normal differentiation of the somatic embryos.

It is arguable if the determination of somatic embryo development is realized in a single cell or in cell clusters. A for-

mation of embryoids *in vitro* from one or several neighbouring cells is assumed to depend on their synchronous embryogenic state and their capacity to interact more effectively in the group. In the carrot cell culture the determination of somatic embryoid development occurs in aggregates of 6-10 cells (Moiseeva, 1991). Such aggregates are considered to be the early stages of embryoid development. Embryonic ryegrass cells contain lipid droplets, protein bodies and plastids with starch grains; carrot nonembryogenic cells contain no starch grains and no protein bodies (Ross, Murphy, 1993). As it is shown by us, embryoids in the ryegrass cell suspension of immature embryo origin arise from single cells.

Thus, a simultaneous addition of growth regulators (2,4-D, picloram and dicamba) to a nutrient medium effectively induces somatic embryogenesis. Somatic embryos arise from single cells. Phytoferritin inclusions in the leucoplasts are a characteristic feature of somatic embryos at globular stage.

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SOMATYCZNA EMBRIOGENEZA W HODOWLI ZAWIESINOWEJ *LOLIUM MULTIFLORUM*

STRESZCZENIE

Z hodowli kalusa powstałego z niedojrzałych zarodków *Lolium multiflorum* otrzymano zawiesiny embrionnych komórek. Somatyczna embriogeneza była indukowana dodatkiem 2,4-D, dicamba i picloram w koncentracji 0,5 mg/l do płynnej pożywki MS. Pokazano, że somatyczne zarodki rozwijały się z pojedynczych komórek. Merystematyczne komórki globularnych embrioidów charakteryzują się obecnością fitoferrytowych inkluzji w leukoplastach.

SŁOWA KLUCZOWE: niedojrzały zarodek, hodowla zawiesinowa, rajgras, embrion somatyczny, fitoferrytyna.