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Cytological study on wheat (*Triticum timopheevi* Zhuk.) protoplasts

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Abstract

Protoplasts were obtained from tetraploid wheat (*Triticum timopheevi* Zhuk.) suspension culture by incubation in solution of 1 % pectinase 500, 1 % driselase and 1 % cellulase and cultivated in Schenk and Hildebrandt medium. Freshly isolated protoplasts contained dense cytoplasm and constricted organellae exhibited negative contrast of their membranes. Together with normal protoplasts huge multinucleate protoplasts were present in the population. 3 h after plating, the cytoplasm showed normal appearance, the negative contrast of membranes was not evident any longer. Cisternae of endoplasmic reticulum and Golgi apparatus were numerous. There were some vesicles and fibres on the protoplast surface. 8 d after plating, many dividing cells were found out and cell clumps arosen in this way were present in the culture. Some of the protoplasts particularly those originally multinucleate ones were upset.

Introduction

Procedure of protoplast isolation has been established for many plant species. However the cultivation of protoplasts of such important food crops as cereals remains still difficult. Only limited success was achieved in isolation and cultivation of wheat protoplasts, as well. In some cases protoplasts were capable of cell wall regeneration, sustained division and colony formation (Li *et al.* 1980, Sethi and Maeda 1983, Bandyopadhyay and Ghosh 1986).

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Abbreviations: Ch - chromosome; Cw - cell wall; D - dictyosome; Er - endoplasmic reticulum; M - mitochondria; N - nucleus; No - nucleolus; P - plastid; SEM - scanning electron microscopy; TEM - transmission electron microscopy; V - vacuole;

Recently shoots, roots or whole plants were obtained from such colonies (Maddock 1987, Hayashi and Shimamoto 1988, Harris *et al.* 1988, Vasil *et al.* 1990). Cytological studies on wheat protoplasts are absent.

Material and methods

Protoplasts were obtained from suspension culture initiated from a callus derived from immature embryos of tetraploid wheat *Triticum timopheevi* Zhuk. Cell suspension was cultured in medium of Schenk and Hildebrandt (1972) with 1 mg l^{-1} 2,4-D and 0,1 mg l^{-1} kinetin. For protoplasts isolation cells of 2-d-old culture were incubated in solution consisting of 1 % pectinase 500 1 % driselase (*Sigma*), 1 % Cellulase (*Onozuka R-10*), 0.25 M mannitol and 0.105 M $CaCl_2$ on rotar shaker (1 rps) for 6 h in dark at 22 °C. Released protoplasts were filtrated through a nylon sieve (28 μm pore size) and centrifugated at 100 *g* for 4 min. Pelleted protoplasts were washed twice in solution containing 0.25 M mannitol and 0.105 M $CaCl_2$ and collected always by centrifugation at the conditions mentioned above. Protoplasts were cultured in Schenk and Hildebrandt medium containing 0.4 M saccharose, 1 g l^{-1} 2,4-D and 0.1 mg l^{-1} kinetin in the dark at 25 °C.

For TEM settled cells and cell clumps of suspension culture were fixed with 1 % glutaraldehyde in 0.05 M phosphate buffer for 1 h and then with 3 % glutaraldehyde in the same buffer for 3 h, postfixed with 1 % OsO_4 in phosphate buffer overnight at 4 °C, dehydrated in ethanol and embedded in Araldite resin. Protoplasts taken before plating (0 h), 3 h and 8 d after plating were fixed for 1 h with 1 % glutaraldehyde in washing (0 h) or culture medium (3 h and 8 d) and then for 3 h with 3 % glutaraldehyde in the same medium. After washing with the appropriate solutions protoplasts were fixed with 1 % OsO_4 in 0.05 phosphate buffer overnight at 4 °C and dehydrated in ethanol. Last portion of ethanol was saturated by uranylacetate. Every time protoplasts were collected by centrifugation at 100 *g* for 4 min. After infiltration the protoplasts were embedded in Araldite. Ultrathin sections were prepared on *LKB Nova III* ultratome, stained by uranylacetate and lead citrate and examined in a *Tesla BS 613* or *JEM 2 000 FX* electron microscopes. Semithin sections were stained by toluidine blue and basic fuchsine (Lux 1981). For SEM protoplasts were fixed as for TEM. After dehydration by ethanol they were dried in critical drying apparatus, attached to aluminum strip, coated with gold and investigated on a *JEM 2 000 FX* microscope.

Results and discussion

During exponential phase of growth the wheat suspension was composed of small or larger cell clumps. Cells located at the periphery of clumps were ussually in the interphase and contained central vacuoles. The cells placed in the central parts of clums were more cytoplasmic (Fig. 1a) and often dividing. Cytoplasm of cells was rich in cell structures concentrated mainly arround nuclei. These organellae were round or oval in the shapes and contained usually one compact or ring shaped

nucleolus together with numerous heterochromatin clumps (Fig. 1a). Plastids were present in the form of protoplastids with small vesicles and infrequent plastoglobuli. Many of them exhibited irregular, cup shapes in the sections (Fig. 1b). This type of plastids is typical also for other cells grown in cultures (Jásik and Hudák 1987 and other). Mitochondria were round or oval and possessed well defined cristae. Dictyosomes, profiles of endoplasmic reticulum and ribosomes were frequent. Between cells in clumps there were present differently thick cell walls without plasmodesmata. Generally the ultrastructure of cultured wheat cells was very similar to that found in other cell cultures occurring in the exponential growth phases.

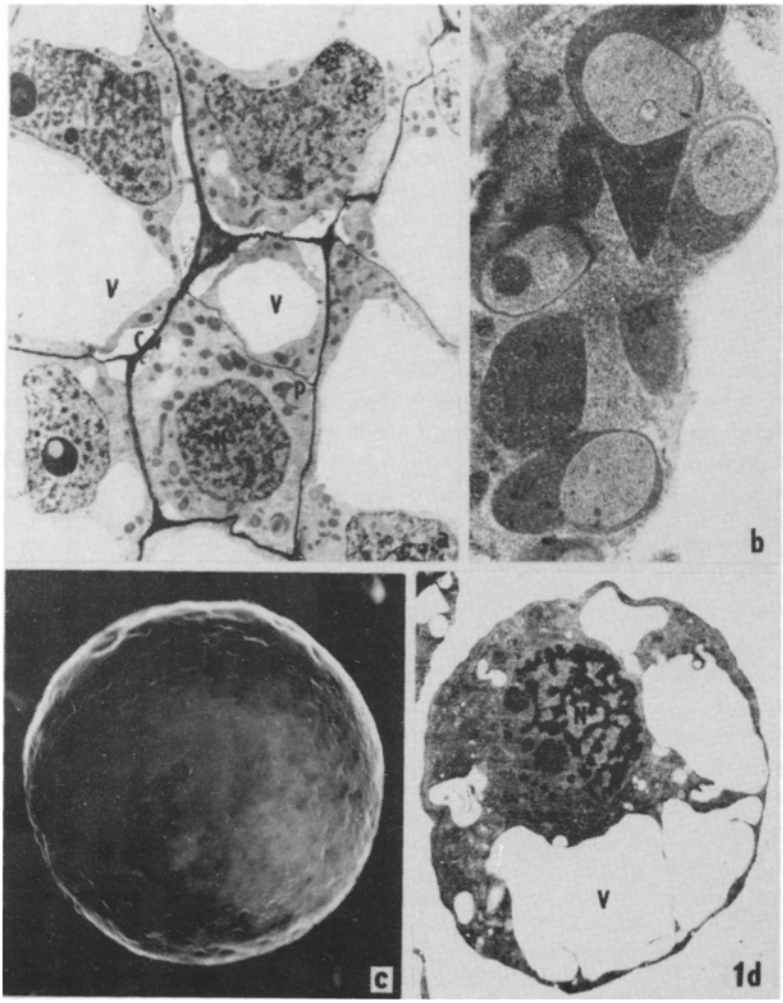

Fig. 1a. Cells in the central part of cell clumps (× 2 000)
1b. Cup shaped plastids in cells of suspension culture (× 14 200)
1c. Freshly isolated protoplast (SEM) (× 2 200)
1d. Freshly isolated protoplast (TEM) (× 2 700)

Freshly isolated protoplasts were round, without striking folds of plasmalema (Fig. 1c,d). Surface of protoplasts was smooth and no fibrils were found out by SEM (Fig. 1c). Size of protoplasts was about 20 μm. However during isolation protoplasts often fused together and huge protoplasts with increased number of nuclei could be seen in population (Fig. 2b,c).

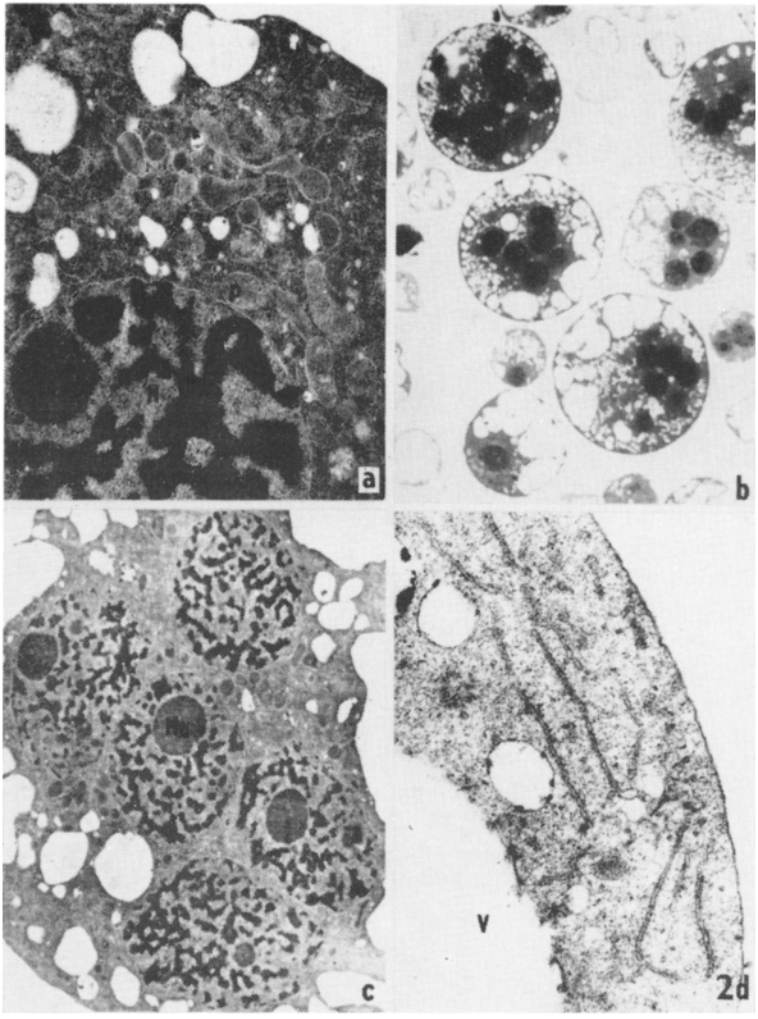

Fig. 2a. Organellae with negative contrast membranes in dense cytoplasm of freshly isolated protoplast (× 6 600)
2b. Semithin section through protoplasts with different number of nuclei (× 330)
2c. Part of cytoplasm with several nuclei (× 2 200)
2d. Well developed cisternae of endoplasmic reticulum in the protoplast 3 h after plating (× 12 800)

The size of these multinucleate protoplasts was much as 70 μm. Spontaneous fusion of protoplasts is well documented (Power *et al.* 1971, Withers and Cocking 1972). According to last authors in the case of protoplasts isolated from leaf tissue the symplastic nature of plant tissue may be the reason of spontaneous fusion. During wall degradation the plasmodesmatal connection between cells enlarge, cytoplasm of neighbouring cells are mixed and multinucleate protoplasts gradually arose in this way.

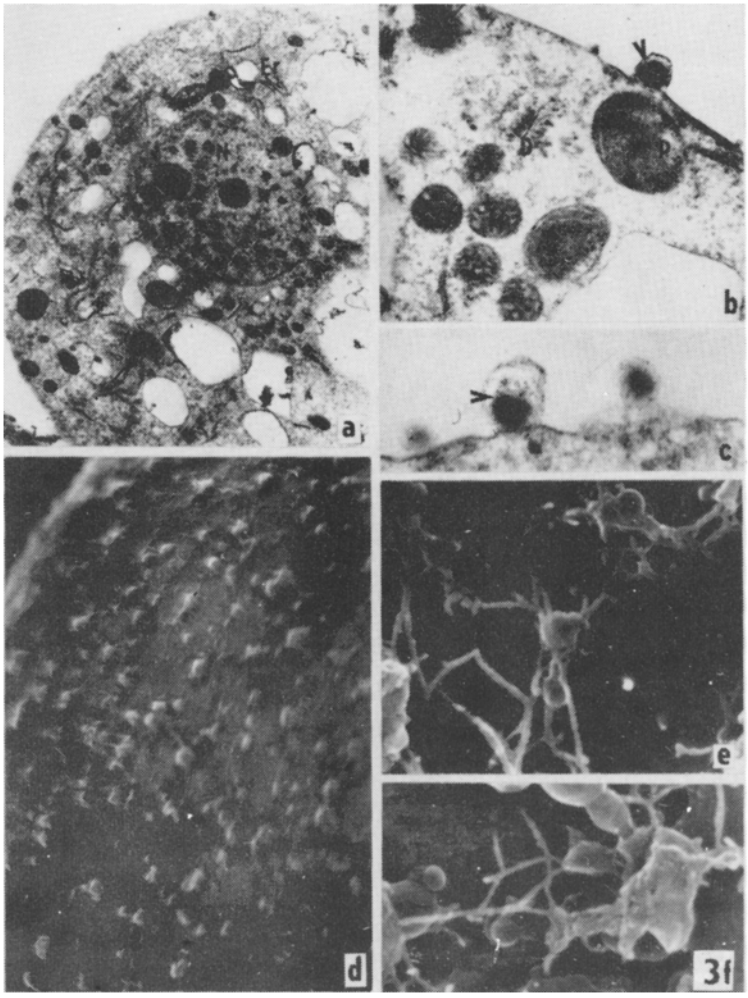

Fig. 3a. Protoplast 3 h after plating (× 4 300)
3b. Dense organellae 3 h after plating, there is a vesicle (arrow) on protoplast surface (× 13 000)
3c. Vesicles on the surface, one of them possess some crystaline like substance (arrow) (× 44 900)
3d. Distribution of vesicles on protoplast (3h, SEM) (× 12 000)
3e. Poor fibril network on the protoplast (3h) (×.45 800)
3f. Fibrils on protoplast surface (3h, SEM) (× 43 300)

However the fusion of protoplasts isolated from cultured cells can not be explained so simply because of usual absence of the plasmodesmata between such cells. On the other hand there are many factors reported to induce aggregation and fusion of protoplasts. Perhaps centrifugation as well as the presence of some salts play a noticeable part in fusion of wheat protoplasts.

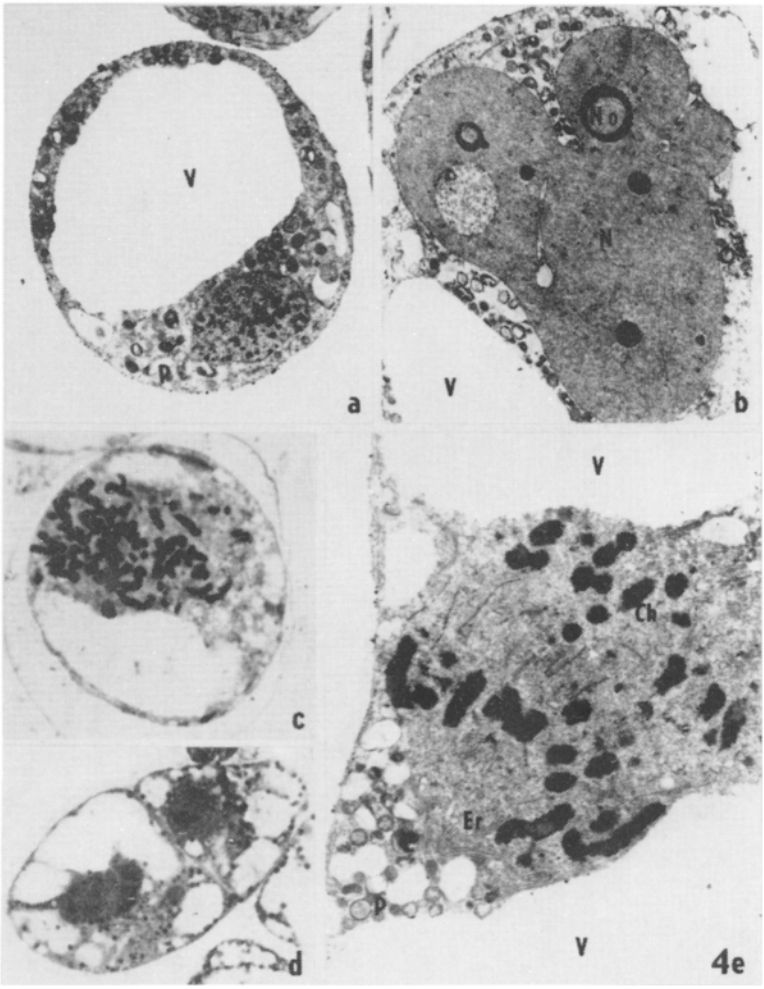

Fig. 4a. Cell derived from protoplast 8 d after plating (× 1 850)
4b. Degrading protoplast 8 d after plating, nuclei had fused before (× 3 100)
4c. Dividing cell derived from protoplast (semithin section) (× 1 250)
4d. Couple of cells originated from protoplast by division (semithin section) (× 750)
4e. Detail of phragmosome with early anaphase chromosomes (× 3 900)

Cytoplasm of the protoplasts was more electrondense than that in suspension cells. Nuclei were located in cytoplasmic thickenings and they were surrounded by

numerous cell organellae (Fig. 1d, 2a). There were highly electrondense clumps of heterochromatin and somewhat less electrondense, compact nucleoli in the nuclei (Fig. 2a,c). Plastids and mitochondria constricted in shapes showed an increase in their matrix density and they were hard distinguishable from the cytoplasm (Fig. 2a). At the same time membranes of protoplasts exhibited negative contrast (Fig. 2a). Similar features were stated for other freshly isolated protoplasts (Gigot *et al.* 1972, Nagata and Yamaki 1973, Taylor and Hall 1978, Protsenko and Radzievskaya 1982) and they can be connected with the plasmolysis of protoplasts caused by placing them in an osmoticum during isolation. Another signs noted by Takebe *et al.* (1973), Gigot *et al.* (1975), Taylor and Hall (1978) as occurrence of osmiophilic droplets in cytoplasm, fragmentation of plastids or formation of crystaline like bodies in plastids were not evident in protoplasts derived from wheat suspension culture.

3 h after plating cytoplasm exhibited normal density (Fig. 2d, 3a) although plastids and mitochondria were fairly electrondense (Fig. 3b). Negative contrast of membranes was not evident any longer. Number of cisternae of endoplasmic reticulum as well as Golgi complex were increased (Fig. 2d, 3a,b) and many vesicles derived from their margins. Some large vesicles jut from the surface of protoplasts (Fig. 3b,c,d). These vesicles varied in size and several of them seemed to be filled with crystalline deposits (Fig. 3c). At the same time separate fibrils and poor fibril network became evident on the surface of protoplasts by SEM (Fig. 3e,f). Appearance of wheat protoplasts after plating reflected their return to more favourable osmotic conditions as well as beginning of the cell wall synthesis. It has been found out this process starts shortly after plating of protoplasts in adequate culture conditions and the first fibres may by detected by SEM within 10-20 min (Williamson *et al.* 1977). More or less complete network arround protoplasts is formed, however, after several hours lag period depending on genus, source of tissue or isolation conditions (Franz and Blashek 1985). Precise pattern of cellulose fibrils synthesis is still matter of dispute. It is obvious, precursors of cell wall are synthetised inside of protoplasts by cisternae of Golgi apparatus and perhaps by endoplasmic reticulum, as well. Both organellae were numerous in the wheat protoplasts 3 h after plating. Vesicles derived from them usually designated as coated fuse gradually with plasmalemma and the content (precursors or enzymes) is released outward (Franz and Blaschek 1985). On the other hand in the case of wheat protoplasts whole vesicles were evidently separed from plasmalemma and they were located outside the protoplasts. According to Belitser *et al.* (1977) surface membranes of such vesicles are formed from plasmalemma which is replaced by membranes of vesicles derived from endoplasmic reticulum or Golgi apparatus. In this way coated vesicles could take place in renovation of plasmalemma.

On the 8th day after plating protoplasts were usually spherical or slightly elongated. Cytoplasm was reduced on layer alongside the cell wall and the central vacuole occupied a great part of the nondividing cells (Fig. 4a). Cytoplasm possessed numerous cell organellae. Nuclei were located in peripheral cytoplasm and contained dispersed chromatin and ring shaped or compact nucleoli. Plastids were present as proplastids or amyloplasts. Mitochondria had well developed cristae. Abundant profiles of endoplasmic reticulum were placed alongside the cell walls. Cell couples or clumps as a consequence of cell division were formed in suspension (Fig. 4d).

Dividing cells contained phragmosomes occupying larger or smaller parts of their volumes and karyokinesis as well as cytokinesis carried out in these central masses of cytoplasm (Fig. 4c,e). Phragmosomes were rich in the cell organellae located at their peripheries mainly arround the poles of mitotic spindles (Fig. 4e). Cell divisions were equal or unequal in the dependence on position of the phragmosomes. The planes of divisions were randomly arranged and cell clumps grew unorganisely in suspension. Pattern of division of regenerated wheat protoplasts was normal. Also according to Fowke *et al.* (1974) protoplasts do not exhibit any abnormalities in division in comparison with the cells *in vivo*. Many cells, in particular originally multinucleate one did not, however, divide and gradual degradation of their organellae were observed (Fig. 4b).

Cytological changes of protoplasts can be explained as their preparation for division. Enlargment of cytoplasm content together with amplification of cell organellae were characteristic features of this stage (Fowke *et al.* 1974, Gigot *et al.* 1975). Striking reprogramming during first days of culture can be observed mainly in the case of mesophyll leaf protoplasts (Nagata and Yamaki 1973, Gigot *et al.* 1975, Belitser *et al.* 1977, Cobianchi *et al.* 1983). These protoplasts convert their autotrophic way of life into heterotrophic one and ultrastructural changes of cell organellae mainly plastids reflected the modification of metabolic pathway. Protoplasts derived from wheat cultured cells did not show such drastic changes in their inner organisation after plating.

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