CYTOHISTOLOGICAL ANALYSIS OF SOMATIC EMBRYOGENESIS
IN CUCUMBER (*CUCUMIS SATIVUS* L.).
I. COMPARISON OF CELL SUSPENSION CONTAINING AND LACKING
NATURAL FLUORESCENCE WITH IN VIVO DEVELOPING EMBRYOS

J.A. TARKOWSKA, D. BRZOSTECKA, W. BURZA¹, S. MALEPSZY¹

Department of Plant Anatomy and Cytology,
Warsaw University, Krakowskie Przedmieście 26/28
00-927 Warsaw, Poland

¹Department of Plant Genetics and Breeding,
Warsaw Agricultural University, Nowoursynowska 166
02-766 Warsaw, Poland

(Received: August 10, 1994. Accepted: November 21, 1994)

ABSTRACT

Under in vivo conditions early-globular embryos occur in cucumber on the 9th day after pollination, heart-shaped ones on the 14th, and morphologically mature embryos appear on the 19th day. Single starch grains already appear in the cells of the globular embryo, and in the heart-shaped one they occur within the forming root cap. In the morphologically mature embryo only the precambium is free from starch. Somatic embryogenesis (SE) in suspension occurs similarly as in vivo, even though the starch localization is somewhat different and torpedo-like embryos occur, which are not observed in vivo. The histological structure of in vitro embryos is similar to in vivo ones, and the greatest morphological difference are the poorly developed cotyledons and their variable number (1 to 3). Aggregates showing fluorescence were found to be composed of cells which differ in morphology from cells not showing fluorescence and appear to be more capable of attaining the mature stages.

KEY WORDS: cell suspension, somatic embryogenesis, *Cucumis sativus*, zygotic embryos, natural fluorescence.

INTRODUCTION

Somatic embryogenesis in in vitro cultures is being observed in an increasing number of monocots and dicots (Evans et al., 1983) and trees (Bonga and Durzan, 1987). This process is being intensively investigated due to the significance of somatic embryos (SE) for biotechnology. The aim is to use SE as a means of vegetative reproduction on a large scale in a technology called somatic seeds. The cucumber is a plant for which such a technology would be desirable (Malepszy et al., 1993).

Somatic embryogenesis in vitro in the cucumber was described for the first time for a culture initiated from leaves (Malepszy et al., 1982) and anthers (Lazarte and Sasser, 1982). Later (Nadolska-Orczyk and Malepszy, 1987) cytological events occurring during the first 7 weeks of leaf explant culture were analyzed on two media (differing in the amount of auxins, stimulating the formation of a characteristic gel-like callus). In suspension culture cucumber somatic embryogenesis was observed for the first time by Malepszy and Solarek (1986), while Burza (1992) observed the occurrence of two types of aggregates in cucumber suspensions. One of them showed a natural green fluorescence, the other one did not. He also found (Burza, unpublished data) that zygotic em-

bryos do not show a fluorescence high enough to be detected under a fluorescent microscope.

The aim of this work was to perform a preliminary cytological characterization of the development of both these aggregates and to compare their development to that of a zygotic embryo.

MATERIALS AND METHODS

Fixation was done in dichromoformalin (0,2-20) or in chromoacetoformalin (CrAF 0.5-1-20). Microtome preparations were stained with Ehrlich's haematoxylin and the PAS reaction for carbohydrates was used (Filutowicz and Kuźdowicz, 1951).

The material for investigations were embryos from immature seeds and a 12-14 month suspension culture of the same inbred cucumber obtained from a monoecious field variety „Borszczagowski” (B). Seeds were taken from manually pollinated plants growing in the greenhouse, at three dates – 8, 14 and 19 days after pollination which, respectively, corresponded to globular, heart-shaped and cotyledon stages (the last stage is a morphologically mature embryo).

The suspension culture was performed under previously determined conditions (Wróblewski and Malepszy, 1992). This was a one-year established culture (8 days after passage) which was inoculated onto a medium stimulating embryo growth.

The material was taken after 4, 8 and 12 days after plating into media choosing two fractions – with and without natural fluorescence.

RESULTS

1. Embryos in vivo

An early globular embryo (9 days after pollination) is built of undifferentiated meristem cells surrounded by a 1-layer protodermis (Fig. 1). Gradually, via an early heart stage, with a clear change in cell shape and the polarization of the direction of their division the embryo assumes a heart shape (14 days after pollination (Fig. 2).

Fig. 1. In vivo cucumber globular embryo: p – protoderm, starch grains – arrows (CrAF, PAS, 900x).

Fig. 2. In vivo cucumber heart-shaped embryo differentiated morphologically: pm – differentiated central cylinder of radicle, s – statolyte starch (CrAF, PAS, 400x).

Its symmetry changes from radial to bilateral and simultaneously histological differentiation into protoderm, embryonic cortex and central cylinder takes place. During this phase of

development the primordium of the radicle (with root cap) can be seen, its central part is taken up by elongated cells of the differentiating procambium. The remaining part of the embryo is made up of isodiametric parenchyma cells. In two distinctly visible cotyledons intense and directional divisions lead to the formation of two strong cotyledons. The mature cucumber embryo (19 days after pollination) is morphologically (radicle, hypocotyl, plumule and cotyledons) and anatomically (preepidermis, preparenchyma and precambium) differentiated. All distinct cells are of the meristemic type.

The location of starch is changed concomitantly with embryo development. Single grains already occur in embryos of the globular stage (Fig. 1) and they occur in the heart-shaped ones in the cells of the forming root cap (statolyte starch) and slightly higher, mainly in the subepidermis (Fig. 2). In the mature embryo only the procambium is free from starch and therefore is an excellent indicator of localization. In the preparenchyma the cells are filled with very numerous starch grains.

2. The nonfluorescent fraction of cell suspension.

Cell aggregates can differentiate in two ways: (1) intense cell proliferation, some of which may secondarily sporadically develop into embryos; (2) directed proliferation, leading directly to the formation of globular and heart-shaped embryos.

Early determination of the cells' fate is difficult, as in both cases small cells with dense protoplasm are seen. The analysis of starch presence in the cells, however, suggests that small grains occur only in the cells which will undergo embryogenesis. Starch is present in all cells in the already clearly forming globular embryo. The number of grains is particularly increased in the cortical parts which is especially clearly visible in the heart-shaped embryo, whose elongating central cells do not accumulate starch to any appreciable extent (Fig. 3).

Fig. 3. Fraction without fluorescence and adventive embryos: a – globular embryo, b – heart-shaped embryo. Starch grains particularly in the cortical layers of both embryos (dichromoformalin, PAS, 130x).

In this fraction the so-called secondary embryogenesis described by Williams and Maheshvari (1986) was observed (Fig. 3).

3. Fraction of the suspension with green fluorescence.

Within a multicellular aggregate two different types of cells are present. Inside, and sometimes at the margins of the aggregate larger, oval, vacuolated cells with a parietal nucleus are found. These cells generally do not contain starch. The second type are smaller cells, with a dense cytoplasm, centrally located nucleus and in general with few starch grains. Clusters of these cells may form protrusions under the top layer. This is the first stage of forming embryo structures which are capable of further development (Fig. 4).

Fig. 4. Fraction with fluorescence- embryos differentiation within aggregate; cells with small starch grains- arrows, vacuolated cells -* (dichromoformalin, PAS, 250x)

Fig.5. Fraction with fluorescence-globular embryo, many starch grains in the central cells are present (CrAF, PAS, 500x).

In the young globular embryo central cells generally accumulate starch grains (on the average 10 per cell), less commonly the starch occurs in the subprotodermis (Fig. 5). The embryo grows fast (particularly its cortical parts) and attains a

form similar to heart-shaped. The location of starch undergoes distinct changes. In heart-shaped embryos starch is deposited in the cortical parts, sometimes only 2-3 layers under the protodermis, and the central parts are free from grains. Analogous changes and starch localization occur in embryos from the fraction without fluorescence (see Fig. 3).

Within spherical aggregates with green fluorescence a strong proliferation of cells was observed, leading to a distinctly layered structure. Such structures in general do not undergo embryogenesis and starch grains occur only sporadically (Fig. 6).

Fig. 6. Fraction with fluorescence-cell proliferation within aggregate, led to a distinctly layered structure. Starch grains occur only sporadically- arrow (dichromoformalin, PAS, 150x).

In further development the heart-shaped embryo may develop into a form similar to a torpedo. This form does not occur in cucumber embryogenesis *in vivo*. The torpedo-shaped embryo is morphologically and histologically differentiated and the observed anomalies above all concern the number and structure of cotyledons.

The hypocotyl is overgrown (Fig. 7) to various degrees (elongated). Two very short cotyledons may differ in degree of development, as well as three cotyledons may develop or only one. In torpedo embryos starch is accumulated in cells of the cortical part of the cotyledon and its amount depends on the degree of cotyledon development.

A characteristic trait of the oldest embryos analyzed is that in the cotyledon part of the explants red chlorophyll fluorescence can be observed. The remaining part of the embryo emits green fluorescence. In general such embryos are morphologically and histologically differentiated, similarly to embryos *in vivo*, but the cotyledons are considerably reduced. In the cap-covered root primordium (with statolyte starch grains) three histogens are present (dermatogen, periblem, plerom). On the opposite pole a plumule is formed between very short and underdeveloped cotyledons. Between these two polar structures a relatively long hypocotyl develops in which within the precambium conducting cells differentiate.

A preliminary comparison of the size of embryos (Table 1) indicates that somatic embryos in the globular and early heart stages differ considerably in size. Embryos with fluorescence in general appear to be larger. The length of morphologically mature embryos was similar.

7

Fig. 7. Fraction with fluorescence-torpedo shape like embryo, pm- cambium, l - very short cotyledons, its cortical cells accumulate starch (CrAF, PAS, 100x).

DISCUSSION

Starch grains are storage material accumulated during cucumber embryogenesis both in vivo and in vitro. In 9-day embryos in vivo single starch grains are present in most cells, but already in the early heart stage they group in the radicle primordium and above - gradually in 1-2 layers beneath the protodermis. In the morphologically mature 19-day embryo starch fills all cells of the parenchyma. Similar changes in starch location take place in embryos in vitro regardless of whether the suspension shows fluorescence. It seems that the presence of starch grains are determinant of the site of embryo formation in aggregates, similarly as was shown by Halperin (1986) for carrot.

The principal difference between the fractions with and without fluorescence concerns the homogeneity of cells forming aggregates from which embryos differentiated. In fluorescent aggregates two types of cells occurred, and in non-fluorescent ones the cells of the forming embryo and the remaining part of the aggregate appeared similar. These dif-

ferences do not appear to exert a significant influence on the embryo formation, however its role seems to be connected with the conversion of embryos into plants. Plants were mainly found to be formed from embryos which show fluorescence (Burza, unpublished). This is so, even though the embryos of both fractions do not differ histologically and also have poorly developed cotyledons.

Underdevelopment of cotyledons appears to be a characteristic trait of most cases of cucumber somatic embryogenesis (Malepszy et al., 1982; Ziv and Gadasi, 1986; Punja et al., 1990). Only in one experiment (Malepszy and Solarek, 1986) in the same line B used for this work were embryos found with giant cotyledons typical for the *Cucurbitaceae*.

ACKNOWLEDGEMENTS

The authors are indebted to M. Sc. B. Bieniak for preparation of photographs. The experiment was partly sponsored by grant 501 40822.

TABLE 1. The comparison of dimensions (μm) between cucumber zygotic embryos and cell suspension derived somatic embryos.

Stage of development	Zygotic embryos	Somatic embryos	
		without fluorescence	with fluorescence
Globular	51,92 49,4 - 54,2	50/57 also 160/178 s.a./ l.a.	57/57 also 270/210 s.a./ l.a.
Early heart	137/148	70/86	312/425
Heart	-	197/228 e.a./c.a.	1125/195 s.a./l.a.
Morphological mature	2503 w.e.l.	-	2800 w.e.l.

Short axis- s.a.; long axis- l.a.; whole embryo length- w.e.l.; cotyledon axis- c.a.; embryo axis- e.a.

LITERATURE CITED

- AMMIRATO P.V., 1983. Embryogenesis, In: Howbook of plant cell culture, vol. 1. Ed. by D.A. Evans, P.V. Ammirato, W.R. Sharp, Y. Yamada., Macmillian Publishing Company, pp 82-123.
- BONGA J.M., 1987. Cell and tissue culture in forestry. Vol. 1. In: General principle of biotechnology. Ed. Don J. Durzan, Martinus-Nijhoff publisher.
- BURZA W., 1992. Wykorzystanie izolacji i kultury protoplastów w biotechnologii roślin rodzaju *Cucumis*. Praca doktorska. SGGW Warszawa. (Use of isolation and culture of protoplasts in plant biotechnology in the genus *Cucumis*. Ph.D. thesis).
- BURZA W., S. MALEPSZY, 1994. In vitro culture of *Cucumis sativus* L. XVIII. Plants from protoplasts through direct somatic embryogenesis. Plant Cell Tissue and Organ Culture (in press).
- HALPERIN W., 1966. Alternative morphogenetic events in cell suspension. Amer. J. Bot. 53(5); 443-453.
- LAZARTE J. E., C.C. SASSER, 1982. Asexual embryogenesis and plantlet development in anther culture of *Cucumis sativus* L., Hort Sci. 17(1); 88.
- MALEPSZY S., NIEMIROWICZ-SZCZYT, J. WISZNIEWSKA, 1982. Cucumber (*Cucumis sativus* L.) somatic embryogenesis in vitro. Acta Biologica 10; 218-220.
- MALEPSZY S., E. SOLAREK, 1986. In vitro culture of *Cucumis sativus*. II. Some aspects of cell culture. Intern. Symp. Plant Tissue and Cell Culture Applic. to Plant Improvement., Olomouc, Czechslovakia; 189.
- MALEPSZY S., 1988. Cucumber (*Cucumis sativus* L.). Biotechnology in Agriculture and Forestry. Vol. 6, Crops II. Ed. Y.P.S. Bajaj, Springer Verlag, Berlin-Heidelberg; 277-293.
- MALEPSZY S., W. BURZA, T. WRÓBLEWSKI, W. GRAJEK, P. KRÓLICZAK, W. CZAJKA, W. LASSOCIŃSKI, 1993. Ogólne założenia pakietu „Biotechnologiczne podstawy produkcji nasiennej u ogórków” oraz wstępne wyniki tematu „Genetyczna i rozwojowa charakterystyka zarodków i powstałych z nich roślin”. I Ogólnopolska Konferencja Biotechnologii Roślin i Przetwórstwa Żywności, Warszawa 27-28 września 1993, 35-38. (General principles of grant „Biotechnological basis of seed production in cucumber” and preliminary results of the grant „Genetic and developmental characterization of embryos and plants derived from them”. I Polish Conference on Plant Biotechnology and Food Processing, Warsaw, September 27-28 1993, 35-38).
- NADOLSKA-ORCZYK A., S. MALEPSZY, 1987. In vitro culture of *Cucumis sativus* L. VI. Histological analysis of leaf explants cultured on media with 2, 4-D and 2, 4, 5-T. Acta Soc. Bot. Polon. 56; 55-60.
- WILLAMS E. G., MAHESHVARI, 1986. Somatic embryogenesis: factors influencing coordinated behaviour of cell as an embryogenic group. Ann. Bot. 57: 443-462.
- WRÓBLEWSKI T., S. MALEPSZY, 1992. In vitro culture of *Cucumis sativus* L. XIV. Established long term culture. Proceedings of Fifth Eucarpia Cucurbitaceae Symp., Skierniewice-Warsaw; 104-107.
- ZIV M., G. GADASI, 1986. Enhanced embryogenesis and plant regeneration from cucumber (*Cucumis sativus* L.) callus by activated charcoal in solid/liquid double-layer cultures. Plant Sci. 47; 115-122.

CYTOHISTOLOGICZNA ANALIZA SOMATYCZNEJ EMBRIOGENEZY
U OGÓRKA (*CUCUMIS SATIVUS* L.)

I. PORÓWNANIE ZAWIESINY KOMÓRKOWEJ ZAWIERAJĄCEJ I NIE ZAWIERAJĄCEJ
NATURALNEJ FLUORESCENCJI Z ZARODKAMI IN VIVO

STRESZCZENIE

W warunkach in vivo u ogórka zarodki wczesno-globularne występują w dziewiątym dniu od zapylenia, sercowate w 14-tym, a morfologicznie dojrzałe zarodki w 19-tym dniu. Pojedyncze ziarna skrobi pojawiają się już w komórkach zarodka globularnego, a w sercowatym występują w obrębie tworzącej się czapeczki radykularnej. W morfologicznie dojrzałym zarodku tylko pramiazga jest wolna od skrobi.

Somatyczna embriogeneza w zawieszynie przebiega podobnie jak in vivo, chociaż lokalizacja skrobi jest nieco inna oraz występują zarodki zbliżone do torpedy. Te ostatnie in vivo nie występują. Budowa histologiczna zarodków in vitro jest podobna do in vivo, a największą różnicą morfologiczną są słabo rozwinięte liścienie i często zmieniona ich liczba (1 do 3). Stwierdzono, że agregaty wykazujące fluorescencję są zbudowane z morfologicznie innych komórek aniżeli agregaty pozbawione fluorescencji i powstałe z nich zarodki wydają się lepiej osiągać stadia dojrzałe.

SŁOWA KLUCZOWE: zawiesina komórkowa, somatyczna embriogeneza, *Cucumis sativus*, zarodki somatyczne, naturalna fluorescencja.