CYTOHISTOLOGICAL ANALYSIS OF SOMATIC EMBRYOGENESIS
IN CUCUMBER (*CUCUMIS SATIVUS* L.).
II. NATURAL FLUORESCENCE AND DIRECT SOMATIC EMBRYOGENESIS
FROM PROTOPLASTS

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ABSTRACT

The development of protoplast derived from somatic embryos and some of their characteristics were compared with embryos from suspension and in vivo in the same B line. Embryos formed in a protoplast culture differed from others that their younger stages contained vacuolated cells, and older ones had altered morphological and histological structure. Somatic embryogenesis is more regular from suspension than from protoplasts. No distinct differences were observed in the rate of embryo development in vivo and in vitro, and in vitro embryos show a larger variation in size at the same stage. Embryos in vitro with fluorescence are generally larger than zygotic ones at each stage. The use of fluorescence is suggested for the selection of heterokaryocytes after protoplast fusion.

KEY WORDS: direct somatic embryogenesis, *Cucumis sativus*, protoplasts, natural fluorescence.

INTRODUCTION

In the cucumber, regeneration of plants from protoplasts of various parts of the plant such as: cotyledons (Deyang and Cocking, 198; Jia et al., 1986; Trulson and Shahin, 1986; Colijn-Hooymans et al., 1988), leaves (Orczyk and Malepszy, 1985), cotyledons and leaves (Punja et al., 1990) has been described. Methods of regeneration from an established suspension culture have been presented, based on the so-called direct somatic embryogenesis from protoplasts (Burza and Malepszy, 1994). In this paper a preliminary cytohistological analysis was performed of the regeneration system described by Burza and Malepszy (1994) taking into consideration the occurrence of two fractions of cell aggregates – showing natural green fluorescence and lacking this property. The phenomenon of natural fluorescence of protoplasts lacking chlorophyll was observed by Burza (1992) and was analyzed cytohistologically in an embryogenic suspension culture (Tarkowska et al., 1994 in press). In this work data concerning the development of zygotic embryos have been studied.

MATERIALS AND METHODS

Protoplasts were obtained from an established embryogenic suspension culture of the same line and using the same procedure and culture conditions presented in an earlier work

(Burza and Malepszy, 1994). The regenerating protoplasts were divided into two fractions. Cell aggregates derived from cell division in a culture of protoplasts showing natural green fluorescence and the second lacked fluorescence. This material was taken for analysis on 22nd day of culture counting from the moment of plating of the protoplasts. Data concerning zygotic embryos from the work of Tarkowska et al. (1994 in press) were used. The procedure of preparing material for cytohistological investigations was also identical.

The number of somatic and zygotic embryos for which sizes were measured was similar and varied from 2-8 and 3-6 for each stage, respectively.

RESULTS

Non-fluorescent fraction

The cell aggregates derived from protoplasts in general form a tissue whose differentiation may proceed in two directions: somatic embryogenesis and sometimes rhizogenesis. Some of the cells in the culture do not differentiate into regular structures. The cells of the developing embryo differ from the surrounding cells of the aggregate by slightly smaller size, more regular shape, and form a spherical structure. The cells of the embryo are still strongly vacuolated similarly to the cells of the aggregate, but in contrast to them they accumulate small starch grains (Fig. 1).

Fig. 1. Fraction without fluorescence- young globular embryo, large vacuolated cells with starch grains (CrAF, PAS, 250x).

In the heart shaped embryo its cortical parts especially in the developing cotyledons are developed from cells which are smaller than the rest, but they are still significantly vacuolated. Starch grains are only accumulated in the cells of the cotyledon part. (Fig. 2).

Fig. 2. Fraction without fluorescence- heart shaped embryo, very small starch grains only in the cotyledonary part (CrAF, PAS, 150x).

In the case of rhizogenesis the radicle is formed from cells with a dense protoplasm but which are still poorly differentiated. Small starch grains accumulate in the initiation center and much larger ones in the vacuolated, larger cells of the cap. The greatest amount of starch is present in the large callus cells which do not undergo differentiation.

Fraction with green fluorescence

The sphere is the primary and most common though not the only shape of the aggregate. The cells are large and vacuolated, some with numerous very small starch grains (Fig. 3).

Fig. 3. Fraction with fluorescence- the aggregate with differentiated embryogenic cells with starch grains- arrow (CrAF,PAS, 500x).

In such aggregates groups of small embryogenic cells become distinct, smaller, less vacuolated and with starch grains. As a result of subsequent divisions this structure is transformed into a globular and later into a heart-shaped embryo. The cells which form it are undifferentiated, without distinct domains containing starch.

Embryogenic cells are formed at various times, therefore in the same aggregate besides embryos new centres appear which undergo the changes described above.

Embryogenic areas form only a part of the aggregate, others remain undifferentiated, or differentiate into areas which in structure and arrangement resemble a young stem. A continuous (or non-continuous) ring of cambium is formed, which may differentiate into conductive cells.

In the cortical cells of the parenchyma a structure in parietal cytoplasm in form of broken bridges starch is accumulated. Such an already histologically differentiated structure contains numerous chloroplasts (emits red fluorescence) (Figs 4, 4a).

Cotyledon embryos also appear in the 22-day culture. Both axes of the embryo are almost equal, very short cotyledons, and the radicle only marked without proper differentiation of cells, a hypocotyl is longer than in the control. Pseudocotyledons may be formed by rows of overlying cells or relatively larger vacuolated cells, some of which contain starch grains.

The size of somatic embryos varies and in the first three stages they are larger than zygotic ones. Moreover, it seems that embryos with fluorescence are always larger than embryos without fluorescence. Zygotic embryos in the morphologically mature stage are larger than both types of somatic embryos, and the differences between the two types of somatic embryos disappear.

DISCUSSION

Somatic embryogenesis in culture of cucumber protoplasts in general is similar to that in cell suspension of cucumber

Fig. 4. Fraction with fluorescence- the aggregate resemble young stem structure. (dichromoformalin, PAS, 130x).

Fig. 4a. Magnification of fragment from Fig. 4.: m- cambium, p- conducting cells, km- parenchymatic cells with starch grains (500x).

and of many other species (Halperin, 1966; Bjohvani and Razdan, 1983), even though some differences have been observed. After neoformation of the cell wall cell aggregates are formed (in general spherical) and strongly vacuolated. Within the aggregates proembryogenic zones with meristematic cell character are distinguished (dense cytoplasm, large centrally located nucleus) which in general undergo embryogenesis (globular and heart shaped stages).

In proembryogenic cells the presence of numerous but small starch grains was observed. However, in further stages no dependence between the stage of embryogenesis and starch distribution was found.

Embryos formed in protoplast culture differed from both embryos from suspensions and from zygotic ones. Their younger stages in many cases contained vacuolated cells and the older ones had some changes in morphological and histological structure. Rhizogenesis occurred rarely, both in the fluorescent and the non-fluorescent fraction.

Comparing somatic embryogenesis in a suspension culture (Tarkowska et al., 1994 in press) with that occurring in the culture of cucumber protoplasts we may note that this process

and the embryos themselves are more regular in suspension. Moreover, in suspension there is no tendency to rhizogenesis. The most significant differences in respect to in vivo embryos concern the proportions of particular embryo parts and underdevelopment of cotyledons. It seems, however, that the rate of embryo development in vivo and in both types of culture (protoplast and suspension) is similar. This indicates that the determination of embryogenicity of a suspension culture is not perturbed by conditions of isolation and culture of protoplasts. These investigations confirm the earlier hypothesis (Burza and Malepszy, 1994) that under the described conditions direct conversion of protoplasts occurs – the so-called direct embryogenesis and a general absence of the callus stage. The effect of fluorescence on embryo development is stronger in the suspension culture where with fluorescence embryos are more numerous and more normal.

On the other hand in the protoplast culture differences occurring between the fractions are not so clear.

The comparison of sizes of the embryos of both fractions seems to indicate that embryos with fluorescence are always larger than zygotic ones in the first three distinguished stages.

TABLE 1. The comparison of dimensions (μm) between cucumber zygotic embryos and protoplast derived somatic embryos with and without natural fluorescence.

Stage of development	Zygotic embryos	Somatic embryos	
		without fluorescence	with fluorescence
Globular	51,92 49,4 - 54,2	208/302 s.a./l.a.	300/320 s.a./l.a.
Early heart	137/148	—	640/700 c.a./s.a.
Heart	—	455/650 e.a./c.a.	830/1240 e.a./c.a.
Morphological mature	2503 w.e.l.	920 e.a.	995/1390 w.e.l./width

Short axis – s.a.; long axis – l.a.; whole embryo length – w.e.l.; embryo axis – e.a.; cotyledonary axis – c.a.

This is similar to embryos from suspension (Tarkowska et al., 1994 in press). A clear difference also exists in the size of embryos of suspension origin and protoplast origin in the first stages. It is both difficult to explain the observed differences and to determine whether the differentiation in size, is linked to the physiological and biochemical state. It seems, however, that the fraction with fluorescence has a tendency to reach a mature stage closer to the zygotic one than the fraction without fluorescence.

The phenomenon observed by Burza (1992) of natural fluorescence in in vitro cultures has not previously been described in the literature. It could be used as a marker for selection of heterokaryocytes in the process of protoplast fusion (Burza 1992). The current investigations indicate that protoplasts showing fluorescence develop into morphologically mature embryos and these structures appear to develop better than in the fraction without fluorescence. This gives a good basis to use protoplasts with fluorescence in the process of somatic hybridization.

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

II. NATURALNA FLUORESCENCJA I BEZPOŚREDNIA SOMATYCZNA EMBRIOGENEZA Z PROTOPLASTÓW

STRESZCZENIE

Porównywano rozwój zarodków somatycznych z protoplastów i niektóre ich cechy z zarodkami z zawiesiny oraz in vivo u tej samej linii B. Zarodki powstające w kulturze protoplastów różniły się od pozostałych tym, że młodsze ich stadia zawierały zwakuolizowane komórki, a starsze miały zmienioną budowę morfologiczną i histologiczną. Somatyczna embriogeneza z zawiesiny jest bardziej regularna niż z protoplastów. Nie stwierdzono zasadniczych różnic w szybkości rozwoju zarodków in vitro i in vivo, a zarodki in vitro wykazują znaczniejsze zróżnicowanie wielkości w obrębie tego samego stadium. Zarodki in vitro z fluorescencją są zwykle większe od zygotycznych w każdym ze stadiów. Sugeruje się wykorzystanie fluorescencji do selekcji heterokariocytów po fuzji protoplastów.

SŁOWA KLUCZOWE: bezpośrednia somatyczna embriogeneza, *Cucumis sativus*, protoplasty, naturalna fluorescencja.