Plant Cell Tissue Organ Culture 3: 221–227 (1984).

Enhanced adventive embryogenesis resulting from plasmolysis of cultured wild carrot cells

D.F. WETHERELL

Biological Sciences Group, University of Connecticut, Storrs, Connecticut 06268, USA

(*Received 27 May 1983*)

Key words: Plasmodesmata, plasmolysis, pre-embryonic cells

Abstract. Adventive embryogenesis in vitro-grown somatic cells of *Daucus carota* L. was increased three-fold by a 45 min plasmolysis pre-treatment using 1M sucrose solutions. A high degree of synchronous development also resulted from this treatment. The enhancement of embryogenesis is interpreted as an increase in the regeneration of cells which have become physiologically somewhat isolated from the tissue of their origin by the plasmolysis-caused rupture of plasmodesmata. Possible causes of the increased synchrony are discussed.

Introduction

Adventive embryogenesis in somatic cell cultures of Daucus carota L. is a special case of the phenomenon of regeneration in which cells at one or more locations in a multicellular cluster give rise to bipolar outgrowths analogous to zygotic embryos. One embryo can emerge from a cluster containing as few as three cells [4]. When the number of cells is large, several to many embryos may arise from each unit. Although all of the cells are assumed to be totipotent the number of embryos formed is small compared to the number of cells in the cluster.

Regeneration has been studied in a variety of lower plants. One of the basic ideas resulting from these studies is that regeneration is initiated by the mechanical or physiological isolation of cells from the integrative forces of the multicellular tissue or organ. It is widely believed that plasmodesmata play an important role in integrative cell–cell communication. Beginning in late 1800's plasmolysis has frequently been used to initiate regeneration in such materials as multicellular algae, moss leaves, fern prothallia and the gemmae of liverworts. This literature has been thoroughly reviewed [1, 8]. The concept of integration of tissues or organs through plasmodesmata seems to have validity for higher plants as well [1, 5]. In the case of natural regeneration through embryogenesis, both the egg-forming meiocyte and the egg itself appear to be isolated from adjacent cells by the absence of functional plasmodesmata, as is the developing zygote and embryo [5].

This paper reports the use of plasmolysis to increase the frequency of regeneration and the degree of synchronous development in suspension cultures of the wild carrot *Daucus carota* L.

Materials and methods

Cells used in these experiments are a subline of a two year old established suspension culture originating from seedling hypocotyl tissue from randomly selected wild seed.

The basic culture medium [15] contains the essential mineral elements, sucrose (5.9×10^{-2} M), thiamine HCl (8.9×10^{-6} M), and 2,4 dichlorophenoxyacetic acid, when present at 2.3×10^{-6} M.

Auxin-grown starting material is cultured in 50 ml shake cultures at 25C with continuous cool white fluorescent light at a fluence rate of 10 μmols m^{-2} s^{-1}). Embryogenesis takes place in auxin-free media in 5 ml roller tube cultures rotated at 12 rpm in the temperature and light conditions given above.

Inoculum for embryogenesis experiments is prepared by sieving and thoroughly washing 7 to 14-day-old auxin-grown cells. The 45-150 micron sieved fraction is inoculated at a packed cell volume of 0.5 μl/ml of culture.

Embryo number is determined by uniformly distributing the contents of a culture within the measured area of a 60 mm petri dish. The entire microscope field at a magnification of 25 x is scored according to stage of embryonic development and the total embryo number calculated from the average counts of five fields.

Culture contents were centrifuged, washed once, and dried at 70 °C for 18 h to obtain dry weight.

Plasmolysis was carried out with filter-sterilized solutions prepared by dissolving the osmotic solutes in culture medium. Drained centrifuge pellets of cells were resuspended in the plasmolyzing solutions and incubated for 45 min. At that time two 50% dilutions were made at five minute intervals with fresh medium and the cells pelleted by centrifugation, washed once, and resuspended in fresh medium for culture. For convenience the concentrations of the sucrose plasmolysing solutions given throughout the paper refer to the amount of sucrose used in addition to that in the basic culture medium. The osmolarity of the basic culture medium is approximately 6.0×10^{-2}.

Results

Microscopic observations

The majority of cells used in these experiments were growing rapidly at the time of treatment. The average cell was roughly isodiametric with a diameter of approximately 25 microns. These cells contained one to many vacuoles surrounded by a thick cytoplasmic layer with many starch grains and plastids. Judging from cell wall configurations nearly all cells were integral parts of clusters in which all members were derived from the same mother cell. Embryos do not arise directly from single free cells.

Cells were examined by brightfield and phase microscopy before, during,

and after exposure to 1.0 M solutions sucrose, sorbitol, or glycerol. With all solutes both protoplast and cell wall shrinkage occurred immediately. Wall shrinkage resulted from randomly oriented folds. Initial wall shrinkage appeared greater in sucrose than in sorbitol or glycerol. Relaxation of the folds occured within 5 min with glycerol and 15 min with sorbitol, but folds were still detectable after one hour in sucrose.

Plasmolysis began in all solutions with the formation of numerous lense-shaped invaginations of the plasmalemma. These regions merged at different rates with different solutes resulting in a transition from highly irregular protoplast shapes to the familiar rounded protoplasts of full plasmolysis. This transition occurred almost immediately with glycerol, within 15–20 min with sorbitol, and in more than one hour in sucrose. When sorbitol was the plasmolyzing agent a gradual swelling returned protoplasts to their original volumes in approximately 30 min. This swelling appeared slower in glycerol and was negligible in sucrose after one hour.

Examination by phase microscopy of the spaces left between the retreating plasmalemma and the wall during early plasmolysis showed large numbers of thin radial strands connecting the protoplast to the wall. These strands were very close to the limit of resolution of 1.3 N.A. 100 x objectives and could not be seen with brightfield illumination. The strands underwent small undulating motions early in the plasmolysis period but appeared to thicken into a beaded form and eventually ruptured leaving small spherical bodies at the inner wall surface. The strands appeared to persist longer in sucrose solutions but were difficult to find after 30 min and were presumed to be ruptured.

Cytoplasmic streaming was easily seen in untreated cells. Streaming ceased at plasmolysis but returned to sorbitol-plasmolyzed cells within 10–15 min. Sucrose-plasmolyzed cells did not regain visible streaming in one hour.

Replacing plasmolyzing solutions abruptly with culture medium resulted in immediate forceful deplasmolysis. Although no cell rupture was observed, the cytoplasmic contents of rapidly deplasmolyzed cells lost contrast and appeared seriously disrupted. Two 50% stepwise dilutions of the plasmolyzing medium at 5 min intervals reduced this effect.

Examination of cells plasmolyzed in 0.5 M and 0.25 M solutions of sucrose and sorbitol showed only quantitative differences from those described for 1.0 M solutions.

Growth and embryogenesis

Plasmolysis pretreatment caused initial growth to lag that of the control by two to three days. At that time treated cultures showed a relatively synchronous burst of embryogenesis in which a large number of globular embryos of uniform origin appeared.

The effect of plasmolysis pretreatment with sucrose solutions of different concentrations upon dry weight growth and yield of embryos after 19 days of

Table 1. The effects of a 45 min. plasmolyzing pretreatment on dry weight growth and adventive embryogenesis in wild carrot cell cultures. Culture period 19 days. Culture volume 5 ml. Inoculum density 0.11-0.14 mg per culture. Results given as the average ± SD of 4 samples

Pretreatment sucrose molarity	A Culture dry weight in mg	B Embryos per culture	Embryo yield B/A
0.0	7.0 ± 1.0	2350 ± 598	340
0.5	6.6 ± 1.0	7620 ± 1180	1200
1.0	5.9 ± 0.7	6040 ± 604	1000
1.5	3.9 ± 0.6	1890 ± 209	490

Table 2. The effect of a plasmolyzing pretreatment on the distribution of stages of embryogenesis in wild carrot cell cultures. Data from same experiment as Table 1. Results given as the average ± SD of 4 samples

Pretreatment sucrose molarity	Distribution of stages of embryogenesis (%)			
	Globular	Heart	Torpedo	Plantlet[a]
0.0	6 ± 2	28 ± 11	46 ± 6	19 ± 7
0.5	7 ± 2	76 ± 7	16 ± 5	3 ± 1
1.0	3 ± 2	70 ± 8	25 ± 7	2 ± 0.5
1.5	8 ± 3	83 ± 5	11 ± 4	2 ± 0.5

[a]Embryos bearing true roots

culture is presented in Table 1. These data show little significant reduction in dry weight growth following plasmolysis in 0.5 M and 1.0 M sucrose solutions, however, plasmolysis in a 1.5 M sucrose solution reduces dry weight growth by half. Plasmolysis in 0.5 M and 1.0 M sucrose solutions causes large increases in the number of embryos produced per culture or per unit of dry weight.

The cultures described in Table 1 were also scored for the distribution of developmental stages of embryogenesis. These data are presented in Table 2 and show a marked increase in developmental synchrony in all three treatments. This experiment was repeated once and gave closely comparable results.

To test the possibility that these effects might be caused by sucrose per se rather than by plasmolysis, 1.0 M sorbitol was used. These results are presented in Tables 3 and 4. Sorbitol effects are intermediate between those of sucrose and control treatments for all parameters measured. Two additional similar experiments, not presented, gave closely comparable results.

Discussion

These results indicate that the number of embryos which can arise by regeneration from a multicellular pre-embryonic unit is not fixed. It is determined,

Table 3. Comparison of the effects of a 45 min. plasmolyzing pretreatment with either 1.0 M sorbitol or 1.0 M sucrose solutions on dry weight growth and adventive embryogenesis in wild carrot cell cultures. Conditions are the same as for Table 1. Results given as the average ± SD of 3 samples

Pretreatment solution	A Culture dry weight in mg	B Embryos per culture	Embryo yield B/A
Control	6.3 ± 0.6	2400 ± 2.6	380
1.0 M sorbitol	5.0 ± 0.6	4320 ± 648	860
1.0 M sucrose	4.1 ± 0.1	5800 ± 297	1400

Table 4. Comparison of the effects of plasmolyzing pretreatment with either 1.0 M sorbitol or 1.0 M sucrose solutions on the distribution of stages of embryogenesis in wild carrot cell cultures. Data from the same experiment as Table 3. Results given as the average ± SD of 3 samples

Pretreatment solution	Distribution of stages of embryogenesis (%)			
	Globular	Heart	Torpedo	Plantlet[a]
Control	12.4 ± 4	43 ± 2	31 ± 2	15 ± 1
1.0 M sorbitol	13 ± 4	69 ± 1	16 ± 3	3 ± 1
1.0 M sucrose	7 ± 2	84 ± 4	8 ± 2	0

[a]Embryos bearing true roots

at least in part, by interactions with adjacent cells. In other systems, for example gemmae of *Lunularia* [11] and *Marchantia* [2, 10] and in fern prothallia [8, 9], strong plasmolysis has greatly increased in frequency of regeneration. These investigators have attributed the effect of plasmolysis to the physiological isolation of single cells or small groups of cells resulting from the distruption of plasmodesmata [8–11]. The same interpretation is given to the data presented here, ie. plasmolysis of pre-embryonic cell clusters disrupts cellular interconnections allowing more cells to develop independently expressing their totipotence through adventive embryo formation. Plasmodesmata are frequent in pre-embryonic cell clusters [6]. The thin protoplasmic strands reported in this paper and interpreted as plasmodesmata stretched across the lumen as the plasmalemma recedes from the wall in plasmolysis. These strands have been observed and similarly interpreted in a variety of materials by several investigators [3, 10–12].

The concept of the developmental role of cell isolation through the disruption of plasmodesmata is strengthened by ultrastructural studies of the developing embryo sac, egg, and early zygote. Several investigators have found that the embryo sac-forming meiocyte is isolated from adjacent cells by a thick wall lacking plasmodesmata [5]. Within the embryo sac of Capsella the egg is at first connected by plasmodesmata to the synergids but these connections disappear after fertilization. The young zygote is not symplastically connected to the other cells of the embryo sac and the sac is isolated from the nucellar cells by the absence of plasmodesmata [13].

In the two most thoroughly studies cases of in vitro somatic embryony a similar situation has been observed. With both *D. carota* [14] and *Ranunculus sceleratus* [7] the tissue from which embryos originate is well interconnected by plasmodesmata. In both cases although the specific cell from which the embryo is derived cannot be conclusively identified, the very early embryo lacks plasmodesmata in all peripheral walls although adjacent walls show numerous plasmodesmata. Early isolation of embryonic cells seems to occur in both cases.

Increased synchrony of embryogenesis following plasmolysis was unexpected. Decreased average globular embryo size and increased size uniformity was noted in the plasmolyzed cultures. This would be expected if a high proportion of the embryos were initiated from single cells and at the same time. Untreated cultures show a large variation in size and time of formation of globular embryos. An alternative explanation which cannot be ruled out without further work is that the higher embryo density of the plasmolyzed cultures intensifies the phenomenon of mutual inhibition [16]. In untreated cultures increased size of inoculum can create a similar situation where the growth of most embryos is arrested in early developmental stages unless released by further dilution of the culture.

In summary, the use of brief strong plasmolysis as a pretreatment significantly improves the yield and the uniformity of adventive embryogenesis in suspension cultures of wild carrot cells. This effect is thought to result from the disruption of plasmodesmatal interconnections between the pre-embryonic cells.

Acknowledgement

This work was supported in part by grant #35-398 from the University of Connecticut Research Foundation.

References

1. Carr DJ (1976) Plasmodesmata in growth and development. In: Gunning BES, Robards AW (eds) Intercellular communication in plants: Studies on plasmodesmata. Heidelberg: Springer pp 243–290
2. Dickson H (1932) Polarity and the productions of adventitious growing points in *Marchantia polymorpha*. Ann Bot 46:683–701
3. Drake GA, Carr DJ, Anderson WP (1978) Plasmolysis, plasmodesmata, and the electrical coupling of oat coleoptile cells. J Exp Bot 29:1205–1214
4. Halperin W (1967) Population density effects on embryogenesis in carrot cell cultures Exp Cell Res 48:170–173
5. Halperin W (1970) Embryos from somatic plant cells. Symp Soc Cell Biol 9:169–191
6. Halperin W, Jensen WA (1967) Ultrastructural changes during growth and embryogenesis in carrot cell cultures. J Ultrastruc Res 18:428–443
7. Konar RN, Thomas E, Street HE (1972) Origin and structure of embroids arising from epidermal cells of the stem of *Ranunculus sceleratus* L. J Cell Sci 11:77–93

8. Miller JH (1968) Fern gametophytes as experimental material. Bot Rev 34: 361–426
9. Nagai I (1914) Physiologische Untersuchungen uber Farnprothallien. Flora 106: 281–330
10. Nagai I (1919) Induced adventitious growth in the gemmae of *Marchantia*. Bot Mag Tokyo 33:99–109
11. Narayanaswami S, LaRue CD (1955) The morphogenic effects of various physical factors on the gemmae of *Lunularia*. Phytomorphology 5:356–372
12. Price SR (1914) Some studies on the structure of the plant cell by the method of dark-ground illumination. Ann Bot 28:601–632
13. Schultz R, Jensen WA (1968) Capsella embryogenesis, the early embryo. J Ultrastruct Res 22:376–392
14. Street HE, Withers LA (1974) The anatomy of embryogenesis in culture. In: Street HE (ed) Tissue culture and plant science. London and New York: Academic Press pp 71–100
15. Wetherell DF (1969) Phytochrome in cultured wild carrot tissue. I. Synthesis. Plant Physiol 44:1734–1737
16. Wetherell DF (1978) In vitro embryoid formation in cells derived from somatic plant tissues. In: Hughes K W et al. Propagation of higher plants through tissue culture. Washington: United States Department of Energy pp 102-124