GROWTH AND MORPHOGENESIS OF GLOBULAR AND OLDER EMBRYOS OF CAPSELLA IN CULTURE[1,2]

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ABSTRACT

RAGHAVAN, V., and J. G. TORREY. (Harvard U., Cambridge, Mass.) Growth and morphogenesis of globular and older embryos of Capsella in culture. Amer. Jour. Bot. 50(6) : 540–551. Illus. 1963.—Early heart-shaped and older embryos of *Capsella bursa-pastoris* (>80 μ long), when cultured aseptically in a semi-solid medium containing mineral salts, vitamins, and 2% sucrose, developed into small plantlets. When grown in a 12-hr light/12-hr dark cycle (light-grown cultures), formation of the primary root system was suppressed in embryos <1000 μ long, while embryos cultured in continuous dark routinely initiated a primary root system and several lateral roots. In embryos >1000 μ long, the inhibition of formation of the root system in light did not occur. Growth of the shoot apex of intermediate and torpedo-shaped embryos was slightly accelerated in light. There was a correlation between the rate of growth of the embryos and their initial length. Better growth in length was observed in light-grown than in dark-grown cultures. Supplementing the basal medium with indoleacetic acid, kinetin, and adenine sulfate permitted successful development of globular embryos (<80 μ long) in vitro. Although a high sucrose concentration or a high salt concentration in the medium partially replaced the effect of the growth factors in inducing development of the globular embryos, no absolute requirement for a high osmotic concentration in the medium was found. The results favor caution in interpreting the inability of small embryos to grow in terms of the osmotic conditions of the culture milieu.

THE FACTORS that control the progressive and orderly development of embryos throughout their ontogeny have attracted the attention of experimental embryologists for some time (Needham, 1942; Brachet, 1950; Wardlaw, 1955). There is now substantial indirect evidence that growth and differentiation of plant embryos may result from an induction by gradients of nutritional substances and hormonal factors in the immediate environment of the developing embryos (Rijven, 1952; Wardlaw, 1955). In the early stages of embryogeny, embryos develop at the expense of the surrounding endosperm tissue, and as far as the evidence goes, such embryos are highly heterotrophic in character. Attempts to culture very small plant embryos, especially at the morphological stages designated as "globular" and "heart-shaped," in chemically defined nutrient media outside the plant tissues have met with little success (Van Overbeek, Conklin and Blakeslee, 1942; Rijven, 1952; Norstog, 1961; Swamy, 1961). It is only after the embryos have developed within the ovular tissues to the beginning of cotyledonary development and the attainment of bilateral symmetry that they become sufficiently independent and autotrophic to lend themselves to culture in vitro even in complex nutrient media. While the nutritional requirements of young embryos are complex, and their responses often highly variable, progressively older embryos show less rigorous requirements and more definitive responses to added nutrients in the medium.

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The genus *Capsella* has been a favorite object for both descriptive and experimental embryology since the work of Hanstein (1870). Later, Famintzin (1879), Schaffner (1906), and Souèges (1914, 1919) made more thorough studies of the embryogenesis of *C. bursa-pastoris*. The first published report of in vitro culture of the embryos of this species was by Rijven (1952). A modification of Rijven's classification and terminology concerning embryo stages will be used in this paper (Table 1). Rijven found that growth in culture of the embryos excised from the ovules during the course of development depended upon the presence in the medium of amino acids which apparently regulated the metabolic processes involved in growth. In addition, Rijven (1952, 1955, 1956) observed that the addition of glutamine produced a stimulation of growth which was even greater than that obtained in the presence of casein hydrolysate or a complex mixture of amino acids of the composition of edestin. Nevertheless, Rijven did not succeed in cultivating embryos less than 100 μ in mean initial length. Embryos at the heart-shaped or torpedo-shaped stages (140–700 μ long) responded consistently to the addition of nutrients only when grown in hanging-drop culture in a medium of high osmotic pressure obtained by the addition of 12–18% sucrose. More recently, using Rijven's experimental procedure, Veen (1961, 1962) studied the effects of substances like gibberellic acid and kinetin on the growth of *Capsella* embryos in culture. Embryos of this species have also been used for cyto-histochemical analysis of embryogenesis (Pollock and Jensen, 1961, 1962).

Rijven's (1952) work leaves the impression that the osmotic value of the nutrient medium is an important factor in the successful development of small embryos of *Capsella* in culture, the high osmotic pressure preventing the appearance of signs of precocious germination associated with cell enlargement and cell elongation. The same conclusion was reached by several other investigators (Ziebur and Brink, 1951; Rietsema, Satina and Blakeslee, 1953; Mauney, 1961; Norstog, 1961; Swamy, 1961). We have found that reproducible normal growth and differentiation can be achieved in immature embryos of *Capsella* when they are grown in vitro in a semi-solid medium containing 2% sucrose; thus the osmotic value of the culture medium may be of relatively little importance in the morphogenesis of the cultured embryos. The results of these studies are described in this paper. In addition, this report presents experiments indicating the importance of growth factors in the culture of globular embryos. These results have significance in support of the view that the transition from heterotrophy to autotrophy during embryogenesis depends upon the availability of specific growth factors to the embryo.

MATERIAL AND METHODS—Embryos of *Capsella bursa-pastoris* Medic. were obtained from plants grown in the greenhouse in a mixture of equal parts of sand, perlite and leaf mold under a supplementary incandescent illumination of ca. 200 ft-c during the daytime. In the spring and summer months, preliminary experiments were done with embryos obtained from plants in nature, with essentially the same results.

Dissection of the embryos for culture was carried out aseptically in a transfer room previously illuminated by UV-sterilamps. Capsules in the desired stages of development were surface-sterilized for 4 min in a 5% filtered solution of "Pittchlor" (manufactured by Columbia-Southern Chemicals Co., Pittsburgh, Pa.) and washed repeatedly in sterile water. Further operations were carried out under a dissecting microscope at a magnification of 90×, keeping the tissues in a depression slide containing a few drops of the liquid medium. The outer wall of the capsule was removed by a cut in the region of the placenta; the halves were pulled apart with forceps to expose the ovules. Torpedo-shaped and younger embryos were confined to 1 longitudinal half of the ovule and were clearly visible through the chalaza either because of their green color (intermediate and torpedo-shaped), or because of the transparent vesicle of their suspensors (globular and heart-shaped). With the help of a sharp, mounted blade, the ovule was split longitudinally to isolate the embryo-containing half. Further manipulations to separate the embryos from the surrounding ovular tissues were performed with #11 surgeon's blades (Clay-Adams). Early globular embryos also could be removed with no apparent injury. A small incision in the ovule, followed by slight pressure with a blunt needle, was enough to free the embryos in advanced stages of development from the ovules; these embryos, without any suspensor cells, floated freely in the medium. Unless otherwise stated, embryos in the globular and heart-shaped stages were cultured with the cells of the suspensor intact. The excised embryos were transferred by micropipettes to standard 10-cm Petri dishes containing 25 ml of the solid medium and/or suspended in a small drop of the liquid medium on a cover-slip over a cavity slide, and sealed with a melted 1:1 paraffin-petrolatum mixture (hanging drop cultures). Usually 6–8 embryos of the desired stages were cultured in a Petri dish or in individual hanging drops. All cultures of globular embryos were in hanging drops; older stages were cultured routinely on agar medium. Each experiment was done 3–4 times, with 6–8 embryos of a stage per treatment. The pooled data from these experiments assured the inclusion of a sufficient number of embryos of the same or closely similar size to allow statistical analysis. The Petri dishes were sealed with masking tape to prevent desiccation of the cultures. Since the number of very small embryos that could be conveniently isolated and handled at a time was limited, in experiments involving many different concentrations of a test substance, there was an interval of several days between setting up the initial and final concentration series.

The basal medium used in this study consisted of macronutrient salts, trace elements, and vitamins in proportions suggested by Dr. H. Castle, Yale University (personal communication, 1961). The composition of this basal medium is as follows:

I. Macronutrient salts (mg/liter): 480 $Ca(NO_3)_2 \cdot 4H_2O$; 63 $MgSO_4 \cdot 7H_2O$; 63 KNO_3; 42 KCl; 60 KH_2PO_4.
II. Micronutrient salts (mg/liter): 0.56 H_3BO_3; 0.36 $MnCl_2 \cdot 4H_2O$; 0.42 $ZnCl_2$; 0.27 $CuCl_2 \cdot 2H_2O$; 1.55 $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$; 3.08 ferric tartrate.
III. Vitamins (mg/liter): 0.1 thiamin hydrochloride; 0.1 pyridoxin hydrochloride; 0.5 niacin.
IV. Sucrose 20 g/liter; specially purified Difco agar (where applicable) 9 g/liter.
V. Glass-distilled water to 1 liter.

All the components of the medium were mixed together and sterilized for 15 min at 15 lb/in^2. A number of growth substances were added separately or in combination to the basal medium in attempts to induce growth in globular embryos. These supplements were mixed with the basal medium and autoclaved. The final pH of the medium was 4.9–5.0.

The cultures were kept in a culture room at 25±1 C and given 12 hr illumination daily by a

TABLE 1. *Stages in the embryogenesis of Capsella. Length was measured from the base of the suspensor to the extreme tip of the embryo, including the cotyledons. (Classification modified from Rijven, 1952)*

Developmental stage	Length, microns
Early globular	ca. 20–60
Late globular	61–80
Early heart-shaped	81–150
Late heart-shaped	151–250
Intermediate	251–450
Torpedo-shaped	451–700
Walking-stick-shaped	701–1000
Inverted-U-shaped	1001–1700
Mature embryo	>1701

combination of cool-white fluorescent tubes and incandescent lamps (light-grown cultures) giving ca. 50 ft-c at the level of the cultures. Parallel sets of cultures were maintained in an incubator at 25±1 C which was kept completely dark except for brief periods of opening for examination (dark-grown cultures). Transfers to fresh media were made at approximately 4-week intervals and the experiments were routinely discontinued after 12 weeks.

Unless otherwise specified, growth measurements were made at the end of 7 days with a dissecting microscope equipped with an ocular micrometer. Although the embryos were still growing after this period, further growth was less regular and unreliable for quantitative evaluation. Growth was expressed as the percentage increase over the initial length in the longitudinal axis of the embryo excluding the length of the suspensor. Since the initial length of the embryos varied, embryos were divided on the basis of their morphological stage of development into definite stage classes (Table 1). For clarity of presentation of results, data on embryos of approximately the same initial length were pooled to determine the average rate of growth.

The morphological changes occurring in the cultured embryos were followed by periodic observations of the cultures under a dissecting microscope. For microscopic examination, samples were fixed in formalin–acetic acid–alcohol. They were dehydrated in n-butyl alcohol series and embedded in Tissuemat. Sections cut at 10 μ were stained in phloxine and fast green.

No systematic attempts were made to rear to maturity seedlings derived from the cultured embryos. In a few trials made, when the growing embryos were transferred to fresh media, many of them produced plantlets.

Statistical methods follow Snedecor (1956).

RESULTS—Early in this work it became apparent that the responses in culture of embryos differing in length, even over a narrow range, varied widely. To evaluate the effects of composition of the medium on the growth of embryos of known developmental stages, a detailed morphological examination was made of embryos varying in length from 50 to 1700 μ. With the help of camera lucida drawings of such isolated embryos, a classification of the developmental stages, based on Rijven (1952), was made (Table 1). Early globular embryos were generally 16–32 cells in number and ca. 40 μ in diameter (Fig. 1). A transition from radial to bilateral symmetry was apparent in the early heart-shaped embryos (Fig. 2), which showed for the first time a visible demarcation between the presumptive shoot and root regions. Embryos in the intermediate and torpedo stages had basically the same morphology (Fig. 3, 4). The axis was short, the cotyledons were well developed, and internally they showed procambial tissue differentiation. In embryos >250 μ long, the cotyledons comprised over $\frac{1}{3}$ of their length. The "walking-stick-shaped" and the "inverted-U-shaped" embryos were further differentiated by the elongation of the hypocotyl and the organization of the meristematic regions of the root and shoot (Fig. 5).

Experiments with heart-shaped and larger embryos—Isolated embryos of *Capsella* >80 μ in length (early heart-shaped; Fig. 2) grew readily in the basal medium described above and differentiated regularly into shoot, root and leaf primordia, although the process was relatively slow. Since reference will be made to the pattern of development of the embryos in the basal medium, a brief description of the sequence will be given. The growth and organization of meristematic regions in the early and late heart-shaped embryos cultured in a semi-solid medium appeared alike. In Fig. 6–9 the external morphological forms of embryos initially 81 μ in length are shown at various times during 2–3 months in culture. The first evidence of growth in the embryos was an increase in diameter due to rapid proliferation of cells during the first week. This was followed by the development of the distal half of the embryo into 2 cotyledonary lobes, at an angle of 45° to the main axis. After 3 weeks in culture, a root apex was initiated at the proximal end. The suspensor cells were sloughed off from the main body of the embryo shortly afterwards. Further development of the embryo was marked by the initiation and increase in size of the shoot apex which was visible externally as a mound of tissue between the cotyledons (Fig. 6–8, 13–15). The shoot meristem showed little internal organization and lacked the characteristic demarcation into cytohistological layers or zones (Fig. 14, 15). The apex developed slowly, producing the first pair of leaves in 4–6 weeks (Fig. 16). By this time the embryos had grown to about 3 mm. Almost simultaneously in dark-grown cultures, the root became active, elongating rapidly to form a primary root several millimeters long and, subsequently, a few laterals (Fig. 9). Elongation of the primary root showed the highest rate observed for any organ of the cultured embryo, averaging

between 200 and 300 μ daily. Development of the primary root was suppressed in embryos grown in light. This inability of embryos cultured in light to initiate a primary root system persisted throughout the period of the experiment. During further development the embryos produced additional leaves rapidly. The leaves were 6–8 mm in length, and were thick and linear and had none of the features of the leaves of the adult plant. Shoot length at this time was less than 1 cm, and the plantlets were characterized by an almost complete absence of internodal elongation (Fig. 9). No evident correlations were obtained between shoot development and the conditions of illumination in the heart-shaped embryos.

These attempts to grow heart-shaped embryos in a simple medium demonstrate that immature embryos are strikingly similar to the mature embryos in their organogenesis. All stages of embryo development through the torpedo stage were normal in embryos cultured in vitro. Thereafter, cultured embryos developed without the characteristic curvature apparent in the walking-stick-shaped and inverted-U-shaped embryos which developed within the restrictions imposed by the ovule. No significant deleterious effect was caused

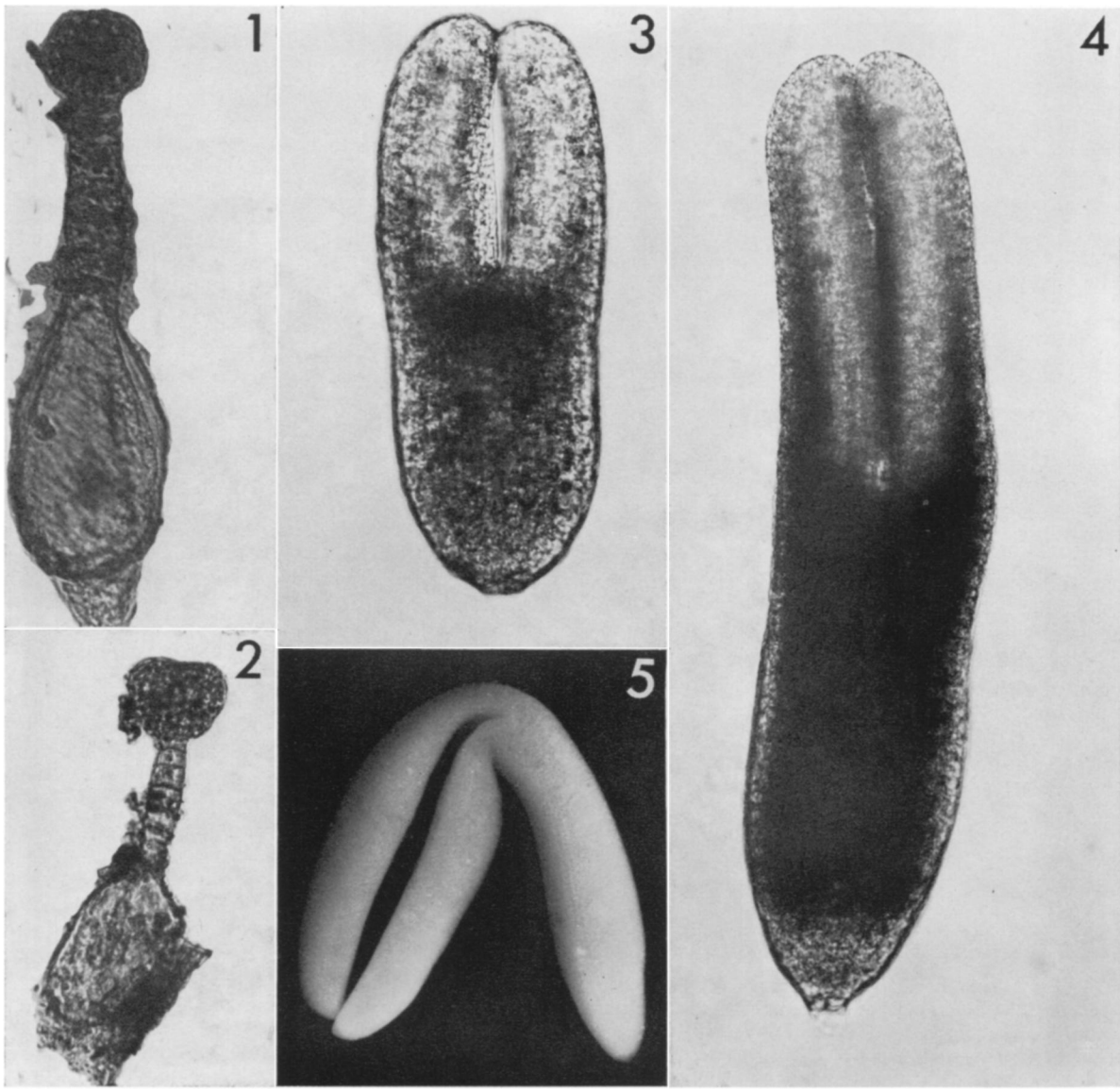


Fig. 1–5. Photographs of whole mounts of embryos of *Capsella* showing the stages at which they were cultured.—Fig. 1. An early globular embryo, including the suspensor. The embryo itself is 54 μ long. ×225.—Fig. 2. An early heart-shaped embryo including the suspensor. The embryo less the suspensor is 81 μ long. ×150.—Fig. 3. An intermediate stage embryo, 405 μ long. ×200.—Fig. 4. A torpedo-shaped embryo, 660 μ long. ×200.—Fig. 5. An inverted-U-shaped embryo, about 1636 μ long. ×55.

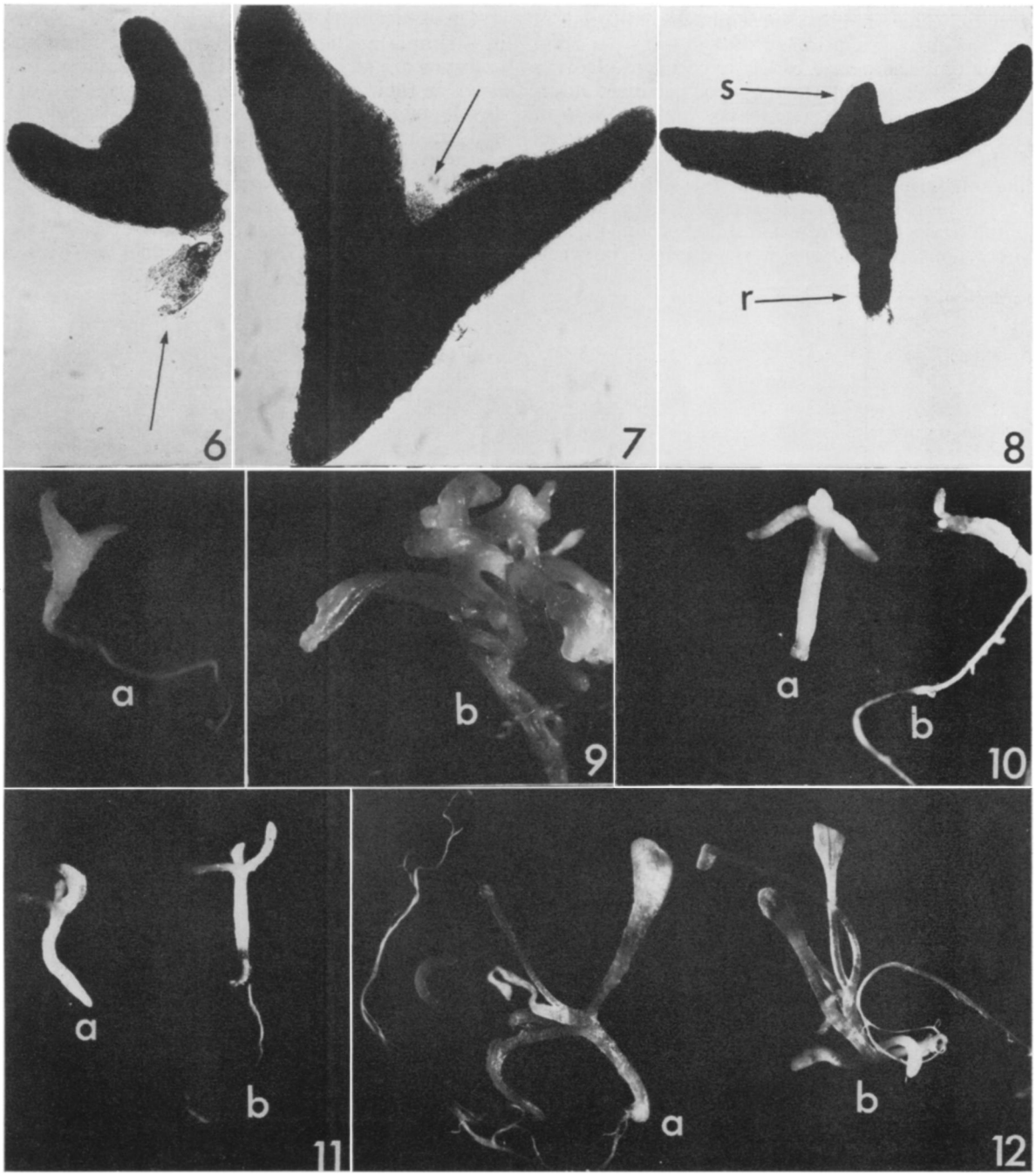


Fig. 6–12.—Fig. 6–9. Development of early heart-shaped embryos in culture.—Fig. 6. An embryo initially about 81 μ long after 2 weeks in culture in the dark. Note the formation of the cotyledonary lobes; the suspensor (arrow) is still attached to the embryo. ×116.—Fig. 7. An embryo at about the same age as in Fig. 6, cultured in alternating 12-hr periods of light and dark. Arrow points to the mound of tissue which later becomes the shoot apex. ×116.—Fig. 8. An embryo after 5 weeks in culture in the dark showing root meristem (r) and shoot meristem (s). ×116.—Fig. 9. Dark-grown cultures of embryos showing the formation of plantlets: (a) formation of the primary root system; (b) plantlet formed from an embryo after about 12 weeks in culture. ×8.—Fig. 10. Intermediate stage embryos after 3 weeks in culture in light (a) and in dark (b). Note the long primary root in (b). ×4.—Fig. 11. Torpedo-shaped embryos after growth for 2 weeks in light (a) and in dark (b). Note the long primary root in (b). ×4.—Fig. 12. Two inverted-U-shaped embryos of closely comparable initial length, after 2 weeks in culture in light (a) and in dark (b). Note the formation of the primary root system in both. ×4.4.

TABLE 2. *Growth of Capsella embryos of different ages in light and dark. Growth data represent increase in length as per cent of initial length ± standard error after 7 days in vitro*

Developmental stage	Dark cultures		Light cultures	
	Initial length, μ	Percent growth	Initial length, μ	Percent growth
Early heart-shaped	88.0	81.9 ± 5.9	88.0	90.4 ± 11.3
Late heart-shaped	180.0	130.8 ± 9.9	208.0	184.3 ± 18.0
Intermediate	333.0	113.1 ± 9.2	277.5	166.4 ± 25.1
	375.0	89.9 ± 8.8	347.0	173.9 ± 10.2
	444.0	100.0 ± 10.4		
Torpedo-shaped	569.0	134.8 + 11.0	569.0	198.1 ± 12.2
	655.0	107.2 ± 8.7	652.0	171.2 ± 9.7
Walking-stick-shaped	777.0	142.7 ± 12.4	832.5	111.6 ± 5.0
	874.0	125.3 ± 9.1	888.0	114.0 ± 9.7
			943.5	150.1 ± 7.8
Inverted-U-shaped	1401.0	132.3 ± 2.4	1540.0	137.8 ± 4.1
			1628.0	128.0 ± 5.9
			1679.0	122.2 ± 6.3

by lack of high cencentrations of sucrose in the medium, as claimed by Rijven (1952).[3]

The pattern of organogenesis in the intermediate and torpedo-shaped embryos (Fig. 3, 4) was more or less similar to that of the heart-shaped embryos. Growth in length of the embryos was initiated within 48 hr after transfer to the medium. This growth represented the over-all growth of the embryos and not merely the growth due to elongation of the hypocotyl. Thus, the initial responses of the embryos, when grown in a semi-solid medium, were profoundly different from the precocious germination described by Rijven (1952). While growth of the primary root was suppressed in light-grown cultures (Fig. 10, 11), a considerable number of embryos in light developed a shoot system a few days ahead of the dark cultures.

Other experiments in which embryos of the walking-stick-shaped, inverted-U-shaped and mature stages were cultured in the basal medium were also of interest with respect to the factors affecting the initiation and growth of the shoot and root primordia. Walking-stick embryos responded in the same way as torpedo-shaped embryos with respect to their morphogenesis in light and dark. On the other hand, when embryos about 1000 μ long (Fig. 5) were planted under both light and dark conditions, the responses were uniform. They showed prolific growth of the primary and lateral roots in 1 week in culture and an elaborate shoot system composed of several linear leaves within 2 weeks (Fig. 12).

[3] In a personal communication Dr. H. Castle (Yale University) informs us that he also succeeded in growing normal plants from *Capsella* embryos isolated at the 16–32-cell stage, and grown in the medium described in this paper (see also Castle, quoted in Naylor, 1952).

Data on the growth of embryos of different ages after 7 days in culture are summarized in Table 2. The results show clearly the greater growth in length of the embryos in light than in dark; in this respect our observations are not in agreement with those of Rijven (1952) who found light inhibitory or without effect on the growth of the embryos. In agreement with Rijven, we observed a correlation between the rate of growth of the embryos and their initial length at excision. Thus, younger embryos did not grow to the same proportional extent as the older ones, suggesting a nutritional limitation. This effect was consistent in embryos up to ca. 700 μ long in light, while in dark-grown cultures and in longer embryos no clear relationship was apparent.

Experiments with globular embryos—Globular embryos (<80 μ long) cultured in the basal medium, either in semi-solid cultures or in hanging drops, did not show any signs of cell division and growth even after prolonged periods in culture. In considering the factors controlling the growth in vitro of these embryos, it became increasingly apparent that the problem was chiefly a nutritional one, the embryos probably being limited in their potentialities for cell division and growth by the lack of one or more specific growth factors in the medium. Indirect support for this view came from observations gathered during attempts to culture globular embryos. Anticipating the fact that the tiny embryos would be particularly sensitive to heavy metal contaminants in the medium, an experiment was run using macronutrient salts specially purified to reduce the total heavy metal content of the medium to less than 10 ppb. Embryos >80 μ cultured in the purified medium surpassed the growth obtained in medium using reagent grade salts, but in no cases were cell divisions

TABLE 3. *Growth of globular embryos in different media. Growth values are expressed as the percentage increase after 10 days in culture in the length of the embryo over the initial length ± standard error*

Composition of media	Initial length of the embryos, microns			
	27.0	40.5	54.0	67.5
Basal medium alone	0(7)[a]	0(9)	75.0(1/24)[b,c]	35.0 ± 5.0(4/18)[c]
Basal medium with IAA: 0.0001 mg/liter	0(3)	0(1)	0(3)	0(3)
0.001 mg/liter	0(1)	0(5)	0(8)	40.0(1/6)[c]
0.01 mg/liter	—	0(6)	25.0 ± 0(2/8)[c]	40.0(1/3)[c]
0.10 mg/liter	0(1)	33.3(1/4)[c]	25.0 ± 0(2/8)[c]	25.0 ± 5.0(4/5)[c]
1.0 mg/liter	—	0(3)	0(5)	0(4)
10.0 mg/liter	—	0(2)	0(1)	0(5)
Basal medium + 0.10 mg/liter IAA and kinetin: 0.0001 mg/liter	—	0(6)	32.1 ± 7.1(7/10)[c]	40.0 ± 0(4/5)[c]
0.001 mg/liter	0(1)	33.3(1/6)[c]	23.0 ± 1.2(5/8)[d]	45.0 ± 5.0(4/4)[d]
0.01 mg/liter	0(6)	—	25.0(1/5)[d]	—
0.10 mg/liter	0(3)	—	0(4)	—
1.0 mg/liter	—	0(5)	—	0(1)
10.0 mg/liter	—	0(6)	—	—
Basal medium + 0.10 mg/liter IAA, 0.001 mg/liter kinetin and adenine sulfate: 0.0001 mg/liter	50.0(1/3)[e]	33.3 ± 0(2/3)[c]	68.8 ± 15.9(4/6)[e]	60.0(1/1)[d]
0.001 mg/liter	0(4)	77.7 ± 29.4(3/4)[d]	154.2 ±46.2(13/14)[e]	287.5 ± 16.4(8/9)[e]
0.01 mg/liter	0(3)	0(1)	50.0 ± 0(2/5)[d]	—
0.10 mg/liter	0(3)	0(2)	—	—
1.0 mg/liter	—	0(3)	0(1)	0(1)
10.0 mg/liter	0(3)	0(2)	0(1)	—
Basal medium with 12.0% sucrose	—	33.0(1/2)[e]	85.0 ± 16.3(10/10)[e]	171.4 ± 18.4(14/14)[e]
Basal medium with 18.0% sucrose	—	0(2)	99.5 ± 23.2(10/10)[e]	150.0 ± 33.6(8/8)[e]
Basal medium with 10× major salts	0(1)	0(3)	60.7 ± 13.2(7/8)[e]	111.4 ± 13.7(7/8)[e]
Basal medium with 0.10 mg/liter IAA, 0.001 mg/liter kinetin and 0.001 mg/liter adenine sulfate, and 12.0% sucrose	—	0(3)	75.0 ± 22.4(5/5)[e]	83.3 ± 15.8(6/6)[e]
Basal medium with 0.10 mg/liter IAA, 0.001 mg/liter kinetin and 0.001 mg/liter adenine sulfate and 10× major salts	—	0(1)	31.6 ± 8.7(6/7)[d]	105.0 ± 3.7(4/4)[d,e]

[a] The figures in parenthesis indicate the number of embryos cultured.

[b] The pairs of figures in parenthesis indicate the number of embryos which showed any measurable increase in size and the number of embryos cultured. Thus, (1/24) means that out of 24 embryos cultured, only 1 had grown to the extent shown on the left of this figure in the table.

[c] Growth due to increase in volume of the embryos without evident cell divisions. These embryos did not show any appreciable growth or morphogenesis even after prolonged periods in culture.

[d] Embryos which develop by both cell enlargement and cell division. Those showing cell division predominantly assumed the shape of a sphere. Often growth was terminated at this stage and no differentiation of cotyledons was observed.

[e] Cell division was rapid during the first 1–2 weeks in culture. Many embryos in this group proceeded to form the cotyledonary lobes and the root and shoot meristems. For description see text.

and growth observed in globular embryos. It was also conceivable that certain of the organic supplements in the medium such as the vitamins were below the level required for optimum growth of the globular embryos. However, even in media with 5× or 10× vitamins, attempts to grow these embryos were unsuccessful. In additional experiments, high or low pH of the medium, high oxygen tension, and high (30 C) or low (20 and 10 C) temperatures were without beneficial effect.

In further attempts to achieve growth of the globular embryos, various mixtures containing the growth factors indoleacetic acid (IAA), kinetin, and adenine sulfate were tested as components of

the basal medium, because their profound influence on organ initiation had been observed in heart-shaped and older embryos. The results of these experiments are summarized in Table 3. Because of the difficulty in handling globular embryos, cultures described in this section were made in hanging drops and kept in the incubator in the dark at 25 C.

When used alone, IAA was generally toxic to the embryos, although in a few cases increases in length of up to 30 μ were noted during the first week in culture. On addition of kinetin several instances of limited growth in size of embryos accompanied by cell division were recorded at 0.0001–0.001 mg/liter kinetin (Veen, 1962). The consistency of the promotive effect of kinetin, although admittedly small, made us look for other substances which, in combination with IAA and kinetin, would sustain cell division and growth in the embryos. As is apparent in Table 3, the addition of low concentration of adenine to the medium had a dramatic effect in inducing continued growth of the embryos. The results show that a combination of IAA, kinetin, and adenine sulfate added as supplements to the basal medium provides the best medium for continued cell division and growth of embryos initially in the range 50–80 μ in length.

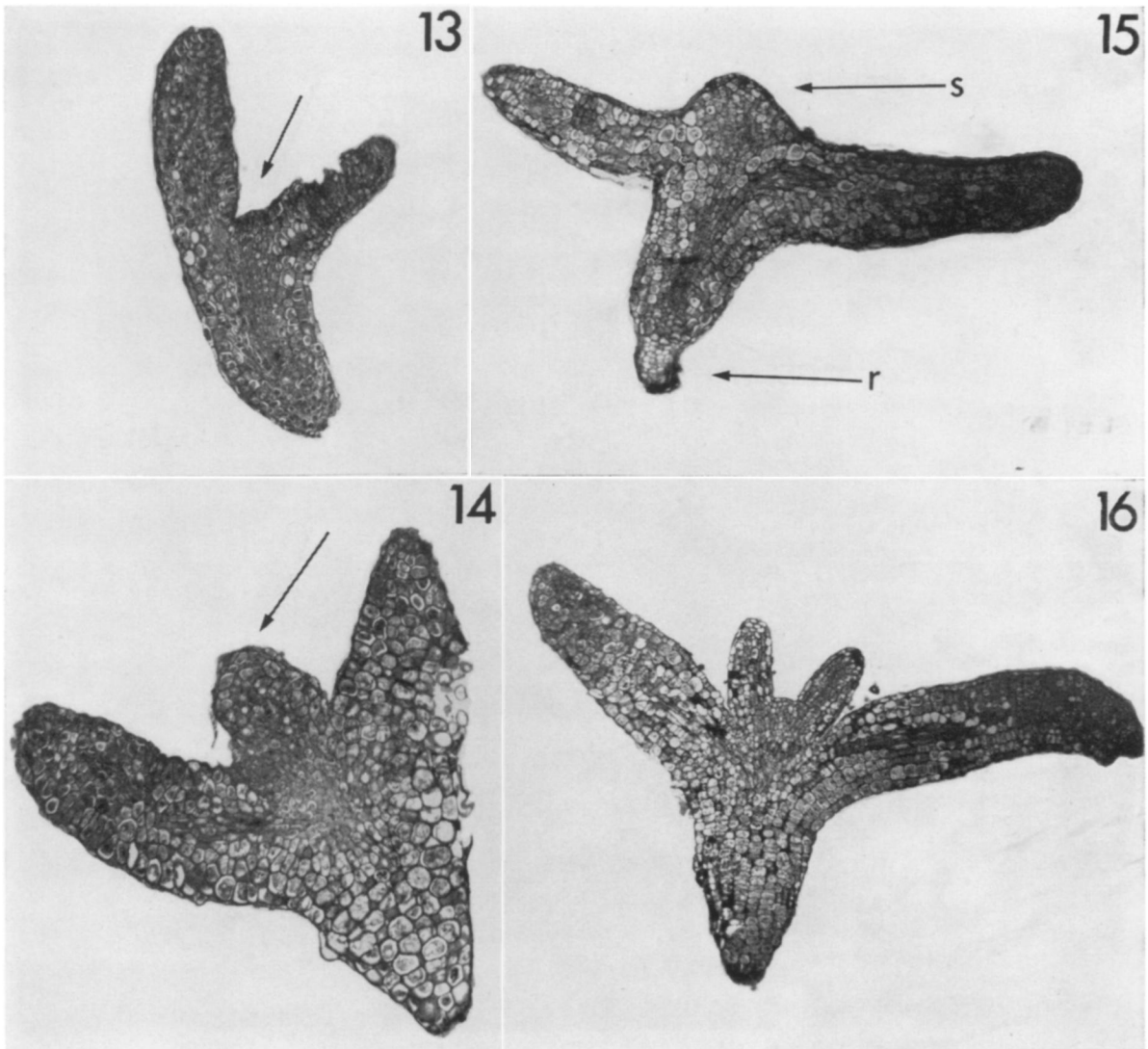
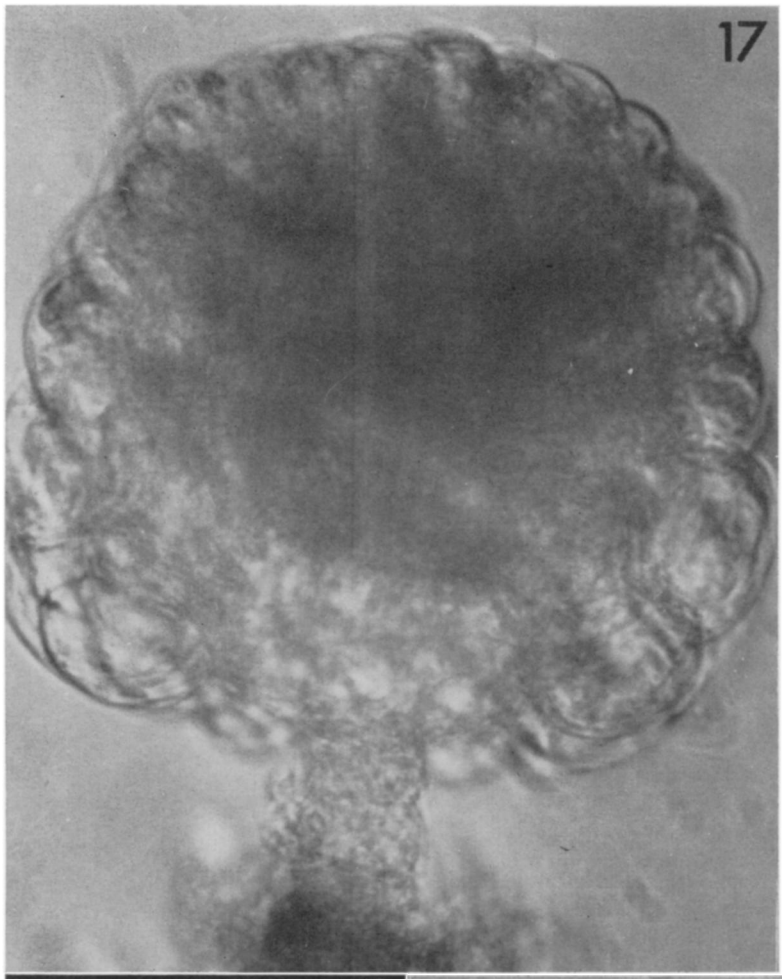


Fig. 13–16. Longitudinal sections of early heart-shaped embryos after being grown for different periods in culture.—Fig. 13. An embryo after 3 weeks in culture in the dark. The position of the shoot apex is indicated by arrow. The root apex is not visible in the plane of sectioning. ×260.—Fig. 14. A light-grown culture of comparable age. The shoot meristem (arrow) is prominent. ×260.—Fig. 15. Section of an embryo after 5 weeks in the dark: (r) root; (s) shoot. Note the lack of cytohistological zonation pattern in the apical meristem. ×130.—Fig. 16. A light-grown embryo 5 weeks old showing the formation of the first pair of leaves. ×130.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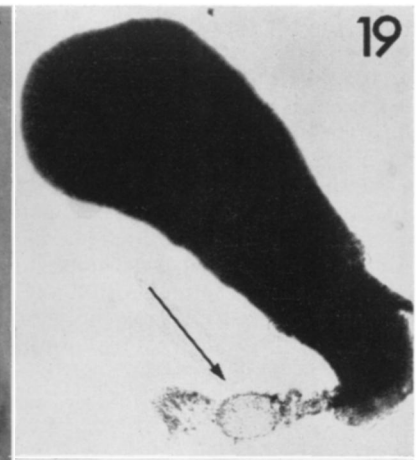
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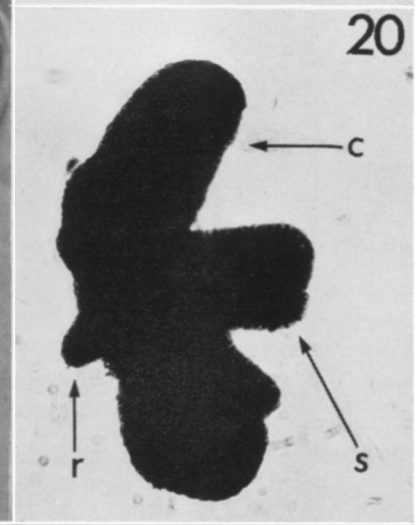
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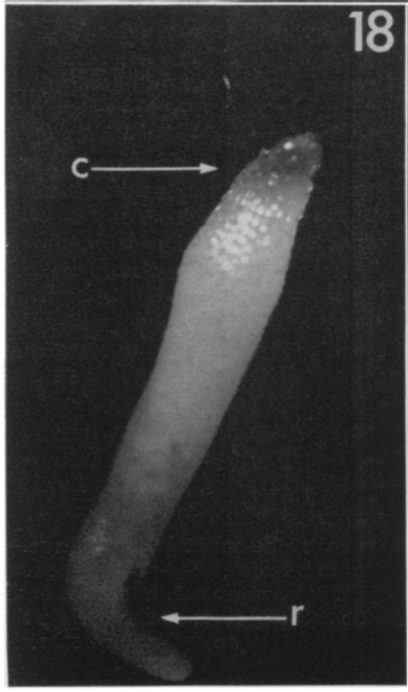
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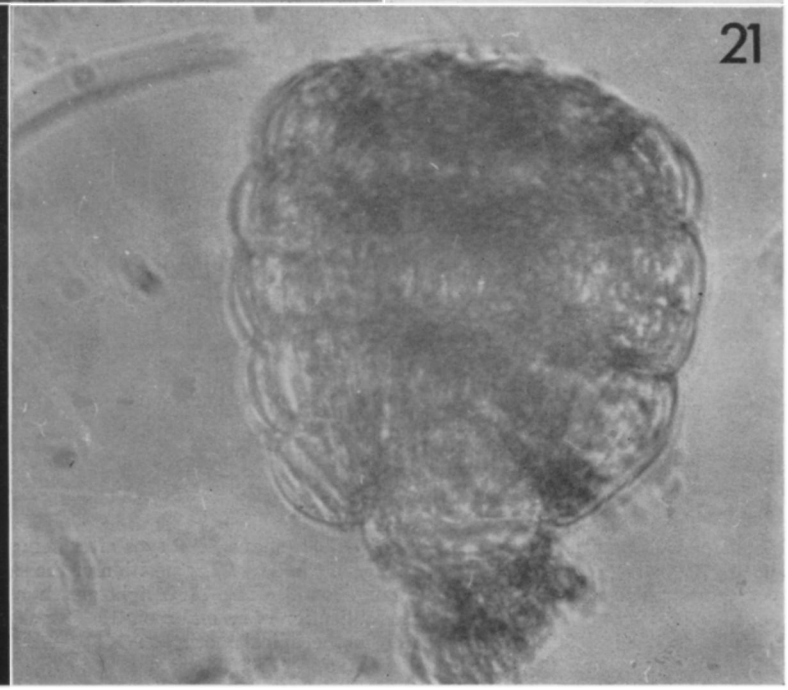
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The only other supplements to the basal medium which supported growth of embryos of comparable length were either high concentrations (12% and 18%) sucrose or 10× the concentration of major salts. Thus, the effect of a delicate balance between IAA, kinetin, and adenine sulfate can be substituted for, in part, by a high sucrose concentration or an increased salt level. However, even these media were inadequate to induce cellular divisions and growth of still smaller embryos.

In Fig. 17, 18 is illustrated the development of an early globular embryo, initially 54 μ long (cf. Fig. 1), in the basal medium supplemented with IAA, kinetin and adenine at 0.1, 0.001 and 0.001 mg/liter respectively. During the first 7–10 days in culture, the embryo increased in size as a sphere by irregular cell divisions and cell enlargement (Fig. 17). The lack of an organized division sequence was especially evident in the outer layers of cells, some of which protruded from the body of the embryo. After the embryo had grown to a few hundred microns in length, bilateral differentiation was observed with the formation of the cotyledons. Further growth of the embryo was similar to that described earlier for heart-shaped embryos. Occasionally, abnormal structures, reminiscent of no specific stages in the embryogenesis of the species, resulted from continued division at and following the sphere-like stage (Fig. 19, 20). Most embryos <54 μ long at isolation did not grow in this medium, while a few which showed signs of growth did not develop beyond the period of early cell divisions (Fig. 21). The pattern of development observed in the globular embryos when cultured in high sucrose or high salt media was similar to that described above.

Discussion—One important modification of the basal medium made by previous workers in attempts to culture very small plant embryos has been the adjustment of its osmotic value. Improved development of immature embryos following such modification led them to the conclusion that a medium isotonic with the ovular sap is a sine qua non for successful culture of very small embryos. In their work on immature barley embryos, Ziebur and Brink (1951) and Norstog (1961) secured suitably high osmotic pressures by the use of 12.0–12.5% sucrose and succeeded in growing embryos of 60–300 μ initial length. Isolated embryos of *Datura stramonium* of different ages required different minimal sucrose concentrations for development, the optimum for preheart-shaped embryos being 8–12% (Rietsema et al., 1953). Rijven (1952) routinely used 12% and 18% sucrose to grow torpedo-shaped and heart-shaped embryos of *Capsella*. Embryos of cotton (Mauney, 1961) and *Citrus* (Swamy, 1961) have also been shown to be sensitive to the osmotic value of the culture medium. On the other hand, we have found that in a semi-solid medium containing only mineral salts, vitamins, and 2% sucrose, embryos of *Capsella* as small as 81 μ regularly undergo cellular divisions and differentiation without evident dependence on a high osmotic value in the medium.

In view of the implications from our findings, it is necessary to re-examine the above-mentioned reports about the importance of the osmotic balance of the medium in the culture of small embryos. Our findings provide new information in 2 respects: First, the nutritional requirements of heart-shaped and older embryos of *Capsella* are not as complex as reported by Rijven (1952). For example, no requirement was found for an exogenous amino acid supply. Second, the inhibition in growth of embryos in a hypotonic medium observed by Rijven need not be ascribed to the physical state of the culture medium. The growth of heart-shaped embryos into small plantlets in a simple medium indicates that for these embryos the nutritional requirements are surprisingly simple. In such embryos development is sustained by amino acids and growth factors available within the embryos themselves, or the embryos can synthesize them from the mineral salts and sucrose provided in the medium.

Rijven (1952) attempted to explain the observed effects of the osmotic value of the culture medium in terms of the control of growth that occurs in the embryos; in his view, high osmotic pressure prevents cell elongation leading to germination. The pattern of development in vitro of early heart-shaped embryos described in this paper is not germination in Rijven's sense, since it does not involve "incipient elongation" of the hypocotyl. Furthermore, the sequence of development of such embryos is similar to that of the mature ones cultured in vitro. Thus, like mature embryos, even the smallest cultured embryos routinely initiated a root, a shoot apex, and later, several pairs of leaves, in that order.

Our findings show that growth and differentiation in the cultured embryos are regulated, not by the osmotic condition of the culture medium, but by specific constituents of the medium which may act by their control of differential cell division and cell enlargement during development. Results of experiments on the culture of globular embryos (<80 μ long) tend to confirm conclusions concerning the general importance of growth fac-

Fig. 17–21. Cultures of globular embryos.—Fig. 17. Formation of a spherical mass from an embryo initially 54 μ long, after 10 days in culture. ×260.—Fig. 18. Differentiation of the cotyledons (c) and root meristem (r) after 6 weeks in culture. The cotyledons are folded together and not visible separately. ×57.—Fig. 19. Abnormal growth of a globular embryo in vitro. The suspensor (arrow) is still attached to the embryo. Development has proceeded without apparent differentiation into the cotyledons. ×130.—Fig. 20. Another type of abnormal growth in globular embryo in vitro. Note the wing-like cotyledons (c), root (r) and shoot (s) regions. ×130.—Fig. 21. An early globular embryo of 40.5 μ initial length, after 12 weeks in culture. Development terminated after a few early cell divisions. ×260.

tors in stimulating embryonic development. In other studies, we have found that beneficial effects on the development of heart-shaped and older embryos are exerted by varying concentrations of IAA, adenine sulfate and kinetin provided separately. No such effects were observed in the development of globular embryos when these substances were supplied individually in the medium. Such observations on the different sensitivities of embryos of different sizes led to the idea that chemical factors, hormone-like in their action, were involved in the inhibition or stimulation of cell division and cell enlargement in embryos of different developmental stages. Clearly, these controlling systems must be amenable to experimental manipulation through changing the composition of the growth factor components of the medium. For globular embryos, a balance among all 3 factors—IAA, adenine and kinetin—was achieved which permitted development to proceed. Presumably, a different balance of these and perhaps other factors is necessary for normal development of isolated embryos of still younger stages. It is perhaps too simple to visualize kinetin as a cell division factor (Miller, 1961) and IAA as controlling cell enlargement; rather the interaction of these factors on the young embryos in isolation provides the necessary chemical environment for the developing embryo to proceed in its normal development drawing upon its chemical environment.

An especially interesting observation from experiments with globular embryos is that the beneficial effect of added growth factors can be partially replaced by growing the embryos in a medium with 12–18% sucrose or with an increased level of the macronutrient salts. Thus, globular embryos show a reduced requirement for exogenous growth factors like IAA, adenine and kinetin in the presence of high concentrations of sucrose or major salts. Some support for this line of thinking is provided by the works of Van Overbeek et al. (1942) and Rietsema et al. (1953) on the in vitro culture of embryos of *Datura stramonium*. Van Overbeek et al. found that heart-shaped embryos (150–500 μ long) of this species could be successfully grown in a medium containing mineral salts, 1% dextrose and a mixture of physiologically active substances (glycine, thiamin, ascorbic acid, nicotinic acid, pyridoxin HCl, adenine, succinic acid and pantothenic acid) supplemented with non-autoclaved coconut milk. In later experiments (Van Overbeek, 1942; Van Overbeek, Siu and Haagen-Smit, 1944), beneficial effects of the same magnitude were obtained using a purified embryo factor preparation from coconut milk in a dilution of 1:19,000. On the other hand, Rietsema et al. have reported that *Datura* embryos could be grown in a mineral salt medium containing 400 mg/liter casein hydrolysate and an optimum sucrose concentration of 8–12%. Since coconut milk is known to contain "auxins" and substances which specifically stimulate cell division (probably kinins) in the less completely identified "active fraction" (Pollard, Shantz and Steward, 1961), this represents a parallel case where the requirement for growth factors has been satisfied by the high sucrose concentration.

At this stage of investigation, it is not certain whether the high concentration of sucrose in the absence of added growth factors exerts an osmotic effect, or whether it influences the metabolism of the cultured tissues in some other unknown way. Although the embryos develop better in a medium with growth substances plus 2% sucrose than do those cultured in high sucrose or high salt medium, our present evidence does not eliminate a role for sucrose in the in vitro development of these embryos as balancing the osmotic medium of the sap. It is probable that the activity or one component of the balanced hormonal control system is in turn controlled by the high sucrose or salt concentration, perhaps through osmotic processes preventing cell elongation, which determines the optimum balance between cell division and cell enlargement (cf. Adamson, 1962). It is further possible that culture of larger embryos on agar rather than in liquid affects in some way the osmotic contribution of the nutrient medium.

Similarly, the role of high salt concentration in substituting for the added growth factors is not understood. In their ability to dispense with exogenous precursors when grown in a medium with high salt, globular embryos of *Capsella* resemble the crown gall tumor tissue recently described by Wood and Braun (1961). They found that the normal and partially altered tumor cell types of *Vinca rosea* lost their requirement for glutamine, asparagine, cytidylic and guanylic acids, and inositol and glutamine, respectively, when grown in White's medium with increased inorganic salt levels. In the case of embryos, the growth factors were not limiting, since no enhancement in growth occurred in a medium with 12% sucrose or 10× salts to which adenine sulfate, kinetin and IAA were added in the usual proportions.

Although the embryos of a number of angiosperms have been successfully grown in vitro, we have little knowledge of the physiological mechanism of organ initiation in cultured embryos. In the present experiments, even the smallest embryos cultured were potentially capable of producing a shoot and/or a root system characteristic of the adult plant. Complete development of the shoot system is slightly accelerated in light. In isolated embryos $<1000\mu$ long, a primary root system developed only in cultures grown in the dark, while longer embryos produced root systems in both light and dark. To explain this variation in embryos differing only in a few hundred microns, it is suggested that mature embryos produce the necessary stimulus for the growth of the primary root system characteristic of the adult plant when grown in light or dark conditions. In younger embryos if cultured in light, the stimulus is lost or not formed in adequate amount.

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