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On embryos and embryoids

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Abstract. The ontogeny of the embryoids arising from a variety of somatic and male gametophytic tissues has been compared to that of a zygote embryo; it has often been stressed that a free cell *recapitulates* the ontogenetic sequence of stages that are seen in the development of a zygote into an embryo. An embryoid significantly lacks a polarised ontogenetic pattern throughout the gamut of differentiation; it lacks typical centres of polar organization like the hypophysis and the epiphysis in early ontogeny; the protoderm formation is belated and also incomplete; the organization of tap root is suppressed. In spite of assuming exomorphic contours comparable with those of the gross developmental stages recognized in embryogenesis consequential to sexual fusion, the embryoid is devoid of internal differentiation both in degree and pattern. Under these circumstances the ontogeny of an embryoid never stands comparison with that of a zygotic embryo. From the morphological standpoint an embryoid is more closely related to a shoot bud.

Keywords. Embryo; embryoid; shoot bud; embryogenesis; ontogeny; tissue differentiation.

1. Introduction

One of the most remarkable achievements of plant tissue culture in recent years has been the inducement of single cells to develop into mature plants. The developmental stages leading to the establishment of the young sporophyte through this method have been described in terms of *in vivo* embryogenesis. Comparisons have been drawn to the globular, heart-shaped and torpedo-shaped embryos; root and shoot apices, and poles have also been recognized. Questionable comments have been offered in reference to the internal differentiation. Such statements as these have found culmination in categorically asserting that "any diploid cell is potentially totipotent and can behave like a zygote" (Steward and Mapes 1963) and pointed attention is drawn to "the very faithful way in which the growth from the free cells could recapitulate normal embryogeny, with the

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formation of globular, heart-shaped and torpedo and cotyledonary states" (Steward 1968).

It is well-known that the bulk of literature dealing with the normal embryogenesis in angiosperms is restricted to the earlier phases of development. The sequences and plane of cell divisions in the zygote up to the attainment of late globular stage or early heart-shaped stage have been well determined. The late embryogeny, however, is largely described in terms of the heart and torpedo stages without giving serious consideration to their internal differentiation. It must be said that these stages have never received the attention that they deserve in embryological studies. Such being the contemporary situation, it is necessary to examine the force of the argument in reference to a single cell in culture recapitulating the ontogeny of a fertilized egg. The sequence of divisions and of differentiation leading to the embryoids in callus chunks should also be critically studied.

Embryoids have been obtained from cells and tissues of diverse origin and chromosome numbers. Somatic cells ($2n$) from the root to the floral parts have been employed; gametophytic cells (n) from pollen have been raised to embryoids, which are known as androgenic embryoids. The usually triploid endosperm cells have also been made to proliferate from the cells of which embryoids have been raised.

2. Embryoids from callus tissue and single cells

A callus has often been defined as a mass of undifferentiated cells, but this may not be an altogether correct definition. The cells in the callus do show a gradient of cellular organization and behaviour under cultural conditions. Halperin and Jensen (1967) have demonstrated that the superficial layer of cells in the callus are different from the rest in terms of organelle distribution and quantum. Enlarged nuclei, large nucleoli and increase in the intensity of pyronin staining also distinguish them from the more deeper layers (Danilina 1972). Perhaps the reason for this may be that the superficial cells are the ones that are directly exposed to the culture environment. Owing to the differential cytological characters the superficial cells often form the initials for the embryoids.

After the completion of a few divisions, it is quite easy to delimit the outline of the initial embryoid within the callus. The clarity has been interpreted as probably due to the discontinuity of the embryoid cells from the rest of the callus and possibly due to the chemical modification of the external cell walls of the embryoid which border the remaining cells. This often results in the separation of young embryoids from the callus while manipulating them for microtome sectioning and also while the culture vessel is subjected to violent motion. Occasionally spontaneous separation is also noticed in several cases. The delimitation time of an embryoid has not been precisely established nor the stage of the embryoid at which it will be able to survive in spite of separation. The separation may be effected from single-celled condition to the torpedo stage. Perhaps it varies in one and the same culture vessel. McWilliam *et al* (1974) state that in carrot the separation of survivable embryoids occurs when they are in the late globular stage but more commonly when they are at heart or torpedo stages.

2.1. *Sequence and patterns of divisions in the initiating cells and the attainment of exomorphic contour*

The initials for embryoids that arise in callus are usually peripherally located. Interior cells of the callus have also been reported to develop into embryoids (Sussex 1972). The superficial cells are roughly cylindrical in shape and are densely cytoplasmic. Single cells lying freely in the culture vessel after their separation from the callus (either from superficial or deeper layers) are potential initials for embryoids; they vary in shape and size depending upon the plant or sometimes even within the same culture vessel.*

The first division in the embryoid initial is usually transverse in most cases but the walls laid down in vertical or oblique planes are also generally met with. The transverse division often takes place parallel to the surface of the callus and results in two unequal cells. The first division plane in isolated embryoid initial is also usually transverse, but this bears no relationship whatsoever with the division planes in adjacent cells or to the culture vessel. Daughter cells of equal volumes are formed in several cases.

In carrot, the next division appears to be vertical in the "terminal cell" and transverse or vertical in the "basal cell", the resulting product assuming a filamentous outline. Instances where the "terminal cell" also undergoes a transverse division are not uncommon. The derivatives of the smaller "terminal cell" form the embryoid proper and of the larger one, the "basal cell" the "suspensor". The sequence of divisions in the "terminal cell" giving rise to the early globular embryoid of eight or more cells has been described as being similar to the planes of cleavage occurring in a quasifluid system in a way strikingly paralelled by the segmentations leading to the formation of glandular hairs in certain plants (McWilliam *et al* 1974; see also Steward and Mohan Ram 1961). However, it should be mentioned that these divisions are not often predictable *contra* to the situation in normal embryogenesis. In fact, in a number of instances where the ontogeny of t e embryoid has been traced, the divisions leading to the globular stage are merely described as "irregular" or as taking place "in all planes". Similarly the divisions in the "suspensor" initial cells are all irregular contrary to the normal situation.

The most neglected aspect of the embryoid ontogeny has been the tracing of the planes and sequence of divisions leading from the early globular, to the late globular, to the subsequent heart and torpedo stages of the embryoid. However, it is obligatory at this point to stress the important differences between the embryo and embryoid ontogenies with particular reference to the sequences and pattern of division activity as may be gleaned from published work.

The outstanding feature of the *in vivo* embryogeny is the orderly and predetermined sequence of cell divisions that lead to the exomorphic globular contour of the embryo, characteristic of the given species. All such divisions strictly obey

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* Some authors question whether there is any true instance of single cells, freely suspended in the culture medium, giving rise to embryoids (personal communication from Professor Street). Since such embryoids have also been reported in the literature (see Danilina 1972), they are considered in the present article.

the physical laws that govern cell division in general. The most important of these are those of Sachs and Errera : (i) Sach's law that new walls intersect the old ones at right angles, (ii) Sach's law of equal masses in the daughter cells and (iii) Errera's law that the new wall is formed along the, minimal area. However, the cell divisions that lead to the embryoid formation in cultured cells and tissues do not often follow these laws. They "discourage geometrical or mathematical analysis of the division of freely suspended cells in terms of either the directions of the new wall or the shapes and proportions of the daughter cells" (Steward 1958). Steward further suggests that "the divisions seem to reflect the highly variable nature of these cells and to be determined by intrinsic rather than extrinsic factors". In other words, more than the culture environment to which the cells are subjected, it is the inherent nature of these cells that is largely responsible for their variable behaviour in respect of their segmentation pattern.

However, one should not ignore the importance of external factors in controlling the segmentation pattern. In fact, spatial factors, constituents of the cultural medium and their concentration, and the presence or absence of polar forces in the environment are all very important. If the cell is in equilibrium with the neighbouring cells and their environment, its dividing phase proceeds in an orderly manner with a predictable pattern. Such is the exact condition under which the zygote is located. But a cell in a culture vessel is not in equilibrium with its neighbours and its environment. As a result, the division pattern here discards all physical laws and developmental norm that govern it. As a result, there is no common pattern of division sequence, new walls being laid down in varied planes (Sussex 1972).

Variations are noticed in cells present in the same culture vessel with respect to their behaviour and division patterns when it gives rise to a globular, heart-shaped and torpedo-staged mass of cells. We may not be able to pinpoint that it is this particular ontogeny that a cell has undergone during its presumed development into an embryoid. In some cell units there may be a progressive increase in cell number without cell enlargement (Sussex 1972) while in others cell enlargement proceeds without significant increase in cell number. "In a large measure, therefore, the individual cells grow in an irregular and highly individual fashion each independently exhibiting sequence of enlargement and division" (Steward 1958).

A number of investigators have traced the embryoid development in carrot and they report not one but several ontogenetic sequences. None of them correspond with the developmental steps as illustrated by Borthwick (1931) for the normal carrot. McWilliam *et al* (1974) themselves agree that the normal carrot embryogeny is of the Solanad or Rubiad type while that of the embryoid is of the Cruciferad or Onagrad type. No correspondence is seen between the embryo and embryoid ontogenies in plants like *Petunia, Atropa, Arceuthobium, Dendropthoe, Ranunculus, Nicotiana,* etc., in which both the *in vitro* and *in vivo* ontogenies are known. In all these instances the ontogenetic sequence of the embryoid has been accepted to be unpredictable. It appears as if the stages are picked out from many in the medium and have been used to draw comparison and to build up the semblance of a sequence. The very fundamental assumption of these authors is that the developmental sequence of embryoids is constructed on the basis of various 'stages' seen in the culture medium. Whether the sequence is

arranged by the author in his own subjective style or whether it is really the sequence of events cannot be verified.

One of the serious objections that can be raised concerns the two-dimensional filamentous stages seen in culture flasks being labelled as filamentous stages of the embryoids.* Many of these filaments are uniseriate with the presence of longitudinal divisions in some of the cells located usually in the middle part. The frequency of divisions is more in this region which means that the main body of the embryoid is derived from intercalarilly positioned cells of the filaments. This situation is totally unlike that of the filamentous stage of the normally developing embryo where the cells located at one end (chalazal) alone give rise to the embryonal body.

2.2 *The question polarity*

The most significant aspect of *in vivo* "embryogenesis" is the phenomenon of polarity which becomes manifest in the zygote itself. There is an unequal distribution of the cytoplasmic organelles coupled with the location of the nucleus nearer the densely distributed locus. This is followed by an asymmetric division of the zygote to result in a small, densely cytoplasmic embryonal initial and a sparsely cytoplasmic suspensor initial again indicating the presence of strong polarizing forces. In the subsequent stages also the polar forces are maintained which become manifest by the organization of the hypophysis at the root pole and epiphysis at the shoot pole with the intervening part forming the axis of the embryo.

In all cases of embryoid development, whether from single cells or from cell groups or callus, there is no evidence of organized polar forces guiding the ontogeny. In single cells that are compared with zygotes, there is no evidence of morphological polarity (Danilina 1972). This is evident not only by the centrally placed nucleus and absence of characteristic vacuole but also by the diverse ways in which the first division of the cell takes place. Either the resultant cells are equal or unequal in size and the wall is laid down transversely, obliquely or vertically there being no uniformity under the same cultural conditions. Also, the distribution of cytoplasm to the daughter cells does not indicate the action of polarity.

In this connection it is pertinent to examine the cases of embryoid ontogeny where polarity is reported to be manifest (see Street 1976). Embryoids developed from callus clumps are reported to be "uniformly polarized"; the root pole is towards the centre of the callus while the shoot is towards the outside. This distinction is reported to be initiated during the first division of the embryoid initial. The mere fact that the first division seggregates the two poles of the embryoid does not mean that the polarizing forces continue to manifest all through the further ontogeny (see the forthcoming paragraphs of the present paper). Even this presumed initial evidence of polarity is absent in microspore embryoids which start their ontogeny by an equal and symmetrical division of the microspore. However, in these embryoids it was assumed that polarity in the globular embryoid arises spontaneously (Street 1976). Haccius and Bhandari (1975) also

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* Street (1976), however, considers them not as a part of the embryoid development but as merely cell aggregates derived from free cells of the culture.

conceive of the establishment of polarity in non-zygotic embryos (including the embryoids) belatedly because of their indefinite position in relation to their environment; this is quite, contrary to the stand taken by Street (1976) who, as said earlier, states that the first division of the embryoid initial itself segregates the two poles.

The lack of polarity in the initial stages of the development of an embryoid from single cells is continued in later phase also. In fact Halperin and Wetherall (1964) observe that it is not known how or at what stage of development the polarity of the root-shoot axis is determined. We doubt whether there is any polarity at all at any stage of embryoid development since from the very start an organized radicular pole is never in evidence. There is also no hint for the operation of possible external polarizing forces in the callus cultures. In none of the embryoids described till now is there an organization of the hypophysis and epiphysis respectively at the root and shoot apices. Hanstein and Souèges, who first recognized these centres for the first time were impressed by their consistent differentiation at the two poles of the embryo although they could not fully appreciate the significance. The organization of hypophysis and epiphysis takes place almost simultaneously and very early in zygotic embryogenesis and this fact emphasizes the operation of equal polar forces at the two ends of the embryo.

2.3. *Factors in tissue differentiation*

Protoderm is initiated in the zygotic embryo immediately after the octant stage in the terminal tier through periclinal divisions. Delayed initiation is not observed in normal zygotic embryogenesis. In embryoids, however, there is not only belated differentiation of the protoderm (see also Haccius and Bhandari 1975) but also it is often incomplete. A careful examination of the published illustrations of the 'globular' embryoids reveals the absence of a typical protoderm. In carrot investigated by McWilliam *et al* (1974) "the delineation from within the cell mass of an epidermal layer enclosing a central group of cells does not take place until there are 32 or more cells in the embryoid proper", and in those studied by Halperin and Wetherall (1964), the "protoderm appears to arise in a patch work random fashion and not as a uniform layer". In the latter case, the protoderm may differentiate even after the development of the cotyledonary node or simultaneously with it. In carrot investigated by Sussex (1972), "there is no stratified surface layer of cells and divisions in surface cells may be in any plane". The absence of a protoderm continues for a prolonged period so that the shoot apex which differentiates later on possesses an incomplete outer tunica layer or with the cotyledons and hypocotyl with an incomplete epidermal layer (Sussex 1972).

Precocious differentiation of vascular tissues is reported in a few cases including *Cichorium endivia* (Vasil and Hilderbrandt 1960). The initiation of vasculature has been reported, as early as the 'globular' stage of the embryo in this species. In fact, long before the organization of a root meristem (adventive root?) its vasculature has developed! Only later on, it is connected with that of the main body of the embryoid, an instance that is quite analogous to that seen in adventitious root buds. Sussex (1972) reported an early differentiation of sieve tubes and tracheary elements in the "cotyledons" and "hypocotyl" in carrot embryoid. It may be mentioned that vascular differentiation in normal embryogenesis in

carrot is initiated only after germination (unpublished observations of the first author).

The organization of a typical suspensor comparable with that of the zygotic embryo is never encountered in the embryoid of any species. However, any cell or a group of cells attached to the so-called radicular pole of an embryoid has been given the name suspensor. The use of this term has been rather indiscriminate in embryoid literature mainly because of the preconceived notion that an embryoid is equivalent to an embryo developed from zygote.

3. Embryoid to "seedling"

The most neglected aspect of the study of embryoid is the transition stage between the embryoid and its "seedling". Nearly all the investigations where young plants have been obtained from embryoids, at best contain exomorphic descriptions of the transitional stages, and even this has never been done in adequate detail. The anatomical aspects of root-shoot "transition" has not been studied. The purpose of this section is to pose some consequential problems worthy of further investigations.

(i) Do the shoot and root (radicular) meristems attain their typical structural features at least in the early "seedling" stages? This is an important issue since in none of the embryoids described till now there is the organization of a typical root apex with the formation of hypophysis or quiescent centre initial. Similar is the case with the shoot apex where there is no epiphyseal differentiation. As mentioned elsewhere, the hypophysis and epiphysis are very important both from morphological and physiological standpoints (Swamy and Krishnamurthy 1975, 1977). If the organisation of these centres is belated, at what stage and in what method do they become organized?

(ii) The second problem that has to be paid attention to, is the way in which vascular differentiation takes place in the "germinating" embryoid. Especially important is the mechanism of the establishment of vascular continuity between the stem and root of the embryoid—"seedling". What is the nature of the first xylem element that differentiated in the germinating embryoid and what is its locus in the axis? This information is important not only in view of the absence of a typical procambial system in the embryoid but also in view of the absence of elongation (at least not to the same extent as in *in vivo* seedling) in a germinating embryoid. If there is no elongation, the first xylary element may not belong to the protoxylem category, but on the contrary to a "secondary xylem", differentiated from a parenchymatous cell.

4. Morphologieal nature of the embryoid

In literature on tissue culture several alternative terms have been employed to designate the embryoid in spite of the repeated usage of the latter term : embryo bud, embryonal bud, neomorph, pseudobulbil and plantlet. This raises questions, as to the morphological nature of the embryoid. Also are in use terms such as *embryogenesis* to describe the embryoidal ontogeny because of the assumption

that embryoids are nothing but embryos in every respect. While the zygotic embryo is always produced after sexual fusion, the embryoid is obtained in a variety of tissue cultures involving both diploid and haploid inocula ; embryoids have been cultured even from zygotic embryos. Therefore it is very important to clearly distinguish the embryoids from the sexually produced embryo (Vasil and Hildebrandt 1966).

It is clearly seen that none of the sequences of embryoid ontogeny in the culture conditions (figures 1B to D) bear any similarity to those of the zygotic embryogenesis. Morphological similarity may be exhibited at certain stages of ontogeny, especially the filamentous, globular, heart—and torpedo—stages. However, none of these stages show any internal organization typical of the sexually developed embryo. Vasil and Hilderbrandt (1966) have already pointed out that the "two phenomena are completely different, not only because one is a sexual and the other asexual, but also because the two structures may have different genetic composition and they develop under very different physiological and morphological conditions ".

All the embryoids that develop under tissue culture or free cell culture conditions exhibit greater similarities to the adventive embryos and shoot buds that arise on diverse parts of the plant body. In the latter instances there is a precocious origin of the shoot part, the roots arising later on from the basal part of the shoot in an adventitious manner. In fact, it is such structures that are very frequently met with in tissue cultures. From the surface of the callus two prophyll-like structures are produced enclosing a shoot meristem (for example, in Vasil and Hilderbrandt 1966). Later on they develop into new plants with several adventitious roots arising at the base. Very few of the shoots immediately after formation are directly associated with the roots, but become later on connected by vascular strands developed in the intervening part. Even those freely lying embryoids (in the culture flask) that possess typical torpedo shape are more akin to the adventitious buds than to the corresponding stage of the sexually produced embryo because in none of the embryoids there is a typical organization of the radicle; when these "germinate", there is no tap root formation, but only the development of adventitious roots. Even if tap root-like structures are seen, they are not strictly comparable to typical tap roots of sexually produced seedlings in their internal organization.

It may thus be concluded that the embryoid of any origin and shape is nothing but an adventitious shoot bud containing an axis and a shoot meristem enclosed by two prophylls, thus resembling a mature embryo in exomorphic form. Even in a few monocotyledon tissue culture where structures resembling embryoids have been obtained, they contain two "cotyledonary" structures and *not one* as should be the case if they are really akin to the zygotic embryo. Distinct "dicotyledonous" embryoids are produced, for example, in the callus cultures of garlic (Abo El-Nil 1977). In bulbil-forming monocotyledons (some members of the Liliaceae) the bulbils or adventive buds initally possess two prophylls and *not one*.

Instances where pseudobulbil-like structures become transformed into "embryoids" under culture have been reported. For example, in the cultured post-fertilization ovules of *Citrus microcarpa* (containing many nucellar 'embryos'), the tumeroid outgrowths, designated as pseudobulbils, are developed all over the

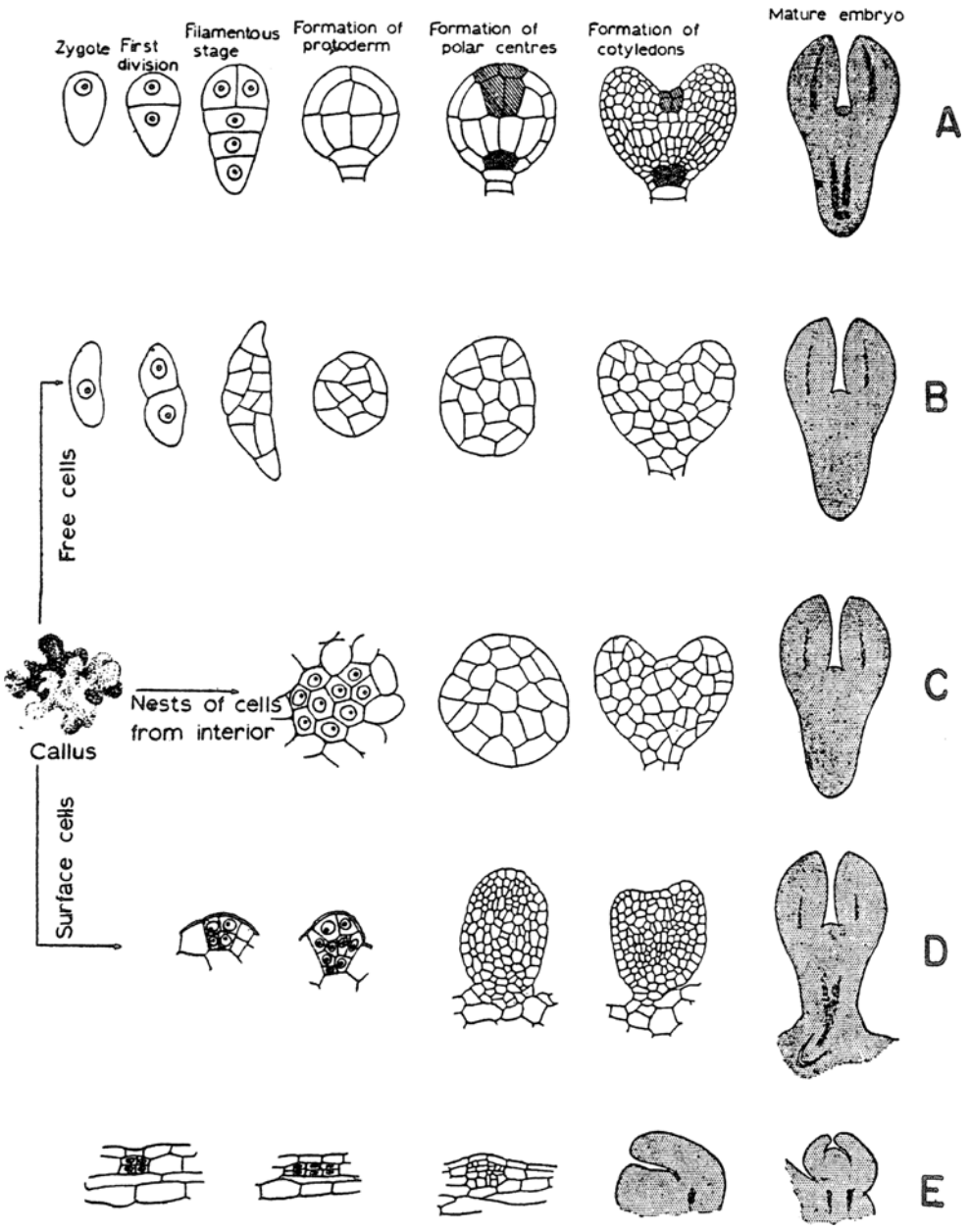

Figure 1. Diagrammatic representation of the development of a sexually produced typical dicotyledon embryo (A), of embryoids (B, C and D) and of an adventitious shoot bud (E). For further explanation see text.

surface of the nucellus (Rangaswamy 1961). Frequently the pseudobulbils became transformed into 'embryos' that even produce 'seedlings'. It is pertinent here only to remark that pseudobulbils and 'embryos' (embryoids) actually belong to one and the same morphological category.

The ontogenetic sequences in the development of embryoids in figure 1 are reconstructed from the works of several authors. The bud arises either from a single cell or from groups of cells in the callus but separated from the mass of surface or deeper layers of the callus. Consequently the 'root tip' part is in continuation of the shoot tip in the first two sequences (B, C) or separated from it by tissues of the callus in the third (d) sequence. In all these instances, however, there is no typical radicle or tap root, all roots arising later being of the adventitious category. In the first two sequences the adventitious roots arise from the 'radicle' directly or after calussing in that part. In the later sequence, the roots arise independently from the callus tissue and the connection with the shoot part is established much later. A point of further significance is that all the three sequences are noticed in one and the same culture vessel containing the growing tissues of *Atropa belladona* (Konar *et al* 1972). This again indicates that the resultant product is an adventitious bud of diverse shapes and origin. We therefore suggest either the total abandonment of the term embryoid and replace it with 'adventive bud' or retain the term with the caution that no similarity to the zygotic embryo should be drawn or implied.

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