On embryos and embryoids

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The literature on embryo and embryoid is critically reviewed in order to verify the assumption of several investigators that the two are identical. It is concluded that there are more differences than similarities between the two and that the embryoids are adventitious buds produced *in vitro* in the various explants, or their derivative calli. The terms relevant to zygotic embryogenesis should differ from those relating to embryoid ontogeny.

THE prediction that somatic cells of vascular plants are totipotent, made at the dawn of the present century[1], was proved correct in the 1950s when Reinert[2] and Steward *et al.*[3] had independently described the production of whole plants from *in vitro* cultured cells of carrot root. This was followed by over 2000 papers using many cell types from diverse taxa of angiosperms and gymnosperms confirming the discovery.

Two basically different pathways of whole plant regeneration were described to be involved in *in vitro* development, one through 'organogenesis' and the other through 'somatic embryogenesis'. Active cell divisions in the cultured explant result in a callus, in which shoot buds differentiate with the concurrent, belated or prior production of root meristems; these calli are called 'organogenic'. The shoot bud and root meristem are not in direct contact with one another, but are always separated by an intermediate region of callus cells. The two together subsequently give rise to a new plant after establishing vascular connections between them. In somatic embryogenesis, either single cells of the explant give rise to an embryo-like structure directly without an intervening callus stage (= 'direct embryogenesis') or such embryo-like structures are produced from single cells of the callus derived from the explant (= 'indirect somatic embryogenesis'); the latter are called 'embryogenic calli'.

The embryo-like structures produced *in vitro* were first designated as 'embryoids' by early workers who appreciated the basic differences (including conceptual) between such structures and true embryos. Subsequently, however, terms such as embryo, proembryo, embryogenesis, and even seedling were increasingly used in embryoid literature inasmuch as that these terms have now almost totally replaced respectively, words such as embryoid, proembryoid, embryoidogenesis and plantlets. Gray[4] has in fact gone to the extent of asserting that the suffix 'oid' should be dropped. He further argues that distinctions between zygotic and non-zygotic embryos have become blurred. Such an assumption has already been reflected in statements such as the following: 'Somatic embryos undergo developmental events similar to those that occur within the embryo-proper region of zygotic embryos'[5], and 'the similarity between zygotic and somatic embryogenesis is both striking and remarkable'[6]. In fact, Zimmerman[6] has used the somatic embryo system as a potential model for studying early events in plant embryo development.

This paper examines the validity of such observations, after critically reviewing the available literature on embryos and embryoids (for full literature see Sankara Rao[7], Modhorst *et al.*[8] and Raghavan[9]).

Zygote vs embryoid-initial cell

The literature on somatic embryogenesis abounds in statements such as this: 'Any diploid cell is potentially totipotent and can behave like a zygote'[10]. Are zygotes and embryoid-initiating cells really similar? If, so, how similar? If, not, in what respects do they differ from each other?

The zygote is *always* a product of sexual fusion between one of the male gametes (carried by the pollen tube) and the egg. It is, therefore, diploid. It is always present in a predetermined location in the micropylar milieu of the embryo sac, and in a 'privileged' location[11]. The zygote also shows distinct polarity through an unequal distribution of the cytoplasmic organelles and metabolites, with the location of the nucleus in the densely cytoplasmic chalazal region and of a large vacuole in the poorly cytoplasmic micropylar region. This spatial asymmetry of cellular contents is derived from the egg cell, which in turn is located in the polarized embryo sac. This asymmetry appears to be a genetic feature, because a mutation in the GNOM/EMB 30 (GN) gene of *Arabidopsis* produces a non-elongate and almost symmetric zygote[12]. Many properties of the egg change drastically immediately after its fertilization, and these changes are triggered by the latter[11,13]. A characteristic feature of the zygote is the extremely limited/absence of *de novo* transcription. The source of mRNA, which is abundantly present and engaged by the ribosomes, and which is 'parcelled out' to code for

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the first proteins of the zygote is unknown[11-13]. Experiments conducted on tagging JIM 8 monoclonal antibodies to zygotic embryo during its development showed the presence of positive arabinogalactan protein epitopes for this antibody in the zygote and two-celled proembryo in species of *Brassica*[14].

Although diploid, the somatic cell, the progenitor of the embryoid, is not the product of sexual fusion. Its position in the explant, callus or culture vial is not predetermined and is therefore, obviously not 'privileged'. The somatic cell (stage 1) becomes a *competent cell* (stage 2); this becomes embryogenic, and still requires externally applied stimuli such as auxin treatment[15-17]. The embryogenic cell (stage 3) does not require the external stimulus[18]. In the carrot system, these three stages have been reported in phase O under the influence of auxins[16,17], followed by phases I, II and III. The embryogenic cell divides repeatedly and develops into a globular and heart-shaped structure. The embryogenic cells were designated *proembryogenic* cells by Halperin[19]. In *Cichorium* species the competent cells become invested with a callose wall before becoming the embryogenic cell[20]; other changes reported in this taxon are: (i) Decrease in its cytoplasmic content with the formation of a large vacuole, (ii) Enlargement of its nucleus, and (iii) Formation of thick radial strands of cytoplasm. It is not clear whether all these changes occur in other taxa (that have expressed somatic embryogenesis). Komamine and Kawahara[21] reported active DNA synthesis as a characteristic feature of embryogenic cells, in contrast to the absence of such synthesis in non-embryogenic cells. Expression of the somatic embryogenic receptor-like kinase (SERK) gene marks carrot cells that are competent to form somatic embryos[22]. Although its function and cellular localization are not known, the predicted SERK protein sequence resembles leucine-rich repeat receptor kinases. Recent experiments employing cell-tracking of JIM 8 antibody-labelled cells have shown that there is no direct correlation between the presence of JIM 8 epitope (for arabinogalactan proteins) on the cell wall and the ability of the cell to develop into somatic embryo[23].

The involvement of embryogenic glycoproteins (E-proteins) during transformation of somatic into embryogenic cells was suggested by Sung and Okimoto[24]. Three polypeptides *a*, *b* and *c* and a 21D7 protein were reported to be operative during this transformation in carrot system[21]. Other required changes reported are: more RNA and protein synthesis with the involvement of both transcription and translation[11], enhancement of the levels of polyamines (such as putrescine) and the associated enzyme arginine decarboxylase (which converts arginine to putrescine)[25,26], higher peroxidase activity[27], and changes in the isoenzymes of glutamate dehydrogenase. Komamine and Kawahara[21] reported the localization of poly(A)$^+$ RNA through *in situ* hybridization techniques, as well as of free calcium ions, in the embryogenic cells.

The initiating cells of the embryoid do not show any evidence of either morphological or cytological polarity[28]. The nucleus is centrally placed; the vacuole, characteristic of zygote, is often absent. The cytoplasm is often dense and evenly distributed throughout the cell.

In ovular, but non-zygotic embryogeny, haploid unfertilized cells of the embryo sac (other than antipodals) or diploid cells from nucellus or integument form the initiating cells of the embryo. In such cases too, there is no polarity in the distribution of cytoplasm in the embryo-initiating cells. They also do not have a specific and 'privileged' location[29].

The data shown above clearly indicate that, even if we equate the embryogenic cell to the zygote, to become comparable to the zygote, several complicated changes are a prerequisite. In other words, what fertilization process promotes in one stroke in the egg (which becomes the zygote), is attained by several changes in a somatic cell. In the light of this, can any somatic cell be considered as equivalent to a zygote?

The division of zygote

The division in the zygote is transverse (an almost longitudinal/oblique division is reported in some taxa like Loranthaceae, Balanophoraceae, *Scabiosa* of Dipsacaceae, and Piperaceae. In most of these instances, as well as in members of Orobanchaceae and many taxa of Orchidaceae, the embryo does not have organ differentiation at the time of seed shedding. This is perhaps due to lack of transverse division in the zygote which in other cases leads to the formation of two cells with distinct developmental potentials. At least in some of these cases, normal plant development requires colonization by symbiotic fungi/vicinity of host surface) and asymmetrical to result in a larger vacuolated and sparsely cytoplasmic *basal cell* (micropylar cell) and a smaller and densely cytoplasmic *apical cell* (chalazal cell). This is already reflected in the asymmetric distribution of the protoplasmic contents in the zygote. The present author feels that all reports of a symmetric division and formation of equal-sized daughter cells from the zygote[30] are likely to be either erroneous or such reports are based on observing two-celled embryos long after the first division is over. The apical cell enlarges and the basal cell often shrinks due to the loss of its large vacuole. It is not yet very clear whether the polarized organization and the pre-localized regulatory factors within the egg initiate a 'cascade of events' leading to the asymmetric division, or the zygotic genome, because of fertilization, directs the *de novo* synthesis of regulatory

factors that are distributed asymmetrically to the cells on division[5] of the zygote.

An asymmetrical division is very important for the co-ordinated subsequent development of the embryo[17,31]. The two cells have distinct developmental roles[5]. The basal cell produces the suspensor and the radicular meristem, whereas the apical cell gives rise to the embryo proper. How the different fates of the apical and basal cells are established is not known, although an activation of different gene sets in the two cells was contemplated leading to their 'autonomous specifications'[32–35]. This difference is highlighted, for example by the accumulation of mRNA from the *Arabidopsis thaliana* Meristem Layer 1 (ATML 1) gene in the apical but not the basal daughter cell[36].

In the *gn* mutant of *Arabidopsis*, the almost symmetric zygote divides transversely to result in two equal cells, the basal is reported to produce a short suspensor[12]. However, a careful scrutiny of this work shows that the suspensor of such mutant embryos is not comparable to that of the control embryos. The claim by Mayer *et al.*[12] that a clear-cut asymmetric division of zygote is not required to establish the fate of the daughter cells is not acceptable because I contend that a marked asymmetry is a prerequisite not only for the proper and full differentiation of a true suspensor and the 'nucleus' of the radicular meristem from the basal cell, but also for the proper development of the remaining part of the embryo from the apical cell. As we shall see later, the *gn* embryo does not undergo organized development.

The division in the embryoid initial (= embryogenic cell) may be transverse, longitudinal or oblique and is variable in the same taxon, in the same culture. The division may be either asymmetrical[37–40], equal and symmetric[41–43] or variable[20,44] in the same system. Based on these cases, as well as on those zygotes which were reported to show an equal division, Toonen and de Vries[17] concluded that an asymmetrical division is not an essential requirement either for zygotic, or somatic embryogenesis. According to them, there does not appear to be a correlation between the regularity or the lack of it in the first division of zygote and embryoid-initial in the same species. However, it should be stressed that an asymmetric division is very vital and that an asymmetric division comparable to that observed in the zygote is absent in the embryoid-initial; neither the two daughter cells derived from symmetric division are programmed properly to their respective roles.

A special mention must be made about the gymnospermous taxa in which early stages of somatic embryogenesis have been studied. Asymmetric cell division has been shown in the embryoid-initial cell to result in a distal small cell with dense cytoplasm (which is reported to develop subsequently into the embryo proper) and a large vacoulated basal cell (which is shown to develop into the suspensor)[45,46] in *Picea* species and in *Gnetum ula*[47]. In none of the gymnosperms the zygote shows a division with the prompt accompaniment of a cell wall between the two daughter nuclei; the subsequent few divisions are also free nuclear. How can we accept the reports of a faithful reproduction of events of the zygote in developing embryoids for these gymnosperms? The somatic cell is not equivalent to a zygote, which undergoes only free nuclear divisions in the beginning.

The division in the cells involved in non-zygotic embryony is again variable both in reference to the plane of the dividing first wall and to the size of the two daughter cells, which may be equal or unequal; invariably the two daughter cells are equal.

As a summary, it can be stated that the division in the zygote is almost always transverse and asymmetric, with the resulting daughter cells programmed to different fates; the division in the embryoid-initial is very variable with reference to its plane as well as to the size of the resultant daughter cells. The basal cell is not programmed in embryoids, consequently leading to the absence of a typical suspensor (both morphologically and functionally) as well as to the absence of a nucleus for the radicular meristem.

Suspensor

Do somatic embryos have suspensors? If yes, do they have the same constitution as of zygotic embryos? If no, can the somatic embryos develop into plantlets by-passing suspensor development? These are some of the very pertinent questions that have been addressed in this section.

In zygotic embryogeny, the suspensor is a highly specialized, terminally differentiated embryonic structure. Some have even considered the suspensor as an organ of the embryo[5]. Even mutants have not eliminated suspensors or have made them insignificant as is revealed by the six suspensor mutant types (*twin*, *sus1*, *sus2*, *sus3* and *raspberry1* and *2*) in *Arabidopsis*[5].

Available studies on zygotic embryogeny have shown that the following are some of the several specific and characteristic roles of suspensors: (i) orienting the embryo in close proximity to the source of nutrients, (ii) involving itself in short-distance transport of metabolites, as evidenced by the transfer-cell morphology of its cells, (iii) possessing high template activity, and polyteny and endoduplication of its nuclei, which suggest its role in the production of some gene product necessary for controlled embryo growth, and (iv) accumulating hormones, which are probably necessary for self-regulation as well as for the control of embryo growth[13,48]. JIM 8 monclonal antibody tagging experiments, conducted with the zygotic embryos of *Brassica* species, showed the

location of epitopes for arabinogalactan proteins in the two-celled embryo, while epitopes for it were found only in the suspensor cells of the 8-celled embryos[14], again indicating that the suspensor is a specialized structure of the embryo.

Invariably, the cells of the suspensor senesce after the heart-shaped stage and are not functional components in the mature embryo. Suspensor mutants in *Arabidopsis* indicate that the persistent and enlarged suspensors beyond the globular stage arrested the further development of the embryo; the probable mechanisms in the arrest of suspensor activity beyond globular stage embryo are detailed in Goldberg *et al.*[5].

The organization of a typical suspensor comparable with that of the zygotic embryo is never encountered in the somatic embryos of any species so far investigated[29,49]. However, any cell or a group of cells attached to the so-called radicular pole of an embryoid has been given the name suspensor[50–52]. Therefore, the use of this term has been rather indiscriminate in embryoid literature. Yeung and Meinke[48] have also stated as follows: 'Although the suspensor appears to play a critical role in zygotic embryogenesis, it usually fails to develop when somatic embryos are produced in culture. The suspensor should, therefore, be viewed as a specialized structure that functions primarily to facilitate the continued development of the embryo proper within the seed.' '... when structures that superficially resemble a suspensor are found (in somatic embryos), they typically lack specialized features characteristic of normal suspensors'. A suspensor is not formed during the early divisions in *Cinchorium* species[17]. A multiseriate suspensor-like stalk was reported in *Cicer arietinum*, but again only superficially resembling the zygotic suspensors[53]. In many embryoids, such structures become especially prominent subsequent to the heart-shaped stage, thus differing from the early senescing suspensors of zygotic embryos.

This section, therefore, reveals the categorical absence of a structurally and functionally typical suspensor in the embryoid and that structures/cells at the basal pole of the embryoid have been indiscriminately named as suspensors.

Polarity

The most significant aspect of zygotic embryogenesis is the phenomenon of polarity, which, as already stated, becomes manifested in the zygote itself, as well as in its asymmetric division. Although the basic polarity of the zygote is inherited from the precursor egg cell, syngamy further accentuates this[49]. The polarity of the egg and zygote is in turn due to the polar electric gradients that already exist in the embryo sac, which were formed during megasporogenesis and megagametogenesis[49,54–56]. Different regions of the embryo sac are subjected to different morphogenetic fields; such an effect was designated by Evenari[57] as topophysic effect. Only the zygote and the synergid, if it gets fertilized, are subjected to the specific topophysic effect of the micropylar milieau and, therefore, come to possess polarized gradients.

In the subsequent developmental stages of the embryo also, the polar forces are maintained and become manifested by the simultaneous organization of the *hypophysis* at the root pole from some of the derivatives of the basal cell of the two-celled embryo, and of the epiphysis at the shoot pole, with the intervening part forming the axis of the embryo[29,49,58,59]. Making no mention about epiphysis, Goldberg *et al.*[5] have also emphasized that the presence of hypophysis at the basal end of the globular embryo was responsible for the establishment of an apical-basal polarity (see also Laux and Jürgens[60]). In the absence of the differentiation of epiphysis and hypophysis, no transition from globular to the cordate stage in dicots (bilaterally symmetrical) and from globular to an unilaterally symmetrical structure in monocots takes place, i.e. the cotyledon(s) do(es) not get initiated[49,61].

During embryoid development, there is no evidence of organized polar forces guiding its ontogeny. Halperin and Wetherall[62] stated that it is not known how or at what stage of development the polarity of the root–shoot axis is determined in the embryoid. Street[63] believed of a spontaneous origin of polarity in the globular embryoid, whereas Haccius and Bhandari[64] conceived of the establishment of polarity in non-zygotic embryos (including embryoids) belatedly because of the indefinite position of the embryoids in relation to their environment.

In none of the embryoids described so far, there is an organization of the polar structures, hypophysis and epiphysis, at the globular stage or subsequently. Consequently, we still do not know how the longitudinal polarity is determined in the embryoid. This is reflected by very vague statements such as this: 'Somatic embryos specify their longitudinal apical-basal and radial tissue-type axes by different mechanisms than zygotic embryos'[5].

Adventive embryo initials such as antipodal, nucellar and integumentary cells are subjected to different topophysic effects due to their location in fields other than the micropylar milieu. They are not polarized from the beginning and the embryos derived from them also are not polarized structures; they do not possess the hypophysis and epiphysis organization at the globular stage[29].

In summary, it can be stated that while zygotic embryos are polarized entities from the beginning until the end, embryoids are not polarized, at least till very late stages in their development; hypophysis and epiphysis organization does not take place at the two poles of the globular embryoid.

Cell divisions, pattern formation and symmetry changes

The zygote (and its products) is (are) considered as a complex and specific reaction system(s) that function(s) in conformity with the laws of physical chemistry and mathematics[54]. It is also a gene-determined reaction system operating under the sustaining environmental conditions prevailing at the micropylar milieu of the embryo sac/endosperm. Therefore, the chains of reactions involved in it are collectively determined by its genome after interaction with the prevailing environmental milieu.

As already mentioned, the division of the zygote is transverse and asymmetric and the embryo is formed by subsequent divisions in these two cells. There is considerable variation in the extent of the relative contributions of the two cells and their derivatives to the embryo, on the basis of which five major types and many sub-types of embryogeny have been recognized[65,66]. In other words, the outstanding feature of the zygotic embryogeny is the orderly and almost predetermined sequence of cell divisions noticed. All such divisions strictly obey the physical laws that govern cell divisions in general. The most important of these are Sachs' and Errera's laws[49,67]. Cell divisions proceed in such an orderly manner that it is almost possible to predict at each stage of embryogenesis of a given species, how and where the next divisions will take place. This enabled Souéges[68] to establish his 'Laws of Embryonomy' and has led to the 'Cell lineage concept' or 'Mosaic theory'[63], its essence has been spelt out by Johansen[65]: 'Each and every cell (in the embryo) has a reason for its existence, its origin can be demonstrated, its destination determined and its position is invariably the same. A superfluous cell would seriously upset the harmonious balance'. Therefore, according to the cell lineage concept (i) great significance is to be attached to the order and planes of division, (ii) during these divisions, the individual cells of the embryo inherit different cytoplasmic potentialities from different regions of the zygote, and (iii) these differences determine the exact role they and their daughter cells play in organizing the embryo and its parts.

The cell lineage concept has gained much support in recent years due to the works on mutant embryos, especially of *Arabidopsis*. Mutation in the GNOM/EMB 30 (GN) gene in *Arabidopsis* expresses in such a way that the zygote divides almost symmetrically; probably because of the loss of asymmetric division, the apical cell subsequently divides very irregularly unlike in wild embryos[12]. The expression of the lipid transfer protein (LTP) gene, which is normally restricted to the apical end of the later stage embryo in control plants, was invariable along with the apical-basal axes in the GNOM embryos[69]. This shows that regularity of cell division patterns and cell lineage, as well as polarity of embryo, were absent in GNOM embryos; this also implies the intimate relationship between cell lineage and polarity during embryo development. Transformation studies with tobacco embryos containing chimeric β-glucoronidase (*GUS*) reporter genes driven by soybean embryo-specific gene promoters showed that a globular embryo is organized into distinct, non-overlapping, transcriptional regions or territories[5]. The longitudinal axis of such embryos contains at least three non-overlapping transcriptional territories: (i) the chalazal, (ii) the equatorial, and (iii) the micropylar. The suspensor represents an additional transcriptional domain along the long axis. Presumably, each such domain sets in motion a cascade of events leading to the differentiation of specific embryo regions later in embryogenesis. Each of these regions can develop independently of the other two regions, as revealed by several of the *Arabidopsis* pattern mutants such as GURKE (GK), MONOPTEROS (mp) and FACKEL (FK), etc.

From the above discussion it is evident that in zygotic embryogeny there is a controlled pattern formation involving those factors and events which cause cell types, tissues and organs to originate at specific locations in the late globular embryos, i.e. the body organization of the seedling is laid down during embryogenesis. Essentially two phases of development have been identified[70,71]: (i) Primary body plane is organized in globular-heart stage transition through specific developmental processes, and (ii) the size of the embryo increases and maturation of embryo completed prior to dormancy. The first represents 30% of embryo development, while the second phase represents the rest of embryo development.

With reference to symmetry, the dicot embryo experiences three distinct morphological phases during its ontogeny – the filamentous, globular and cordate phases. There is an apparent radial symmentry in the globular stage and it becomes bilaterally symmetrical in the heart-shaped stage. In monocot embryos, the filamentous and globular stages occur, but later the embryo assumes a different contour (not cordate) due to the formation of a single cotyledon. A unilateral symmetry results due to this feature. What is the cause of these regular transitions in the symmetry of the developing embryo? Either the embryo itself exerts self-regulation or the control emanates from the surrounding milieu, especially the endosperm. Some evidences for the latter possibility are given by Krishnamurthy[72]. The establishment of auxin asymmetries in the embryo-proper region of globular embryos is shown to be responsible for the transition to bilateral symmetry at the heart stage[5]; globular embryos have very rich auxin contents.

Cell divisions leading to embryoids *in vitro* as well as to adventive embryos that develop in fields other than that prevailing in the micropylar milieu of the

embryo sac do not obey the physical laws operating in zygotic embryogeny, and so discourage geometrical or mathematical analysis[29,73]. This behaviour is essentially due to the absence of forces that cause a polarizing gradient in the initiating cells which consequently are not kept in equilibrium with the neighbouring cells and their immediate milieu[29]. The sequence of divisions in early embryoid development has been described as similar to the planes of cleavage occurring in a quasi-fluid system[74,75]. However, it should be mentioned that these divisions are not often predictable *contra* to the situation in zygotic embryogeny. In fact, a number of instances where the ontogeny of the embryoid has been traced, the divisions leading to the globular stage were merely described as 'irregular', or as taking place 'in all planes', without following any common pattern or sequence[76]. It is often difficult to pin-point that it is this particular ontogeny that the cells have followed or is likely to follow subsequently in an embryoid. In some embryoids there is a progressive increase in cell number without cell enlargement, while in others cell enlargement proceeds without significant increase in cell number.

In the carrot system, which has been studied by several investigators, there have not been one but several ontogenetic sequences reported during embryoid development. None of them correspond to the developmental pattern illustrated by Borthwick[77] for the zygotic embryogeny. Similarly, no correspondence is seen between the zygotic embryogenesis and embryoid ontogeny in any taxon for which both the *in vitro* and *in vivo* sequences are known so far. But embryoid ontogeny is assumed to be similar to zygotic ontogeny merely based on the observation of apparently globular, cordate, torpedo and mature configurations in the former. In other words, the hard-core embryoidologists reinforce the notion that the end-product rather than the means of achieving it is of greater importance, i.e. they firmly believe that different developmental sequences and cell lineages can result in identical end structures[78]. However, the question still remains: are they really identical?

In view of the above facts, embryoidologists have come out with an alternative theory to the cell lineage concept, called 'Regulative theory of embryo organization'[63]. According to this theory, segmentation patterns in developing embryos (and embryoids) have no taxonomic significance. During early development, the constituent cells of the embryo do not inherit distinct and specific cytoplasmic potentialities but remain undetermined and uncommitted. An embryogenic 'field' may exist in combination with a position effect, i.e. a given cell acts in the embryo (and embryoid) in relation to the surrounding cells. In other words, it is not the cell or cell group itself that determines the future histogenic regions of the embryo it gives rise to, but the position that the cell or cell group occupies in the developing embryo (and embryoid). This idea is often called 'the positional information' or 'Wolpert model'[79–81]. This school of workers therefore believe that embryogeny (as well as embryoidogeny) should not be looked upon as adhering to laws concerned with parsimony, origin, numbers, disposition and destination of cells segmented.

Symmetry changes from the radial globular to bilateral heart-shaped embryoids were not noticed in the carrot system in the continued presence of auxin[62,82]. It was suggested that new gene products are needed for the transition to the heart stage and that these new products are synthesized only when exogenous auxin is removed. However, it was suggested[83,84] that not only the presence of auxins but their proper polar transport is a prerequisite for normal morphogenesis beyond the globular stage. Treatment of globular somatic embryos with auxin transport inhibitors resulted in blockage of morphogenesis to the cordate stage, i.e. no cotyledon initiation resulted (see details in Zimmerman[6]).

It is evident from this section that cell divisions and cell lineages not only follow physical laws but are also predictable in zygotic embryogeny whereas in somatic embryogeny such predictions cannot be made as the cell divisions are not regular and do not obey mathematical laws; cell lineages cannot be traced. It is also evident that while in zygotic embryos symmetry changes are both intrinsically and extrinsically controlled, as well as dependent on polar distribution of auxins, the mechanism of symmetry changes in embryoids is not yet clear; also results on the involvement of auxins in these processes are contradictory and inconclusive, resulting in the proposal of only speculative explanations[6].

Tissue and organ differentiation

A mature zygotic embryo contains two principal organ systems – the axis and cotyledon(s)– and these organs are composed of three basic or primordial tissue zones – protoderm, procambium and ground meristem – which will respectively become the epidermal, vascular and parenchymatous tissues of the seedling. Such tissue differentiation is believed to take place in the globular–heart transition stage itself[5]. Studies on transgenic tobacco embryos also reveal that at the globular–heart transition stage, each of these three tissue zones have distinct transcriptional programs[5]. 2S2 albumin mRNA accumulates within parenchyma cells while, neither EP2 nor 2S2 mRNA is detectable within the procambium.

Protoderm differentiation seems to be an important morphogenetic event in zygotic embryogenesis[85,86]. Protoderm is the first tissue region to differentiate through periclinal divisions in the zygotic embryo when the latter's terminal tier is in the octant stage; its differentiation is completed, on any account, prior to the 16-celled

stage depending on the taxon. This is indicated by the uniform expression of the carrot EP2 lipid transfer protein mRNA in the protoderm from the globular stage onwards[87]. ATML 1 gene is expressed first in the apical daughter cell of the zygote and subsequently in all cells of the 8-celled embryo proper of *Arabidopsis*. However, after the tangential divisions, the expression of this gene becomes restricted to the protoderm layer and is no longer detectable in the inner cells[36]. The genetic basis of protoderm and other tissue differentiation is also shown by the mutation in the Knolle (*KN*) gene of *Arabidopsis*, which perturbs the segregation of protoderm and the fate of the inner cells[88]. Similarly, the epidermis-defective mutants of carrot, *tsll*, and of pea do not show the normal morphogenesis during embryo development[36].

The ground meristem cells are distinctly segregated from protoderm very early in the late globular stage but not later than the early heart stage[5]. This has been demonstrated through localization studies with a soybean kunitz trypsin inhibitor mRNA, designated as *kti3*. The blocking out of ground meristem from provascular meristem takes place in the heart stage, through cell enlargement, decreased stainability, and increased vacuolation. Further details on ground meristem and its activity are discussed by Krishnamurthy[49]. The ground meristem cells in the cotyledon and axis become highly specialized and accumulate large amounts of storage proteins (and oils) that will be utilized as food source by the seedling.

Our knowledge on procambialization and vascular differentiation in embryos is very meagre[49,89,90]. A detailed review on this aspect is provided by Krishnamurthy[49]. It can be summarized from this review that the procambial initiation commences independently in the cotyledon(s) and radicle, while the hypocotyl exhibits a conspicuous developmental lag in the differentiation of procambium. Vascular differentiation from procambium normally occurs during seed germination, and takes place independently (with independent waves of transverse course of differentiation) in the radicle and cotyledon(s). The two systems (having collateral arrangement of xylem and phloem in the shoot and radial arrangement in root) are connected by belated vascular differentiation in the transition region of the hypocotyl from differentiated parenchyma cells and not from procambial tissue.

In embryoids and non-zygotic embryos, there is not only a belated differentiation of protoderm, but it is often incomplete[64]. A careful examination of the published illustrations of the globular embryoids reveals the absence of a typical protoderm. In carrot, 'the delimitation from within the cell mass of an epidermal cell layer enclosing a central group of cells does not take place until there are 32 or more cells in the embryoid proper'[74], and the 'protoderm appears to arise in a patchy work random fashion and not as a uniform layer'[62]. In the latter case, the protoderm may not differentiate even after the development of the cotyledonary node or may do so simultaneously with it. In the carrot system investigated by Sussex[76], 'there is no stratified surface layer of cells and divisions in surface cells may be in any plane'. In this instance the absence of a protoderm continues for a prolonged period so that the shoot apex which differentiates later on has an incomplete outer tunica, and the cotyledons and hypocotyl possess an incomplete epidermal layer.

A precocious differentiation of vascular tissues was reported in a few embryoids including *Cichorium endivia*[91]. In this species, the initiation of vasculature has been reported as early as in the globular embryoid; 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 similar to that seen in adventitious buds. Sussex[76] reported an early differentiation of sieve tubes and tracheary elements in the cotyledons and hypocotyl of carrot embryoids. It may be mentioned here that vascular differentiation in zygotic embryos of carrot is initiated only after seed germination[29].

The axis of the zygotic embryo terminates in shoot and root apical meristems at its opposite poles. The hypophysis, derived along with the suspensor from the basal cell of the two-celled proembryo, forms the nucleus around which the radicular meristem becomes organized; the hypophysis itself is not fully responsible for the production of the constituent cells of the radicular meristem[58] as has been assumed by many investigators (see for example, Goldberg *et al.*[5]); it is partly derived from *ca* and partly from *cb*. The fact that no root meristem is formed in the 'hypophyseal group' of mutants (e.g. *hobbit*) in which the first recognizable defect is the aberrant development of hypophysis[92] suggests that a root meristem cannot be established unless the hypophysis cell group is correctly specified. The epiphysis, which concurrently differentiates along with hypophysis, forms the nucleus around which the shoot apical meristem becomes organized and therefore, the assumption that the shoot meristem is formed belatedly when compared to root meristem[5,93,94] is erroneous. The importance of epiphysis in shoot apical meristem organization (as well as in cotyledon formation) is highlighted by mutants in tomato[61]. Meristem differentiation is independent of other regions of the embryo; in fact differentiation of shoot and root meristems is also independent of each other. This is evident from a study of several meristem mutants not only in *Arabidopsis* but in other taxa as well. In other words, meristems represent independent submodules within the apical and basal regions of the zygotic embryo.

The differentiation of shoot and root apical meristems in embryoids has not received any attention at all in

the previous studies. Investigators have merely mentioned about these meristems in a very casual way and in nearly 90% of embryoid literature these structures have not been mentioned at all, as the descriptions of embryoids are merely based on external morphology. In many instances, their occurrence is merely assumed at the two poles of the embryoid. In the light of this finding, it is very difficult to make any reasonable comparison between the shoot and the root apical meristem of embryos and embryoids. A critical scrutiny of the few available illustrations of embryoids by this author indicates that a reasonably organized shoot apical meristem is present in the mature embryoids in many cases, whereas the same cannot be said of root apical meristem. The latter is not present in a form comparable to radicular meristems of zygotic embryos.

The cotyledons form the principal organs of the zygotic embryo. The cotyledon is a terminally differentiated organ and invariably accumulates food reserves for use during seedling development, after which it usually senesces. In dicots, the cotyledons are specified from two lateral domains in the apical region of the axis, while the only cotyledon of the monocots is differentiated from a single domain at the apical region of the embryonal axis. Barring cases with chlorophyllous embryos, cotyledons of the majority of angiosperms are non-green structures while within the seed. The cotyledons are specially designed not only to assist the process of dormancy at maturity of the embryo through ABA and LEA protein interactions but also in germination through the provision of nutrients from its stored reserves. The leaves, on the other hand, have roles subsequent to seed germination and are primarily concerned with photosynthesis and transpiration. The *lecl* mutants of *Arabidopsis* have leafy cotyledons; they consequently do not have dormant mature embryos, do not synthesize storage and LEA proteins and germinate precociously; probably they are also deficient in ABA synthesis[94–97]. In other words, the leafy cotyledons lose their specialized functions.

Are cotyledons present in the embryoid? If so, are they equivalent in size, structure and function to those of zygotic embryos? These two questions are worthy of examination. A survey of the embryoid literature has indicated that the majority of workers have reported the presence of cotyledons in the embryoids. The basis of such reports is the fact that the embryoids show the same morphological sequence of contour changes – globular, heart-shaped and torpedo stages – as in zygotic embryos; they have also been impressed by the position of the cotyledon like in the mature embryoids. If they are truly equivalent to cotyledons, mere morphological similarities and topography are not sufficient. In the case of dicotyledons, two structures resembling cotyledons are invariably produced in the embryoids. Sometimes, additional similar-looking structures are also found in the same embryoid and these have been conveniently ignored when it comes to interpretation. In embryos of zygotic origin, the two cotyledons are of equal size and perfectly opposite to one another; barring cases with chlorophyllous embryos, the cotyledons are invariably non-green. A scrutiny of the embryoid literature and the illustrations contained therein indicates that the two 'cotyledons' are often of unequal size and are not perfectly inserted at the same locus; in other words, they are slightly alternate in position and not opposite. In the majority of embryoids so far studied, the so-called cotyledons are green.

In those monocotyledons, in which somatic embryogenesis has been studied in detail, barring a few cases such as of some grasses, embryoids often show two 'cotyledon-like' structures. Monocotyledonous embryoids, if they are truly equivalent to zygotic embryos, should show only one such structure and not two. This fact indicates that the structures so formed in the embryoids are not equivalent to cotyledons but are only leafy structures. In those monocotyledons, such as grasses, where a single 'cotyledon' has been shown in the embryoids, the single structure is not really the cotyledon although named so by the investigators. In monocots that shows distichous type of leaf phyllotaxy in the mature plant, the embryoid also shows two cotyledon-like structures almost opposite to one another, while in grasses with alternate phyllotaxy, the embryoid also shows a single cotyledon-like structure with a sheathing base simulating morphologically the scutellum. In many grass embryoids, additional leafy structures with sheathing base are also formed in quick succession, giving a false resemblance to the coleoptile.

In many embryoids the so-called cotyledon is not dorsiventrally flattened although the cotyledon of the zygotic embryo may not have that morphology. Its internal structure is not similar to that of the cotyledon of the zygotic embryo. In 'cotyledons' of none of the embryoids, reserve food materials typical of those of zygotic embryos have so far been reported, although expression of genes coding for LEA proteins has been reported to be present (see, however, discussion in a subsequent section).

In the case of gymnosperms, the embryoids have been shown to have multiple cotyledons as in the case of zygotic embryos[4,46]. These appear to be similar in morphology also to those of zygotic embryos. In gymnosperms, so far embryoids have been initiated only in about a dozen cases, mostly conifers and one species of *Gnetum*. In all these cases, the explant used for this purpose is the young embryo itself. It is a known fact that in Gymnosperms even under natural conditions typical cleavage polyembryony is very characteristically present, where the young embryo splits into more units

each with its own suspensor system and multiple cotyledons. Since only embryos have been used as explants in gymnosperms and since their cells have potency to form additional embryos (by cleavage polyembryony) the same tendency is repeated *in vitro* when the embryo is used as an explant. In those few angiosperms where young embryos have been used as explants (e.g. some melons and grasses[4]), their cells produce several embryos, through a process akin to cleavage polyembryony, all resembling the zygotic embryos. In other words, the potency to form polarized embryos as a result of fertilization is retained by the cells of the zygotic embryo up to early cotyledon stage.

From the discussion in this section, it may be concluded that in embryoids (i) tissue initiation and differentiation is erratic spatially and temporally, (ii) there is no organization of root apical meristems, although the shoot meristem is fairly well-organized, and (iii) the so-called cotyledons are not structurally and functionally equivalent to cotyledons of zygotic embryos; they are more comparable to leaves.

Dormancy

Mature zygotic embryos enter into dormancy until conditions are favourable for post-embryonic development, i.e. embryogenesis terminates with a dormancy period. At this stage, the cells of the embryo become dehydrated and desiccated as result of induced water loss; metabolic activities cease. There is also a repression of genes encoding for storage proteins and LEA proteins and remain transcriptionally quiescent; other cytological features of dormant embryos are listed in Villiers[98].

As already indicated, normal cotyledon development, differentiation and morphology (including its constitutive storage and LEA proteins and growth regulators) are absolutely necessary for the onset of maturation and dormancy of zygotic embryos. Abscissic acid (ABA) regulates and maintains embryo dormancy. ABA mutants of *Arabidopsis* germinate precociously and are desiccation intolerant; they are also defective in the synthesis of storage proteins and LEA proteins[99]; treatment of ABA avoids precocious germination[100]. Whether dormancy is controlled by the embryo itself or by the surrounding tissues is a moot question[101,102], although there are more evidences for the latter possibility.

It has been stated that embryoids are similar to zygotic embryos except that 'they do not become dormant'[5]. They grow and differentiate continuously and germinate directly into plantlets[6]. They are also believed to express a number of genes that are shown to be ABA inducible and that are generally associated with desiccation tolerance (e.g. LEA genes) (see next section). If desiccation tolerance is an intrinsically controlled phenomenon, embryoids should also have entered into dormancy, but they do not. So, dormancy programme is induced extrinsically, possibly through a maternal signal, which could be simply a high concentration of ABA. Ammirato[103] in fact induced dormancy in mature embryoids through exogenous ABA supply.

It can, thus, be concluded that embryoids do not enter into dormancy so typical of seed embryos; they, therefore, do not express the associated structural and physiological features.

Gene expression and molecular markers

Approximately 15,000 diverse genes are reported to be active in the embryos of plants as different as soybean, *Arabidopsis* and cotton[104]. Many of these genes are expressed both spatially and temporally in specific cell types, regions and organs of the embryo[104,105]. It has been claimed that 'spatial and temporal gene expression programmes appear to be similar in somatic and zygotic embryos'[5,6,15,105]. This appears to be a sweeping statement, because a critical study of earlier literature points to the contrary.

Attention must first be drawn to genes that are related to a class of proteins called late embryogenesis abundant (LEA) proteins[106–108]. These are highly hydrophilic proteins expressed abundantly very late in embryogenesis in many plant species including cotton, barely, rice, rape and wheat and are ABA-inducible. Because of the latter, they are presumed to protect the embryo from desiccation. Four groups of LEA proteins are so far known.

The LEA genes isolated from carrot somatic embryos are reported to include representatives of all four described groups of LEA proteins and are listed in Zimmerman[6]. Out of the 20 genes listed in table 1 of Zimmerman[6] from the somatic embryos of carrot, only six are shared between somatic and zygotic embryos. Among these six, expression of the genes at a comparable stage of development of the embryo and embryoid is limited to three only; even among these three, expression of two genes is also seen in the earlier stages of somatic ontogeny (abundant in the proembryogenic mass/globular embryo but not in subsequent stages; therefore, not LEA proteins in the strictest sense of the word), while they are expressed in zygotic embryos only subsequent to the heart stage. With reference to EMB – 1 LEA gene expression (which is expressed only in the embryos of the seed but from globular stage onwards in somatic embryos), Wurtele *et al.*[109] indicated that the expression was triggered in very late zygotic embryos by a secondary signal (possibly a pulse of ABA) from the maternal environment, but even without a trigger in somatic embryos, that too, very early in ontogeny.

Isozyme patterns of zygotic embryos between torpedo stage up to 5-day-old seedling have been compared with

those of similar stages in somatic embryogeny and plantlet in carrot. The following seven enzyme systems were analysed: arylesterase, glucose phosphate isomerase, phosphogluconate dehydrogenase, alcohol dehydrogenase, isocitrate dehydrogenase, aspartate aminotransferase and phosphoglucomutase[110]. It was found that profiles of somatic embryos were generally different from those of zygotic embryos; a similarity was found only in aspartate aminotransferase patterns between the two systems at the torpedo stage. It was further noticed that alcohol dehydrogenase 4 and phosphoglucomutase 1 and 7 were stage-specific markers for zygotic embryos but no somatic embryogenesis-specific isozyme could be located.

It may, therefore, be concluded that gene expression and molecular marker studies, although very limited, have indicated greater differences than similarities between zygotic embryos and embryoids.

Germination

The mature zygotic embryo germinates and produces a seedling. In the dicots the radicular meristem gives rise to a tap root while in monocots, the radicle grows into a very short-lived tap root, but several adventitious roots are produced soon.

The most neglected aspect of the study of embryoids is the transition stage between the embryoid and its plantlet. Nearly in all the investigations in which plantlets have been obtained from embryoids, only exomorphoic descriptions (that too incomplete) of the transitional stages are provided. It has already been emphasized that the embryoids do not have an organized radicular meristem at the pole opposite to the shoot apical meristem. This is because of the fact that a hypophysis is never organized in the embryoid. Consequently the embryoid is a unipolar structure and not bipolar. In no instance, the embryoid 'germinates to produce a radicle and tap root'. A careful examination of the embryoid literature by the present author convinces him that the root(s) that emerge(s) from such germination is (are) only adventitious. Even in those cases of embryoids where a single root emerges initially, this has its origin adventitiously at the basal part of the embryoid.

Morphological nature of the embryoid

In an earlier article this author along with late B. G. L. Swamy[29] discussed this problem at length and concluded that the embryoid is not equivalent to the zygotic embryo and that it should be best considered as an adventitious bud. The information accumulated subsequent to that paper and discussed in the earlier pages of this review is summarized in Table 1. It is evident from this table that the earlier conclusion – that the embryo and embryoid cannot be equated – is much more strongly supported by recent information. The embryoids exhibit greater similarities to the adventitious shoot buds that arise in diverse parts of the plant body.

There are a series of morphological expressions of organogenesis in *in vitro* systems. There are cases where shoot buds originate at one part of the callus with root meristems originating concurrently/precociously/belatedly at regions of the callus opposite to the shoot buds and the two systems are interconnected subsequently to produce new plants. At the other extreme are instances where a shoot meristem and the adventitious root-producing callus region (with fewer constituent cells) are brought very close to one another in the form of an embryoid. When these embryoids germinate, there is no tap root formation but adventitious roots in variable numbers are produced at the basal end of the shoot meristem. This opinion is substantiated by surgical experiments carried out in carrot somatic embryos[111–113]. If the upper part of the somatic embryo is separated from the lower part by surgical manipulation, the half having the shoot meristem regenerated a new adventitious root system provided this half has at least 25–90% of the original length of the embryoid. On the other hand, the regeneration of the shoot pole with its meristem was difficult from the half having the so-called root pole of whatever length. Although the authors have explained these observations on the basis of the competence of the somatic embryos with different parts with different developmental capabilities, the striking result from these studies is that the shoot pole is able to regenerate the root pole fully but the converse is very difficult. In other words, this result supports the present author's contention that there is no root meristem organized in the pole opposite to the shoot in the embryoid and consequently regeneration of shoot pole becomes difficult while the opposite is easy. These experiments also show that all roots that are produced in the surgically operated shoot half, are adventitious, again emphasizing the adventitious bud nature of the embryoid.

Adventitious shoot apical meristems are formed either naturally or under induction in a number of different locations in a plant, display different degrees of activity and acquire a variety of different fates during development[114]. All these adventitious shoot buds show the invariable presence of two prophylls. The cultured explant is one such location where there is a conducive environment for the expression of totipotency and plasticity by its cells to organize adventitious shoot meristems which are distributed in the callus here and there or in specified locations such as in the embryoids. In other words, it may be concluded that the embryoids of any origin are nothing but adventitious shoot buds in an unusual place containing an axis and a shoot meristem

Table 1. Major events of embryogenesis and embryoidogenesis

Embryo	Embryoid
Zygote	**Embryoid-initial cell**
Immediate product of fertilization	Not an immediate product of fertilization
Diploid	Diploid, haploid or otherwise
Privileged location in the micropylar milieu of embryo sac	Not in a privileged location
Polarized with distinct cytoplasmic asymmetry	Not polarized in the strict sense (very often lacks cytoplasmic asymmetry)
Proembryo	**Proembryoid**
Division of zygote always asymmetric	Division of embryogenic cell sometimes asymmetric and highly variable in the same system
Terminal and basal cell differentiation follows distinct patterns	Absence of terminal and basal cell differentiation
Differentiation of suspensor	'True' suspensor absent
Apical-basal polarity continues	Apical-basal polarity often absent
Formation of embryo proper	Simulate formation of zygotic embryo proper
Cell divisions obey physical and mathematical laws	Cell divisions usually defy mathematical and geometrical analysis
Globular stage	**Globular stage**
Cell divisions and lineages obey 'laws of embryonomy'	Cell divisions and lineages often do not adhere to 'laws of embryonomy'
Establishment of radial symmetry	Establishment of radial symmetry
Differentiation of protoderm and axial tissue	Tissue differentiation is erratic, spatially and temporally
Simultaneous differentiation of hypophysis and epiphysis at the two opposite poles of the embryo	No differentiation of hypophysis and epiphysis at the opposite poles of the embryoid
Initiation and progress of pattern formation with the establishment of distinct non-overlapping domains or territories	Pattern formation is not evident
Heart stage	**Heart stage**
Establishment of bilateral (dicots)/unilateral (monocots) symmetry	Establishment of bilateral/unilateral symmetry irrespective of monocots or dicots
Symmetry changes intrinsically and extrinsically controlled by and dependent on polar distribution of auxins	Mechanism of symmetry changes not clear; results on auxin involvement are contradictory and inconclusive
Differentiation of cotyledon(s)	Typical cotyledons absent ; cotyledons are really 'prophylls'
Visible appearance of shoot-root axis	Visible appearance of a long axis with only the shoot meristem
Further differentiation of shoot and root apical meristems	Further differentiation of only shoot meristem; radicular meristem not organized
Initiation of senescence of suspensor	The so-called suspensor continues to remain prominent
Torpedo and mature stages	**Torpedo and mature stages**
Further development of cotyledon(s)	Further development of prophyll(s)
Further differentiation of axis	Further differentiation of axis
Blocking out of ground meristem and provascular tissues	Erratic blocking out of ground meristem and provascular tissues, spatially and temporally
Further development of root and shoot meristems	Further differentiation of only shoot meristem
Synthesis of storage proteins and/or lipids in cotyledons	No synthesis of storage proteins and/or lipids in the cotyledons
Synthesis of LEA proteins	Synthesis of only 6 out of 20 LEA proteins reported in zygotic embryos shared; even these are not LEA proteins in the strictest sense of the word
Cessation of RNA and protein synthesis	RNA synthesis continues
ABA activity prominent	ABA activity insignificant
Loss of water	No loss of water
Induction of dormancy	Absence of dormancy
Inhibition of precocious germination	Promotion of precocious germination
Stage-specific isozyme markers present	Stage-specific isozyme markers absent
Germination	**Germination**
Germinates after dormancy and results in a seedling	Produces a plantlet by precocious 'germination'
A tap root is formed, which is short-lived in monocots and long-lived in dicots	A tap root is never formed, whether in dicots or monocots
A typical transition between root and shoot vascular dispositions is noticed in the hypocotyl (transition) region	Transition between shoot and adventitious root vascular systems is through differentiation of parenchymatous cells of callus origin directly to vascular tissues

enclosed by two prophylls (some times one or more prophylls), thus resembling a mature embryo in exomorphic form; they should not be considered even as 'facsimiles of embryos' as Raghavan[13] considered them. Even if they show apparent similarity to embryos and result in similar end products (production of new plants) they should not be described by terms which are relevant only to zygotic embryogeny; otherwise it is like considering a humanoid as a human being, however close the former may be to the latter.

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ACKNOWLEDGEMENTS. I thank P. K. Pius for his help in the preparation of the manuscript.

Received 20 June 1998; revised accepted 27 October 1998