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A Review Article

IN VITRO SOMATIC EMBRYOGENESIS IN ANGIOSPERMS WITH SPECIAL REFERENCE TO MONOCOTYLEDONS

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Introduction

In sexual reproduction of plants, a male and a female gamete fuse to from a single cell, the zygote, which develops into a new plant through an embryo. Formation of an embryo is known as "Embryogenesis" and formation of an embryo from a zygote is "Zygotic embryogenesis" (Esau, 1977). The resulting plant is a new individual arising from a single cell and has characters of both male and female plants. Another type of embryogenesis is possible by the induction of somatic (vegetative) cells. This is known as "Somatic embryogenesis" (or non-zygotic embryogenesis) and the embryos are known as "Somatic embryos" (or non-zygotic embryos). This may occur in nature (as seen in citrus family) or may be induced by *in vitro*. The latter is known as "*In vitro* somatic embryogenesis". This is reported in a wide variety of angiosperm species, both form reproductive tissues such as nucellus and synergid cells, and from true vegetative (somatic) tissues such as leaf margins (Veyret, 1974; Raghavan, 1976: Tisserat *et al,* 1979, Litz & Gray, 1992).

Somatic embryogenesis in general can be defined as "The development of embryos from somatic haploid or diploid cells, without fusion of gametes". Somatic embryogenesis has been reported in dicotyledons more than in monocotyledons despite the fact that the latter consist most of the world major food crops. This is also true for *in vitro* somatic embryogenesis. It is reported that many difficulties of *in vitro* culture in monocotyledons have hindered such studies (Williams and Maheswaran, 1986). However, *in vitro* somatic embryogenesis and plant regeneration have been reported in

many of the economically important cereal grasses (Vasil and Vasil, 1982, 1986) and in some palms (Tisserat De mason, 1980; Schwendiman *et al,* 1988). This suggests that the possibility also exists in monocotyledons but needs more attention.

Present status of somatic embryos

Somatic embryos of monocotyledons even though in limited crops, are now automated and produced in large numbers. They are coated to form "artificial seeds" which make them an efficient delivery system of embryos (Murashige, 1978; Drew, 1979; Redenbough *et al,* 1989, 1991, 1994). Therefore, micropropagation via somatic embryogenesis offers significant advantages over that via organogenesis for large scale plant production (Atree & Fowke, 1991; Becwar *et al,* 1990; Wevste *et al,* 1990). With the better understanding of developmental control mechanisms, genetic engineering and transformation of monocotyledon crop improvement has recently being enhanced (Vasil, 1993; Aryon *et al,* 1991 a, 1991 b).

Zygotic and somatic embryogeny

The developmental stages of the zygotic embryo in dicots can be generalized into globular, heart shape and torpedo stages (Esau, 1977; Johri, 1984). It has been reviewed that in dicotyledons *in vitro* somatic embryogenesis follows a very similar pattern of their zygotic counterparts (Steward *et al,* 1958; Thomas *et al,* 1972; Reinert *et al,* 1977; Johri, 1984). However, the situation is different in monocotyledons where the general stages of the dicotyledon embryogenesis are not found. A different development pattern may be involved, making generalization difficult. A particular course of development may be specific to a species or a genus or even to an entire family (Poddubhaya, 1967; Natesh & Rau, 1984) l. Such observations indicate the possibility of a variation between somatic and zygotic embryogenesis of the same species in monocotyledons. This possibility cannot be ruled out since there are only very few reports on the comparison of the two processes in monocotyledons. The difficulty in identifying developmental stages may have hindered zdetailed studies on *in vitro* somatic embryogenesis in monocotyledons.

In many reported studies on somatic embryogenesis of monocotyledons, only morphology of the structures are reported and discussed. Also, characters of a somatic embryo are not identified in the structures.

The objective of this review is to identify the features of proembryonal and embryonal stages, and to understand the embryogenesis in monocotyledons. It would then be possible to gain a knowledge on similar

and different structures involved in somatic embryogenesis processes in angiosperms in general. This could be used in experiments on somatic embryogenesis in monocotyledon crops.

Embryo and bud

In angiosperms, the zygote is an unique cell which is located at the micropylar pole of embryo sac with its basal portion attached to the embryo-sac wall. Even under a light microscope, this cell presents a characteristic polarized appearance with the mycroplylar pole vacuolated and chalazal pole containing the nucleus and most of its cytoplasm. The division of the zygote results in tetrad, quadrant, octant etc. Thus forming a globular proembryo (Fig 1). This would lead to differentiation of the protoderm in the globular proembryo, followed by the development of cotyledons and epicotyl loci in the shoot pole and, the radicle together with the hypocotyledonary region at the root pole. Swamy and Padmanabhan (1962) stated that no organ of the embryo possess an obligation to any particular proemb-ryonic tier.

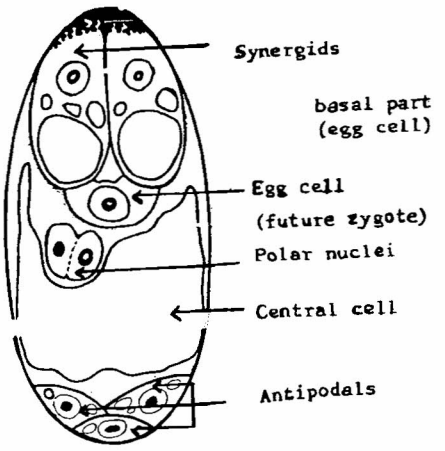

Fig 1. The zygote and proembryonal stages (From Johri, 1984)

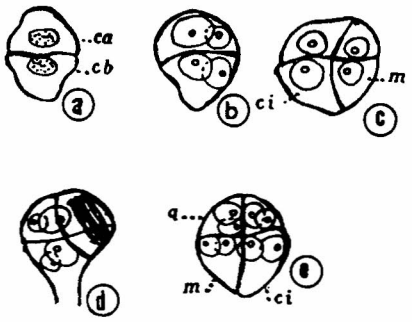

Fig 1b. The zygote and proembryonal stages (From Johri, 1984)

An embryo by definition has a shoot and a root apex (the embryonal axis) in the same structure. A bud has only a shoot apex. But under *in vitro,* a radicle may be present at a later stage. Buds (axillary or non axillary) have procambial strands that are connected with the vascular system of the mother plant during development. In contrast to a bud, the embryo is an individual from its inception. It does not establish vascular connections with the other plant at any time of its life but, has vascular connections between its shoot and root apex. According to Haccius, this feature could be the most distinctive character of an embryo. Accordingly, structures arising from callus, with procambial strands connected to the vascular strands already dispersed within the callus are adventitious buds (Fig 2). Similar structures without such connections are "somatic embryos of a callus". Practically it is not easy to observe the continuation of procambial strands between the two epics even though they are present. To prove this, a considerable number of serial sections of the embryo has to be studied.

Williams and Maheswaran (1986) reported that frequently, somatic embryos from calli lack a clear line of separation and are connected to the mother tissue over a broad area of the root pole or root apex region. Therefore, they cannot be separated without damaging their basal end, despite the absence of vascular connections with the mother callus. This suggests that somatic embryos, even though have an individual inception, need not necessarily be separated individual structures.

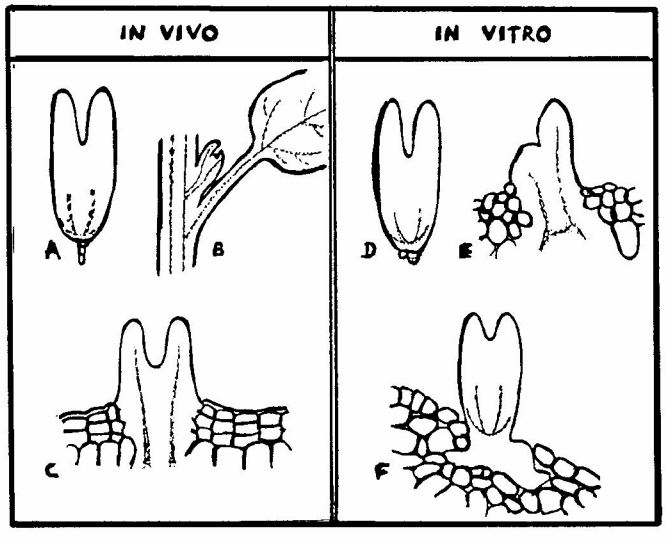

Fig 2. An illustration of characters in organogenesis and embryogenesis (From Haccius, 1978) A, D & F: embryos, B, E & C: buds

These facts show the difficulty of characterizing and defining the process of *in vitro* somatic emryogenesis. Therefore the definition given by Williams and Maheswaran (1986) can be suggested to be the most suitable one: the process by which haploid or diploid somatic cells develop into embryos through characteristic embryonal stages but without fusion of gametes.

However this definition appears to be more suitable to dicotyledons than to monocotyledons since comparative studies on the two processes are lacking in the latter. Also, these criteria still cannot be applied to many monocotyledons since the characteristic zygotic embryonal stages have not been properly reported.

Organogenesis and Somatic Embryogenesis

Christianson (1987) distinguished the two patterns of *in vitro* differentiation, i. e. organogenesis and somatic embryogenesis. These are distinctly different processes that can also occur in nature.

Different developmental characters of the two processes are:

1. Embryogeny of zygotic and somatic embryos progress from a single cell through several proembryonal stages. On the other hand, organogenesis results in the formation of either shoot or root meristems with the subsequent development of true leaves associated with the shot meristem.

2. Somatic embryos must meet the basic criteria of a bipolar structure with closed vascular system. In organogenesis, vascular tissue is continuous between adventitious and the underlying callus (Haccius, 1978) (Fig 2.).

3. Embryo specific proteins occur in cotyle dons of somatic embryos but are not found in leaves originating in adventitious shoots (Crouch, 1982).

However, the developmental abnormalities or neomorphs may blurr the differences between the two (Krikorian & Kann, 1981). Many published reports have described globular and heart shaped structures as somatic embryos, although neither the histology has been studied nor the ability to regenerate complete plants have been shown. Also somatic embryos can develop incompletely so that parts like cotyledons, shoot meristems and even hypocotyl are absent (Litz & Gray, 1992). It is possible to interpret these structures in embryogenic cultures as examples of organogenesis. The ontogeny can be better determined by histological analyses also because biochemical evidence is lacking.

Origin of somatic embryos

A frequent question encountered in somatic embryogenesis studies in its single cell or multiple cell origin, Even though direct development of embryos from single cells in suspension cultures have been illustrated in very limited cases (Steward *et al*, 1958; Williams and Maheswaran, 1986), it has been suggested that somatic embryos could arise either form single cells or from group of cells. Proof of single cell origin of somatic embryos in callus in the presence of a suspensor while when the origin is from a group of cells, the embryos are based with a broad area of parental tissue. On the other hand, somatic embryos have been described as originating for proembvryonic masses of cells that develop from single cell (Halperin, 1966). Haccius (1979) also suggested that a single somatic embryo arising from a cleaving cell complex may not be from an individual cell, but the entire cell complex has been formed from a segmenting single cell thus suggesting a single cell origin of somatic embryso.

However, as mentioned earlier (Tisserat *et al,* 1980), a single cell origin had not been demonstrated in many cases of somatic embryogenesis. Bipolar embryoids in many reported cases have been formed from aggregates of cells resembling meristematic cells. In such a situation, the necessity of physiological and physical isolation of a somatic cell/s preceding somatic embryogenesis has been suggested (Sannasgala, 198). This physical isolation is mostly by forming a "thick" polysaccharide cell wall (Fig 3). This character can be compared to the zygote which is separated within the ovule in this manner. The physical isolation results in cleavages within the cell complex.

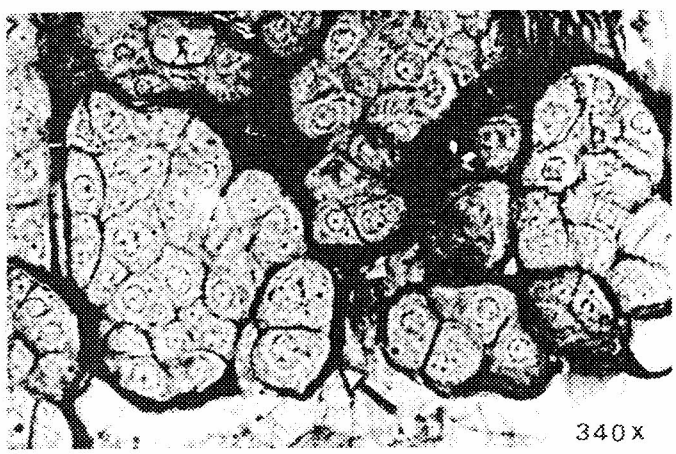

Fig 3. Physical isolation in *vitro* somatic embryogenesis (From Tisserat & De Mason, 1980)

Initiation of somatic embryos from calli very often shows formation of multiple embryos. This is also found in zygotic embryos which is termed as "monozygotic polyembryony: (or cleavage polyembryony). Thus cleavage within the cell mass in not a new development specific to *in vitro* somatic embryogenesis, but a process that is also found in nature.

It has been reported that a polyembryonal cell complex is present in angiosperm ovule but has become rudimentary and performs other functions. If the embryo is removed, the suspensor cells regain their mitotic activity and produce one or more new embryos (Haccius, 1978). This is in agreement with the hypothesis proposed by Sharp *et al* (1980) and Evans and Sharp (1981) where cells of the suspensor have been considered as pre-embryonic determined and their ability to form new embryos proves that they could be released from the inhibitory influence of the embryos formed initially.

It is not yet known why in one case the somatic embryos develop directly from a single cell and in the other, from a preliminary proembryonal cell complex as reported in Ranunculus (Konar *et al,* 1972). This is probably due to many factors, one of which is reported as the effect of polarizing influence of the local environment of embryogenic cells (for e. g. the stage at which the embryonic process begins). However, very few studies have been reported in this area.

Development pattern of somatic embryo

The inintial step in the somatic embryogenesis either *in vitro* or *in vivo,* is a change in the determination or :fate: of certain cells within a tissue. This is the "inductive stage: and is in response to environment and culture conditions. However, the acquisition of competence leading to induction of somatic embryos has been demonstrated more in dicotyledons than in monocotyledons.

A hypothesis for the occurrence of two different patterns in *in vitro* so matic embryogenesis, direct and indirect, has been put forward (Sharp *et al,* 1980) and further elaborated by several other authors (Evans and Sahrp, 1981; Sharp *et al,* 1982; Williams and Maheswaran, 1986). According to this hypothesis, in direct somatic embryogenesis, cells are already determined for embryogenic development (known as pre-evbryonic determined cells: PEDC), requiring only favourable conditions (e. g. growth regulators at suitable concentrations) to initiate cell division and to express embryogenesis. They include cells of reproductive tissues such as nucellus, synergids and hypocotyl. By contrast, indirect somatic embryogenesis

requires redetermination of vegetative (somatic) cells into callus, callus proliferation and development of the embryogenic determined state. These cells are known as :induced embryogenic determined cells: (IEDC). For these cells, growth regulators are required not only to initiate cell division but also toe determine the embryogenic state. However, it is also hypothesized that the embryogenic determination is at the molecular level and that a cell can lose its embryogenic potential at any stage of the embryo development (Sannasgala, 1989). On this basis, it could be expected that in *in vitro,* unless suitable conditions prevail throughout the development of a somatic embryo, the somatic proembryonal stages may result in other developments or abnormal structures (Krikoria & Kann, 1981; Williams and Maheswaran, 1986; Ammirato, 1987; Sannasgala, 1989). *In vitro* somatic embryogenesis is usually induced by an auxin-in most cases 2,4-D (Steward *et al,* 1964; Litz Cover, 1980; Ammirato, 1987; Litz, 1986; Polito *et al,* 1989, Novak *et al,* 1989; Figueira *et al,* 1991; Rance *et al* 1994). However, in very limited cases, induction is also reported on basal medium without any growth regulators (Vieitez & B, 1990). somatic embryos may form direclty from the explant without an intermediary callus formation. Also, secondary embryos may be formed from somatic embryos and has been shown in *Carica papaya* (Lits & Con, 1980; Litz *et al,* 1992) and also in *prunus persica* (Raj *et al* 1990) and *pinus taeda* (Becwar *et al,* 1990). Usually these do not require the presence of plant growth regulators in the growing medium but may be stimulated by auxin.

According to Halperin (1966) and Mc William *et al* (1974), planes of early cell division in *in vitro* somatic embryogenesis are often highly irregular and may differ from the zygotic embryogenesis.

Haccius and Bhandari (1975) compared somatic embryogenesis to the late differentiating zygotic embryogenesis which is shown by Orchids (Vyret, 1974) and some other plants. They suggested that a zygote differs from the first cell of somatic embryos by its defined place at the micropylar end of the embryo sac. They also suggested that a well marked polarity of the embryo sac brings about an early and direct differentiation in young embryos. According to them, the establishment of polarity in somatic embryos is belated because of their indefinite position in relation toe the environment.

It has been shown in rice that somatic embryognesis recapituates zygotic embryogenesis. Many anatomical similarities with early stages of zygotic embryogenesis have been shown. For e. g. the intial division of the zygote and the epithelial cell in somatic embryogenesis istransverse. Forma-

tion of a "notch" between coleoptile and root apex is also reported. However, initiation or organs in somatic embryogenesis is reported to be slower (Jones & Rost, 1991; Uchmiya & Torigama, 1991)

In monocotyledons, only a few reports demonstrat the formation of a cotyledon duritng somatic embryogenesis (Escalant, 1987; Escalant and Teisson, 1988 both in Musa; Schwendiman *et al,* 1988 in oil palm). In the zygotic embryo of mMusa, a major part of the cotyledon is the haustorium. The reported functions of a haustorium is to provide nutrients and growth regulators are available in the surrounding media, the need of a hustorium is questionable.

The occurrence of abnormal embryos

In nature, it is known that the stages of the embryo development are regulated by characteristic levels of hormones. Under in vitro, to induce the passage from one stage to the next, it is also necessary to have concentrations of particular exogenous hormones especially auxins at various levels. In the absence of these requirements, underdeveloped and irregularly shaped somatic embryos could be formed. These structures are known as :abnormal embryos" or " aberrant forms". Such embryos have been reported in many plant species under *in vitro* conditions (Steward *et al* 1958; Nitsch, 1969; Kohlenbach, 1978; Ammirato, 1974, 1987; Couch, 1982; Mukherjee *et al,* 1988). Such structures would not grow into complete plants. Two reasons have been suggested fro their inability to grow into plants:

1. the embryos are not matured to develop into plants

2. lack of suitable environmental conditions (chemical & physical).

In general, the zygote would undergo embryogenic stages and develop into an embryo which at maturity would germinate and produce a complete plant. But under *in vitro,* it is reported that unless the conditions remain suitable, the development pattern of the proembryo at any stage, may be change. This may results in abnormal or aberrant embryos (Sannasgala, 1989). Abscisic acid has shown to be beneficial in modifying the formation of all the developmental aberrant forms (Ammirato, 1974, 1977, 1983; Vasil & Vasil, 1981, 1982; Ranch *et al,* 1972; Ammirato, 1985). These include: a) underdeveloped shoot apex, b) formation of multiple embryos resulting in multiple shoots, c) malformed cotyledons (differ in number or the morphology), d) direct development into plants without seed maturation thus resulting in malformed plants.

Storage substances in zygotic and somatic embryos

Very few reports are available on the storage substances present during the development of a zygotic embryo and in embryogenic ells of callus and cell suspension cultures.

Presence of starch grains is reported as a characteristic feature of embryogenic cells in suspension (Halperin and Jensen, 1967; Mc William *et al,* 1974; Williams and Maheswaran, 1968; Karlsson & Vasil, 1986). It is also reported as a striking feature in the germinating zygotic embryo of some spp., especially in Musa (Mc Gahan, 1961). However, accumulation and disappearance of starch are also reported during bud formation in some dicotyledons (Thorpe, 1980; Arnold, 1987).

Protein and lipid bodies have so far been reported in *in vitro* embryogenic cells only in the family Brassicaceae, 1982; Jones, 1974). The same protein has been identified in their zygotic embryogenic cells.

Lipo-protein storage material is reported in oil palm both in zygotic and somatic embryogenic cells (Jones, 1974; Schwendima *et al,* 1988). They have not been present in non-embryogenic callus and mristematic cells of shoots. However, in *panicum maximum* and *pennisetum purpureum* of Gramineae, only starch has been reported and no protein or lipid have been observed (Karlsson & Vasil, 1986). But in *Triticum aestivum* L., an endosperm specific protein is reported (Aryan *et al,* 1991; Aryan & Thomas, 1991).

This suggest that the type of storage substance in embryogenic cells vary in monocotyledons and may also differ among different tissues in the embryo of the same plant sp. But a similarity exit in zygotic and somatic embryogenesis of the same plant species. Therefore it appears that unless the storage substance in identified in the zygotic embryognesis, it cannot be used as a criterion of *in vitro* embryogenic cells.

Characterization of proembryonal stages

In many reports on *in vitro* somatic embryogenesis, the authors appear to have used the term "proembryo" arbitrarily. The reason may be due to the confusion with respect to criteria for an *in vitro* embryo. Before identifying the criteria of an *in vitro* "proembryo", it is necessary to define the criteria of the proembryo in nature.

Natesh and Rau (1984) supported by Johri (1984) considered the tetrad stage (4 celled stage) as the beginning of a proembryo. Esau (1977)

described "proembryo" as the stages before the protoderm is initiated. On this basis, the term "Proembryo" can be given to stages between tetrad to the stage just before the protoderm is initiated. Many authors also consider the stage with a protoderm as a proembryo. But the presence of a protoderm is not an essential criterion.

Criteria to identify proembryonal stages

The above information suggest that the term proembryo is not used for one particular stage but, for many stages in the formation of an embryo.

* In all these stages, the proembryo histologically is a mass of non differentiated and differentiated cells. They lack an epidermis and vascular strands which contain differentiated cells.

* Cells of the proembryo have dense cytoplasm, a large nucleus and prominent nucleolus, small number of vacuoles or non at all (Halperin and Jansen, 1962; Thomas *et al,* 1972; Mc William *et al,* 1974; Williams and Maheswaran, 1986).

* Presence of protoderm is not a definite criterion BUT

Under *in vitro* conditions where somatic cells are involved, an initial demarcation appears. This is a physical isolation by which the embryonic cells are distinguished from their non-embryonic cells (Fig 3). This physical isolation is a prerequisite and has been reported in monocotyledons (Tisserat and De Mason, 1980; Schwendiman *et al,* 1972; Mc William *et al,* 1974). This demarcation is not necessarily by an epidermis, but by the arrangement of the outermost cells of the group of embryogenic cells, to give the appearance of a boundary layer. This boundary layer has a "thick outer cell wall" (this is only a technical term) which is positive to Periodic acid Schiff staining.

* There may be many other criteria for a proembryo, but a structure, especially in *in vitro* can finally be confirmed as a proembryo, only if it develops into an embryo.

Charaterization of the *in vitro* somatic embryo

When the development pattern of the zygotic embryo of different species in monocotyledons is considered, it is possible to say that no particular tier in the proembryo attributes to a particular part.

In an embryo, the important parts to from a plant are the shoot and the root apex (embryonal axis) which are collectively termed as the "embryo proper" by Natesh and Rau (1984). It was suggested to use the term

"supplementary organs" for the other structures of the embryo such as cotyledon, haustorium, hypocotyl, susspensor etc. (Sannasgala, 1989). The supplementary organs are required to provide nutrients, growth regulators and protection, thus enabling the embryo proper to develop into a plant. They are also essential in nature, for the development of the embryo proper into a plant. In nature, depending on the environment of the embryo proper, these supplementary organs remain simple or become specialized to carry-out their functions. For example, the cotyledon can have varying degrees of differentiations. But in all plants, the end result is that the shoot apex becomes covered by the cotyledon, In nature, such covering is needed for the survival of the embryo proper (Sannasgala, 1989). Since the functions of a supplementary organs are carried out artificially in *in vitro* culture, the need of these organs is questionable. Therefore, the embryo prefer together with the procambial connections can be considered as the :essential parts" of a somatic embryo *in vitro.* It is possible that different conditions are needed to induce the expression of the supplementary organs, as the formation of different organs can expect to be controlled by different genes. Therefore, *in vitro* conditions which induce somatic cells to from an embryo proper, may not induce the formation of the supplementary organs as well.

It is quite possible that somatic embryos do not need a cotyledon or a suspensor, as they develop in a nutrient rich media. This suggests that the somatic embryo is relatively less differentiated into organs when compared with their zygotic counterparts. But undifferentiated embryos are also found in nature. For example, all orchids are reported to have undifferentiated embryos thus lacking organs.

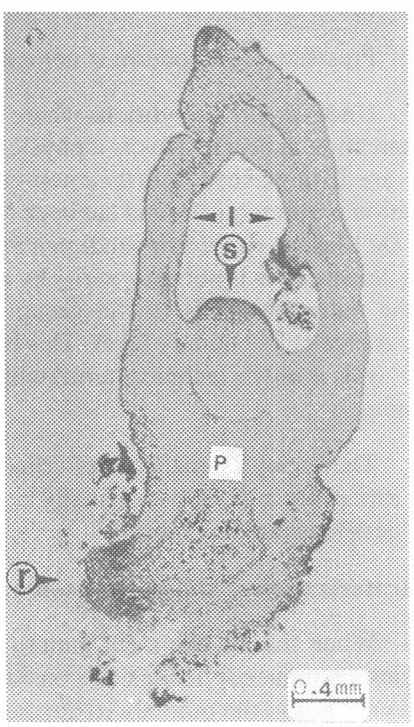

Fig 4. A somatic embryo illustrating the characteristic features (s: shoot meristem, r: root meristem, ct: cotyledo, p: procambial strands)

Criteria to identify a somatic embryo *in vitro*

a) Bipolarity: presence of a shoot and root apex in a unit structure proven by histological studies.

b) Presence of continuous procambial strands between the two meristems (Fig 4).

Characterization of the *in vitro* somatic embryogenesis

It is seen that among different plant species, there are many similar features in the development pattern of somatic embryos. This tends to conclude that in angiosperms, there are prominent stages in the development of somatic embryos. But, these stages may show a different degree of specialization.

The prominent stages in monocotyledons appear to include formation of meristematic loci with demarcations from the neighbouring non-embryonic cells (physical isolation) Fig 3), cleavage polyembryony and late differentition. It is interesting to note that some of these prominent stages are found not only in somatic embryogenesis, but also in zygotic embryogenesis of certain species. Thus it appears that in monocotyledons, there is a resemblance between zygotic and somatic embryogenesis processes even though a close resemblance between the two processes in the same species is not found.

Four major differences were identified between somatic and zygotic embryognesis in monocots (Sannasgala, 1989).

In somatic embryogenesis:

* the initial stages are not easy to identify. They could be identified as meristematic cell clusters.
* proembryos show late differentiation into an embryo. This is presumably due to lack of polarity in the initial cell cluster.
* supplementary organs of the embryo may not be developed.
* a common feature of cleavage polyembryony is shown.

Due to difficulties of identification of structures in somatic embryogenesis of monocotyledons, a set of working hypotheses have been adapted in research. They are

* the inial stages in sonlatic embrgageuesis cannot be distinguisued from normal cell divisiou of meristematic cells (Mc william et al 1974; Tisserat et al, 1980)

* There may be several patterns of somatic embryo development. There may be individual and isolated somatic embryos or clusters which are connected to the mother tissue.

* Morphological and physiological differences between somatic and zygotic emgryogenesis can be expected as, in each case, embryos are formed under different physical and chemical environments (Borthwick, 1931; Abo El Nl, 1977)

Conclusion

Somatic embryogenesis recapitulates zygotic embryogenesis in dicotyledons. But in monocotyledons the above factor cannot be generalized. It appears to depend on the plant species where in some a similar pattern is present while in others a difference is reported. The difficulty appears to be in identifying structures *in vitro* of monocotyledons. Some criteria to identify the somatic embryogenesis are reported. These may be used *in vitro* somatic embryogenesis in monocotyledous is important as they comprise major food crops of the world today.

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