HISTOLOGICAL STUDIES ON THE ORIGIN AND PROCESS OF PLANTLET DIFFERENTIATION IN SUGARCANE CALLUS MASS

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ABSTRACT

A histological study has been made of organogenesis in sugarcane callus grown on two kinds of media: One was that of Murashige-Skoog, containing 3 ppm 2,4-D and designated non-organ-forming medium; the other was the same, without 2,4-D but with kinetin, NAA, and casein hydrolysate for shoot differentiation. Organogenesis did not usually take place under non-organ-forming conditions unless the callus was in earlier passages. Proembryoids characterized by smaller, isodiametric cells with densely staining nuclei could be formed as early as 8 days after subculturing under shoot-forming conditions. They later developed a shoot-apex structure which was ontogenetically comparable with a seed-embryo derived from a zygote but structurally, more nearly analogous to a vegetative shoot apex such as is found in the cane tip. Polyembryonies were not uncommon. The cells in the portion of callus, not far from the area which bore shoot apices, became differentiated into protoxylem elements. Two patterns of differentiation in callus were observed. In one shoots appeared first and roots were initiated later, in the other only roots formed, and there was a complete loss of totipotency. Schenk-Hildebrandt medium was very effective in promoting root differentiation in vitro. An embryoid was believed to be of unicellular origin as shown by our observation as well as the relevant published papers.

INTRODUCTION

The sequential development of whole carrot plantlets from callus cultures was reported first by Wiggans more than 20 years ago. Reinert was a pioneer in demonstrating the formation of embryoids from higher plant tissues in vitro. Steward et al. first generated plants from cell suspension cultures of carrot. Konar and Nataraja initiated an embryoid from the stem epidermis of Ranunculus sceleratus L. Heinz and Mee, and Barba and Nickell derived complete sugarcane plantlets from callus tissue in 1969. Subsequently regeneration of whole plants from cell cultures, callus or segments from different parts of the plant body has been a relatively common phenomenon in many higher plant species. Embryo-like structures, embryoids or whole plants, or both, have been induced from such highly differentiated cells as the pollen grain, triploid endosperm cells, and the epidermal cells from leaves and stems. These achievements constitute an important means of vegetative propagation and are of great importance to both the horticulturist and the plant breeder.

Reinert et al. and Johri have demonstrated that the sequence of development of the embryoid in culture is generally comparable to that of the zygotic embryo. The embryoid is originated from a single cell, among the millions of cells in callus, which has the exceptional capability of proliferating into a group of cells before an embryoid is formed.
Mass production of sugarcane plantlets in vitro has been carried out since the establishment of the Tissue Culture Laboratory at this Institute in 1970 with a production capacity of 2 to 3 hundred plants per month. For the past 3 years through studies on shoot differentiation from sugarcane callus tissue, it has been concluded that shoot differentiation is influenced by (a) the donor's genotype, (b) the previous culture medium, (c) the number of subcultures (unpublished data of our laboratory). However, the exact origin of a shoot primordium and the processes of embryogenesis in callus cultures are still obscure. Considerable endeavor is needed to clarify the precise internal physiological and biochemical changes which must take place before a cell can exhibit its totipotency, or acquire embryological competence like that of a zygote. An experiment was therefore started to trace histologically the origin and the sequential development of a plantlet from the callus mass.

**MATERIALS AND METHODS**

Initiation of callus from subapical meristems and rolled young leaves was achieved on a modified Murashige and Skoog (MS) medium as described in a previous paper. At 3rd or 4th passages the callus masses of F 162 or F 170, growing on a modified MS medium supplemented with 3 ppm 2,4-D, were separately inoculated on 2 media. The first medium was that mentioned above and designated non-organ-forming; the second was the same without 2, 4-D but supplemented by the following substances at the concentrations indicated, in milligrams per liter: kinetin, 1; naphthaleneacetic acid (NAA), 1; casein hydrolysate, 400. This was designated organ-forming medium. After inoculation, a piece of callus 2 x 4 x 4 mm in size was taken after 0, 4, 8, 11, 14, 17, 20, 23, 26 days in culture, and fixed in FPA or CRAF fixatives. The fixed specimens were dehydrated with a series of tertiary butyl alcohol and embedded in paraffin. Sections were cut at 10 microns, by a rotary microtome, and stained with safranin-fast green, or safranin, tannic acid and orange G.

**RESULTS AND DISCUSSION**

Fig. 1 is a general view of callus growing on a non-organ-forming medium. Such a piece of callus was composed of relative large parenchymatous cells which were randomly distributed in the central portion, and numerous smaller and isodiametric cells with conspicuous nuclei which usually occurred in the peripheral region. Organogenesis in callus did not usually take place under non-organ-forming condition except in earlier passages in which the callus was strongly influenced by the differentiated tissues of the donor. Therefore, all meristemoids or embryo-like structures discussed in the following paragraphs were products of the organ-forming medium.

A callus sample taken from a 14-day-old culture demonstrated nodule-like meristemoids, conforming to Torrey's term, on its outer surface (Fig. 2). The meristemoid was composed of numerous cells in orderly development. The cells were smaller, isodiametric in appearance, lacking apparent vacuoles, and with densely staining nuclei. The meristemoids were virtually proembryoids which might develop as early as the 8th day after subculturing. Their cross-sections showed cells arranged concentrically (Fig. 3). Meristemoids could later develop into multi-shoot-apex structures as shown in Fig. 4.
FIGURE 1. Parenchymatous callus mass on a non-organ-forming medium.

FIGURE 2. Longitudinal section of nodule-like meristemoids. m = meristemoid.

FIGURE 3. Cross section of meristemoids seen in Fig. 2.

FIGURE 4. Multiple shoot apices, arising from callus 20 days after subculturing. s = shoot apex, m = meristemoid.
Figs. 5 and 6 show two typical shoot apices developed from a callus mass. The structure in Fig. 5 is analogous to that of an embryo as seen in sagittal sections of a mature sugarcane seed, developed from a zygote, as illustrated by Artschwager et al. Like the apical structure of a seed-embryo, the apical meristem, which usually protruded into an empty space, was in the center and flanked by embryonic leaves which were, in turn, enclosed by a coleoptile-like structure (Fig. 6). The developmental stage in Fig. 6 was much later than that of Fig. 5, as demonstrated by the mature appearance of cells in the surrounding coleoptile-like structure. Ontogenetically, a callus-embryo is comparable to an embryo derived from a zygote as discussed by Reinert et al.

FIGURE 5 and FIGURE 6. Longitudinal section of 2 typical shoot apices with apical meristem at the center and leaf primordia on both sides; coleoptile-like structure.

FIGURE 7. Surface view of a shoot apex corresponding to the stages of Figs. 5 and 6. s = shoot apex.
and Johri. Judging from the appearance of Figs. 6 and 11, the coleoptile-like structure seemed to be formed earlier than the embryonic leaves which is characteristic of grass embryos. However, histologically, it has not been possible

**FIGURE 8.** Longitudinal section of double apical meristems flanked by embryonic leaves: d = double apical meristems.

**FIGURE 9.** Two apical meristems developing from callus under embryonic leaves, or coleoptile-like structures: a = apical meristem.

**FIGURE 10.** Surface view of a polyembryoid corresponding to the stage of Fig. 11. s = shoot.
to find a shoot-root axis or other structures such as radicle and coleorhiza, which are integral parts of a seed-embryo. The coleoptile in callus-embryoids is considered to be functionless because its original role of protecting the delicate plumule while it penetrates the soil is unnecessary under the circumstance of sterile culture. As far as the structure is concerned, a callus-shoot-apex is therefore much simpler than a seed-embryo, and more resembles a vegetative shoot apex.

Probably due to less restriction in the development of a shoot-meristem in callus than in the intact cane tip, the leaf primordia may not be arranged as regularly as those of a vegetative shoot apex, so that there may be more leaf primordia, or more apical meristems in a shoot-apex-unit as seen in the lower parts of Figs. 4, 8 and 9.

Fig. 7 is a surface view of a shoot apex protruded out of a callus mass. Its age corresponded to those shown in Figs. 5 and 6. Some layers of green cells can clearly be seen in the embryonic leaves.

Fig. 8 is a longitudinal section of a double apical meristem whose cells were densely stained and are flanked by two embryonic leaves. Presumably any cell at the periphery of a callus could develop into a shoot primordium. Therefore polyembryonies were not uncommon in the process of shoot differentiation. Johri reported polyembryony in the callus culture of *Ranunculus sceleratus* L. He attributed this phenomenon to cleavage and/or budding of the embryonic mass at different stages of callus development. Sometimes several embryonic leaves could be seen at the same time in a shoot-apex unit (Fig. 8).

**FIGURE 11.** Triple shoot apex: s = shoot apex, c = coleoptile-like structure.
This phenomenon could be caused by the overlapping of several apical meristems.

Two apical meristems (deeply stained) were able to develop from the callus mass under the covering of embryonic leaves, or the coleoptile-like structure in the outer layer which grew earlier than the apical meristem (Fig. 9). This shows that the development of a callus-shoot-meristem was not as regular as that of a vegetative shoot apex in a cane tip.

A surface view of polyembryoids is presented in Fig. 10, the interior structure of which is shown in longitudinal section in Fig. 11. This is a model of a triple shoot apex in which each apical meristem is flanked by leaf primordia and covered by the coleoptile-like structure, giving it the shape of an arch.

Cross sections in the portion of a callus not far from the area where a shoot apex had developed, showed that some of the callus cells had differentiated into typical protoxylem cells (Fig. 12). Some of them demonstrated secondary wall thickening in a scalariform-reticulate pattern which could be clearly distinguished at a higher magnification (Fig. 13). Tracheid elements and phloem cells have also been reported in a callus mass,\textsuperscript{10, 14}

Fig. 14 is a cross section of a shoot apex. The apical meristem with cells in an orderly arrangement and deeply stained is in the center and surrounded by leaves. Some vascular bundles have already formed in the outer layers of leaves.

A general view of a young shoot is shown in Fig. 15, which was photographed in the developmental stage several days later than that of Fig. 10. It appeared to be growing vigorously as shown by the broad, green leaves. Such a growth pattern was especially common when callus was in its earlier passages. However, in some sugarcane varieties newly-emerged shoots had a needle-like form which prevailed after the seventh passage.

Another differentiation pattern in callus is the formation of roots (Figs. 16-18). Fig. 16 is a root primordium just initiated from a callus mass. The center cells are still meristematic in appearance, and arranged in several rows indicating a typical root tip. The initiation pattern was analogous to that of a lateral branch root derived from the taproot of a normal plant, as discussed by Esau\textsuperscript{6}. Fig. 17 shows a root system developing from callus mass grown on a Schenk-Hildebrandt (SH) medium (28) which is effective in stimulating root initiation and is now in routine use in our laboratory for rooting plantlets in vitro. Some plantlets, although they had grown for 6-8 weeks after initiation still lacked a root system. Consequently they would not survive after having been transferred to vermiculite. Poor rooting caused considerable difficulty in raising seedlings although SH medium helped to solve this problem and contributed a great deal to our scheme of mass production of sugarcane plantlets through callus culture. Fig. 18 is a surface view of some roots which were developing from callus mass. They were very tender in texture and white in color.

Organogenesis in sugarcane callus appears to proceed in either of 2 ways, according to our laboratory results. One pattern was that a shoot appeared first and a root initiated later, usually after plantlets had grown on sterile culture for 4-6 weeks. Some sugarcane genotypes failed to form roots at all (Fig. 15). Another pattern was for a callus piece to form only a root. Such roots usually appeared on the surface of a callus mass (Fig. 18). The callus
FIGURE 12. Cross section of a piece of callus showing that the cells have differentiated into protoxylem elements.

FIGURE 13. Magnified view of tracheid elements characterized by the secondary wall thickening: $t =$ tracheid.


FIGURE 15. General view of a young shoot at a stage several days later than that of Fig. 10.
FIGURE 16 and FIGURE 17. Longitudinal sections of root primordia.

FIGURE 16. Root primordium just initiated from a callus 14 m days after subculturing: \( r = \) root primordium.

FIGURE 17. Root system, originating from callus cells, effectively promoted by Schenk-Hildebrandt medium. \( r = \) root system.

FIGURE 18. Surface view of a developing root system derived from a callus 21 days after subculturing.
appeared to have lost its capability to initiate shoots and was generally regarded as useless because it would never be differentiated into shoots.

Concerning the origin of embryoid in callus, although it is difficult to determine whether a single cell or an aggregate could develop into a complete embryoid, some indirect evidence in the literature led us to believe that a meristemoid is originated from a particular single cell in a callus mass. The available data which support this hypothesis are as follows: Muir et al. reported that isolated single cells could successfully be grown on a nurse culture, separated by a filter paper, and these gave rise to a callus mass. Later, Visil and Hildebrandt demonstrated that completely isolated single cells of tobacco could grow in microchambers to form small clumps of cells which could then be differentiated to form adult tobacco plants. Konar and Nataraja stated that epidermal cells of Ranunculus sceleratus L. were able to form an embryoid. Reinert et al. also found that the formation of an embryoid from a single carrot cell could be followed directly by serial microscopical observations. These, and other data, would support the hypothesis that an embryoid in callus arises originally from a single cell. Still to be determined is what kind of cells can exhibit this totipotency, and how much physiological and biochemical change occurs in the cell before initiation of an embryoid. It is suggested that techniques of histochemistry and electron microscopy might be employed to identify the proembryonic cells in callus masses.

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REFERENCES


All photographs were taken with either 35 mm photo head or luminars of Zeiss Ultraphot II. Each scale division equals 100 μ.
ESTUDIOS HISTOLÓGICOS SOBRE EL ORIGEN Y DESARROLLO DE LA DIFERENCIACIÓN DE PLANTULAS EN CALLOS DE CAÑA DE AZÚCAR

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RESUMEN

Se efectuó un estudio de organogenesis en callos de caña de azúcar desarrollados en dos tipos de medios. Uno de ellos fué el de Murashige-Skoog suplementado con 2,4-D 3 ppm, y designado como un medio no formador de órganos; el otro medio fué el mismo anterior pero sin 2,4-D y con agregado de kinetina, ácido naftalen acético y caseína hidrolizada para diferenciación de brotes. La organogénesis no tuvo lugar usualmente en la condición de no formación de órganos, excepto el callo en los primeros pasajes. En la condición de formación de brotes y a los 8 días desde el sub-cultivo se formaron tejidos proembriodes caracterizados por células más pequeñas, isodiamétricas y con nucleos muy teñidos. Posteriormente ellos desarrollarían en estructuras de apice, la cual es comparable desde el punto de vista ontogenético al embrión semilla, pero más parecido estructuralmente al apice vegetativo en el punto de crecimiento de la caña de azúcar. El fenómeno de poliembrina fué bastante común. Las células en la porción del callo cercana al nacimiento de los apices diferenciaron en elementos del protoxilema. Se observaron 2 patrones en la diferenciación de los callos. Uno de ellos fué brotes primeros, y raíces iniciadas más tarde. El otro fué la formación solamente de raíces en el callo, con la pérdida absoluta de la capacidad de formar brotes. El medio de Schenk-Hildebrandt fué muy efectivo en provocar diferenciación radicular in vitro. Un embriode fué considerado como originado de una sola célula como evidenciaron nuestras observaciones como así la literatura disponible.