1987

Initiation and growth of embryogenic callus and suspension cultures of Zea mays L.

Donna Jean Spannaus-Martin

Iowa State University

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Initiation and growth of embryogenic callus and suspension cultures of *Zea mays* L.

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Iowa State University, 1987
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Initiation and growth of embryogenic callus and suspension cultures of *Zea mays* L.

by

Donna Jean Spannaus-Martin

A Dissertation Submitted to the Graduate Faculty in Partial Fulfillment of the Requirements for the Degree of DOCTOR OF PHILOSOPHY

Department: Biochemistry and Biophysics

Major: Molecular, Cellular, and Developmental Biology

Approved:

Signature was redacted for privacy.

In Charge Of Major Work

Signature was redacted for privacy.

For the Major/Department

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For the Graduate College

Iowa State University
Ames, Iowa

1987
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INTRODUCTION

The concept that somatic plant cells could give rise to a plant embryo that would be capable of forming a complete plant has its beginnings in the latter part of the nineteenth century with the work of G. Haberlandt (1), a German plant physiologist. Haberlandt hoped to achieve continued cell divisions in explanted tissues on nutrient media and attempted to grow single isolated cells in aseptic culture. In 1902, he predicted that isolated cells should be capable of developing as 'artificial embryos.' Haberlandt's attempts at developing plants from single cells in culture were unsuccessful. Pfeffer (2) stated that the failure to form a new plant from a single isolated cell was "obviously due to the difficulty of presenting such a cell with a supply of nutriment of appropriate quality and quantity."

The year 1904 marked the beginning of plant embryo culture, when Hannig (3) removed mature embryos from seeds of Raphanus and Cochlearia and placed them in a medium containing mineral salts and sucrose. These excised embryos were able to develop into young plantlets. The relatively large size of mature embryos and the ease with which they can be isolated facilitated their use in early embryo studies. Mature embryos have simple nutrient requirements, while immature embryos are dependent on the diffusion of
metabolites from the surrounding endosperm. Culture of immature embryos was not possible until 1941, when van Overbeek et al. (4) reported that the addition of the liquid endosperm from coconut to the culture medium was effective in supporting the growth of immature embryos from coconut palm and other species. van Overbeek et al. (5) stated that coconut milk contained at least three factors: 1. embryo factor, 2. leaf growth factor, and 3. auxin. Many of the active components of coconut milk have now been identified as sugar-alcohols such as myoinositol, leucoanthocyanins, cytokinins, auxins, and gibberellins. This knowledge has resulted in the ability to culture and induce the formation of embryos on synthetic, chemically-defined media in the absence of liquid endosperm (6).

Until 1958, the only observations of plant embryos that had been made were those that had developed within the plant from a fertilized egg cell. Steward et al. (7) described the formation of normal appearing plant embryos from suspension cultures of Daucus carota. Embryoids were described on the surface of carrot cultures grown on an agar medium by Reinert (8).

Embryo cultures have been useful for studying the growth, differentiation, and nutritional requirements of developing plant embryos. They have provided a way to obtain plants from hybrids that would be incapable of surviving
under normal conditions. Because the somatic embryos produced from embryogenic callus can show some genetic variation (9), there are comparisons that can be made between the hundreds of potential plants represented in a few Petri dishes containing embryogenic callus and the hundreds of developing plants represented in a field plot. Following the addition of exogenous DNA to plant cells, it is usually desirable to be able to grow the resulting transformed plant. A greater understanding of somatic embryogenesis could facilitate this. For all of these experiments, it is preferable that the maximum number of somatic embryos possible be obtained from the callus culture. For this reason, it is important to understand the development of early somatic embryogenesis; to know what environmental factors can induce cells to become embryogenic; and to manipulate these factors to maximize the production of somatic embryos.

The Morphology of Early Embryogenesis

Normal zygotic embryo development has been described for several plant species. The first division of the zygote is unequal, resulting in the formation of a terminal cell and a large basal cell (10). In most cases, the small terminal cell goes through repeated cell divisions to form the embryo
proper, while the large basal cell divides to form the suspensor. In cotton, the terminal cell has been described as containing more plastids, larger mitochondria, and more variably shaped mitochondria than the basal cell. The basal cell contains dense, membranous vacuoles. Associated with these vacuoles are small vesicles, apparently fusing with them to form large vacuoles. The small terminal cell stains darkly for nucleic acids and proteins, while the larger basal cell stains relatively lightly (11).

Shepherd's purse, Capsella bursa-pastoris, is often used as an example of embryogenesis that is typical of many plant species. The zygote of Capsella contains large polysome complexes and active dictyosomes. Starch and lipid deposits can be observed after fertilization. The zygote undergoes its first division by producing a transverse wall resulting in the formation of a large, highly vacuolate basal cell and a small, densely-staining, cytoplasmic terminal cell. The terminal cell appears to contain more ribosomes and less endoplasmic reticulum than the basal cell. The nucleus of the terminal cell is surrounded by a shell of mitochondria and plastids. As the terminal cell divides, the starch that accumulated during the zygote stage is rapidly utilized. The cells contain few dictyosomes and little endoplasmic reticulum, but are rich in ribosomes that are grouped into small polysomes (12). The terminal cell continues to go
through a series of divisions, eventually forming the 'globular,' 'heart-shaped,' 'intermediate,' 'torpedo-shaped,' 'walking stick-shaped,' 'inverted U-shaped,' and mature embryonic stages of development. Ultrastructural differences are not detectable between the cells of the developing embryo until the late 'globular' or early 'heart-shaped' stage when the embryonic leaves begin to form.

Soon after it is formed by the first unequal division, the basal cell of *Capsella* begins a series of transverse divisions resulting in the formation of a large basal cell connected to a series of smaller suspensor cells that in turn join the developing embryo. The basal cell contains a conspicuous central vacuole in which a highly lobed nucleus is suspended within a strand of cytoplasm traversing the vacuole (13). The suspensor cells are rich in dictyosomes, mitochondria, plastids, and spherosomes. During the globular stage, there is a decrease in quantity of ribosomes and in the concentration of nucleic acids and proteins. At the 'heart-shaped' stage, cytoplasmic degradation of the suspensor cells begins, including the depletion of ribosomes, and the loss of nucleic acids and proteins. The large vacuolate cell remains active for some time after the other cells have degenerated.

Early stages of somatic embryogenesis appear to closely resemble the corresponding stages of zygotic embryo
development. Bachs-Hüsemann and Reinert (14) were able to follow embryoid formation from a single carrot cell. These cells underwent an initial unequal division and then gave rise to a group of cells consisting of both embryonic and more internal parenchymatous cells. These observations supported the view that a multicellular aggregate is necessary to initiate embryoid formation. The primary action of these cells adjacent to the embryonic cells is thought to involve the transport of nutrients to the embryo, and possibly to provide an environment that would induce the polarity of the initial embryonic cell. Even though other cells may be required to induce embryogenesis, the somatic embryo itself is still thought to arise from a single cell (15). Kuo and Lu (16) were able to identify single cells on the surface of Zea mays callus derived from microspores that appeared to be different from the majority of the other callus cells. They were able to follow the development of these cells, which followed a pattern similar to that of normal zygotic embryos. Most of these cells could be seen in the surface layers of the callus, although a few were found in the inner layers. This embryo-forming cell contained denser cytoplasm and a larger nucleus than the other cells of the callus. The first division of this cell resulted in the formation of a basal cell and a terminal cell. The terminal cell divided longitudinally and then transversely, and
eventually could go on to form an embryo. The basal cell went on to form the suspensor.

The ultrastructural development of embryoids from *Ranunculus sceleratus* L. cultures was described by Konar *et al.* (17) and Thomas *et al.* (18). Small cells were observed containing dense cytoplasm and a large nucleus containing highly condensed chromatin. Greater nuclear and nucleolar volume density has also been observed in embryogenic pollen grains of *Hyoscyamus niger* (19). Many ribosomes and mitochondria are present in the *Ranunculus* embryos, as well as plastids containing starch grains. A vacuole can be seen which persists until the two-cell stage of development. When the terminal cell begins dividing, this central vacuole appears to break up into smaller vacuoles which are grouped around the nucleus. Plasmodesmata are observed between the embryonic cells and the mature epidermal cells. If these embryonic cells are found adjacent to one another, the wall between them contains many more plasmodesmata. There are reports in other species that few cell-cell connections between embryonic cells and nonembryonic callus cells exist. In *Citrus*, early embryonic cells have thick walls (3-5 μm) either lacking plasmodesmata or containing what appear to be nonfunctional plasmodesmata that separate these cells from the nonembryonic callus (20). Howarth *et al.* (21) observed suberin lamellae in the walls of cells surrounding
meristematic regions of *Lotus corniculatus* callus. These findings support the hypothesis that physiological isolation is necessary to induce embryo formation. The cells of embryogenic carrot cultures have been described as containing numerous vesicular bodies apparently derived from dictyosomes and small lipid bodies (22).

The unequal division that occurs in zygotic embryo development is also observed in embryonic carrot cultures (14). However, the first division of an embryonic cell does not always follow the pattern of normal zygotic development. The first cell division of embryogenic pollen grains is usually equal (19). This appears to be related to whether or not the embryo is likely to form a suspensor connecting the embryo to the callus. Somatic embryos derived from pollen cultures tend not to form suspensors. If an embryogenic cell is going to form a suspensor, it will initiate that formation within the first few cellular divisions (23).

The embryo continues to divide and develops into a globular stage embryo. Halperin and Jensen (22) state that the most obvious difference between the cells of the embryo and the suspensor cells is the tremendous increase in the number of free ribosomes in the embryonic cell. Other differences described include a higher concentration of rough endoplasmic reticulum, dictyosomes and starch in the suspensor cells. The amount of endoplasmic reticulum in
embryonic cells decreases and the amount of smooth endoplasmic reticulum increases. These characteristics are very similar to those seen in zygotic embryo development.

The presence of many ribosomes and a large nucleus tend to be common to all embryonic cells. Other structures that have been described in the cells of the globular embryos of *Ranunculus* include numerous spherosomes arranged in a single layer around the cell wall, plastids containing globular centers that are surrounded by what appears to be lipid droplets, myelin-like bodies in the cytoplasm and in vacuoles, and an increase in the number of starch grains contained in the amyloplasts. As the embryo begins to form the 'heart-shaped' and 'torpedo-shaped' stages, the superficial cells expand and become highly vacuolate, and only the central core of cells remains intensely staining and cytoplasmic (17).

Anatomical studies on the initiation of embryogenic callus in the immature embryos of *Zea mays* have been done (24, 25, 26, 27). Springer et al. (27) reported that by the third and fourth days in culture, the epithelial layer of the scutellum became more cytoplasmically dense. The coleorhizal end of the scutellum showed rapid cellular proliferation and enlargement by the fourth day. By the ninth and tenth days, random locations at the scutellar surface became intensely meristematic and showed differentiation of distinct cell
types. These areas eventually developed into shoot apices, complete with two leaf primordia and an apical meristem. The development of this type of callus is considered to be organogenic, of type I callus formation (Appendix 1).

The anatomical changes that occur in the formation of somatic embryos in maize were described by Vasil et al. (25). Meristematic regions in the coleorhizal end of the scutellum are often associated with the procambial cells of the immature embryo. These meristematic regions became organized into globular stage embryos. From these results, they concluded that somatic embryos in maize cultures were multicellular in origin. McCain and Hodges (26) observed similar meristematic regions developing from the scutellar cells. By eight to nine days in culture, these broad-based meristematic regions had organized to form somatic embryos containing an apical dome and a coleoptilar notch. The development of well-formed somatic embryos on suspensors is referred to as type II callus. Their results also point to a multicellular origin for Zea mays somatic embryos.

Lu et al. (24) used immature embryos of Zea mays to produce embryogenic callus cultures. By the eighth to ninth day in culture, early somatic embryos could be seen on the coleorhizal end of the scutellum. By days 11-12, well differentiated coleoptiles which showed either a terminal pore or longitudinal slit could be found along the periphery
of the scutellar proliferation. This corresponds to the 'heart-shaped' stage of zygotic development. Each coleoptile was enveloped by a single, well developed scutellum. Each coleoptile usually contained one shoot meristem. Unless the auxins are removed from the medium, the somatic embryos will usually not develop past this stage. If the auxins are removed from the medium, the embryos will germinate forming roots and leafy structures.

Factors Affecting Embryogenesis

Many physical and chemical conditions can affect the production of embryos from somatic plant tissues. Much of the work in determining how the environment affects embryogenesis has been done using anther cultures rather than immature embryo cultures. Embryogenic anther cultures have often been used to obtain haploid plants. The development of an embryo-forming microspore is similar to the development of somatic embryos derived from immature embryos. The conditions that influence the frequency of somatic embryo formation in one type of explant could provide some insight into other ways of increasing the embryogenic potential of the other type of explant.

The condition of the plant at the time the explants are taken can determine if embryogenesis will occur. The light
intensity at which the plant was grown and the age of the plant have been shown to affect embryo induction in cultured anthers of tobacco (28). Tsay (29) reported that the amount of nitrogen supplied to donor tobacco plants influenced the embryogenic ability of cultured anthers.

The ability of somatic tissues from gramineaceous species to produce embryos may be related to the extent of differentiation that has occurred in the explant. Wernicke and Brettell (30) found that cultured leaves of Sorghum bicolor could only be induced to dedifferentiate and produce callus tissue if the leaf cells had not passed a critical stage of differentiation. The greater the 'distance' (spacial and temporal) from the apical meristem, the less likely a piece of leaf would be to produce somatic embryos (31).

Nitrogen levels and nitrogen sources in the culture media appear to be very important for embryo development. Embryo development in wild carrot cultures could only be initiated if the medium contained reduced nitrogen (32). In wheat anther cultures, the addition of glutamine to the medium increased the frequency of embryo appearance, and these embryos formed earlier than those on a medium without glutamine (33). Another amino acid, L-proline, may be more important in the initiation of friable, embryogenic callus cultures in grasses. L-proline has been shown to stimulate
embryogenesis in anther cultures of triticale (34). Green et al. (35) were able to increase the frequency of somatic embryo formation from immature maize embryos on N6 medium from zero to 65% by the addition of 50 mM proline. The optimum proline concentration to initiate friable, embryogenic calli was found to be 25 mM. L-glutamine was not found to promote somatic embryogenesis in these cultures.

The presence or absence of hormones in the medium can be essential for the production of embryogenic callus. The induction of somatic embryogenesis requires the presence of auxin. Vasil and Vasil (36) reported that levels of 2,4-dichlorophenoxyacetic acid (2,4-D) between 0.25 mg/L and 2.5 mg/L supported the rapid proliferation of embryogenic cells, while 2,4-D levels of 0.5-1.0 mg/L were required in order for these cells to organize into globular embryos. If these levels exceeded 4-5 mg/L, no embryogenic callus was formed at all (37). Development of embryos past the coleoptilar stage will usually not occur unless all of the 2,4-D is removed (35). Kinetin does not affect the embryonic axis of an embryo, but it will induce the scutellum of Pennisetum typhoideum to form callus (38), and the epiblast of immature barley embryos to form embryoids (39). Gibberellin has been shown to inhibit somatic embryogenesis (40) and induce germination of immature barley embryos (41). The presence of abscisic acid has reduced the incidence of
precocious germination (42).

The presence of some energy source, usually sucrose, seems to be required to induce embryogenesis. Lu et al. (24) reported that embryogenic callus formation increased 65% when immature Zea mays embryos were placed on a medium containing 12% sucrose, compared with a medium containing 3% sucrose. This high sucrose concentration also inhibited the germination of cultured embryos. The increase in the formation of somatic embryos in a medium containing a high sucrose concentration may be the result of the osmotic effects of the carbohydrate in addition to its effects as an energy supply. Brown et al. (43) found that a concentration of 3% sucrose produced the maximum induction of shoot formation in tobacco cultures, but a concentration of 2% sucrose could show the same amount of shoot formation if mannitol was added to the medium to provide the same water potential as the medium containing 3% sucrose. It is known that mannitol can enter callus cells, but it cannot be metabolized by tobacco cells. A sucrose concentration below 2% at the same water potential as the medium containing 3% sucrose resulted in a decrease in the quantity of shoots formed. Wetherell (44) reported that cultured wild carrot cells showed a great increase in the number of embryoids produced if the cells were pretreated with 1 M sucrose for one hour before culturing. This effect is thought to result
from the disruption of plasmodesmatal interconnections between the pre-embryonic cells. Rupturing of thin, radial, cytoplasmic strands between the cell wall and the cell membrane could be observed using light microscopy during the time of plasmolysis. This supports the hypothesis that cellular isolation is required before embryogenesis can occur.

Cold shock or heat shock have been used to induce embryo formation from anthers of several species. Rice anthers incubated at 10°C for seven days before culturing showed a two-fold increase in callus production (45). Cold pretreatment of Brassica napus anthers did not increase embryo production, but a pretreatment at 35°C did result in increased embryo formation (46). The increased response in the cold treated anthers is believed to be linked to the slow degradation of the tapetum during the pre-treatment. The heat pretreatment may disrupt the normal developmental processes of the anther tissue and the microspores, synchronizing the physiological states of the two tissues, stimulating embryo induction. Heat pretreatment may synchronize the cells so that they are at a stage of development that is more susceptible to induction when the temperature is reduced.
Embryogenic Suspension Cultures

Embryogenesis in suspension cultures was first reported by Steward et al. (7) when they described the formation of structures in suspension cultures of Daucus carota that followed a course of development similar to that seen in zygotic embryo formation. Embryogenic suspension cultures of grasses have been difficult to produce. An embryogenic suspension culture of Bromus inermis was obtained by Gamborg et al. (47). However, the few plants regenerated from these cultures were all albino plants. It has only been recently that stable and long-term embryogenic suspension cultures of Gramineae have been established that are capable of the regeneration of normal plants (48, 49, 50, 51). These cultures are rapidly growing, finely dispersed cultures, and are free of callus pieces, organized tissues, meristems and meristemoids. Cultures were comprised primarily of cell aggregates of about 50 cells, with each aggregate comprised of individual and discrete groups of 2-6 cells. These cells were predominantly small, richly cytoplasmic and starch-containing meristematic cells (52).

Suspension cell cultures provide a unique system for the study of embryogenesis, cell growth, and tissue differentiation. A high percentage of the cells are in direct contact with the liquid culture medium. This
diminishes the establishment of gradients within the culture that occurs with callus cultured on solid medium. There are additional considerations that must be examined in the establishment of liquid cultures. The concentration of nutrients and growth regulating factors usually needs to be modified due to the more intimate contact between the cells and the culture medium. There is also a lower oxygen concentration within the liquid medium, so that the culture requires some form of agitation to obtain adequate aeration. This agitation facilitates the fragmentation of the cell clusters, leading to free cells and smaller cell clusters.

Explanation of Dissertation Format

In this dissertation, the alternate format is used. Two papers are included. Paper 1 is being submitted to the American Journal of Botany. Paper 2 has been submitted to In Vitro. Both papers are collaborative work with Dr. Dwight T. Tomes of Pioneer Hi-Bred International, Incorporated. All of the field and greenhouse work, most of the tissue culture work, and the initial sample preparation was done at Pioneer Hi-Bred in the laboratories of Dr. Tomes, Dr. Sheila Maddock, and Dr. Marc Albertsen.
PART I.

DEVELOPMENT OF TYPE I AND TYPE II

EMBRYOGENIC CALLUS IN ZEA MAYS
DEVELOPMENT OF TYPE I AND TYPE II EMBRYOGENIC CALLUS IN ZEA MAYS

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ABSTRACT

The immature embryos of maize genotype H99 can be induced by dicamba to produce primarily type I embryogenic callus, that is firm, compact, relatively slow-growing. Immature embryos of one (G35 X B73)s line cultured in the presence of 2,4-dichlorophenoxyacetic acid (2,4-D) yield type II embryogenic callus, a rapidly-growing, highly friable callus exhibiting well-formed somatic embryos. A selected responsive (G35 X B73)s line can be induced to yield a high frequency of type II callus whereas embryos from a second selected nonresponsive (G35 X B73)s line cannot be induced to form somatic embryos under any known set of conditions.

Immature embryos from these three corn lines were cultured, fixed at various times during the culture period, and embedded in paraffin and/or resin. The H99 embryos showed a generalized area of meristematic tissue that appeared to be initiated in the subepidermal layers of the scutellum. The immature embryos of the responsive (G35 X B73)s line showed distinct groups of meristematic cells at the scutellar surface. These groups were only 6-8 cells in size in contrast to the large meristematic regions observed in H99 immature embryos. The scutellar cells of the nonresponsive (G35 X B73)s hybrid did not develop meristematic regions, and the cells of both the epidermal and
subepidermal layers became vacuolate. The location and small number of meristematic cells in the responsive hybrid line suggests that somatic embryogenesis proceeds via a different pathway in the responsive \((G35 \times B73)_s\) line than in H99 embryos. The results suggest that embryo formation in the responsive \((G35 \times B73)_s\) hybrid originates in the epidermal layer from a single cell or from a very small number of isolated cells.

All three genotypes cultured on media containing an auxin source showed the development of a large air space separating the epidermal and subepidermal layers from the remainder of the scutellum and the embryonic axis. Immature embryos cultured in the absence of auxin did not develop this air space, but developed protein bodies within the scutellar cells. The large air spaces formed may be involved in the isolation of scutellar cells to allow the formation of embryogenic callus.
INTRODUCTION

The process of zygotic embryo formation in maize has been described by light microscopy (1,2) and by transmission electron microscopy (3,4,5). It has only been in recent years that somatic embryogenesis has been observed in maize explants (6,7). Vasil et al. (8) described the development of somatic embryos at the node of the scutellum and in its basal region in close proximity to the root meristem and the procambium of the embryo axis when the embryos were cultured on a medium containing 6% sucrose. Embryos cultured on a medium containing 2% sucrose produced globular embryoids arising from the epidermal and subepidermal layers of cells (8). McCain and Hodges (9) observed the presence of broad based meristematic regions that organized to form the somatic embryos. Both of these studies implicate a subepidermal origin for the somatic embryo with a large mass of cells organizing to form the embryo.

Somatic embryos arising from a single scutellar cell in maize cultures have also been described. Kuo and Lu (10) observed cells at the surface of the callus derived from microspores that possessed denser cytoplasm and a larger nucleus than the other cells of the callus. These cells exhibited a pattern of development similar to that observed in zygotic embryo development.
Genotypic differences have been observed in the ability of maize inbred lines to undergo somatic embryogenesis (11). The formation of two types of embryogenic callus have been observed from immature embryos of maize lines. Type I callus is firm, compact, translucent, and relatively slow-growing under the proper culture conditions. Rapidly growing callus that is a highly friable with well-formed somatic embryos is designated type II (12),(Appendix A).

In this study, the cellular changes that occur during the initial culture period of immature embryos are compared for three different genotypes. The embryos of each genotype give a high frequency of either a type I response, type II response, or do not form organized callus under the appropriate culture conditions. The morphological changes occurring in each response were compared to determine the cellular origin of each response. An understanding of the development of each type of callus formed may provide insight as to how culturing methods could be manipulated in order to increase the frequency of a particular type of response.
Ears from three lines of maize, H99 and two \(F_5\) lines of the cross G35 × B73 were surface sterilized in a 20% sodium hypochlorite solution containing three drops of Tween 20 for 10 minutes and were rinsed three times in sterile water. The crown of the kernel was cut off, and immature embryos, 1-2 mm in length, were dissected out of the kernel. The embryos were placed with the embryo axis in contact with the media surface. Immature embryos from H99 were cultured on medium described by Duncan et al. (13) containing 1.4 g/L proline and 3.3 mg/L dicamba. Immature embryos from the two \((G35 \times B73)_5\) hybrid lines were cultured on a modified N6 containing 1.4 g/L proline and 0.75 mg/L 2,4-D. Some of the embryos of the \((G35 \times B73)_5\) hybrid were also cultured on a modified N6 medium that did not contain 2,4-D, but did contain 1.4 g/L proline. At various times during the culture period, embryos were fixed in 4% paraformaldehyde in 0.05 M cacodylate buffer, pH 7.2 if the embryos were to be used for both cytochemistry and general staining, or in formalin-acetic acid-ethanol if they were to be used only for general staining, for 24 to 48 hours. The sections were dehydrated through a graded ethanol and xylene series, infiltrated and embedded in Paraplast (melting point 56°C), or the embryos were fixed in 3% glutaraldehyde in 0.05 M cacodylate buffer,
pH 7.2 for two hours, washed three times in buffer for twenty
minutes each wash, post-fixed in 1% osmium tetroxide in 0.05
M cacodylate buffer for 1½-2 hours, dehydrated in a graded
acetone series and embedded in Medcast resin. Seven-
micrometer thick paraffin sections were cut, mounted and
stained with hemalum (14). One-half micrometer thick resin
sections were cut and stained with 1% toluidine blue O with
1% borax for 30 minutes at room temperature (14). An
additional 10-30 embryos from each ear were cultured for two
weeks. At the end of this time the embryos were examined and
classified as giving a type I or type II embryogenic
response, or not eliciting an embryogenic response. Only
embryos from ears in which 30% or greater of the cultured
embryos produced type I callus in H99, and type II callus in
the responsive (G35 X B73)s line were sectioned and examined
for this work.

For protein staining of paraffin sections, consecutive
sections were mounted on three separate slides. The slides
were then deparaffinized in xylene, coated with 0.4% formvar
in dichloroethane, and hydrated through a graded ethanol
series. One slide was incubated in either 0.4% pepsin or
0.4% trypsin in 0.02 N HCl at 37°C for 48 hours. A second
slide was incubated in 0.02 N HCl at 37°C for 48 hours. A
third slide was incubated in distilled water at 37°C for 48
hours. All three slides were then placed in 1% aniline blue
black in 7% acetic acid for 10 minutes at 58°C as described by Fisher (15). Slides were dehydrated to xylene and mounted with Permount.

Nucleic acid staining was done using Azure B following a method described by Jensen (16). Consecutive sections were mounted on four separate slides and rehydrated to H₂O. Slide one was placed in 1 N perchloric acid at 4°C for 24 hours to remove the RNA. Slide two was placed in 0.5 N perchloric acid at 70°C for 30 minutes to remove the DNA. Slide three was treated by both of these methods to remove both RNA and DNA. Slide four was placed in distilled water during this time. After these treatments, all four slides were placed in 0.025% Azure B in 0.5 M citrate, pH 4 at 55°C for 30 minutes. The slides were then rinsed in running tap water for five minutes, blotted dry, and placed in tert-butyl alcohol for ten minutes. Slides were then placed in xylene and mounted.

Carbohydrate staining was done using the periodic acid-Schiff's reaction (PAS) described by Jensen (16). Consecutive sections were mounted on four separate slides and rehydrated to H₂O. Slide one was placed in a sterile coplin jar and covered with a filter-sterilized solution of 0.5% α-amylase in phosphate buffered saline 37°C for 48 hours. Slide two was placed in phosphate buffered saline 37°C for 48 hours. Slide three and four were placed in water for 48 hours. Slides one, two, and three were placed in 0.5%
periodic acid for 20 minutes at room temperature. All four slides were washed in running water for ten minutes and placed in Schiff's reagent at 4°C for 15 minutes. The slides were rinsed in tap water, placed in 2% sodium bisulfite for 2 minutes, washed in tap water for ten minutes, dehydrated to xylene and mounted.
RESULTS

Somatic embryogenesis in immature embryos of *Zea mays* was examined in the three genotypes, each of which was known to have a high frequency of a particular type of response under the proper culture conditions. Sixty-five percent of the H99 embryos cultured formed type I embryogenic callus. The remainder of the embryos did not produce an organized callus. Thirty-five to sixty percent of the responsive (G35 X B73)₅ embryos cultured in the presence of 2,4-D produced a type II embryogenic response (Table 1). None of the nonresponsive (G35 X B73)₅ embryos produced either type I or type II callus. Embryos cultured on medium that did not contain 2,4-D did not produce callus, but exhibited precocious germination or a swelling and hardening of the scutellum.

At the time of removal from the kernel, the morphology of the embryos from each genotype was identical (Figures 1-3). The scutellum was comprised of large irregularly shaped parenchyma cells, and a distinct epithelial layer was observed over the entire surface of the scutellum.

By the fourth day in culture on medium containing an auxin source, the epidermal layer of the responsive (G35 X B73)₅ embryos contained cells in which each cell possessed a large nucleus (Figure 4). At this time, cells of
Table 1. Response of H99 Ears

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<th>TYPE II</th>
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Table 2. (G35 X B73)₅ Embryos Cultured for Fourteen Days

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Plate I  Day zero immature embryos, approximately 1.5 mm in length.

Figure 1.  H99 embryo.  Bar equals 0.1 mm.

Figure 2.  Responsive (G35 X B73)₅ embryo.
Bar equals 0.2 mm.

Figure 3.  Nonresponsive (G35 X B73)₅ embryo.
Bar equals 0.1 mm.
Plate II

Figure 4. Responsive (G35 X B73)_5 embryo four days after culturing. Arrow points to large epidermal cell possessing a large nucleus. Bar equals 0.02 mm.

Figure 5. Resin section of a responsive (G35 X B73)_5 embryo five days after culturing. Bar equals 0.02 mm.

Figure 6. H99 embryo five days after culturing. Coleorhizal end of the embryo is to the right. Bar equals 0.5 mm.

Figure 7. Coleorhizal end of the scutellum of an H99 embryo five days after culturing. Bar equals 0.05 mm.

Figure 8. Coleorhizal end of the scutellum of an H99 embryo six days after culturing. Bar equals 0.05 mm.
the scutellum close to the zygotic embryo began to deteriorate, resulting in a separation of the epithelium and subepidermal layers of the scutellum from the remainder of the embryo.

By the fifth day in culture, the responsive (G35 X B73)_5 embryos had small, distinct groups of cells in the epithelial layer (Figure 5). These groups, consisting of six to eight cells, exhibited a polarity of the cells within the group. Smaller, more cytoplasmic cells were observed at the surface of the scutellum, whereas larger, more vacuolate cells were closer to the subepidermal cells of the scutellum.

The epithelial layer of the H99 embryos stained intensely and showed no evidence of vacuolation by the fifth day in culture (Figures 6, 7). The scutellar cells directly beneath these cells also stained more intensely, and many mitotic nuclei were observed in this region. The nonresponsive (G35 X B73)_5 embryos did not contain any meristematic regions within the scutellum. Cells in both the epidermal and subepidermal layers of the nonresponsive embryos appeared swollen and vacuolate.

By the sixth day in culture, the H99 embryos showed broad-based areas of meristematic cells (Figure 8). These meristematic regions appeared to be primarily in the subepidermal regions. The appearance of these meristematic regions corresponded with the appearance of type I callus in
this genotype; 65% of the embryos from this genotype formed type I callus with 75% of the embryos sectioned demonstrating densely meristematic regions by day six.

The responsive (G35 X B73)_5 embryos exhibited distinct groups of meristematic cells that appeared to be isolated from the other cells of the scutellum (Figure 13). These cells were located at the surface of the scutellum, but a portion of the group extended into the subepidermal region. Of the two ears used for day six embryos, 60% of the embryos produced type II callus. The isolated groups of cells were observed in 70% of the embryos sectioned. This correspondence suggests that the type II callus is derived from these isolated cell clusters.

The air spaces separating the epidermal and subepidermal layers from the remainder of the embryo became more pronounced by the sixth day of culture in all three genotypes (Figures 9-11). The formation of the air spaces was observed in almost all of the embryos cultured in the presence of an auxin, including embryos that appeared to be nonresponsive. Embryos cultured in the absence of an auxin source did not develop this separation of the epidermal and subepidermal layers (Figure 12).

By the seventh day in culture, areas of the scutellum of the responsive (G35 X B73)_5 line developed several of these groups of isolated cell clusters (Figure 14). In addition,
Plate III  Embryos cultured in the presence of an auxin source for six days. Coleorhizal end is to the left. Bars equal 0.5 mm.

Figure 9.  H99 embryo.

Figure 10.  Responsive (G35 X B73)5 embryo.

Figure 11.  Nonresponsive (G35 X B73)5 embryo.
Plate IV

Figure 12. (G35 X B73)$_5$ embryo cultured in the absence of an auxin source. Bar equals 0.2 mm.
Plate V

Figure 13. Responsive (G35 X B73)$_5$ embryo six days after culturing. Bar equals 0.05 mm.

Figure 14. Responsive (G35 X B73)$_5$ embryo seven days after culturing. Bar equals 0.1 mm.

Figure 15. Responsive (G35 X B73)$_5$ embryo seven days after culturing showing globular stage somatic embryo. Bar equals 0.05 mm.

Figure 16. Responsive (G35 X B73)$_5$ embryo eleven days after culturing showing globular stage somatic embryos. Bar equals 0.1 mm.

Figure 17. Responsive (G35 X B73)$_5$ embryo fourteen days after culturing showing globular stage somatic embryos. Bar equals 0.5 mm.

Figure 18. Responsive (G35 X B73)$_5$ embryo fourteen days after culturing. Bar equals 0.1 mm.
globular stage somatic embryos could be observed emerging from the surface of the scutellum (Figure 15). By day eleven of culture, groups of globular stage somatic embryos were found at the coleorhizal end of the embryo on the surface of the scutellum (Figure 16). Clusters of globular stage somatic embryos were present at fourteen days after culturing (Figure 17). All of the embryos sectioned that demonstrated globular stage somatic embryos on the scutellar surface also contained large areas of several small isolated cell groups on the surface of the newly formed callus (Figure 18).

Embryos cultured in the absence of 2,4-D accumulated structures within the scutellar cells presumed to be protein bodies because of the readily apparent staining with 1% aniline blue black in 7% acetic acid (Figures 20, 22). These structures were apparent by the sixth day of culture, and increased in size with increasing time in culture (Figure 26). However, treatment with trypsin or pepsin failed to significantly decrease the extent of staining within these putative protein bodies (Figure 24). These enzymes did reduce the staining present in the cytoplasm of the embryos examined. Embryos cultured in the presence of 2,4-D did not develop these protein bodies (Figures 19, 21, 23, 25).

Cytochemical staining procedures for carbohydrates and for nucleic acids did not reveal any observable differences between the embryos of the three genotypes.
Plate VI  Protein staining of responsive (G35 X B73)5 embryos. Bars equal 0.05 mm.

Figures 19, 21, 23. Embryos cultured in the presence of 2,4-D for six days.

Figures 20, 22, 24. Embryos cultured in the absence of 2,4-D for six days.

Figures 19, 20. Embryos incubated in distilled water at 37°C for 48 hours prior to staining.

Figures 21, 22. Embryos incubated in 0.02 N HCl at 37°C for 48 hours prior to staining.

Figures 23, 24. Embryos incubated in 0.4% pepsin in 0.02 N HCl at 37°C for 48 hours prior to staining.

Figure 25. Embryo cultured in the presence of 2,4-D for eleven days.

Figure 26. Embryo cultured in the absence of 2,4-D for eleven days.
DISCUSSION

The results of this study have demonstrated that type II callus arises from small clusters of cells located at the scutellar surface. Single cells containing large nuclei were observed on the scutellar surface of embryos producing primarily a type II response. These cells were similar in appearance to those observed by Kuo and Lu (10) on the surface of maize microspore cultures. These cells may be the precursors to the isolated cell clusters that were seen in the later stages of cell culture. Distinct cell clusters were located primarily at the scutellar surface. The subepidermal layer consisted primarily of vacuolate cells. The presence of meristematic cells in the epithelium coupled with the absence of meristematic cells in the subepidermal layer suggests that type II embryogenic callus originates from the epidermal layer. Isolated cell groups were observed in a high frequency of embryos that exhibited a similar high frequency of type II callus formation.

This mechanism represents a second pathway, different from the pathway described by Vasil et al. (8) and McCain and Hodges (9), by which somatic embryogenesis can occur in the immature embryos of Zea mays. Somatic embryos developing from the epithelial cells become isolated from the rest of the scutellar cells and begin developing in a pattern similar
to that observed in zygotic embryo development. Polarity is established early within the group of cells, with those cells closest to the scutellum becoming vacuolate and forming a suspensor, and the cells at the surface becoming cytoplasmic to form a globular embryo. Single cell origins of somatic embryos from immature embryos in other species of Gramineae have been reported (17, 18). Jones and Rost (18) observed that single cells of the epithelium underwent divisions resulting in the formation of a proembryo similar to that seen in zygotic embryo development.

The H99 genotype developed an intensely staining epidermal layer followed by the development of a large area of meristematic cells in the subepidermal region. The development of a intensely staining, cytoplasmic epithelial cell layer upon culturing of immature embryos of Zea mays was previously described by Springer et al. (7). The initial meristematic activity of callus formation in these cultures occurred in the epithelium and subepithelial layers of the scutellum. Vasil et al. (8) also reported the development of zones of meristematic cells that would then further organize into groups of embryogenic cells. McCain and Hodges (9) observed similar broad-based meristematic regions, which they interpreted as evidence for the multicellular origin of somatic embryos. Somatic embryogenesis derived from the subepidermal layers of the scutellum appear to be
multicellular in origin, resulting from a generalized meristematic region, which then organizes to form embryoids.

Although the differences in the response of the responsive (G35 X B73)₆ and the H99 may be due in part to differences in environmental conditions, specifically a different auxin source, the genotypic differences exert a stronger effect. Previous results from this laboratory (Tomes, unpublished results) demonstrate that H99 embryos cultured with 2,4-D as the only auxin source also produce primarily a type I response.

The results reported in this study also demonstrate the separation of the epidermal and subepidermal layers from the remainder of the zygotic embryo. This separation developed as a result of the deterioration of the interior scutellar cells and was seen in almost all (greater than 95%) of the embryos (from all of the genotypes) cultured in the presence of an auxin. This separation is not observed in embryos cultured in the absence of an auxin and so must be caused, at least in part, to the presence of the auxin. The separation of the epidermal and subepidermal layers from the remainder of the scutellar cells was also reported by Vasil et al. (8). The isolation of these cell layers may play an important role in eliciting an embryogenic response.

The embryos cultured in the absence of 2,4-D developed what appeared to be protein bodies within the scutellar
cells, although incubation with pepsin failed to extract them. If these structures are protein bodies, it is not clear what prevented the loss of staining intensity upon treatment with the proteases. These protein bodies may simply be deposits of protein which appear as denatured material to the proteases and cannot be digested. The development of protein bodies in the scutellar cells of embryos cultured in the absence of an auxin source indicates that these cells are differentiating to form mature scutellar cells.

The absence of differences between genotypes in staining for carbohydrates or nucleic acids does not mean that these differences are not there. It does imply that if there are differences in the nucleic acid content of the cells of the embryo, that these differences are qualitative and not quantitative.
REFERENCES


PART II.

COMPARISON OF GROWTH CHARACTERISTICS OF TWO

EMBRYOGENIC MAIZE SUSPENSION CULTURES
COMPARISON OF GROWTH CHARACTERISTICS OF TWO EMBRYOGENIC MAIZE SUSPENSION CULTURES

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and M.C. ALBERTSENv

§Journal Paper No. J-12795 of the Iowa Agriculture and Home Economics Experiment Station. Project No. 2817. Supported in part by a grant from Pioneer Hi-Bred International, Inc.

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Histological and growth rate studies were carried out on embryogenic suspension cultures of two genotypes, B73 and B73<2G35. Suspensions were sampled at various times between zero and eight days after subculture, both before and after sieving at four days through a screen of 500-μm pore size. Suspension cells were either fixed and embedded in paraffin for light microscopy or plated on solid medium for growth rate studies. Samples taken from the B73<2G35 suspension culture showed a consistent, high growth rate both before and after sieving. Cell clusters consisted of small groups of meristematic cells in which divisions occurred primarily at the surface. This culture exhibited a strong negative correlation between the average size of the cell clusters and the mitotic index. The growth rate of the B73 culture was generally lower than that of the B73<2G35 culture and was further reduced after sieving. The B73 suspension culture contained large groups of cells in which divisions occurred more often in the innermost cell layers. This culture exhibited a positive correlation between the average size of the cell clusters and the mitotic index. The significance of these growth characteristics for the use of such suspension cultures in selection and transformation studies is discussed.
INTRODUCTION

Embryogenic maize suspension cultures are potentially suitable material for use in in vitro selection and/or transformation studies. Such suspensions consist of relatively small groups of cells, which can be more uniformly exposed to selective agents in the liquid culture medium or to transformation vectors. In addition, they have the ability to regenerate plants (1). The distribution of the dividing cells within the cell groups may be important in determining the value of such suspensions for selection and transformation studies. Dividing cells near the surface will be more accessible, whereas internal cells will be less accessible for selection and transformation procedures. We have therefore carried out histological and growth rate studies with two maize suspension lines, over a period of eight days after subculture, to investigate the relationship between morphological features and growth characteristics.

Sieving suspension cultures provides a means of decreasing cluster size and, hence, increasing the proportion of exposed cells on the surface of the cluster. However, sieving may also have deleterious effects, either due to mechanical damage caused to the cell groups or because a certain minimum cluster size may be necessary for optimal growth. The effect of sieving suspension material through
screens of 500-μm pore size has, therefore, also been examined in this study.
MATERIALS AND METHODS

Two embryogenic maize suspension cultures of the genotypes (G35 X B73) X B73 (designated B73<2G35) and B73 were used in this study. Both were derived from 'type II' embryogenic callus originating directly from cultured immature embryos (2). At the time of the experiments, the B73 line had been in suspension for 7-10 months and, the B73<2G35 line, for 5-8 months. The callus lines from which the suspensions originated had been initiated approximately 6 months (B73) and 10 months (B73<2G35) before transfer to liquid culture.

Suspensions were grown in a liquid medium consisting of Murashige and Skoog (3) salts and vitamins, sucrose (30 g/L), and 2,4-D (2 mg/L) (pH 5.8 before autoclaving). Cultures were maintained as 60-ml aliquots in 250-ml baffled flasks and were incubated at 27°C in the dark on a gyrotary shaker (100 rpm). Suspensions were subcultured at 3- to 4-day intervals at 1:1 or 1:2 dilutions, dependent on the growth of the cells. At each subculture, the cell groups were first washed twice by removing and replacing 30 ml of the culture medium.

Two separate experiments were carried out, each beginning at the time of subculture (Day 0). Cells were inoculated into culture medium at 10% cell volume. After
four days, the cultures were sieved through a screen of 500-μm pore size by using a foam sponge, and the cells were placed in fresh culture medium at 10% cell volume. In the first experiment, samples were taken at day 1, day 4 (presieving and postsieving), and day 6. In the second experiment, samples were taken immediately after subculture (Day 0), and at days 2, 4 (presieving and postsieving), 6, and 8 (2 and 4 days after sieving). These samples were fixed in formalin-acetic acid-ethanol for 24 hr, dehydrated in an ethanol series, embedded in paraffin, and sectioned at 7 μm. Sections were stained with hemalum according to the method described by Berlyn and Miksche (4).

The number of mitotic figures and the total number of cell nuclei per cell cluster were determined from the fiftieth and seventy-fifth sections for each time point sampled. The location of the mitotic figures within the cell cluster was also noted. Mitotic figures that were in the first or second cell layers of the cell cluster were considered to be surface cells. The mitotic index and average cell cluster size were calculated as:

\[
\text{Mitotic Index} = \frac{\# \text{ Mitotic Cells}}{\text{Total \# Nuclei}} \times 100
\]

\[
\text{Average Cell Cluster Size} = \frac{\text{Total \# Nuclei Counted}}{\# \text{ Cell Clusters}}
\]
Average cell cluster size represents the average number of nuclei through a single section of the slide and does not represent the total number of cells in a cluster. However, an average of these values provides a relative value for the size of the cell clusters within the suspension.

In the second experiment, additional samples were taken to measure the growth rate of the cultures on solid medium. At each sampling time, ten replicates of 150 mg of cells were placed on teflon discs on the top of solid medium. The weight of the cells supported by each disc was recorded daily for 14 days.
RESULTS

The general appearances of the two cultures were different. The B73<2G35 suspension culture consisted of relatively small, uniformly sized cell clusters (Figure 1). The B73 suspension contained cell groups of a greater range of sizes (Figure 2), the majority of which were much larger than those seen in the B73<2G35 culture. The paraffin sections of the B73<2G35 culture revealed several small ribbons of cells in addition to the solid clusters of cells (Figure 3). This culture morphology is similar to that observed in other maize cultures (1). Although the B73 culture also had ribbons of cells, their frequency was much lower (Figure 4). At each sample time before sieving, the average cell cluster size of the B73 culture was larger than that observed for the B73<2G35 culture (Table 1).

The mitotic index of the B73<2G35 culture decreased with time in culture before sieving. After sieving, the mitotic index increased to a value that was equal to or higher than that observed early in the culture period before sieving.

The mitotic index of B73 culture increased from day 1 to day 4 in Experiment 1. Two days after sieving (day 6), the mitotic index showed a sharp decrease. In Experiment 2, the mitotic index was never as high as in Experiment 1, and sieving did not have as great an effect on mitotic index as
Figures 1-4  Morphology of two maize suspension cultures. (Bars equal 1 mm).

Figure 1. Fresh mount of the B73<2G35 suspension culture.

Figure 2. Fresh mount of the B73 suspension culture.

Figure 3. Paraffin section of B73<2G35 culture. Micrograph shows cell suspension during Experiment 1, day 4 presieving sample. Arrow points to a "ribbon" of cells.

Figure 4. Paraffin section of B73 culture. Micrograph shows cell suspension during Experiment 1, day 4 presieving sample.
Table 1. Average Cell Cluster Size and Mitotic Index of B73<2G35 and B73 Suspension Cultures

<table>
<thead>
<tr>
<th>DAY</th>
<th>CELL LINE</th>
<th>AVERAGE CLUSTER SIZE</th>
<th>MITOTIC INDEX</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>EXPERIMENT 1</td>
<td></td>
</tr>
<tr>
<td>ONE</td>
<td>B73&lt;2G35</td>
<td>32.9</td>
<td>1.45</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>50.7</td>
<td>0.49</td>
</tr>
<tr>
<td>FOUR</td>
<td>B73&lt;2G35</td>
<td>34.5</td>
<td>1.02</td>
</tr>
<tr>
<td></td>
<td>Presieving B73</td>
<td>74.3</td>
<td>1.33</td>
</tr>
<tr>
<td>FOUR</td>
<td>B73&lt;2G35</td>
<td>20.0</td>
<td>1.06</td>
</tr>
<tr>
<td></td>
<td>Postsieving B73</td>
<td>30.1</td>
<td>1.30</td>
</tr>
<tr>
<td>SIX</td>
<td>B73&lt;2G35</td>
<td>21.5</td>
<td>1.43</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>29.4</td>
<td>0.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EXPERIMENT 2</td>
<td></td>
</tr>
<tr>
<td>ZERO</td>
<td>B73&lt;2G35</td>
<td>21.6</td>
<td>1.88</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>38.8</td>
<td>0.50</td>
</tr>
<tr>
<td>TWO</td>
<td>B73&lt;2G35</td>
<td>27.7</td>
<td>1.13</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>28.9</td>
<td>0.76</td>
</tr>
<tr>
<td>FOUR</td>
<td>B73&lt;2G35</td>
<td>29.2</td>
<td>0.72</td>
</tr>
<tr>
<td></td>
<td>Presieving B73</td>
<td>32.8</td>
<td>0.56</td>
</tr>
<tr>
<td>FOUR</td>
<td>B73&lt;2G35</td>
<td>17.0</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>Postsieving B73</td>
<td>17.3</td>
<td>0.82</td>
</tr>
<tr>
<td>SIX</td>
<td>B73&lt;2G35</td>
<td>15.9</td>
<td>2.07</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>23.4</td>
<td>0.26</td>
</tr>
<tr>
<td>EIGHT</td>
<td>B73&lt;2G35</td>
<td>17.4</td>
<td>2.18</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>26.9</td>
<td>0.44</td>
</tr>
</tbody>
</table>
in Experiment 1. The size of the average cell cluster of the B73 culture was also larger in the first experiment than in the second experiment. The mitotic index fluctuated during Experiment 2, but was at the lowest level at day 6 (2 days postsieving). By day eight, the mitotic index had increased, suggesting that the B73 culture had begun to recover from the effects of sieving.

An inverse relationship between the mitotic index and the average cell cluster size was observed for B73<2G35, and linear regression analysis revealed a correlation coefficient of -0.81 for this relationship, as shown in Figure 5. Linear regression analysis revealed a positive correlation coefficient of 0.80 between average cell cluster size and mitotic index for the B73 line (Figure 6).

The B73 culture contained a higher percentage of mitotic figures in the interior of the cell cluster (Table 2), whereas almost all the mitotic figures observed in the B73<2G35 culture could be found on the exterior surface of the cell clusters. This is partly because that, with a smaller cell cluster size, the B73<2G35 culture has a higher percentage of cells on the surface.

The plated cells of the B73<2G35 culture grew at a comparable rate when plated on solid media at all times during the culture period, both before and after sieving (Figure 7). The B73 culture did not grow as rapidly as the
B73<2G35

$r = -0.81$

Figure 5. Correlation of mitotic index and average cell cluster size of B73<2G35.
Figure 6. Correlation of mitotic index and average cell cluster size of B73.
Table 2. Number and Location of Mitotic Cells Within the Clusters of B73 and B73<2G35 Suspension Cultures

<table>
<thead>
<tr>
<th>DAY</th>
<th>CELL LINE</th>
<th>TOTAL # NUCLEI CELLS</th>
<th># CELL CLUSTERS</th>
<th>% INTERNAL MITOTIC CELLS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>B73&lt;2G35</td>
<td>3942</td>
<td>57 (0)</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>6083</td>
<td>30 (13)</td>
<td>120</td>
</tr>
<tr>
<td>4 Pre-</td>
<td>B73&lt;2G35</td>
<td>4137</td>
<td>42 (0)</td>
<td>120</td>
</tr>
<tr>
<td>sieving B73</td>
<td></td>
<td>7877</td>
<td>105 (24)</td>
<td>106</td>
</tr>
<tr>
<td>4 Post-</td>
<td>B73&lt;2G35</td>
<td>2082</td>
<td>22 (3)</td>
<td>104</td>
</tr>
<tr>
<td>sieving B73</td>
<td></td>
<td>3513</td>
<td>47 (7)</td>
<td>120</td>
</tr>
<tr>
<td>6</td>
<td>B73&lt;2G35</td>
<td>2575</td>
<td>37 (2)</td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>B73</td>
<td>3524</td>
<td>3 (0)</td>
<td>120</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>4 Pre-</td>
</tr>
<tr>
<td>sieving B73</td>
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<tr>
<td>4 Post-</td>
</tr>
<tr>
<td>sieving B73</td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>8</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

The number in parentheses is the number of these cells in the interior of the cell cluster.
B73<2G35 culture before sieving (Figure 8), and after sieving, the growth rate showed a dramatic decrease.
Figure 7. Growth curve of B73<2G35 suspension culture plated on solid medium.
Figure 8. Growth curve of B73 suspension culture plated on solid medium.
DISCUSSION

The suspension culture environment is a useful one for the study and utilization of plant cells *in vitro*. Cell cluster size and the location of mitotic cells are factors that are relevant to the use of suspension cultures in transformation or selection studies. A small cluster size and the presence of actively dividing cells at the surface increases the proportion of dividing cells that are in direct contact with the culture medium and are accessible to selective or transforming agents. A high percentage of dividing cells at the surface allows more possibilities for the introduction of foreign DNA into these cells by electroporation (5), microinjection (6), the use of a particle gun (7), or transformation by *Agrobacterium* (8). Maize suspension cultures may also be used as a source of protoplasts (9,10). A culture morphology of small cell clusters with dividing cells near the surface should result in more ready release of mitotic cells during protoplast isolation, and these cells may show a greater ability to divide upon culture.

In the present study, the two suspension phenotypes differed in the proportion of cells that were at the surface of the clusters and in direct contact with the culture media. The B73 cell line averaged a greater number of cells in each
cluster and therefore a lesser proportion of cells in contact with the culture medium. The B73<2G35 cell line averaged a smaller number of cells in each cell cluster and exhibited more ribbon-like cell groups. These morphological features result in a greater proportion of the cells in this suspension that are in direct contact with the culture media. This may be a contributing factor in the findings that the B73<2G35 culture had a higher mitotic index than the B73 culture and that a higher percentage of the dividing cells were located at the surface of the cell cluster. In soybean suspension cultures of very small cell cluster size, it has been reported that the cells found in the center of a cluster, which do not have direct contact with the culture medium, cease to divide and enter a quiescent stage (11). The authors of that study believe that this growth characteristic may be due to the early differentiation of the cells when they become completely surrounded by other cells in the cluster.

Recently, our laboratories have observed B73 suspension cultures that exhibited the same culture phenotype as the B73<2G35 culture described in this paper and a B73<2G35 culture that reverted to a phenotype similar to that observed in the B73 culture. These observations and the fact that the two maize cultures are genetically similar (pedigree relationship approximately 75%) suggest that the differences
observed between the two cultures are developmental rather than genetic differences or differences due to hybrid vigor. It is unlikely that genotype alone accounts for the differences observed in culture phenotype.

The correlation of the mitotic index with the average cell cluster size reveals that smaller cell cluster sizes in the B73<2G35 line result in a higher mitotic rate in the suspension culture. Inasmuch as the B73<2G35 culture grows as smaller cell clusters, passing these small clusters through a 500-μm sieve would be expected to do less damage to them than to the larger clusters of the B73 culture. This would allow the small cluster size to be maintained within the culture. After sieving, the mitotic rate of the B73<2G35 culture increased to the previous level or a higher rate. The location of small groups of dividing cells near the surface of the clusters of the B73<2G35 material may result in them being less disrupted by sieving.

The correlation of mitotic index with cell cluster size in the B73 cell line reveals that this culture achieves a higher mitotic rate as the size of the cell cluster increases. In the B73 line, sieving may result in more disruption of the larger cell clusters and cause greater damage to meristematic regions in the interior. This could result in more adverse effects on growth because many of the dividing cells are in this location.
The culture phenotype of the B73<2G35 cell line has characteristics that make it useful for experimental purposes. It is a rapidly growing culture, the cell cluster size is smaller, and the dividing cells are on the surface of the cell cluster. The growth rate of the culture is unaffected by sieving the cells so that small cell cluster size can be maintained. Selection of an appropriate culture morphology may prove an important step in the use of suspension cultures in transformation, selection, and regeneration studies.
REFERENCES


SUMMARY

The application of tissue culture techniques provides a mechanism to systematically examine somatic embryogenesis. Understanding the process of somatic embryogenesis may prove useful for the improvement of techniques utilized in the regeneration of plants.

Immature maize embryos about 1.5 mm in length can be induced to form embryogenic callus by culturing them in the presence of an auxin. This can result in the formation of two types of embryogenic callus. Type I callus is firm, compact, translucent, and relatively slow-growing, whereas type II callus is rapidly growing and highly friable with well-formed somatic embryos. Type I callus has been suggested to represent organogenesis. Type II callus, in contrast, is considered embryogenic.

Previous investigations of somatic embryogenesis have provided evidence that meristematic tissue originates within the subepidermal layer (25, 26). The development of the somatic embryo was proposed to arise from a region of meristematic cells that organizes to form a somatic embryo, and not from a single cell. A single cell origin for somatic embryos in maize microspore cell cultures, however, has been reported in one study (16). It has also been reported in immature embryo cultures of two other Gramineae species (53,
The present work examined callus formation from cultures of three corn lines with different genotypes. Each of the genotypes responded differently to the culture conditions.

Immature embryos of line H99 generated type I callus. Light microscopic analysis revealed a subepidermal, multicellular, origin for the embryogenic type I callus. A large mass of meristematic cells was observed. These observations are similar to those previously reported in the literature for somatic embryogenesis in maize (25, 26, 27).

In contrast, analysis of responsive (G35 X B73)$^5$ hybrid revealed the presence of single cells containing large nuclei in the epithelial layer of the scutellum. Small groups (6-8 cells) of meristematic cells could be found at the surface of the scutellum by the fifth day of culture. These observations are similar to those made in microspore-derived callus of maize (16). This observation suggests that embryos derived from the epithelial layer originate from a single embryogenic cell or a very small group of cells.

These studies suggest that two mechanisms exist for the generation of somatic embryos from immature embryos of Zea mays. Further, the initial pathway for the formation of embryogenic callus may predetermine the subsequent type of callus produced. Immature embryos from the responsive (G35 X B73)$^5$ produced primarily a type I embryogenic
response. The presence of only a small number of isolated meristematic cells found in the epidermal layer in this line suggests a single cell origin for somatic embryos originating from the epithelial cells. Type I embryogenic callus formation in cultures of corn line H99 seems to be consistent with the previously reported findings. The large mass of meristematic cells in the subepidermal layers observed in these embryos is consistent with a multicellular origin of somatic embryos developing from this region.

Two factors are primarily responsible for eliciting an embryogenic response from an immature maize embryo. Environmental factors, specifically the auxin sources, are important in influencing the type of embryogenic callus produced by an immature embryo. There is also a strong relationship between the genotype of the maize line and the frequency that a specific embryogenic response is observed. Immature embryos from the H99 line have a high frequency of a type I response and a very low frequency of a type II response, even when cultured on medium containing 2,4-D as an auxin source.

A separation of the epidermal and subepidermal layers from the remainder of the scutellum was evident after four days in culture in all of the genotypes cultured in the presence of an auxin. The isolation of the scutellar cells from the remainder of the zygotic embryo may be a necessary
step in the formation of embryogenic callus.

Embryos cultured from both the responsive and nonresponsive (G35 X B73)$_5$ lines in the absence of an auxin accumulated structures presumed to be protein bodies because of the readily apparent staining with 1% aniline blue black in 7% acetic acid. However, treatment with trypsin or pepsin failed to significantly decrease the extent of labelling within these putative protein bodies. These enzymes did reduce the staining present in the cytoplasm of the embryos examined. If these structures are protein bodies, it is not clear what prevented the loss of staining intensity upon treatment with the proteases. These protein bodies may simply be deposits of protein which appear as denatured material to the proteases and cannot be digested. The development of these protein bodies suggests that in the absence of an auxin source, the cells of the scutellum begin to differentiate into mature scutellar cells.

Friable embryogenic callus derived from immature maize embryos can be utilized to produce embryogenic suspension cultures. The growth characteristics of these rapidly growing, embryogenic cultures are relevant to their use for in vitro selection and/or transformation. Since embryogenic suspension cultures are composed of small clusters of cells, they allow selective agents and exogenous DNA much more access to a greater proportion of the cultured cells. For
suspension cultures to be utilized for their maximum application, it is desirable to be able to obtain plants from them. In order to optimize the regeneration of maize plants from cultured cells, it is necessary to obtain a basic knowledge of the growth patterns of the cultured cells.

Suspension cultures of Zea mays somatic embryos have also been shown to be strongly influenced by the environment of the corn line examined. Suspension cultures of a B73 maize line reveal a relationship between the cell cluster size and the mitotic index of the culture. There is, seemingly, a positive influence on the mitotic index by increasing cluster size. Suspension cultures of a B73<2G35 line demonstrate an opposite effect. An inverse relationship between cluster size and mitotic index is observed. Cell clusters for this hybrid consisted of small groups of meristematic cells in which divisions occurred primarily at the surface. In contrast, the B73 suspension culture contained large groups of cells in which divisions occurred more often in the innermost cell layers.

These and similar corn lines may provide a basis for the development of suspension cultures with well-defined growth properties. The generation of suspension cultures with desirable, well-defined growth properties may prove invaluable for the potential use of these culture in in vitro selection and/or transformation studies.
REFERENCES


Scanning electron micrographs showing development of type II callus.

Figure 1, 2. Day zero embryo.
Bar equals 0.2 mm.

Figure 3. Day two embryo.
Bar equals 0.2 mm.

Figure 4. Immature embryo after eight days in culture in the presence of 2,4-D.
Bar equals 0.5 mm.

Figure 5. Immature embryo after eleven days in culture in the presence of 2,4-D.
Bar equals 0.5 mm.

Figure 6. Immature embryo after fourteen days in culture in the presence of 2,4-D.
Bar equals 0.5 mm.
APPENDIX B
Figure 1. Type I embryogenic response from an embryo cultured in the presence of 2,4-D. Bar equals 0.2 mm.

Figure 2. Type II embryogenic response from an embryo cultured in the presence of 2,4-D. Bar equals 0.2 mm.

Figure 3. Precocious germination of an embryo cultured in the absence of 2,4-D. Bar equals 0.5 mm.

Figure 4. Scutellar swelling in an embryo cultured in the absence of 2,4-D. Bar equals 0.5 mm.
Embryogenic maintenance callus exhibits genotypic differences in the ability to produce regenerated plantlets when the auxin source is removed. A study of the morphology and cytochemistry of three genotypically and phenotypically different corn lines was done to determine a possible biochemical basis for these observed variations.

Callus from a B73 line contained many somatic embryos, and regenerated well. Hemalum stained sections (Figure 1) revealed many cytoplasmic cells arranged in small, distinct groups at the surface of the callus. These groups of cells were usually either surrounded by vacuolate cells or isolated from the remainder of the callus cells. These groups of cells were similar in appearance to those observed in the responsive \((G35 \times B73)_5\) line described previously. Carbohydrate staining revealed a strand-like polysaccharide contained in the fluid-filled spaces of the callus. Fine starch grains could be seen throughout the callus. Certain cells and groups of cells at the callus surface stained very intensely for proteins. These areas often demonstrated a high concentration of RNA in the same cells, but this did not always occur.

Callus from 3377, a Pioneer hybrid, contained many somatic embryos, but the embryos did not regenerate well when
the 2,4-D was removed. The meristematic cells of this callus were organized in much larger groups, and well-defined globular embryos on suspensors could be seen (Figure 2). Carbohydrate staining revealed the presence of extremely fine starch grains. Some of the cells of the callus appeared to have a pink-stained cytoplasm, presumably due to the fine dispersal of starch grains. The smaller starch grains were found in the cells at the surface, and larger starch grains were found in the interior cells of the callus. Some strand-like material similar to that observed in the B73 callus was also observed. Staining of the callus with aniline blue black showed areas of high protein concentration. These areas appeared to be in distinct, small groups of cells, resembling those observed in the B73 cultures. RNA was also found in higher concentrations in localized groups.

Callus from a (G35 X B73) X B73 hybrid was similar in macroscopic appearance to the other two genotypes, but the callus regenerated slowly, if it regenerated at all. Paraffin sections revealed very few cytoplasmic cells on the surface of the callus, although a few embryo-like structures were observed (Figure 3). Cytochemical analyses for carbohydrates revealed almost no detectable starch in the callus, and little amorphous material present. Some distinct groups of cells could be seen using the stain for proteins. There was very little detectable difference in cells of
callus stained for nucleic acids.

Observations on maintenance callus are similar to those made on callus initiation in immature embryos. Distinct groups of cells are observed on the callus surface of all three genotypes. These cell groups are seen more frequently on callus that regenerates well. Callus that does not regenerate well was found to not have as many distinct cell groups or meristematic regions.

The callus cultures examined in this study had been in culture for different lengths of time, so it is difficult to ascertain if the observed differences are related to genotype, or if they are due to the age of the culture. The observations do suggest that the ability of a particular callus culture to regenerate plants is directly related to the quantity of small, isolated groups of cells.
Embryogenic maintenance callus stained with hemalum. Bar equals 0.1 mm.

Figure 1. B73 callus.

Figure 2. Pioneer brand 3377 callus.

Figure 3. (G35 X B73) X B73 callus.
ACKNOWLEDGMENTS

I would like to thank Dr. Darryll E. Outka for his encouragement during the onset of this project. His enthusiasm for electron microscopy and plant cell culture was an inspiration to me, and his confidence in me provided the motivation to pursue this degree. I would like to thank Dr. Carl L. Tipton for taking over as my major advisor when Dr. Outka's health did not allow him to continue in that capacity.

I am especially grateful to Dr. Dwight Tomes for his guidance and patience during this study. I have enjoyed working with Dr. Tomes, the members of his lab group, and the other members of the Department of Biotechnology Research at Pioneer Hi-Bred. This study would have been much more difficult without their professional advice and willingness to provide assistance.

I thank Dr. Harry T. Horner, Dr. Bernard White, Dr. Malcolm Rougvie, and Dr. Ethan Hack for serving as members of my graduate committee.

I am especially thankful for Bill Glass and Dave Lewis for maintaining the microscopes in the laboratory in excellent working order.
I would also like to thank Pioneer Hi-Bred and the ISU Graduate College for their financial support during my graduate work.

I would like to thank the many friends that I have made during my years at Iowa State that have made the time so memorable. Although there are too many to name them all, a few deserve special mention. Special thanks go to Diane McDonald, Dr. Sandra K. Frank, Dr. Thomas Cheetham, Joseph Slater, and Bruce Wagner, who provided support in the bad times, and many enjoyable diversions whenever they were necessary. I am grateful to Dr. Alan Myers, and Margaret and Helen Myers for their friendship and kindness during the completion of this dissertation.

I extend a loving thank you to my family for their support and encouragement through the years.

Finally, and most important, is a special thank you to someone who has been a fellow scientist to bounce ideas off of, a proofreader, a gentle push when necessary, a shoulder to lean on, and the best husband any woman could ask for. This work would never have been completed without the love and encouragement of my husband, Dr. Bruce L. Martin.