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# AN ULTRASTRUCTURAL STUDY ON EARLY CALLUS DEVELOPMENT FROM IMMATURE EMBRYOS OF THE MAIZE STRAINS A188 AND A632

P. F. FRANSZ* and J. H. N. SCHEL

Department of Plant Cytology and Morphology, Agricultural University, Wageningen

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SUMMARY

Immature embryos of two maize inbred lines (A188 and A632) have been examined during the first three days of in vitro culture using light and electron microscopy. The first day of culture is characterized by an increase in the amount of organelles, changes in vacuolation and in nucleolar morphology. These ultrastructural events take place in both inbred lines and are not influenced by the presence of 2,4D. After one day of culture variation in developmental patterns occurs, which is dependent on genotype and culture conditions. It is found, that in the scutellum of A188 embryos proliferation is activated strongly within two days of culture, followed by the appearance of a broad scutellar meristematic zone. A second meristematic region is observed around the coleorhiza. In A632 embryos only a coleorhizal meristematic zone developed, while in the scutellum proliferation was hardly observed.

Therefore we have subdivided the first period of maize embryo culture into two phases. The first phase is called the shock response and takes about one day, while the second phase starts after one day and is called growth response.

In concurrent cultures of A188, which were prolonged for several weeks, white compact callus developed accompanied by somatic embryos, while in the A632 cultures non-embryogenic callus was produced.

## 1. INTRODUCTION

Plant regeneration with maize has been obtained most commonly from immature embryos (GREEN and PHILLIPS, 1975; for recent reviews, see e.g. GREEN, 1983; VASIL, 1983; TOMES, 1985). In most cases the scutellum was the origin of embryogenic callus formation; histological studies (SPRINGER *et al.*, 1979; VASIL *et al.*, 1985) revealed that the initiation starts in the epidermal and the subepidermal region towards the coleorhizal end of the scutellum. In both studies morphological changes were observed after the third day of in vitro culture. No cytological study, however, has been done of the first days of maize embryo culture. Electron microscopical investigations about this early period have been carried out almost exclusively on dicotyledonous systems (YEOMAN & STREET,

* Correspondence and reprints: Department of Plant Cytology and Morphology, Arboretumlaan 4, 6703 BD Wageningen, The Netherlands.

1977; see also HOWARTH *et al.*, 1981, 1983). With respect to the monocotyledons, KOTT *et al.* (1985) investigated the callus origin sites in haploid barley embryos and described some ultrastructural changes of the epidermal and subepidermal scutellar region.

It has been reported earlier that the maize strain A188 produces embryogenic callus with a high regeneration efficiency (GREEN & PHILLIPS, 1975). On the contrary, in cultures from strain A632 only non-embryogenic callus developed without regeneration capacity (GREEN & PHILLIPS, 1975; GREEN & RHODES, 1982; but see also DUNCAN *et al.*, 1985). The aim of the present study was to investigate the events occurring during the early (0–3 days) stages of culture of these different genotypes using cytological techniques. Some observations were made on cultures with modified conditions, especially with modified levels of 2,4D and sucrose (see LU *et al.* 1983).

## 2. MATERIALS AND METHODS

### 2.1. In vitro culture

Maize inbred lines A188 and A632 (kindly supplied by Dr. C. E. Green, Minnesota) were grown in the greenhouse. Pollination and harvesting took place in the late summer of 1985. Within two weeks after pollination whole cobs were broken into three or four parts and surface sterilized with 70% ethanol (30 sec), followed by commercial bleach diluted to 1.5% hypochlorite to which a drop of Tween 80 was added (5–10 min). After three rinses with sterile water immature embryos (1–2 mm) were excised and placed on an agar nutrient medium with the embryo axis in contact with the medium. The medium was composed of the inorganic components of MURASHIGE & SKOOG (1962) and the organic compounds as described by GREEN & PHILLIPS (1975). Sucrose was added in two different concentrations (2% and 6%); in most cases 2,4D (2,4 dichlorophenoxy acetic acid) was added (2 mg/l, abbreviated as GP2; medium without 2,4D: GP0). The embryos and resulting callus cultures were incubated at 28–30 °C with a 16/8 hours light/dark regime. Subculturing took place after two to three weeks.

### 2.2. Light and electron microscopy

After various periods of in vitro culture embryos were sampled and fixed for microscopical examination.

Light microscopy (LM):

Specimens were fixed in 4% glutardialdehyde in 0.1 M sodium cacodylate buffer, pH 7.2, for 1 hr at room temperature. After dehydration through an ethanol series (50–70–90–100–100%) the specimens were embedded in 2-hydroxyethylmethacrylate (Technovit 7100, from Kulzer, Wehrheim, Germany). Sections (5 μm thick) were stained with toluidine blue.

Transmission electron microscopy (TEM):

Specimens were fixed in 2% glutardialdehyde in 0.1 M sodium cacodylate buffer, pH 7.2 for 2 hrs at room temperature. Postfixation, sectioning and staining pro-

cedures used were as described by SCHEL *et al.* (1984).
Scanning electron microscopy (SEM):
Fixation and postfixation took place as for TEM. The specimens were further processed for SEM using the OTO-method, according to POSTEK & TUCKER (1977). After the last rinsing period the specimens were dehydrated and critical point dried. The dried samples were examined with a Jeol ISM-35C scanning electron microscope.

## 3. RESULTS

### 3.1. General morphology

Immature embryos from the A188 and the A632 lines had a scutellum length of 1–2 mm at the onset of culture and showed a well defined shoot-root axis. We have subdivided the scutellum into a top, middle and basal region (*fig. 1*).

Embryos of both strains, cultured on GP0 medium, germinated within a few days and the swollen scutellum became green. In the presence of 2,4D embryos from the A188 line developed protuberances within one week and a green spot appeared at the scutellar top. Subsequently, leafy structures were recognized in the middle and basal region. Opaque scutellar-like bodies appeared at the base of these structures, while friable non-embryogenic callus developed near the coleorhiza. Elongation of the coleoptile often took place. Young leaves emerged from the scutellar-like bodies after three weeks and developed into plantlets. In general, no embryoids could be observed in these cultures. However, in some cases soft and friable embryogenic callus was formed with typical globular embryoids.

When these A188 embryos were cultured with a raised sucrose level (GP2-6% medium) the roof of the swollen scutellum broke open after 3 to 4 days at the centre or the edges of the basal and middle part, but never at the top (*fig. 2a*). A green spot appeared at the top and within 10 days a completely disrupted scutellum was present (*figs. 2b* and *c*). When the scutellum roof remained intact, 'trumpet-like' structures with knurled edges appeared (*fig. 2d*). White compact embryogenic callus developed at the base of these structures or from under the

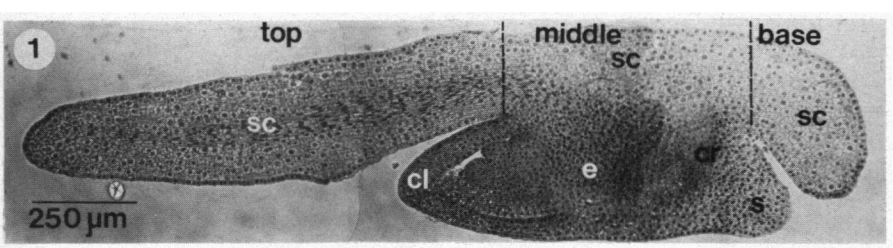


Fig. 1. Tangential longitudinal section of an A188 embryo at the onset of culture (t = 0). The scutellum is subdivided into three regions: top, middle and base. e, embryo axis; cl, coleoptile; cr, coleorhiza; sc, scutellum; s, suspensor.

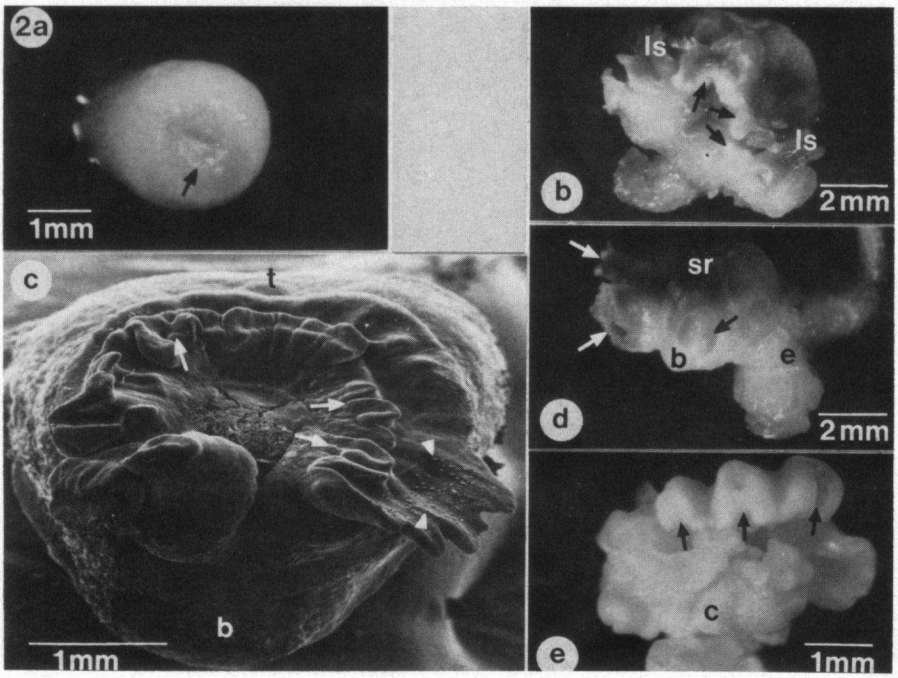


Fig. 2. a. Three days cultured A188 embryo on GP2 with 6% sucrose showing the scutellar roof. Note the beginning of disruption (arrow).
b. The same embryo after 10 days of culture. The scutellar roof is completely disrupted. White compact scutellar bodies (arrows) arise at the base of the green leafy structures.
c. Scanning electron micrograph of a five days cultured A188 embryo. Note the presence of scutellar bodies (arrows). Trichomas (arrowheads) develop on the leafy structures. b, scutellar base; t, scutellar top.
d. Ten days cultured A188 embryo on GP2 with 6% sucrose. Note the appearance of trumpet-like structures (arrows). sr, scutellar roof; b, scutellar base; e, embryo axis.
e. After three weeks the scutellar-like bodies have developed into somatic embryoids (arrows). c, compact callus.

disrupted scutellum roof. After several weeks shoots and roots and somatic embryos (*fig. 2e*) were observed. Subculturing was then needed to prevent excessive root formation.

If A632 embryos were put on GP2-medium with 6% sucrose the events as described above were not observed. These embryos generally became yellow to brown; friable non-embryogenic callus was produced starting near the coleorhiza from under the scutellum. This callus mainly produced roots; in one case, a white scutellar-like body was observed, but regeneration could not be obtained.

## 3.2. Cytomorphology

In previous studies by SPRINGER *et al.* (1979) and VASIL *et al.* (1985) it has been reported that scutellar embryogenic callus develops in the (sub)epidermal region

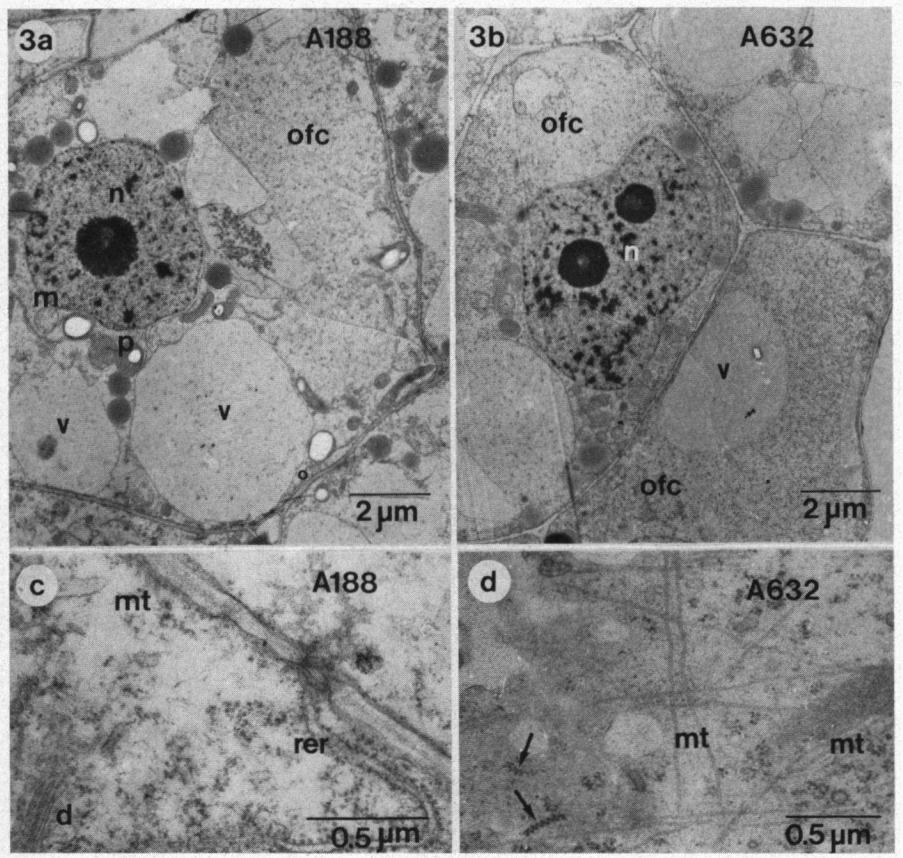


Fig. 3. a. Electron micrograph of a scutellum cell at the basal region from A188. n, nucleus; v, vacuole; m, mitochondrion; p, plastid; ofc, organelle-free cytoplasm.
b. As in *fig. 3c*, but now from A632.
c. Detail near the cell membrane of an A188 scutellum cell. Many ribosomes are present. rer, rough endoplasmic reticulum; d, dictyosome; mt, microtubules.
d. Detail near the cell membrane of an A632 scutellum cell. Note the microtubules (mt) and the helical polysomes (arrows).

near the base of the scutellum. Therefore, we focussed in this study on the middle and basal scutellar region.

3.2.1. Observations on both strains at the onset of culture (t = 0)
As shown in *fig. 3a*, cells of the line A188 contained a round nucleus, whereas the nucleus of A632 cells was more or less lobed (*fig. 3b*). Several mitochondria and plastids were present. Ribosomes were observed free as well as bound to the endoplasmic reticulum (*fig. 3c*). Cells of strain A632 also contained many polysomes (*fig. 3d*, arrows). The cell wall in both strains had a firm appearance except for newly formed cell walls which were slightly irregular. Plastids some-

times contained starch granules. The nuclear chromatin was diffuse with patches of condensed chromatin. The nucleolus was characterized by a peripheral granular region and a central fibrillar region. The vacuoles were small or medium-sized and cytoplasmic areas were observed in which no organelles other than some ribosomes were found (*figs 3a,b*). This organelle-free cytoplasm was encircled by vacuoles, small vesicles and endoplasmic reticulum, but not by a continuous membrane. Cortical microtubules, randomly arranged, were abundant (*figs 3c,d*).

3.2.2. Cytological changes during the first days of culture on GP2 medium containing 6% sucrose

The cytoplasm of A188 embryos, studied by light microscopy, was no longer uniformly stained after 30 min of culture. This was mainly due to the vacuolation, which was more evident. Mitotic activity was strongly reduced. After one day of culture vacuolation had decreased, while proliferating cells were present in the whole scutellum. Day 2 embryos showed an increased mitotic activity in the whole scutellum, especially in the middle and basal region. At day 3 a broad meristematic zone was present in the middle part of the scutellum and also around the coleorhiza (*fig. 4a*). Tracheary elements developed in the top of the scutellum.

The A632 scutellum cells showed an identical pattern as A188 after 30 min of culture: an increased vacuolation accompanied by a reduced mitotic activity. After one day vacuolation decreased while mitotic figures reappeared in the scutellum, especially in the vascular region. At day 3 the embryos showed a meristematic zone around the coleorhiza (*fig. 4b*). Little meristematic activity is seen in the scutellum.

The ultrastructural observations confirm the increase in vacuolation of the A188 scutellum cells after 30 min of culture (see *table 1*). Many polysomes with helical configurations appeared in the cytoplasm. Organelle-free cytoplasmic regions were still present. In the nucleolus the fibrillar region was surrounded by the granular part, although in some cells these regions were intermingled. Dictyosomes were frequently observed while microtubules could not be found.

After one day of culture the scutellum cells showed a reduced vacuolation (*table 1*). The small vacuoles contained electron-dense material and sometimes osmiophilic droplets. Areas of organelle-free cytoplasm were no longer detected. The nucleolus showed intermingled granular and fibrillar regions, often with a so-called nucleolar vacuole (JORDAN, 1984). The cytoplasm was filled with high concentrations of polyribosomes, numerous mitochondria, dictyosomes and plastids, some of which contained starch (*fig. 5a*). Microtubules were observed adjacent to the cell membrane. The cell wall shape of the scutellum cells was more or less irregular.

Scutellum cells of day 2 embryos showed meristematic characteristics: cells were relatively small, had a central nucleus and a high cytoplasmic density. The vacuoles contained electron-dense material. In the nucleolus the fibrillar and granular regions were segregated or intermingled. One large or several small

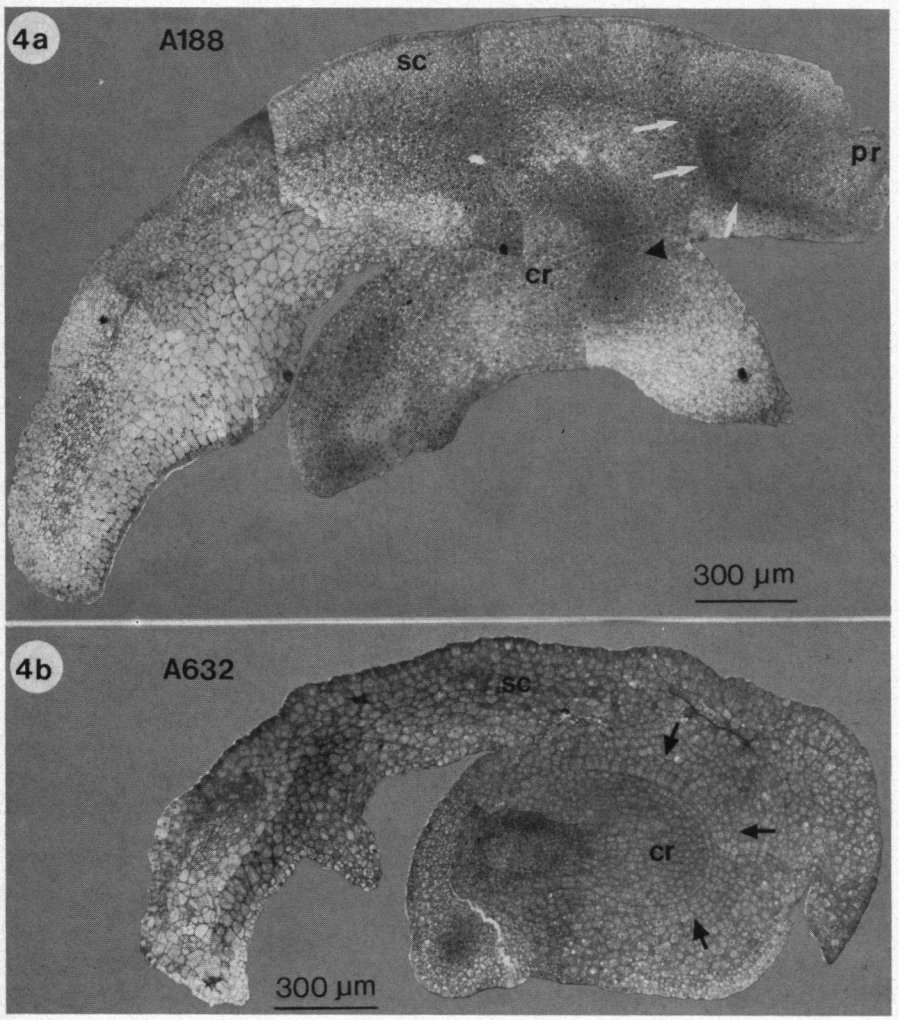


Fig. 4. Tangential longitudinal section of an A188 embryo (a) and an A632 embryo (b) after three days of culture on a GP2 medium with 6% sucrose. Note the meristematic zones (white arrows) in the scutellum and around the coleorhiza (cr, arrowhead) from A188 and around the coleorhiza (black arrows) only in the A632 embryo. Also note the protuberance (pr) at the basal end of the A188 embryo.

nucleolar vacuoles were often observed. In the middle part of the scutellum, amyloplasts were numerous, and the shape of the cell wall was very irregular. Intercellular spaces were prominent (*fig. 5b*). At the scutellar base the cell wall was thin and wrinkled. Here no intercellular spaces were observed (*fig. 5c*). After three days of culture vacuolation increased in the basal scutellum but reduced in the middle region (*table 1*). The vacuoles contained no electron-dense material (*fig. 5d*). The amount of starch granules was decreased.

Table 1. Surface density* of vacuoles in scutellum cells during the first three days of culture. Data expressed as means ± standard error.

| time in culture | A188 GP2 6% | A188 GP0 | A188 GP2 | A632 GP2 6% |
|---|---|---|---|---|
| 0 | 0.203 ± 0.055 | | | 0.180 ± 0.091 |
| 30 min | 0.391 ± 0.133 | 0.831 ± 0.053 | | 0.794 ± 0.065 |
| 1 d | 0.085 ± 0.031 | 0.501 ± 0.178 | 0.294 ± 0.078 | 0.114 ± 0.048 |
| 2 d | 0.058 ± 0.026[a]<br>0.232[b] | | 0.439 ± 0.139 | |
| 3 d | 0.138 ± 0.067 | 0.374 ± 0.050 | 0.348 ± 0.115 | 0.023 ± 0.012 |

* Surface density ($s^{vac}/s^{cell}$), calculated from several cells, is expressed in units of $\mu m^2/\mu m^2$.
[a] Surface density at the basal region.
[b] Surface density in a representative cell at the middle region.

In the A632 scutellum cells the vacuoles were significantly enlarged after 30 min of culture (*table 1*)a. No change in nucleolar morphology was observed. The high concentrations of ribosomes caused a high cytoplasmic density. After one day of culture the same characteristics as in the A188 cells were observed. Vacuolation was decreased and vacuoles contained electron-dense material. Many helical polysomes, dictyosomes and mitochondria were present and plastids sometimes contained starch. The nucleolus, however, still had the segregated arrangement of granular and fibrillar regions. The cell wall was irregularly formed.

After three days the cells of the meristematic zone around the root apex (see *fig. 4b*) had penetrated into the scutellum and showed a high cytoplasmic density. They did not differ in morphology from the subepidermal cells of the middle and basal scutellum. In contrast to the A188 cells the cells of the A632 strain contained very small vacuoles (*fig. 6a*). In the nucleolus the fibrillar and granular regions were generally intermingled although incidently segregation occurred. Nucleolar vacuoles were infrequently observed. Plastids contained starch.

### 3.2.3. Cytomorphogenesis of A188 embryos cultured on GP0 and GP2 medium with 2% sucrose

At the light microscopical level A188 embryos cultured on GP2 medium resembled the GP2–6% cultured embryos during the first days of culture, although the degree of vacuolation differed. Embryos cultured on GP0 medium at first showed no differences as compared with the GP2 cultures. However, after two days no extensive increase in mitotic activity, as observed in the presence of 2,4D, took place, while vacuolation increased in the basal part of the scutellum. After three days no meristematic zone was observed.

At the electron microscopical level embryos of all cultures showed enlarged vacuoles after 30 min of incubation (*table 1*). During the following period GP2 embryos cultured at the low sucrose concentration differed from those cultured at the high sucrose concentration. For example, vacuoles were completely filled with electron-dense material and cell walls were very irregular (*fig. 6b*). Embryos

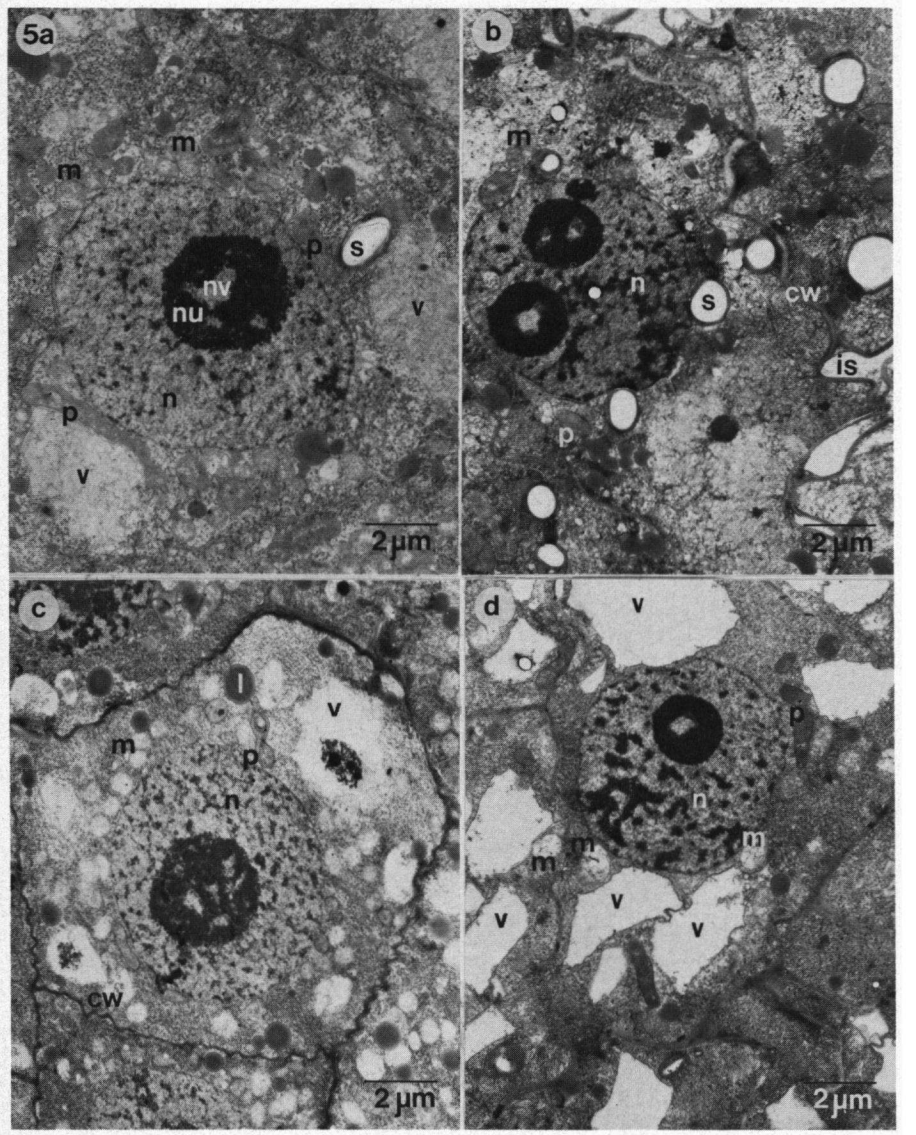


Fig. 5. Scutellum cells of A188 embryos cultured on a GP2 medium with 6% sucrose. a, one day culture; b, two days culture at the middle region; c, two days culture at basal region; d, three days culture middle/base. n, nucleus; nu, nucleolus; nv, nucleolar vacuole; v, vacuole; m, mitochondrion; p, plastid; s, starch; l, lipid droplet; cw, cell wall; is, intercellular space.

cultured on GP0 medium, also contained darkly stained vacuoles. After three days of culture they tended to the t=0 situation: a lower cytoplasmic density,

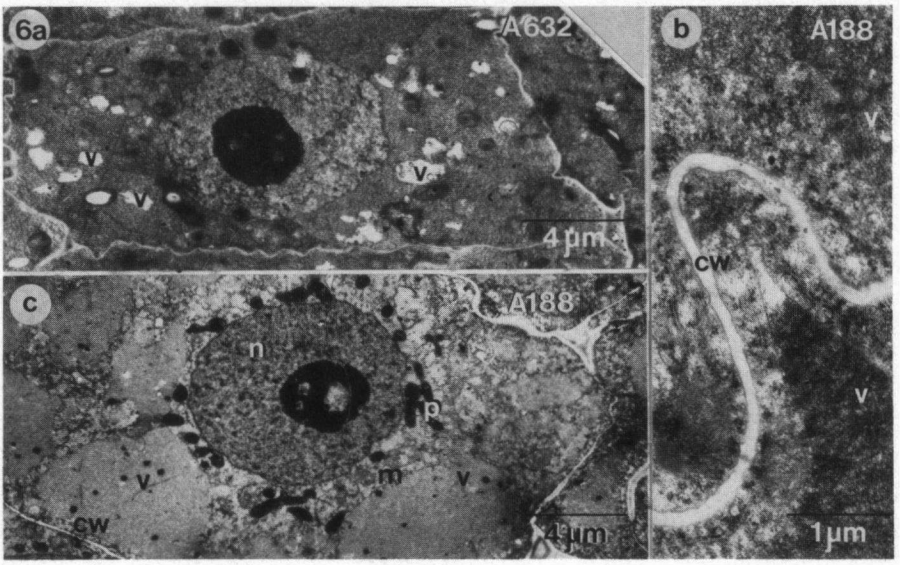


Fig. 6. a. Embryo of the A632 line cultured on a GP2 medium with 6% sucrose for three days. Scutellum cell in middle region. Note the presence of many small vacuoles (v).
b. Embryo of the A188 line cultured on GP2 medium with 2% sucrose for one day. Detail of the vacuoles of two adjacent cells, containing electron-dense material. Also note the irregularly shaped cell wall.
c. Embryo of the A188 line cultured on a GP0 medium with 2% sucrose for three days. n, nucleus; v, vacuole; m, mitochondrion; p, plastid; cw, cell wall.

medium-sized vacuoles and a decrease of the amount of mitochondria and plastids. The plastids showed a darker groundplasm (*fig. 6c*).

## 4. DISCUSSION

White and compact callus tissue is an important stage in maize regeneration through tissue culture. GREEN & PHILLIPS (1975) and FREELING *et al.* (1976) regenerated maize plants from immature embryo cultures by organogenesis, while LU *et al.* (1982, 1983) and NOVAK *et al.* (1983) obtained somatic embryogenesis. This callus, also called type I callus (ARMSTRONG & GREEN 1985), may be transformed into soft embryogenic callus which can be maintained for longer periods (type II callus; see also VASIL *et al.* 1984).

Our histological observations are in agreement with the work of SPRINGER *et al.* (1979) and VASIL *et al.* (1985). The embryogenic callus originated in the scutellar region towards the coleorhiza. However, the pattern of development, leading to plant regeneration, may differ. SPRINGER *et al.* (1979) obtained regeneration by organogenesis, which started in the epidermal and subepidermal

layers. A cambium produced typical radial seriation. It is unlikely that this development results into white and compact callus, because prominent shoot apices originate directly from this cambial zone. VASIL *et al.* (1985) reported the establishment of somatic embryogenesis through the white compact callus. Proliferation started in cells near the procambial strands. We conclude from these and our data that different types of callus initiation patterns give rise to different types of callus. It is likely, that both embryogenic and non-embryogenic callus is capable to develop in the immature embryo and may do so simultaneously from different origins. However, several factors, e.g. genotype, culture conditions and physiology of the explant tissue may influence this development. This suggests that 'difficult' maize lines are also capable in producing regenerable callus, which is confirmed by DUNCAN *et al.* (1985), who reported regenerable callus 'in appearance' from 199 maize inbred lines. However, for strain A632 the authors were unable to show regeneration in their 1983 experiments, while their claim for regeneration capacity in the 1984 experiments was only based on visual appearance of the calli. Regeneration from these calli was not further attempted.

Our ultrastructural observations indicate that the period of callus initiation can be subdivided into two phases. The first phase takes about one day, while the second starts after one day of culture. During the first phase changes occur in nucleolar morphology, degree of vacuolation and cell wall shape, while several organelles increase in number. This accumulation of organelles is a general feature in callus initiation (ISRAEL & STEWARD 1966; HALPERIN & JENSEN 1967; FOWKE & SETTERFIELD 1968; YEOMAN & STREET 1977; KOTT *et al.* 1985). The second phase starts with the recovery of mitotic activity. During this phase ultrastructural variation becomes visible between the different ways of culture. These differences comprise the degree in vacuolation, cytoplasmic density and organelle structure.

The importance of nucleolar morphology in growth-induced cultures has been shown by ZWAR & BROWN (1968), who reported the accumulation of $^{14}C$ labelled 2,4D in nucleoli of dividing cells in cultured explants from the Jeruzalem artichoke tuber. Nucleolar modifications also occur during activation of quiescent root cells from *Zea mays* (DELTOUR & BRONCHART 1971; DE BARSY *et al.* 1974; DELTOUR *et al.*, 1979) and in *Allium cepa* (RISUENO & MORENO-DIAZ DE LA ESPINA 1979). In cells with low activity the fibrillar and granular regions are compact and segregated. In contrast, nucleoli of proliferating cells contain less compact and intermingled regions (NAGL 1976; YEOMAN & STREET 1977). The term 'nucleolar vacuole' is used to describe the nucleolar areas with a generally nucleoplasmic appearance, containing loosely dispersed pre-ribosomal-like particles and fibrils (JORDAN 1984). DELTOUR & DE BARSY (1985) suggest that the nucleolar vacuoles, observed during early seed germination of maize, participate in the increase in the nucleolus-nucleoplasm exchange interface and are established when the nucleolar volume decreases, while the output of ribonucleoproteins increases rapidly. Taking into account these data, nucleolar activity and probably RNA synthesis in embryo cultures of maize start between 30 min and

one day of incubation. Because the polysomes accumulate within 30 min of culture, they are either formed from pre-existing cytoplasmic ribosomes and/or from pre-existing ribosomal subunits stored in the nucleolus. Similar observations on nucleolar modifications have been reported in cultured Jeruzalem artichoke tuber tissue (FOWKE & SETTERFIELD 1968; YEOMAN & STREET 1977). VASIL (1973) also observed a vacuolated nucleolus in callus cell nuclei of the hypocotyl of an in vitro germinated pea seedling in the presence of 2,4D. Fibrillar and granular regions, however, became segregated in the presence of 2,4D.

Vacuolation and cell wall morphology are parameters indicative for the changes in water potential in the plant tissue. Cell expansion and turgor depend on cell wall elasticity, while the vacuole serves as an osmotic system that develops turgor pressure (see monograph of KRAMER 1983). Before culture the vacuoles of the scutellum cells are in an early stage of development as can be noticed from the areas of organelle-free cytoplasm (AMELUNXEN & HEINZE 1984; HILLING & AMELUNXEN 1985). After 30 min. of culture an inward diffusion of water leads to an increase in vacuolation and turgor pressure, especially in embryos cultured on medium with a low sucrose concentration. The walls of the young scutellum cells are very elastic and, as a result, the cells expand as the turgor pressure increases. However, some doubt exists wether the primary cause of cell enlargement is turgor pressure or the difference in water potential (RAY *et al.* 1972; KRAMER 1983).

The observed ultrastructural changes are in good agreement with the cytological process of wound-healing in injured plants tissue, which is known from early studies (BARCKHAUSEN 1978). Two distinct stages have also been described by MACLEOD *et al.* (1979) who studied the response of Jeruzalem artichoke tissue when cultured on medium containing 2,4D. The first stage was called the wound response and occurred in the presence or absence of 2,4D. The second stage was called the growth response and was initiated by 2,4D. The wound response was hardly influenced by seasonal variation, while the growth response showed seasonal dependency. Our results confirm Macleods two-component hypothesis with electron microscopical evidence; however, in the case of maize embryo culture there is hardly any wounding. The embryo can be excised very easily from the caryopsis without being disrupted, because it is not grown together to the endosperm. At most some deletion of the suspensor may take place, but in embryos of 13 DAP the functional role of this tissue seems to be less important (SCHEL *et al.* 1984). Although we cannot really speak of a wound response, the scutellar cells show similar ultrastructural characteristics. Therefore, we prefer to use the term shock response instead of wound response.

The proces of callus initiation is an important step of plant regeneration through somatic embryogenesis as well as through organogenesis. We conclude that the pattern of development leading to any callus type is influenced by several main factors: genotype, culture conditions and physiology of the explant. This pattern is effected during the second phase of callus initiation, after a first period of culture. No distinct ultrastructural parameters were observed which might be characteristic for embryogenic callus.

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