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PROTOPLASMA

Origin and Development of Somatic Embryos in *Medicago sativa* L. (Alfalfa)

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Summary

Embryoids which developed from callus obtained from leaves of *Medicago sativa* L. (alfalfa) were studied with the light and scanning electron microscopes; embryoids are not common in leguminous plants. Sections showed that in organised callus embryoids apparently originated from groups of embryonic cells, whereas in friable callus, and in the epidermis of cotyledons, hypocotyls and roots of callus-derived plantlets, they often originated from single cells. Many early stages of embryogenesis (1, 2, 3- and 4-celled stages) were observed, particularly in epidermal sites. Stages in somatic embryogenesis, though variable, resembled those described for the normal zygotic embryos of alfalfa. A suspensor-like structure was sometimes present in the embryoids. Numbers of cotyledons, up to six, were observed. The SEM provided confirmation of the many stages of development and the very large number of embryoids present, as well as evidence of the range of form resulting from differential growth of regions of the embryos.

Keywords: Alfalfa; Embryogenesis; Embryos; Histology; *Medicago sativa;* SEM.

1. Introduction

Since the first somatic embryos *in vitro* were described by Reinert (1958), such structures have been observed in 80 different species in 33 families (Wetherell 1978), and somatic embryogenesis is recognised as a widespread phenomenon. However, in the *Fabaceae*, somatic embryogenesis is not a common event, although embryoids have been reported in alfalfa (*Medicago sativa* L.) (Saunders and Bingham 1972, Kao and Michayluk 1980, Santos *et al.* 1980, Johnson *et al.* 1981), and red clover (*Trifolium pratense*) (Phillips and Collins 1980).

In general, it has been observed that embryoids develop either from callus or cell suspension cultures, or as adventive structures developing without an intervening callus phase from cotyledons and hypocotyls of existing embryoids and young plantlets (see reviews by Raghavan 1976, Reinert *et al.* 1977).

From observations of late globular, heart-shaped and torpedo-shaped stages, and of young carrot seedlings *in vitro*, various workers have stressed the close similarities between zygotic embryos and somatic embryos arising in culture. However, published literature contains few descriptions of the origin of embryoids from single cells of embryogenic tissue, and the sequence of early developmental stages has not often been satisfactorily related to normal embryogenesis from the egg cell. Backs-Hüsemann and Reinert (1970) described the development of somatic embryos from single cells, and small groups of cells, in tissue cultures of carrot. The origin of embryoids from the stem epidermis of *Ranunculus sceleratus* (Konar and Nataraja 1965, Konar *et al.* 1972b) was also unusually clearly established.

The present investigation traces, for the first time, the origin and development of embryoids on callus tissue, and of secondary adventive embryoids on the cotyledons of embryoids from callus and from the hypocotyl and root surfaces of young plantlets of a leguminous plant (*Medicago sativa*, alfalfa) *in vitro*.

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2. Material and Methods

In order to obtain callus induction and embryoid formation peeled leaflets with the mesophyll exposed were incubated on agar medium with 2,4-D and kinetin as previously described (SANTOS *et al.* 1980).

Histological studies were carried out on callus and on 0.5 mm long portions of cotyledons, hypocotyl and roots from plantlets regenerated *in vitro*. Callus and other specimens were fixed in formalin-acetic acid-ethyl alcohol (FAA), dehydrated through an ethanol series and embedded in paraplast (SASS 1966). Sections were cut 8–10 μm thick, stained in tannic acid and iron alum with safranin and orange G (SHARMAN 1943), and mounted in Canada balsam. Some of the material fixed in FAA was prepared for scanning electron microscopy by treatment in a Polaron critical point dryer after dehydration through a graded alcohol series to absolute alcohol. Specimens were mounted on stubs with double-sided tape, and coated with a thin layer of gold in an Edwards S 150 sputter coater for 2.5 minutes. They were observed and photographed in a Cambridge Stereoscan 150 scanning electron microscope at 10 kV.

3. Results

Peeled leaflets incubated on UCHIMIYA and MURASHIGE's (1974) agar medium with 2.0 mg/l 2,4-D and 0.25 mg/l kinetin developed friable callus which gave rise to clusters of green embryoids on its surface (see SANTOS *et al.* 1980). These clusters originated from a common compact nodule of callus tissue, consisting of parenchymatous cells with an external layer of meristematic cells competent to give rise to embryoids. Observations with the scanning electron microscope showed embryoids in various stages of development emerging on the callus surface. Fig. 1 shows a general view of a portion of callus with embryoids in globular, early and late heart-shaped, torpedo and cotyledonary stages of development. Fig. 2 shows in greater detail some of the youngest, approximately globular embryoids on the piece of callus in Fig. 1. The arrangement of the surface cells suggests that both single and small groups of multiple embryoids originated from the callus.

Embryoids derived from callus and then placed on hormone-free agar medium gave rise to further embryoids on their cotyledons, hypocotyl (especially in a swollen proximal region closer to the root), and root. In these organs of the plantlets, two distinct types of epidermal cells occurred: non-embryogenic epidermal cells thinly lined with cytoplasm, and highly cytoplasmic embryogenic cells which stained densely and occurred singly in small groups of two or more, or in long bands (Fig. 3).

A general description of the origin of embryoids, and the stages of development observed, will be given, although slight differences occurred according to the site of origin.

In the more friable types of callus, longitudinal sections revealed meristematic nodules embedded in the friable tissue. Each nodule consisted internally of parenchymatous tissue surrounded by 2–3 layers of densely cytoplasmic cells (Fig. 4). Single or small groups of embryogenic cells became detached from the outermost layer of cells, which was in contact with the friable callus. These remained embedded in the tissue (Figs. 5 and 6). Such cells were easily distinguishable by their dense staining reaction, and gave rise to embryoids which were thus surrounded by the friable callus.

Single embryogenic cells were observed in sections of the hypocotyl (Fig. 7), and small groups on cotyledons. These were somewhat palisade-like in shape when situated on the lower epidermis facing the midvein or at the margin of the cotyledons, but elsewhere, on most of the surface of the cotyledon, were isodiametric (Fig. 8). Evidence for the embryogenic nature of these epidermal cells comes primarily from observations on the plane of their first division, which was periclinal to form a 2-celled proembryoid (Fig. 9), in which the apical cell (*a*) was larger than the basal cell. The second division was again periclinal (Fig. 10), forming a 3-celled proembryoid. In some instances it appeared that the

Fig. 1. Scanning electron micrograph (SEM) of a piece of callus bearing embryoids in various stages of development. The arrow indicates the region of developing globular stages shown in Fig. 2 (× 24)

Fig. 2. SEM of the region of callus arrowed in Fig. 1, showing the cellular arrangement in groups of globular embryoids (× 140)

Fig. 3. T.S. cotyledon of an embryoid showing a single layer of embryogenic cells (*ec*) at the margin of the cotyledon (× 180)

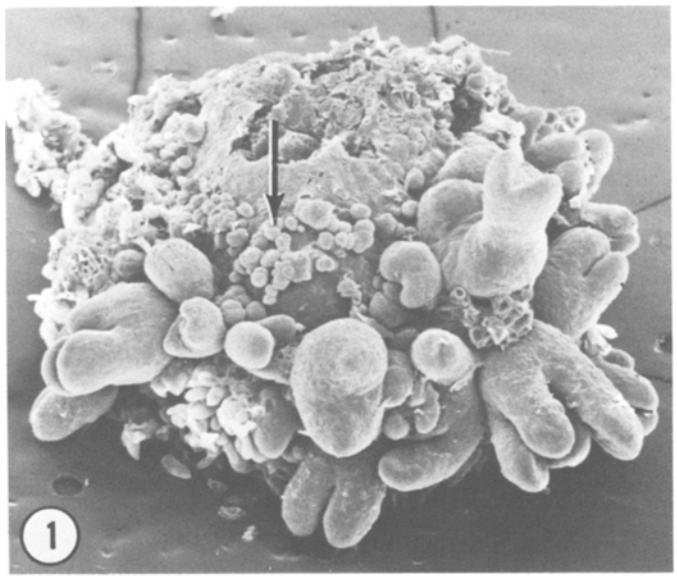
Fig. 4. L.S. meristematic nodule embedded in friable callus. Two to three layers of embryogenic cells overlie parenchyma (× 260)

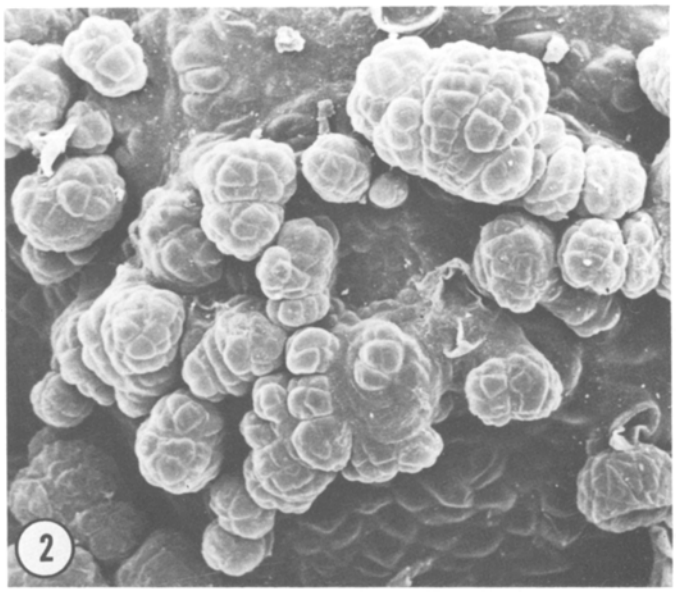
Fig. 5. Densely cytoplasmic cells being released from the surface of meristematic nodules (× 495)

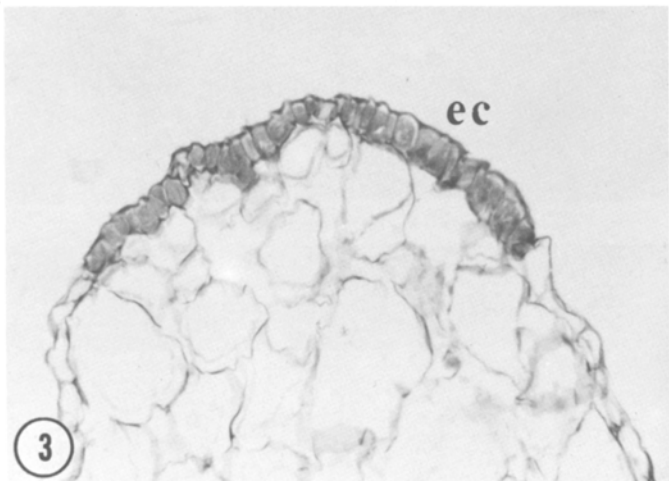
Fig. 6. Group of 4 embryogenic cells embedded in friable callus, having been released from a meristematic nodule (× 485)

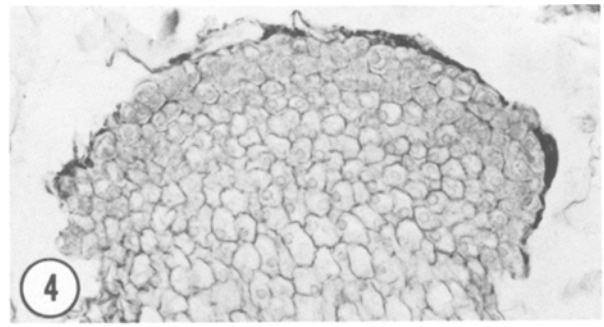
Fig. 7. A single embryogenic cell in the hypocotyl surface layer (× 585)

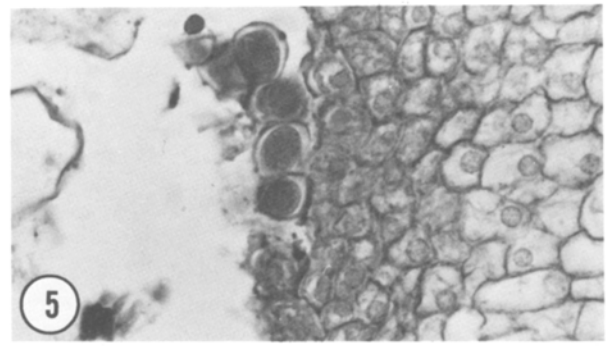
Fig. 8. T.S. cotyledon of embryoids which themselves have embryogenic epidermal cells. These are relatively isodiametric (× 610)

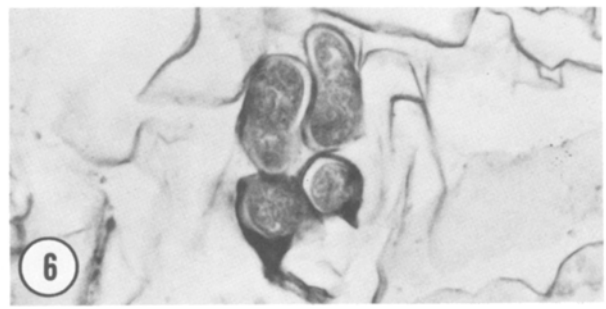


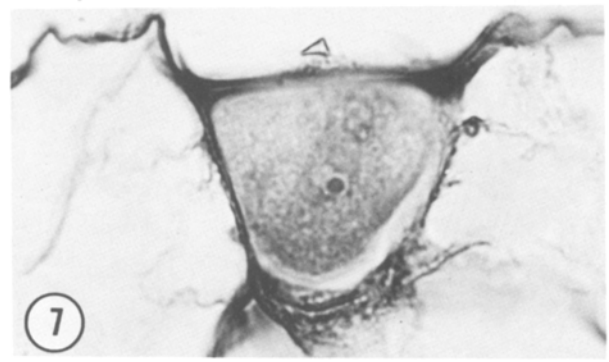


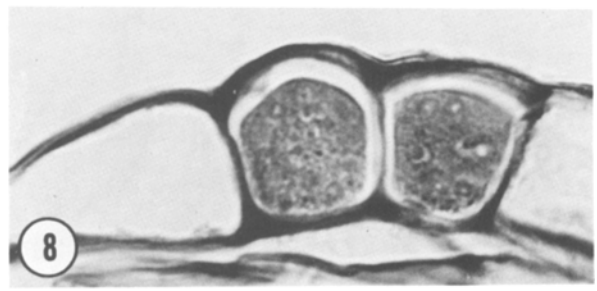












Figs. 1-8

second division occurred in the basal cell, and in others (*e.g.* Fig. 10) in the apical cell. During embryogenesis in the hypocotyl, the cuticle was sometimes disrupted and the plane of the first division was not uniformly orientated. The 4-celled stage of embryogenesis was frequently observed in the hypocotyl (Fig. 11); more complex globular structures developed from this. Fig. 12 shows a group of 7 adjacent globular embryoids on a piece of hypocotyl. In some globular embryoids, for example that shown in Fig. 13, which originated from a cotyledon, a suspensor-like structure (*s*) attached the embryoid to the epidermis.

Early heart-shaped stages of embryogenesis are shown in section and as seen with the SEM in Figs. 14 and 15, both originating in callus. The region of attachment of some of these embryoids was narrow and readily damaged in sectioning (Fig. 14); in other instances, embryoids were attached to the callus by a thick, solid stalk. These may possibly have originated from a group of cells rather than a single embryogenic cell; early stages of embryogenesis (1-4 celled) were not observed in callus though they could have been present.

Embryoids eventually developed cotyledons, and groups of embryoids with two (Fig. 16) and with four to six cotyledons (Fig. 17) were observed on callus cultures. Fig. 16 also shows a suspensor-like structure (*s*). An embryoid with well developed cotyledons and the thick region of attachment referred to above is shown in section in Fig. 18; it contrasts with the somewhat lateral attachment to the cotyledon of the embryoid in Fig. 20. Somewhat abnormal embryoids also occurred on the cotyledon surface, with two cotyledons which were unusually prominent in relation to the body of the embryoid (Fig. 19). Fig. 21 shows a longitudinal section of a hypocotyl bearing embryoids in different stages of development. On the left hand side of the section the epidermis consisted totally of embryogenic cells (*ec*), which formed a double layer in some parts. Towards the base of the section can be seen a mass of smaller embryoids (*e*). On the right hand side, at the top of the section, non-embryogenic epidermal cells are present, while closer to the torpedo-shaped embryoid the epidermal cells are embryogenic.

On the root surface embryoids originated from single cells, and became embedded in the hormone-free agar medium in which the plantlets were growing.

4. Discussion

Somatic embryogenesis was observed on cultured callus tissue, and on the surface of cotyledons, hypocotyls and roots of induced plantlets of *Medicago sativa* L. Our observations suggest, but do not conclusively establish that on organised callus embryoids originated from groups of apparently homogeneous meristematic cells, while on friable callus they originated from single embryogenic cells distinguishable from the non-embryogenic ones by their staining reactions.

Anatomical studies of somatic embryogenesis in callus tissue have been carried out in only a few cases. Embryogenic cells have been found deeply embedded in callus parenchyma of *Tylophora indica* (RAO and NARAYANASWAMI 1972), while embryogenic cells were also located in the peripheral layers of callus of this plant. In callus of *Ranunculus sceleratus* embryoids originated either at the periphery or from deep seated cells (KONAR and NATARAJA 1969). Densely staining initial cells of embryoids were observed in suspension cultures of *Atropa belladonna* (KONAR *et al.* 1972 a), and in callus cultures of carrot (REINERT 1959, DANILINA 1972). However, HALPERIN and JENSEN (1967) and MCWILLIAM *et al.* (1974) were unable to identify such initials in cultures of wild carrot and cultivated varieties respectively. THOMAS *et al.* (1972) likewise failed to observe initial cells in embryogenic callus of *Ranunculus sceleratus*.

The present work provides evidence for the origin of embryoids from single cells in the epidermis of cotyledons, hypocotyls, and roots of induced plantlets as well as in friable callus of *Medicago sativa*. Indeed,

Fig. 9. T.S. cotyledon of embryoid with embryogenic cells, some of which have divided; *a* larger apical cell (× 590)

Fig. 10. As Fig. 9, but one cell has divided again to give a 3-celled embryoid (× 590)

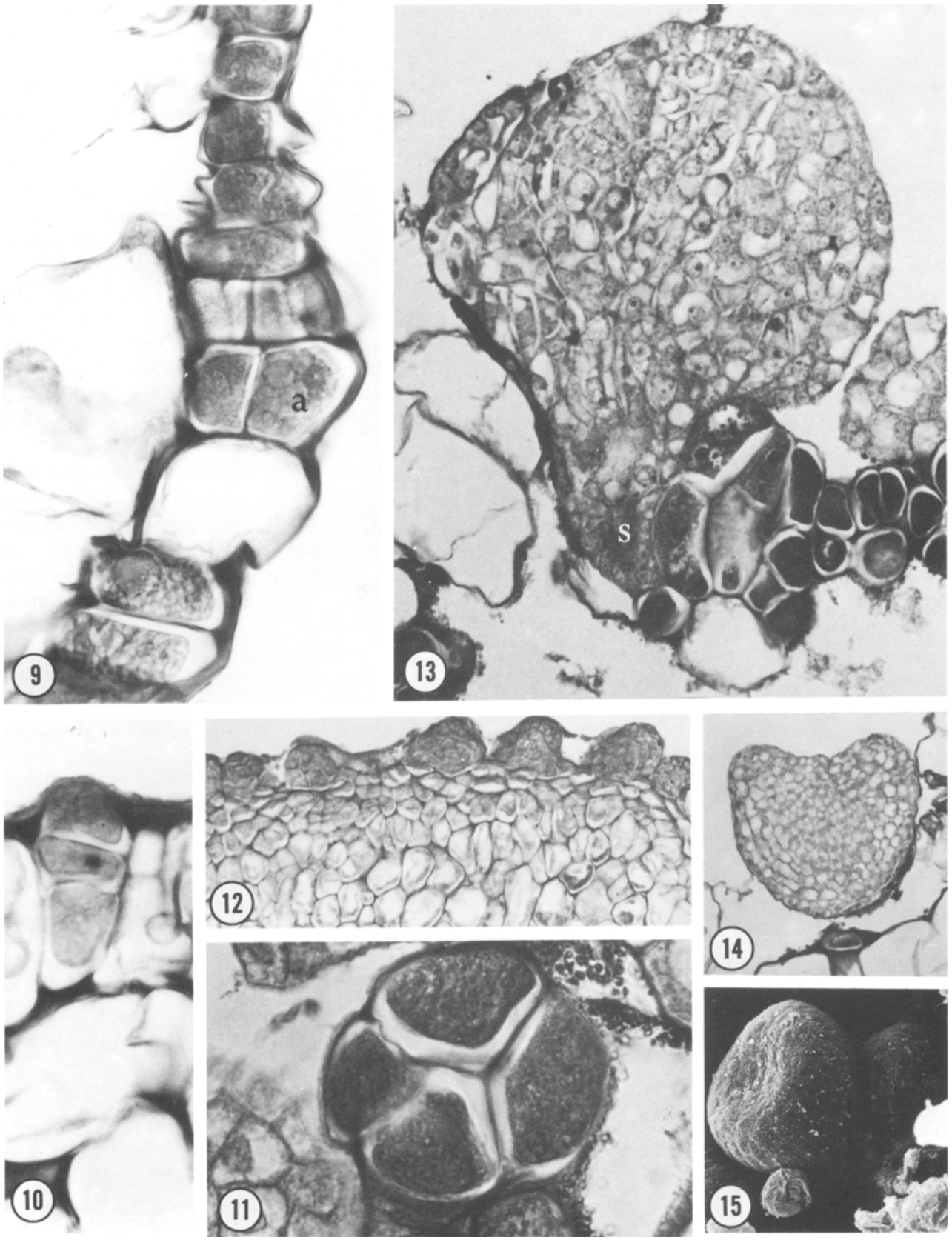
Fig. 11. A four-celled (or possibly 8-celled) globular embryoid developing on a hypocotyl (× 585)

Fig. 12. Seven adjacent globular embryoids at the surface of the hypocotyl (× 85)

Fig. 13. A globular embryoid with a multicellular suspensor, *s*, from T.S. cotyledon of an existing embryoid (× 315)

Fig. 14. L.S. embryoid in friable callus, showing an early heart-shaped stage (× 190)

Fig. 15. SEM of globular and early heart-shaped embryoids on callus (× 212)



Figs. 9-15

early stages of somatic embryogenesis were more readily observed in these epidermal sites. The ability of epidermal cells to form embryoids along the hypocotyl of plantlets derived from callus cultures was also demonstrated in *Ranunculus sceleratus* (KONAR and NATARAJA 1965, KONAR *et al.* 1972 b), *Daucus carota* (HACCIUS and LAKSHMANAN 1969), and *Brassica napus* (THOMAS *et al.* 1976). BROERTJES (1972) observed division of epidermal cells at the base of the petiole of *Saintpaulia*, which superficially resembled early stages in embryoid development from cotyledons of *Medicago*. But adventitious buds developed in *Saintpaulia*, and although they originated ultimately from a single epidermal cell the subsequent pathway of development differed fundamentally from that of embryoids. Embryoids arising on the cotyledon surface were also reported in cultures of zygotic embryos of different stages in *Biota orientalis* (KONAR and OBEROI 1965), and in *Ilex aquifolium* (HU and SUSSEX 1971, HU *et al.* 1978). In most instances the origin and development of the embryoids were not traced histologically.

Observations of somatic embryogenesis in *Medicago* with both light and scanning electron microscopes have indicated the close similarities between zygotic embryos and embryoids arising in culture, at all stages of development. Preliminary information on the development of the early stages of the zygotic embryos in alfalfa can be found in MARTIN (1914). Later, COOPER (1935) and FRIDRIKSSON and BOLTON (1963) traced in detail the zygotic embryology of *Medicago sativa*. The zygote divides periclinally to form a 2-celled proembryo consisting of an enlarged basal cell and a much smaller apical cell (COOPER 1935). Subsequently, a 3, 5, and 6-celled linear proembryo is formed (COOPER 1935, FRIDRIKSSON and BOLTON 1963).

The first and subsequent divisions observed in somatic embryogenesis of *Medicago sativa* were also periclinal, forming 2, 3, and 4-celled proembryoid stages, well seen in structures which arose from cotyledon surfaces. The main difference, compared to zygotic embryogenesis, was that the first division produced either two cells equal in size, or an apical cell which was slightly larger than the basal cell. It appears that the globular embryo is formed from the apical cell, and that the subapical cells together with the basal cell divide to form the short suspensor. The 3-celled linear proembryoid stage was frequently observed on the surface of cotyledons. However, on the hypocotyl it was not always possible to define the plane of the first division. A 4-celled spherical stage was commonly observed, and the 3-celled linear proembryoid was not seen as frequently. The 3-celled proembryo as described by COOPER (1935), consists of an enlarged basal cell, a central cell and a smaller cell. Protoderm and provascular strands became differentiated in the embryoid in the same way as in the zygotic embryo (COOPER 1935).

Variation in the development of embryoids occurs in *M. sativa*, again in agreement with the pattern of embryo development *in vivo* (COOPER 1935).

In zygotic embryos the suspensor is filiform, whereas in globular, heart and torpedo-shaped embryoids it was reduced and multicellular. However, a distinct demarcation between embryo and suspensor is not always clear even *in vivo*; for example, in *Trifolium pratense* the suspensor was observed to be a massive structure (MARTIN 1914).

The SEM has not yet been widely used in studies of somatic embryogenesis. Recently, it has been applied, as a tool, to follow somatic embryogenesis in several monocotyledons, for example *Pennisetum purpureum* (HAYDU and VASIL 1981) and *P. americanum* (VASIL and VASIL 1982), *Panicum maximum* (LU and VASIL 1981), and *Triticum aestivum* (OZIAS-AKINS and VASIL 1982). It was also used to observe more closely the ontogeny of subsequent stages of somatic embryogenesis in *Nicotiana tabacum* (WEATHERHEAD and GROUT 1979) and *Coffea arabica* (SONDAHL *et al.* 1979). Embryoids which developed from pollen in cultured anthers of *Hyoscyamus niger* were also observed (DODDS and REYNOLDS 1980). In the present work on *Medicago* the SEM proved useful both to study early globular stages of embryoid development on callus and on the surface

Fig. 16. SEM of callus with well developed torpedo shaped embryoids with 2 cotyledons; *s* suspensor (× 16)

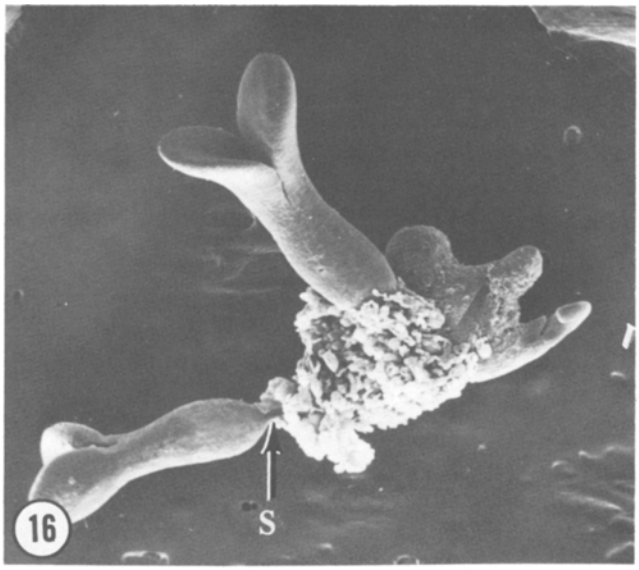
Fig. 17. SEM of callus and embryoids similar to those in Fig. 16, except that the embryos have 5 or 6 cotyledons (*c*) (× 18)

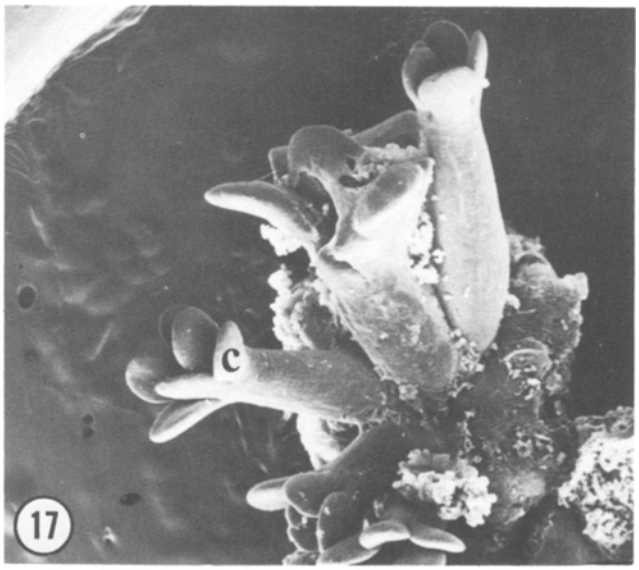
Fig. 18. L.S. embryoid with well developed cotyledons. Note the thick region of attachment to the callus (× 66)

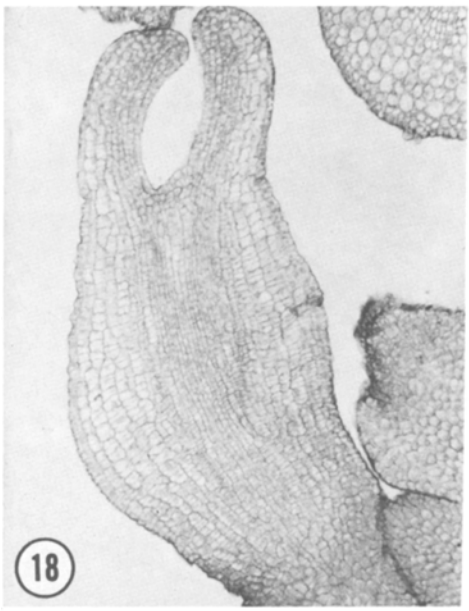
Fig. 19. SEM of two slightly anomalous embryoids on the cotyledon surface. The region of attachment (*r*) is broad, and the embryoids are dominated by their cotyledons (× 160)

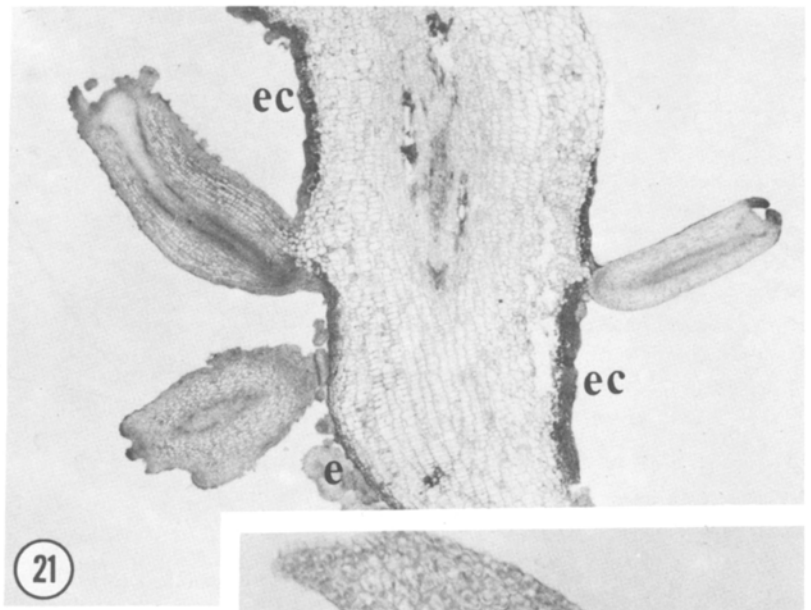
Fig. 20. An embryoid in the cotyledonary stage, attached somewhat laterally to a cotyledon (× 120)

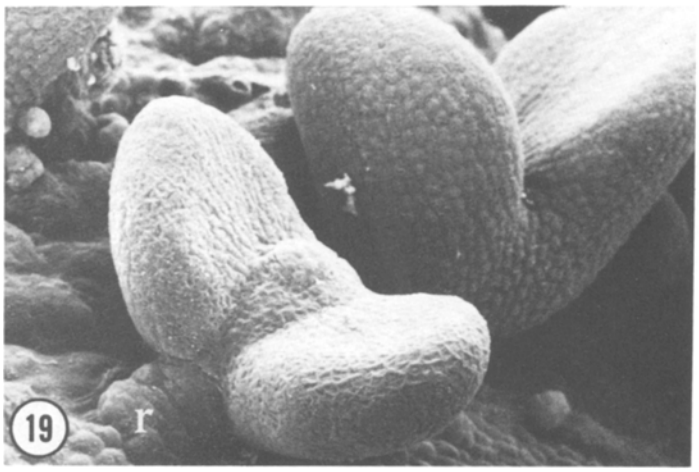
Fig. 21. L.S. hypocotyl with adventive embryoids in various stages; *e* mass of young embryoids; *ec* embryogenic cells. (× 16)

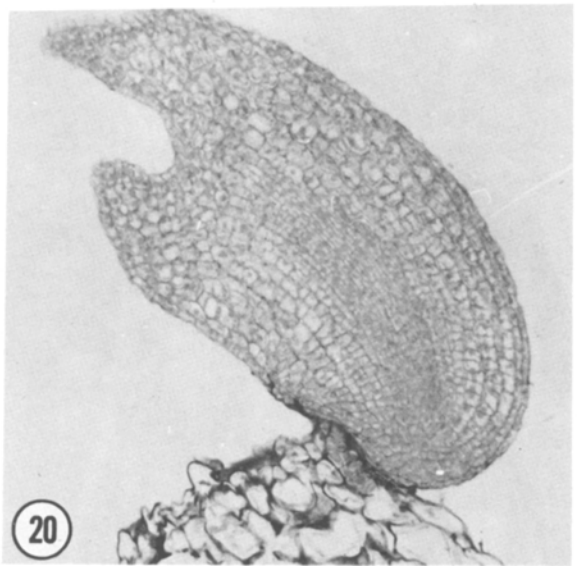












Figs. 16-21

of plantlets, and to confirm similarities between the pathway of development of zygotic embryos and embryoids. In particular, study with the SEM emphasised the large number of embryoids, and the diversity of stages of development, and provided a way to recognise the occasional abnormalities to be found on a small piece of tissue grown in culture. Scanning electron micrographs undoubtedly provide some additional insight into developmental studies of somatic embryogenesis.

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References

Backs-Hüsemann, D., Reinert, J., 1970: Embryobildung durch isolierte Einzelzellen aus Gewebekulturen von *Daucus carota*. Protoplasma **70**, 49–60.

Broertjes, C., 1972: Use in plant breeding of acute, chronic or fractionated doses of X-rays or fast neutrons as illustrated with leaves of *Saintpaulia*. Agric. Res. Repts. 776, Centre for Agricultural Publishing and Documentation, Wageningen.

Cooper, D. C., 1935: Macrosporogenesis and embryology of *Medicago*. J. Agric. Res. **51**, 471–477.

Danilina, A. N., 1972: Morphogenesis in tissue cultures of *Daucus carota*. Phytomorphology **22**, 160–164.

Dodds, J. H., Reynolds, T. L., 1980: A scanning electron microscope study of pollen embryogenesis in *Hyoscyamus niger*. Z. Pflanzenphysiol. **97**, 271–276.

Fridriksson, S., Bolton, J. L., 1963: Development of the embryo of *Medicago sativa* L. after normal fertilization and after pollination by other species of *Medicago*. Can. J. Bot. **42**, 23–33.

Haccius, B., Lakshmanan, K. K., 1969: Adventiv-Embryonen – Embryoide – Adventiv-Knospen. Ein Beitrag zur Klärung der Begriffe. Österr. bot. Z. **116**, 145–158.

Halperin, W., Jensen, W. A., 1967: Ultrastructural changes during growth and embryogenesis in carrot cell cultures. J. Ultrastruct. Res. **18**, 428–443.

Haydu, Z., Vasil, I. K., 1981: Somatic embryogenesis and plant regeneration from leaf tissues and anthers of *Pennisetum purpureum* Schum. Theor. appl. Genet. **59**, 269–273.

Hu, C. Y., Ochs, J. D., Mancini, F. M., 1978: Further observations on *Ilex* embryoid production. Z. Pflanzenphysiol. **89**, 41–49.

– Sussex, I. M., 1971: *In vitro* development of embryoids on cotyledons of *Ilex aquifolium*. Phytomorphology **21**, 103–107.

Johnson, L. B., Stuteville, D. L., Higgins, R. K., Skinner, D. Z., 1981: Regeneration of alfalfa plants from protoplasts of selected Regen S clones. Plant Sci. Lett. **20**, 297–304.

Kao, K. N., Michayluk, M. R., 1980: Plant regeneration from mesophyll protoplasts of alfalfa. Z. Pflanzenphysiol. **96**, 135–141.

Konar, R. N., Nataraja, K., 1965: Experimental studies in *Ranunculus sceleratus* L. Development of embryos from the stem epidermis. Phytomorphology **15**, 132–137.

– – 1969: Morphogenesis of isolated floral buds of *Ranunculus sceleratus* L. *in vitro*. Acta Bot. Neerl. **18**, 680–699.

– Oberoi, Y. P., 1965: *In vitro* development of embryoids on the cotyledons of *Biota orientalis*. Phytomorphology **15**, 137–140.

– Thomas, E., Street, H. E., 1972a: The diversity of morphogenesis in suspension cultures of *Atropa belladonna* L. Ann. Bot. **36**, 249–258.

– – – 1972b: Origin and structure of embryoids arising from epidermal cells of the stem of *Ranunculus sceleratus* L. J. Cell Sci. **11**, 77–93.

Lu, C., Vasil, I. K., 1981: Somatic embryogenesis and plant regeneration from leaf tissues of *Panicum maximum* Jacq. Theor. appl. Genet. **59**, 275–280.

Martin, J. N., 1914: Comparative morphology of some *Leguminosae*. Bot. Gaz. **58**, 154–167.

McWilliam, A. A., Smith, S. M., Street, H. E., 1974: The origin and development of embryoids in suspension cultures of carrot (*Daucus carota*). Ann. Bot. **38**, 243–250.

Ozias-Akins, P., Vasil, I. K., 1982: Plant regeneration from cultured immature embryos and inflorescences of *Triticum aestivum* L. (wheat): evidence for somatic embryogenesis. Protoplasma **110**, 95–105.

Phillips, G. C., Collins, G. B., 1980: Somatic embryogenesis from cell suspension cultures of red clover. Crop. Sci. **20**, 323–326.

Raghavan, V., 1976: Experimental Embryogenesis in Vascular Plants. London-New York: Academic Press.

Rao, P. S., Narayanaswami, S., 1972: Morphogenetic investigations in callus cultures of *Tylophora indica*. Physiol. Plant. **27**, 271–276.

Reinert, H. J., 1958: Untersuchungen über die Morphogenese an Gewebekulturen. Ber. dtsch. bot. Ges. **71** (15).

– 1959: Über die Kontrolle der Morphogenese und die Induktion von Adventivembryonen an Gewebekulturen aus Karotten. Planta **53**, 318–333.

– Bajaj, Y. P. S., Zbell, B., 1977: Aspects of organization–organogenesis, embryogenesis, cytodifferentiation. In: Plant Tissue and Cell Culture (Street, H. E., ed.), pp. 389–427. Oxford-London: Blackwell Scientific Publications.

Santos, A. V. dos, Outka, D. E., Cocking, E. C., Davey, M. R., 1980: Organogenesis and somatic embryogenesis in tissues derived from leaf protoplasts and leaf explants of *Medicago sativa*. Z. Pflanzenphysiol. **99**, 261–270.

Sass, J. E., 1966: Botanical microtechnique. Ames, Iowa: Iowa State College Press.

Saunders, J. W., Bingham, E. T., 1972: Production of alfalfa plants from callus tissue. Crop. Sci. **12**, 804–808.

Sharman, B. C., 1943: Tannic acid and iron alum with safranin and orange G in studies of the shoot apex. Stain Technol. **18**, 105–111.

Sondahl, M. R., Salisbury, J. L., Sharp, W. R., 1979: SEM characterization of embryogenic tissue and globular embryos during high frequency somatic embryogenesis in coffee callus cells. Z. Pflanzenphysiol. **94**, 185–188.

Thomas, E., Hoffmann, F., Potrykus, I., Wenzel, G., 1976: Protoplast regeneration and stem embryogenesis of haploid androgenetic rape. Molec. gen. Genet. **145**, 245–247.

THOMAS, E., KONAR, R. N., STREET, H. E., 1972: The fine structure of the embryogenic callus of *Ranunculus sceleratus* L. J. Cell Sci. **11**, 95–109.

UCHIMIYA, H., MURASHIGE, T., 1974: Evaluation of parameters in the isolation of viable protoplasts from cultured tobacco cells. Plant. Physiol. **54**, 936–944.

VASIL, V., VASIL, I. K., 1982: Characterization of an embryogenic cell suspension culture derived from cultured inflorescences of *Pennisetum americanum* (pearl millet, *Gramineae*). Amer. J. Bot. **69**, 1441–1449.

WEATHERHEAD, M. A., GROUT, B. W. W., 1979: A scanning electron microscope study of embryoid development in the anthers of *Nicotiana tabacum* L. Ann. Bot. **44**, 631–632.

WETHERELL, D. F., 1978: *In vitro* embryoid formation in cells derived from somatic plant tissues. In: Propagation of higher plants through tissue culture: A bridge between research and application. U.S.A. Dept. of Energy. 102–124.