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Somatic Embryogenesis and Plant Regeneration from Leaf Tissues of *Panicum maximum* Jacq.*

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Summary. Somatic embryogenesis was induced in proliferating leaf segments of *Panicum maximum* Jacq., cultured on Murashige and Skoog's nutrient medium containing 2,4-dichlorophenoxyacetic acid and coconut milk. The embryoids gave rise to plants on a medium containing gibberellic acid. The plants were successfully transplanted to soil and grown to maturity. Histological examination of proliferating leaves showed that the embryogenic callus tissue was formed by divisions in cells of the lower epidermis as well as the mesophyll tissue. The regenerated plants showed the normal chromosome number of 2n = 4x = 32.

Key words: Cereals/grasses – Guinea grass – *Panicum maximum* Jacq. – Plant regeneration – Somatic embryogenesis

Abbreviations

BAP, benzylaminopurine; CM, coconut milk; GA, gibberellic acid; NAA, naphthaleneacetic acid; 2,4-D, 2,4-dichlorophenoxyacetic acid

Introduction

Plant regeneration has been reported from tissue cultures derived from embryos, seedlings or inflorescences of most of the important species of cereals and many grasses (Thomas et al. 1979; I.K. Vasil and Vasil 1980). In most instances regeneration takes place by the organization of shoot meristems and buds (Rangan 1974; Chen et al. 1977; Nakano and Maeda 1979; Shimada and Yamada 1979; Springer et al. 1979), which tend to be multicellular in origin. The culture of excised leaf tissues has proved most difficult (Cocking 1978), the only major exception being *Saccharum officinarum* (sugarcane) where callus tissue cultures derived from young leaves can be routinely induced to undergo organogenesis in vitro leading to plant formation (Heinz and Mee 1969). In cereals, Saalbach and Koblitz (1978) reported obtaining a single shoot from cultured young leaf tissues of *Hordeum vulgare* (barley). These experiences, and the extreme recalcitrance of cereal/grass mesophyll protoplasts to undergo sustained cell division in vitro, have given rise to serious doubts regarding the totipotency of cells of cereal/grass leaves (Potrykus 1980; Wernicke and Brettell 1980). The recent reports of somatic embryogenesis and plant regeneration from leaves of *Sorghum bicolor* (Wernicke and Brettell 1980) and *Pennisetum purpureum* (Haydu and Vasil 1981) are, therefore, encouraging. In this report we describe the induction of somatic embryogenesis and the recovery of normal plants from the leaves of *Panicum maximum* Jacq. (Guinea grass).

Materials and Methods

Three genotypes of *Panicum maximum* Jacq. (PM44, PM49 and PM53) were used in the present experiments. These genotypes were selected because they reproduce sexually, and not apomictically, as most of the genotypes of this species. The plants were grown in pots in the green house. Shoots removed from adult plants were sterilised, after removing the outermost leaves, in 70% ethanol for 1 min., followed by 20% chlorox for 10 min. The sterilised shoots were washed five times with sterile distilled water. After removal of several of the outer layers of leaves, the inner whorled leaves were cut into 2 mm thick transverse segments, starting at the level of the shoot meristem and going above. Segments of 1-4 of the youngest whorled leaves were placed together in culture, and no parts of the stem, shoot meristem or any axillary buds were used. Murashige and Skoog's (1962) nutrient medium (MS), containing 3% sucrose and gelled with 0.8% agar, was used in all the experiments. The pH of the medium was adjusted to 5.8, and it was sterilised by autoclaving and then distributed in 35 × 10 mm Falcon Petri dishes. Induction of callus tissues from leaf explants was obtained in MS medium supplemented with

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2,4-D and CM, in the dark at 27°C. Embryoids formed in this medium were transferred to MS medium containing 1 mg/l GA, and incubated at 27°C with 16 hr of light, to obtain plantlets. The plantlets were transferred to MS medium with only half the concentration of inorganic components for the establishment of a vigorous root system. Finally, the plants were transplanted to potting soil, acclimatised for a few days in the growth chamber, and then moved to the green house and grown to maturity.

For histological studies tissues were fixed in formalin-acetic-alcohol, dehydrated in a tertiary-butyl alcohol series, and embedded in paraplast. Serial sections were cut at 10 μm, and stained with safranin-fast green. Small pieces of cultured leaf segments as well as embryoids formed in vitro were fixed in 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) for 2 hr. at room temperature. The fixed tissue was rinsed with buffer for 30 min., and post-fixed in 1% osmium tetroxide overnight. After dehydration through a graded alcohol series, the samples were critical point dried and coated with gold. The specimens were examined and photographed in a Hitachi S-450 Scanning Electron Microscope, operating at 20 KV.

Results

Callus tissue first became visible at the cut ends of the leaf explants within a week on MS medium containing 2.5-10 mg/l 2,4-D and 5-15% CM. No significant differences were found in callus formation within the range of concentrations of 2,4-D and CM used, but no organised callus tissue was formed in the absence of 2,4-D, with or without CM. Addition of CM was not essential for organised growth of callus. A 3 cm long region of the leaf, starting from about the level of the shoot meristem, was found to be most responsive for the induction of callus formation. After one week, the area of the veins also became swollen, followed by the appearance of callus tissue along the abaxial (lower) surface of leaf segments (Figs. 1-4). No callus tissue was formed on the adaxial (upper) surface. When more than one whorled leaf was cultured, callus tissue was formed primarily on the abaxial surface of the outermost (oldest) leaf, while the inner and younger leaves gave rise to little, if any, callus tissue. The initial callus tissue formed at the cut ends of the leaves was soft and transluscent, but a compact callus tissue was formed on the abaxial surface of the leaf segments opposite to the vascular bundles. After about two weeks, localised areas of a white and embryogenic callus and many embryoids appeared on the surface of the compact callus tissue. The white color of the compact and embryogenic callus tissue was probably caused by the accumulation of starch grains in its cells. In 3-4 weeks after the initiation of culture, numerous embryoids at different stages of development were seen scattered along the surface of the embryogenic callus tissue on the abaxial surface of the leaf. Each leaf segment gave rise to 20-40 embryoids, which showed the typical organization of grass embryos (Figs. 4, 5). The callus tissue obtained from the leaf explants was sub-cultured at two week intervals, and has retained its capacity to form embryoids for more than three months without any significant loss of embryogenic potential.

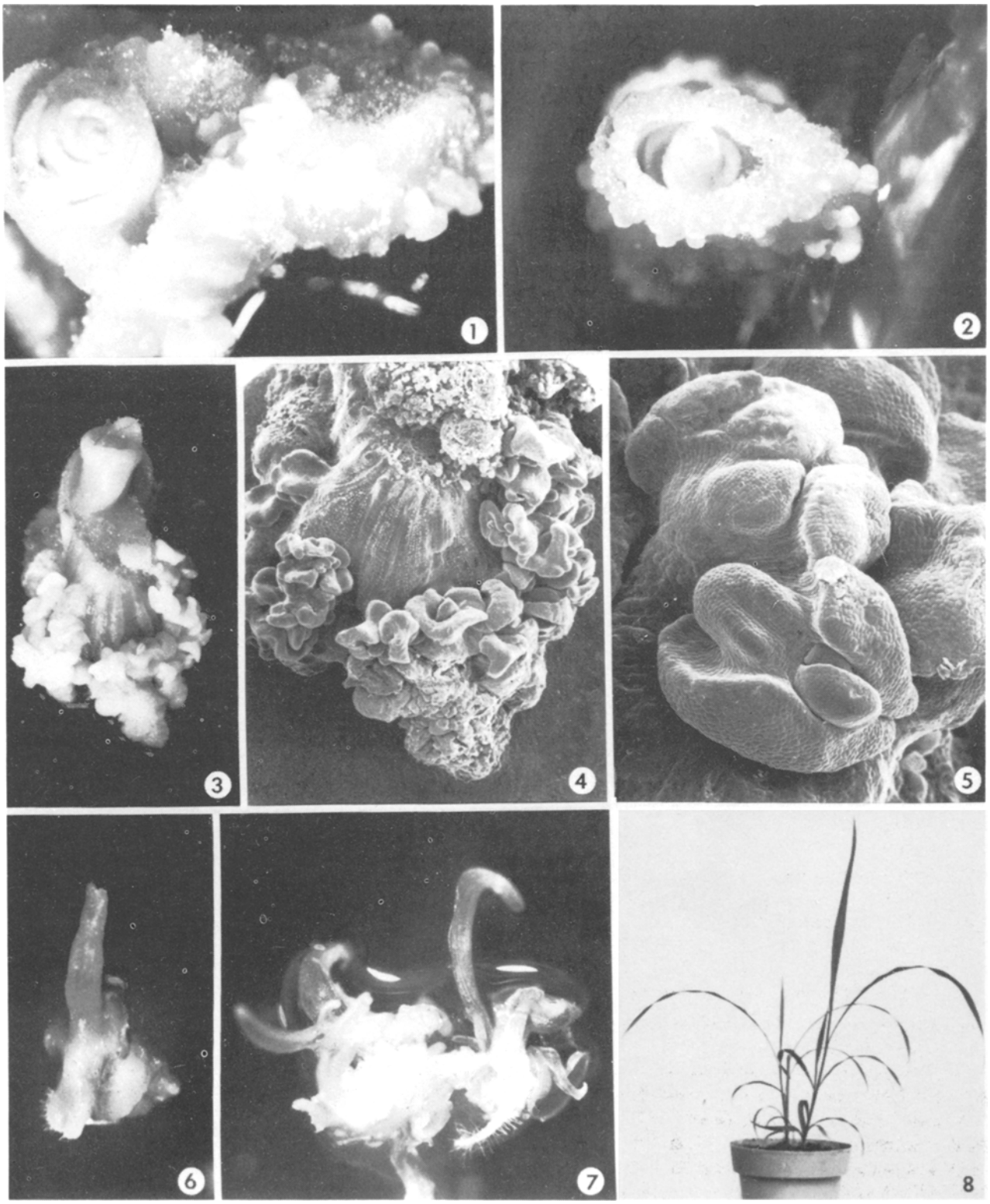
The embryoids formed on the medium containing 2,4-D and CM were transferred to MS medium supplemented with 1 mg/l GA. Most of the embryoids matured and germinated to form plantlets (Figs. 6, 7). Addition of CM to this medium did not support the continued development and germination of embryoids. On a medium supplemented with 0.2 mg/l NAA, only about 20% of the embryoids germinated. The plantlets obtained on the GA medium were transferred to culture tubes containing MS medium with half the amount of inorganic components. After one to two weeks the plantlets had formed a vigorous root system and several leaves, and were transplanted to potting soil (Fig. 8). Finally, the plants were moved to the green house and grown to maturity. The regenerated plants were shown to have the normal chromosome number of $2n = 4x = 32$. No phenotypic variation was observed.

Histological examination of cultured leaf segments showed no cell divisions in the upper epidermis or the tissues contained in the upper half of the leaf blade (Figs. 9-16). The compact, white and embryogenic callus tissue was formed by cell divisions in the lower epidermis, as well as in the mesophyll cells located on either side of the vascular bundles. In some leaves the mesophyll tissue formed the compact organised callus while the epidermal cells did not divide. In other instances cells of the mesophyll tissue formed a soft and friable callus tissue (Fig. 15), which showed no organised growth. Organised structures and embryoids were formed only on the surface of the compact callus tissue (Figs. 14, 16, 17).

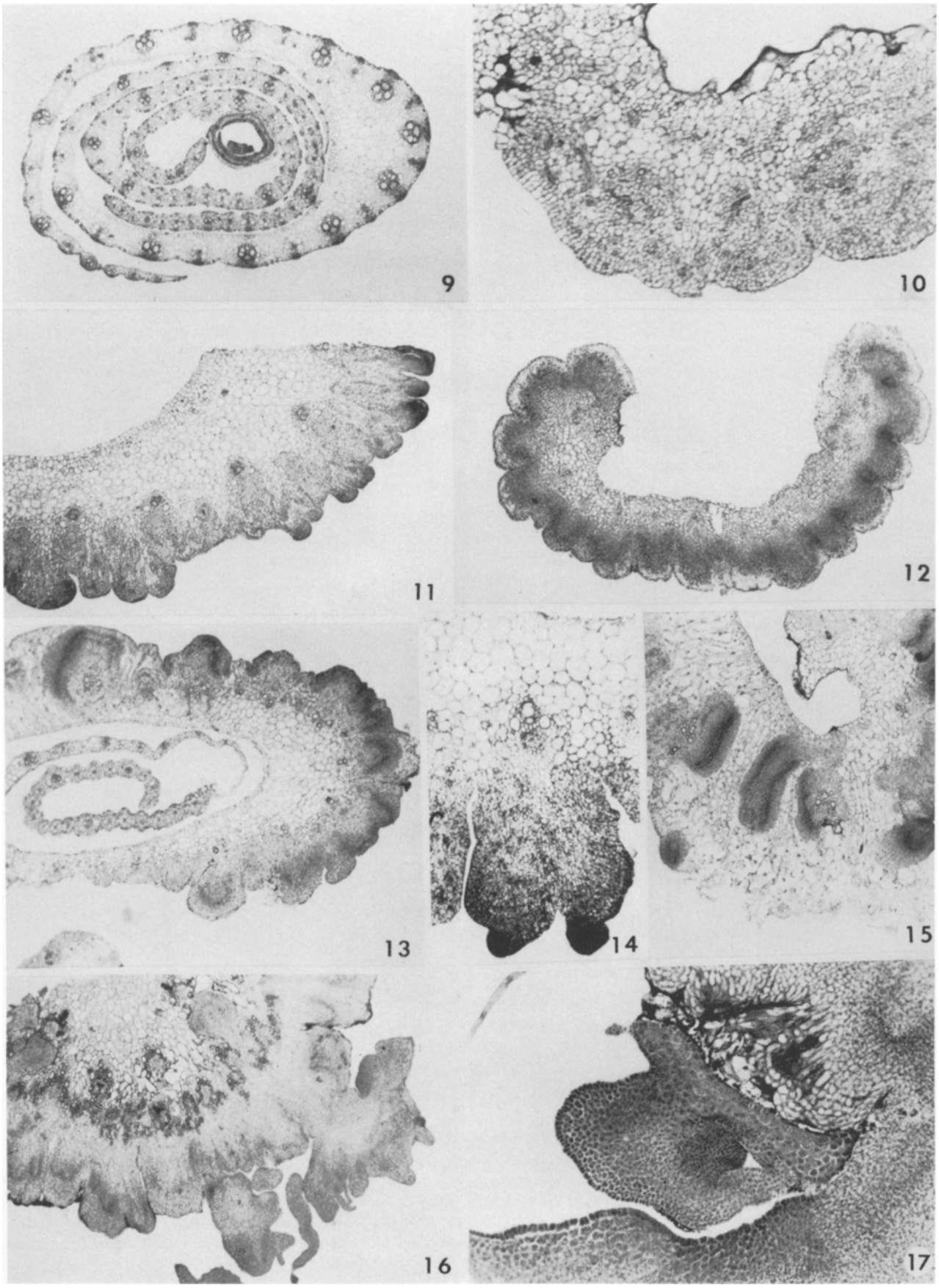
Leaves which gave rise to callus, embryoids and plants were yellowish-green in color and structurally developed, showing a well-developed mid-rib, vascular bundles, characteristic groups of sclerenchyma cells, and mesophyll cells with plastids (Fig. 9). They were still in a whorled condition and not fully expanded. Younger leaves, in which structural development had not been completed, did not form any callus (Fig. 13). The third and the fourth leaves, away from the shoot apex, appeared to be

Table 1. Callus formation from leaves of different genotypes of *Panicum maximum* cultured on Murashige and Skoog's (1962) medium containing 10 mg/l 2,4-dichlorophenoxyacetic acid and 15% coconut milk

Genotype	% leaf segments forming callus	% leaf segments forming embryogenic callus
PM44	50	16
PM49	48	2
PM53	1.2	0



Figs. 1-8. Somatic embryogenesis and plant regeneration from leaf segments of *Panicum maximum*. **1, 2** Two week old cultured leaf segments. White and compact embryogenic callus can be seen on the abaxial surface of the outermost leaves (× 20; × 12); **3** Three week old cultured leaf segments showing swollen veins and many embryoids (× 10); **4** Scanning electron micrograph of part of the leaf shown in Figure 3 (× 18); **5** Typical well organised embryoid with a scutellum, coleoptile and coleorhiza (× 112); **6** Germination of embryoid after three days on MS medium containing GA (× 38); **7** Plantlets after one week on MS medium with GA (× 10); **8** Plants regenerated via somatic embryogenesis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most responsive to the induction of callus and morphogenesis leading to the formation of embryoids and plants. Although segments obtained from the leaf blade as well as the leaf sheath gave rise to callus, embryoids and plants, the leaf sheath region appeared to have a better capacity for callus formation and somatic embryogenesis.

The three genotypes used in our experiments showed marked differences in their ability to produce callus tissues from leaf segments (Table 1).

Discussion

The white, compact and embryogenic callus tissue obtained from cultured leaf segments of *Panicum maximum* is similar to the callus tissues obtained from the scutellum of immature embryos and young inflorescences of *P. maximum* described by us earlier (Lu and Vasil 1981a). The embryoids obtained from the leaf callus tissue show the typical organization of cereal/grass embryos.

Cells of the lower epidermis and mesophyll cells from the lower half of the leaf divide to produce callus tissue. Cells of the upper epidermis or in the upper half of the leaf did not show any capacity for cell proliferation. Also, when more than one whorled leaf was placed in culture, only the outermost leaf formed callus while the inner and younger leaves failed to proliferate. Very young leaves, which had not completed structural differentiation, did not form any callus tissue. The reasons for these patterns of behaviour are not understood.

Clonal propagation of many horticultural species is now routinely achieved by the culture of leaf segments and the formation of shoot buds either directly or after an intervening callus stage. However, the induction of callus formation from leaves of cereals/grasses has proved most difficult, and it has been suggested that the difficulties encountered in the culture of cereal mesophyll protoplasts might be better understood or resolved by the successful culture of leaf segments and by determining the conditions required for good growth of the resulting callus tissue (Cocking 1978). The present experiments clearly show that cells of the leaves of *Panicum maximum* not only can be induced to proliferate in vitro, but can also be induced to form somatic embryos and plants. It will be interesting to see if embryogenic suspension cultures – like those obtained from immature embryos and inflorescences of *P. maximum* (Lu and Vasil 1981b) – can also be isolated from leaf callus. Such cultures provide a good source of protoplasts as shown by the successful culture of protoplasts isolated from embryogenic suspension cultures of *Pennisetum americanum* (V. Vasil and Vasil 1980).

The formation of somatic embryos, which are said to be of single cell origin (Haccius 1978), is of rare occurrence in cereals/grasses. In a number of recent reports from our laboratory, we have described the successful induction of somatic embryogenesis and plant regeneration from tissue cultures obtained from immature embryos, inflorescences and suspension cultures of *Pennisetum americanum* and inflorescences of *P. americanum* × *P. purpureum* (V. Vasil and Vasil 1981a, b), immature embryos, inflorescences and suspension cultures of *Panicum maximum* (Lu and Vasil 1981a, b), and leaf tissues of *Pennisetum purpureum* (Haydu and Vasil 1981). These results emphatically establish the fact that many differentiated cells of the cereal/grass plant, like those of numerous dicotyledonous and monocotyledonous species, are indeed totipotent.

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Figs. 9-17. Histology of somatic embryogenesis in *Panicum maximum* leaf culture (transections of leaves with abaxial surface facing down). **9** Whorled leaves at time of culture (× 106); **10** Radial files of cells produced by divisions in the lower epidermis (× 270); **11** Swollen veins resulting from divisions in cells of the lower epidermis and the mesophyll tissue (× 105); **12** Callus formed by mesophyll tissue only (× 105); **13** Callus formation on the abaxial surface of the outermost leaf. The younger inner leaf did not form any callus (× 105); **15** Soft and unorganised callus formed by mesophyll tissue (× 105); **14, 16** Organised structures and embryoids on the surface of the compact callus (× 225, × 105); **17** Embryoid attached to the surface of compact leaf callus (× 270)

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