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Somatic Embryogenesis and Plant Regeneration from Leaf Tissues and Anthers of *Pennisetum purpureum* Schum.*

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Summary. Explants obtained from leaves of *Pennisetum purpureum* Schum. gave rise to callus tissues when cultured on Murashige and Skoog's nutrient medium containing only 2,4-dichlorophenoxyacetic acid (2,4-D), or 2,4-D supplemented with naphthalene-acetic acid and/or 6-benzylaminopurine. Embryoids were formed in the white, compact and highly organised areas of the callus and developed into plants. Histological examination of cultured leaf segments showed that the callus tissue was formed by division in cells of the mesophyll tissue as well as the lower epidermis. A rapidly growing embryogenic callus tissue was also obtained apparently from the somatic tissues of anthers of *P. purpureum*, and induced to form plants through somatic embryogenesis. Plants obtained from leaf and anther culture were grown to maturity, and were shown to have the normal chromosome number of 2n = 4x = 28.

Key words: Cereals/grasses – Elephant or napier grass – *Pennisetum purpureum* – Plant regeneration – Somatic embryogenesis

Introduction

Leaves of graminaceous species provide a good material for the isolation of protoplasts. However, the culture of cereal leaf protoplasts has proved to be most frustrating, and sustained cell divisions have not been obtained from mesophyll protoplasts of any cereal/grass species (Potrykus et al. 1976; Galston 1978; Potrykus 1980; I.K. Vasil and Vasil 1980). It has not been possible in most instances even to induce callus formation from cereal leaves (Bhojwani and Hayward 1977; Bhojwani et al. 1977; Cocking 1978; O'Hara and Street 1978). Such extreme recalcitrance of cereal leaf cells to divide in vitro has given rise to doubts about their totipotency (Wernicke and Brettell 1980), which must be expressed if cereal leaf cells are to be used successfully in somatic hybridization and genetic modification experiments. Saalbach and Koblitz (1978) reported the formation of callus from cultured leaf explants of *Hordeum vulgare*, and were able to obtain a single plant in their experiments. More recently, Wernicke and Brettell (1980) have obtained somatic embryos and plantlets from segments of seedling leaves of *Sorghum bicolor*. This paper describes the formation of callus, embryoids and plants from leaf and anther tissues of *Pennisetum purpureum* Schum., Napier or Elephant grass. The induction of somatic embryogenesis and plant regeneration from cultured leaf tissues of *Panicum maximum* has also been described from our laboratory (Lu and Vasil 1981c).

Materials and Methods

Shoots of twenty-one genotypes of *Pennisetum purpureum* Schum. (PP1 to PP10 and PP12 to PP22, selected and maintained by Dr. S.C. Schank), Elephant or Napier grass, were obtained from adult plants growing in the green house or the field. All of the older and fully mature leaves were removed, and the remaining part of the shoot – including two visible nodes and several of the younger leaves which were still whorled around each other and were pale yellow in color – was sterilised by immersion in 70% ethanol for 30 sec., followed by 4 min. in 20-50% Clorox solution and washed five times with sterile distilled water. Leaves present above the level of the shoot meristem were cut into 3-5 mm thick segments, and placed on Murashige and Skoog's (1962) nutrient medium (MS) in Falcon Petri dishes (35 × 10 mm) or Pyrex culture tubes (25 × 100 mm). The medium contained 3 to 6% sucrose, 0.8% agar, and was sterilised by autoclaving after adjusting the pH to 5.8. In various experiments the medium was supplemented with one or more of the following: 2,4-dichlorophenoxyacetic acid (2,4-D), naphthalene acetic acid (NAA), benzylaminopurine (BAP), 5% coconut milk and 0.4 mg/l thiamine. Expanded green leaves obtained from plants formed in vitro in anther and leaf callus tissue cultures were also cut into 3-5 mm segments and cultured.

Young inflorescences, obtained at or soon after the flag leaf stage, were sterilised by the same procedure as used for shoots. Anthers were dissected out from individual florets, and cultured on

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MS medium containing half the concentration of mineral salts, 6% sucrose, 5% coconut milk, 0.4 mg/l thiamine, and supplemented with various combinations of 2,4-D (0.25 and 0.5 mg/l), 1 mg/l NAA, 0.5 mg/l BAP and 0.01 mg/l kinetin.

Chromosome counts were made from squashed root tips obtained from regenerated plants and pretreated with a saturated solution of α-bromonaphthalene for 1 hr, fixed in 3:1 ethanol:acetic acid for 24 hr, and stained with Feulgen solution. For histological studies tissues were fixed in formalin-acetic-alcohol, dehydrated in a tertiary-butyl alcohol series, embedded in Paraplast, sectioned at 10 μm, stained with safranin-fast green, dehydrated and mounted in Permount.

For scanning electron microscopy, tissues were fixed in 2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) for 2 hr at room temperature, rinsed with buffer for 30 min., and post-fixed overnight in 1% osmium tetroxide. After dehydration through a graded alcohol series, the samples were critical point dried and coated with gold. The material was examined and photographed in a Hitachi S-450 Scanning Electron Microscope, operating at 20 KV.

Results

1 Callus Induction and Plant Regeneration from Leaf Explants

Callus tissue was formed from cultured leaf segments in 4-15 days on MS medium supplemented with 0.5-10 mg/l 2,4-D (Figs. 1-5). The frequency of callus formation varied considerably, but in a majority of the experiments 70-100% of the cultured leaf segments formed callus at all of the concentrations of 2,4-D used. Callus was obtained from leaf segments of all 21 genotypes used.

Three types of callus tissue, with varying morphogenetic potential, were obtained: (a) Compact Callus – a white, compact and highly organised callus tissue which was embryogenic in nature. (b) Soft callus – a pale-gray and/or yellowish-brown soft and friable callus. (c) Gelatinous callus – a yellowish-brown soft callus producing a gelatinous substance on its surface. The compact callus was formed on MS medium containing 0.5 mg/l 2,4-D. The soft callus was formed in a wide range of concentrations of 2,4-D (0.5-10.0 mg/l), but occasionally appeared to give rise to localised areas of white and compact callus when transferred to a medium containing 0.5 mg/l 2,4-D. Although the entire sequence of development from leaf to plant regeneration could be obtained on a medium containing 0.5 mg/l 2,4-D, the optimum results were obtained when the medium contained 0.5 mg/l 2,4-D, 0.5 mg/l BAP, 1 mg/l NAA and 5% CM (the same medium was used for the sub-culture and maintenance of the embryogenic callus tissue). The compact callus was comprised of tightly packed small and richly cytoplasmic cells. Disc and cup-shaped structures were seen to arise on the surface of the compact callus tissue (Fig. 10). These differentiated into the scutellum of embryoids. Later a tubular coleoptile appeared in the centre of the scutellar cup (Figs. 6, 11). The embryoids formed in vitro germinated to give rise to plants (Fig. 7). Almost all of the embryoids formed in vitro germinated precociously. Young plantlets (1-5 cm) were transferred to a hormone-free medium in culture tubes to establish a more vigorous root system, and after two weeks were transferred to a mixture of soil-vermiculite in pots and placed in a growth chamber for acclimatisation. Many of the regenerated plants were grown to maturity in the green house and/or in the field (Figs. 8, 9). The regenerated plants had the normal tetraploid chromosome number of $2n = 4x = 28$, and did not show any phenotypic abnormalities.

Rarely, white organised structures also appeared on the surface of callus formed at higher concentrations of 2,4-D (1.0-10.0 mg/l), but these failed to develop into organised embryos or plants. The soft and friable callus tissue did not normally undergo any organised growth, However, in some instances white and organised masses of callus tissue appeared on the surface of the soft callus and gave rise to embryoids and plants. The gelatinous callus tissue never showed any organised growth.

Histological examination of cultured leaf segments showed that the callus tissue was formed by divisions of mesophyll cells located in the lower half of the leaf blade, and cells of the lower epidermis. In rare instances divisions were seen in mesophyll cells present in the upper half of the leaf, and contributed to the formation of callus, but cells of the upper epidermis were never seen to divide. Cells of the lower epidermis formed only the compact type of callus tissue, while the mesophyll cells gave rise to soft as well as the compact callus tissue. The conditions which determine this dual behaviour of the mesophyll cells could not be determined, but are probably related to their developmental stage at the time of culture.

The basal 5-8 centimeters of the youngest 3-4 leaves were found to be most suitable for culture. The leaves were yellowish-green in color, and not fully expanded. However, they had completed their normal structural development and organisation, and showed fully formed vascular bundles, bundle sheaths, and groups of sclerenchyma cells. The leaves obtained from plants regenerated in vitro were fully expanded and green in color. Both types of leaf material proved to be conducive to the induction of callus formation and somatic embryogenesis. The compact embryogenic callus tissue showed vigorous growth in vitro. For example, from a single 5 mm leaf explant, more than 30 embryogenic callus cultures were obtained in six weeks, with the callus being sub-cultured every ten days. The embryogenic nature of the callus tissues remained unchanged during more than ten months of routine sub-culture (somatic embryos and plants from leaf tissues were first obtained in January, 1980). Somatic embryogenesis was observed in all genotypes used, except PP6 and PP7.

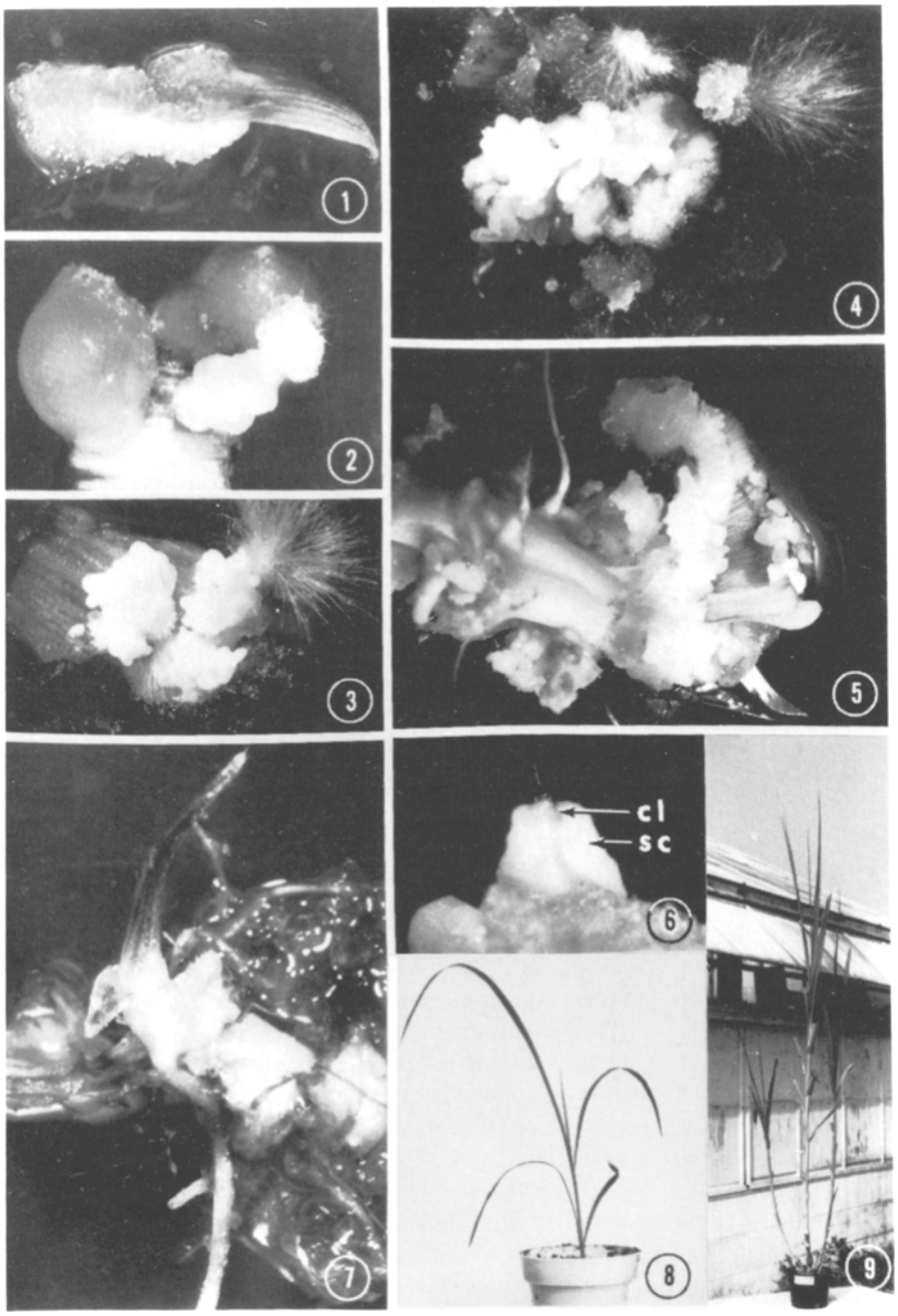
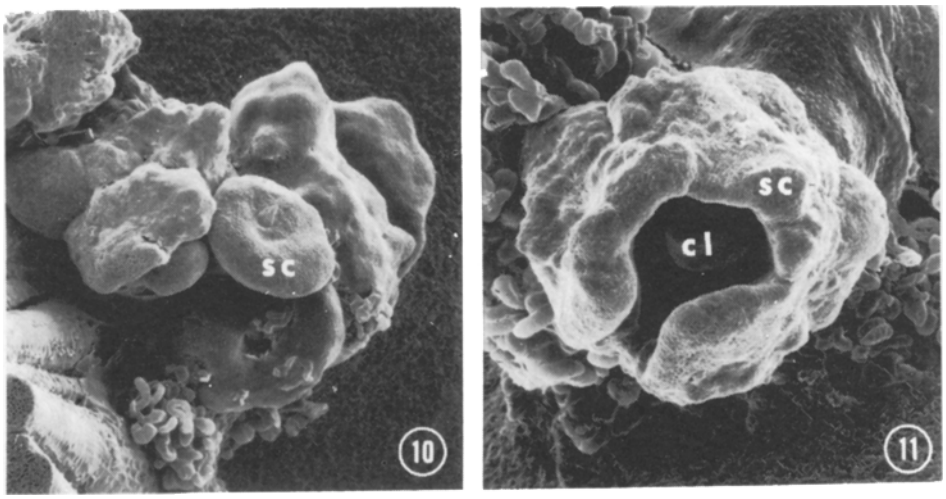


Fig. 1-9. Callus induction, somatic embryogenesis and regeneration of plants from leaf explants of *Pennisetum purpureum*. **1** Formation of soft callus tissue from expanded green leaf obtained from plants regenerated in vitro; **2** White and compact callus, and gelatinous callus obtained from leaf segment; **3-5** White, compact and embryogenic callus tissue obtained from leaf explants; **6** Organization of embryoid with coleoptile (cl) and scutellum (sc); **7** 'Germination' of embryoid and plantlet formation; **8,9** Plants obtained from leaf callus and embryoids



Figs. 10-11. Scanning electron micrographs of embryoid formation. **10** Organization of disc-shaped scutellum (sc). Note original leaf tissue in lower left corner (X 35); **11** Cup-shaped scutellum (sc) enclosing the coleoptile (cl) (X 60)

2 Callus Induction and Plant Regeneration from Anthers

Anthers were obtained from inflorescences at the flag leaf stage, and these were shown to contain microspores at about the time of first microspore mitosis. Callus tissue was formed in only 1% of the cultured anthers. All of these were non-morphogenic in nature, except the callus tissue obtained from a single anther on MS medium supplemented with 0.5 mg/l 2,4-D, 0.5 mg/l BAP and 1.0 mg/l NAA, which showed embryogenic competence. The callus tissue was white in color, compact and highly organised, like the compact callus tissue obtained from leaf segments, and formed hundreds of embryoids and plants in two months (December, 1979 to January, 1980). At least 90 of the plants were transferred to soil and grown in the green house. Root tip squashes of the regenerated plants showed $2n = 4x = 28$ chromosomes. No haploid, aneuploid or tetraploid plants were discovered in the regenerated population, and all of the plants showed normal morphological characteristics of the donor plants. It is, therefore, most likely that the callus was formed from the somatic tissues of the anther. The embryogenic callus tissue obtained from anthers has been subcultured for almost one year, and has shown no decline in its embryogenic potential.

Discussion

In *Sorghum bicolor* cells of the 'developing mesophyll, bundle sheath and occasionally epidermal cells close to lesions caused by excision' were shown to proliferate (Wernicke and Brettell, 1980). Optimum response was shown by the two youngest leaves in 10-day old seedlings. In *Hordeum vulgare* callus tissue originated from mesophyll tissue (Saalbach and Koblitz 1978). In both *Panicum maximum* (Lu and Vasil 1981c) and the present study, somatic embryos and plants were obtained from callus tissue formed by divisions in both the mesophyll tissue and the lower epidermal layer. The upper half of the leaf, particularly the upper epidermis, did not proliferate. Callus tissue was formed throughout the cultured leaf segments, and was not restricted to the areas of excision. Both in *Panicum* and *Pennisetum* the leaves were obtained from mature plants showing complete structural development of vascular bundles, mesophyll tissue, etc.; and in the latter even green and expanded leaves obtained in vitro were used successfully. These results clearly demonstrate the totipotency of cereal leaf tissues, including mesophyll cells. Whether information gained from these experiments can be usefully applied to the difficult problem of the culture of cereal mesophyll protoplasts remains to be seen. However, renewed efforts need to be made to isolate mesophyll protoplasts from the developmental stages of leaves that proved to be so useful in plant regeneration from leaf segments or from leaves formed in vitro. It would also be worthwhile to isolate an embryogenic suspension culture from the compact and embryogenic leaf callus tissue. Such an embryogenic suspension culture, isolated from immature embryos, proved to be crucial in the successful culture of protoplasts of *Pennisetum americanum* and the formation of somatic embryos and plantlets (V. Vasil and Vasil 1980).

In most of the work carried out in our laboratory on the culture of cereal species, we have found that the induction and isolation of a compact, white and organised callus tissue is the most critical factor in obtaining somatic embryogenesis in vitro. Such embryogenic callus tissues have been isolated from protoplasts, leaf explants, inflorescences, and immature embryos (Lu and Vasil 1981a, b, c; V. Vasil and Vasil 1980, 1981a, b). These results show that

totipotent cells are indeed present in the various tissues and organs of a cereal/grass plant during various stages of its development, and that these can be 'cloned' and maintained in vitro. In all instances embryoids were formed in the white and compact callus tissue, which closely resembled the callus tissue formed from proliferating scutellum of immature embryos.

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