SOMATIC EMBRYOGENESIS IN Panicum maximum Jacq. (GUINEA GRASS)

BY

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KEY TO ABBREVIATIONS

ABA = abscisic acid
BAP = benzylaminopurine
CM = coconut milk
2,4-D = 2,4-dichlorophenoxyacetic acid
2iP = 6(γ,γ-dimethylallylamino)-purine
GA = gibberellic acid
IAA = indole-acetic acid
KM = Kao and Michayluk's medium
MS = Murashige and Skoog's medium
NAA = naphthaleneacetic acid
MES = 2-(N-Morpholino)-ethane sulfonic acid
SLS = sodium lauryl sulfate
The principal objectives of this study were to obtain embryogenic tissue cultures from different explants of Panicum maximum (Guinea grass), to establish embryogenic suspension cultures, and to obtain totipotent protoplasts from these cultures for culture and regeneration.

Tissue cultures were initiated from leaves, immature embryos, mature embryos and young inflorescences of Panicum maximum Jacq., on Murashige and Skoog's medium supplemented with 2.5, 5, and 10 mg/1 2,4-dichlorophenoxyacetic acid (2,4-D). The resulting compact and white callus tissues were embryogenic in nature and formed many embryos. Calluses with embryos were transferred onto the same nutrient medium with gibberellic acid and 0.2 mg/1 2,4-D, or without 2,4-D. In cultures derived from leaf segments, immature embryos and young inflorescences, plantlets were produced via somatic embryogenesis after 3-5 weeks. These young plants were successfully transplanted to pots and grown in the greenhouse. Plant development in callus obtained from mature embryos took place through the organization
of shoot meristems. The regenerated plants were shown to have the normal tetraploid chromosome number of \(2n = 4x = 32\).

Embryogenic calluses derived from cultured immature embryos and young inflorescences of Guinea grass were placed in Murashige and Skoog's liquid medium supplemented with 1 mg/l 2,4-D and 2.5% coconut milk, to initiate suspension cultures. Suspensions were comprised of two types of cells, namely: small, richly cytoplasmic and starch-containing embryogenic cells and large vacuolated non-embryogenic cells. A presumed sequence of developmental stages from single embryogenic cells to globular and heart-shaped stages of embryogenesis was observed in the suspension cultures. Plantlets were produced when the suspensions were plated in an agar medium without any hormone or with only 0.2 mg/l 2,4-D or naphthaleneacetic acid. Embryogenic suspension cultures derived from immature embryos as well as from inflorescence segments gave rise to plants which also showed the normal somatic chromosome number of \(2n = 4x = 32\).

The embryogenic suspension cultures were treated with an enzyme mixture to remove the cell wall and release the protoplasts. The protoplasts were cultured in Kao and Michayluk's liquid medium supplemented with 0.2 mg/l 2,4-D and 0.5 mg/l zeatin. Cell divisions leading to embryoid and colony formation occurred in the liquid medium. Plantlets were formed via somatic embryogenesis when the cell colonies were transferred to MS agar medium.
GENERAL INTRODUCTION

The recent upsurge of interest and activity in plant cell and tissue culture techniques has been greatly stimulated by their presumed usefulness in the improvement of crop plants. Nevertheless, for the attainment of most of these oft-mentioned objectives—rapid multiplication of desired genotypes and the maintenance of genetic purity, selection of mutant/variant cell lines, incorporation of foreign genetic information into recipient cells, somatic hybridization, etc.—it is essential not only to be able to manipulate single plant cells but also to have the ability to regenerate plants from single cells. Some of these objectives have been achieved in model plant species like carrot, petunia, tobacco, etc., but important crop plants like the cereals and the legumes have proven to be extremely recalcitrant in vitro.

Plant regeneration from tissue cultures of most of the important species of cereals and many of the grasses has been reported (Vasil and Vasil, 1980a). Such regeneration is often sporadic and short-lived in nature. Plant regeneration takes place either by the development of axillary buds on pre-existing shoot meristems or rarely by the organization of multicellular shoot meristems in callus tissues. Both developmental processes yield plants which can be chimeral in nature and thus unsuitable for genetic studies. On the other hand, regeneration of plants from single cells, through the formation of somatic
embryos, would be of great advantage because such embryos—like their zygotic counterparts—arise from single cells.

Somatic embryogenesis has been described only rarely in tissue cultures of cereals and grasses. The most exhaustive and definitive study on this subject has been made by Vasil and Vasil (1980b, 1981a,b,c), who have described somatic embryogenesis and plant regeneration from callus and cell suspension cultures derived from immature embryos and inflorescences of *Pennisetum americanum*. The purpose of this study was to ascertain whether other species of cereals and grasses, such as *Panicum maximum* (Guinea grass), possess similar embryogenic competence.
CHAPTER ONE
LITERATURE REVIEW

Somatic embryogenesis is the process of embryo initiation and development from cells that are somatic in origin. Induced somatic embryogenesis in vitro provides a useful tool for rapid and uniform clonal propagation of plants which is a prerequisite for most applied uses of plant tissue cultures, including somatic hybridization and genetic modification (Vasil and Vasil, 1980a). Formation of asexual embryos from somatic cells or certain unfertilized gametic cells has been reported in 59 families of angiosperms that encompass 138 genera and 239 species. Somatic embryogenesis in vitro was first observed in *Daucus carota* by Reinert (1958) and Steward et al (1958). Recently Tisserat et al. (1979) have listed 32 families, 81 genera and 132 species that show asexual embryogenesis in vitro. Most of these species belong to the dicotyledons, especially the families Ranunculaceae, Solanaceae and Umbelliferae. Very few examples of somatic embryogenesis occur in the monocotyledons, particularly the cereals and grasses.

**Somatic embryogenesis and Plant Regeneration from Immature Embryos and Inflorescences**

In the Gramineae callus cultures have been established from a variety of tissues; namely: root, inflorescence, seedling, and mature and immature embryos (Dudits et al., 1975; Bhojwani and Hayward, 1977; Chen et al., 1977; Chin and Scott, 1977; Dale and Deambrogio, 1979). Such cultures are obtained by placing the explant onto a
nutrient agar medium supplemented with 2,4-D. Cultures initiated from immature embryos and inflorescences have been shown to have the greatest capacity for plant regeneration. Tissue cultures initiated from young inflorescences of *Andropogon gerardii*, *Sorghastrum nutans* and *Agropyron cristatum* have been used as an alternative means for the cloning of selected superior genotypes within a short period of time (Chen et al., 1977, 1979; Lo et al., 1980).

Plant regeneration has been achieved from cultured tissues of most of the important species of cereals and grasses (Vasil and Vasil, 1980a). Regeneration takes place by the organization of multicellular shoot meristems and the development of axillary buds, followed by the formation of adventitious roots (Table 1). This type of regeneration can lead to the formation of chimeral or mixoploid plants which are not desirable. Formation of "embryo-like structures" and somatic embryos has been reported only in a few instances: (a) immature embryos of *Hordeum vulgare* (Norstog, 1970), *Sorghum bicolor* (Thomas et al., 1977), *Lolium multiflorum* (Dale, 1980) and *Pennisetum americanum* (Vasil and Vasil, 1981b). (b) immature inflorescences of *Sorghum bicolor* (Bretell et al., 1980), *Pennisetum americanum*, *P. americanum* x *P. purpureum* hybrid (Vasil and Vasil, 1981b), and *P. purpureum* (Wang and Vasil, 1981).

**Somatic Embryogenesis and Plant Regeneration from Leaves**

Extensive efforts have been made for many years to isolate and culture mesophyll protoplasts of cereals and grasses (Potrykus, 1980; Vasil and Vasil, 1980c). No convincing evidence of cell division has been obtained so far. Thus, some authors have questioned whether
Table 1. Graminaceous species in which plant regeneration through the organization of shoot meristems has been reported in cultures derived from immature embryos and inflorescences.

<table>
<thead>
<tr>
<th>Species</th>
<th>Source tissue</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agropyron cristatum</td>
<td>young inflorescence</td>
<td>Lo et al. (1980)</td>
</tr>
<tr>
<td>Agropyron smithii</td>
<td>young inflorescence</td>
<td>Lo et al. (1980)</td>
</tr>
<tr>
<td>Alopecurus arundinaceus</td>
<td>young inflorescence</td>
<td>Lo et al. (1980)</td>
</tr>
<tr>
<td>Andropogon gerardii</td>
<td>young inflorescence</td>
<td>Chen et al. (1977)</td>
</tr>
<tr>
<td>Avena sativa</td>
<td>immature embryo</td>
<td>Cummings et al. (1976)</td>
</tr>
<tr>
<td>Bromus inermis</td>
<td>young inflorescence</td>
<td>Lo et al. (1980)</td>
</tr>
<tr>
<td>Hordeum vulgare</td>
<td>immature embryo</td>
<td>Dale and Deambrogio (1979)</td>
</tr>
<tr>
<td>Lolium multiflorum x L. perenne</td>
<td>immature seed</td>
<td>Ahloowalia (1975)</td>
</tr>
<tr>
<td>Panicum virgatum</td>
<td>young inflorescence</td>
<td>Lo et al. (1978)</td>
</tr>
<tr>
<td>Saccharum officinarum</td>
<td>inflorescence</td>
<td>Heinz and Mee (1969)</td>
</tr>
<tr>
<td>Saccharum sinensis</td>
<td>young inflorescence</td>
<td>Liu et al. (1972)</td>
</tr>
<tr>
<td>Stipa viridula</td>
<td>young inflorescence</td>
<td>Lo et al. (1980)</td>
</tr>
<tr>
<td>Sorghastrum nutans</td>
<td>young inflorescence</td>
<td>Chen et al. (1979)</td>
</tr>
<tr>
<td>Sorghum bicolor</td>
<td>immature embryo</td>
<td>Gamborg et al. (1977)</td>
</tr>
<tr>
<td>Triticum aestivum</td>
<td>immature embryo</td>
<td>Gosch-Wackerle et al. (1979)</td>
</tr>
<tr>
<td>Zea mays</td>
<td>immature embryo</td>
<td>O'Hara and Street (1978)</td>
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<td></td>
<td></td>
<td>Shimada (1978)</td>
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<tr>
<td></td>
<td></td>
<td>Green and Phillips (1975)</td>
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<tr>
<td></td>
<td></td>
<td>Freeling et al. (1976)</td>
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<tr>
<td></td>
<td></td>
<td>Torne et al. (1980)</td>
</tr>
</tbody>
</table>
the leaf cells of the Gramineae are actually totipotent (Wernicke and Brettell, 1980).

Bhojwani and Hayward (1977) reported that all attempts to induce callusing of leaf segments of wheat proved unsuccessful, irrespective of the medium tried and the age of the leaf. O'Hara and Street (1978) also could not get any callus from leaf segments of wheat. Similar difficulties were encountered by Cocking (1978) in the initiation of callus cultures from certain cereal species.

Saalbach and Koblitz (1978) obtained callus formation and a single shoot from cultured barley leaf segments. Microscopic preparations showed that the callus originated from mesophyll cells. Callus formation and subsequent plant regeneration have been achieved from young leaves of two species of *Sachcharum*. Heinz and Mee (1969) induced callus formation from leaves of *S. officinarum* in modified Murashige-Skoog's (1962) medium supplemented with 2,4-D and coconut water. Transfer of the callus to a 2,4-D-free medium led to plant regeneration. Callus was also obtained by Liu et al. (1972) from leaves of *S. sinensis*. Shoots and roots were formed on a medium containing dalapon.

Callus formation and plant regeneration have been obtained from seedling leaves of *Oryza sativa* (Henke et al., 1978; Bhattacharya and Sen, 1980), *Sorghum bicolor* (Wernicke and Brettell, 1980), and from leaves excised from adult plants of *Pennisetum purpureum* (Haydu and Vasil, 1981). In the presence of different concentrations of 2,4-D the leaf pieces proliferated to form an embryogenic callus which, after transfer to an auxin-free medium, formed plantlets. Only in *Sorghum bicolor* and *Pennisetum purpureum* was somatic embryogenesis observed during the process of plant regeneration.
The results described above provide ample evidence of the totipotency of cereal and grass leaves.

**Somatic Embryogenesis and Plant Regeneration from Freely Suspended Cells**

In order to exploit the full potential of plant cell culture technology, plants must be regenerated from single cells or through the formation of somatic embryos which are known to arise from single cells. The establishment of embryogenic suspension cultures would be an important step in this direction and may also be of use in mutant selection and somatic hybridization.

Suspension cultures have been established in several species of cereals and grasses, namely: *Triticum monococcum*, *Triticum vulgare*, *Hordeum vulgare* (Gamborg and Eveleigh, 1968); *Bromus inermis* (Gamborg et al., 1970); *Oryza sativa* (Ohira et al., 1973); *Phragmites communis* (Sangwan and Gorenflot, 1975); *Sorghum bicolor* (Brar et al., 1979); *Zea mays* (Brar et al., 1979; Polikarpochkina et al., 1979) and *Pennisetum americanum* (Vasil and Vasil, 1979). These cultures are non-morphogenic in nature and are generally comprised of cells that are elongated, ellipsoidal or oval in shape.

Somatic embryogenesis and plant regeneration from suspension cultures has not been reported, except in *Bromus inermis* where only albino plants were recovered (Gamborg et al., 1970), and more recently in *Pennisetum americanum* (Vasil and Vasil, 1981 a,c). The embryogenic suspension cultures of *Pennisetum* were obtained from immature embryo or inflorescence callus. By manipulating duration of culture and dilution ratios cultures were obtained which consisted...
predominantly of embryogenic cells. Such cultures have proven to be the only source of totipotent protoplasts of cereals and grasses (Vasil and Vasil, 1980b).

Isolation and Culture of Protoplasts

The advances in cell and tissue culture technology in the last two decades have opened up new avenues for conducting basic genetic research on higher plants at the cellular level and have provided powerful tools for improving plants through somatic hybridization and incorporation of foreign organelles, DNA or microorganisms (Vasil et al., 1979). A prerequisite for the utilization of most of this technology is the regeneration of plants from protoplasts. Presently, plant regeneration has been achieved in 7 families and 11 genera (Vasil and Vasil, 1980a; Binding and Nehls, 1980; Kao and Michayluk, 1980; Santos et al., 1980). Most of the species in which plants have been regenerated from protoplasts belong to the dicotyledons, especially the Cruciferae and the Solanaceae. Only two genera belong to monocotyledons, namely: *Asparagus officinalis* (Bui-Dang-Ha and MacKenzie, 1973), and *Pennisetum americanum* (Vasil and Vasil, 1980b).

Protoplasts have been isolated from leaves, coleoptile, stem, endosperm, suspension and callus cultures of many species of monocotyledons (Evans et al., 1972; Ruesink and Thimann, 1965; Motoyoshi, 1971; Nemet and Dudits, 1976; Potrykus et al., 1979). Sustained cell division and callus formation have been reported in many species, namely: *Triticum monococcum* (Nemet and Dudits, 1976); *Hordeum vulgare* (Koblitz, 1976); *Oryza sativa* (Deka and Sen, 1976); *Zea mays* (Potrykus et al., 1977, 1979; Brar et al., 1979); *Sorghum bicolor* (Brar et al.,...
1979); *Pennisetum americanum* (Vasil and Vasil, 1979); *Saccharum*
sp., (Maretzki and Nickell, 1973; Evans et al., 1980). No shoots
or plants have been obtained from such protoplast-derived callus
tissues. Mesophyll protoplasts of cereal or grass species have been
unsuccessfully cultured. Plant regeneration from protoplasts of
cereals and grasses has so far been obtained only in *Pennisetum*
americanum (Vasil and Vasil, 1980b). The protoplasts were isolated
from an embryogenic suspension culture, and gave rise to plantlets
through the formation of somatic embryos.
CHAPTER TWO
SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM THE MATURE EMBRYO, IMMATURE EMBRYO AND INFLORESCENCE OF Panicum maximum Jacq.1

Introduction

Regeneration of plants from tissue cultures of most of the important species of cereals and several grasses has been reported. In Hordeum vulgare, Cheng and Smith (1975) reported plant regeneration from callus derived from apical meristems, while Dale and Deambrogio (1979) obtained only roots from the culture of meristem tip, root, immature embryo and mature embryo. Whole plants were regenerated only from immature embryo callus. In Zea mays, plant regeneration has been achieved using immature embryos (Green and Phillips, 1975; Freeling et al., 1976) and mesocotyl tissue (Harms et al., 1976; Torne et al., 1980). In millets, callus formation and subsequent plant regeneration have been established for several genera, namely: Eleusine coracana, Paspalum scrobiculatum, Panicum miliaceum, and Pennisetum americanum (Rangan, 1974, 1976; Vasil and Vasil, 1981b).

In Oryza sativa, plant regeneration has been reported from leaf-, root-, seed- and seedling-derived calli (Nishi et al., 1968; Mascarenhas et al., 1975; Henke et al., 1978). In Sorghum bicolor, callus with leafy shoots was obtained from immature embryo

1. The information contained in this chapter has been accepted for publication in Amer. J. Bot. (1981).
(Gamborg et al., 1977) and shoots (Masteller and Holden, 1970), while shoot and embryo-like structures were induced from immature embryos (Thomas et al., 1977) and young inflorescences (Brettell et al., 1980). In *Triticum aestivum*, callus induction and subsequent plant regeneration have been described from the culture of inflorescences (Dudits et al., 1975; Gosch-Wackerle et al., 1979; Akins, 1981), immature embryos (Shimada, 1978; Akins, 1981) and mature embryos (Chin and Scott, 1977). In the case of grasses, young inflorescences proved to be the most useful explant for the cloning of desirable genotypes of several species, namely: *Andropogon gerardii* (Chen et al., 1977), *Sorghastrum nutans* (Chen et al., 1979), *Agropyron cristatum*, *A. smithii* and *Alopecurus arundinaceus* (Lo et al., 1980). Other explants (caryopses, immature embryos, internode) have been used to propagate *Dactylis glomerata* (Conger and Carabia, 1978), *Lolium* spp. (Ahloowalia, 1975) and a hybrid between *Lolium multiflorum* and *Festuca arundinacea* (Kasperbauer et al., 1979).

In this chapter, the culture of immature embryos, mature embryos and young inflorescences of *Panicum maximum*, and the regeneration of plants by the formation of somatic embryos are described.

**Materials and Methods**

Immature and mature embryos, as well as the young, unemerged inflorescences of Guinea grass (*Panicum maximum* Jacq. 199, selection from U.S.D.A. PI-167241, provided by Dr. R. L. Smith) were collected from field-grown plants. Palea and lemma of the seeds were removed under a Zeiss dissecting microscope. Outer leaves of the inflorescences were removed. All the materials were surface sterilized with 70%
ethanol for 1 minute, followed by 20% Clorox for 20 minutes, and
washed five times with sterilized distilled water. Whole mature
seeds and excised immature embryos (0.5-1 mm in size) were dissected
and three were placed to a Falcon Petri dish (35 x 10 mm). In-
florescence segments were cut into 2 mm cross sections before culture.

Murashige and Skoog's (1962) medium supplemented with 2,4-D
(2.5, 5 and 10 mg/l) was used for callus induction. MS medium con-
taining only coconut milk (15%), or supplemented with coconut milk
(15%) and either IAA, NAA or 2,4-D was used for plant regeneration.
The medium was adjusted to a pH of 5.8 with 1N NaOH before auto-
claving, solidified with 0.8% agar, and distributed in Petri dishes,
5 ml/dish. Cultures were maintained in the dark at 27°C for the
initiation of callus, and at 27°C with 16 hr of diffuse light for
plant regeneration. After 2-3 weeks in the regeneration medium
plantlets were transferred to culture tubes containing half-strength
MS medium for the establishment of a more vigorous root system.
The plants were then transplanted to potting soil and covered with a
beaker to maintain the humidity, and were finally moved to a greenhouse.

Root tips were pretreated with α-bromonaphthalene for 1 hour
and then fixed in 3:1 ethanol-acetic acid. Chromosome counts were
made on cells from squashed root tips stained with Feulgen solution
and observed with a Zeiss phase contrast microscope.

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.
Embryogenic callus at different stages of development and embryoids 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 at 20 KV.

Results

1. Culture of Immature Embryos

Excised immature embryos were placed on the agar medium so that the embryo axis faced down and was in contact with the medium, while the scutellum was exposed (Figs. 1, 2, 9). Vigorous callus formation started within 4-5 days. No significant difference in the quantity or the quality of callus produced was noted at different concentrations of 2,4-D (2.5-10 mg/l). A soft and translucent callus was produced from the embryo axis, apparently from the coleorhiza and coleoptile. The scutellum produced a rapidly growing, compact and white callus which caused extensive infoldings in the peripheral parts of the scutellum within 9 days (Figs. 11, 12). During further proliferation of the scutellar callus many cup-shaped structures with tube-like protuberances in their centers were formed by the end of the third week (Figs. 3, 4, 13-15). Histological examination of the cup-shaped structures showed them to be similar to the scutellum in morphology and structure. The tube-like coleoptile enclosed the shoot meristem, while the root meristem was covered by the coleorhiza
Frequently the scutellum of the newly-formed embryoids proliferated to form tertiary embryoids (Fig. 16).

Reduction in the amount of auxin in the medium, or its complete omission, resulted in the germination of the embryoids, and the emergence of leaves from the coleoptile and roots from the coleorhiza (Fig. 5). Germination of embryoids occurred even when they were left in an auxin medium for two or three months. This was probably because of the gradual depletion/destruction of auxin from the culture medium. After plantlets were formed, they were transferred to culture tubes containing half-strength MS medium. Young plants were successfully transplanted into soil in pots and acclimatized in a growth chamber for several days before transfer to the greenhouse (Fig. 6). Root-tip squashes from plants regenerated from immature embryo callus showed the normal tetraploid chromosome number of $2n = 4x = 32$ (Fig. 7).

Immature embryos (0.5-1 mm in length) showed a morphologically well-developed shoot-root axis which was enclosed by a coleoptile at the shoot end and a coleorhiza at the root end. Vascular tissue was evident as an array of elongated cells running from the scutellar node into the scutellum. The latter had a single layer of epidermis over its entire surface. The cells of the scutellum contained large and prominent starch grains (Fig. 17).

Excised embryos showed almost no visible morphological changes for the first two days in culture except the elongation of the embryo axis. By the fourth day the cells in the outer epidermal layer of the scutellum began to elongate at right angles to the surface of the scutellum (Figs. 10, 18, 19). The cells of the epidermis,
Figs. 1, 2. Guinea grass immature embryos at the time of excision and culture. Fig. 1. scutellar view. x22. Fig. 2. axis view. x20. Embryo was placed onto the surface of agar medium in face-down position shown in Fig. 1.

Figs. 3, 4. Proliferating immature embryo callus showing organization of numerous embryoids (Cl=coleoptile, Sc=scutellum). x4.

Fig. 5. Germination of embryoids. x8.
Fig. 6. Plants regenerated from immature embryo callus via somatic embryogenesis. x0.26.

Fig. 7. Chromosome complement from root tip of plant regenerated in vitro (2n = 4x = 32). The two arrows indicate extraneous background material. x1250.

Fig. 8. Shoot development in callus from mature embryo. x10.
Figs. 9-14. Scanning electron micrographs of immature embryos from 0 to 20 days after culture.

Fig. 9. Day 0. x43.

Fig. 10. Outer epidermal cells of the scutellum begin to elongate after 3 days of culture. x40.

Fig. 11. The scutellum produced a compact, white callus after 6 days of culture; the soft callus at the bottom was produced by the embryo axis. x33.

Fig. 12. Extensive infoldings in the scutellar callus after 10 days of culture. x28.

Figs. 13, 14. Organization of embryoids after 20 days of culture. Figs. 13. x20; Fig. 14. x34.
Fig. 15. Scanning electron micrograph showing mature embryoids produced in vitro. x120.

Fig. 16. The scutellum of the newly-formed embryoids proliferating to form tertiary embryoids. x78.
and later some subepidermal cells divide repeatedly and rapidly to form the embryogenic callus (Fig. 20). Internal segmenting divisions, which appeared to represent the early stages of embryogenesis, were observed on the periphery of the embryogenic callus (Figs. 25-29). A cambium-like zone was often formed within organized parts of the callus, and divided rapidly to form radial files of cells (Figs. 21-23). Cells located away from this zone were enlarged and accumulated starch (Fig. 24). These cells looked like scutellar cells and may give rise to embryoids, but this was not determined. Embryoids at different stages of development were observed on the surface of three to four week-old callus (Figs. 30-32). These embryoids were identical to zygotic embryos in structure and organization, but had a marked tendency towards premature germination.

2. Culture of Mature Embryos

Mature sterilized seeds were placed directly on MS medium containing 2.5-10 mg/l 2,4-D. Callus was produced by 40-50% of the embryos, and was usually visible after 2 or 3 days of incubation. The soft, translucent and friable callus was maintained on MS medium with 5 mg/l 2,4-D for eight months by sub-culture every 3-4 weeks. Over 90% of the cultures produced roots when the callus was transferred to a medium containing 0.2 mg/l 2,4-D or no 2,4-D.

Shoot formation in callus tissue obtained from mature embryos (Fig. 8) was observed under the following conditions: (a) in MS medium containing 15% coconut milk and 0.2 mg/l 2,4-D. (b) when the callus was first transferred to MS medium with 0.2 mg/l 2,4-D, and then to MS medium containing 1 mg/l 2iP and 15% coconut milk.
Fig. 17. Longitudinal section of an immature embryo at the time of culture. (Oe = outer epidermis, ie = inner epidermis). x220.

Fig. 18. Immature embryo after 4 days in culture. x132.

Fig. 19. Enlarged view of part of the scutellum from Fig. 18. The outer epidermal cells are elongated at right angles to the surface of the scutellum. x660.

Fig. 20. Immature embryo after 6 days in culture showing formation of embryogenic callus. x132.
Figs. 21, 22. Differentiation of cambium-like zones in organized structures found in the embryogenic callus. Fig. 21. x132; Fig. 22. x330.

Fig. 23. Radial files of cells produced by the cambium-like zone. x660.

Fig. 24. Cells located away from the cambium-like zone were enlarged and accumulated starch. x330.
Figs. 25-27. Internal segmenting divisions in cells at the outer periphery of embryogenic callus, possibly representing the early stages of embryogenesis. x660.

Figs. 28-32. Embryoids at different stages of development. x330.

Fig. 33. Longitudinal section of embryoid undergoing precocious germination (Cl = coleoptile, Cr = coleorhiza, R = root, Sc = scutellum). x108.
In all instances plant regeneration was achieved through the organization of shoot buds followed by the differentiation of adventitious roots. In some of the cultures a compact and white callus tissue, reminiscent of the scutellar callus from immature embryos, was seen near the periphery of the soft callus but the formation of any of the characteristic grass embryonic structures like the coleoptile, the scutellum or coleorhiza, was never observed. The regenerated plants were successfully transplanted to soil.

3. Culture of Young Inflorescence Segments

The developmental stage of the inflorescence at the time of excision and culture was found to be critical for the induction of embryogenic callus tissue. When the stage was not right, only a soft, friable and translucent callus was produced from the inflorescence axis and from each individual floral primordium. Such callus tissue did not show any potential for embryogenesis. Best results were obtained from inflorescences in which the floral primordia were just being formed and had not fully differentiated. Segments from such inflorescences were placed on MS medium with 2.5-10 mg/l 2,4-D. The young floral primordia as well as the rachis produced both friable and compact callus (Figs. 34-36). The friable callus formed many roots (Figs. 34, 35). The compact and white callus, again similar to the scutellar callus from immature embryos, continued to proliferate on the 2,4-D medium. When the callus was transferred to a medium containing only 0.2 mg/l of 2,4-D, embryoids with the typical organization of grass embryos were formed on the surface of the callus (Fig. 37). These germinated to form plantlets (Fig. 38) which were
Figs. 34, 35. Callus formation from segments of young inflorescences. Note the compact and white callus, and the translucent and friable callus and roots. xl2.

Fig. 36. Callus formation from rachis of young inflorescence. x20.

Fig. 37. Differentiation of embryoids in compact and white callus (Cl = coleoptile, Sc = scutellum). xl2.

Fig. 38. Plantlets formation from embryoid. x8.
successfully transferred to soil. Generally, 20-30 plants were obtained from a single immature embryo in 8-10 weeks, and 15-20 plants from each inflorescence segment in about 6 weeks. The procedures described here should, therefore, be useful in the rapid clonal propagation of desirable genotypes.

Discussion

Specific explants can be used as a source of tissue for initiating callus cultures capable of regeneration of whole plants. Among these different sources, apical meristems produce cultures highly capable of regeneration, but are difficult and time-consuming to excise (Cummings et al., 1976). Immature embryos and young inflorescences are two useful sources from which large numbers of plants can be produced either via somatic embryogenesis or shoot formation (Green and Phillips, 1975; Chen et al., 1977; Gamborg et al., 1977; Thomas et al., 1977; Brettell et al., 1980; Vasil and Vasil, 1981b and present study), but weeks or months are needed to obtain flowering material. Although mature embryos are easy to manage, the callus produced from them generally forms only roots, and even in those cases where plants are produced, they are few in number and are formed by the organization of shoot meristems and not somatic embryos (Conger and Carabia, 1978 and present study). Recently leaves of Sorghum and Pennisetum were shown to form plants through somatic embryogenesis (Wernicke and Brettell, 1980; Haydu and Vasil, 1981).

The results of the present experiments emphasize the importance of selecting a suitable explant at a defined stage of development to ensure a specific developmental pattern in vitro. This is shown by
the significantly different nature and behavior of callus tissues obtained from immature embryos, mature embryos and inflorescence segments cultured at different stages of development.

The synthetic auxin 2,4-D has proved to be the most suitable growth regulator for callus induction, growth and even embryo formation in cell and tissue cultures of cereals and grasses (Green, 1978). For cereals, in most instances a concentration of 1 to 5 mg/l was found to give the best results (Shimada, 1978; Dale and Deambrogio, 1979; Vasil and Vasil, 1981b). Brettell et al. (1980) used 0.2 and 1 mg/l of 2,4-D for inflorescence cultures of sorghum, and found that cultures could be more successfully established by increasing the concentration of 2,4-D to between 2 and 5 mg/l. Generally a higher level of 2,4-D is needed in grass tissue cultures. For example, 5 mg/l was found to be the best concentration for Indian grass (Chen et al., 1979), and 15 mg/l for Orchard grass (Conger and Carabia, 1978). In the present study, 2.5, 5 and 10 mg/l of 2,4-D was used, but no significant differences in the quantity or quality of the callus produced were observed. Guinea grass probably has a wide tolerance to different concentrations of 2,4-D.

Embryogenic callus tissue obtained from young inflorescences has the same morphology and morphogenetic potential as the scutellar callus from immature embryos. The organization of somatic embryos in both the immature embryo- and inflorescence-derived callus cultures was similar to the descriptions provided for *Sorghum bicolor* and *Pennisetum americanum* (Brettell et al., 1980; Vasil and Vasil, 1981b).
Embryoids arise from the superficial cells of the embryogenic callus. Differences between the superficial cells and cells located deep within the embryogenic callus at the light microscope level are difficult to determine. The ultrastructure of carrot embryogenic clumps has been investigated by McWilliam et al. (1974) and Street and Withers (1974). Their observations showed that the superficial cells destined to become embryos are highly cytoplasmic and possess a large diffusely-staining nucleus with a single darkly-staining nucleolus. These cells also show intense staining for proteins and RNA.

Starch grains were noticed in all embryogenic calli irrespective of their origin, although the role of storage starch in somatic embryogenesis is unclear. No starch grain accumulation was observed in the soft, friable callus obtained from mature embryos.

Cells of the outer and inner epidermis of the scutellum differ morphologically and cell divisions occur only in the outer epidermis. This is also the case in maize (Springer et al., 1979), and sorghum (Dunstan et al., 1978). Using \(^3\)H-thymidine incorporation and Feulgen staining technique, Akins (1981) has shown that DNA synthesis and mitosis occur in the outer epidermal and subepidermal layers of the scutellum of wheat during the third day of culture. This is probably also true in Panicum maximum, since elongation of the outer epidermal cells of the scutellum could be observed during the third and fourth day of culture, although no determination of DNA synthesis and mitosis has been made.
CHAPTER THREE

SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM THE LEAF TISSUES OF *Panicum* maximum Jacq.²

**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; Vasil and Vasil, 1980a). In most instances regeneration takes place by the organization of multicellular shoot meristems and buds (Rangan, 1974; Chen et al., 1977; Nakano and Maeda, 1979; Shimada and Yamada, 1979; Springer et al., 1979). The culture of excised leaf tissues of cereals has proven most difficult (Cocking, 1978), although callus tissue cultures have been derived from the young leaves of *Saccharum officinarum* and have routinely undergone 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*. These experiences, and the extreme difficulty of cereal and grass mesophyll protoplasts to undergo sustained cell division in vitro, have given rise to serious doubts regarding the totipotency of cells of cereal and grass leaves (Potrykus, 1980; Wernicke and Brettell, 1980). The

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² The information contained in this chapter has been accepted for publication in *Theoretical and Applied Genetics* (1981).
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. This chapter describes experiments on 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 provided by Dr. W. W. Hanna) were used. 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 greenhouse. Shoots removed from adult plants were sterilized after removing the outermost leaves, in 70% ethanol for 1 min., followed by 20% chlorox for 10 min. The sterilized shoots were washed five times with sterilized 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 proceeding distally toward the apex of the leaf. Segments from 1-4 of the youngest whorled leaves were placed together in culture. Murashige and Skoog's (1962) nutrient medium, 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 the medium was sterilized by autoclaving and then distributed in 35 x 10 mm Falcon Petri dishes. Induction of callus tissues from leaf explants was obtained in MS medium supplemented with different combinations of 2,4-D (2.5, 5, and 10 mg/l) and CM (5, 10, and 15%), 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 half-strength MS medium for the establishment of a vigorous root system. Finally, the plants were transplanted to potting soil, acclimatized for a few days in the growth chamber, and then moved to the greenhouse 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 at 20 KV.

Results

Callus tissue first became visible at the cut ends of the leaf explants within a week on MS medium containing different combinations of 2,4-D (2.5, 5, and 10 mg/l) and CM (5, 10, and 15%). No significant differences were found in callus formation within the range of concentrations of 2,4-D and CM used, but no organized callus tissue was formed in the absence of 2,4-D, with or without CM. Addition
of CM was not essential for organized 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 became swollen, followed by the appearance of callus tissue along the abaxial (lower) surface of the leaf segments (Figs. 39-41, 43). 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 (Fig. 58). The initial callus tissue formed at the cut ends of the leaves was soft and translucent. After about two weeks localized areas of white and embryogenic calluses and many embryoids appeared on the surface of the compact callus tissue. The white color of the compact and embryogenic callus tissue was caused by the accumulation of starch grains in its cells. The embryogenic callus tissue was sub-cultured at two week intervals, and has retained its capacity to form embryoids for more than five months without any significant loss of embryogenic potential (Fig. 42). 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 (Figs. 43, 44). Each leaf segment gave rise to 20-40 embryoids. The embryoids produced are similar to zygotic embryos in having a shoot (coleoptile)-root (coleorhiza) axis, and a scutellum typical of grass embryos (Figs. 45, 46).

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
Figs. 39, 40. Two week old cultured leaf segments. White and compact embryogenic callus can be seen on the abaxial surface of the outermost leaves. Fig. 39. x15; Fig. 40. x20.

Fig. 41. Three week old cultured leaf segments. x12.

Fig. 42. Embryogenic callus obtained from leaves. x15.
Fig. 43. Three week old cultured leaf segments showing swollen veins and many embryoids. xl2.

Fig. 44. Scanning electron micrograph of part of the leaf shown in Fig. 43. x23.

Fig. 45. Scanning electron micrograph of part of the leaf segment with embryoids. x36.

Fig. 46. Typical, well-organized embryoid with a scutellum, coleoptile and coleorhiza. xl40.
the embryoids matured and germinated to form plantlets (Figs. 47, 48). Addition of CM to MS medium did not support the continued development and germination of embryoids. On MS medium supplemented with 0.2 mg/l NAA, only about 20% of the embryoids germinated. The plantlets obtained on the MS + GA medium were transferred to culture tubes containing half-strength MS medium (Fig. 49). After one to two weeks the plantlets had formed a vigorous root system and several leaves, and were transplanted to potting soil (Fig. 50). Finally, the plants were moved to the greenhouse 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 that leaves which gave rise to callus, embryoids and plants were structurally well-developed and showed vascular bundles, characteristic groups of sclerenchyma cells, and mesophyll cells with plastids (Figs. 51, 52). They were still in a whorled condition and not fully expanded. No cell divisions were seen in the upper epidermis or in the tissues contained in the upper half of the leaf blade (Figs. 53-58). The compact, white and embryogenic callus tissue was produced by cell divisions in the lower epidermis, and in the mesophyll cells. Periclinal divisions occurred either in one or two lower epidermal cells (Fig. 53) or in several lower epidermal cells resulting in the formation of radially oriented files of cells (Fig. 54). Cell divisions in the mesophyll tissue were restricted to cells located in lower half of the leaf (Fig. 55), especially those on either side of the vascular bundles (Figs. 56, 57). In some leaves the
Fig. 47. Germination of embryoids after three days on MS medium containing GA. x40.

Fig. 48. Plantlets after one week on MS medium with GA. x12.

Fig. 49. Plantlet in culture tube with half-strength MS medium. x0.6.

Fig. 50. Plants regenerated from leaves via somatic embryogenesis. x0.33.
Fig. 51. Whorled leaves at time of culture. x120.

Fig. 52. Cross section of leaf at time of culture showing well-developed vascular tissue, sclerenchyma, parenchyma, and mesophyll tissue. x600.

Fig. 53. Periclinal divisions in some of the lower epidermal cells. x300.

Fig. 54. Radial files of cells produced by divisions in the lower epidermis. x300.

Fig. 55. Embryogenic callus formed by mesophyll tissue only. x120.

Fig. 56. Soft and unorganized callus formed by mesophyll tissue. x120.
mesophyll tissue formed the compact organized callus while the epidermal cells did not divide (Fig. 55). In other instances cells of the mesophyll tissue formed a soft and friable callus tissue (Fig. 56), which showed no organized growth. Organized structures and embryoids were formed only on the surface of the compact callus tissue (Figs. 59-62). The third and the fourth leaves, away from the shoot apex, appeared to be most responsive to the induction of callus and morphogenesis leading to the formation of embryoids and plants.

The three genotypes (PM44, PM49, PM53) used in this experiment showed marked differences in their ability to produce callus tissue from leaf segments (Table 2).

**Discussion**

The synthetic auxin 2,4-D was found to be very effective in inducing callus formation and somatic embryogenesis in cultured leaf segments. The fact that no significant differences were found at the different concentrations of 2,4-D used indicate that embryogenesis could be initiated over a wide range of 2,4-D concentrations. However, for the germination of the embryoids and plantlet formation, the embryoids must be transferred to a medium containing GA but devoid of 2,4-D. Coconut milk was not required for the induction of embryoids, although they could be formed in the presence of coconut milk.

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 in Chapter Two. The embryoids
Fig. 57. Divisions in cells located on either side of the vascular bundle. x600.

Fig. 58. Callus formation on the abaxial surface of the outermost leaf. The younger inner leaf did not form any callus. x120.

Fig. 59. Leaf segments with proliferating embryogenic callus. x120.

Figs. 60, 61. Organized structures and embryoids on the surface of the compact callus. x300; x120.

Fig. 62. Embryoids attached to the surface of compact leaf callus. x300.
Table 2. Callus formation from leaves of different genotypes of *Panicum maximum* Jacq. cultured on Murashige and Skoog's (1962) medium containing 10 mg/l 2,4-dichlorophenoxoacetic acid and 15% coconut milk.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% leaf segments forming callus</th>
<th>% leaf segments forming embryogenic callus</th>
</tr>
</thead>
<tbody>
<tr>
<td>PM 44</td>
<td>50</td>
<td>16</td>
</tr>
<tr>
<td>PM 49</td>
<td>48</td>
<td>2</td>
</tr>
<tr>
<td>PM 53</td>
<td>1.2</td>
<td>0</td>
</tr>
</tbody>
</table>
obtained from the leaf callus tissue also show the typical organization of cereal and grass embryos. These data show that cells of the scutellum, inflorescence, and leaf are totipotent, at least until certain stages in their development, and that they can be dedifferentiated in vitro to form rapidly dividing populations of embryogenic cells. The recent work with sorghum leaves also show that leaf tissue can express totipotency even to the extent of forming somatic embryos (Wernicke and Brettell, 1980).

In the present experiment, 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. In sorghum, the embryogenic callus tissue is also formed by mesophyll and/or epidermal cells. 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. Older leaves formed only soft callus. The reasons for these patterns of behavior are not understood. Perhaps a gradient of endogenous hormone concentrations is found in leaves of different ages, and favorable concentrations for embryogenic callus formation are available only at specific stages of development.

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 and grasses
has proven 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. If embryogenic suspension cultures can be isolated from such leaf callus, this suspension can serve as a good source of protoplasts as shown by the successful culture of protoplasts isolated from embryogenic suspension cultures of Pennisetum americanum (Vasil and Vasil, 1980b).

The genotype of Panicum maximum (PM 199) used for the culture of immature embryos and inflorescences is an apomict (Chapter Two), and the embryos produced in nature are of somatic origin. It will not be of any advantage, therefore, to clonally propagate this genotype by tissue culture techniques. However, the knowledge gained from that study was used in the clonal propagation of two genotypes (PM 44, PM 49) which reproduce sexually and are difficult to maintain because of their heterozygous nature. The three genotypes showed different degrees of response in culture indicating that genotype as well as the developmental stage of the explant play important roles. Bhattacharya and Sen (1980) also found that only two out of eleven genotypes of rice respond favorably.
CHAPTER FOUR

SOMATIC EMBRYOGENESIS AND PLANT REGENERATION FROM THE FREELY SUSPENDED CELLS OF Panicum maximum Jacq.3

Introduction

The recent upsurge in interest in plant cell and tissue culture research and its possible uses in the improvement of plants has been caused by the development and utilization of some novel techniques of plant cell and tissue culture (Vasil, 1980 a,b). In order to exploit the full potential of such technology, the regeneration of plants from single cells or from the formation of somatic embryos which are said to arise from single cells is necessary.

The capacity of somatic plant cells to undergo embryogenesis has been well documented (Vasil and Vasil, 1972; Kohlenbach, 1977, 1978; Street, 1979). Somatic embryogenesis in suspension culture was first demonstrated in Daucus carota (Steward et al., 1958), and is now known in many dicotyledonous species (Ranunculus sceleratus: Konar and Nataraja, 1965; Cichorium endivia: Vasil and Hildebrandt, 1966; Atropa belladonna: Konar et al., 1972b). Somatic embryogenesis in suspension cultures of cereals and grasses has never been observed, except in Bromus inermis where only albino plants were recovered (Gamborg et al., 1970), and more recently in Pennisetum americanum (Vasil and Vasil, 1981a). Somatic embryogenesis and plant regeneration

3. The information contained in this chapter has been accepted for publication in Ann. Bot. (1981).
tion from cultured immature embryos, inflorescences and leaves of *Panicum maximum* Jacq. have been described in Chapters Two and Three, and the same phenomenon in suspension cultures is now given in this chapter.

**Materials and Methods**

Embryogenic callus cultures were obtained from immature embryos (0.5-1 mm) and young inflorescences of *Panicum maximum* Jacq. (selection 199 from U.S.D.A. PI-16724, kindly provided by Dr. Rex Smith), on Murashige-Skoog's (1962) agar medium with 2.5-10 mg/1 2,4-D. The calluses originated from the scutellum of the immature embryos, and from the young floral primordia and rachis (Lu and Vasil, 1981). Three to six weeks old calluses were dissected into small pieces and placed in liquid MS medium with 2,4-D (1 mg/1) and coconut milk (2.5%), in 250 ml Erlenmeyer flasks on a gyrotory shaker at 150 rpm, to initiate suspension cultures. The suspension was maintained on the same medium by adding approximately 10-15 ml of the cell suspension to 35 ml of fresh medium every 3-4 days. The cultures were left in the dark at 27°C. For plating unwashed suspension cultures were mixed with equal amounts of 1.6% agar medium at 45°C without any hormone or with 0.2 mg/l 2,4-D or NAA, and poured into Falcon Petri dishes. In another experiment 0.5 ml of 5-day old cell suspension was dispensed on top of the MS agar medium with different concentrations of 2,4-D (0.25, 0.5, 1, and 2 mg/l). The Petri dishes were sealed with Parafilm and kept in a growth chamber at 27°C, with 16 hours of diffuse light. Plantlets formed in culture were transferred to half-strength MS medium for the establishment of a
more vigorous root system. The plants were then transplanted to potting soil and were later moved to the greenhouse. Chromosome counts were made from squashed root tips, pretreated with α-bromonaphthalene for 1 hr and fixed in 3:1 ethanol:acetic acid for half an hour, and stained with Feulgen solution.

Results

Embryogenic callus was initiated in MS agar medium with 2.5-10 mg/l 2,4-D from the scutellum of immature embryos (0.5-1 mm in size), and from the young floral primordia and rachis of unemerged inflorescences. The embryogenic calluses were compact in texture and white in color. When the calluses were cut into small pieces and placed in MS liquid medium with 5 mg/l of 2,4-D and 15% coconut milk, they remained tight and non-friable. However, if the concentration of 2,4-D was reduced to 1 mg/l, the embryogenic calluses began to break up and grew rapidly. The suspension culture was thus maintained in MS liquid medium with 1 mg/l of 2,4-D and 2.5% coconut milk.

The suspension culture was comprised of two types of cells: (1) small, embryogenic cells with dense cytoplasm and often containing prominent starch grains and (2) large, vacuolated, non-embryogenic cells (Fig. 63). The embryogenic cells were generally present in compact small groups, and only occasionally occurred as single cells. The non-embryogenic cells were elongated or ellipsoidal in shape and seldom divided. The proportion of the two types of cells in a culture depended on the volume of the inoculum used during each sub-culture and the duration of sub-culture. Usually the larger the volume of the inoculum, the larger the number of elongated cells in the suspension
**Fig. 63.** Suspension culture showing two types of cells: small, embryogenic cells and vacuolate, non-embryogenic cells. x1005.

**Figs. 64-67.** Randomly selected stages of embryoid formation in suspension cultures derived from immature embryos. Fig. 64. x1005. Figs. 65-67. x314.
culture. The suspension culture grew very slowly when less than 5 ml of inoculum was used for sub-culture.

Single embryogenic cells and various stages of embryoid formation up to the differentiation of a lateral notch were seen in the suspension cultures (Figs. 64-71). A well-defined, multiseriate and multicellular suspensor was formed (Figs. 68-71). The notch formation is similar to that seen in the development of zygotic embryo and represents the site of shoot apex and coleoptile formation. Embryogenesis in suspension cultures did not proceed beyond this stage of development.

When an unwashed suspension culture was mixed with an equal amount of 1.6% agar medium without any hormone or with only 0.2 mg/l of 2,4-D or NAA and plated in Falcon Petri dishes, fully differentiated embryoids were formed in three weeks (Figs. 76). After the suspension culture had been maintained for six months, embryoids were not formed unless the cell masses were transferred again to MS agar medium with 2,4-D (0.25, 0.5, 1, and 2 mg/l). Scanning electron micrographs showed different stages of embryoid development starting from globular embryoids to fully differentiated embryoids with a well-defined shoot-root axis, a scutellum and a coleoptile (Figs. 72-75). The embryoids germinated (Fig. 77) to form plantlets with shoots and roots when transferred to MS agar medium with or without 1 mg/l GA. The plantlets were transferred to culture tubes containing half-strength MS medium to establish a more vigorous root system (Fig. 78). Finally the plants were transplanted to potting soil and moved to the greenhouse (Fig. 79). The regenerated plants showed the normal chromosome number of 2n = 4x = 32.
Figs. 68-71. Randomly selected stages of embryoid formation in suspension cultures derived from immature embryos. x314.
Figs. 72-75. Scanning electron micrographs of the different stages of embryoid development in suspension cultures derived from young inflorescence.

Fig. 72. Globular stage. x62.

Figs. 73, 74. Early cotyledonary stage. (Arrows indicate formation of notch which represents the site of shoot apex and coleoptile). Fig. 73. x52; Fig. 74. x140.

Fig. 75. Fully differentiated embryoid with a well-defined shoot-root axis, a scutellum and a coleoptile (partly broken). x74.
Fig. 76. Organization of embryoids with tubular coleoptile surrounded by the scutellum in plated suspension culture. (Cl = coleoptile, Sc = scutellum). x7.

Fig. 77. Germination of embryoid with the first leaf protruding from the coleoptile. x7.

Fig. 78. Plantlets in culture tube. x0.6.

Fig. 79. Plant regenerated from somatic embryoid formed in suspension culture. x0.1.
Hundreds of embryoids were present in each suspension culture, but not all completed the typical embryogenic development. In some no roots were formed and in others no shoots were formed. Whenever 2,4-D level was higher than that needed for embryogenesis to proceed, the embryoids proliferated to produce new embryogenic cells (Fig. 80). Polyembryony was thus common (Figs. 81-83), either because of secondary proliferation of the organized embryoids, or as a result of a simultaneous development of several embryoids in a proembryonal mass of embryogenic cells.

**Discussion**

Suspension cultures derived from immature embryos as well as from young inflorescences yielded normal green plants. However, after about three months of sub-culture only albino plants were obtained from suspension culture of immature embryo origin, while green plants were recovered from inflorescence-derived suspension cultures after more than one year of sub-culture. The appearance of albino plants may be due to physiological reasons or owing to the selection of mutant albino cells. In Bromus inermis only albino plants were recovered from suspension cultures (Gamborg et al., 1970).

Plant regeneration through somatic embryogenesis has also been reported from suspension cultures and callus cultures derived from immature embryos and inflorescences of Pennisetum americanum, inflorescences of P. americanum x P. purpureum (Vasil and Vasil, 1981a, b), and leaves and inflorescences of P. purpureum (Haydu and Vasil, 1981; Wang and Vasil, unpublished). Since somatic embryos arise from single cells (Konar et al., 1972a; McWilliam et al., 1974;
Figs. 80-83. Secondary proliferation of embryoids and polyembryony in suspension culture. x314.
Haccius, 1978), such a course of regeneration can be of considerable use in mutation and selection studies. Plant regeneration has been reported from tissue cultures of many species of cereals and grasses through the organization of shoot meristems (Rangan, 1974; Chen et al., 1977; Nakano and Maeda, 1979; Shimada and Yamada, 1979; Springer et al., 1979). Since shoot meristems are generally understood to be multicellular in origin, plants derived from them can be chimeral in nature.

It is clear that 2,4-D is involved in somatic embryogenesis in both suspension and callus cultures of *Panicum maximum*. There is a wide range of 2,4-D requirement. It is essential for the induction of embryogenesis and for the development of embryoids up to about the globular stage in both *Panicum maximum* and *Pennisetum americanum* (Vasil and Vasil, 1981c).

The usefulness of many of the modern techniques of somatic cell genetics depends on the availability of totipotent protoplasts. Embryogenic suspension cultures, such as those described here, are the only source of totipotent cereal and grass protoplasts available at this time (Vasil and Vasil, 1980b; Chapter Five). Embryogenic suspension cultures also provide an ideal system to study the factors controlling embryogenesis. Furthermore, suspension cultures provide an additional method for rapid clonal propagation.
CHAPTER FIVE
ISOLATION AND CULTURE OF PROTOPLASTS

Introduction

Plant protoplasts are an extremely valuable material for use in genetic modification experiments, such as the uptake of cell organelles, microorganisms or other foreign genetic materials, and in somatic hybridization. Therefore, the ability to regenerate plants from protoplasts is an important requirement for the utilization of such novel techniques. Plant regeneration from protoplasts has been achieved in several dicotyledonous species, particularly those belonging to the Solanaceae (Vasil and Vasil, 1980c). Monocotyledons, especially cereals and grasses, have proven to be a rather recalcitrant group in tissue culture (Thomas and Wernicke, 1978; Potrykus, 1980; Vasil and Vasil, 1980c). Despite extensive efforts sustained cell divisions resulting in callus formation have been obtained from protoplasts in only six species, namely: Oryza sativa (Deka and Sen, 1976; Cai et al., 1978); Hordeum vulgare (Koblitz, 1976); Triticum monococcum (Nemet and Dudits, 1976); Zea mays (Potrykus et al., 1977, 1979); Sorghum bicolor (Brar et al., 1979) and Pennisetum americanum (Vasil and Vasil, 1979). Organized development of embryoids and plantlets has been obtained only in Pennisetum americanum (Vasil and Vasil, 1980b). In this chapter the isolation and culture of protoplasts from embryogenic suspension
cultures of *Panicum maximum* Jacq., and their subsequent growth and differentiation into embryoids and plantlets is described.

**Materials and Methods**

Young, unemerged inflorescences were cut into 2 mm segments and grown on MS nutrient medium supplemented with 2.5-10 mg/l 2,4-D and 5-15% coconut milk, to induce the formation of embryogenic callus tissue. The latter was placed in MS liquid medium with 2 mg/l 2,4-D and 5% coconut milk to initiate suspension cultures. These were subcultured every 4-5 days by adding approximately 10-15 ml of the cell suspension to 35 ml of fresh medium in 250 ml Erlenmeyer flasks, placed on a Gyrotory shaker at 150 rpm, in the dark at 27°C.

One week before the isolation of protoplasts, the suspension cultures were provided with 35 ml of fresh medium every other day. Protoplasts were isolated by mixing 1.5 ml of suspension culture (0.4-0.5 g fresh weight) with 8 ml of a filter-sterilized enzyme mixture (2% Cellulysin, 1% Macerozyme, 0.5% Driselase, 0.5% Rhozyme, 0.15M sorbitol, 0.15M mannitol, 0.7mM NaH$_2$PO$_4$·H$_2$O, 7mM CaCl$_2$·2H$_2$O, 3mM MES buffer at pH 5.7) in 50 ml Erlenmeyer flasks on a Gyrotory shaker at 60 rpm at 25°C for 5-6 hours.

The protoplast/enzyme mixture was filtered through a layer of Miracloth, and through 100 and 50 µm stainless steel filters to remove undigested cells and other cellular debris. Protoplasts were collected and washed three times by low speed centrifugation (100g x 3 min). The medium used for washing and culture of protoplasts was Kao and Michayluk's (1975) nutrient medium without nucleic acid bases, vitamin B$_{12}$, alanine, cysteine and glutamic acid, but
containing 0.3M glucose, 0.2 mg/l 2,4-D and 0.5 mg/l zeatin. Protoplasts were cultured in liquid droplets (0.1-0.15 ml) or in a very thin layer of nutrient medium in Falcon Petri dishes (50 x 9 mm) at a density of 1-3 x 10^5/ml. Petri dishes were sealed with Parafilm, incubated in dark in a growth chamber at 27°C for three days, and then in diffuse light with a 16/8 day/night cycle. Fresh medium was added to the culture after 7 days and then continued at 3-4 day intervals. After three weeks the resulting cell masses were transferred to MS agar medium with different concentrations of 2,4-D to induce the formation of embryoids, which were then transferred to a hormone-free MS medium. Within 7-10 days plantlets were formed and transferred to culture tubes with half-strength MS medium.

Results

The suspension cultures are composed of two types of cells: (a) small, embryogenic cells with dense cytoplasm and prominent starch grains. (b) large, vacuolated non-embryogenic cells. By controlling the time of sub-culture and the volume of the inoculum used during each sub-culture the suspension culture can be manipulated to consist predominantly of embryogenic cells. Such a culture was used for the isolation of protoplasts (Fig. 84). After 5-6 hours of incubation and subsequent filtration and washing, cell walls were totally removed from 95% of the cells. This was confirmed by treating the washed protoplast preparations with 20% SLS in 0.15M sorbitol and 0.15M mannitol. All the protoplasts were lysed by SLS while the cells remained intact.

In order to determine whether the remaining cells were capable of cell division, they were washed three times to remove SLS and cultured. None of the cells was ever seen to grow or divide.
Three types of protoplasts could be seen in culture (Figs. 85, 86), namely: (a) vacuolated protoplasts. (b) small, densely cytoplasmic, embryogenic protoplasts and (c) spontaneous fusion products of the latter. Owing to the highly embryogenic nature of the suspension used for the isolation of protoplasts, only rare vacuolated protoplasts were seen. Neither the vacuolated protoplasts, nor the fused coenocytic protoplasts, ever divided.

Embryogenic protoplasts regenerated cell walls within 2–4 days of culture. The first cell division took place after 5–6 days of culture (Figs. 87,88). By the eighth day of culture, 4- and 8-celled colonies were observed (Figs. 89-92). Further cell divisions followed in rapid succession, without significant cell enlargement, resulting in the formation of either tightly-packed cell aggregates or globular embryoids (Figs. 93-101). Up to 36 cell aggregates and 40 embryoids were formed in each culture drop (Figs. 100, 101). It was not possible to identify and count a number of smaller cell colonies which were also present. This precluded the determination of plating efficiency. Somatic embryogenesis did not proceed beyond the early cotyledonary stage (Fig. 101) in liquid culture despite the use of KM medium with starch (Kao and Michayluk, 1980), sucrose, ABA and casein hydrolysate. The embryoids underwent further cell proliferation from their surface layers to form many cell colonies.

Protoplast-derived cell colonies were transferred to MS medium with various supplements to induce callus formation. Of the several media tested, MS medium with 2,4-D (0.25, 0.5, 1, and 5 mg/l) best promoted callus formation (Fig. 102). Cells turned black and died
Fig. 84. Inflorescence-derived suspension culture, consisting predominantly of embryogenic cell aggregates, from which protoplasts were isolated. x466.

Figs. 85, 86. Freshly isolated protoplasts (arrows indicate spontaneous fusion products). Fig. 85. x242; Fig. 86. x774.

Figs. 87, 88. First cell division in regenerated cells from protoplasts. x1150.

Fig. 89. 4-celled colony. x736.
Figs. 90-95. Stages of colony formation from protoplasts. Figs. 90-94. x736; Fig. 95. x450.
Figs. 96-99. Globular embryoids regenerated from protoplasts. Fig. 96. x500; Fig. 97. x242; Fig. 98. x300; Fig. 99. x242.

Figs. 100, 101. Protoplast-derived cell colonies and embryoids in liquid culture medium. x96.
in two weeks in MS medium with ABA (0.1 mg/l). Cells divided a few times and then turned brown in MS medium without any hormones or with only coconut milk (10 and 15%). Cytokinin did not support cell proliferation. Cells grew slowly in MS medium with 2,4-D provided in combination with one of the cytokinins (BAP, zeatin or kinetin). Embryoids were formed (Fig. 103) in MS medium with low concentrations of 2,4-D (0.25, 0.5, or 1 mg/l) directly, or after transfer from a medium with higher 2,4-D (5 mg/l) to lower 2,4-D (0.25, 0.5, and 1 mg/l). Embryoids germinated precociously after transfer to MS medium with either zeatin (1 mg/l) or 2iP (1 mg/l). Embryoids and green plantlets were formed on hormone-free MS medium (Figs. 104-106).

Discussion

Embryoids formed in protoplast cultures of Pennisetum americanum (Vasil and Vasil, 1980b) and Panicum maximum (present study) do not directly form plants. Instead they produce an embryogenic callus which gives rise to embryoids and plantlets. It might be possible to control this and get plant regeneration directly from protoplast-derived embryoids.

The embryoids formed in vitro are similar in morphology to zygotic embryos. However, they appear to accumulate less starch in the scutellar cells than the zygotic embryos, and appear less dense. The amount of starch accumulated in embryoids developing in vitro may depend to some extent on their origin. Those embryoids developing from immature embryo cultures accumulate the most starch, those from the cell suspension cultures less, and those embryoids
Fig. 102. Protoplast-derived callus. x7.

Fig. 103. Formation of embryoids. x7.

Fig. 104. Mature embryoids with shoot-root axis enclosed by scutellum. x8.

Figs. 105, 106. Germination of embryoids and formation of green plantlets. Fig. 105. x4; Fig. 106. x3.
which arise from protoplasts accumulated the least starch. The more closely the amount of starch in the scutellum of these embryoids approaches that found in the scutellum of zygotic embryos, the more readily can vigorous plants be regenerated from them. Thus, protoplast-derived embryoids generally give rise to weaker plantlets.

Although plants could be regenerated from protoplasts of *Panicum maximum*, several problems still need to be overcome for the maximum exploitation of this system: (1) the regenerated plants are not vigorous and turn yellow after transfer to culture tubes. This is probably due to the incomplete maturation of the embryoids. (2) The plating efficiency is quite low compared to some of the dicotyledons.
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Chin-yi Lu was born on July 29, 1948, in China. She moved to Taiwan in 1949. She received her high school diploma from Taipei First Girls' High School in June 1966, and Bachelor of Science degree in Botany from National Taiwan University in 1970. In 1971, she entered the Botany Department of NTU at the graduate level and earned the Master of Science degree in 1973. From 1970 to 1975, she worked as a teaching assistant in the Department of Botany, NTU. She was promoted to lecturer in 1975. In 1977, she entered the Department of Botany of the University of Florida for studies toward the degree of Doctor of Philosophy. She is married to Yuen-Po Yang and they have a son.
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