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Tissue Culture of the Forage Grass Little Bluestem (Schizachyrium scoparium (Michx) Nash)

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TISSUE CULTURE OF THE FORAGE GRASS LITTLE BLUESTEM

(*Schizachyrium scoparium* (Michx) Nash)

by

David D. Songstad

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(Schizachyrium scoparium (Michx) Nash)

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable for meeting the thesis requirements for this degree. Acceptance of this thesis does not imply that the conclusions reached by the candidate are necessarily the conclusions of the department.

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LIST OF ABBREVIATIONS

Abbreviation	Term
AD	Apical Dome
Cp	Coleoptile
Cr	Coleorhiza
FL	First Leaf
LP	Leaf Primordium
MC	Meristematic Core
R	Radicle
S	Scutellum
Sh	Shoot
SA	Shoot Apex
VS	Vascular System
WN	Whitish Nodules

INTRODUCTION

Little bluestem, Schizachyrium scoparium (Michx) Nash, a warm-season perennial native grass, is widely distributed over the temperate portion of North America (Hitchcock, 1971), extending from Quebec to Alberta and southward to Florida and Arizona. It is a major upland component of the tall and mixed grass prairies and furnishes substantial amounts of palatable forage throughout the summer. Little bluestem has also been used for soil conservation purposes and its reseeding characteristic makes it especially useful in range and conservation plantings.

Although the plant can be propagated vegetatively by dividing crowns, due to its bunch-type growth, the conventional way of propagation as such is not efficient. In the previous research conducted in this laboratory, segments of young inflorescences of some forage grass species were explanted onto a modified MS medium supplemented with 2,4-D and initiated calluses. After subcultured on the basal medium with auxin removed, the calluses differentiated into numerous somaclone plantlets (Chen, Stenberg and Ross, 1977; Chen, Lo and Ross, 1979; Lo, Chen and Ross, 1980). Such methods of rapid cloning would considerably shorten a breeding program.

Another aspect of grass tissue culture research is focused on induction of mutations. Since most forage grass

plants are heterozygous due to natural cross-fertilization and self-incompatability, desirable recessive characteristics are likely masked by the dominant genes. Induction of recessive mutations from the allelic dominant genes would uncover the desirable traits. Mutation treatment of cells in vitro followed by induction of plant regeneration from these cells would be more accurate in obtaining homozygous recessive plants. However, plant regeneration in callus cultures may take place either through germination of somatic embryos, which are of single cell origin and genetically homogenous, or the development of shoots, which according to Wang and Vasil (1982), might be reorganized by many existing cells in the callus tissue. In the latter case, genetical heterogeneity, which would be obstructive to in vitro mutation studies, would result.

In this research, the method previously used in cloning of forage grasses in this laboratory was applied to culture little bluestem. The calluses grown on the morphogenetic medium were histologically investigated to determine the pattern of differentiation.

LITERATURE REVIEW

Plant regeneration from tissue culture of forage grasses was first reported by Gamborg, Constabel and Miller (1970) in smooth bromegrass (Bromus inermis Leyess). In that experiment, calluses were initiated from mesocotyls which were excised from germinating seeds and explanted on an agar medium supplemented with 0.5 mg/liter 2,4-dichlorophenoxyacetic acid (2,4-D), 2.0 mg/liter p-chlorophenoxyacetic acid (pCPA) and 0.1 mg/liter kinetin. Cell suspension cultures were then established from the callus in liquid B_5 medium containing 1 mg/liter 2,4-D, from which somatic embryos evolved. Plantlets that developed from the embryos were albino. All cultures were incubated at 28°C in darkness until the plantlets were visible.

Atkins and Barton (1973) induced calluses from orchardgrass (Dactylis glomerata L.) primary roots or germinating seeds cultured on RM (Linsmaier and Skoog), Miller's and White's media containing various levels of 2,4-D or indole-3yl-acetic acid (IAA) in combination with two levels of kinetin. RM medium supplemented with 5.0 mg/liter 2,4-D was far superior to White's and slightly better than Miller's in induction of callus from root cultures. The germinating seeds, when cultured on RM medium containing 1.0 mg/liter 2,4-D, dedifferentiated into callus, indicating that different explants responded to the hormone

differently. No differentiation from those cultures was reported.

Orchardgrass calluses were also obtained by Conger and Carabia (1978) from cultured whole caryopses on modified SH medium containing 15.0 mg/liter 2,4-D and 2.15 mg/liter kinetin. The same basal medium with the 2,4-D level reduced to 5.0 mg/liter was used for callus maintenance. Callus differentiation was found to be sporadic when subcultured on the medium containing 1.0 mg/liter 2,4-D. Hanning and Conger (1982) further initiated calluses from orchardgrass young leaf segments on SH medium containing 30 μM 3,6-dichloro-o-anisic acid (dicamba). Embryogenesis occurred directly in the primary cultures.

Callus initiation was observed by Chen, Chen, Lo and Ross (1982) in segments of young inflorescences of orchardgrass cultured on RM media supplemented with 2,4-D or NAA up to 2.26×10^{-2} mM. The callus tissue was not totipotent, but plants regenerated from the axillary buds within the spikelets of the inflorescence explants.

Ahloowalia (1975) cultured immature F_1 seeds of the hybrid Lolium multiflorum X Lolium perenne on a modified NW (Niizeki and Oono) medium supplemented with 1.5 mg/liter 2,4-D, 6.5 mg/liter IAA and 0.25 mg/liter zeatin. The calluses were maintained on RM medium with the same hormone combinations except that zeatin was replaced by kinetin at

2.15 mg/liter. Shoots and roots differentiated from calluses subcultured on half-strength RM medium containing 0.75 mg/liter 2,4-D, 3.25 mg/liter IAA and 1.075 mg/liter kinetin. Embryo-like structures were initiated from cell suspension cultures.

Big bluestem (Andropogon gerardii Vitman) was cultured in vitro by Chen, Stenberg and Ross (1977). Segments of unemerged inflorescences, 5 to 10 mm long, were explanted on RM basal medium supplemented with 5.0 mg/liter 2,4-D in combination with 0 or 0.2 mg/liter kinetin. Friable calluses were initiated in both of the treatments. The calluses, which were maintained on RM medium containing 5 mg/liter 2,4-D, differentiated into shoots after cultured on the same basal medium containing less than 2.0 mg/liter 2,4-D. Histological studies of the differentiating callus tissue revealed a monopolar pattern of differentiation, a characteristic of organogenesis. Similar results were obtained in this laboratory with smooth bromegrass, creeping foxtail, crested wheatgrass, green needlegrass and western wheatgrass by Lo, Chen and Ross (1980).

Conger, Carabia and Lowe (1978) initiated callus from excised embryos of tall fescue (Festuca arundinacea Schreb) grown on MS medium supplemented with 2,4-D or 2,4,5-T at concentrations ranging from 10 to 60 μM. Two,four-D was found to be better in callusing than 2,4,5-T.

Tall fescue plantlets were regenerated sporadically by

Lowe and Conger (1979) from calluses placed on MS medium containing 0.5 mg/liter 2,4-D. The poor morphogenetic response of the calluses was attributed to genotypic effects.

Chen, Lo and Ross (1979) initiated calluses from unemerged inflorescences of ten indiangrass (Sorghastrum nutans L. Nash) genotypes cultured on RM medium containing 5.0 or 10.0 mg/liter 2,4-D in combination with 0 and 0.2 mg/liter kinetin. The calluses grew faster on the medium supplemented with 5.0 mg/liter 2,4-D, regardless of the kinetin concentration supplemented. Plantlets regenerated from the calluses subcultured on RM medium with 2,4-D levels less than 1.0 mg/liter. Differences in morphogenetic response were observed.

Dale (1975) explanted ryegrass (Lolium multiflorum) meristem tips on MS medium containing 0.2 mg/liter kinetin. No calluses were initiated, but rather plantlets regenerated directly from the explant. Similar results were obtained from meristem tip cultures of orchardgrass, timothy (Phleum pratense), Lolium and Festuca species (Dale, 1977a, 1977b).

Immature embryos from ten ryegrass genotypes initiated callus when cultured on MS medium supplemented with 2.0 mg 2,4-D and 0.2 mg BAP/liter (Dale, 1980). Scutellum-derived callus differentiated white nodules which were then subcultured on RM or B_5 basal media and developed into embryoids and shoots.

In guinea grass (Panicum maximum), Lu and Vasil (1982) obtained calluses from young inflorescences, and immature and mature embryos grown on MS medium supplemented with 2.5 to 10.0 mg/liter 2,4-D. Most of the calluses originated from the scutellum of the embryos and the young floral primordia of the inflorescence. The calluses, which were maintained on the MS basal medium containing 5.0 mg/liter 2,4-D, were found to be embryogenic on the same medium with reduced levels of 2,4-D. Similar results were obtained by Lu and Vasil from cultured leaves and protoplast-derived callus tissue (1981a, 1983c) and freely-suspended cells (1981b) of the same species.

Vasil and Vasil (1981) cultured immature embryos and young inflorescence segments of pearl millet (Pennisetum americanum). The embryos, 7 to 15 days after pollination, were orientated so the embryo axis faced the medium. Calluses initiated from the embryos on MS media containing 1.0 to 10.0 mg/liter 2,4-D, and from the inflorescence segments on MS medium supplemented with 2.5 mg/liter 2,4-D. The calluses proliferated best from inflorescences showing recognizable individual spikelets and floral parts. Coleoptile-like structures emerged from organized areas resembling scutellums after subculturing the callus on MS medium without any hormones. Vasil and Vasil (1982) also reported similar findings from cell suspension cultures of pearl millet.

Haydu and Vasil (1981) obtained calluses from cultures of young leaf segments of elephant grass (Pennisetum purpureum Schum.) on MS medium supplemented with 0.5 to 10.0 mg/liter 2,4-D. Embryogenesis was observed from the calluses maintained on MS medium containing 0.5 mg/liter 2,4-D, 0.5 mg/liter benzylaminopurine (BAP), 1.0 mg/liter naphthaleneacetic acid (NAA) and 5.0 percent coconut milk (CM). Similar findings were reported in inflorescence cultures of elephant grass by Wang and Vasil (1982).

Heyser and Nabors (1982) induced calluses from immature embryos, mature seeds, mesocotyls and leaf and adjoining stem segments of proso millet (Panicum miliaceum L.) explanted on RM medium containing various concentrations of 2,4-D and 2,4,5-T. Except for mature seeds and mesocotyls, which initiated calluses equally well on both of the auxins used, 2,4-D appeared to be more effective in callus induction for the other two explants. Embryoids developed from the calluses and eventually germinated into plantlets on RM medium containing no hormones.

In creeping bentgrass (Agrostis palustris Huds.), Krans, Henning and Torres (1982) initiated calluses from cultured caryopses on MS medium supplemented with 1.0 and 10.0 mg/liter 2,4-D. The calluses proliferated on MS medium containing 5.0 mg/liter 2,4-D and differentiated into shoots on the medium supplemented with 0.1 or 1.0 mg/liter kinetin.

It appeared that the auxin 2,4-D could be the sole hormone supplement to various basal medium for callus initiation and maintenance of forage grass cultures. The same callus induction medium with 2,4-D removed was usually applied to induce plant regeneration. However, in some cases, kinetin fortified to the auxin free medium might enhance morphogenetic induction.

Though morphogenic calluses were initiated from immature and mature embryos, the frequency of plant regeneration from these calluses was low. Young inflorescences seemed to be the most effective explants used in in vitro plant regeneration of forage grasses.

Embryogenesis was first described by Steward, Mapes and Mears (1958) in carrot suspension cultures which required coconut milk to initiate regeneration. Shoot and root apexes differentiated simultaneously from a proembryo cell mass, which resulted in bipolar appearance. Embryos in forage grass cultures were first observed by Gamborg, Constabel and Miller (1970) in bromegrass suspension cultures.

Recently, many reports have described the formation of somatic embryos in forage grasses. Vasil and associates regenerated plants through embryogenesis in cultures of protoplasts, immature embryos, leaves and inflorescences of several grass species (Vasil and Vasil, 1980, 1981, 1982; Haydu and Vasil, 1981; Lu and Vasil, 1981a, 1981b, 1981c,

1982). Dale (1980) observed somatic embryos from cultured immature embryos of ryegrass. Embryoid formation in an orchardgrass leaf callus was seen by Hanning and Conger (1982). McDaniel, Conger and Graham (1982), in a histological study of differentiating orchardgrass callus, found both patterns of regeneration.

Unlike somatic embryos, which are of single cell origin and appear similar to zygotic embryos, regeneration of plants via organogenesis arises from multicellular cell clusters which develop into shoot meristems (Wang and Vasil, 1982). Prior to 1980, most grass cultures regenerated plantlets through organogenesis. Ahloowalia (1975) observed shoots initiating from ryegrass calluses. Chen and associates initiated shoots from differentiating calluses of big bluestem (Chen, Stenberg and Ross, 1977), indiangrass (Chen, Lo and Ross, 1979), bromegrass (Lo, Chen and Ross, 1980), and directly from orchardgrass cultures (Chen, Chen, Lo and Ross, 1982). Conger and Carabia (1978) initiated shoots from orchardgrass calluses. Similar results were obtained from callus cultures of tall fescue by Lowe and Conger (1979) and of creeping bentgrass by Krans, Henning and Jones (1982).

MATERIALS AND METHODS

In a preliminary experiment, RM formula (Linsmaier and Skoog, 1965), supplemented with 1.25, 2.50 and 5.00 mg 2,4-D or NAA/liter was prepared for primary culturing. Later, the RM medium fortified with 5 mg 2,4-D/liter was adopted for callus induction and proliferation. The media were adjusted to pH 5.8 and solidified with 8 grams of agar per liter. Twenty milliliters of medium were dispensed into a 2.5 x 15 cm screw-capped test tube. The media were autoclaved at 132°C and 1.05 kg/sq cm for 15 minutes, and cooled to a slant. For subculturing, RM medium supplemented with various concentrations of 2,4-D and/or kinetin was prepared. Unless otherwise stated, the media were dispensed into 125-ml Erlenmeyer flasks, 40 ml each. The flasks were capped with a double layer of aluminum foil before being autoclaved.

Seven field-grown individual plants of little bluestem (Schizachyrium scoparium (Michx) Nash), each representing a genotype, were selected for this research. Young inflorescences, collected from these plants at the stage when the flag leaf was just emerging, were used as explants. For explanting, a leaf whorl was removed from the tiller furnishing inflorescence, until only one leaf sheath remained. The stems were then surface disinfected with 70% ethanol and placed in a sterile petri dish. Each stem was split longi-

tudinally with a pair of scalpels so that the inflorescence was exposed. The inflorescence was then removed from the stem and cut into 5 to 10 mm segments. One segment was inoculated to each culture tube. The primary cultures were incubated at 25 ± 1°C in the dark.

Calluses of two genotypes were used to determine the callus maintenance medium for this species. Approximately 100 mg of callus were transferred to a 125 ml flask, containing 40 ml of RM solid medium supplemented with 1.25, 2.50, or 5.00 mg 2,4-D/liter. Ten replications were made in each of the hormone treatments. Fresh-weight increases were determined from each of the treatments after one month of incubation in the dark at 25 ± 1°C.

Morphogenetic response of genotype 5 callus was tested first by subculturing it on RM medium containing various levels of 2,4-D and kinetin. The hormone combination which showed the most effectiveness on shoot differentiation from the callus was then adopted to compare the shoot-forming capacity among the calluses of the five genotypes. Shoot counts from each treatment were made from six flasks after one month incubation in the dark at 27 ± 1°C and one week in a growth chamber with 16 hour photoperiods (cool-white fluorescent light at 2 Klx).

In the histological studies of callus differentiation, a piece of callus grown on the differentiation medium in the

dark at 27 ± 1°C was collected at one-week intervals for five weeks and fixed in the Nawaschin fluid for 48 hours. The fixed tissues were then dehydrated through an ethanol/butanol series and embedded in paraffin blocks which were serial-sectioned at 10 μm in thickness and stained with safranin- or Feulgen-fast green combination. The detailed procedure was described by Sass (1958).

RESULTS

In the primary cultures of little bluestem inflorescence segments, the auxin NAA supplemented to RM basal medium was not effective on callus initiation. All of the explants grown on these media showed browning three weeks after inoculation and eventually wilted. All segments of the young inflorescences inoculated on the medium supplemented with 2.5 or 5.0 mg 2,4-D/liter initiated compact, yellow calluses which started to grow at the cut-ends of the pedicel and on the surface of spikelets one week after explanting. Calluses of little bluestem, which proliferated much slower than those of big bluestem, required about two months of primary culturing before isolation and subculturing of callus could be made. Figure 1A shows the callus spread and covered the explants cultured on RM medium supplemented with 5.0 mg 2,4-D/liter.

The calluses isolated from the primary cultures were temporarily propagated on RM medium supplemented with 5.0 mg 2,4-D/liter in 125-ml flasks, each of which contained 40 ml of medium. The cultures were maintained at 25±1°C in the dark. The texture of the callus in the first passage of subculturing was soft and slightly slimy, but became firm and healthy looking after one to two transfers (Figure 1B). The formula RM medium supplemented with 5.0 mg 2,4-D/liter was then adopted for callus initiation and propagation of

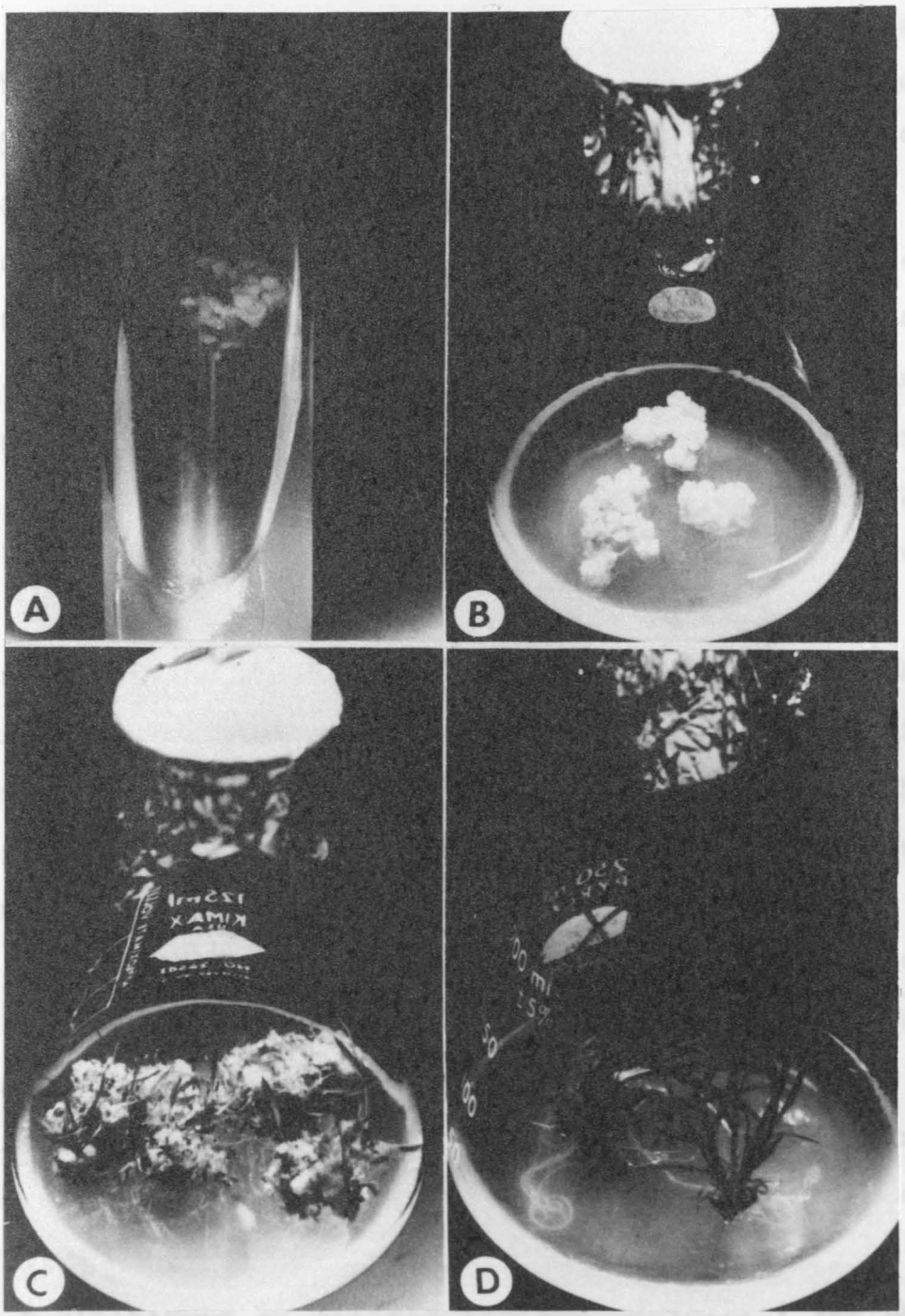
Fig. 1. Plant regeneration from inflorescence cultures of little bluestem:

(A) Callus initiation from inflorescence segments

(B) Callus tissue proliferation

(C) Shoot differentiation from callus tissue

(D) Adventitious root formation from shoots


A
B
C
D

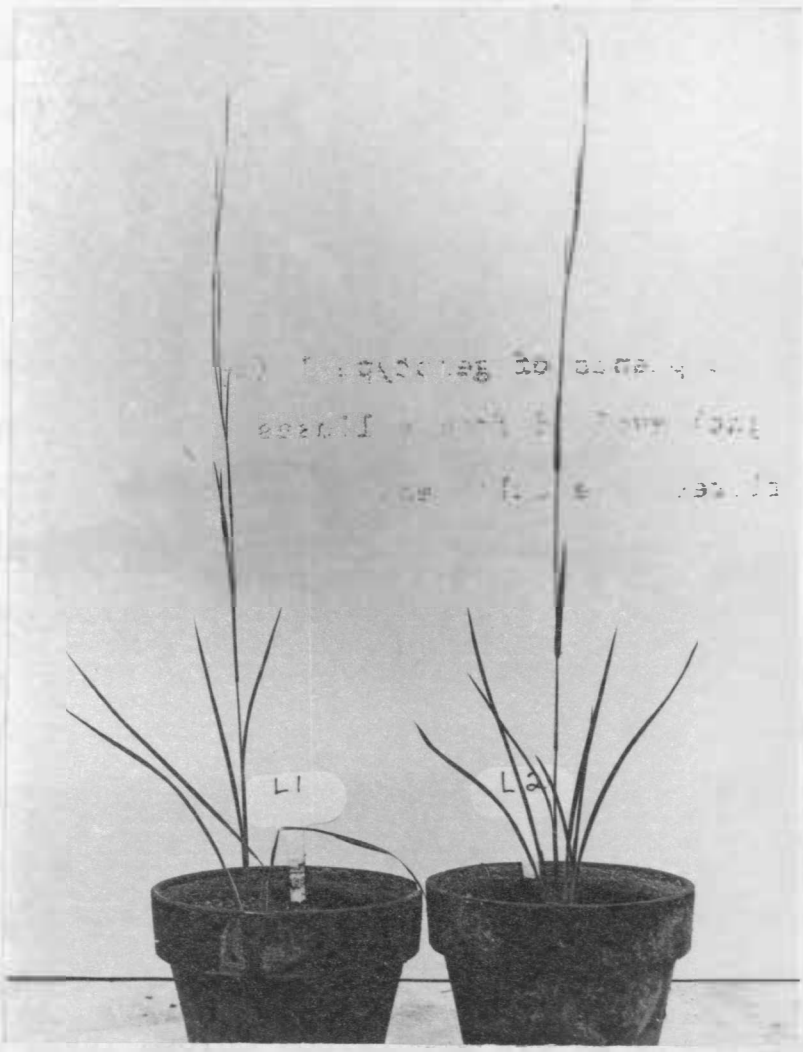
the other genotypes of little bluestem.

A test for totipotency of the propagated calluses was made by transferring them on RM medium, devoid of hormones, in 125-ml flasks. The cultures were incubated at 25±1°C in the dark. Two weeks after transfer, whitish nodules appeared on the surface of the callus, signaling that differentiation might have been taking place in the callus cells. That was verified later by the emergence of numerous shoots from the callus tissues four weeks after incubation. These cultures were then moved to a growth chamber at 27±1°C with 16 hour photoperiods (cool-white fluorescent light at 2 Klx). The shoots became green and grew normally under light (Figure 1C). The shoots were transferred together with calluses at their bases onto 250-ml flasks, each of which contain 50 ml RM basal medium, free of hormones, to enhance root growth. Thus, *in vitro* plant development was completed (Figure 1D).

At the three leaf stage, the plantlets were removed from culture flasks and transplanted either into pots containing vermiculite, which were nourished with diluted Hoagland's solution, or directly into potting soil. In greenhouse condition, the plants reached maturity six months after transplanting (Figure 2).

A difference in differentiation capability was noted between the two genotypes of little bluestem calluses tested. Shoot counts in ten flasks, each of which initially

Fig. 2. Mature plants of genotype 1 (left) and genotype 2 (right) evolved from calluses of little bluestem inflorescence cultures.


L1
L2

contained 100 mg of callus of genotype 1, totaled 66 with a range from 1 to 14 shoots per flask, whereas those of genotype 2 reached 172 with a variation from 7 to 26 shoots per flask (Table 1).

Since auxin and cytokinin might interact on plant regeneration (Skoog and Miller, 1957), the callus of genotype 5 was inoculated on RM medium supplemented with 0.0, 0.1, 1.0, or 5.0 mg 2,4-D and kinetin/liter, making 16 possible treatments. Every treatment consisted of six flasks, each of which contained four approximately 100-mg pieces of callus which had been subcultured for four passages. Shoot counts were made after four weeks of incubation in the dark at 27±1°C followed by one week in a lighted growth chamber.

The auxin 2,4-D present in the RM medium exerted an unfavorable effect on shoot initiation of genotype 5 callus. Irrespective of the kinetin concentrations tested, the calluses subcultured on RM medium containing 1.0 mg 2,4-D/liter initiated 15 shoots, with a range of 0 to 13 among the four kinetin treatments. The calluses cultured on the same basal medium containing 5.0 mg 2,4-D/liter initiated 1 shoot, which occurred only on the medium further supplemented with 5.0 mg kinetin/liter. Numerous shoots were initiated from the calluses grown on RM medium with no or 0.1 mg 2,4-D/liter. More shoots were counted from the calluses on 2,4-D free medium than those grown on the same

Table 1. Shoot differentiation in two little bluestem calluses cultured on RM medium containing no hormones.

Genotype	No. shoots per 10 calluses	Range
L_1	66	1 - 14
L_2	172	7 - 26

medium containg 0.1 mg 2,4-D/liter. Additions of kinetin up to 5.0 mg/liter to the medium containing no or 0.1 mg 2,4-D/liter enhanced shoot initiation. The degree of enhancement was parallel to the amount of kinetin present in the medium. On the other hand, 2,4-D at high concentrations gave adverse effects on callus differentiation into shoots as well as aerial roots. The calluses grown on the media with no 2,4-D generally initiated more aerial roots than those on higher 2,4-D media. Kinetin at high concentrations tended to inhibit aerial root formation (Table 2).

The effects of high kinetin on differentiation were then tested involving five genotypes of calluses. The calluses subcultured for five passages were grown on RM medium supplemented with 0.0 or 5.0 mg kinetin/liter. Each treatment consisted of five flasks. The handling of cultures was essentially the same as in the previous experiment.

Calluses of genotypes 4 and 6 failed to initiate shoots on either medium, while genotype 7 callus did not differentiate shoots on the medium with no kinetin, but three shoots were counted in one flask containing kinetin.

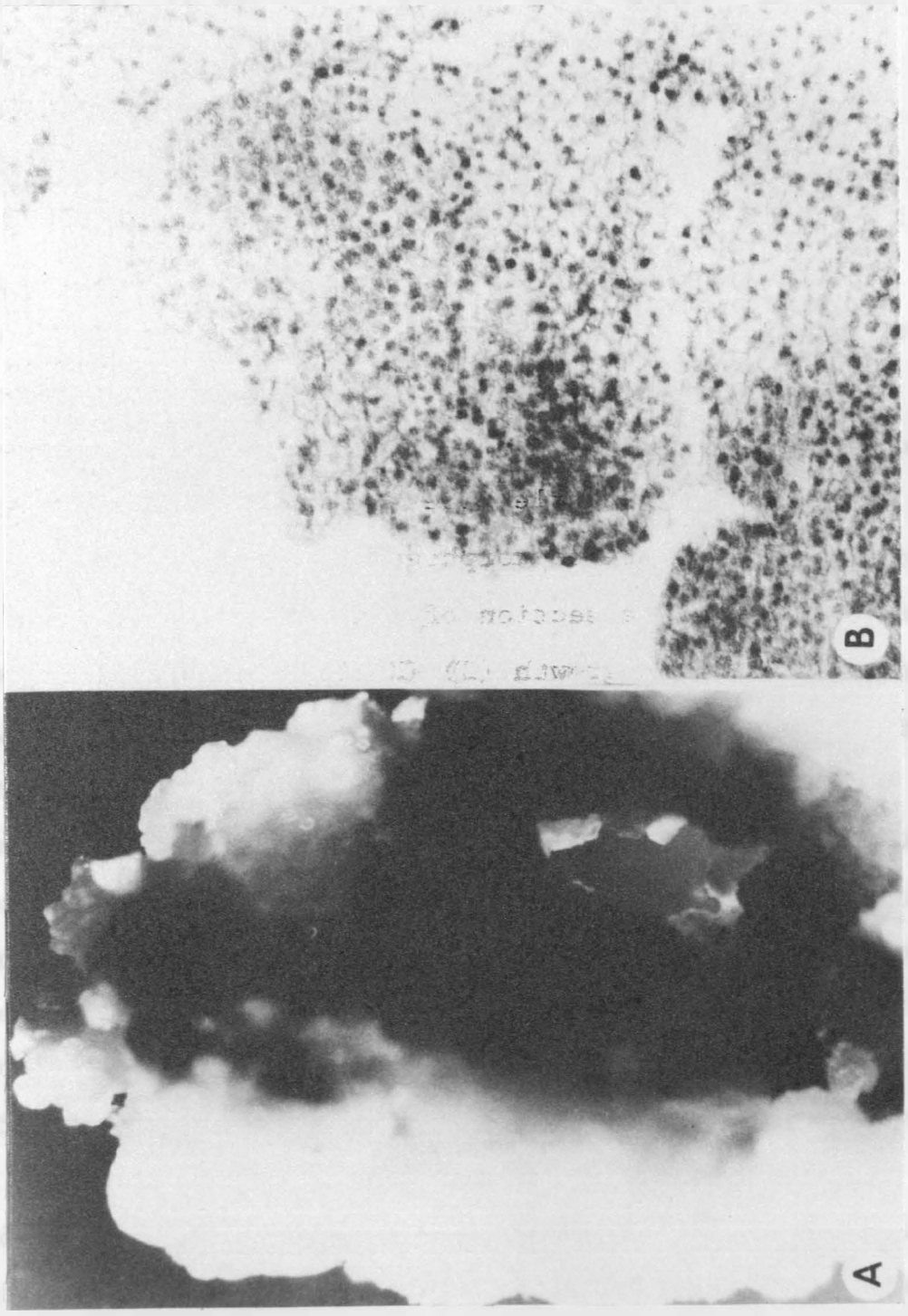
Wide differences in shoot counts between the two treatments were noted in the calluses of genotypes 3 and 5. In genotype 3, 83 shoots were counted from the culture grown on kinetin medium whereas only 12 on RM basal medium. In

Table 2. Morphogenetic effects of 2,4-D and kinetin on callus cultures of little bluestem genotype 5.

2,4-D (mg/l)	0				0.1				1.0				5.0			
Kinetin (mg/l)	0	0.1	1.0	5.0	0	0.1	1.0	5.0	0	0.1	1.0	5.0	0	0.1	1.0	5.0
Number of shoots	57	75	86	147	15	34	53	43	1	1	0	13	0	0	0	1
Root initiation	+++	+++	++	++	+++	++	++	+	+	+	+	+	-	-	-	-

+++ = intense aerial root initiation
++ = moderate aerial root initiation
+ = minimal aerial root initiation
- = no aerial root initiation

Fig. 3. A callus of little bluestem genotype 5 at the time of subculture on morphogenetic induction medium (A) (X 20) and a section of the callus showing unorganized growth (B) (X 300).


B
A

genotype 5, 110 versus 15 shoots were counted in these treatments. In comparison to the previous experiments the lower counts in this case might reflect a decline of totipotency.

The results from these two experiments might imply that besides genotypic differences in differentiation potential, decline of totipotency in calluses seemed to be also genotype-dependent. Kinetin might have an effect on either restoring or prolonging totipotency in the callus of genotype 7 (Table 3).

Differences in aerial root density were shown among the calluses grown on RM medium containing 0.0 or 5.0 mg kinetin/liter. Except genotype 4, in which no differences in growth of aerial roots were noted, calluses grew less aerial roots when cultured on RM medium containing 5.0 mg kinetin/liter than on RM basal medium. Calluses of genotypes 3 and 5, which differentiated numerous shoots, initiated no aerial roots when grown on the medium containing kinetin. The effects of kinetin on retardation of root initiation seemed to be inversely related to potential of shoot differentiation, which appeared to be genotype-dependent.

Previously, RM basal medium, devoid of hormones, was used in this laboratory as morphogenetic medium for callus differentiation of many forage grass species (Chen, Stenberg and Ross, 1977; Chen, Lo and Ross, 1979; Lo, Chen and Ross,

Table 3. Genotypic differences in morphogenetic response to RM medium supplemented with 0 or 5 mg/l kinetin.

Kinetin (mg/l)	0		5.0	
	Number of shoots	Root initiation	Number of shoots	Root initiation
Genotype				
3	12	+	85	-
4	0	+	0	+
5	15	++	110	-
6	0	+++	0	+
7	0	+++	3*	++

+++ = intense aerial root initiation
++ = moderate aerial root initiation
+ = minimal aerial root initiation
- = no aerial root initiation
* = one callus initiated 3 shoots

1980). This formula, which was also effective on morphogenetic induction of little bluestem callus as indicated in the preceeding experiments, was then used in studies of morphological and structural changes in the differentiating calluses. Calluses of three genotypes of little bluestem, after subcultured for three passages on maintenance medium, were transferred onto the morphogenetic medium. The calluses were then examined with a stereomicroscope, photographed and fixed at one week intervals for five weeks.

No evidence of shoot or root initiation was seen in callus tissues at the time of subculturing on the morphogenetic induction medium (Figure 3A). Sections made from these calluses showed unorganized growth. All the cells were similar in size and contained dense cytoplasm with a large nucleus (Figure 3B).

Calluses grown seven days on the morphogenetic induction medium developed a rough texture with small protrusions throughout their surfaces (Figure 4A). Sections of the calluses showed meristematic cores consisting of small isodiametric cells surrounded by larger irregular shaped callus cells (Figure 4B). Each cell of the meristematic core contained a large nucleus within a dense cytoplasm.

Whitish nodules, which appeared to be shoot primordia, emerged from a callus of genotype 5 cultured for fourteen days on the morphogenetic induction medium (Figure 5A). Shoot differentiation was evident in the sectioned tissues.

Fig. 4. A callus of little bluestem genotype 5 subcultured seven days on morphogenetic induction medium showing a rough texture (A) (X 20) and a section of the callus showing a meristematic core (B) (X 300).

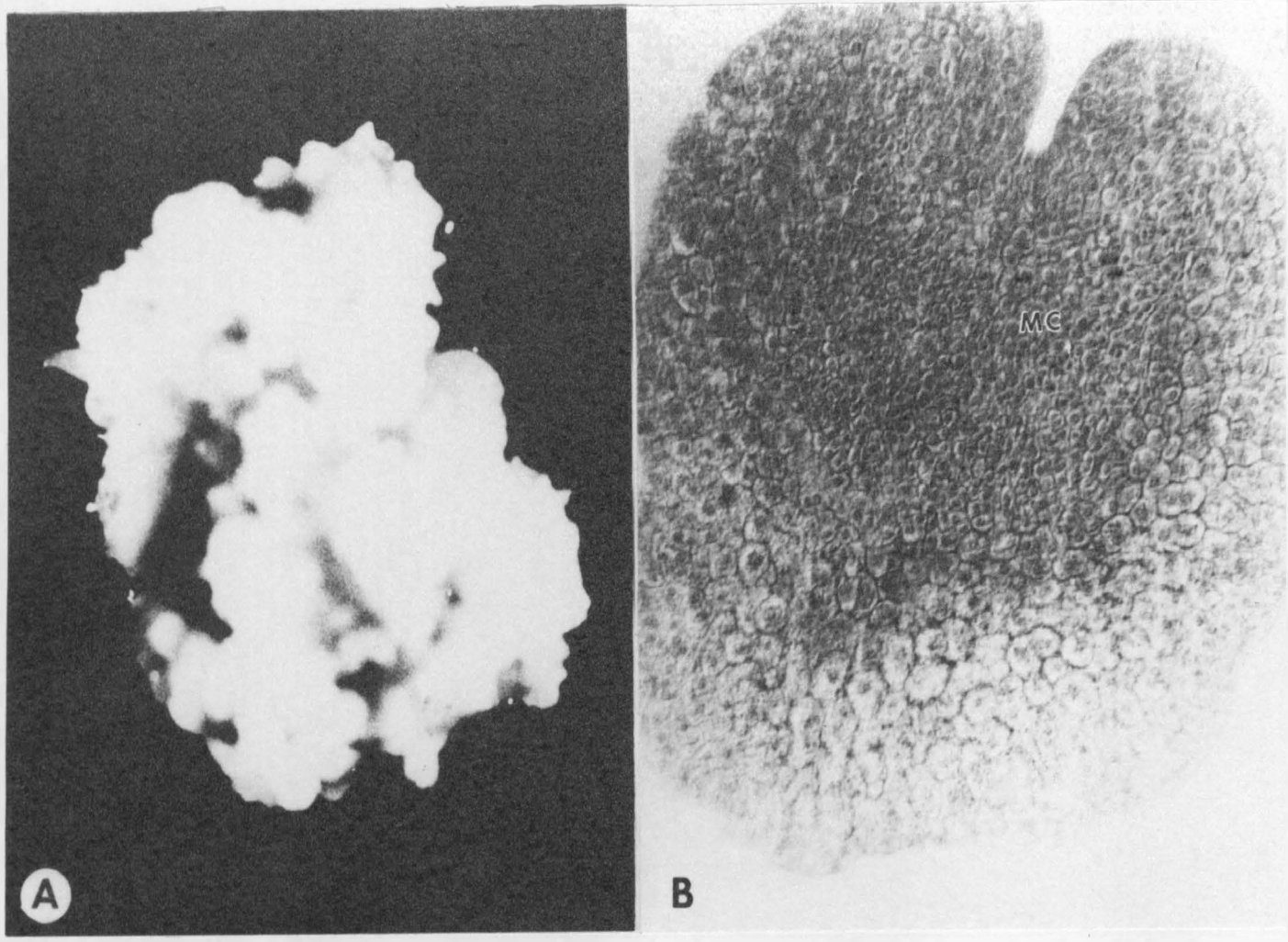
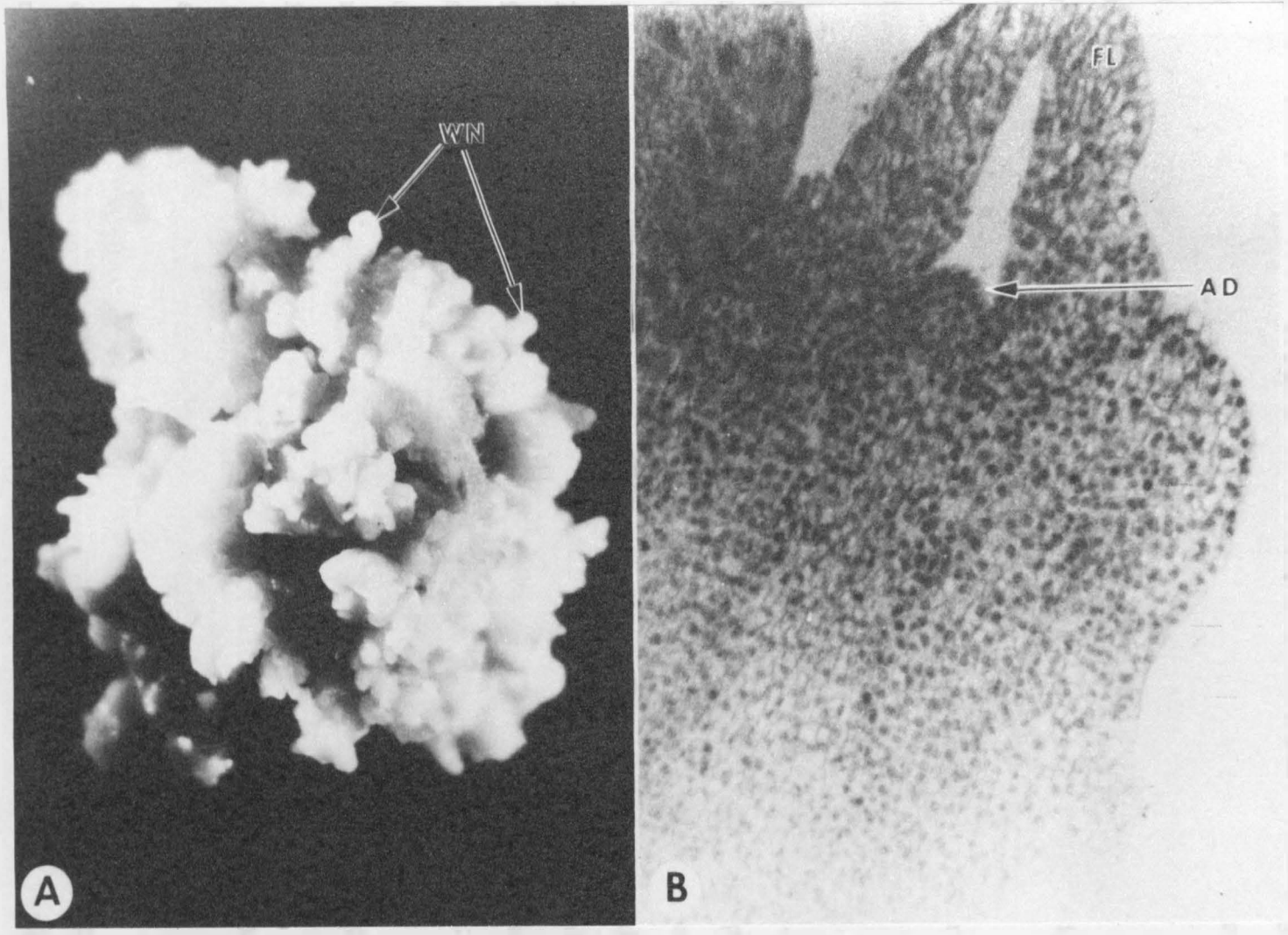

A
MC
B

Fig. 5. A little bluestem callus of genotype 5 fourteen days after subcultured on morphogenetic induction medium showing whitish nodules (arrows) on surface (A) (X 20) and a section of the callus showing a shoot primordium consisting of the first leaf which encapsulates the apical dome (B) (X 300).


WN
A
FL
AD
B

Each shoot consisted of a coleoptile-like structure encapsulating an apical dome. Further specialization in the coleoptile resulted in vacuolated cells near its outer margins. The cells of the apical dome possessed large nuclei and dense cytoplasm. No leaf primordia were initiated from the apical dome at this stage (Figure 5B). Lack of root primordia at the opposite pole of this structure indicated a monopolar pattern of differentiation.

Embryo-like structures were found in a callus of genotype 1 after twenty-one days of incubation (Figure 6A). The embryoid possessed a scutellum-like structure, a coleoptile which enclosed an apical dome, and a root on the same axis. The coleoptile and the scutellum-like structures showed large cells with small nuclei, implying that cellular differentiation was occurring. Meristematic cells with dark stained nuclei were located throughout the apical dome. No clear demarcation was noted between the coleorhiza and the radicle in the root apex, indicating that they were not fully differentiated.

The callus of genotype 1 also initiated shoot-like structures which were characteristic of organogenesis (Figure 6B). The monopolar pattern of differentiation was evident since no root emerged from the same axis. Root primordia which differentiated independently from the callus were also observed (Figure 6C). These structures eventually developed into aerial roots.

Fig. 6. Sections of little bluestem genotype 1 callus after subcultured twenty-one days on morphogenetic induction medium showing (A) an embryo-like structure with scutellum, coleoptile, apical dome, radicle and coleorhiza (X 150), (B) a shoot-like structure with first leaf, apical dome and leaf primordium (X 75), and (C) a root primordium (X 300).

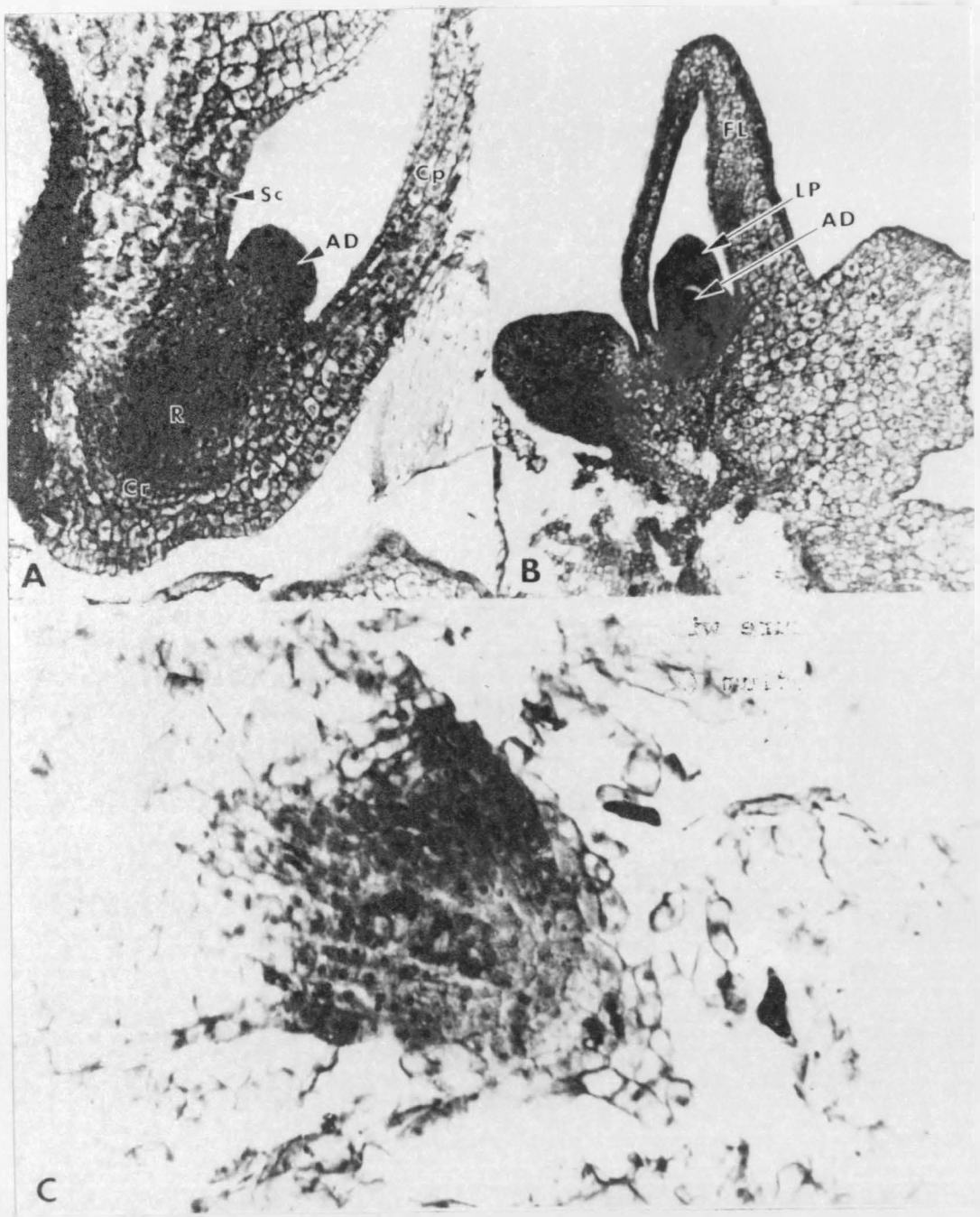
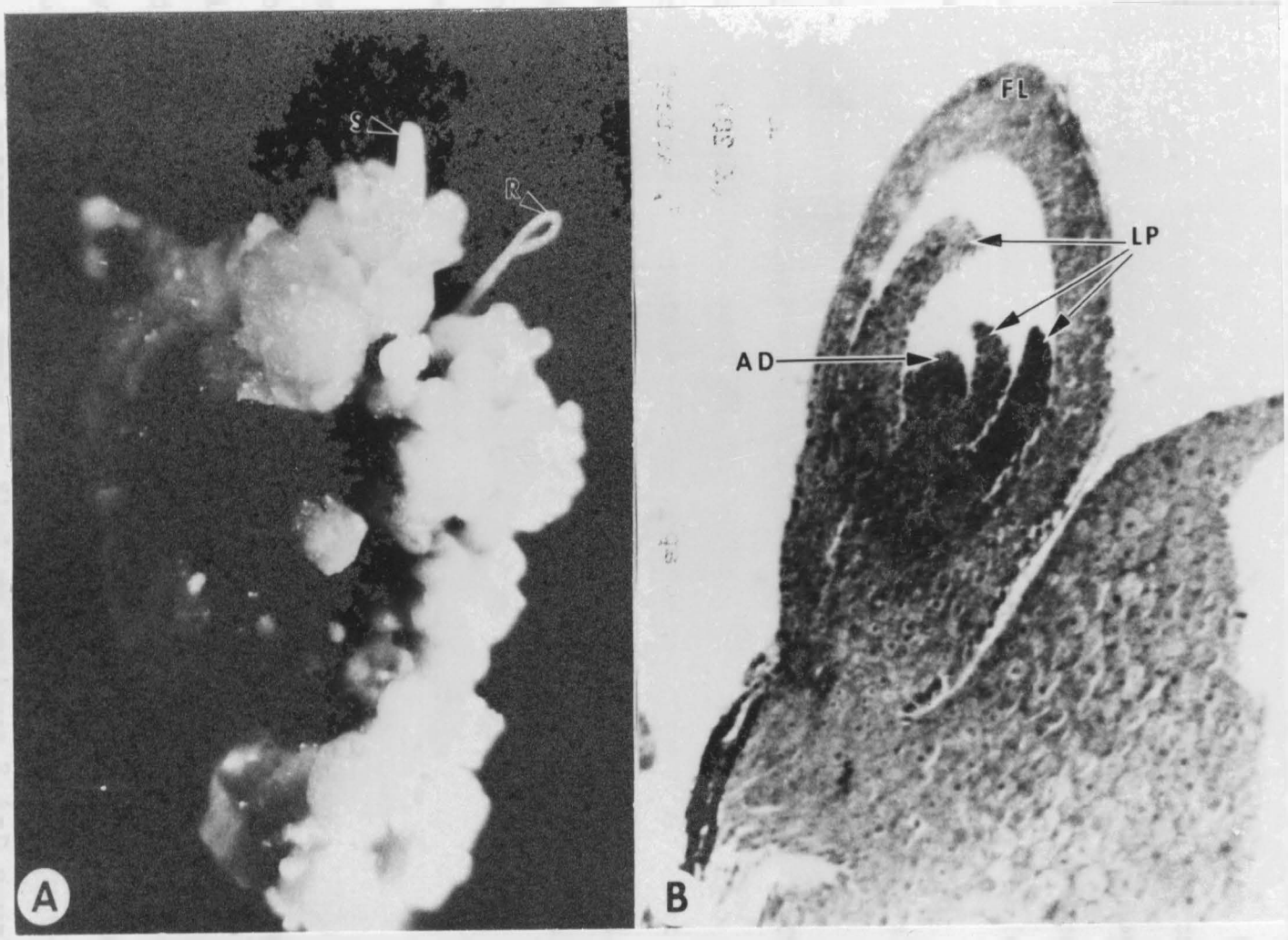

Sc
AD
Cp
R
Cr
A
FL
LP
AD
B
C

Fig. 7. A callus of little bluestem genotype 5 subcultured for twenty-eight days on morphogenetic induction medium showing shoots and roots (A) (X 20) and sectioned shoot apex with first leaf, apical dome and leaf primordia (B) (X 125).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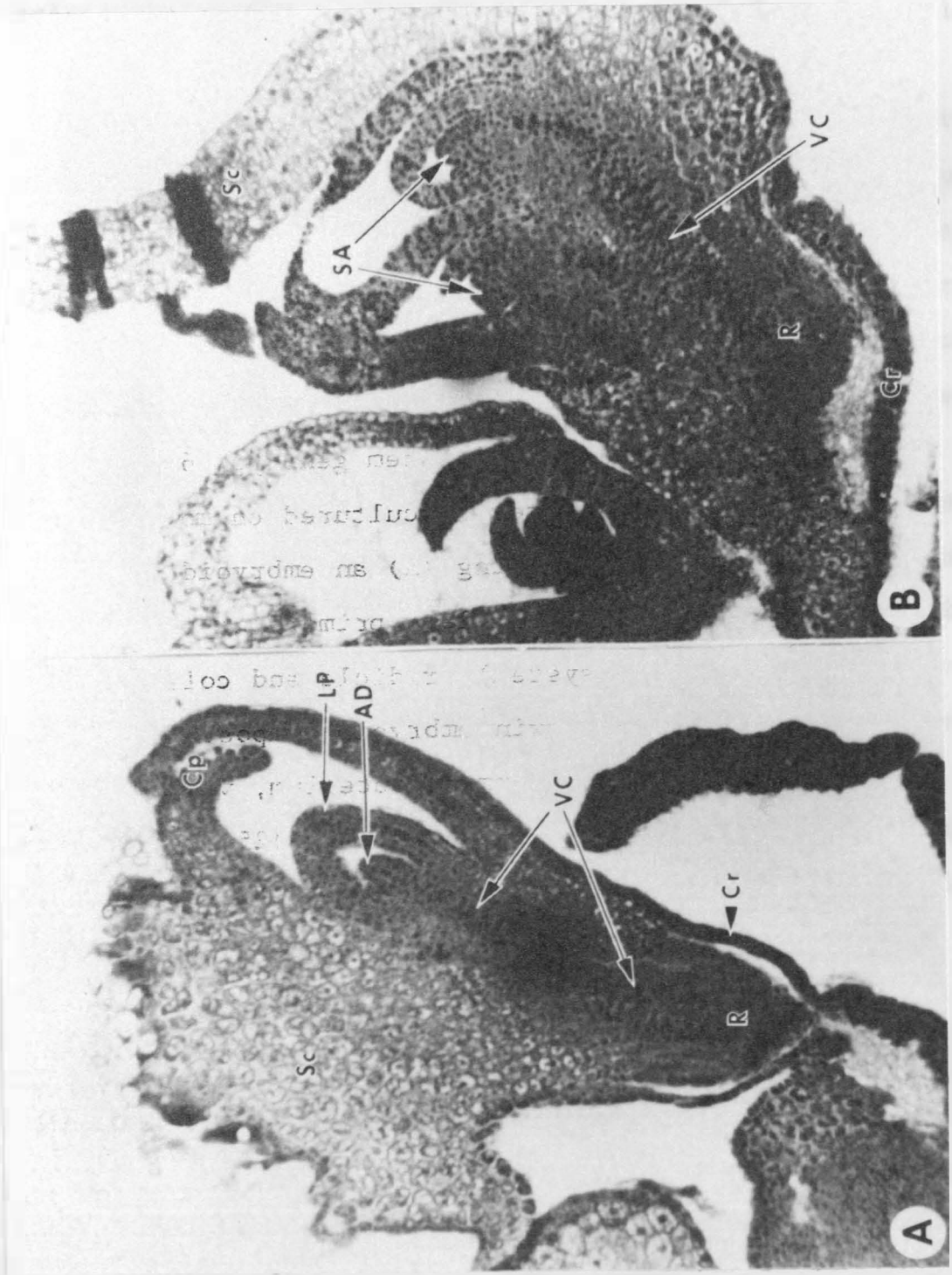
Further development of shoots was seen on the surface of genotype 5 callus cultured for twenty-eight days on the morphogenetic induction medium (Figure 7A). Histologically, a shoot apex bore three leaf primordia which were enclosed by the first leaf. The lack of an associated root primordium on the same axis suggested that the shoot was differentiating through organogenesis (Figure 7B).

Well developed somatic embryos were noted in a callus tissue of genotype 6 fixed thirty-five days after subculturing on the differentiation medium (Figure 8A). The shoot apex was composed of an apical dome and a leaf enclosed by a coleoptile-like structure. The root apex consisted of the coleorhiza and the radicle. The coleorhiza and the scutellum-like structures were not in fixed positions, as they are in little bluestem zygotic embryos (Reeder, 1957). A vascular system was developing.

In the same callus, a twin embryoid sharing one scutellum-like structure was observed (Figure 8B). The twin embryoid had separate shoot apices, but a common root apex. This apex was composed of a distorted coleorhiza and a radicle, possibly resulting from a merger of the shoots. A vascular system extended from both shoot apices to the root apex.

An attempt was made to find an optimum 2,4-D concentration in RM basal medium for little bluestem callus maintenance. Calluses of genotypes 1 and 2, which were

Fig. 8. Sections of a little bluestem genotype 6 callus thirty-five days after subcultured on morphogenetic induction medium showing (A) an embryoid with scutellum, coleoptile, leaf primordium, apical dome, (vascular system), radicle and coleorhiza (X 125) and (B) a twin embryoid composed of two shoot apices and a common scutellum, vascular system, radicle and coleorhiza (X 125).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originally grown on RM medium containing 5.0 mg 2,4-D/liter were also transferred onto RM medium with 2,4-D concentrations reduced to 1.25 and 2.50 mg/liter. Each treatment consisted of ten flasks. A statistical analysis was made according to the simple factorial experimental design. The only significant F value (7.4675) at 5% level occurred among treatments (Table 4). No differences between genotypes or interactions between genotype and treatment were observed.

The Tukey's D-test (Snedecor, 1957) was then used to compare the average fold-increase of callus fresh weight between any two hormone treatments in genotypes 1 and 2 (Table 5). No significance was determined among the treatments of genotype 2 calluses, whereas genotype 1 differed between 5.0 and 1.25 or 2.50 mg 2,4-D/liter. However, the lower 2,4-D levels failed to sustain undifferentiated callus growth, henceforth the medium containing 5.0 mg 2,4-D/liter was used for callus proliferation.

Table 4. Analysis of variance of little bluestem callus proliferation on RM medium supplemented with three levels of 2,4-D.

Source of Variation	D.F.	SS	MS	F
Genotype	1	8.2304	8.2304	2.0836
Treatment	2	58.9935	29.4967	7.4675*
Genotype x Treatment	2	2.9873	1.4946	.3783
Error	54	213.3024	3.9500	
Total	59	283.4156	4.8037	

*significance at 5% level.

Table 5. Fold-increase of little bluestem callus growth on RM medium supplemented with three levels of 2,4-D.

Genotype	2,4-D Concentration mg/liter	Fold increase Mean S.E.
L_1	1.25	8.02 ± 0.39*
	2.50	7.49 ± 0.54*
	5.00	5.24 ± 0.37
L_2	1.25	8.25 ± 1.03*
	2.50	8.17 ± 0.66*
	5.00	6.55 ± 0.55

*Shoot differentiation was noted in at least three calluses one month after subculture.
Expected D = 2.15

DISCUSSION

Young inflorescences of little bluestem plants used as explants initiated totipotent callus cells. Effectiveness of inflorescence explants in regeneration of plantlets has been reported in tissue cultures of many other forage grass species (Chen, Stenberg and Ross, 1977; Chen, Lo and Ross, 1979; Lo, Chen and Ross, 1980; Vasil and Vasil, 1981, 1982; Lu and Vasil, 1982; Wang and Vasil, 1982). Calluses have been initiated from caryopses and immature embryos, but differentiation capacities ranged from ten percent for those of orchardgrass (Conger and Carabia, 1978) to thirty percent for pearl millet (Vasil and Vasil, 1981). Recently, young panicles and spikes have also been adopted in in vitro cultures of cereal crops such as sorghum (Brettell, Wernicke and Thomas, 1980), triticale (Nakamura and Keller, 1982) and wheat (Ozias-Akins and Vasil, 1982). Morphogenetic quality of the calluses seemed to be related to developmental stage of the inflorescence.

In grass cultures, texture of totipotent callus tissues usually was firm. However, some little bluestem calluses showed a viscous material on their periphery, similar to what was described by Haydu and Vasil (1981). Repeated subculturing on the same medium yielded compact calluses which were then differentiated into plantlets after transferral onto morphogenetic induction medium.

As reported in other gramineous species (Rangan, 1974; Green and Phillips, 1975; Chen, Stenberg and Ross, 1977; Chen, Lo and Ross, 1979; Conger and Carabia, 1978; Vasil and Vasil, 1981; Ozias-Akins and Vasil, 1982), 2,4-D was also effective in induction and maintenance of little bluestem callus. The morphogenetic inhibiting effect of this auxin on little bluestem callus seemed to be much lower than on those of other grass species studied in this laboratory, since shoot differentiation occurred in the calluses cultured on the media containing 2,4-D at a concentration as high as 2.5 mg/liter (Table 4).

In view of the fact that slimy callus texture was shown in primary cultures on the medium containing 5 mg 2,4-D/liter, the 2,4-D concentration appeared to be maximum for callus induction and maintenance, since lowering from this level would induce differentiation in the calluses (Table 5). Furthermore, a high 2,4-D concentration in medium might induce cytological instability by hindering normal spindle formation in cultured cells (Street, 1977). Such karyological abnormal cells might lose morphogenetic capacity (Bayliss, 1973).

An addition of kinetin to the morphogenetic induction medium enhanced shoot and retarded root initiation in little bluestem calluses. The increased initiation of shoots appeared to be the result of kinetin stimulating cell division (Fox, 1969). Other gramineous species that showed a

similar morphogenetic response included oats (Carter, Yamada and Takahashi, 1967), rice (Henke, Mansur and Constantin, 1978), green needlegrass (Lo, Chen and Ross, 1980) and creeping bentgrass (Krans, Henning and Torres, 1982). However, this effect was not universal since Lo (1979) noted that calluses from bromegrass and crested wheatgrass did not show an elevated response.

The morphogenetic effect of kinetin has also been studied in various lily species. Chen, Jones and Songstad (1983) obtained buds from leaf and bulbscale cultures of Lilium longiflorum and L. formosanum on MS medium supplemented with high levels of kinetin. High incidences of bulblet initiation were also reported in L. auratum and L. speciosum cultures by Takayama and Misawa (1982). These results agreed with the high cytokinin-low auxin model proposed by Skoog and Miller (1957).

Except in smooth bromegrass culture where Gamborg, Constabel and Miller (1970) observed embryoid formation, during the 1970's plant regeneration through organogenesis was noted in almost all successful cultures of gramineous species (Rangan, 1974; Green and Phillips, 1975; Ahloowalia, 1975; Chen, Stenberg and Ross, 1977; Conger and Carabia, 1978; Chen, Lo and Ross, 1979; Lowe and Conger, 1979). In this pattern of morphogenesis, shoots and roots differentiated independently from callus tissues. Rhizogenesis occurred more frequently than caulogenesis. However, plant

regeneration would not be completed unless adventitious roots initiated from the base of the shoots. Roots which were from a callus never initiated shoots.

The ontogeny of shoots or roots has not been clearly understood. According to Sacristan and Melchers (1969) and Ogura (1976), variations of chromosome number were observed in root tip cells of regenerated tobacco plants and in the progeny obtained by selfing. In the tobacco cultures, shoots that formed through organogenesis were of multicellular origin. If genetic variations should have occurred in the cell population, chimeras would have resulted. More recently, shoots initiated directly from chimeric African violet leaf explants (Norris, Smith and Vaughn, 1983) were also chimeras, indicating a multicellular origin. Thus, plants regenerated from a cell culture through organogenetic pattern might not be genetically uniform and vegetative propagation by somatic cell culture through organogenesis would not be practical for cloning. However, Chen, Lo and Ross (1981) noted that cytologically abnormal big bluestem plants derived from a callus culture were visually identifiable and could be removed from the population. Similar results were obtained by Chen, Chen and Ross (1982) where plantlets regenerated from short-term indiangrass callus cultures were genetically uniform.

After the observation of embryoids in smooth bromegrass cell cultures (Gamborg, Constabel and Miller, 1970),

somatic embryogenesis has been reported in forage grass cultures of pearl millet (Vasil and Vasil, 1980), elephant grass (Haydu and Vasil, 1981), guinea grass (Lu and Vasil, 1981a), orchardgrass (Hanning and Conger, 1982) and proso millet (Heyser and Nabors, 1982). Characteristics of this morphogenetic response were the simultaneous development of the shoot and root apices. Embryoid germination resulted in regeneration of complete plants.

The origin of the embryoids has been extensively studied but only somewhat understood. Haccicus (1978) reported that somatic embryos differentiated from a proembryonal cell cluster which appeared to be the result of repeated divisions of a single callus cell. Though no evidence has proved the single cell origin of embryoids, cytological examination of root tips from regenerated plants showed an uniform chromosome distribution, indicating a homogeneous genetic composition (Haydu and Vasil, 1981; Vasil and Vasil, 1981; Lu and Vasil, 1982; Wang and Vasil, 1982).

The early signs of differentiation of gramineous calluses has not been clearly observed, except in orchardgrass where Hanning and Conger (1982) noted presumptive proembryos in sectioned tissues. On the other hand, calluses from the dicots carrot (Chen and Holden, 1973) and celery (Chen, 1976) were shown to exhibit various stages of embryoid development.

Dispersion and differentiation of callus cells in liquid cultures has aided in studying the early developmental stages of bromegrass (Gamborg, Constabel and Miller, 1970), guinea grass (Lu and Vasil, 1981b) and pearl millet (Vasil and Vasil, 1982) embryoids. Similar culture conditions could be applied to the development of little bluestem embryoids.

Somatic embryos forming in one callus and adventitious shoots in another were reported by McDaniel, Conger and Graham (1982) in calluses derived from orchardgrass caryopses. Lo, Chen and Ross (1980) suggested that friable calluses might provide a favorable internal environment for bipolar differentiation whereas compact calluses were only capable of monopolar initiation. Although friability of a callus seemed to be related to the genotype of an individual from which the callus was induced, other factors such as type and age of explants and culture environment would also affect texture of the callus in culture. The occurrence of both organogenesis and embryogenesis in one callus as observed in this study might be caused by a high heterogeneity of texture within the callus.

Nonzygotic embryos evolving from little bluestem callus tissues showed some resemblances to a panicoid sexual embryo, in which the epiblast was missing and the scutellum paralleled the contour of the coleorhiza (Reeder, 1957). The somatic embryos possessed a wider cleft between the

coleorhiza and scutellum.

The future for *in vitro* plant cell and tissue cultures appears promising. Evans and Sharp (1981) cited several advantages for implementing these techniques over conventional breeding practices. Plants that normally develop slowly or are in high demand can be rapidly cloned, thereby shortening the time needed for propagation. Furthermore, *in vitro* breeding enables new varieties to be established when only a few plants are initially available. Desirable qualities such as greater vigor, earlier flowering and higher yields have occurred in plants regenerated from callus cultures.

SUMMARY

Young inflorescence segments of little bluestem (Schizachyrium scoparium (Michx.) Nash), 5 to 10 mm long, explanted on Linsmaier and Skoog's RM medium supplemented with 5.0 mg 2,4-dichlorophenoxyacetic acid (2,4-D)/liter and incubated at 25 ± 1°C in the dark initiated morphogenic calluses. The calluses were maintained on the same medium used for callus induction. Naphthaleneacetic acid was not effective in callus initiation. Morphogenetic responses occurred after the calluses were transferred onto the RM medium with 2,4-D lowered below 2.5 mg/liter. Kinetin up to 5.0 mg/liter added to RM basal medium improved totipotency of the calluses. Genotypic differences in morphogenetic responses among the calluses tested were noted.

Morphological and histological studies revealed that plant regeneration from a callus of this plant cultured on RM medium devoid of hormones might be through either somatic embryogenesis or organogenesis or both. The plantlets developed normally at 27 ± 1°C under 16-hour photoperiods (cool-white fluorescent light at 2 Klx) and reached maturity ten months after transplanted into pots and reared in a greenhouse.

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