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Cloning of Lilium Longiflorum and Andropogon Gerardii by Tissue Culture of Leaf and Floral Parts

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CLONING OF <u>LILIUM</u> <u>LONGIFLORUM</u>
AND <u>ANDROPOGON</u> <u>GERARDII</u> BY TISSUE
CULTURE OF LEAF AND FLORAL PARTS

BY

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A thesis submitted in
partial fulfillment of the requirements
for the degree Master of Science, Major
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1976

CLONING OF LILIUM LONGIFLORUM

AND ANDROPOGON GERARDII BY TISSUE

CULTURE OF LEAF AND FLORAL PARTS

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser   Date

Head   Date
Botany-Biology Department

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Special thanks is given to Dr. Charles McMullen for his help with the photography.

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LIST OF ABBREVIATIONS

Abbreviation	Term
FAA	formalin-acetic acid-alcohol
IAA	indole-3-acetic acid
NAA	alpha-napthalene acetic acid
2,4-D	2,4-dichlorophenoxyacetic acid
MS	Murashige and Skoog medium
RM	Linsmier and Skoog medium

INTRODUCTION

The significance of employing tissue culture techniques in plant propagation was reviewed by Murashige (1974). Rapid cloning of desirable genetic stock by tissue culture for shortening a plant breeding program has been proposed by many workers (Chen, 1976; Nickell, 1973).

The Easter lily, L. longiflorum Thunb. is known not only for its ornamental value, but also for its large chromosomes, making it ideal cytological material.

Although producing seeds readily in some cultivars, this species is in practice propagated vegetatively by the use of bulbscales. To a cross breeding program, however, this method of propagation is not ideal in rapid cloning of superior segregates, since a considerable amount of time would be required for the plants to develop sizable bulbs for this purpose.

Sheridan (1968) obtained embryoids from stem cultures of the Easter lily but the development and establishment of plantlets was not reported. Recently, Chen and Holden (1975) successfully cloned the wood lily, L. philadelphicum by culturing leaf and floral parts. They indicated that these explants contained intercalary meristem tissue, which readily responds to culture media. Earlier, a similar result was obtained by Robb (1957) in cultures of L. speciosum.

Big bluestem, <u>Andropogon gerardii</u> Vitman, is a South
Dakota native grass. It possesses many desirable charac-
teristics and unique adaptations to this region. In a
forage breeding program carried by the Plant Science
Department of this university, large numbers of big
bluestem plants were collected from this and neighboring
states. Superior plants, obtained by recurrent selection
are available for making synthetics (Ross, personal
communication). A large quantity of individuals from these
selected plants should then be cloned within a short period
of time. The development of a method for rapid cloning of
these superior plants has, therefore, been greatly needed.
Successful tissue culture of <u>Lolium</u> species was reported
by Ahloowalia (1975), but a method of culturing big
bluestem has yet to be developed.

This research has primarily dealt with the development
of methods for cloning the species <u>L</u>. <u>longiflorum</u> and <u>A</u>.
<u>gerardii</u> by tissue cluture of leaf and/or floral parts.
The pattern of plantlet differentiation from the cultures
was also histologically studied.

LITERATURE REVIEW

Plant tissue culture involves the excision of plant cells or tissues from a parent plant and the subsequent placement of that tissue into an artificial environment capable of allowing cellular growth and division to occur.

According to Murashige (1974), there are four main areas in which plant tissue culture can serve a useful purpose; 1) the production of pharmaceuticals and other natural products; 2) the genetic improvement of crops; 3) the recovery of disease-free clones and preservation of valuable germ plasm; and 4) rapid clonal multiplication of selected varieties.

As early as 1898 exploratory work was being done in the area of tissue culture by a German scientist, Gottleib Haberlandt. Although when Haberlandt published his work in 1902 he had not yet observed cellular division or growth in culture he realized that such a tool would have great research potential.

A span of thirty years passed with little progress in the field until in 1934 Phillip White established a culture of actively growing tomato roots. Two years previous to this, White (1932), is his less successful experiments with wheat roots had devised a nutrient media. With several modifications of this media and tomato plant tissue, White

(1934) was able to record seemingly indefinite growth of tomato root tips in his liquid nutrient media. Even though callus tissue was not observed, White's experiment was the first to demonstrate that plant tissue could be independent and metabolically active under in vitro conditions for extended periods of time.

In that same year Gautheret (1934), a prominent French scientist, reported that excised cambial tissue from Salix capraea and Populus nigra, when cultured on a solidified medium containing Knop's solution, glucose and cysteine hydrochloride, appeared to proliferate for several months.

The interest in tissue culture intensified and by the year 1937 several important papers were published. White (1937), in the continuance of his root culture studies found that root growth in vitro was greatly enhanced by the addition of B vitamins and auxins to the culture medium. This in turn led Gautheret (1937, 1938) to include these components to his culture medium and thereby obtain more appreciable growth in his Salix cultures.

Another Frenchman by the name of Nobecourt (1937, 1938 a,b) performed some preliminary studies involving the culture of carrot root sections. He was the first to find that carrot tissue culture could differentiate roots.

The following year White (1939a) succeeded in culturing a tobacco variety Nicotiana glauca x N. langsdorffi,

and that same year described the formation of leafy buds upon sequential transfer of the tissue to a liquid media.

Since that time many scientists have used tobacco and carrot culture methods to study callus tissue and organ formation (McWilliam et al., 1974; Murashige et al., 1965; Skoog, 1944) as well as for bioassays involving nutrients, minerals and various plant hormones.

Research lagged in the area of tissue culture until the late 1940's when scientists became interested in the factors affecting vascular differentiation (Camus, 1949; Jeffs and Northcote, 1967; Wetmore and Rier,. 1963; Wetmore and Sorokin, 1955).

Work done in this area was exclusively carried out using dicotyledonous plant tissue for a variety of reasons. In view of the fact that a desirable explant should possess an area of meristematic or cambial activity, dicot tissue, since it has a vascular cambium, would naturally offer a wide variety of specimens. Explants taken from monocots, on the other hand, are necessarily confined to root pericycle, apical meristem, or intercalary meristem at the base of the leaf.

Monocots also require a higher level of callus-inducing hormones to invoke a response than do dicots (Wilmar and Hellerdoorn, 1968; Atkin et al., 1973; Green et al.1974). This difference is further substantiated by

the fact that dicots are much more susceptible to crown
gall tumors (Smith, 1942) than are monocots (Braun and
Stonier, 1958). This evidence appears to indicate that
tumorigenesis is more a trait of dicots than monocots
(Butcher, 1973).

It is a general rule that in order to promote callus
production in dicots, a high auxin, low kinetin ratio is
needed (Skoog and Miller, 1957). The hormonal ratio is
usually reversed to induce differentiation. In many cases
cytokinins are of little importance or are dispensed with
(Atkin and Barton, 1973; Carter et al., 1967; Shimada et al.,
1969; Takatori et al., 1968).

Because of one or more of these factors the culture of
monocotyledonous plants was delayed for a period of years.

In 1945 a successful stem tip culture was reported by
Loo using the monocot, Asparagus officinalis.

The basal end of the tips was submerged in a liquid
nutrient and grew continuously for over 20 transfers with-
out any root formation. There was, however, no callus
tissue formation or plantlet differentiation.

Then in 1951 Morel and Wetmore aseptically excised
cubes of tuber tissue from Araceae viz. Amorphophallus
Rivieri, and inoculated them onto Gautheret's medium con-
taining various concentrations of B vitamins and naphtha-
lene acetic acid. Callus tissue was induced along with

the sporadic appearance of bud initials.

Tissue Culture of Liliaceae

The family Liliaceae contains many ornamental plants as well as economically important vegetables such as asparagus. These plants usually reproduce by bulbs or tubers, a rather slow process making the aspect of rapid vegetative propagation through tissue culture particularly attractive.

In 1957 Lilium speciosum Thun. was firs successfully cultured by Robb. Tissue cylinders were bored out from bulb scales and explanted onto a modified White's solid medium. Cellular proliferation from the explants became evident nine weeks after explantation, followed soon afterward by initiation of bulblets. It was found that regeneration of plantlets occurred in far greater number when the explant was taken from the basal end of the bulb scale which possessed cambial activity. Robb also noted that spring bulbscales exhibited greater plantlet regeneration, indicating that these bulbs possess some factor capable of promoting bulblet formation.

An economically important vegetable, Asparagus officinalis, has generally been propagated via the use of seeds. Asparagus is dioecious and therefore, cross-pollination causes extreme variation in yielding ability between individuals in a variety. In view of these factors, a method of rapid vegetative propagation was desirable.

After Loo (1945) succeeded in culturing asparagus stem tips, his methods were later expanded on by Gorter (1965) and Andreasson et al. (1967), but the method was still limited to one plant per growing point.

In 1968, Takatori et al. devised a method, which by using spear sections inoculated on a solidified Murashige and Skoog (1962) (MS) medium containing 5 ppm NAA and coconut milk, callus tissue could be induced to form. The medium was then changed by adding 50 ppm adenine sulfate to induce plantlets. The final step involved the transfer of the plantlet to a medium containing no added growth factors. That same year, Wilmar and Hellendoorn successfully cultured asparagus using the hypocotyl of germinated seeds. The explant was cultured on Linsmier and Skoog (1965) (RM) medium supplemented with 1 ppm 2,4-D and 0.315 ppm kinetin. Even though the end result of their experiment was very similar to that of Takatoris', they were the first to discover the formation of embryos in monocot tissue culture.

Majumdar and Sabharwal (1968) successfully cultured Haworthia turgida young inflorescence on White's media supplemented with coconut milk, IAA and kinetin. Two years later Kaul and Sabharwal (1970), cultured the young in-florescence of several varieties of Haworthia using, again, White's media supplemented with coconut milk.

Fujino, Fujimura and Hamada (1972), cultured tissues

of _Iris_ _hollandica_, taken from axillary buds, on kano medium supplemented with NAA.

Chen and Holden (1972) cultured young petals of the daylily, _Hemerocallis_ _flava_ on MS solid medium supplemented with 6 ppm NAA to induce adventitious roots. These roots were then induced to form callus tissue when transfered to MS solid media containing 5 ppm 2,4-D. Plantlet differentiation occurred upon subculture of the callus tissue to MS media plus 1 ppm 2,4-D and 1 ppm kinetin. A similar result was reported by Heusen and Apps (1976).

Chen and Holden (1975) also succeeded in culturing the woodlily (_Lilium_ _philadelphicum_), a South Dakota native prairie plant, using leaf bases and floral parts as a source of explants. MS solid medium supplemented with 5 ppm NAA and 0.1, 0.5 or 1 ppm kinetin was found to be effective in the formation of roots and shoots. A related species, the Easter lily (_Lilium_ _longiflorum_ Thunb.), was cultured by Sheridan in 1968 using stem apicies as inoculum. The explants were cultured on RM solid medium supplemented with 2 ppm IAA and exhibited callus formation after three weeks in culture. When explants were placed on medium lacking IAA buds appeared on the tissue surface. It was also found that callus grew equally well in basal liquid medium with no IAA or kinetin added.

Even though _L_. _longiflorum_ has, therefore been cultured,

Sheridan's choice of inoculum was not ideal. Each source plant would yield a low number of explants which would raise the cost of the method considerably if used commercially. The chances of losing valuable germ plasm would also be increased using this explant source. Another factor involved would be the fact that each explant would represent a different genotype. These factors would be decreased if a number of explants could be taken per plant.

Tissue Culture of Gramineae

The family Gramineae contains many important cereal crops and forage grasses. Breeding programs involved with these plants could be greatly aided with tissue culture techniques for several reasons: 1) recovery of pathogen-free plants, 2) the production of haploid or homozygous diploid plants in large quantities within a short period of time through anther culture, and 3) the somatic hybridization of plants through protoplast fusion.

It has been only since 1967 that any culture of Gramineae species has produced both callus tissue and plantlets, even though a great amount of research regarding these species had been done previously. As far back as 1932, White attempted to culture excised wheat roots. It was not until 1967 that Carter, Yamada and Takahashi succeeded in culturing Avena sativa on RM medium supplemented with 2,4-D.

Callus tissue was induced from excised seedling roots. A second RM media containing no growth factors induced plantlet formation. Kinetin appeared to show little or no influence on either callus or plantlet induction.

Successful culture of rice (Oryza sativa) was reported by Nishi, Yamada and Takahashi (1968). RM solid media were used with 10^{-5} M 2,4-D added as the only growth regulator. Dehusked seeds of rice were used as inoculum, the root system of which soon formed a yellow callus. The same procedure for plantlet induction was used as for oats; that was, transfer of callus cells to media containing no growth factors.

In 1969 three more important papers were published, two dealing with sugarcane culture and one with wheat culture.

Barba and Nickell (1969), in their work with several varieties of Saccharum, found that White's medium was far superior to MS medium in supporting callus tissue growth. This situation was reversed for the induction of shoots and roots. A hormonal concentration of 5 ppm 2,4-D was sufficient to induce callus formation on the stalk explant, whereas 2,4-D concentrations of 0, 0.05 or 0.5 ppm usually induced roots and/or shoots with some varietal difference. Neither kinetin nor coconut milk showed any effect on growth or differentiation.

Heinz and Mee (1969) also cultured several varieties of _Saccharum_ using shoot apices, inflorescences and leaves as inocula. Again the hormone 2,4-D was used in conjunction with MS solid medium and coconut milk. Callus tissue usually formed along the cut edges of the explants. Callus proliferation was particularly active at the base of the pedicel.

Differentiation of roots and shoots was again brought about by transfer of callus tissue to solid basal medium without any growth regulators.

Since wheat is one of the most important cereal crops many attempts were made to culture it. Trione, Jones, and Metzger (1968) succeeded in inducing callus tissue using cotyledonary nodes of wheat seedlings. A modified Hildebrandt's "D" medium was used (Hildebrandt _et al_. 1946).

Shimada, Sakakuma and Tsunewaki (1969) were able to induce callus tissue and plantlets from seedling roots of wheat. Kinetin exhibited little or no effect, while 2,4-D in 1 to 10 ppm or IAA in 50 ppm induced callus formation when added to White's basal medium. Callus tissue explanted on media containing 2,4-D concentrations of 1 ppm or higher caused callus growth only. Shoots were formed on 6 calli but did not correspond to any combination or concentration of growth factors.

Another important member of the family _Gramineae_

Sorghum bicolor, was cultured in 1970. Mastellar and Holden of South Dakota State University succeeded in culturing young shoots of sorghum on MS solid medium. Two, four-D concentrations of 1 to 5 ppm were found to be optimal in producing callus tissue on the first node of the excised shoots. IAA or NAA at concentrations equal to or higher than 2,4-D failed to induce callus formation. Coconut milk was also added to the medium and was found to appreciably increase the growth rate. Kinetin, substituted for coconut milk, permitted only very slow callus growth. When pieces of callus tissue were transfered to media containing NAA at 5 ppm and placed in a lighted incubator bud initials were soon formed along with roots. The plants were subsequently planted in vermiculite and reached maturity under greenhouse conditions.

Norstog (1970) used immature barley embryos as inoculum. These embryos were cultured on medium J (Norstog, 1967) plus 0.1 ppm kinetin. Embryo-like outgrowths occurred in the first node opposite the scutellum of the cultured embryos. As development progressed the outgrowths became vascularized and shoot and root primordia became evident. Embryogenesis is rare in monocot culture and so it was a coincidence that that same year Gamborg, Constabel, and Miller (1970) published their results on embryogensis of albino plants in Bromis inermis. Using the mesocotyl

of brome seedlings, callus tissue had been established and maintained on agar medium containing mineral salts, sucrose, casein hydrolyzate and vitamins. Growth regulators includ-ed 0.5 ppm 2,4-D, 2 ppm p-chlorophenoxyacetic acid and 0.1 ppm kinetin. The callus tissue was then transfered to liquid medium with 1 ppm of 2,4-D as the only growth factor and incubated in the dark. Embryos were soon produced. Albino plantlets were harvested from the culture within one month. Neither changes in sucrose or nitrate levels, addition of gibberellic acid nor exposure to light induced chlorophyll formation.

Although attempts to culture Zea mays have been made by many workers (Green, Phillips and Kleese, 1974; Gresshoff and Day, 1973; La Rue, 1949; Linsmaier-Bednar and Bednar, 1972; Robbins and White, 1936; Sheridan, 1975; Sternheimer, 1954; Straus, 1954, 1958; Straus and La Rue, 1954; Sun and Ullstrup), Green and Phillips (1975) first succeeded in obtaining both callus tissue and plantlets using immature embryos 14 to 24 days after pollination. The embryos were placed on solid MS medium containing various vitamins from which callus tissue was induced. Plantlets differentiated from scutellar callus tissue near the end of the monthly culture period presumably after some depletion of 2,4-D.

Forage grasses of Gramineae have also been receiving

attention within the last few years. Atkin and Barton (1973) attempted to culture some temperate species. The growth of callus tissue ranged from good in the case of Dactylis glomerata, Agrostis tenuis, Cynosurus cristatus and Poa trivialis to poor in several Lolium species and varieties. Most cultures developed root primordia which sometimes grew into roots, but shoot primordia, none of which grew into shoots, occurred only in Lolium multiflorum var. westerwoldicum. RM medium supplemented with 2,4-D caused callus proliferation, but kinetin exhibited no marked influence.

Regeneration of ryegrass plants in culture was done by Ahloowalia in 1975. He placed ryegrass seeds in a modified medium (NW) of Niizeki and Oono (1968) supplemented with IAA, 2,4-D and Zeatin. Callus appeared to originate from the plumule of the seedling. Shoots were induced on MS media and NW media containing 1 ml of coconut water per liter and 2 ppm zeatin.

MATERIALS AND METHODS

Tissue Culture of L. longiflorum

The Murashige and Skoog formula (MS) (Murashige and Skoog, 1962) was adopted for preparing culture media. For primary culturing, the basal medium was supplemented with a combination of 5 or 10 ppm NAA and 0, 0.1, or 1 ppm kinetin, making a total of six hormonal treatments. The acidity of the media was adjusted to pH 5.8 with either 0.1 N NaOH or HCl before autoclaving. Eight grams of granulated agar were added to each liter to gel the media, which were dispensed into 125 ml Erlenmeyer flasks, 40 mls each. The culture flasks were then capped with a double layer of aluminum foil. The culture media used in subculturing will be described in the results.

Pot plants of the Easter lily cultivar, 'Nellie White' were used to furnish the culture materials. For leaf culture, young leaves, 2 to 8 cm long, were stripped near the base of the flower buds and disinfected in 1% filtered calcium hypochlorite solution being continuously stirred for 15 minutes to insure thorough disinfection. The leaves were then rinsed in three changes of sterile double distilled water, blotted with sterile filter paper and explanted on medium, one per culture flask. If a leaf exceeded 2 cm in length, a transverse cut was made and only

the basal region used as inoculum.

For the culture of floral parts, young floral buds, 1.5 to 2 cm long were dipped in 70% ethanol for 5 seconds and then submerged in 7% filtered calcium hypochlorite solution for 3 minutes. The procedure of disinfection was the same as described by Chen and Holden (1975). After rinsing in three changes of sterile double distilled water, the buds were aseptically opened exposing the stamens and pistil, which were removed for explanting. In both primary cultures of leaf and floral parts, each hormonal treatment consisted of 12 culture flasks. Unless stated otherwise, the cultures were kept in the dark in an incubator maintaining a temperature of 25 $\pm$ 1° C.

Histological investigations on the origin of the plantlets from leaf cultures were made by sampling the cultures at 0, 5, 10 and 15 day intervals of incubation. The leaves were aspirated and fixed immediately in FAA for 48 hours and then dehydrated through a normal-butanol series before being imbedded in paraffin. Serial sections, 10 microns in thickness, were cut with a rotary microtome, mounted on slides and stained using the triple tri-arch method obtained from Turlox Biological Supplies Company. The procedures described above were essentially taken from Sass (1958). The sections were examined and photographed using a Nikon microscopic camera.

The Feulgen squash technique described by Darlington and LaCour (1970) was employed in making root-tip chromosome counts of the plantlets.

Tissue Culture of A. gerardii

Young unemerged inflorescence from two clones of Andropogon gerardii were taken at the stage when the tip of the flag leaf was just emerging and used as inocula. For disinfection, the outer leaves were peeled off and the inner leaves surface-sterilized by swabbing them with 70 per cent ethanol. A longitudinal split was then made through the remaining leaves with a scalpel, exposing the young inflorescence which was removed and placed in a sterile petri dish. It was cut into 1.5 cm pieces before being inoculated on Linsmier and Skoog (1965) (RM) medium supplemented with 5 ppm of 2,4-D and 0 or 0.2 ppm of kinetin. The media were adjusted to pH 5.8 and solidified with 8 grams of agar per liter before autoclaving. Twenty milliliters of the media were dispensed into 2.5 x 15 cm screw capped test-tubes, and cooled at a slant. Each treatment consisted of 17 tubes. The cultures were placed in the dark at 25 ± 1° C. The RM basal medium was also used for subculturing. Hormonal concentrations and culturing conditions will be described in the results.

The procedures for making histological investigations of differentiating callus were essentially the same as

those used for the leaf culture of <u>L</u>. <u>longiflorum</u>.

RESULTS

Tissue Culture of L. longiflorum

Initiation of small translucent callus tissue was noted at the basal region of most of the lily cultures two weeks after explanting (Figure 1A). In leaf culture, the callus tissue which did not further proliferate reorganized to form adventitious buds or roots (Figure 1B). Cultures initiating buds were subcultured on solid MS medium, free of hormones, and placed in a growth chamber with alternating 16-hour light (2,000 lux) and 8-hour dark periods at 25 ± 1° C. Chlorophyll formation usually became evident within 48 hours after exposure to light while roots gradually initiated from the bud (Figure 1C). The plantlets continued growing (Figure 1 D and E) and were allowed to establish sufficient root systems before being transferred to vermiculite. The plants were nourished with one quarter strength of Hoagland solution. Several weeks later, the plantlets were transferred to potting soil and developed normally in the greenhouse (Figure 1F). Several plantlets produced a full-sized flower eight months after transplantation to the soil. The plants were only about 15 cm high at the time of flowering, making them disproportionate in size to the flower (Figure 2).

The response of the leaf cultures to the various hormonal treatments as recorded three months after
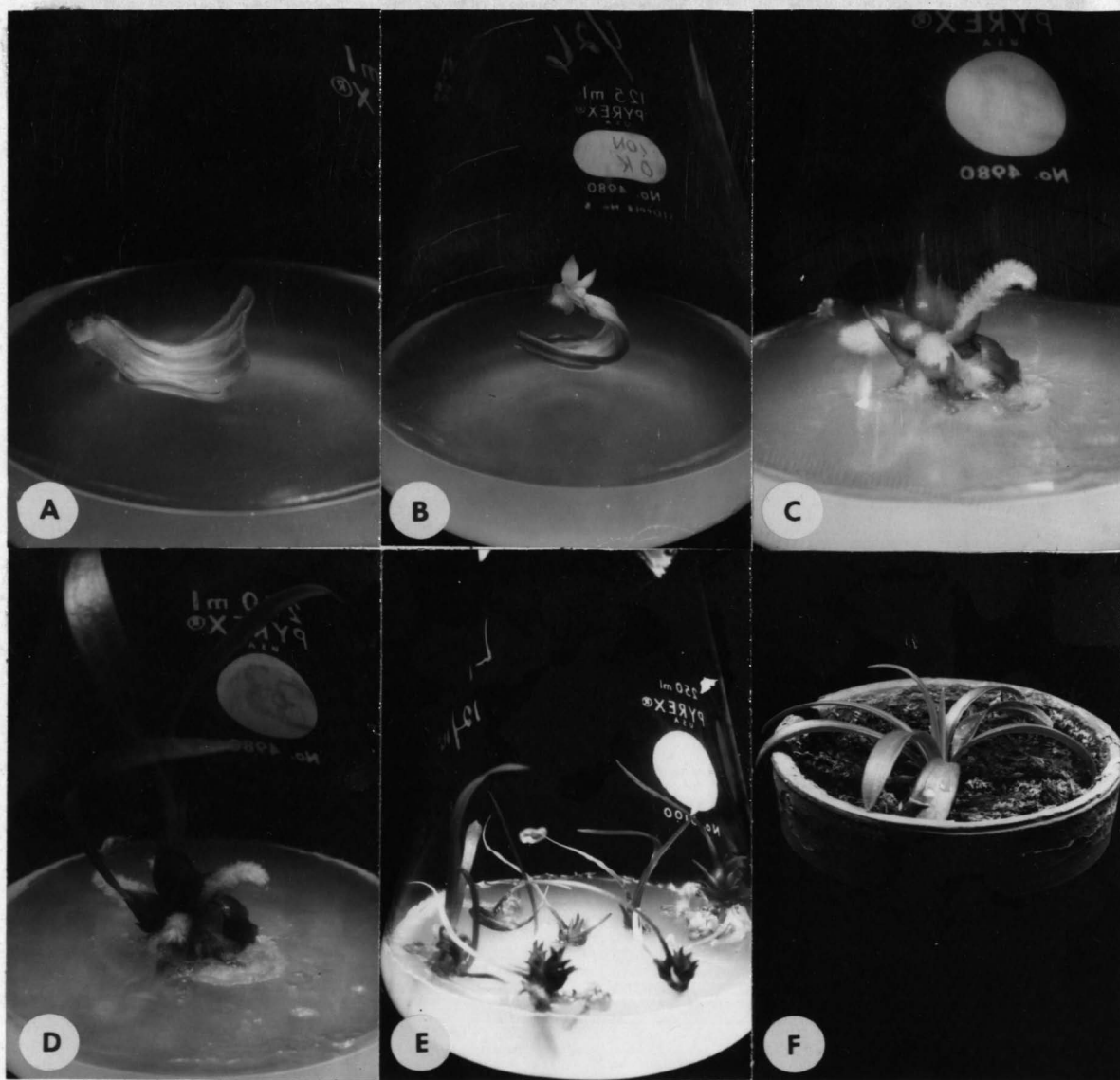
Figure 1. A sequential development of the leaf culture of L. longiflorum. (A) callus initiation at the leaf base (B) formation of adventitious buds from the intercalary region of the leaf base (C) initiation of roots from the bud (D) leaves emerging from the bud (E) plantlets nourished on MS medium, free of hormones (F) a plant developing from the leaf culture grown in potting soil.

Figure 2. A flowering L. longiflorum plant
eight months after transfer to potting soil.

explanation is summarized in Table 1.

Table 1. Response of L. longiflorum leaf cultures to
MS solid media containing NAA and kinetin.*

NAA (ppm		5			10	
Kinetin (ppm)	0	0.1	1	0	0.1	1
Buds	7	8	8	6	7	10
Roots only	4	2	3	4	5	1
Callus only	1		1	1		1
Curling only		2		1		

*12 bottles per treatment.

All of the hormonal treatments tested were effective
in bud formation. Since a limited number of explants were
cultured, it would not be possible to differentiate statis-
tically the effectiveness among the treatments. No
apparent difference in bud initiation between the two
composite NAA concentrations was noted. However, it
appeared that a synergistic effect between 10 ppm NAA and
1 ppm kinetin concentrations existed. The relatively high
hormonal concentrations gave the best result in bud
formation but the least response in initiation of adventi-
tious roots. The inhibitory effect of kinetin on root

formation and its promotive effect on bud initiation were reviewed by Fox (1969). Leaves which did not exhibit growth usually curled and became a deep green color compared to explants showing organogenesis. These explants died after prolonged culturing.

Histological investigations of the leaf bases sampled at 0, 5, 10, and 15 days after explanting revealed no potential buds existing anywhere on the leaf taken at the time of explanting (Figure 3A). Sections made from leaves cultured 5 days appeared slightly swollen near the leaf base, indicating that either cellular dedifferentiation or callus initiation had begun (Figure 3B). By the tenth day of culturing, regeneration of meristematic tissue which was localized at the edge of the leaf base was noted (Figure 3C). Leaves cultured 15 days showed reorganization of these meristematic cells into shoot primordia. Many of the cells were in various stages of mitosis (Figure 3D).

Culture of anther filaments and pistils usually initiated callus and, thereafter, adventitious roots (Figure 4). No adventitious buds were noted in the primary cultures at the time of subculturing. However, numerous buds developed from callus tissue two weeks after transferral to solid MS medium containing either 0.1 or 1 ppm NAA. The buds were then separated from the callus tissue and transferred to MS basal media, free of hormones, for
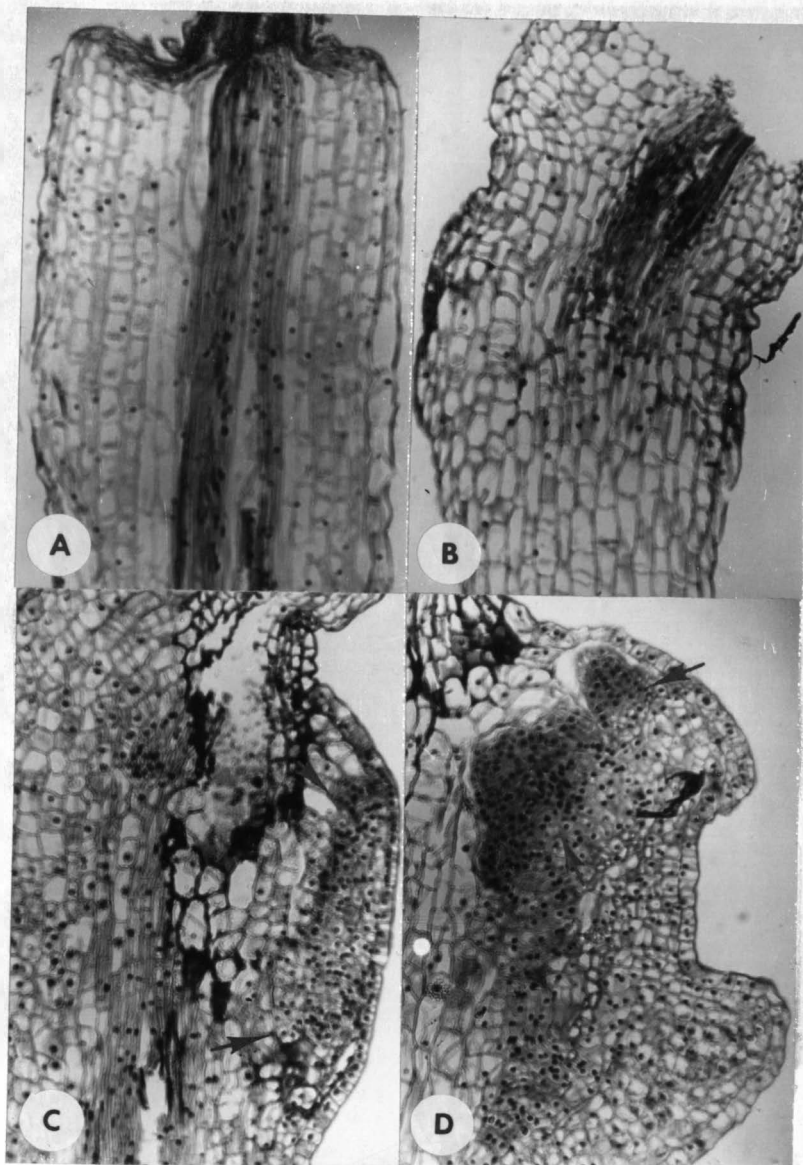
Figure 3. Longitudinal sections of L. longiflorum
leaf cultures. (A) a section made at the time of
explanting (B) a section made 5 days after explant-
ing showing a slight increase in size (C) a section
made 10 days after inoculation showing distinct
meristematic regions (indicated by arrows) (D) a
section made 15 days following explanation exhib-
iting shoot primordia (indicated by arrows). (34 x)

Figure 4.   A differentiating callus
derived from floral culture.

full development of plantlets.

Fifteen plants were randomly chosen for root-tip chromosome counts. No deviation from the normal chromosome number of 2n=24 was noted (Figure 5).

Tissue Culture of A. gerardii

Unlike the lily explants from which adventitious buds were directly induced, in primary cultures of big bluestem, callus tissue was initiated from the rachis of the cultured inflorescence in all hormonal combinations tested (Figure 6A). The callus, light yellow in color and friable in texture, proliferated vigorously (Figure 6B). It was transferable after an incubation period of four weeks. Since no apparent differences in growth rate were noted among the normonal treatments, kinetin was omitted from the media used for subcultivation.

RM solid basal media supplemented with 0, 0.1, 1 and 5 ppm of 2,4-D were then prepared for testing the morphogenetic induction of the callus. The subcultures in the various hormonal treatments were grown in 125 ml Erlenmeyer flasks containing 40 mls of medium and kept in the dark at 25 ± 1° C. Two weeks after transfer, whitish nodules appeared on the surface of the calli cultured on media containing 0, 0.1 or 1 ppm 2,4-D (Figure 6C). These nodules later developed into shoots. The callus grown

Figure 5. A root-tip cell of a plantlet evolving
from the leaf culture of L. longiflorum showing
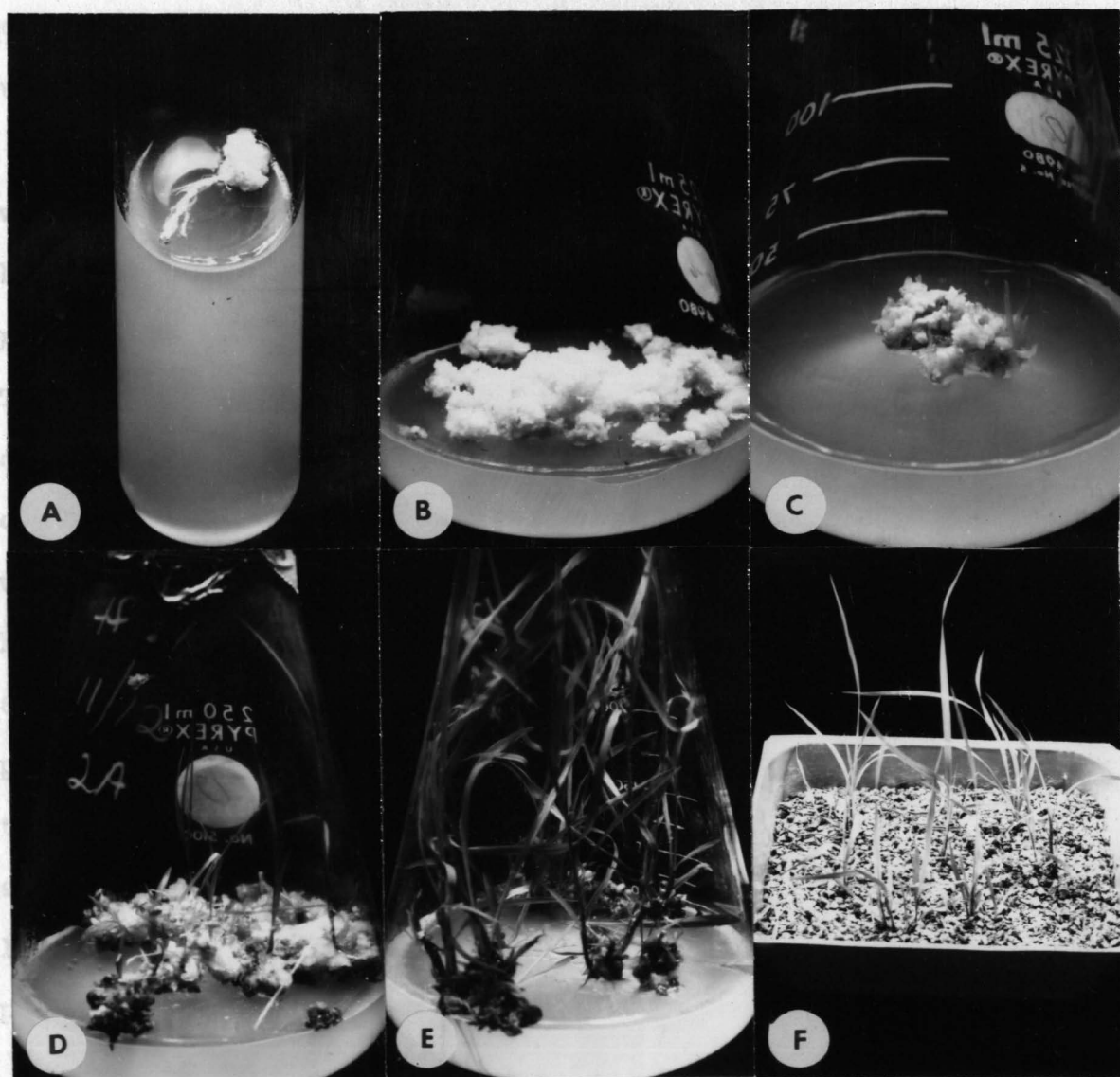the normal chromosome number 2n=24. (3,000 x)

Figure 6. A sequential development of A. gerardii
inflorescence culture. (A) callus initiation at
the rachis of the inflorescence (B) proliferation
of callus tissue (C) shoot differentiation via
organogenesis (D) growth of shoots and root form-
ation (E) further development of leaves and roots
(F) plants transferred from culture to vermiculite.

on the medium containing 5 ppm 2,4-D still maintained an undifferentiated condition.

When shoots were visible, the differentiating callus was then transferred to RM solid basal medium, free of hormones, and placed in a growth chamber at 27° C with a photoperiod of 16-hour light (2,000 lux) and 8-hour dark. The shoots turned green within 3 to 4 days and slowly developed roots (Figure 6D and E). Plantlets with sufficient root systems were planted in vermiculite which was watered with one quarter strength of Hoagland solution, and kept under a long day condition in the greenhouse (Figure 6F). After 3 to 4 weeks the plants were transferred to potting soil. It was later found that plants could be transferred directly from the culture flasks to sterile soil with an 85 per cent survival rate. In either case, leaf blades were usually cut back at the time of transplanting to reduce transpiration.

Microscopic examinations of the serial sections of differentiating callus tissue revealed that the pattern of differentiation was monopolar, i.e. shoot and root primordia arose independently from undifferentiated callus cells as shown in Figure 7A and B. Thus, under the culture conditions described, organogenesis was the mode of development for big bluestem cultures.

Even though callus tissue appeared to grow well on all
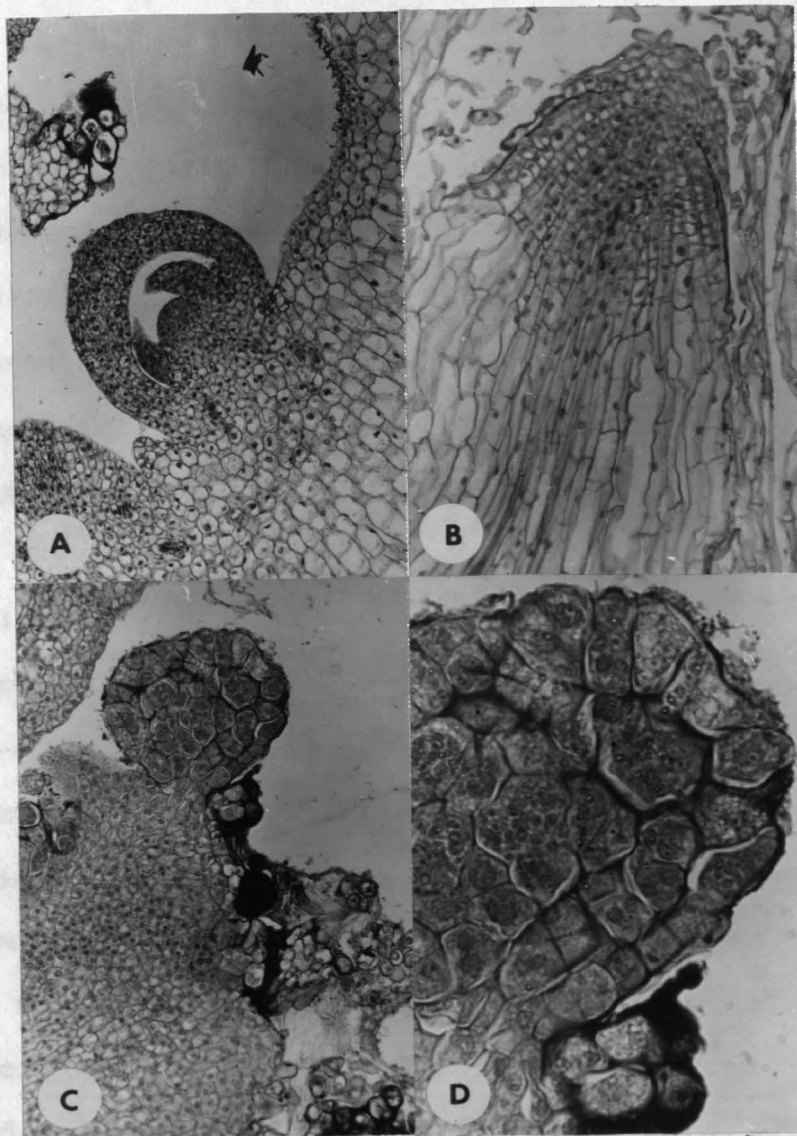
Figure 7. Histological sections of differentiating
A. gerardii callus tissue. (A) a section showing
organogenesis of a bud (B) a section exhibiting
root organogenesis (C) a region of polynucleate
cells (130 x) (D) a higher magnification of the
polynucleate cells (250 x).

callusing media tested, an optimal callus maintenance medium, on which the tissue culture would proliferate the best without cellular differentiation, was sought.

A preliminary experiment was first conducted in order to determine if initial callus size in a subculture affected the growth rate. Varying amounts of A. gerardii callus tissue were randomly inoculated on RM solid basal medium supplemented with 1, 2, 5, and 10 ppm 2,4-D. The cultures were incubated in the dark at 25 $\pm$ 1° C for one month and reweighed. The net gains of fresh weight were calculated (Appendix 1).

An analysis of correlation between initial weight and net weight-gain in the subcultures of callus revealed a significant r-value of -0.81, indicating that the rate of proliferation of callus subcultures was negatively related to their initial weights; a smaller amount of callus in a subculture would result in a higher net fold-increase.

Reinders (1942) found that hormones such as IAA and 2,4-D could cause cells to take up excess water. Any measurement would, therefore, need to include dry tissue weights in order to be fully accurate.

About 300 mg of callus tissue were then inoculated in each of the flasks containing the same media as were used for the preliminary experiment. Each hormonal treatment consisted of 10 culture flasks.

After a 30-day culture period, the callus tissue of
each flask was carefully removed, placed in aluminum pans
and weighed to obtain final fresh weight. The tissues were
then dried at 95° C for 48 hours and reweighed to obtain
final dry weights. The initial dry weights within each
treatment were estimated by using the following formula:

$$\text{initial dry wt} = \frac{\text{average final dry wt}}{\text{average final fresh wt}} \times \text{initial fresh wt}$$

The increase in folds of fresh and dry weights are
illustrated in Figure 8.

Analysis of variance was made in order to determine if
any significant difference in callus growth existed among
the four treatments. Although no significant F-value
(2.07) for the fresh weight fold-increase among the
treatments was noted, a significant F-value of 3.13 at the
5 per cent chance level was calculated for dry weight in-
crease (Table 2A and B), indicating that differences in dry
weight increase existed among the four treatments.

Table 2A. Analysis of variance for fresh weight increase
of A. gerardii calli grown on four 2,4-D treatments.

Source of Variation	d.f.	SS	MS	F
Treatments	3	8.75	2.92	2.07
Error	36	50.77	1.41	
Total	39	59.52		
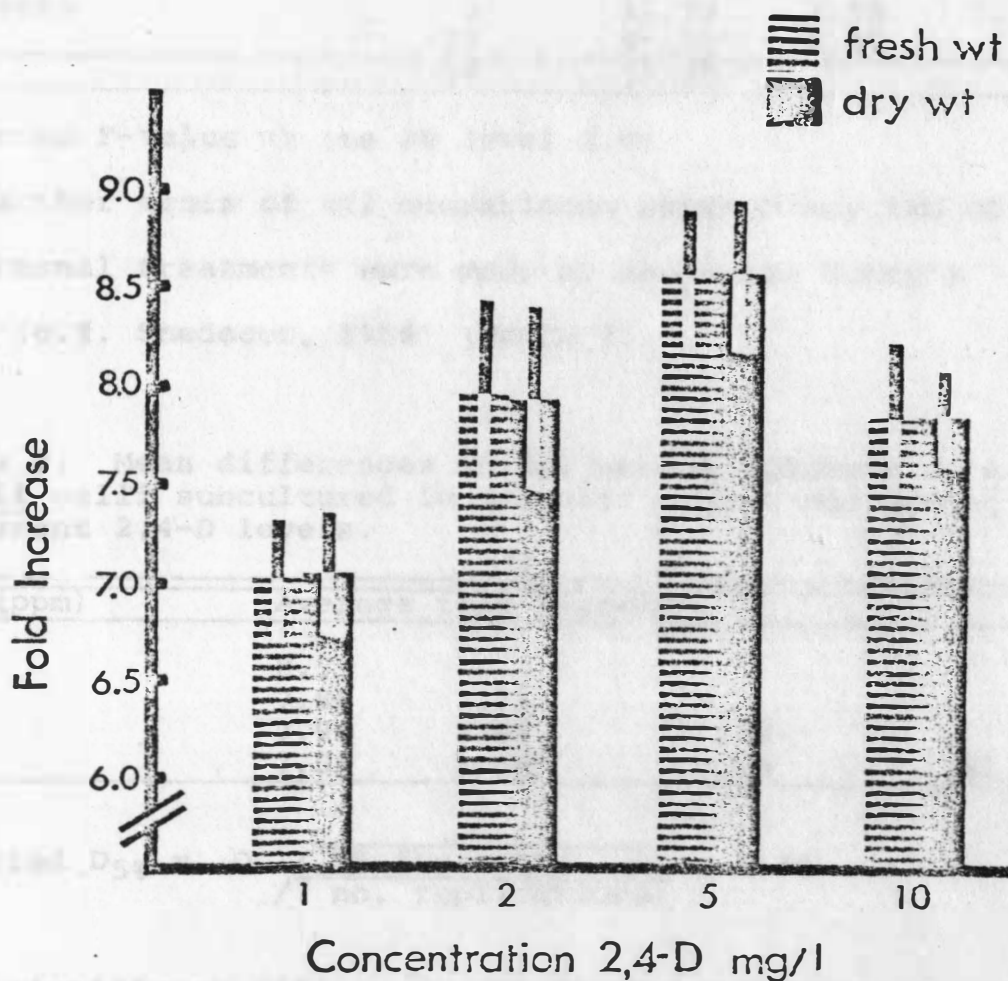
Figure 8. Growth rate of A. gerardii
callus tissue on RM media containing
four different 2,4-D concentrations.

Table 2B. Analysis of variance for dry weight increase
of A. gerardii calli grown on four 2,4-D treatments.

Source of Variation	d.f.	SS	MS	F
Treatments	3	11.93	3.98	3.13*
Error	36	45.87	1.27	
Total	39	57.80		

* Expected F-value at the 5% level 2.88

Further tests of all comparisons between any two of
the hormonal treatments were made by employing Tukey's
D-test (c.f. Snedecor, 1956) (Table 3).

Table 3. Mean differences in dry weight increase of A.
gerardii calli subcultured in RM solid medium containing
4 different 2,4-D levels.

2,4-D (ppm)	Average fold increase			
5	8.59			
10	7.86	.73		
2	7.94	.65	.08	
1	7.05	1.54*	.89	.81

* Expected $D_{5\%}$ = Q $\sqrt{\dfrac{MS \text{ for error}}{\text{no. replications}}}$ = 1.36

A significant difference was found to exist only be-
tween 1 ppm and 5 ppm treatments. Since organogenesis also
occurred consistently in the callus grown on the medium
containing 1 ppm, and sporadically on 2 ppm of 2,4-D, these
media with low 2,4-D concentrations should not be used for
callus maintainence. The medium supplemented with 5 ppm
2,4-D, therefore, was adopted for supporting callus growth.

At the time of preparing this thesis, the callus has been subcultured 11 times without loss of totipotency.

DISCUSSION

Explants used to establish a tissue culture desirably possess a meristematic region such as cambial tissue, for example, parenchyma has also been cultured (Nitsch and Nitsch, 1956). In monocots, however, the stem apex is usually deeply sunken and tightly sheathed by leaves, making excision difficult. The absence of vascular cambium in most monocots further limits the range of readily available explants to root-tips, embryos and the regions where intercalary tissue exists. Though Shimada, Sakakuma and Tsunewaki (1969) obtained callus tissue, and thereafter plantlets, from a root culture of wheat, the use of this organ carries with it problems in obtaining adequate disinfection without inflicting damage to the young meristematic tissue. Other researchers have used plumule (Lolium spp., Ahloowalis, 1975), primary root (Avena sativa, Carter et al.; 1967), and embryos (Hordeum vulgare, Norstog; 1970 and Zea mays, Green et al.; 1974). The tissue of an active seed usually respond readily in vitro. However, the risk of losing valuable germ plasm is high if the seed possesses a unique genotype. On the other hand, regions of intercalary tissue such as leaf bases or floral parts have been used in monocot culture by Chen and Holden (1972, 1975) in daylily

and wood lily; by Heinz and Mee (1969) in sugarcane; and by Kaul and Sabharwal (1972) in Haworthia species. Leaves of some species in Liliaceae are easily obtained and disinfected. If floral parts or unemerged inflorescence are used, the unopened bud or the flag leaf need only be surface-sterilized since the inocula are germ-free.

Tran (1973) found in his experiments with tobacco cultures that epidermal layers taken from different regions of a parent plant and cultured under identical conditions responded differently. A similar result has been obtained in cultures of L. longiflorum. Primary cultures of leaf directly produced bulblets while cultures of floral parts produced abundant callus tissue and numerous adventitious roots on the same media. Subculture of the callus tissue on a medium containing a lower hormonal concentration was required for bud initiation. Thus, leaf cultures of the Easter lily produced bulblets in a much shorter period of time compared to the floral explants. Floral callus tissue, however, tended to produce a greater number of plantlets per explant than leaf cultures.

Differences in morphogenetic response of a plant tissue cultured in vitro may result from the types of hormone used. IAA and its analogue NAA, have been widely used in rooting and production of parthenocarpic fruits. These hormones were considered as organ-forming substances

by Chen and Holden (1973). In many cases, regeneration of plantlets directly from explants cultured on the medium containing such hormones were noted (Chen, 1976; Chen and Holden, 1972, 1975; Kaul and Sabharwal, 1972; Kato and Takeuchi, 1963). The same tendency has been observed in the leaf culture of L. longiflorum.

According to Chen and Holden (1973), herbicidal hormones such as 2,4-D and tordon tended to induce callus and maintain the cells in an undifferentiated condition. Cultures grown on high concentrations of such hormones must be subcultured on a medium with hormone concentrations lowered or omitted in order for morphogenesis to occur (Steward et al., 1970). The behavior of A. gerardii cultures followed this pattern.

Prolonged subculture of callus tissue might accumulate chromosomal variation which would effect cytological homogenity of the culture (Dudits, et al. 1976; Mitra et al., 1960; Partanen, 1959; Torry, 1967). A decrease in totipotency of prolonged cultures has, in part, been attributed to the chromosomal changes. Murashige and Nakano (1967) found a definite increase with time in the number of aneuploid cells in tobacco cultures. They suggested that a loss of totipotency in prolonged cultures was due more to aneuploidy than euploidy, since polyploid plants could arise spontaneously in culture. Polyploid plants were also induced

after colchicine treatments of sugarcane suspension culture (Heinz and Mee, 1969) and daylily callus cultures (Chen and Goeden 1974). Shimada et al. (1969) also hypothesized that a rather strong selection against aneuploidy operated during differentiation. In callus tissue of A. gerardii a region of large multinucleate cells were found, suggesting that endomitosis might have occurred. Due to a limitation of time, chromosome counts on either the callus cultures or the plantlets was not made. In L. longiflorum, however, no deviation from the normal 2n = 24 chromosome number was found upon examination of plantlet root-tips, although chromosomal variations could have been present in the callus tissue.

Histological examination of morphogenesis in L. longiflorum leaf bases and A. gerardii callus tissue showed definite regions of small, highly cytoplasmic cells containing prominent nuclei, indicating active cellular division and growth. These regions were usually surrounded by larger vacuolated cells, which in the case of L. longiflorum, contained numerous starch grains. According to McWilliam, et al. (1974) the presence of numerous starch grains in a callus culture was an indication of differentiation. Thomas et al. (1972) stated that variation in starch deposits was common among various plant species and that if starch grains were present, the number was

usually decreased in cells of the meristematic regions. Amyloplasts were not evident in the differentiating callus tissue of A. gerardii.

Kaul and Sabharwal (1972) and Partanen (1959) noted the development of small vessel elements interdispersed among callus cells in differentiating tissue. In leaf sections of L. longiflorum, vessel-like cells were seen in the greatest number among the larger vacuolated cells immediately surrounding the meristematic regions.

Tissue culture of L. longiflorum has practical value from two standpoints: first, it is a popular ornamental plant making its rapid propagation desirable commercially. Secondly, the Easter lily possesses a low number of large, easily distinguishable chromosomes, making it valuable for cytological experimentation.

The culture of big bluestem is particularly important to South Dakota since it is a native forage grass of this region. The rapid cloning of superior plants within a relatively short period of time makes the creation of synthetic varieties possible. Also, rapid propagation of large numbers of plants from a big bluestem clone would interest horticulturalists involved in landscape design.

SUMMARY

Leaf blades obtained immediately below the flower buds and stamens or pistils taken from the buds, 1-2 cm long, of L. longiflorum c.v. 'Nellie White', were separately cultured in the dark at 25° c in Murashige and Skoog (MS) solid medium supplemented with all possible combinations of NAA at 5 and 10 ppm levels and kinetin at 0, 0.1 and 1 ppm levels. Growth occurred on all media via initiation of adventitious buds arising from the intercalary region of the leaves. Floral parts did not initiate bulblets directly but when subcultured on MS medium plus 0.1 or 1 ppm NAA, bulblets were produced in large numbers.

Histological studies of the leaf bases of L. longiflorum revealed no potential buds present at the region of intercalary tissue at the time of explanting; but in a leaf sectioned 15 days after explanting on MS medium containing 5 ppm NAA, meristematic-like cells, which were reorganizing into shoot tips, were found. In leaf culture, bulblets normally occurred about 3 weeks after explanting. Root-tip chromosome counts made from 15 plantlets revealed no variation in chromosome number.

Calluses of two genotypes of Andropogon gerardii Vitman, collected from colonies in southeastern South Dakota, were induced from 0.5 to 1 cm segments of young inflorescences cultured on Linsmaier and Skoog (RM) solid

medium supplemented with 5 ppm of 2,4-dichlorophenoxyacetic acid (2,4-D) and 0 or 0.2 ppm kinetin in the dark at 25° C. The callus condition was maintained best by subculturing it on RM solid medium supplemented with 5 ppm of 2,4-D, but differentiation by organogenesis into numerous plantlets was induced from calluses grown on the same basal medium with the concentration of 2,4-D reduced below 2 ppm. The plantlets showed a normal development if grown under light on RM solid basal medium, free of hormones. At the two-leaf stage, the plantlets were transplanted into sterile soil in plant-bands with a survival rate as high as 85 per cent. This technique will be applied to the rapid cloning of superior genotypes of this species obtained by recurrent selection for making synthetic varieties.

APPENDIX 1

2,4-D Treatment	Initial Weight	Fold Increase
10 ppm	0.0096	128.89
	0.0473	33.84
	0.0506	56.20
	0.1123	15.30
	0.1201	17.87
	0.0164	52.40
	0.0690	21.01
	0.0965	10.14
	0.1373	13.23
5 ppm	0.0038	277.82
	0.0617	21.28
	0.1518	11.33
	0.0836	9.09
	0.1876	5.87
	0.0379	22.14
	0.2188	6.80
	0.1264	8.41
	0.1508	9.30
2 ppm	0.0689	17.23
	0.0776	16.65
	0.0323	37.20
	0.0865	12.65
	0.1165	13.80
	0.0564	16.70
	0.0523	24.29
	0.1202	10.41
	0.1340	11.28
1 ppm	0.0200	61.31
	0.0948	15.66
	0.1840	8.94
	0.0655	19.71
	0.1234	10.72
	0.0215	72.00
	0.0618	15.10
	0.0979	17.91
	0.0758	13.28

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