DEVELOPMENT AND EVALUATION OF TECHNIQUES FOR REGENERATING PLANTLETS FROM SOMATIC AND GAMETOPHYTIC TISSUES OF SELECTED GRASSES

By

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INTRODUCTION

This dissertation is composed of four parts. Part I contains general information and a Review of Literature pertinent to the subject matter. Parts II, III and IV are prepared as separate and complete manuscripts to be submitted for publication in a professional journal. The format of each manuscript conforms to the style of Plant Cell, Tissue and Organ Culture.

PART I

GENERAL INFORMATION AND LITERATURE REVIEW

GENERAL INFORMATION AND LITERATURE REVIEW

Plant cell and tissue culture techniques may be used as powerful and innovative tools to supplement conventional methods of plant improvement and genetic and physiologic investigation. The induction and growth of callus and subsequent regeneration of viable plantlets which can be grown to maturity are essential and primary prerequisites for practical utilization of cell and tissue culture. Generally, development of in vitro techniques for monocotyledonous plants, including families in the Gramineae family has lagged behind that for dicotyledonous plants. However, plantlet formation from callus has been reported for all major cereals (Gray and Conger, 1985).

Until recently the development of plant tissue culture technology for grasses and cereals has lagged far behind that of many other agronomic crops because plant regeneration has frequently been of low frequency and of short duration (Gray and Conger, 1985).

Until recently, a typical response for grass tissue cultures was a low frequency of regeneration from callus, which decreased with increasing time and number of subcultures (Conger, 1981). These problems are being circumvented by attention to culture media components, use of certain responsive cultivars and genotypes and visual inspection of cultures to select for promising material used in subcultures (Nabors et al., 1983). Plantlet formation from callus cultures of somatic tissue was first reported for hybrids of Italian

ryegrass, Lolium multiflorum Lam, perennial ryegrass, Lolium perenne L., (Ahloowalia 1975), orchardgrass, Dactylis glomerata L., (Chin and Scott, 1977; Conger and McDonnell, 1983), and big bluestems, Andropogon gerardii Vitman, (Chen et al., 1977). Although Gamborg et al. (1970) reported plant regeneration via embryogenesis of bromegrass (Bromus inermis L.), this report led only to the production of albino plants. Within the past five years there have been several reports of embryogenesis from in vitro cultures of gramineae species (Dale, 1980; Gray and Conger, 1985; Vasil, 1983; Gray et al., 1985). A primary advantage of embryogenesis is that cultures maintain totipotency for very long periods of time compared to regeneration with organogenesis. Also, culture work is less labor-intensive because the embryos contain a complete plant axis and, in contrast to shoots produced via organogenesis, do not have to be further manipulated to induce development of other essential organs such as roots. The selection of useful mutants can be facilitated because of the single cell origin of non-zygotic embryos (Gray and Conger, 1985; Ahloowalia, 1983). It has been suggested that plants regenerated via somatic embryogenesis are genetically stable (Hanna et al., 1984; Vasil, 1983). The production of large numbers of plants that do not exhibit phenotypic or chromosomal changes could be extremely useful in the improvement of forage grasses with a "bunch" type growth habit which do not possess plant parts such as rhizomes or stolons for mass vegetative propagation.

A number of media have been successfully employed to obtain embryogenic cultures. These include modifications of MS (Murashige and

Skoog, 1962), LS (Linsmaler and Skoog, 1965), SH (Schenk and Hildebrant, 1972) and N-6 (Chu et al., 1975). Several auxins have been used to produce an embryogenic response, with 2,4-D (2,4-dichlorophenoxyacetic acid) being the most common. Dicamba (Hanning and Conger, 1982) and 2,4-5T (2,4,5-trichlorophenoxyacetic acid) are also effective (Heyser and Nabors, 1982). Weaker auxins such as IAA (Indole acetic acid) and NAA (Naphthaleneacetic acid) are relatively ineffective. Cytokinins may actually inhibit embryogenic responses in some species (Gray and Conger, 1985; Conger et al. 1982).

In grasses, somatic embryo development generally follows the transfer of cells or callus to media lacking auxin. However, somatic embryo initiation and maturation occurs on the primary medium. Transfer to a secondary medium is needed for their growth into plants (Vasil and Vasil, 1981a & b; Breiman, 1985; Brettel et al., 1980).

Sucrose appears to be the most effective reduced carbon source for somatic embryogenesis, although raising the sucrose concentrations benefited the formation of embryogenic callus in Zea mays. Ahn, et al. (1985) found nonsignificant differences between 20 and 60 gr L^{-1} sucrose in increasing callus fresh weight of bermudagrass.

For many monocotyledonous plants and especially for members of the Gramineae, regions of actively dividing cells seem to respond most readily in culture (Dale, 1980; Conger, 1982).

Chandler and Vasil (1984) found in Napier grass that both the maximum proliferation of embryogenic callus and the yield of plants was maximal for callus from frequently (about 2 weeks) subcultured cultures as contrasted with infrequent subculture (3-4 weeks or longer).

Several factors are potentially important for inducing embryogenesis in grass tissue cultures including genotype, medium and explant sources (Raghavaram and Nabors, 1985; Ahloowalia, 1982). There is conflicting evidence as to whether nonzygotic, or somatic, embryogenesis is under genetic control. Green et al. (1984) found that in Zea mays that response originally occurred in only a few genotypes. In Dactylis glomerata, only seven percent of 330 genotypes tested showed an embryogenic response from the outer anther wall and from within anthers (Gray and Conger, 1985). Embryogenic cell suspension cultures have been reported for only four forage grasses (Gray et al., 1985; Green et al., 1983, Lu and Vasil, 1981a; Vasil and Vasil, 1981a).

Nonzygotic embryogenesis from tissue culture of haploid floral parts is potentially useful for developing pure breeding lines (Wu et al., 1985). Plants possessing gametic chromosome numbers have been obtained from inflorescence cultures of Festuca arundinacea Schreb (Kasperbauer et al., 1980) but not via embryogenesis.

Immature embryos, young inflorescences, and young leaves are ideal sources for the initiation of embryogenic callus cultures. The developmental and physiological stages of the donor tissues are also critical for the initiation of stable embryogenic cultures (Sharma et al., 1984; Tyagi et al., 1985; Chen et al., 1977; Tsung et al. 1985). The formation of somatic embryos has recently been described in Dactylis glomerata by McDaniel et al. (1982); Panicum miliaceum by Rangan and Vasil (1982); Pennisetum americanum by Vasil and Vasil (1981a); Triticum aestivum by Ozias-Akins and Vasil (1982); and Zea mays by Lu et al. (1982). Earlier investigations described only shoot

morphogenesis in these species. It is likely that in many of the earlier studies compact and slow growing embryonic tissue was also formed, but it was either inadvertently or deliberately discarded in favor of the more common, relatively faster growing and friable nonembryogenic tissues. In other instances, the phenomenon of somatic embryogenesis was either not recognized or was misinterpreted (Vasil I.K., 1983; Cobb et al., 1985).

Since the early experiments with Datura innoxia Mill, the culture of whole anthers and the subsequent development of haploid plants has been successfully demonstrated in at least 79 species of angiosperms. Three new varieties of rice and three of tobacco, that originated from plantlet regeneration from anther culture, have been released in China; and in Japan a superior tobacco variety, F211, resistant to bacterial wilt has been obtained through anther culture (Evans et al., 1983).

Haploids may be utilized to facilitate the detection of mutations and the recovery of unique recombinants. Haploids possess only one allele at each locus; so it is possible for recessive mutants to be detected. Furthermore, doubling of the chromosome number of haploids offers a method for the rapid production of homozygous plants, which in turn may be used as parents for production of hybrids (Evans et al., 1983). Haploids may be grouped into two broad categories: monoploids, which possess half the number of chromosomes from a diploid species; and polyhaploids, which possess half the number of chromosomes (gametophytic set) from a polyploid species. Haploids may occur spontaneously in nature or they may be induced experimentally. One way to obtain haploids is by culturing excised anthers. Haploid plantlets

are formed in two distinct ways: by direct androgenesis (embryos originating directly from the microspores without callusing), or by organogenesis from haploid callus tissue. The percentage of regenerated plants of androgenic origin and the total number of regenerated plantlets per anther vary greatly, depending on the cultivar, medium, and various other endogenous and exogenous factors (Evans et al., 1983; Lo et al., 1980). Haploids occur naturally and may be induced experimentally following some trauma. Sunderland et al. (1981) found that cold pretreatment of barley anthers increased the frequency of callus formation. Conger (1985) concluded that orchardgrass anthers pretreated for 3 weeks at 4°C produced embryos directly from the somatic tissue of the anther wall and division of microspore nuclei was stimulated in anthers pretreated 6 weeks at 4°C resulting in multinucleate microspores.

The composition of the medium is one of the most important factors affecting not only the success of anther culture but also the mode of development (Heberle-Boss 1980). Ye et al. (1985) found that the combination of 2,4-D and Zeatin in their optimal ranges significantly increased callus induction in anthers of two barley cultivars.

Callus induction and root formation from mature caryopses of bermudagrass have been reported (Krans 1981; Ahn et al., 1984). Plants of bermudagrass have been regenerated from immature inflorescences through somatic embryogenesis (Ahn et al., 1985).

Tissue culture research with perennial grasses has been limited. Of special potential importance is the development of haploids or polyhaploid plants from gametophytic tissues. Such plants would have

value in genetic studies and could be used to rapidly develop homozygous plants for further use in breeding improvement or genetic study.

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PART II

EFFECTS OF AUXIN CONCENTRATION ON EMBRYOGENIC CALLUS INDUCTION FROM CULTURED YOUNG INFLORESCENCES OF FIVE GENOTYPES OF OLD WORLD BLUESTEM (*BOTHRIOCHLOA* SP.) AND BERMUDAGRASS (*CYNODON* SP.)

EFFECTS OF AUXIN CONCENTRATION ON EMBRYOGENIC CALLUS INDUCTION FROM CULTURED YOUNG INFLORESCENCES OF FIVE GENOTYPES OF OLD WORLD BLUESTEM (*BOTHRIOCHLOA* SP.) AND BERMUDAGRASS (*CYNODON* SP.)[1]

Keywords: *Bothriochloa ischaemum*, *Cynodon dactylon*, forage grasses, auxin, tissue culture, somatic embryogenesis

Abstract

Objectives of this research were to induce callus formation from immature inflorescences of plants (genotypes) of two Old World bluestem, *Bothriochloa ischaemum* (L.) Keng. Var. *ischaemum*, accessions (A-8793 and A-8911c) and three bermudagrass, *Cynodon dactylon* (L.) Pers., accessions (A-10978b, A-12164, and 'Brazos') and to determine the optimum auxin concentrations for inducing and promoting embryogenic (E) callus in the respective genotypes. Immature inflorescences < 9 mm in length were plated on modified Murashige-Skoog (MS) agar medium containing 0, 1, 3, or 5 mg L^{-1} of 2,4-dichlorophenoxyacetic acid (2,4-D). Explants of all genotypes produced callus by the end of a 4-week dark incubation period at 25°C. When subcultured onto fresh media and maintained at 25°C with a 16 hr photoperiod, calli became

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embryogenic within 8 weeks of inoculation. Three mg L^{-1} of 2,4-D in the media maximized E callus production in both bluestem genotypes and in A-10978b and A-12164 bermudagrass genotypes. Maximum E callus production from Brazos bermudagrass resulted from the 1 mg L^{-1} treatment. Somatic embryos developed after subculture under light. Embryos showed scutellum-like structures and coleoptile-coleorhiza bipolar organization. Plantlets were regenerated from all genotypes except Brazos, whose embryoids failed to germinate. All callus from Brazos eventually senesced. Light and scanning electron microscopy confirmed regeneration through somatic embryogenesis.

Introduction

A major constraint in the application of tissue culture to grass improvement has been relatively low frequency plant regeneration (6). The use of a single medium system to produce callus and shoots, as in tobacco and some other dicotyledonous plants, has not proven generally applicable to grasses. In the latter group the medium that produces callus rarely promotes regeneration. In gramineous species, calli derived from young inflorescences have been shown to produce somatic embryos, or embryoids (5,9,10,11,12,13,14,15,16). Manipulation of the culture medium components, particularly type and concentration of plant growth regulators and use of specific cultivars have, in some cases, resulted in increases in the number of regenerated plants and/or in the development of shoots from E callus (4,7). Thus, production of E callus and subsequent plant regeneration in grasses may be improved by altering auxin content in initiation media.

Bermuda [Cynodon dactylon (L.) Pers.] and Old World bluestem [Bothriochloa ischaemum (L.) Keng.] grasses are perennial warm-season plants widely used for pasture and/or turf in the southern USA. Callus induction from immature inflorescence explants, and plantlet regenerations via somatic embryogenesis have been reported in these species (2,3,8), but the studies involved few plant genotypes and little information was presented on the effects of different levels of auxin in the media on induction and growth of embryogenic (E) callus. Specific objectives of this research were to determine the best auxin

concentrations in the media for the induction and growth of E callus in respective genotypes.

Materials and Methods

Shoots containing young inflorescences were collected at the beginning of the blooming season (June of 1985 and 1986) from three field-grown bermudagrass [Cynodon dactylon (L.) Pers.] genotypes (A-10978b, A-12164 and 'Brazos'), and two greenhouse-grown Old World bluestem (Bothriochloa ischaemum (L.) Keng. Var. ischaemum) genotypes (A-8793 and A-8911c).

Shoots were surface sterilized in 10% commercial clorox for 2 min, 70% ethanol for 1 min, and rinsed 5 times in sterile distilled water. Immature inflorescences (6-9 mm) were excised aseptically and separated into their corresponding racemes (4-6 racemes per inflorescence). To ensure precision and homogeneity in the experimental unit, racemes of approximately the same size (6-9 mm in length), and from the same inflorescence, were used as explants for each experimental replication. The explants were cultured separately on multiwell plates (Corning 25820) filled with 1 ml of half strength Murashige and Skoog (MS) medium (1) supplemented with 0, 1, 3, or 5 mg L^{-1} of 2,4 dichlorophenoxy acetic acid (2,4-D) and 1 mg L^{-1} filter sterilized indole-3-acetic acid (IAA). The pH of the media was adjusted to 5.8 prior to addition of agar (7 g L^{-1}). All components of the media, except IAA, were autoclaved at 121°C for 15 min. The experimental design was a randomized complete block with split plots and 15 replications. Genotypes constituted main plots and auxin treatments

sub-plots. Blocking was accomplished by maintaining complete sets of treatment combinations in respective containers (multiwell plates or plastic containers for petri dishes) incubated in a Percival environmental chamber. Thus, there were 15 such containers in the growth chamber. Genotypes were randomly arranged within the blocks.

Cultures were maintained in the dark at 27°C for 4 weeks and then transferred to plastic petri dishes (35 x 10 mm) containing fresh media with the same components as the initial media. They were cultured for 4 more weeks at 25°C with 16 hour/day of cool-white fluorescent light at an intensity of ca 3,000 lux. After a total of 8 weeks incubation the proportion of embryogenic callus in each culture plate was visually estimated using percentages in five ranges 0, 1-25%, 26-50%, 51-75% and more than 75%.

After the observations were made, calli were transferred to plastic petri dishes (55 x 15 mm) containing half-strength MS medium with 0.7% agar, 0.5 mg L^{-1} of 2,4-D and 1 mg L^{-1} of zeatin for embryo germination and plantlet formation. Cultures were incubated at 24°C in a growth chamber under 16 h/day of fluorescent light at approximately 3,000 lux.

Specimens for scanning electron microscopy (SEM) examination were placed in 2% (V/V) glutaraldehyde (0.2 M sodium cacodylate, pH 7.3) for 2 h, washed in 0.05 M sodium cacodylate buffer (pH 7.3), and postfixed in 2% (V/V) osmium tetroxide (0.2 M sodium cacodylate, pH 7.3) for 2 h at room temperature. Tissue samples were subsequently washed in buffer, dehydrated in graded ethanols, critical point dried in CO_2, affixed to aluminum stubs with silver paint, and coated with

approximately 40 nm of gold-palladium. Specimens were examined with a JSM 35 U SEM. Light micrographs were taken with a stereomicroscope.

Regenerated plantlets were transferred to basal medium in petri dishes and allowed to grow. When the leaves were more than 2 cm long plantlets were transferred to soil in pots. Acclimatization was accomplished by either covering the pots with a transparent plastic cover and maintaining them in a growth chamber for one week or by placing them in a greenhouse for several weeks.

Results and Discussion

Young immature inflorescence explants 6-9 mm in length contained many spikelets (Fig. 1a-d). Individual florets were recognizable with their accessory organs at an early stage of development. Very young stamen and pistils were evident in the individual juvenile florets.

After 1 week in culture, explants of the two bluestem and A-12164 bermudagrass genotypes increased in size on medium containing 3 or 5 mg L^{-1} 2,4-D, and exhibited external signs of callus formation. The same was true for explants of Brazos bermudagrass on medium containing 1 mg L^{-1} 2,4-D. Callus production in A-10978b bermudagrass explants was greatest on medium containing 3 mg L^{-1} 2,4-D but also was present on both the 1 and 5 mg L^{-1} treatments. There were no visible signs of swelling or callus formation in explants on basal medium without auxin. After 4 weeks in culture, soft, translucent, non-embryogenic (NE) callus was formed from explants of the two bluestem and A-12164 bermudagrass genotypes on medium containing 3 or 5 mg L^{-1} 2,4-D, from A-10978b bermudagrass explants on 1,3 and 5 mg L^{-1} 2,4-D treatments and

from explants of Brazos bermudagrass on medium with 1 mg L^{-1} 2,4-D. Explants of the bluestem and A-12164 bermudagrass genotypes cultured in 1 mg L^{-1} of 2,4-D and 1 mg L^{-1} of IAA appeared to remain alive, but exhibited no signs of swelling or callus formation. Explants of Brazos on medium containing 3 or 5 mg L^{-1} 2,4-D responded similarly but produced no callus. Explants cultured on basal medium without growth regulator turned brown and died.

During the second 4-week culture period, after the calli had been subcultured on fresh media containing the same respective growth regulator treatment levels, but incubated under cool-white fluorescent light, differences in their appearance and composition became evident. Bermudagrass E callus formed later in the 8-week culture period than E callus of the bluestem genotypes. Calli from old world bluestem and bermudagrass were composed of two distinct tissue types (non-embryogenic and embryogenic). However, the appearance and composition of callus from the two species differed. Callus from old world bluestem explants was composed of a soft, compact, translucent, and non-embryogenic component, and a yellow-to-light-yellow, friable, embryogenic, portion (Fig. 2a-b). Calli from the three bermudagrass genotypes consisted of a soft, friable, translucent, and non-morphogenic portion and a light-yellow-to-white, compact, slow-growing, embryogenic portion (Fig. 3a-b).

Medium with 3 mg L^{-1} of 2,4-D produced the greatest amount of E callus in both old world bluestem genotypes and in two of the three bermudagrass genotypes (Table 1). E callus production in Brazos bermudagrass was greatest at the 1 mg L^{-1} 2,4-D level. It is well

known that the yield of embryoids due to culturing varies from one genotype to another. The earliest visually detectable E callus was in old world bluestem cultures which occurred in about 2 weeks after subculturing to fresh media in the presence of light. Induction of E callus in our cultures probably was correlated with decreases of auxin level in the media that we assumed is brought on by photoinactivation. After the development of mature embryoids in E calli (Figs. 2-4), cultures were transferred to 1/2 strength MS media, either lacking in plant growth substances, or with 1 mg L^{-1} of zeatin to induce the germination of embryoids and the formation of plantlets. The embryoids formed in vitro from old world bluestem and bermudagrass had characteristic features of grass embryos, including a well defined epiblast, scutellum, coleoptile, and coleorrhiza (Fig. 4). Hundreds of regenerated plants (Fig. 5) from the two bluestem genotypes and from A-10978b and A-12164 bermudagrass genotypes were successfully transplanted into potting soil in the greenhouse. No plantlets were obtained from the E callus of Brazos bermudagrass. The E calli clusters of Brazos appeared healthy several weeks following induction but no embryoids were detected and the calli eventually senesced and died. Also a very high frequency (ca. 90%) of plantlets from bermudagrass accession 10978b were albinos.

In summary, our results demonstrate that the concentration of 2,4-D in culture medium significantly affects E callus production in both old world bluestems and bermudagrass and that genotype by 2,4-D concentration interactions can be expected. The results suggest that about 3 mg L^{-1} 2,4-D will be optimal for some genotypes.

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Table 1. Effect of 2,4-Dichlorophenoxyacetic acid level on embryogenic callus production from young inflorescence explants of old world bluestem and bermudagrass genotypes.

	2,4-D Concentration (mg L^{-1})			
Genotype	0	1	3	5
	---------- % E callus* ----------			
A-8793 OWB	0c**	0c	70a	5b
A-8911c OWB	0c	0c	55a	7b
A-12164 bermuda	0b	0b	43a	0b
Brazos bermuda	0b	42a	0b	0b
A-10978b bermuda	0c	7b	50a	7b

*Mean of 15 reps.
**Means within a row (genotype) followed by the same letter are not significantly different by Duncan's multiple range test (P = 0.01)

Table 2. Analysis of variance for 2,4-Dichlorophenoxy acetic acid concentrations in media on (E) callus induction from young inflorescence explants of old world bluestem and bermudagrass genotypes.

Source	D.F.	Sum of Squares	Mean Square	F-Ratio
Reps	14	1.9000E-01	1.3571E-02	
Treatments				
Genotype	4	4.0389E-01	1.0097E-01	4.20
Main Plot Error E (a)	56	1.346	2.4038E-02	
Treatment	2	6.980	3.490	282.34
Genotype x Treatment	8	5.789	7.2368E-01	58.54
Error	140	1.731	1.2361E-02	
Total	224	16.440		

Coefficient of variation (main plot) = 47.11%
Coefficient of variation (subplot) = 58.52%

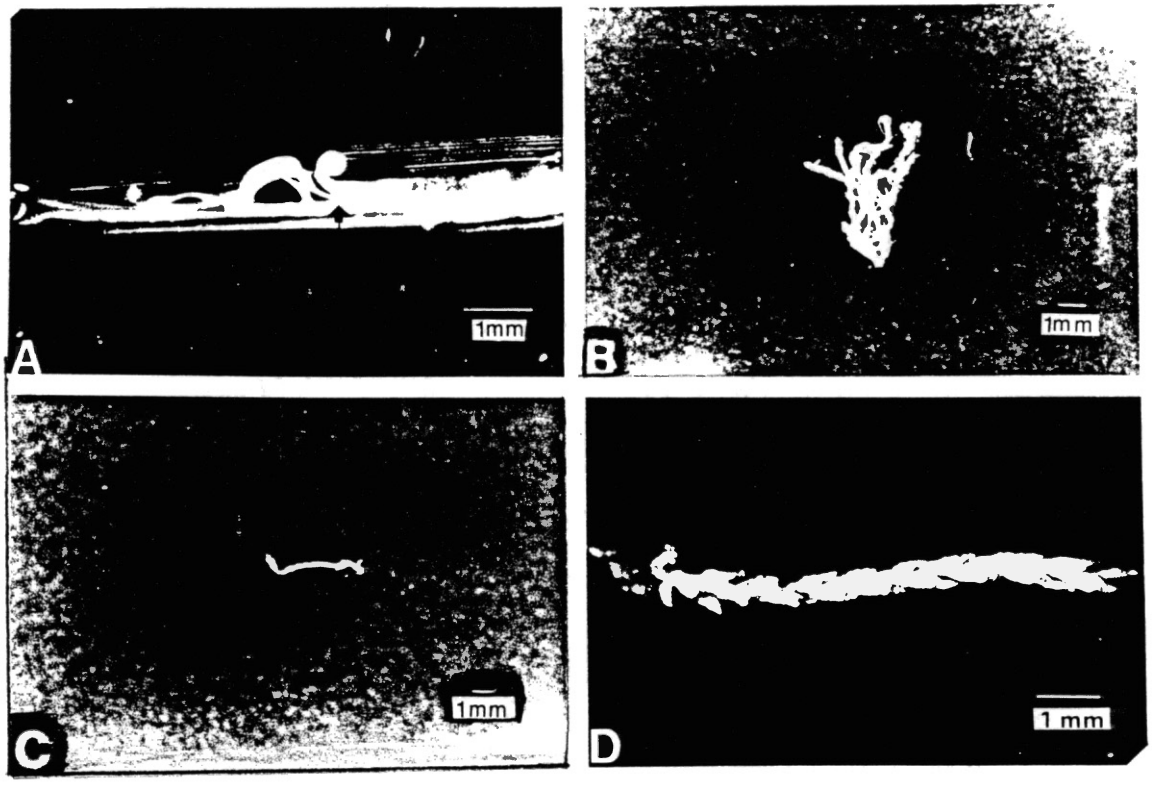

Fig. 1. Typical inflorescence size from bluestem and bermudagrass genotypes used as explant sources. A. Portion of a seed stalk with inflorescence still in boot. B. An excised inflorescence ca 6 mm in length. C. A single raceme from the inflorescence in B. D. Close-up of a young raceme showing individual florets.

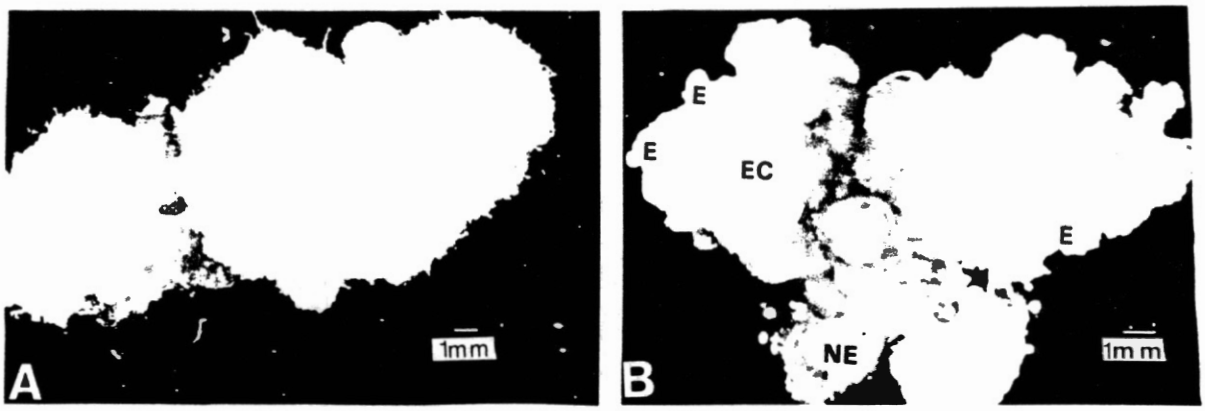

Fig. 2. Basic types of callus obtained from old world bluestem explants. A. White, soft, compact non-morphogenic callus. B. Nonembryogenic (NE) and embryogenic (EC) callus and developing embryoids (E).

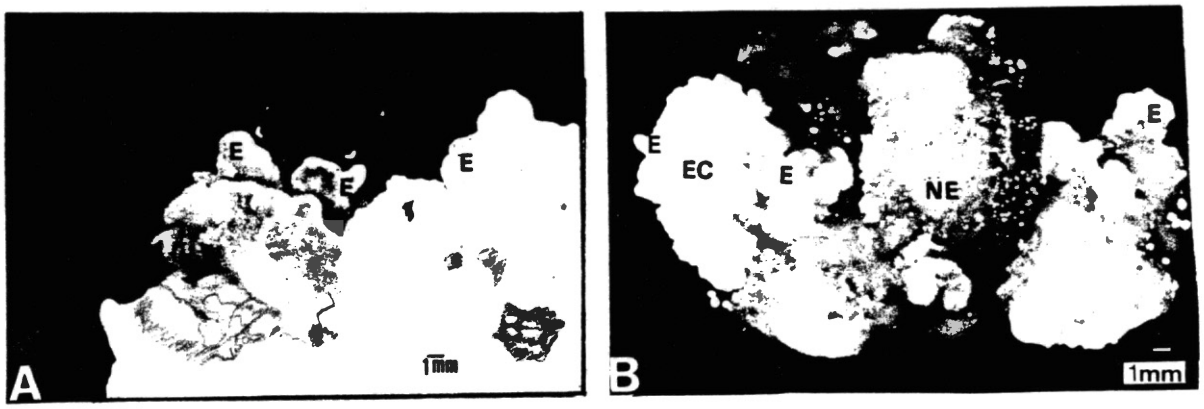

Fig. 3. Basic calli types obtained from bermudagrass explants. A. Soft, friable, translucent and non-morphogenic callus. B. Compact, light-yellow, embryogenic callus (EC) and developing embryoids (E).

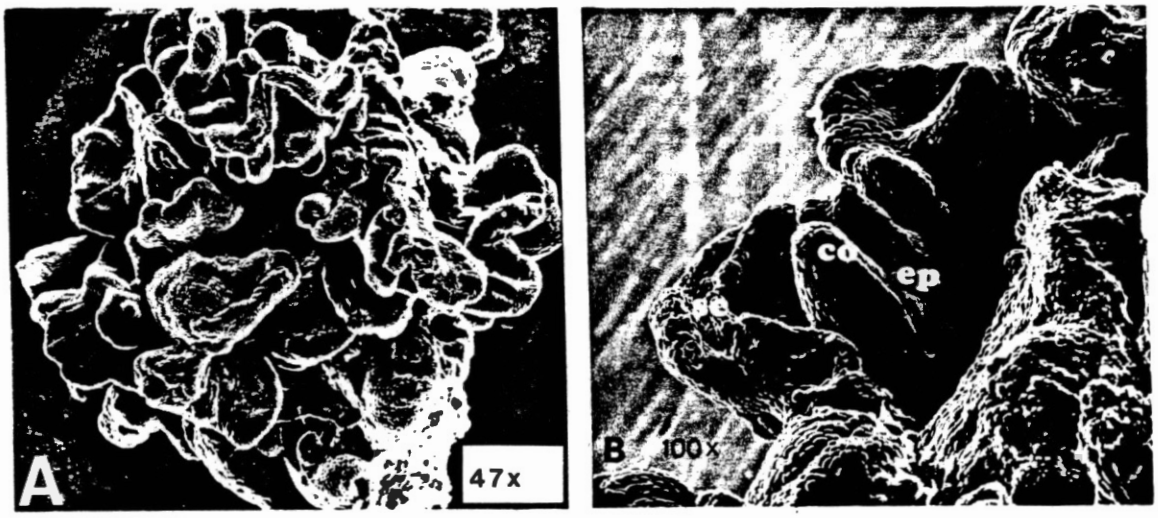

Fig. 4. Scanning electron micrographs of embryogenic (E) callus of old world bluestem genotypes. A. Numerous embryoids. B. Close-up of an embryoid showing the scutellum (sc), coleoptile (co), and epiblast (ep).

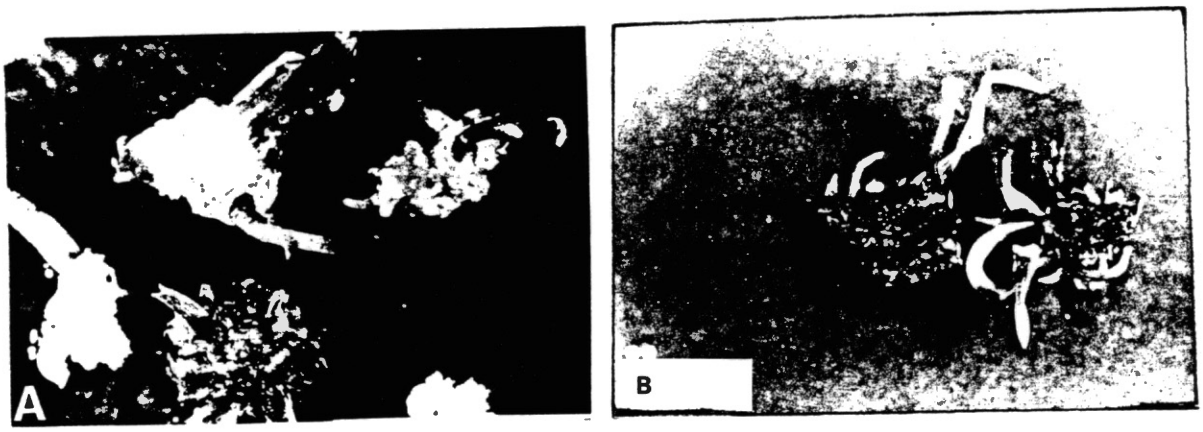

Fig. 5. Regenerating plantlets of old world bluestems. A. Shoots developing from different embryoids. B. Young plants with shoots and roots.

PART III

EFFECTS OF CASEIN HYDROLYSATE AND AUXIN 2,4-D CONCENTRATION ON INDUCTION AND GROWTH OF EMBRYOGENIC CALLUS FROM IMMATURE INFLORESCENCES OF ZEBRA BERMUDAGRASS [*CYNODON DACTYLON* (L.) PERS.]

EFFECTS OF CASEIN HYDROLYSATE AND AUXIN 2,4-D CONCENTRATION ON INDUCTION AND GROWTH OF EMBRYOGENIC CALLUS FROM IMMATURE INFLORESCENCES OF "ZEBRA" BERMUDAGRASS [*CYNODON DACTYLON* (L.) PERS.][1]

Abstract

Primary objectives of this research were to evaluate the effect of casein hydrolysate (CH) on induction and growth of embryogenic (E) callus from immature inflorescence-explants of a variegated bermudagrass, "Zebra", [*Cynodon dactylon* (L.) Pers.], and to determine the level of 2,4-D (2,4-Dichlorophenoxyacetic acid) concentration in the medium for induction and maximum amount of growth of callus. Immature inflorescences about 6 mm in length were inoculated in petri dishes containing modified Murashige and Skoog (MS) agar medium (Ahloowalia medium).

A split plot experiment consisting of two levels of CH (0, 200 mg L^{-1}) and four auxin 2,4-D treatments (1, 3, 5, 7 mg L^{-1}) were randomly assigned to main and subplots, respectively. The experimental design was a randomized complete block with 10 replications. Following inoculation, cultures were maintained in the dark at 25°C for 4 weeks, subcultured in fresh media containing identical nutrient levels and maintained at 25°C under cool white fluorescent light for 4 more

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weeks. After 8 weeks, calli were scored on the basis of fresh weight and percentage of E callus was visually evaluated. Presence of CH and 3 mg L^{-1} of 2,4-D maximized both fresh weight and percentage of E callus. The effect of CH cannot be analyzed alone because the statistical analysis showed CH interaction with 2,4-D; but within each level of 2,4-D the presence of CH showed statistical significant differences in both variables analyzed (fresh weight and % of E callus) Duncan test at 5%. Light and scanning electron microscopy confirmed regeneration through somatic embryogenesis.

Additional index words: Tissue culture, somatic embryogenesis, embryoids, "Zebra" bermudagrass (cynodon dactylon), casein hydrolysate, 2,4-D, grasses.

Introduction

Although plant regeneration from callus cultures has been reported for several forage grasses, consistent, long term, high frequency regeneration in a wide range of species and genotypes remains a major problem (Conger, 1985, 1984; Gray and Conger, 1985).

Until recently, a typical response for grass tissue cultures was a low frequency of regeneration from callus which decreased with increasing time and number of subcultures (Conger, 1982).

Among turfgrass and forage grass species, plant regeneration through somatic (non-zygotic) embryogenesis has been reported in annual ryegrass, Lolium multiflorum Lam.; red fescue, Festuca rubra L.; and orchardgrass, Dactylis glomerata L.; (Conger et al., 1982); Guinea grass, Panicum maximum Jacq.; (Lu and Vasil, 1981), Pearl millet, Pennisetum americanum; (Vasil, 1983a), Proso millet, Panicum miliaceum; (Heyser and Nabors, 1982), Little bluestem, Schizachyrium scoparium Michy. Nash; (Songstad, D.D., 1983), Common bermudagrass, Cynodon dactylon (L.) Pers.; (Ahn et al., 1984, 1985) and old world bluestem, Bothriochloa ischaemum Linn. Keng; (Doye, B., 1986).

"Zebra" bermudagrass [Cynodon dactylon (L.) Pers.], is a variegated plant found among an F_1 progeny population growing on the Agronomy Research Station at Stillwater, Oklahoma. The variegated "Zebra" pattern consists of alternating green and chloratic transverse stripes across the leaf blade (Johnston and Taliaferro, 1975). In a previous experiment we tested the ability of eleven different bermudagrass genotypes to produce callus and regenerate plants: 1.

HN1-14, Stratford common, 2. HN1-20, Brazos (SS 16), 3. HN2-5, 10978b, 4. HN1-2, Midland, 5. HN1-3, Oklon, 6. HN2-2, 959, 7. Turf Nurs 6-1, PI 291585, 8. Turf Nurs 7-3, PI 295339, 9. Turf Nurs 13-4, Tifgreen II, 10. Turf Nurs 30-5 OP from PI 295339, and 11. "Zebra" bermudagrass. "Zebra" bermudagrass had the highest score in callus induction and growth (unpublished). Because this genotype is a prolific producer of seed heads in the field and greenhouse throughout most of the year, a consistent supply of inflorescences for explants is assured. Also the "Zebra" pattern serves as a marker with potential usefulness in assessing somoclonal variegation among regenerated plants.

Although callus induction and plantlet regeneration through somatic embryogenesis has been reported in common bermudagrass (C. dactylon), the possibility of increasing the production of E callus by modifying 2,4-D concentration on the media was not investigated.

Recently, manipulations of the culture-medium components (particularly type and concentration of plant growth regulators) and use of certain cultivars have, in isolated instances, resulted in increasing the number of, and extending the duration of, the regeneration of plants (Conger and McDonnell, 1983; Armstrong and Green, 1985; Heyser et al., 1982).

Addition of casein hydrolysate (CH) to the culture medium has been demonstrated to inhibit precocious germination in barley embryos, presumable because it raises the osmotic value of the medium (Inomata, 1978b). It has been suggested that the sodium chloride or amino acids, which are components of CH, are the ingredients responsible for the resultant high osmorality and inhibition of early germination of

embryos (Cameron and Duffos, 1977). More recently CH has been effective in checking precocious germination of immature embryos and has been responsible for intense growth and differentiation in barley and rice embryo cultures (Showe and Bhaduri, 1982).

Often, subtle changes in the culture medium facilitate long-term maintenance of embryogenic capacity. In maize the initiation of callus containing embryoids (E callus) was optimized with addition of CH and proline to the medium (Armstrong and Green, 1985). Also Gray and Conger (1984) working with callus suspension cultures of Dactylis glomerata, found CH essential for embryogenesis. They found that embryogenic competence can be turned on or off by adding or deleting CH.

Specific objectives of this research were: 1. evaluate the effect of CH on induction and growth of E callus, 2. determine the level of auxin concentration in the media which maximizes E callus, 3. characterize growth and differentiation under regulated conditions.

Materials and Methods

Tillers containing young inflorescences of "Zebra" bermudagrass were obtained from plants growing in the greenhouse of the Agronomy Research Station at Stillwater, OK. After removing the outermost leaves, the material was surface sterilized in 10% commercial clorox for 2 min and 70% ethanol for 1 min and rinsed 5 times in three times distilled water. Immature inflorescences from 4 to 6 mm long were excised aseptically under a stereomicroscope in a laminar flow cabinet and separated into their corresponding racemes (4-6 racemes per

inflorescence). To insure precision and homogeneity in the experimental unit, racemes of approximately the same size and from the same inflorescence were used as explants for each experimental replication. Explants were inoculated at random on 6 cm diameter plastic petri dishes filled with 20 mls of half strength Murashige and Skoog's (MS) medium (Ahloowalia, 1982) supplemented with 0 or 200 mg L^{-1} CH and 1, 3, 5, or 7 mg L^{-1} 2,4-D. Petri dishes were then sealed with parafilm tape.

The pH of all media was adjusted to 5.8 prior to addition of agar (7 g L^{-1}). All components of media except CH were autoclaved at 121°C for 15 min.

Cultures were maintained in the dark at 25°C for 4 weeks, then transferred to plastic petri dishes (100 x 15 mm) on fresh media with the same components as the initial media and cultured at 25°C with 16 hour/day of cool-white fluorescent light having an intensity of approximately 3,000 lux. After 4 more weeks of incubation, calli were scored on the basis of callus fresh weight and percentage of callus containing embryoids, or E callus. Observations were compared statistically by analysis of variance and Duncan's multiple range test at 1% and 5% probability levels.

After the observations were made, calli were transferred to plastic petri dishes, 100 x 25 mm, on half strength MS medium containing 7 g L^{-1} agar, 0.5 mg L^{-1} of 2,4-D and 1 mg L^{-1} of Zeatin for embryo germination and plantlet formation. Cultures were incubated in a growth chamber under 16 h/day of diffused light at 25°C.

The experiment was designed as a split plot with the two levels of CH assigned to main plots and the four concentrations of 2,4-D to subplots and 10 immature inflorescence replications cultured per treatment combination of growth regulator. The experimental unit was considered to be a petri dish. The response variables were fresh callus weight and the percentage of E callus expressed as percent of total callus within each petri dish. In order to have a good quantitative estimation of plantlet regeneration, regardless of the number of shoots or roots, we counted all the plantlets regenerated within each individual petri dish as a single unit.

Developing plantlets were transferred to basal media and allowed to grow. When the leaves were several cm long, the plantlets were transferred to soil in pots. Acclimatization was accomplished by either covering the pots with a transparent plastic cover and maintaining them in a growth chamber for one week or by placing them in a greenhouse for several weeks.

Specimens for scanning electron microscopy (SEM) were placed into 2% (V/V) glutaraldehyde (0.2 sodium cacodylate, pH 7.3) for 2 h, washed in 0.05 M sodium cacodylate buffer (pH 7.3), and postfixed in 2% (V/V) osmium tetroxide (0.2 M sodium cacodylate, pH 7.3) for 2 h at room temperature. Tissue samples were subsequently washed in buffer, dehydrated in graded ethanols, critical point dried in CO_2, affixed to aluminum stubs with silver paint, and coated with approximately 40 mm of gold-palladium.

Photographs were taken using a stereomicroscope with transmitted light. Samples for SEM were examined with an JSM 35 U scanning electron microscope.

Results and Discussion

After one week in culture, the explants began to swell. Because we used only inflorescence segments in which the floral primordia were just being initiated, a portion of the spikelet primordia initially continued developing as they would in the intact plant with the outer glumes enclosing the primordia of the stamens and pistil. After the second week, first outgrows from the explants consisted of small groups of translucent cells, followed by the formation of white tissue on many of the explants. As the callus grew, it became dark yellow in color and the lobbed surface became more pronounced.

After 5 weeks, two types of proliferations were observed on almost all the cultures: one watery, firm and translucent and the other friable, white and opaque (Fig. 1). Plantlets were not observed to regenerate from the watery callus. The friable white proliferation spontaneously formed embryo-like structures (embryoids) and eventually plants on its surface.

Proliferating embryogenic tissue was clearly visible 6 weeks after culture when it had a smooth texture which later became modular and produced embryoids. Well developed embryoids with a distinctly visible purple coleoptile, germinated on media with low levels of 2,4-D and Zeatin. These embryoids were identical to zygotic embryos in structure and organization but had a tendency for premature germination.

Analysis of these embryoids at the SEM level showed that in Zebra bermudagrass embryoid development is similar to that reported by Vasil and co-workers for several grass varieties (Vasil 1982). At any given time several stages of embryoid formation were evident with the smooth folded structures the most obvious (Fig. 2a and c). These spathulate structures were similar to structures considered to be scutellar tissue in other grasses undergoing somatic embryogenesis (Vasil 1983b; Vasil and Vasil 1982a; Vasil 1982).

The first indication of embryoid germination was the formation of green pigment around the edges of the scutellar tissue. The first sign of shoot production was the growth of a coleoptile-like structure from the center of the scutellar tissue which, in turn, was followed by emergence of a shoot from the coleoptile. At the same time the scutellar tissue became more similar to a leaf (Fig. 2c and d). Limited root growth also occurred in culture, although well established shoots with none or very low numbers of roots were successfully transplanted to a mixture of commercial soil and vermiculite and mature plants were produced.

Further proliferation resulted in the development of organized structures some of which possessed a clearly defined root-shoot axis and thus resembled sexually formed embryos. After 5 to 6 weeks some of the cultures had many of embryo-like structures which "germinated" and produced a prolific mass of shoots (Fig. 2c).

Fresh weight: The fresh weight of the individual callus masses was greatest on medium with 3 mg L^{-1} of 2,4-D and CH. This pattern of better response with casein hydrolysate in the media was similar in 5

and 7 mg L^{-1} of 2,4-D (Fig. 3). The interaction effect between CH levels and 2,4-D levels was highly significant ($P < .01$).

Increasing the concentration of 2,4-D had the effect of maintaining the proliferation of embryo-like structures for a longer time and inhibiting the formation of shoots, but simultaneously encouraged the development of glutinous root-derived callus. The proliferation of organized embryo-like structures could be maintained through at least 6 subcultures by transferring the compact callus to fresh media every 4 weeks. However, even under these conditions, a tendency towards the production of shoots could not be avoided.

Percentage of E callus: E callus showed a similar pattern of response obtained in the fresh weight. Thus, the greater percentage of E callus was obtained when the media contained 3 mg L^{-1} of 2,4-D. Within levels of 2,4-D, the media in which casein hydrolysate was added presented the best response (Fig. 4). The CH x 2,4-D interaction effect was also highly significant ($P < .01$) for E callus. Addition of CH to the medium greatly increased E callus production at the 3 mg L^{-1} 2,4-D concentration, less at the 5 mg L^{-1} concentration and none at the 1 and 7 mg L^{-1} concentrations.

Plants were regenerated from 45% of the cultures. Thirty-one percent were albino and 66% appeared to have the normal R_o plant phenotype. Albino plants died when embryogenic food reserves were exhausted.

The development of scutellar tissue and the production of a coleoptile during germination indicates that regeneration is predominantly initiated by the formation of somatic embryos. The

embryoids formed in vitro from Zebra bermudagrass showed characteristic features of grass embryos, including a well defined epiblast, scutellum, coleoptile, and coleorrhiza (Fig. 2b).

The finding of somatic embryogenesis in cultures initiated from immature inflorescence explants demonstrates that these explants are embryogenically competent. These results are in agreement with Ahn et al. 1985 and Doye, B., 1986.

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Table 1. Analysis of variance for effects of casein hydrolysate (CH) and 2,4-Dichlorophenoxyacetic acid on callus production (fresh weight).

Source	D.F.	Sum of Squares	Mean Square	F-Ratio
Reps	9	1.356E-001	1.507E-002	
Treatments				
Casein	1	1.485	1.485	141.29**
Main Plot Error E (a)	9	9.460E-002	1.051E-002	
Treat	3	6.955	2.318	200.76**
Casein x Treat	3	1.448	4.826E-001	41.79**
Error	54	6.236E-001	1.155E-002	
Total	79	10.742		

Coefficient of variation (main plot) = 14.70%
Coefficient of variation (subplot) = 30.81%

Table 2. Analysis of variance for effects of casein hydrolysate (CH) and 2,4-Dichlorophenoxyacetic acid on embryogenic (E) callus production (in percentage).

Source	D.F.	Sum of Squares	Mean Square	F-Ratio
Reps	9	732.500	81.389	
Treatments				
Casein	1	2531.250	2531.250	27.41**
Main Plot Error E (a)	9	831.250	92.361	
Treat	3	14692.500	4897.500	59.68**
Casein x Treat	3	5226.250	1742.083	21.23**
Error	54	4431.250	82.060	
Total	79	28445.000		

Coefficient of variation (main plot) = 46.88%
Coefficient of variation (subplot) = 88.38%

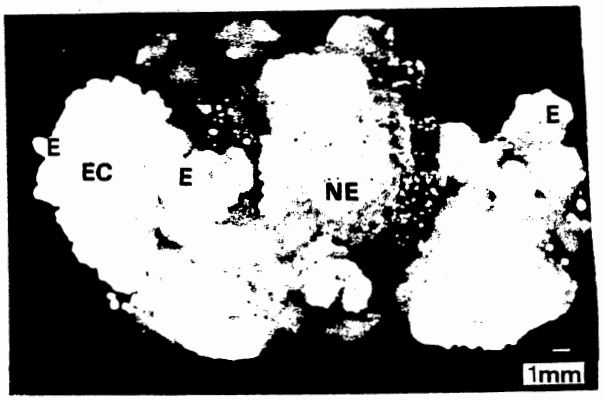

Fig. 1. Close up of embryoid (E) and non-embryoid (NE) callus containing regions. E callus is usually produced in small sectors surrounded by (NE) callus.

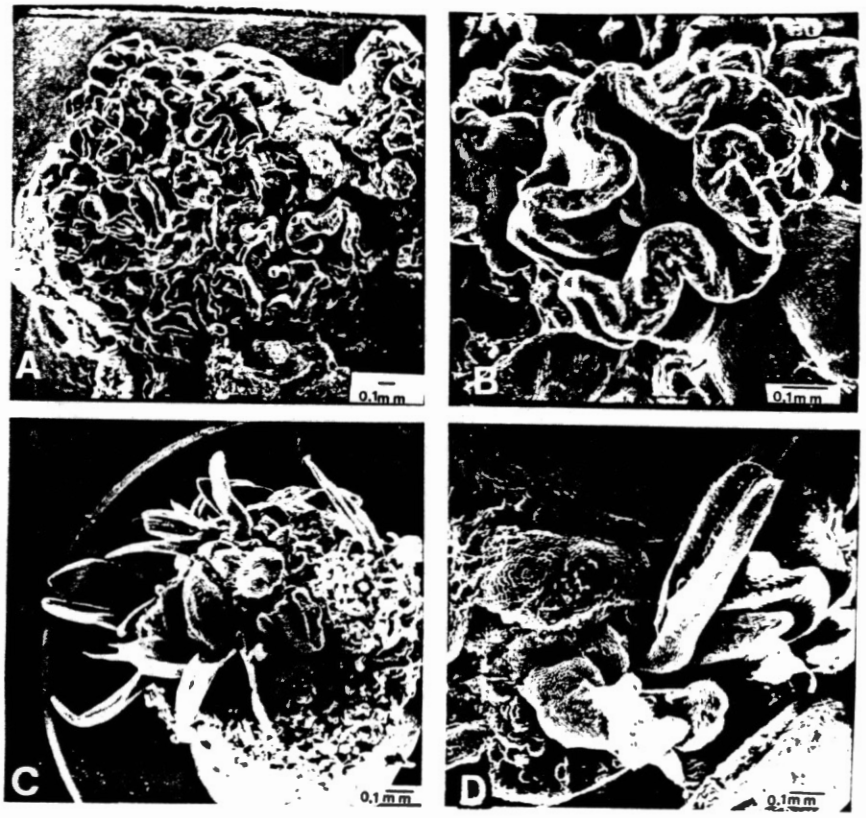

Fig. 2. A. Scanning electron micrograph showing embryoids structures arising from callus surfaces. B. Scanning magnification of the embryoid shown in A. Two shoot meristems appear to be enclosed in a single coleoptile (atypical embryoid). C. Scanning electron micrograph (SEM) showing embryoids structures and embryoids germinating. D. Scanning magnification of embryoid germination stage showed in Fig. C. Shoots start to elongate through the coleoptile (upper left) evidence of scutellar notch.

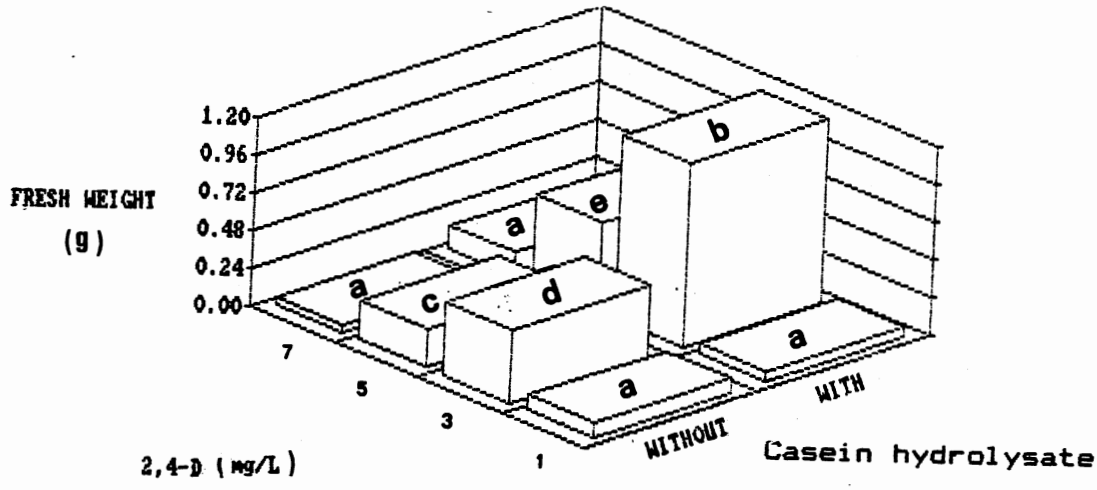

Fig. 3. Effect of casein hydrolysate and 2,4-D concentrations on callus production (Fresh Weight) from young inflorescence-explants of "Zebra" bermudagrass. Bars with the same letter are not significantly different as indicated by the Duncan multiple range test ($\alpha = 0.05$).

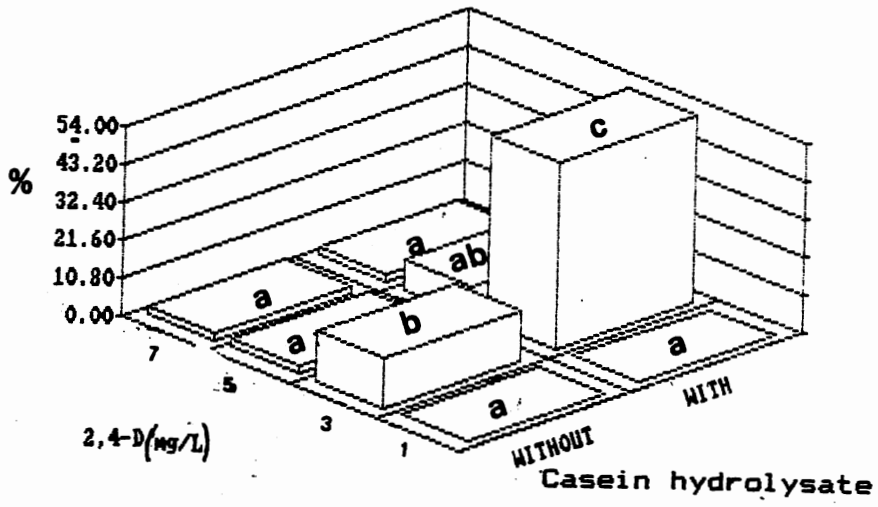

Fig. 4. Effect of casein hydrolysate and 2,4-D concentrations on embryogenic (E) callus production (in % of the total callus that is E callus). Bars with the same letter are not significantly different as indicated by the Duncan multiple range test ($\alpha = 0.05$).

PART IV

THE INFLUENCE OF COLD PRETREATMENT ON INDUCTION AND GROWTH OF CALLUS FROM ANTHERS OF OLD WORLD BLUESTEM [*BOTHRIOCHLOA ISCHAEMUM* (L.) KENG.]

THE INFLUENCE OF COLD PRETREATMENT ON INDUCTION AND GROWTH OF CALLUS FROM ANTHERS OF OLD WORLD BLUESTEM (*BOTHRIOCHLOA ISCHAEMUM* (L.) KENG.)[1]

Keywords: anther culture, cold pretreatment, tissue culture, forage grasses

Primary objectives of this research were to evaluate the effect of cold temperature pretreatment on callus induction from anthers of Old World Bluestem plants (*Bothriochloa ischaemum* L. Keng.) and to determine the effect of light or dark culture on callus growth. Eighteen hundred anthers were collected when the microspores were judged to be in the uninucleate stage of development and were inoculated in petri dishes containing modified Murashige and Skoog MS agar medium (Ahloowalia medium) with 200 $\mu g\ L^{-1}$ of caseine hydrolysate and 3 $mg\ L^{-1}$ of 2,4-D (2,4 dichlorophenoxyacetic acid). A randomized complete block experimental design in split plot arrangement, consisting of two culture environments (dark vs light) in the main plots and three 7 day temperature pretreatments [control-no pretreatment, cultured on nutrient medium in growth chamber, 5°C, 0°C] as subplots were conducted. The experimental unit was a petri dish containing 100 anthers with three replications. Cultures were maintained for 6 weeks either in the dark or under 16 h/day of cool

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1. To be submitted to publication to *Crop Science*.

white light. After 4 weeks the number of anthers in each petri dish with callus was determined and at the end of the 6 week incubation period the fresh weight was measured. Anthers pretreated at 0°C for 7 days before culture had the highest level of callus induction, while illumination during culture produced the highest level of callus growth (Duncan 5% sig. level). Over 97 plants were differentiated from anthers cultured in vitro. Light and scanning electron microscopy confirmed regeneration through somatic embryogenesis. The results demonstrate the feasibility of the anther culture techniques in this obligate apomitic species.

Introduction

Since Guha and Maheshwari (1964) reported the development of embryos from microspores of Datura innoxia, interest in the use of anther and microspore cultures has progressively increased. The culture of whole anthers and the subsequent development of haploid plants through androgenesis was confirmed by Nitsch and Nitsch (1969) with Nicotiana tabacum L. While several haploid producing methods are available (Swartz, 1982), anther culture at present appears to be the most promising one. Doubled haploids provide immediate homozygosity, permit the rapid recovery of recessive allels, and might allow the use of controlled crosses in various schemes to maximize heterozygosity. Theoretical models for such schemes have been published for Medicago sativa L. (Bingham et al., 1979) and should be applicable for many polyploid outcrossing forage grasses. Although progress in the development of anther-derived haploids in major cereal grains has been relatively rapid in recent years, it has been much slower for forage grasses (Conger, 1985).

Old World Bluestems, Bothriochloa ischaemum (L.) Keng, are warm-season, apomictic bunchgrasses that possess good forage potential for the southern Great Plains. Callus induction and plantlet regeneration from culture of immature inflorescences has been reported in this species (Doye, B., 1986).

One of the factors most important in anther culture success is genotype of the anther donor material. Niizeki and Oono (1968), working with rice anthers, found significant differences in the

frequency of callus formation. They tested 10 varieties and only 2 produced anther callus. Plants were regenerated from both genotypes.

Studies on cold pretreatments applied to flower buds to improve the efficiency of the anther culture process were initiated with Datura innoxia (Guha and Maheshwari 1964). Generally, cold pretreatment involves subjecting buds, inflorescences, panicles, spikes, or anthers to cool temperatures (-5 to 15°C) for several days followed by conventional culture procedures. The pretreatment of excised spikes or panicles has been the most prevalent method used in cereals (Chaleff and Stolarz, 1981; Genovesi and Magill, 1979; Sunderland and Evans, 1980). However, for previous experiments on Old World Bluestem (unpublished) we found that excised anther pretreatment has been easy and with less contamination problems than the pretreatment of the whole immature inflorescence in inducing embryogenic callus when compared at the same pretreatment temperature and duration.

This study was initiated to determine and characterize the influence of different cold pretreatment temperatures and concentrations of 2,4-D on induction of callus from anther explants.

Materials and Methods

Shoots containing young unemerged inflorescences of Old World Bluestem genotype 8793 (Bothriochloa ischaemum L. Keng.) were obtained from plants growing in the greenhouse at the Agronomy Research Station, Stillwater, OK. The tillers were collected when the microspores were judged to be in the uninucleate stage of development. After removing the outermost leaves, the inner sheating leaves were surface sterilized

in 10% commercial clorox for 1 min, swabbed with 70% ethanol, and rinsed 5 times in three times distilled water, before aseptic removal of the immature inflorescences which ranged in length from 20 to 30 mm.

Panicles were excised aseptically using a stereo microscope (X10) in a laminar flow cabinet. Panicles were separated into individual spikelets. Spikelets were placed in 15 mm x 100 mm petri dishes filled with three times distilled water to prevent desiccation. Anthers 1.0 to 2.0 mm long were considered suitable for culturing. Anthers were excised from a floret, placed in a petri dish containing sterile distilled water, then chosen at random and used in the various treatments. Anthers were either inoculated directly onto the medium with growth regulators (100 anthers per petri dish) or pretreated in the dark for 7 days at either 5°C or 0°C. During cold pretreatment, anthers were cultured on half strength of Murashige and Skoog's (MS) Basal media (Ahloowalia, 1982). Anthers were cultured in 60 x 15 mm plastic dishes filled with this medium in which neither 2,4-D nor caseine hydrolysate were added. Petri dishes were sealed with parafilm tape. The directly plated anthers were cultured either in the dark or in 16 hour/day of cool-white fluorescent light at approximately 40 µmol photon $m^{-2}s^{-1}$, 25°C, in half strength MS media supplemented with 200 mg L^{-1} of caseine hydrolysate and 3 mg L^{-1} of 2,4-D and 1 mg L of IAA (indole-3-acetic acid). Pretreated anthers were plated on the same medium as the directly plated anthers and incubated under the same conditions.

The pH was adjusted to 5.8 prior to addition of agar (5 g L^{-1}). All components of media except caseine hydrolysate and IAA were autoclaved at 121°C for 15 min. Caseine hydrolysate and IAA were filter sterilized.

After 4 weeks of incubation, the number of anthers with callus in each petri dish was counted and two weeks later, the fresh weight of the callus was measured. Observations were compared statistically by analysis of variance and Duncan's multiple range test procedure at 1% and 5% significance levels (Steel and Torrie, 1980).

After the observations were made, calli were transferred to plastic petri dishes (100 x 25 mm) on half strength MS medium containing 5% agar, 0.5 mg L^{-1} of 2,4-D and 1 mg L^{-1} of Zeatin for embryo germination and plantlet formation. The temperature throughout was 25°C and cultures were incubated in a growth chamber under 16 hour/day of cool white fluorescent light (40 µmol photon $m^{-2}s^{-1}$).

The experiment was designed as a randomized complete block design in a split-plot arrangement, the two environmental types (dark or light environment) were randomly assigned to whole plots and anther cold treatment were randomly assigned to subplots. For each of the six treatment combinations there were three replications. A total of 1800 anthers were cultured. Developed plantlets were transferred to basal media and allowed to grow. When the leaves were several cm long, the plantlets were transferred to soil in pots. Acclimatization was accomplished by either covering the pots with a transparent plastic cover and maintaining them in a growth chamber for one week or by placing them in a greenhouse for several weeks.

Specimens for scanning electron microscopy (SEM) were placed into 2% (v/v) glutaraldehyde (0.2 M sodium cacodylate, pH 7.3) for 2 h, washed in 0.05 M sodium cacodylate buffer (pH 7.3), and postfixed in 2% (v/v) osmium tetroxide (0.2 M sodium cacodylate, pH 7.3) for 2 h at room temperature. Tissue samples were subsequently washed in buffer, dehydrated in graded ethanols, critical point dried in CO_2, affixed to aluminum stubs with silver paint, and coated with approximately 40 mm of gold-palladium. Samples for SEM were examined with an JSM 35 U scanning electron microscope.

Results and Discussion

Effect of Environment (Light vs Dark) and Cold Pretreatment on Callus Induction of Anthers

More of both the control and cold pretreated anthers produced callus when they were cultured under lighted conditions (Fig. 2). Differences in number of anthers producing callus as affected by light and dark conditions were statistically significant ($P < 0.001$). Also, because the ANOVA Table showed no statistical interaction between environment (light or dark) and temperature pretreatment we can safely assume that the better responses on callus induction caused by the light environment are consistent and independent of low temperature treatment. The pretreatment temperatures however, also influenced anther response. The higher percent anther response was obtained when anthers were pretreated at 0°C. Anthers pretreated at 5°C tended to remain rather small during culture and retained the green color during almost all the culture period. Anthers with no cold pretreatment

(control) enlarged with culture and turned golden brown after a few weeks on agar medium.

Effect of Environment (Light vs Dark) and Cold Pretreatment on Fresh Weight of Anther-Callus

Analysis of variance showed that variation associated with effects of environment (light vs dark) and temperature pretreatments interaction was highly significant. The significant interaction suggests that callus growth is environment dependent and that either the treatments light vs. dark need to be analyzed within each temperature pretreatment (0°C, 5°C or control) or temperature treatments within dark or light environments (Fig. 2). Anthers cultured directly onto the media without cold pretreatment (control) had the lowest callus fresh weights. The highest production of callus was obtained when anthers were pretreated at 0°C and cultured under light. The 5°C cold pretreatment response was relative to control vs. 0°C and again light environment maximized the callus growth. In general within each pretreatment illumination appeared to be a favorable practice compared with dark treatment.

Plant Regeneration

Plants were regenerated from all 6 treatments. Albino and green plants, with and without roots, as well as "root only" have been regenerated. The control treatment had the poorest yield of regenerated plants. However all the plants from the control treatment were green. The highest yield of regenerated plants, green and albino,

came from 0°C pretreatment. Out of 97 plants that were regenerated in this study, 19 were albino, 36 weak green plants died after being transferred to greenhouse, and 42 plants are growing to maturity for cytological analysis. In general, cold pretreatments appeared to be very effective when compared to direct culture. This observation applies only to the procedures, temperatures, durations, and genotypes used in this experiment. Significant interactions between culture-environment (light and dark treatment) and anther cold pretreatment temperatures indicated that light culture treatment may be worthwhile for anther callus induction while depending on temperature pretreatment for callus growth. As Marsolais and collaboraters (1984) stated, the high frequency of albino regenerated plants can be due to the culture temperature during the initial stage of in vitro microspore division. Although we were able to induce anther callus and regenerate plants, further analysis is required to determine ploidy level of the regenerated plants.

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Table 1. Analysis of variance for cold pretreatment and culture conditions on anther callus induction.

Split Plot Anova

Field Name: % Description: Percent of Anther With Callus

Source	DF	Sum of Squares	Mean Square	F-Ratio
Reps	2	2.333	1.167	
Treatments				
Env	1	128.000	128.000	768.00
Main Plot Error E (a)	2	3.333E-001	1.667E-001	
Treat	2	553.000	276.500	184.33
Env %Treat	2	16.333	8.167	5.44
Error	8	12.000	1.500	
Total	17	712.000		

Coefficient of Variation (Main Plot) = 1.09%
Coefficient of Variation (Subplot) = 5.65%

Table 2. Analysis of variance for cold pretreatment and culture conditions on fresh weight of callus.

Split Plot Anova

Field Name: WT Description: Calli Fresh Weight

Source	DF	Sum of Squares	Mean Square	F-Ratio
Reps	2	1.074E-002	5.372E-003	
Treatments				
Env	1	1.115	1.115	330.65
Main Plot Error E (a)	2	6.744E-003	3.372E-003	
Treat	2	31.325	15.663	1693.77
Env %Treat	2	9.027E-001	4.514E-001	48.81
Error	8	7.398E-002	9.247E-003	
Total	17	33.435		

Coefficient of Variation (Main Plot) = 1.90%
Coefficient of Variation (Subplot) = 5.44%

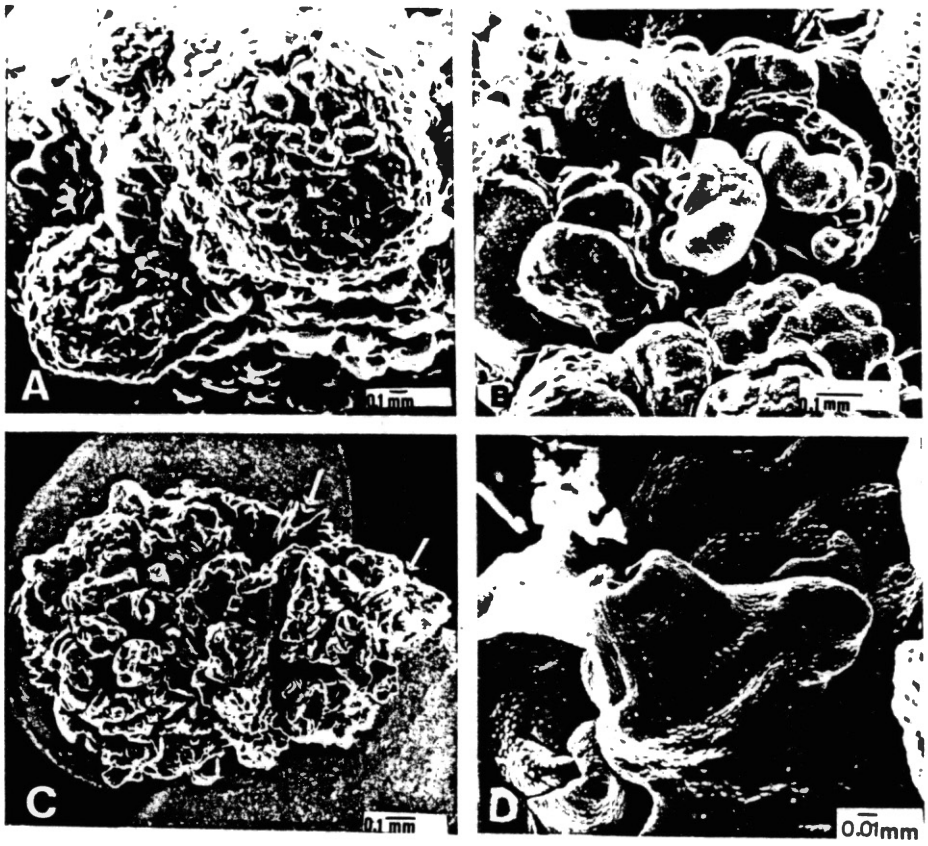

Fig. 1. A. Scanning electron micrograph SEM of callus containing early embryoid formation regions. B. SEM magnification showing the early embryoid formation (black arrows) and well developed embryoids (white arrow). C. SEM of E callus containing embryoid germination (white arrows). D. SEM magnification showing a well developed scutellum enclosing a coleoptile.

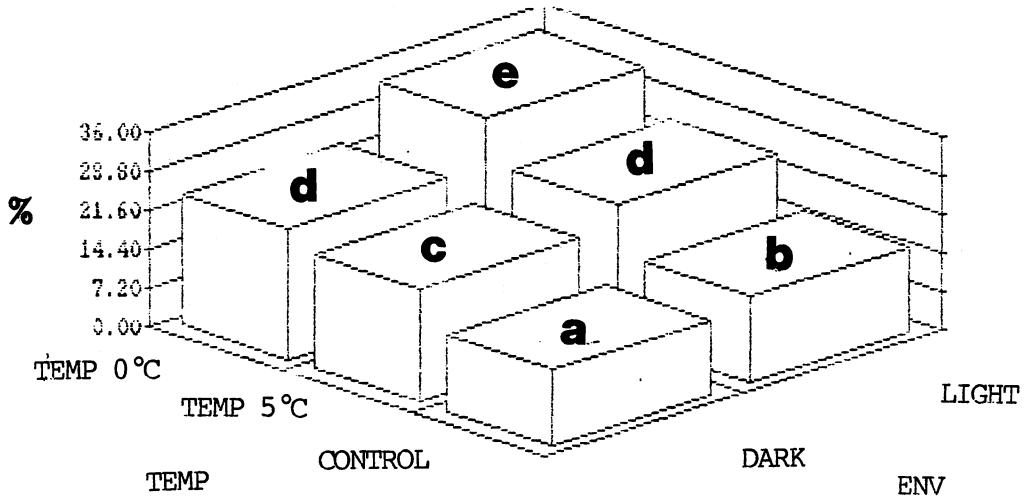

Fig. 2. Effect of cold pretreatment and culture conditions (dark vs light) on callus induction from anther explants of old world bluestem grass. Bars with the same letter are not significantly different as indicated by the Duncan multiple range test ($\alpha = 0.05$).

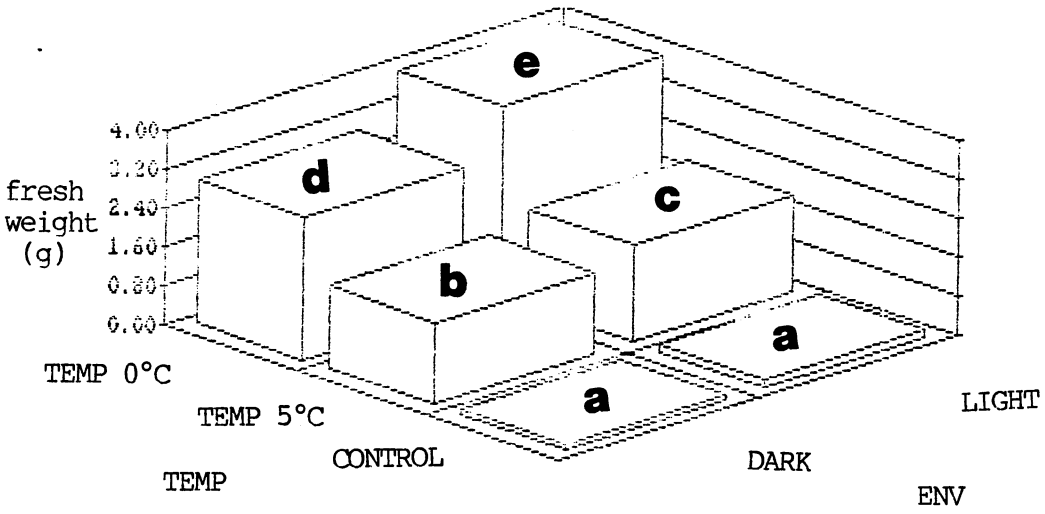

Fig. 3. Effect of cold pretreatment and culture conditions (dark vs light) on callus growth (fresh weight) from anther explants of old world bluestem grass. Bars with the same letter are not significantly different as indicated by the Duncan multiple range test ($\alpha = 0.05$).

VITA

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