IN VITRO CULTURE AND SELECTION STRATEGIES FOR SELECTED FORAGE GRASSES

By

MOHAMED CHAKROUN

Diplome d'Ingenieur
Ecole Superieure d'Horticulture de Chott-Mariem
Sousse, Tunisia
1981

Master of Science
Oklahoma State University
Stillwater, Oklahoma
1984

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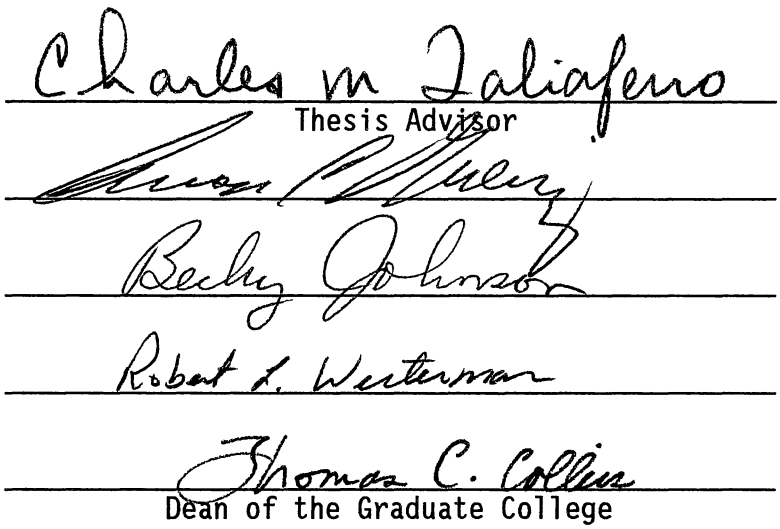

Thesis Advisor

Dean of the Graduate College

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INTRODUCTION

Plant cell and tissue culture, a key component of plant biotechnology, has demonstrated usefulness in increasing the efficiency and effectiveness of cultivar improvement programs. It has been used as a means of virus eradication through meristem tip culture, rapid clonal propagation of specific genotypes, induction of haploid plantlets from anther and pollen culture, and induction and selection of mutants (Conger and Gray, 1984).

The usefulness of tissue culture for plant improvement programs depends on the ability to predictably regenerate plants in vitro. Progress has been achieved in inducing callus and regenerating plants in many species of the Gramineae family. 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. To overcome this problem, research has focused on identifying responsive cultivars or genotypes, explants, and culture media components, especially hormones, which are the major determinant factors for inducing callus and ultimately formation of shoots and roots. Also, careful visual inspection of cultures and selection of promising callus is necessary.

Plant cell and tissue culture techniques are being increasingly utilized in selection for traits of agronomic and economic importance including resistance to low and high temperatures, diseases, and

tolerance to salinity and herbicides. Knowledge of the optimal conditions which favor differentiation under in vitro conditions is a prerequisite for the utilization of these techniques.

This dissertation involves two unrelated studies: 1) development of tissue culture protocols for callus formation and plant regeneration using mature bermudagrass (Cynodon spp.) caryopses as explants, and 2) development of in vitro selection strategies for herbicide tolerance in Old World bluestem (Bothriochloa sp.) grass.

Chapter I contains a review of literature pertinent to the two studies. Chapter II reports the results of studies of the induction of embryogenic callus and plant regeneration using mature bermudagrass caryopses as explants. A protocol for effectively sterilizing and germinating bermudagrass caryopses in vitro is reported in this chapter. Chapter III concerns the effects of accessions, basal media, auxin types and auxin concentrations on embryogenic callus induction and regeneration from germinating bermudagrass caryopses. Chapter IV examines the establishment of dose-response curves for Old World bluestem callus cultures subjected to imazapyr herbicide concentrations. Also included in this chapter are descriptions of callus initiation, maintenance, and protocol for callus exposure to the herbicide. It also reports the step-wise in vitro selection and characterization of nonselected, imazapyr tolerant, and imazapyr sensitive calli of Old World bluestem accessions A-8953 and A-8918c.

Chapters II, III, and IV will be presented in a form acceptable for publication by the Crop Science Society of America journal (Anonymous, 1988). This format is also accepted by other professional journals reporting plant cell and tissue culture research.

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CHAPTER I

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Plant cell or tissue cultures are prerequisites for application of most aspects of plant biotechnology. Plant callus cultures are also useful for induction or isolation of somaclonal variants, screening of tissue for resistance to stress and other selecting agents, and production of somatic hybrids. A wide range of applied biotechnology depends on the ability to initiate and establish callus and cell cultures and the capability to regenerate plants. In this chapter, a review of literature with regard to somatic embryogenesis in grasses and *in vitro* selection for herbicide tolerance is presented.

Somatic embryogenesis in grasses

In the past 10 years, rapid progress has been made in *in vitro* regeneration of monocots in general and specifically in various Gramineae species. However, the limited application of tissue culture techniques in grass improvement programs is mainly due to the low frequency of plantlet regeneration. Callus initiation and plant regeneration can be influenced by several factors such as culture medium (basal salt composition, auxin and sucrose level), genotype, and explant source (Gray and Conger, 1985).

In grasses, regeneration via somatic embryogenesis has been achieved from immature inflorescences, immature and mature embryos, shoot meristems, and seed explants (Conger and Gray, 1984). Immature

tissues, such as immature embryos and young inflorescences are very responsive to tissue culture in several grasses (Dale, 1980; Conger and Gray, 1984; Ahn et al., 1985). Problems associated with use of inflorescences as explants include significant differences in physiological maturity within and among inflorescences, accuracy in identifying the inflorescence part (palea, lemma, pistil) from which the callus is initiated, the totipotency of these parts, and the considerable time required to dissect out explants. Mature caryopses are ideal sources for initiating callus in many grasses. They have the advantages of being available all year and requiring relatively little expenditure of time to put into culture (Johnson and Worthington, 1987). However, a major drawback in their use is contamination. Speakman and Kruger (1983) compared several methods of surface sterilizing wheat seeds. They investigated the use of ethanol, mercuric chloride ($HgCl_2$), sodium hypochlorite (NaOCl), silver nitrate ($AgNO_3$) and terramycin. They recommended that wheat seeds be soaked in a terramycin solution and then immersed in a $AgNO_3$ solution for effective surface sterilization.

In grasses, the use of caryopses as explants to establish embryogenic callus has been reported for red fescue (Festuca ruba L.) (Torello et al., 1984), tall fescue (Festuca arundunacea Schreb.) (Conger and McDaniel, 1983; Lowe and Conger, 1979), Old World bluestem (Bothriochloa ischaemum (L.) Keng) (Johnson and Worthington, 1987), orchardgrass (Dactylis glomerata L.) (Conger and Carabia, 1978), creeping bentgrass (Agrostis palustris Huds.) (Krans et al., 1982), Kentucky bluegrass (Poa pratensis L.) (McDonnell and Conger, 1984), perennial ryegrass (Lolium perenne L.) (Torello and Symington, 1984), and proso millet (Panicum miliaceum L.) (Heyser and Nabors, 1982).

Beside the explant, the genotype plays an important role in the success of tissue culture. In grasses, genotypic differences have been found for efficiency in callus induction, embryogenesis, and plant regeneration (Torello et al., 1984; Ahn et al., 1987).

Improved culture media and conditions have resulted in an increase in growth and differentiation of plant callus and cell cultures. Murashige and Skoog (1962, MS) is the most widely used medium for Gramineae species. Schenk and Helderbrandt (1972, SH) medium has been successfully used to regenerate orchardgrass, tall fescue and Kentucky bluegrass plants (McDonnell and Conger, 1984; Conger and McDaniel, 1983; Conger and Carabia, 1978). Linsmaier and Skoog (1965, LS) medium was used to culture proso millet (Heyser and Nabors, 1982).

In recent years, attention has been focused on the type and lowest concentration of auxin to maximize embryogenesis. Several auxins have been used to produce embryogenic cultures, with 2,4-D (2,4-dichloro phenoxyacetic acid) being the most common. Other less frequently used synthetic auxins include 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), picloram (4-amino-3,5,6-trichloropicolinic acid), NAA (α-naphthalene acetic acid) and dicamba (3,6-dichloro-o-anisic acid). In a study conducted to induce callus and regenerate plants from mature embryo explants of Kentucky bluegrass, McDonnell and Conger, (1984) concluded that dicamba and picloram should be considered as the preferential auxins in future attempts to initiate cultures of this grass species. Torello et al. (1984) reported that 2,4,5-T was as effective as 2,4-D, while NAA was ineffective in red fescue callus induction and growth promotion. Plantlet regeneration is affected by the level of auxin in the medium. Torello and Symington (1984) found that the frequency of

plantlet regeneration was higher for calli maintained in media supplemented with 5 mg L^{-1} 2,4-D than that with 10 mg L^{-1}.

Sucrose has been the most frequently used carbohydrate source for plant tissue culture media, with concentration depending on the species. Ahn et al. (1985) found nonsignificant differences between 20 and 60 g L^{-1} sucrose concentration for callus fresh weight of bermudagrass. Lu et al. (1983) found that increased sucrose concentration promoted somatic embryogeny in maize.

Somatic embryo development, characterized mainly by scutellum formation, occurs after transfer of callus from the primary medium to one with a reduced level of auxin or no auxin. In various grass species, somatic embryo germination and subsequent organogenesis have required supplementing the nutrient medium with a cytokinin. It has been suggested that a cytokinin such as zeatin is required for embryo germination and shoot formation in Old World bluestem and bermudagrass (Artunduaga et al., 1988; Johnson and Worthington, 1987; Metzinger et al., 1987). Apparently, no cytokinin was used by Ahn et al. (1985, 1987) for regenerating bermudagrass plants.

Several investigators have described the two major types of callus; embryogenic (E) and non-embryogenic (NE). The E callus is characterized by a solidified, less friable mass, creamy white to yellow in color, while the NE callus is a brownish, translucent and gelatinous mass, (Nabors et al., 1983; Torello et al., 1984; Johnson and Worthington, 1987). Embryogenic callus cultures and plant regeneration in Old World bluestem was described by Metzinger et al. (1987), using explanted immature inflorescences. Development of suspension cultures from mature seeds of Plains Old World bluestem and plant regeneration

via somatic embryogenesis have been reported (Johnson and Worthington, 1987). A sucrose concentration of 12% (w/v) optimized embryogenic callus formation (Johnson, unpublished). The addition of L-proline to the medium containing 6% sucrose had no significant effect on embryogenic callus formation.

Although it has been suggested that plants regenerated via somatic embryogenesis generally exhibit genotypic fidelity in many species (Hanna et al., 1984), some regenerated plants exhibit genetic variation that is stable and heritable. This type of heritable variation was termed somaclonal variation by Larkin and Scowcroft, 1981. Larkin et al. (1989) summarized the various genetic effects responsible for the altered phenotypes. Those genetic changes are: chromosomal number changes, chromosomal structure rearrangement, DNA amplification, point mutation, altered expression of multigene families and mobilization of transposable elements. Somaclonal variant plants derived from cultures of apomictic Old World bluestem plants have been characterized morphologically and cytogenetically. Differences as indicated by isozyme variations have been identified (Taliaferro et al., 1989). In this example, the somaclonal variant plants may have the potential for improving this grass species.

In vitro selection for herbicide tolerance

In vitro selection techniques are considered as biotechnological alternatives to conventional plant improvement procedures. The development of new genetic variants or mutants using in vitro plant cell and tissue cultures is of major importance to plant breeders.

In a recent review, Widholm (1988) listed several traits of agronomic importance that can be selected using in vitro techniques. These include resistance to herbicides, diseases, salt, metals, cold, heat, drought, insects, high or low pH, low nutrients and increased levels of free amino acids. He suggested that only traits being expressed at the cellular level can be readily identified using plant callus and cell culture systems. However, even if cells with increased resistance to such a trait can be selected in vitro, this does not necessarily mean that the whole plants regenerated from those cells will display the same tolerance. Morphological or physiological characteristics at the plant level may be more important than those of cells. Widholm (1988) suggested that the desirable characteristic, selected in vitro must persist in the regenerated plants and be heritable in order for a selected trait to be agriculturally useful.

At present, the application of herbicides is an important production practice for a large number of crop species. Seedlings of several crop species are sensitive to the present herbicides, prohibiting their use during the establishment stage. Several new, highly potent, and environmentally safe herbicides have been developed by different chemical companies. These include glyphosate (N-[phosphonomethyl]glycine), sulphonylureas, imidazolinones, aminotriasole, and phosphinothricin. They have broad spectrum activity discriminating poorly between weeds and crops and inhibit plant growth by interfering with the biosynthesis of essential amino acids (LaRossa and Falco, 1984).

The use of tissue and cell culture methods to study herbicide tolerance offers several advantages: 1) Characterization at the cellular

level of the biochemical processes involved in herbicide tolerance, 2) elimination of the complication arising from the morphological variability and highly differentiated stage of various tissue of whole plants and 3) permeation of a relatively uniform treatment of tissue.

Selection for herbicide tolerance *in vitro* has been a favored area of research with several reports of tolerance at the cellular level. The plant cell and tissue culture approach has proven to be effective in selecting herbicide tolerant callus and cell lines of several crop species. Chaleff and Ray (1984) selected tobacco mutants that were resistant to the sulfonylurea herbicides, chlorsulfuron and sulfometuron methyl. They reported that the mutant plants expressed over 100-fold greater resistance than normal plants. The resistance was due to a single dominant or semi-dominant mutation at one of the two unlinked loci conditioning response to the herbicide. The mode of action of these two herbicides has been demonstrated through biochemical and genetic studies. Chaleff and Mauvais (1984) reported that acetolactate synthase (ALS), also referred to as acetohydroxyacid synthase (AHAS), was the target enzyme responsible for the biosynthesis of the branched chain amino acid isoleucine, leucine, and valine. Sulfonylurea-resistant mutants of *Datura innoxia* have been isolated using haploid cell suspension culture system. Some sulfonylurea-resistant variants showed cross resistance, while others exhibited sensitivity to the imidazolinones (Saxena and King, 1988). Mutants resistant to chlorsulfuron have been obtained from *Arabidopsis thaliana* by screening for growth of seedlings in the presence of the herbicide (Haughn and Somerville, 1986).

Shaner and Anderson (1985) obtained maize cell lines which showed 100 times higher tolerance to imidazolinones through cell cultures. These cell lines were regenerated into resistant plants in the presence of normally toxic levels of the herbicide. Seed was obtained from these plants. Shaner et al. (1984) showed that the development of tolerance to imidazolinone herbicides in maize is due to the alteration of the AHAS enzyme. Using in vitro microspore mutagenesis and selection, Swanson et al. (1989) regenerated five double-haploid canola plants tolerant to an imidazolinone, 5-ethyl-2-[4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl]nicotinic acid (Pursuit). Evaluation of the mutants under field conditions indicated that the original mutants and hybrids produced could tolerate at least two times the recommended field rate of another imidazolinone, methyl 6-[4-isopropyl-4-methyl-5-oxo-2-imidazoline-2-yl]-m-toluate (Assert).

Using suspension culture selection techniques, glyphosate tolerant carrot cell culture lines were reported by Nafziger et al. (1984). These cell lines were obtained by gradual transfer into medium with higher levels of the herbicide. Glyphosate-resistant cell lines have also been obtained from haploid suspensions of tobacco and the expression of stable resistance has been demonstrated in tobacco calli and regenerated plants (Singer and McDaniel, 1985). Glyphosate inhibits the enzyme 5-enolpyrovyshikimate 3-phosphate synthase (EPSPS), involved in the biosynthesis of aromatic amino acids. The EPSPS is the primary target of the herbicide in plants (Nafziger et al., 1984; Rubin et al., 1984; Mousdale and Coggins, 1984).

A cell suspension scheme was used to isolate seven amitrole tolerant clones from haploid and diploid tobacco plants. This tolerance

was expressed in calli, regenerated plants, and progenies; however, simple Mendelian inheritance patterns were not observed (Singer and McDaniel, 1984).

Alfalfa suspension cell lines resistant to the herbicide L-phosphinothricin have been selected (Donn et al., 1984). These cell lines had over 20- to 100-fold resistance than the wild type cells. L-phosphinothricin inhibits the enzyme glutamine synthase (GS) causing rapid accumulation of ammonia and therefore, toxifying the plant cells (Tachibana et al., 1986).

According to Chaleff (1988), the use of cell culture techniques is limited because of several reasons: 1) inability to regenerate plants from cultured cells and / or protoplasts of many crop species, 2) the most agronomically important traits are genetically complex, 3) trait modification issuing from cultured cells in vitro is a function of the whole plant, and 4) molecular and cellular bases of many agronomically important traits are not known, thus making it difficult or impossible to design selection strategies.

Applying selection pressure generally reduces plant regeneration potential. Tissues or cells to be screened utilize suspension cultures (SC), SC plated in or on agar-solidified medium, protoplasts or pieces of callus. It has been suggested that a dose-response must be determined before imposing selective conditions. Each plant cell line and species has a unique inhibitory level to a selecting agent (Gonzales and Widholm, 1985). In Fig. 1, a generalized scheme for grass in vitro selection is presented.

Due to the high medium-to-tissue contact, embryogenic suspension culture system is more rigorous and has a more rapid growth rate than

solid media. The advantage of cell suspension cultures in selecting for resistance to selecting agents is that it allows large numbers of cells to be relatively easily screened *in vitro*, by applying standard microbial techniques for isolating desired mutants (Gonzales and Widholm, 1985).

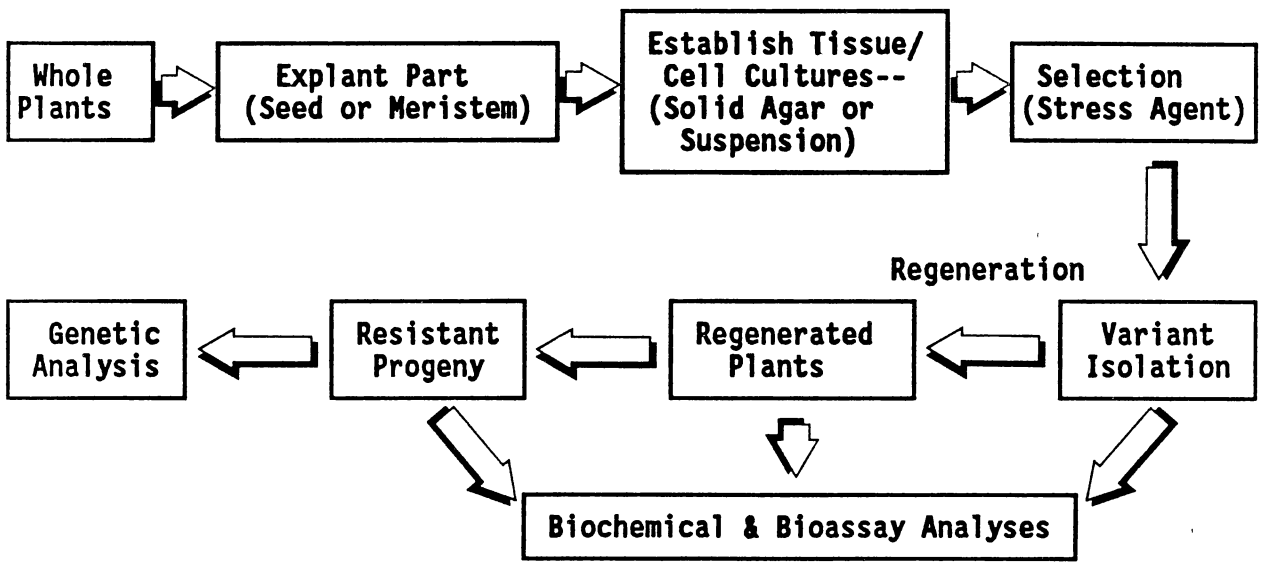

Fig. 1. Steps in tissue or cell culture strategies for increasing resistance of grasses to stress agents.

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CHAPTER II

INDUCTION OF EMBRYOGENIC CALLUS AND PLANT REGENERATION FROM MATURE BERMUDAGRASS CARYOPSES

Induction of Embryogenic Callus and Plant Regeneration from Mature Bermudagrass Caryopses

Key words: Bermudagrass, caryopsis explants, 2,4-D, dicamba, somatic embryogenesis, plant regeneration

ABSTRACT

Regeneration of plants from cultured tissue derived from mature caryopses of Cynodon spp. has not been reported. The ability to use mature caryopses as explants would facilitate tissue culture applications with Cynodon spp. An experiment was performed to assess the potential for embryogenic callus (EC) production and plant regeneration from mature caryopsis explants as influenced by type and composition of basal medium. Sterile germinating caryopses of four cultivars (67X64, Zebra, Guymon, and TN 4-4) were cultured on modified Murashige-Skoog (mMS) medium containing 4.5, 13.5, or 27 μM 2,4-D (2,4-dichlorophenoxyacetic acid), or Schenck-Hildebrandt (SH) medium containing 2.25 μM 2,4-D and 5, 20 or 40 μM dicamba (3,6 dichloro-o-anisic acid). Response variables were callus fresh weight after 5 (FW1) and 11 (FW2) wk culture, estimated percent EC after 5 wk, and number of regenerated plants. Callus was initiated from explants in all treatment combinations. All response variables exhibited significant differences ($P<0.01$) attributable to cultivar and medium. Significant ($P<0.01$) cultivar x medium interactions were present for FW1, FW2 and percentage

EC. FW1 was greatest for Guymon cultured in SH containing 20 μM dicamba. Greatest FW2 was in 67X64 cultured in SH containing 2.25 μM 2,4-D plus 5 μM dicamba. EC was greatest for Guymon and Zebra cultured in SH medium containing dicamba. All calli grown in mMS containing 2,4-D eventually senesced. All cultivars produced EC, but regenerate plants were obtained only from Guymon and Zebra. Greatest numbers of regenerate plants were obtained from SH medium containing 40 μM dicamba. Plants dwarfed primarily by reduction in internode length were found among regenerated Zebra plants.

INTRODUCTION

Cynodon spp. are warm-season, perennial grasses introduced into the southern United States and used for forage, turf and soil-stabilization. The development of new, well-adapted, high yielding Cynodon cultivars may be facilitated by combining conventional breeding procedures and in vitro culture techniques. Application of the latter requires efficient and effective methods for establishing and maintaining cultures and regenerating plants. In grasses, plant regeneration via somatic embryogenesis has been achieved using immature inflorescences, meristem tips, leaf or stem segments, and mature caryopses as explant sources (Conger and Gray 1984). Young inflorescence explants have been successfully used to regenerate plants of several grass species, but their use is not without complicating factors. These include: (1) physiological and maturity differences within and among inflorescences significantly affecting callus initiation, growth, and totipotency, (2) unavailability in nonflowering periods, and (3) difficulty in distinguishing from which part of the inflorescence callus originates. Mature caryopses are an ideal source for callus initiation because they are easily stored, and thus continuously available, require less time to explant, and are physiologically relatively uniform (Johnson and Worthington 1987).

To date, bermudagrass plant regeneration via somatic embryogenesis has been reported only from immature inflorescences. Ahn et al. (1985, 1987) and Artunduaga et al. (1988) used immature bermudagrass inflorescences cultured respectively on N6 and modified Murashige-Skoog (mMS) agar media with 2,4-D (2,4-dichlorophenoxyacetic acid) as an auxin

source. In each case, callus was obtained which ultimately produced regenerate plants via somatic embryoids.

Knowledge of the conditions necessary for callus initiation, growth and plantlet regeneration is mandatory for successful use of in vitro culture techniques. Many factors influence the initiation and growth of embryogenic cultures, the major ones being explant, genotype, medium composition, and auxin type and concentration. Modified Schenk-Hildebrandt (SH) medium, containing dicamba (3,6 dichloro-o-anisic acid), was used to initiate callus from Kentucky bluegrass explants (McDonnell and Conger, 1984). Conger and McDaniel (1983) used SH to produce tall fescue callus from seed explants thereby permitting the screening of seed samples for the fungal endophyte Acremonium coenophialum. The mMS medium with 2,4-D auxin has been successfully used in our laboratory for initiating and maintaining Old World bluestem (Bothriochloa spp.) and bermudagrass callus capable of somatic embryogenesis (Artunduaga et al. 1988). However, plant regeneration from Cynodon species has been limited to cultures derived from young inflorescence explants. The objective of this study was to evaluate growth and type of callus induced from Cynodon caryopses explants as influenced by cultivar and media composition.

MATERIALS AND METHODS

Cynodon cultivars used in the study were Guymon, Zebra, 67X64 (all C. dactylon var. dactylon) and TN 4-4 (C. transvaalensis). Caryopses were surface sterilized by processing through the following steps: (1) washing for 15 min in 50 ml distilled water containing a few drops of Tween 20 (polyoxyethylene sorbitan monolaurate) as a surfactant, (2)

immersing in 70% ethanol for 1 min, (3) washing in a 20% (v/v) solution of commercial Clorox containing 2-3 drops of Tween 20 on a magnetic stirrer for 15 min under vacuum, (4) immersing in a 10% (v/v) daconil fungicide solution for 5 min, and (5) rinsing in autoclaved distilled water 4 to 5 times prior to aseptic germination in 0.2% KNO_3 solution in Erlenmeyer flasks placed on a gyratory shaker for 48 to 72 h.

Germinating caryopses were rinsed once in sterile distilled water and cultured on two media: (1) mMS containing the major and minor minerals of Murashige-Skoog (1962), Gamborg's B-5 vitamins (1968), 3% (w/v) sucrose and either 4.5, 13.5 or 27 µM 2,4-D. The pH was adjusted to 5.8 prior to addition of 0.7% (w/v) agar (Sigma Chemical Co., St. Louis, MO) followed by autoclaving at 121 °C and 1.1 kg cm^{-2} for 15 min and (2) Schenk and Hildebrandt (1972) medium modified with 3% (w/v) sucrose, and supplemented with either 2.25 µM 2,4-D plus 5 µM dicamba, 20 or 40 µM dicamba. The latter media components, pH set at 5.8, contained 0.7% Sigma agar and were sterilized by autoclaving with the exception of dicamba, which was filter sterilized and then added to the autoclaved medium. Treatments were designated by letter as follows: A = mMS + 4.5 µM 2,4-D; B = mMS + 13.5 µM 2,4-D; C = mMS + 27 µM 2,4-D; D = SH + 2.25 µM 2,4-D and 5 µM dicamba; E = SH + 20 µM dicamba; and F = SH + 40 µM dicamba. About 20 ml of medium was dispensed in each presterilized (100 X 15 mm) petri dish.

Treatments were arranged in a randomized block design with unequal number of replications. A replicate consisted of a petri dish (100 x 15 mm) containing 10 germinating caryopses. Cultivars 67X64 and Guymon had each treatment replicated six times. Because of fewer available germinating caryopses, Zebra and TN 4-4 had treatments replicated only

four times. Cultures were incubated for 5 wk in the dark at 27 ± 1 °C (culture period 1). After this period, callus growth (fresh weight in g/petri dish) was determined and the proportion of EC in each culture plate was visually estimated using the scale: (1) 0, (2) 1-25%, (3) 26-50%, (4) 51-75% and (5) >75%.

The six best growing calli per plate were transferred to a petri dish (100 X 20 mm) containing fresh medium with the same components as the initial medium, and were cultured for 6 more wk (culture period 2) at 27 ± 1 °C under cool white fluorescent light at an average intensity of 42 µmol s^{-1} m^{-2} with a 16 h photoperiod. Callus growth (final weight - initial weight) per petri dish during this second culture period was determined.

A portion of each callus mass was transferred to petri dishes containing the same media components without auxin and 6.8 μM of zeatin (mixed isomers, Sigma Chemical Co., St. Louis, MO) for somatic embryo germination and subsequent plantlet formation. Cultures were incubated in a growth chamber as described above. When adequate shoot and root systems had developed, plantlets were transferred to sterile soilless mix (sand-vermiculite-peat moss, 2:2:3) in small pots and acclimated in a growth chamber for 1 wk before being taken to the greenhouse. Data were analyzed using the general linear models (GLM) procedure of the Statistical Analysis System (SAS, 1982). Mean comparisons were made using Fisher's least significant difference (LSD) test at the 0.05 percent probability level.

RESULTS

Callus tissue formed within 2 wk from germinating caryopses of all cultivars on all media treatments. No contamination occurred. Callus appeared to originate from the base of the shoot of the emerging seedlings. Callus initially consisted of non-embryogenic vacuolated cells, somewhat uniform in structure, translucent, gelatinous, and white to brownish in color. After 2 to 4 wk, EC formed in some cultures and was characterized as solid, opaque, creamy white to yellow in color and having a nodular surface (Fig. 1).

All response variables had significant differences ($P<0.01$) attributable to cultivars, medium treatments, and interaction between the two except the cultivar x medium interaction for FW2 (Table 1). Mean FW1 was greatest on medium E and F for Guymon and medium E and A for 67X64 (Fig. 2). FW1 was not recorded for TN4-4 because of the small amount of callus produced. Callus FW1 was also very low for Zebra on all media (Fig. 2). The greatest percentage of EC was obtained from Guymon and Zebra cultured on SH medium supplemented with dicamba (Fig. 3). Although TN 4-4 produced relatively little callus, about 25% of that produced was embryogenic, except for treatment C. Only treatments E and F resulted in EC production for 67X64 and in significantly smaller percentages than for other cultivars. Guymon and 67X64 produced significantly greater FW2 than TN 4-4 and Zebra in all medium treatments (Fig. 4). Treatment D produced the greatest amount of FW2 for both Guymon and 67X64. There were no significant differences in FW2 among media for Zebra and TN 4-4.

The number of regenerated plantlets from callus cultures after transfer to regeneration media is shown in Table 2. When calli were transferred onto auxin free medium supplemented with 6.8 μM zeatin, no plantlets were regenerated from 67X64 or TN 4-4. Calli of these two cultivars turned brown and died. Most of the EC of Guymon and Zebra differentiated into somatic embryoids and subsequetly regenerated into plantlets within 3 to 4 wk (Fig. 5). The highest frequency of plantlet regeneration was from EC cultured on SH with 40 µM dicamba. No significant difference was found between the other two media. No albino (chlorophyll deficient) plantlets were obtained. Variant plants were found among those regenerated from Zebra calli (Fig. 6). Those plants were dwarfed by reduction in internode length compared to normal plants.

DISCUSSION AND CONCLUSIONS

Callus induction from all media and genotypes tested was attributed mainly to the absence of contamination, presence of auxin in the media and to the physiological uniformity of the explants. In a preliminary experiment, a lower concentration of Clorox resulted in a high bacterial contamination rate. The development of embryogenic (regenerable) callus was similar to that previously described for bermudagrass using immature inflorescence as explants (Ahn et al., 1987; Artunduaga et al., 1988). In this study, the influence of basal media (mMS vs. SH) was less than that of auxin type and concentration on the induction of somatic embryogenesis. The substitution of dicamba for 2,4-D gave better callus induction and growth for Kentucky bluegrass (McDonnell and Conger, 1984), maize (Duncan et al., 1985) and wheat (Hunsinger and Schauz, 1987). Dicamba has shown to be a more useful

auxin than 2,4-D for the induction of regenerable callus from species such as bermudagrass, in which callus induction is often difficult.

Cultivar affected somatic embryogenesis significantly and Guymon and Zebra responded better than 67X64 and TN 4-4. Similar cultivar or genotype responses have been reported in maize (Tomes and Smith, 1985), red fescue (Torello et al., 1984) and wheat (Sears and Deckard, 1982; Maddock et al., 1983). The differences among genotypes may be due to the presence of genes responsible for the ability to form somatic embryos. Previous research utilizing mature caryopses of bermudagrass as explants resulted in the recovery of non-regenerable callus cultures (Ahn et al., 1985; Krans, 1981), this may have been due to the utilization of non-responsive cultivars or genotypes. This study demonstrates that somatic embryogenesis and plantlet regeneration can be achieved by in vitro culture of explanted mature bermudagrass caryopses if a responsive cultivar is utilized. Therefore, regeneration from caryopsis explant can now be efficiently used for different in vitro bermudagrass plant improvement methods. The conditions in which the parental material is raised are still of major importance. The lack of some of the assumed EC to develop into embryoids and subsequently shoots suggests that visual selection of callus for plantlet regeneration potential may not be sufficient.

In conclusion, this research shows that callus fresh weight was greatest in Guymon and 67X64 plated on SH media supplemented with dicamba for initial growth. SH medium containing the lowest concentrations of dicamba and 2,4-D produced the greatest callus growth during the second growth period.

Percent EC was greatest (approx. 50%) in Guymon and Zebra with SH media containing 40 µM dicamba. This combination resulted in the largest number of plants being regenerated. Percentage of EC and plantlet regeneration were significantly and positively correlated. Differences in cultivar response to media indicate that further testing is needed to optimize use of dicamba as an auxin source. Plants that have been regenerated from Zebra are now being evaluated for somaclonal variation and will be the subject of a future study.

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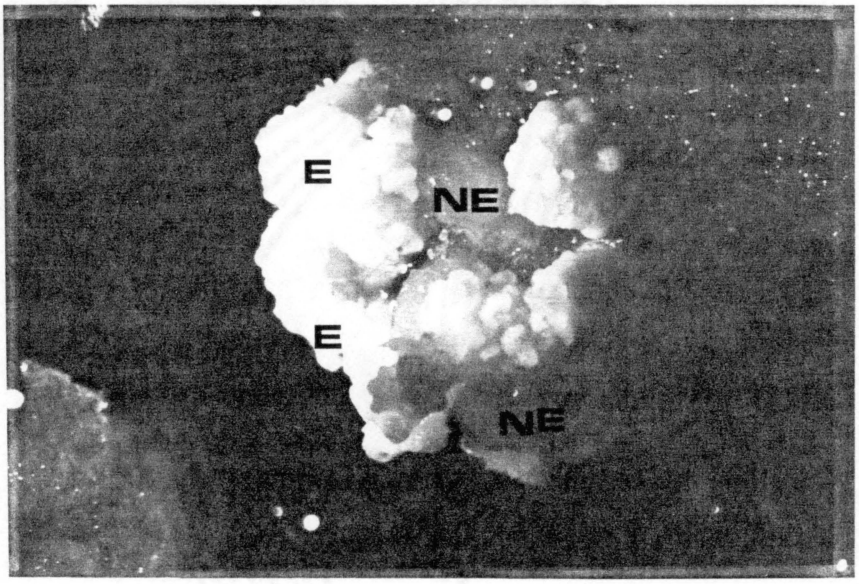

Fig. 1. Callus with embryogenic (E) and non-embryogenic (NE) regions derived from cultured germinating bermudagrass caryopses after 5 wk of culture on SH medium containing 40 μM dicamba.

Table 1. Analyses of variance for fresh weight (FW1), percentage of embryogenic calli (EC), fresh weight (FW2), and plantlet regeneration (PLR) with cultivars (Cul) and media (Med) effects and their interaction.

		Mean square			
Source	df	FW1	% EC	FW2	PLR
Cul	3 (2)†	127.53**	7.35**	2930.12**	202.92**
Rep(Cul)	16 (13)	2.74	0.62	163.42	5.95
Med	5 (5)	82.73**	4.71**	140.72**	174.33**
Cul x Med	15 (10)	24.67**	1.64**	144.11	61.65**
Error	80‡ (65)	1.70	0.32	9.09	5.32

** Indicates significance at the 0.01 level probability.

† The df in parenthesis is for FW1.

‡ The df of the error source is 78 for FW2.

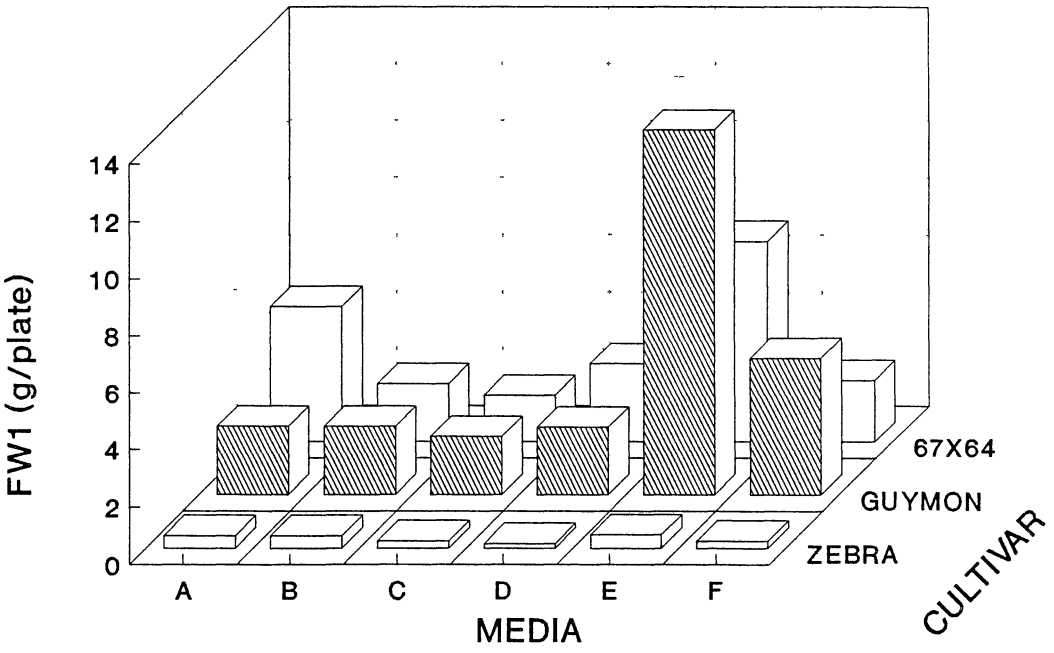

Fig. 2. Mean fresh weight (FW1) of calli per petri dish after 5 wk of culture in darkness.
LSD (0.05) = 3.5, 3.9 for comparisons between media means at each genotype and genotype mean at each medium, respectively.
A = mMS + 4.5 µM 2,4-D; B = mMS + 13.5 µM 2,4-D; C = mMS + 27 µM 2,4-D; D = SH + 2.25 µM 2,4-D and 5 µM dicamba; E = SH + 20 µM dicamba; and F = SH + 40 µM dicamba.

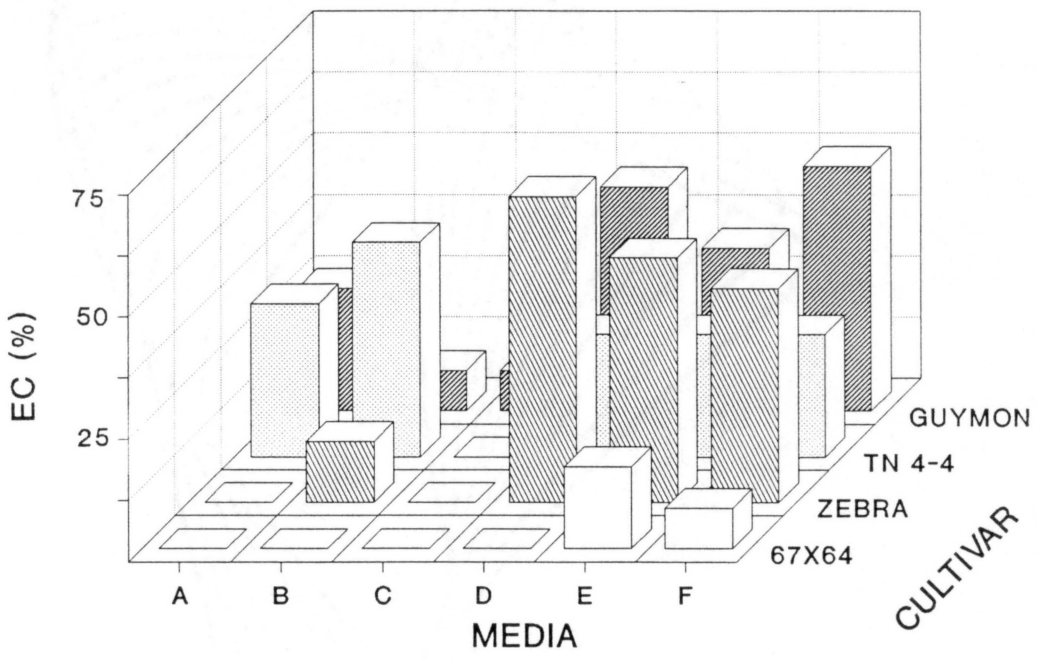

Fig. 3. Mean percentage of EC per petri dish after 5 wk of culture in darkness.

LSD (0.05) = 20 and 21 for comparisons between media means at each genotype and genotype mean at each medium, respectively.

A = mMS + 4.5 µM 2,4-D; B = mMS + 13.5 µM 2,4-D; C = mMS + 27 µM 2,4-D; D = SH + 2.25 µM 2,4-D and 5 µM dicamba; E = SH + 20 µM dicamba; and F = SH + 40 µM dicamba.

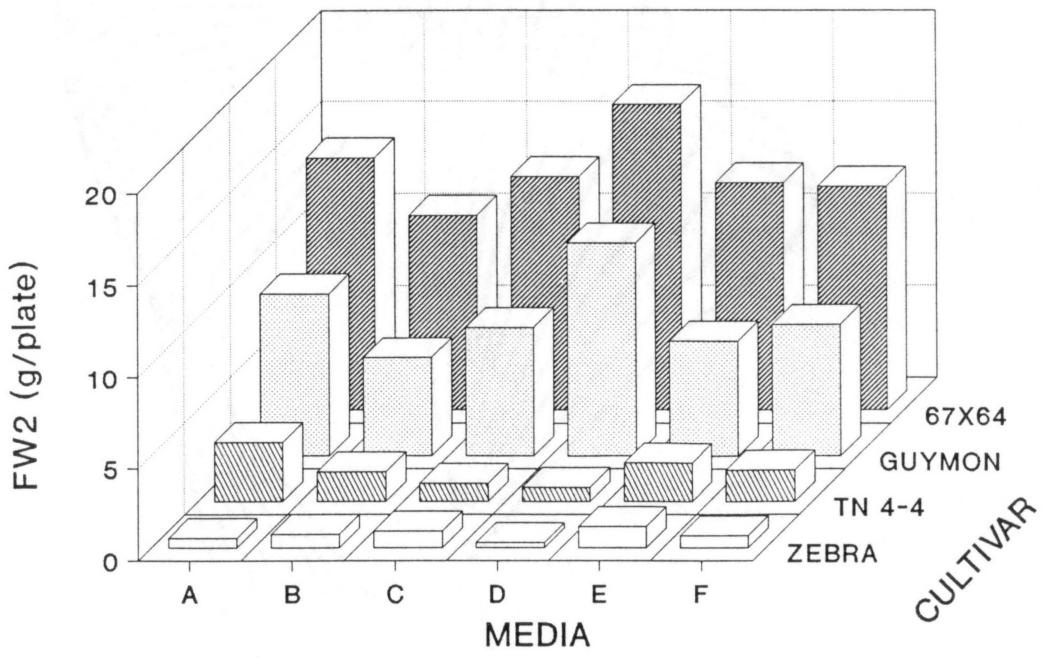

Fig. 4. Mean fresh weight (FW2) of calli per petri dish for the second growth period.

LSD (0.05) = 4.26 and 4.32 for comparisons between media means at each genotype and genotype mean at each medium, respectively.

A = mMS + 4.5 µM 2,4-D; B = mMS + 13.5 µM 2,4-D; C = mMS + 27 µM 2,4-D; D = SH + 2.25 µM 2,4-D and 5 µM dicamba; E = SH + 20 µM dicamba; and F = SH + 40 µM dicamba.

Table 2. Mean number of regenerated plantlets per petri dish from callus cultures after 4 to 6 wk on regeneration media.

	Medium Type†		
Cultivar	D	E	F
	- - - - - No. Reg. Plt./Plate - - - - -		
67X64	0	0	0
Guymon	4 a*	7 a	13 b
Zebra	6 a	7 a	18 b
TN 4-4	0	0	0

* Means in row followed by the same letter are not significantly different at the 0.05 percent level. LSD (0.05) = 3.2 and 3.3 in rows and columns, respectively.

† D, E, and F are media in which callus was cultured before being transferred to regeneration media.

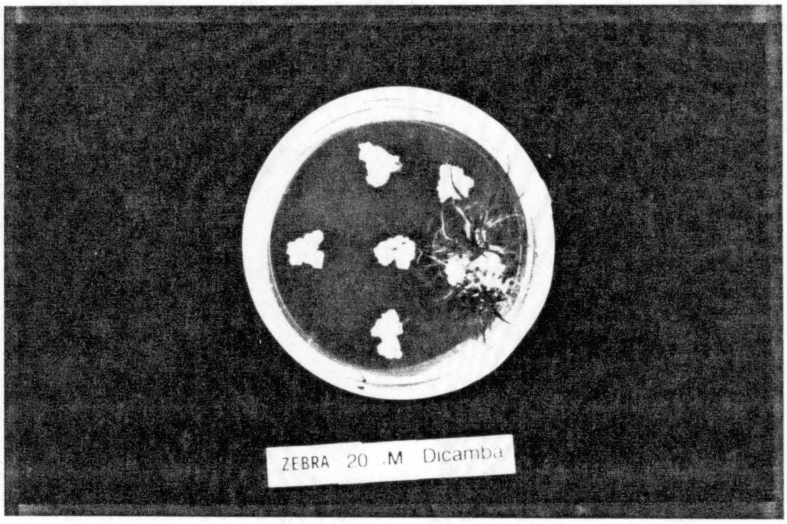

Fig. 5. Plantlet regeneration after transfer to SH regeneration medium.

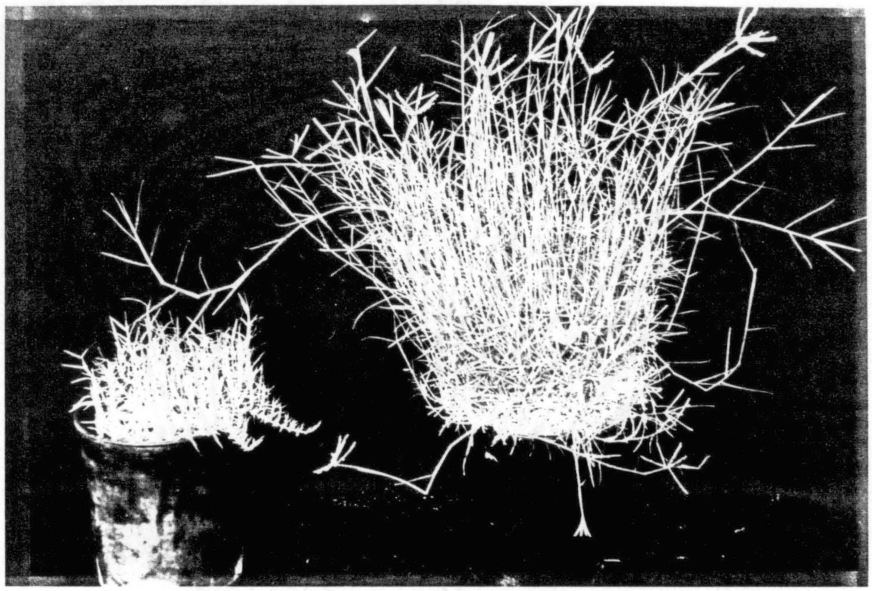

Fig. 6. Phenotypic variation (differences in height) observed in regenerated plants of Zebra bermudagrass. Normal plt. (right) vs. dwarfed plt. (left).

CHAPTER III

EFFECTS OF BASAL MEDIA AND AUXINS ON EMBRYOGENIC CALLUS INDUCTION AND REGENERATION FROM THREE BERMUDAGRASS ACCESSIONS

Effects of Basal Media and Auxins on Embryogenic Callus Induction and Regeneration from Three Bermudagrass Accessions

ABSTRACT

Bermudagrass, Cynodon dactylon (L.) Pers., callus cultures frequently consist only of fast-growing non-embryogenic tissue with no regeneration potential. In order to identify materials or procedures that enhance regenerability, a factorial experiment was conducted evaluating three accessions (A-9959, SS-18, and SS-19), two media [modified Murashige-Skoog (mMS) and N6], and two auxin types [2,4-D (2,4-dichlorophenoxyacetic acid) and dicamba (3,6-dichloro-o-anisic acid)] supplied at three concentrations (5, 15, and 30 μM). Explants were germinating caryopses. Response variables were callus fresh weight (FW) and estimated percent embryogenic callus (EC) after 7 wk dark culture. Callus relative growth rate (RGR) was determined during the ensuing 6 wk from the 6 best calli masses selected at the end of the first 7 wk period. The mMS medium produced more FW than N6 ($P<0.01$), but percentage EC was not different for the two media. Both FW and EC were affected by accession, type and concentration of auxin, and 1st and 2nd order interactions involving medium, auxin type and concentration. Greatest EC production was on mMS medium containing 30 μM dicamba. Somatic embryogenesis was documented using scanning electron microscopy. Plants were regenerated from two of the accessions.

INTRODUCTION

Plant regeneration via somatic embryogenesis from a variety of genotypes and explant sources is desirable in the application of non-conventional techniques to bermudagrass, Cynodon dactylon (L.) Pers., improvement.

Medium composition including type and concentration of auxin, genotype, and explant source are major factors influencing the induction of somatic embryogenesis. The most commonly used auxin for initiation and maintenance of grass callus cultures has been 2,4-D. Dicamba (3,6-dichloro-o-anisic acid), another synthetic growth regulator, has also been successfully used to induce somatic embryoids in grass callus cultures. Using dicamba as an auxin source, McDonnell and Conger (1984) reported callus induction and plantlet formation in Kentucky bluegrass from mature embryo explants while Conger and McDaniel (1983) used callus cultures derived from mature caryopses to screen tall fescue seed samples for an endophyte.

Attempts by Krans (1981) and Ahn et al. (1985) to regenerate plants from cultured mature bermudagrass caryopses were not successful. However, Ahn et al. (1985, 1987) and Artunduaga et al. (1988) successfully regenerated bermudagrass plants using young inflorescence explants cultured on N6 and mMS media, respectively. Both groups used 2,4-D (2,4-dichlorophenoxyacetic acid) as an auxin source.

In a preliminary experiment, we tested treatments that resulted in in vitro bermudagrass plant regeneration via somatic embryogenesis from cultured germinating mature caryopses. However, most of the callus cultures consisted of fast growing non-embryogenic tissue with little or

no regeneration potential. This experiment was conducted to: (1) better define the effects of accession (genotype), medium, and type and concentration of auxin on callus induction, growth, and regeneration potential of callus from bermudagrass caryopses explants and (2) characterize mode of regeneration from this callus.

MATERIAL AND METHODS

The study was conducted as a randomized complete block design with a factorial arrangement of treatments (3 accessions x 2 media x 2 auxins x 3 concentrations). Mature caryopses from field-grown bermudagrass accessions A-9959, SS-18, and SS-19 were surface sterilized as follows: (1) wash for 15 min in 50 ml distilled water containing a few drops of Tween 20, (2) immerse in 70% ethanol for 1 min, (3) wash in a 20% (v/v) solution of commercial Clorox containing 2-3 drops of Tween 20 on a magnetic stirrer for 15 min under vacuum, (4) immerse in a 10% (v/v) daconil fungicide solution for 5 min, and (5) rinse in sterile distilled water 4 to 5 times prior to aseptic germination in 0.2% KNO_3 solution in Erlenmeyer flasks placed on a gyratory shaker for 48 to 72 h.

Modified Murashige and Skoog (mMS) and N6 media were used (Table 1). The mMS medium included the macro and micro-salts of Murashige and Skoog (1962), B-5 vitamins (Gamborg et al., 1968), 3% (w/v) sucrose, and 2,4-D or dicamba at 5, 15 or 30 μM concentration. The N6 medium (Chu et al., 1975) was supplemented with 3% (w/v) sucrose and the same auxin treatments. All media were adjusted to pH 5.8 with 1N NaOH. Agar (0.7% w/v) was added prior to sterilization by autoclaving at 121 °C and 1.1 kg cm^{-2} for 15 min. Dicamba was filter-sterilized through a 0.2 μm filter and added to the autoclaved medium. Ten sterile germinating caryopses of each accession were cultured on each of the twelve media/auxin treatment combinations in 100 X 15 mm polystyrene petri dishes. Petri dishes were wrapped with Parafilm strips. All treatment combinations were initially replicated five times, but one replicate of SS-18 was lost to contamination.

Table 1. Composition of basal media used to culture bermudagrass mature caryopses.

Constituents	Media	
	mMS	N6
Macro-minerals (mg L^{-1})		
NH_4NO_3	1650	-
KNO_3	1900	2830
$MgSO_4.7H_2O$	370	185
KH_2PO_4	170	400
$(NH_4)_2SO_4$	-	463
$CaCl_2.2H_2O$	440	166
$FeSO._4H_2O$	27.8	27.8
Na_2EDTA	37.3	37.3
Micro-minerals (mg L^{-1})		
$MnSO_4.H_2O$	16.9	4.4
$ZnSO_4.7H_2O$	8.6	1.5
H_3BO_3	6.2	1.6
KI	0.83	0.8
$Na_2MoO_4.2H_2O$	0.25	-
$CuSO_4.5H_2O$	0.025	-
$CoCl_2.6H_2O$	0.025	-
Vitamins + Other organics (mg L^{-1})		
Thiamine-HCl	10.0	1.0
Pyridoxine-HCl	1.0	0.5
Nicotinic acid	1.0	0.5
Myo-inositol	100.0	-
Glycine	-	2.0

EDTA-ethylenediaminetetraacetic acid.

After incubation in the dark at 27 ± 1 °C for 7 wk, callus fresh weight (FW) per petri dish was recorded and percentage of embryogenic callus (EC) was visually estimated. One half of the tissue from each of the six best calli in each petri dish was subcultured onto fresh maintenance medium identical to the original. The remaining half of the tissue from the six best calli masses was subcultured onto two shoot induction media containing the components of mMS or N6, except the auxin, which was replaced by 6.8 μM zeatin. Calli on maintenance media were cultured for an additional 6 wk after which the relative growth rate (RGR) was determined (Singer and McDaniel, 1986). At the end of this period, pieces of callus from selected treatments were put on shoot induction media as previously described. All cultures after the first 7 wk period were maintained in a growth chamber under 16 h photoperiod provided by cool white fluorescent tubes (about 42 μmol s^{-1} m^{-2}) at 27 ± 1 °C. To promote root growth, plantlets were transferred to Magenta GA-7 culture vessels (Magenta Corp., Chicago, IL) with 50 ml of half-strength mMS without growth hormones.

Response data were analyzed using the GLM procedure of SAS (SAS, 1982). Means were compared by Fisher's protected least significant difference (LSD) test.

For scanning electron microscopy, callus samples were fixed in 1.6% glutaraldehyde dissolved in 0.12 M sodium cacodylate at pH 7.4 for 2 h, washed three times in 0.1 M sodium cacodylate buffer, and postfixed in 1% osmium tetroxide dissolved in 0.1 M sodium cacodylate for 2 h. This was followed by two rinses with 0.1 M sodium cacodylate buffer for 20 min each, and left in the same buffer overnight. The samples were then dehydrated through a graded ethanol series to 100% (each step was

20 min). They were critical-point dried from liquid CO_2, mounted on aluminum stubs with double-sided tape, and then coated with gold-palladium film. Samples were viewed on a JEOL JSM-35 U scanning electron microscope and photographed on Polaroid-type 55 Pos./Neg. film.

RESULTS AND DISCUSSION

All treatments resulted in callus growth from caryopses explants within 2 wk of culture. Growth, morphology and embryogenic capacity of callus differed with various treatments (Fig. 1). Callus morphology was significantly influenced by type and concentration of auxin. High concentrations of 2,4-D and low concentrations of dicamba resulted in the production of white translucent callus. Low 2,4-D and high dicamba concentrations produced callus which, after 4 wk, became either slow-growing, yellowish-white and compact, or fast-growing, dark yellow and friable in appearance. N6 medium containing 5 μM dicamba promoted rhizogenesis but not embryogenesis.

Callus Fresh Weight

Differences in FW were attributable to all main effects and to several interactions including the 3rd order interaction (Table 2, Fig. 2, Appendix A). A-9959 produced more FW than either SS-19 or SS-18 within a particular medium and auxin. The basal media (mMS vs. N6) used for callus initiation and proliferation from mature caryopses greatly influenced FW. The mMS medium supplemented with either auxin was superior to N6 for FW. mMS medium supplemented with 5 μM dicamba produced the greatest FW. This is in contrast to results of Ahn et al. (1985) who found that calli originating from immature inflorescences grew larger on modified N6 than on MS. Thus, different explant sources may require different media. Although accessions differed in FW, their trend in response to other treatment variables was generally similar.

Embryogenic Callus

Significant differences ($P<0.0.1$) in EC were attributable to accession, auxin type, and auxin concentration (Table 2, Fig. 3, Appendix B). Two of the three 2nd order interactions were significant ($P<0.05$ or $P<0.01$), but the 3rd order interaction was not significant ($P>0.05$). The greatest percentage EC occurred in A-9959 and SS-19 cultures subjected to 30 μM dicamba on both mMS and N6 media. Within 2,4-D concentrations, EC was greatest for A-9959 and SS-19 at the 5 and 15 μM concentrations, respectively. SS-18 produced EC only on mMS supplemented with 30 μM dicamba. Auxin type and auxin concentration effects on EC induction were accession dependent. More EC was induced at lower concentrations of 2,4-D and at the highest concentration of dicamba. Although the molecular basis of EC induction is not well understood, Hahne et al. (1988) using different biochemical procedures [silver-staining of proteins, in vivo labeling of proteins with (^{35}S) methionine and in vitro translation of poly $(A)^+$ RNA] identified several proteins that were specific for embryos or callus under various culture conditions. Thus, somatic embryogenesis is under genetic control.

Scanning electron microscope (SEM) examination of the types of callus observed in this study showed that EC was composed of globular and nodular masses (Fig. 5a), while the non-NE was composed of elongated cells (Fig. 5b). The friable EC observed (Fig. 5c, 5d) appeared comparable to that obtained in oat by Bregitzer et al. (1989). Friable EC is considered very good material for establishing embryogenic cell suspension cultures to be used for genetic manipulations.

Relative Growth Rate

Variation in RGR was attributable to medium, auxin type, and auxin concentration (Table 2, Fig. 4, Appendix C). The accession main effect and most interactions with accession were not significant ($p>0.05$) with the exception of accession x auxin and accession x medium x auxin ($P<0.05$). The greatest difference in RGR was due to medium with mMS resulting in almost twice the amount as N6.

Plant Regeneration

Upon transfer to regeneration medium, only calli that were capable of forming somatic embryos germinated and developed plantlets. Within 2-3 wk, embryoids developed purple coleoptiles. An embryo possessing the major organs of a typical zygotic embryo, such as coleoptile and scutellum, is shown in Fig. 6a. Poly-embryos having multiple shoot primordia frequently appeared (Fig. 6b). Plant regeneration was characterized by the development of a single shoot or multiple shoots with varied numbers of roots (Fig. 6c). Variable numbers of roots without shoots frequently formed and ultimately senesced. Numerous plantlets were obtained from accessions A-9959 and SS-19 from most of the treatments producing EC. The SS-18 EC eventually died without embryoid germination and plantlet development.

CONCLUSIONS

Mature bermudagrass caryopses may be used as explants to produce callus with plant regeneration capability. Growth, morphology, and plant regeneration potential of callus from caryopses explants are dependent on main and interaction effects of accession (genotype),

medium type, and type and concentration of auxin. Regeneration potential of callus was most strongly influenced by accession and auxin concentration. Embryogenesis and plant regeneration was optimized using accession A-9959 with mMS medium supplemented with 30 μM dicamba.

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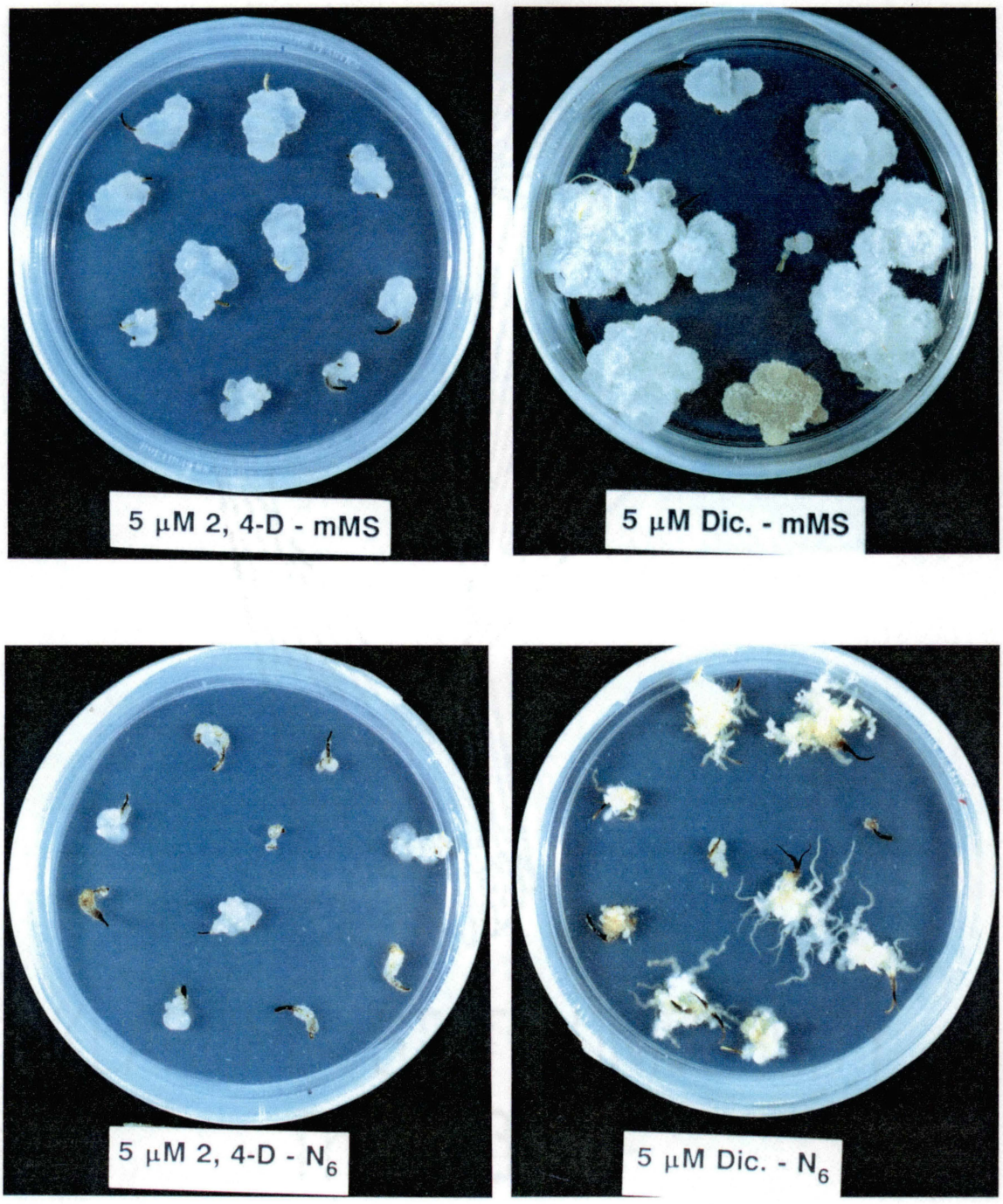

Fig. 1. Effects of media, auxin type and auxin concentrations on callus induction and growth from germinating caryopses of A-9959 bermudagrass. Photographs were taken after 6 wk of culture.

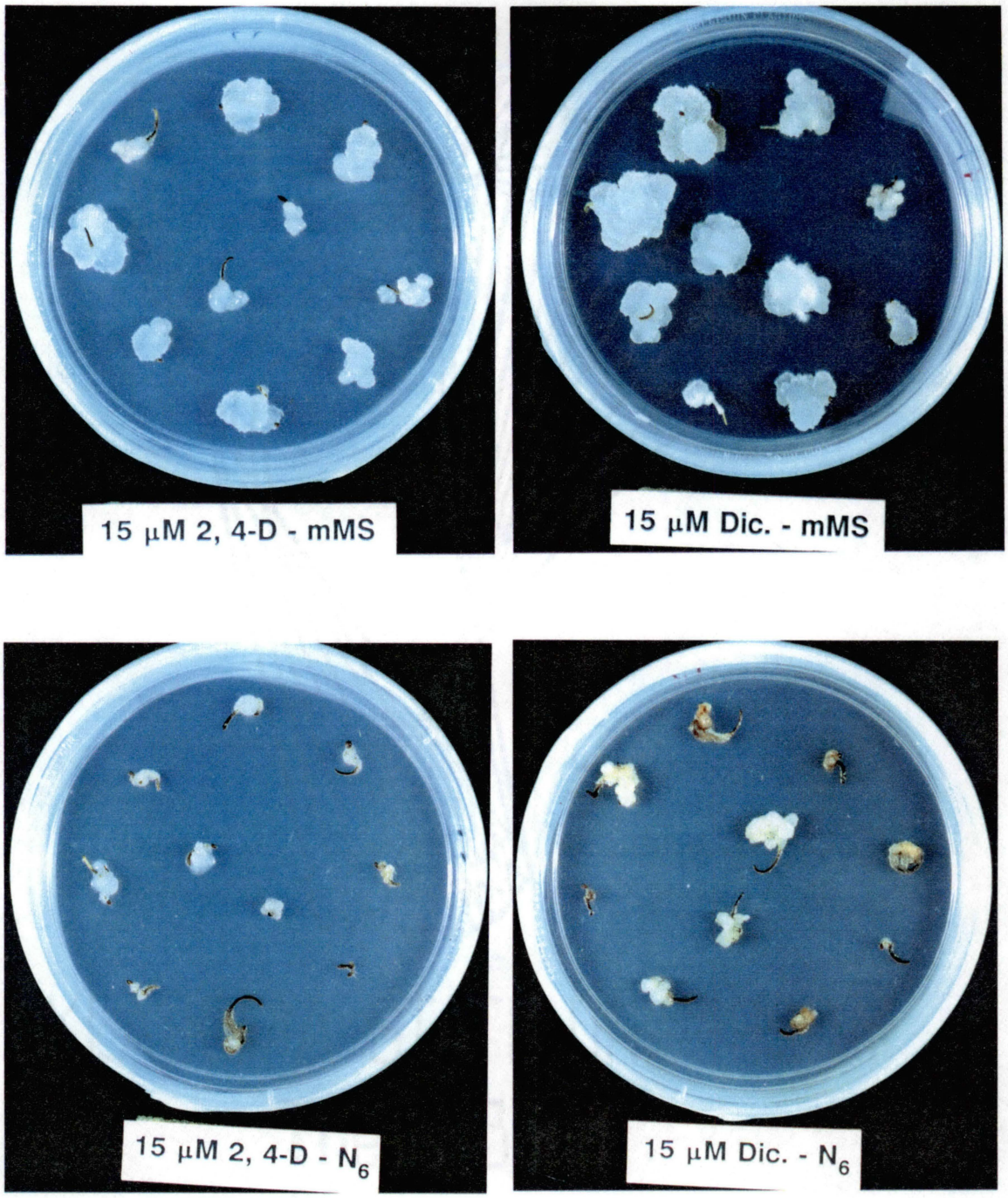

Fig. 1. (Continued).

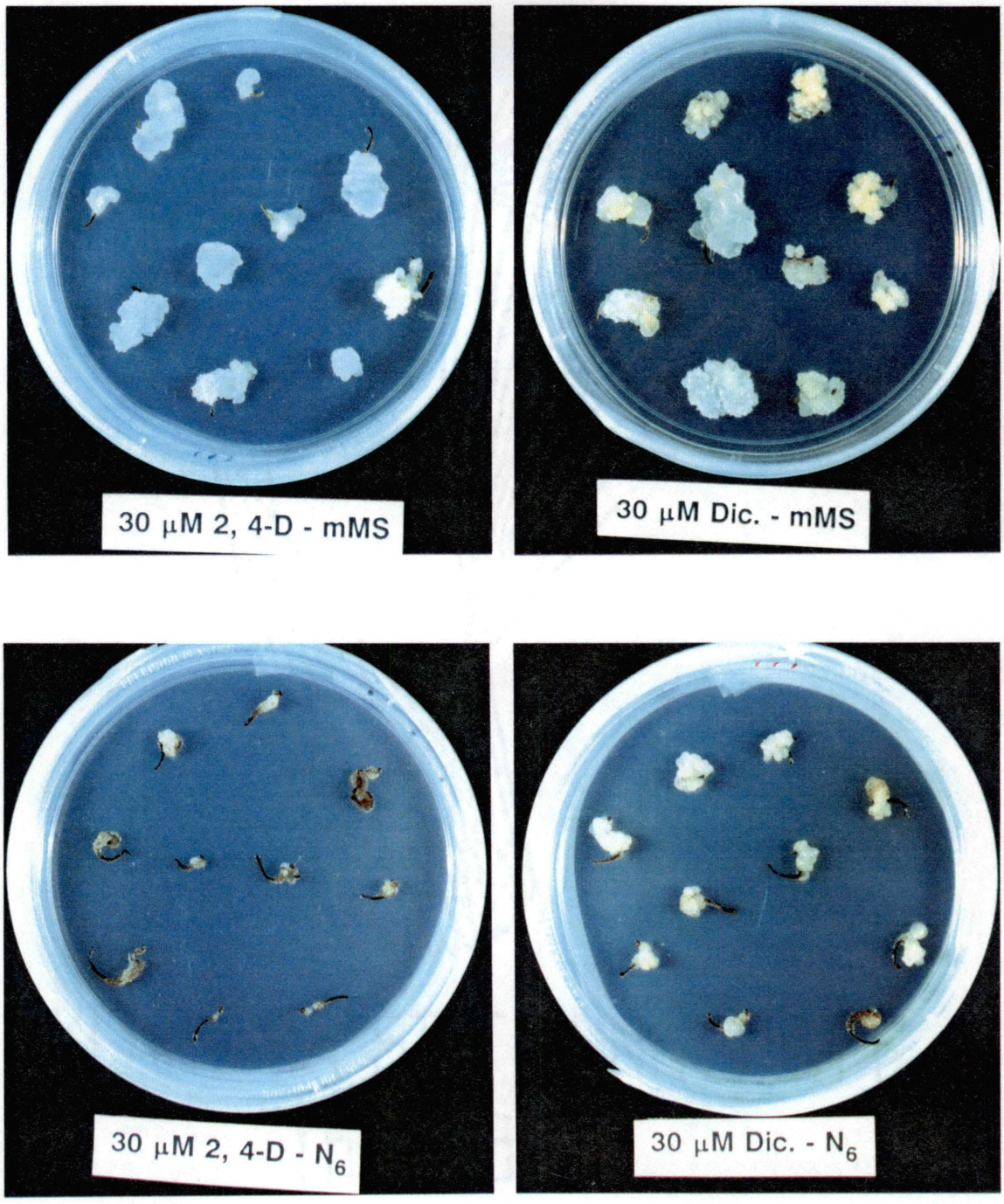

Fig. 1. (Continued).

Table 2. Analyses of variance and mean squares of callus fresh weight (FW), percentage of embryogenic callus (EC) and relative growth rate (RGR) from cultured germinating caryopses of three bermudagrass accessions.

Source	df	FW	EC	RGR
Acc	2	42.226**	5.524**	0.032
Med	1	837.237**	0.631	21.761**
Acc x med	2	22.108**	0.025	0.016
Aux	1	83.052**	3.360**	0.767**
Acc x Aux	2	2.360	0.599*	0.217*
Med x Aux	1	33.484**	1.165*	1.832**
Acc x Med x Aux	2	0.693	0.547	0.250*
Con	2	96.782**	2.036**	0.190*
Acc x Con	4	2.402	0.291	0.053
Med x Con	2	60.251**	0.041	1.470**
Acc x Med x Con	4	3.437*	0.401	0.048
Aux x Con	2	4.080	8.915**	0.418**
Acc x Aux x Con	4	8.683**	1.917**	0.120
Med x Aux x Con	2	1.909	0.794*	0.463**
Acc x Med x Aux x Con	4	8.120**	0.128	0.093
Error	127	1.413	0.200	0.093

*, ** significant at 0.05, 0.01 levels of probability, respectively.

Acc = accession; Med = medium; Aux = auxin; Con = concentration.

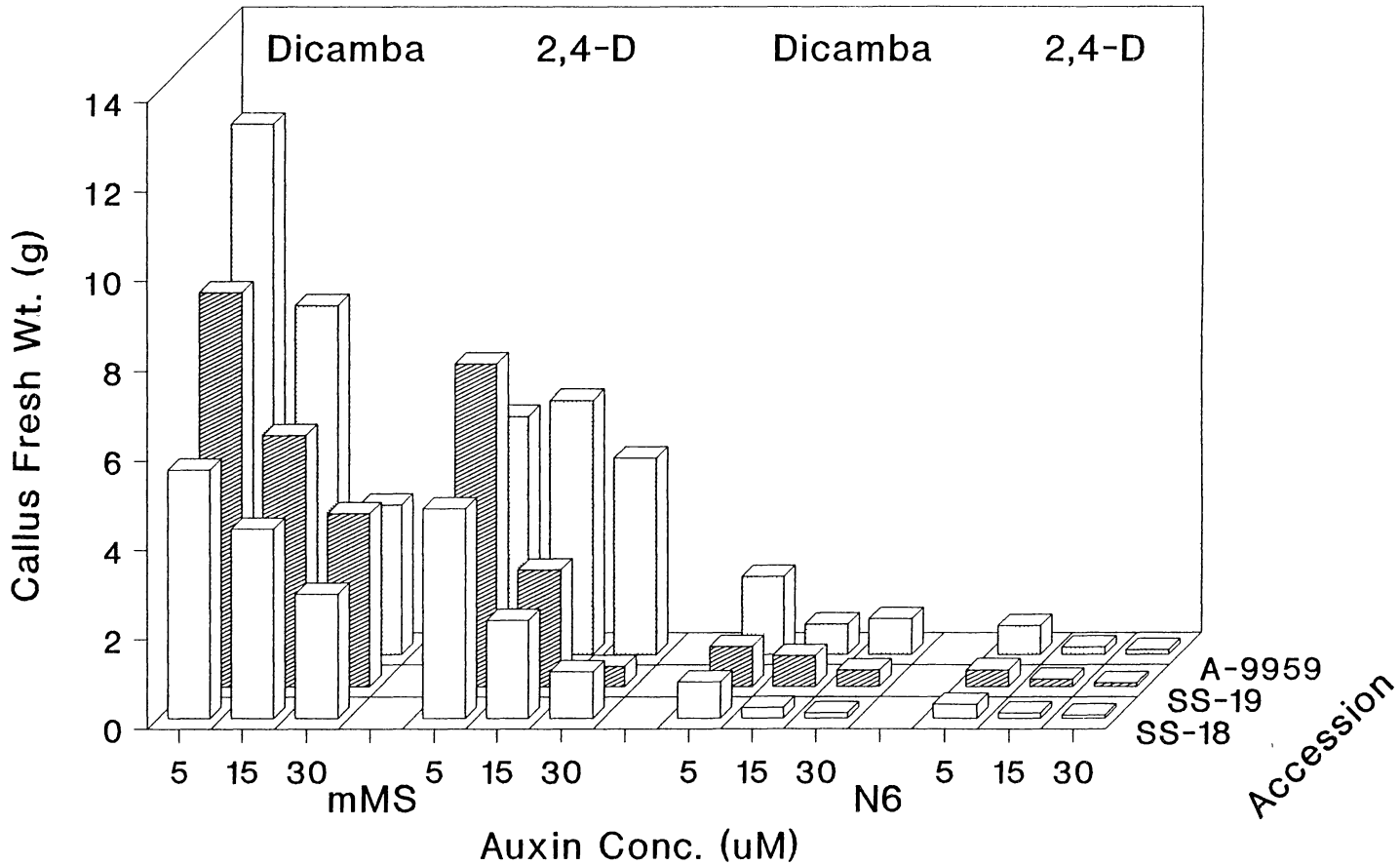

Fig. 2. Effects of media, auxin type and auxin concentrations on callus fresh weight from cultured caryopsis explants of three bermudagrass accessions. LSD (0.05) = 1.6, 1.5 and 1.7 for A-9959, SS-19, and SS-18 respectively.

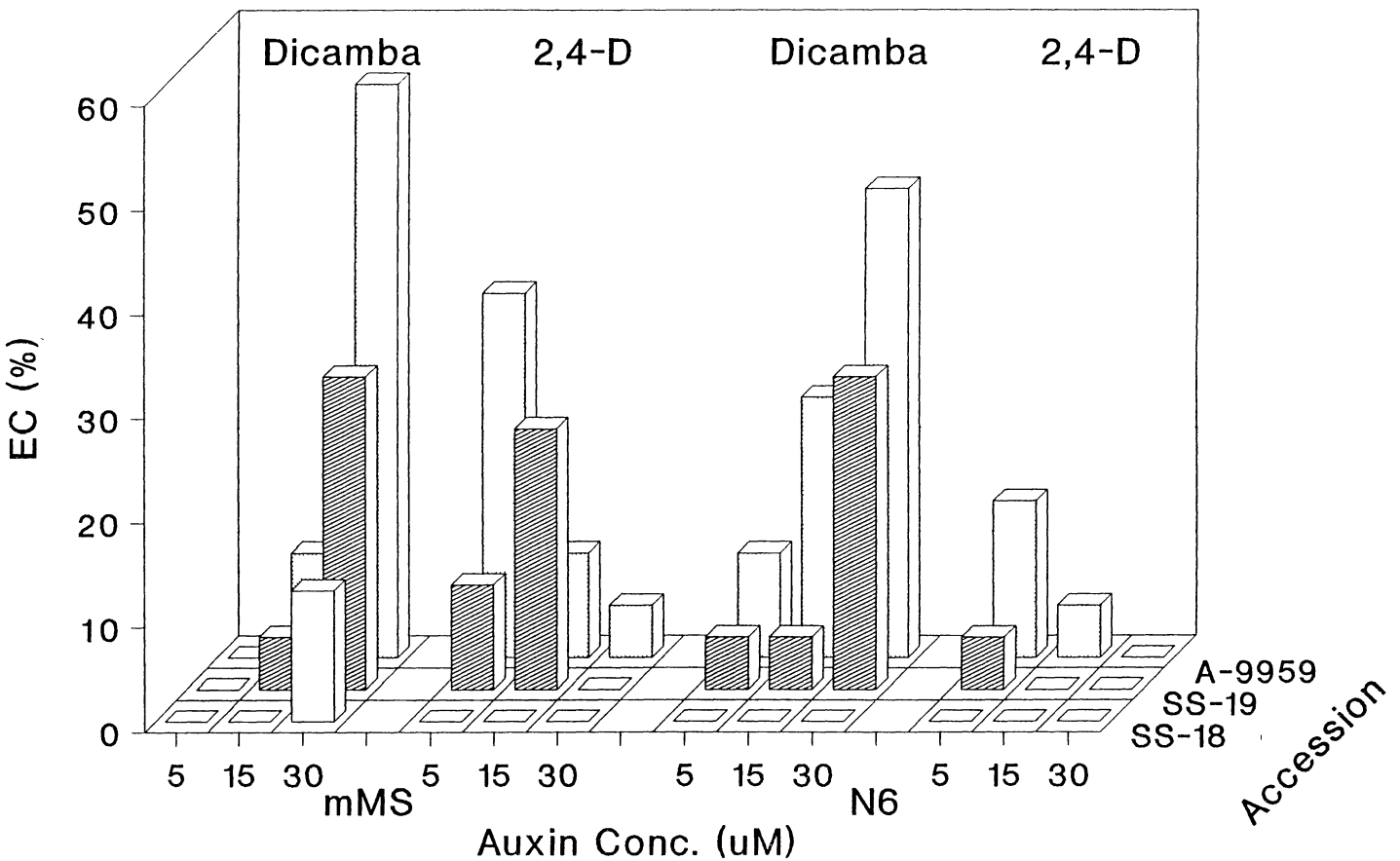

Fig. 3. Effects of media, auxin type and auxin concentrations on percent EC from cultured caryopsis explants of three bermudagrass accessions. LSD (0.05) = 15, 14 and 16 for A-9959, SS-19, and SS-18 respectively.

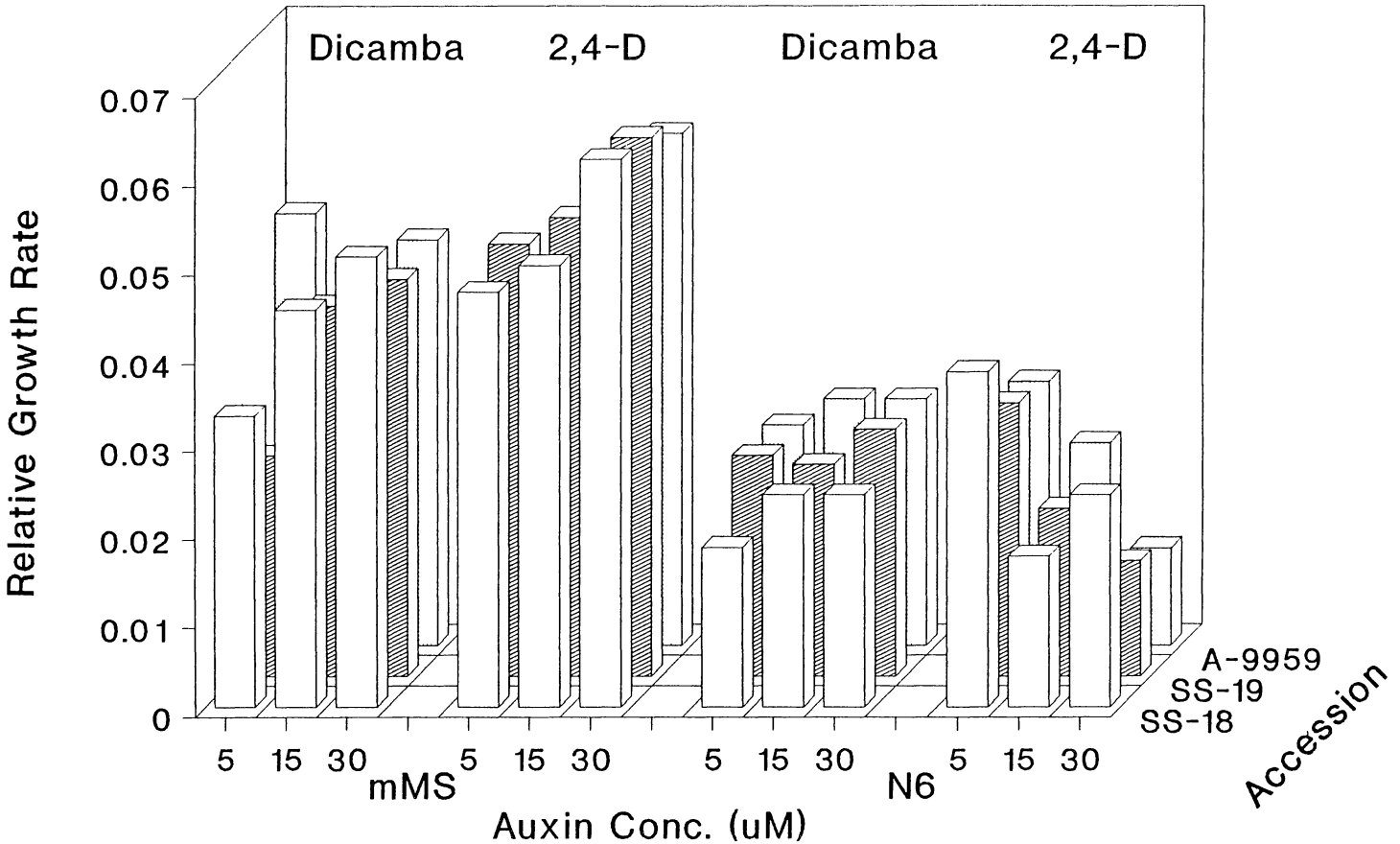

Fig. 4. Effects of media, auxin type and auxin concentrations on callus relative growth rate from cultured caryopsis explants of three bermudagrass accessions. LSD (0.05) = 0.01, 0.009 and 0.011 for A-9959, SS-19, and SS-18 respectively.

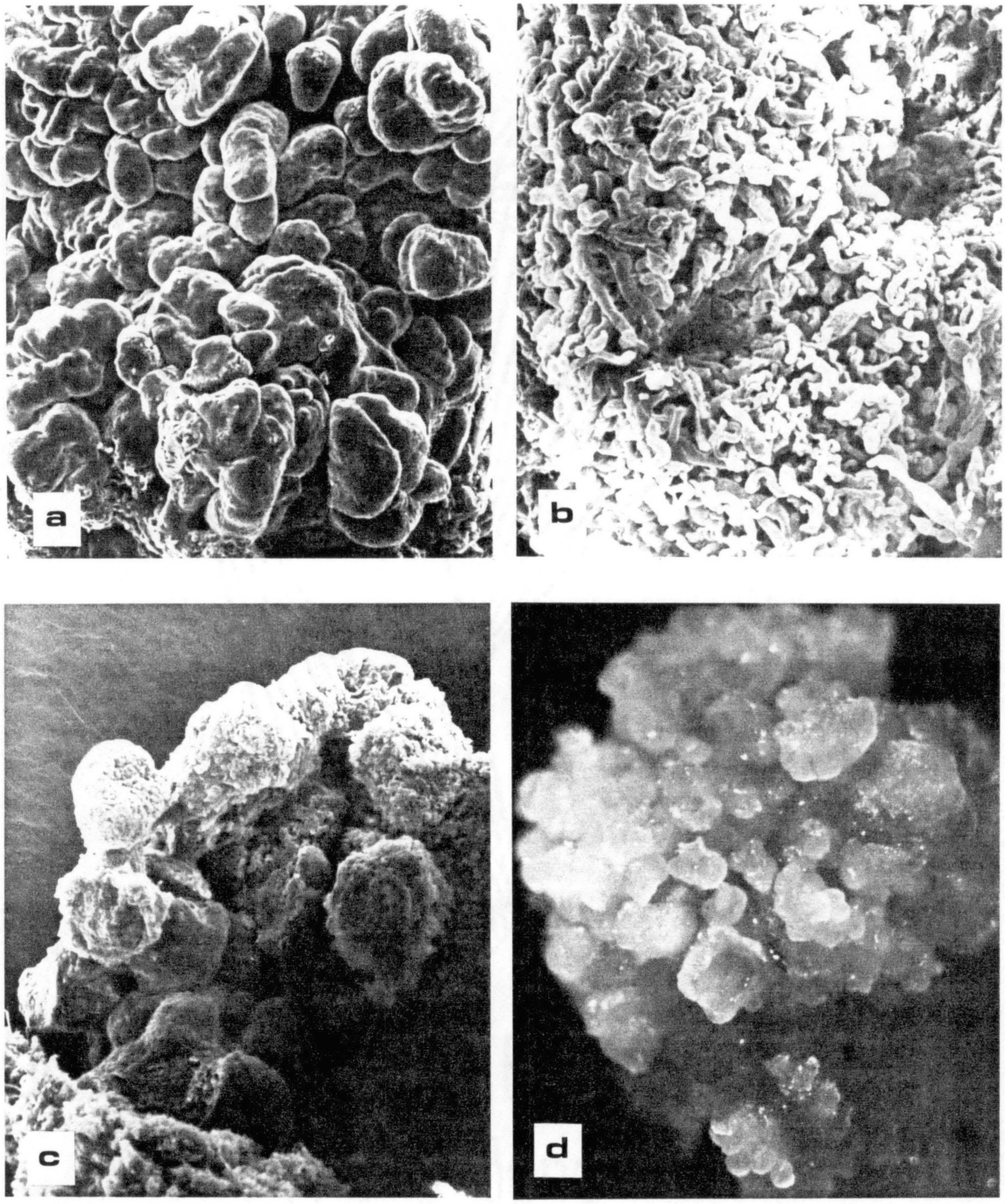

Fig. 5. Scanning electron micrographs of A-9959 bermudagrass callus cultures showing: (a) EC, (b) non-EC, (c) friable EC; (d) friable EC sectors on non-EC. X 30 for a, b, and c.

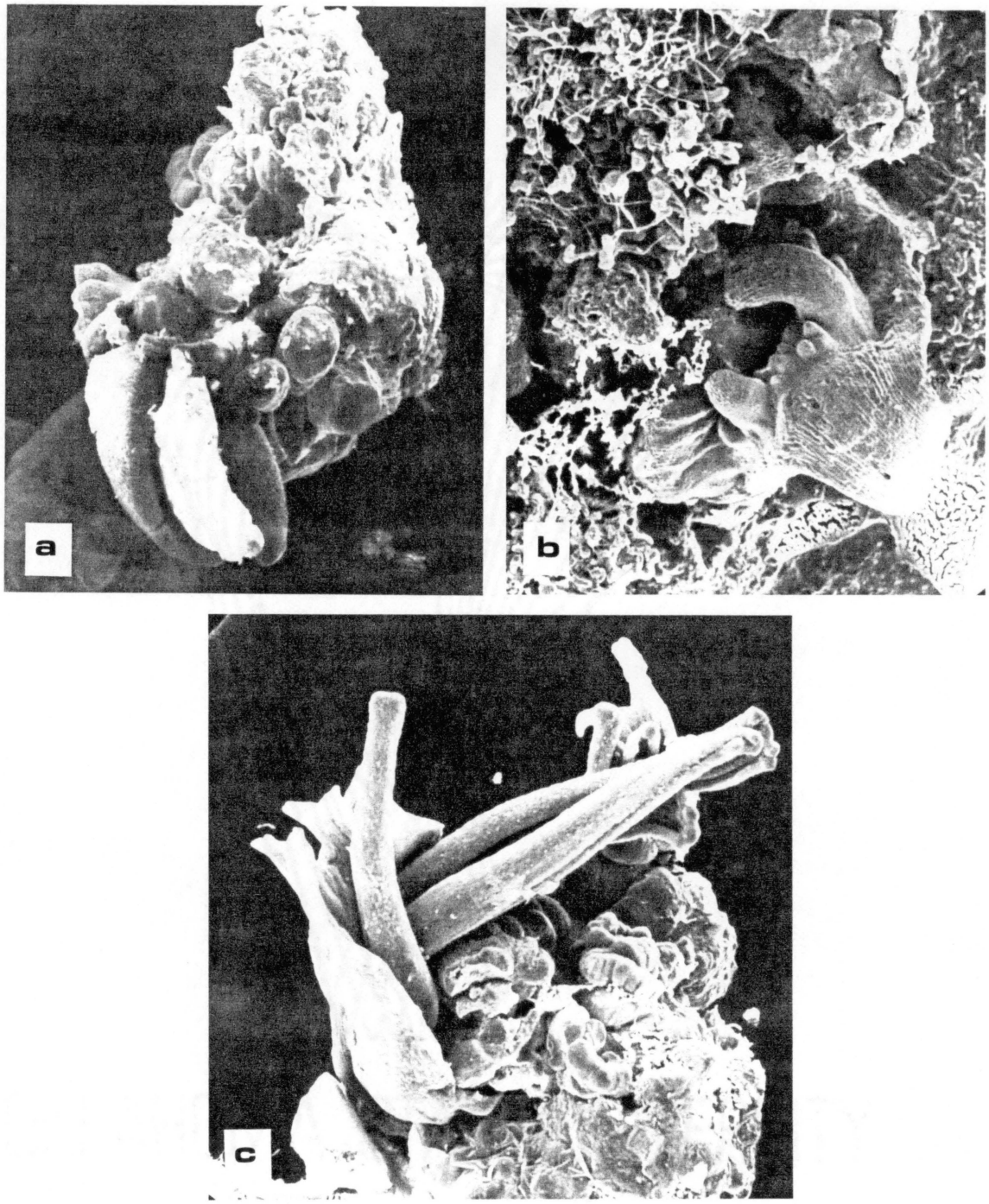

Fig. 6. Scanning electron micrographs of A-9959 bermudagrass callus cultures showing: (a) somatic embryoid starting germination, (b) fused embryo with multiple coleoptiles, and (c) multiple shoots emerging from embryoids. X 30 for a and b, X 20 for c.

CHAPTER IV

USE OF CALLUS CULTURES TO SELECT FOR IMAZAPYR RESISTANCE IN OLD WORLD BLUESTEM (*BOTHRIOCLOA* SP.) GRASS

Use of Callus Cultures to Select for Imazapyr Resistance in Old World Bluestem (Bothriochloa sp.) Grass

ABSTRACT

Herbicide resistance induced via tissue culture selection strategies is of interest and potential importance in many crops. Callus cultures of two tissue culture responsive Old World bluestem (OWB) (Bothriochloa sp.) accessions, A-8953 and A-8918c, were initiated from mature caryopses on a modified Murashige-Skoog (mMS) medium and used in screening for resistance to imazapyr (ARSENAL) herbicide. Imazapyr is a broad spectrum herbicide that inhibits plant growth by interfering with the biosynthesis of valine, isoleucine, and leucine. Dose-response curves were established by growing A-8953 callus masses on mMS media containing 0, 0.01, 0.05, 0.1, 1, 10, or 100 μM imazapyr for 10 wk. During the 10 wk period, calli were transferred to fresh media every 2 wk. After 65 days, calli relative growth rates, relative to the control, were inhibited by 53 and 33 percent at 1 and 0.1 μM imazapyr concentrations, respectively. Step-wise in vitro selection was initiated with embryogenic callus of both accessions placed on media containing 0, 0.1, or 1 μM imazapyr. Callus was subjected over time to progressively higher concentrations, up to 8 μM. Mean callus relative growth rate and the number of tolerant calli decreased with increasing imazapyr concentration. Some tolerant calli persisted at all concentrations. Isoleucine concentrations in unselected (check) and

tolerant callus were not different ($P>0.05$), except in one case, and were higher than those of sensitive callus. Leucine and valine concentrations of tolerant callus were intermediate to those of unselected and sensitive calli. Regenerated plants are now being evaluated for imazapyr resistance.

INTRODUCTION

In vitro selection for herbicide resistant cell lines and plants has been practiced using a number of different species and classes of herbicides. Herbicides and plant species subjected to selection protocol include aminotriazole with tobacco (Singer and McDaniel, 1984), L-phosphinothricin with alfalfa (Donn et al., 1984), glyphosate with carrot, tobacco, and petunia (Nafziger et al., 1984; Singer and McDaniel, 1985; Steinrucken et al., 1986), sulfonylureas with tobacco (Chaleff and Ray, 1984) and canola (Swanson et al., 1988), and imidazolinones with maize (Shaner and Anderson, 1985; Anderson and Georgeson, 1989), canola (Swanson et al., 1989) and wheat (Heering et al., 1992).

Imazapyr (AC 243,997), 2-[4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl]nicotinic acid, is a new herbicide of the chemical class imidazolinone developed by the American Cyanamid Company under the trade name ARSENAL. It is highly potent to plants at low application rates and is environmentally safe with low mammalian toxicity. It has broad spectrum activity, discriminating poorly between weeds and crops. Imazapyr inhibits plant growth by interfering with the enzyme acetohydroxyacid synthase (AHAS) which is required for the synthesis of the branched chain amino acids valine, leucine, and isoleucine (Shaner et al., 1984).

Applying selection pressure to cultured plant tissues generally reduces regeneration potential. Gonzales and Widholm (1985), indicated that a dose-response curve should be developed to aid in determining appropriate levels of stress inducing agents. Each plant cell line and

species may have a unique inhibitory level to a selecting agent.

Old World bluestems (OWB), Bothriochloa sp. are warm-season, perennial grasses used in the southern United States for forage and soil stabilization. Presently, there is no labelled preemergent herbicide available to aid in control of weeds during establishment of OWB stands. OWB seedlings are sensitive to present herbicides, prohibiting their use during the establishment stage. Tissue culture may provide an effective means of selecting for herbicide resistant cell lines and recovering resistant plants from OWB. The objectives of this study were: 1) to establish dose-response curves for imazapyr herbicide for Old World bluestem callus cultures, 2) to initiate a step-wise selection to recover resistance and 3) to characterize the selected calli with regard to their free pool amino acids.

MATERIAL AND METHODS

Initiation and maintenance of embryogenic callus

Mature caryopses of OWB accessions A-8953 and A-8918c were surface sterilized by: (1) pre-washing for 15 min in 50 ml distilled water containing Tween 20 surfactant, (2) 1 min immersion in 70% (v/v) ethanol, (3) 15 min washing in 20% (v/v) Clorox under vacuum and with agitation, (4) 5 min washing in a 10% (v/v) daconil fungicide solution, and (5) rinsing five times with autoclaved distilled water. The sterilized seeds were germinated aseptically in a 0.2% KNO_3 solution in Erlenmeyer flasks placed on a rotary shaker (120 rpm) for 48 to 72 h. Ten germinating caryopses were plated in 15 x 100 mm petri dishes (at least 10 replicates) containing modified Murashige-Skoog (mMS) medium previously successfully used with OWB (Johnson and Worthington, 1987). This medium contained Murashige-Skoog major and minor salts (Murashige and Skoog, 1962), B-5 vitamins (Gamborg et al., 1968), 6 % (w/v) sucrose, and 3 mg L^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D). The pH was adjusted to 5.8 with 1N NaOH and 0.7% (w/v) agar (Sigma Chemical Co., St. Louis, MO) was added, prior to sterilization by autoclaving at 121 °C and 1.05 kg cm^{-2} for 15 min. Calli were subcultured 5 wk after initiation and maintained on the same medium for 14 additional wk, being transferred to fresh medium at 4 to 5 wk intervals. Calli were maintained throughout the culture period in a growth chamber at 28 ± 1 °C with a 16 h photoperiod providing a light intensity of about 42 μmol m^{-2} s^{-1}.

Establishment of dose-response curves

Vigorous, mostly embryogenic, callus was used in establishing the

dose-response curves. Calli were exposed to mMS medium prepared as described above and supplemented with imazapyr at 0, 0.01, 0.05, 0.1, 1.0, 10.0 or 100.0 μM concentrations. Imazapyr was filter sterilized (0.2 μm pore opening, Gelman Sci. Ann Arbor, MI) and added aseptically to autoclaved media. For each treatment, four calli per petri dish were used, each being approximately 0.3 g fresh wt. The experimental design was a randomized complete block with seven replicates. Petri dishes were dark incubated in a growth chamber at 27 ± 1 °C. Calli were subcultured every 2 wk on fresh media with the same concentration of imazapyr for five transfers. Callus tissue in each petri dish was weighed under sterile conditions at the end of the each culture period and data were logarithmically transformed. Relative growth rate was calculated as: (ln final weight - ln initial weight)/ transfer days (Singer and McDaniel, 1986). Data were analyzed using the GLM Procedure of SAS (SAS, 1982). Means were compared by the LSD test.

To quantify cell survival in the callus exposed to the above herbicide concentrations, cell viability was tested after the fifth transfer following a modified fluorescein diacetate (FDA) technique (Widholm, 1972). Calli (5 mg fresh wt.) were macerated and gently suspended in 1 ml distilled water. Ten μl of FDA solution (10 mg of FDA diluted in 1 ml acetone solution) were mixed with 500 μl suspended cells, and then two drops of cell suspension were placed on a microscope slide and observed under fluorescent lighting with an OLYMPUS microscope utilizing an Osram Mercury Short Arc Lamp with a dichroic mirror (DM 580), exciter filter (BP 545) and barrier filter (0590). Viable cell count data are based on two counts per replicate and four replicates for each treatment.

For plant regeneration, healthy, well-growing calli were transferred to new medium of the same composition except without 2,4-D and with 6.8 μM zeatin. Regenerated plantlets were transferred to pots and acclimated for about 2 wk before being taken to the greenhouse.

Step-wise selection scheme

Pieces of embryogenic callus weighing approximately 0.3 g fresh wt. were put on modified Murashige and Skoog (mMS) media supplemented with 0, 0.1 or 1 μM imazapyr. Four calli per plate and six plates (replicates) per treatment were subcultured every 2 wk on fresh mMS media with progressively higher concentrations of imazapyr until reaching the level of 8 μM, then concentrations were stepped down to 5 μM. Plates were dark incubated in a growth chamber at 27 to 28 °C. Callus tissue in each plate was weighed under sterile conditions at the end of the each culture period and callus weight data were logarithmically transformed. Relative growth rates were calculated as described above. Data were analyzed using the ANOVA Procedures of SAS (SAS, 1982). Based on the relative growth rate, tolerant calli were identified using prediction intervals generated by the equation:

$$P[\bar{Y}-t_0.025\sqrt{s^2\frac{(n+1)}{n}} \leq Y \leq \bar{Y}+t_0.025\sqrt{s^2\frac{(n+1)}{n}}]=0.95$$

where Y = predicted sample mean, $\bar{Y}$ = sample mean, $t_{0.025}$ = tabulated value of Student's t, n = number of observations, and s^2 = sample variance (Steel and Torrie, 1980). At the end of each transfer, some of the well-growing calli were placed onto shoot induction medium. Regenerated plantlets were maintained as described above.

Free amino acid analysis

Imazapyr tolerant and sensitive callus from cultures grown on 4, 5 and 6 μM concentrations were sampled for free amino acid extraction using the method of Bieleski and Turner (1966). Tissue samples from control cultures were also included. Approximately 0.1 g of fresh wt. callus was sampled. After adding 50 000 pmol norleucine (Nor) as an internal standard, tissue was suspended in extraction solvent of methanol, chloroform, and water (12:15:3 by volume) and homogenized for 30 s. Homogenized tissue was agitated for 24 h on a platform reciprocal shaker (110 rpm) at 4 °C. After adding 2.5 ml of chloroform and water (1:1.5, v/v), the mixture was vortexted and centrifuged at 10 000 xg for 20 min. The top methanol-water-phase was removed from each sample, placed in a new test tube, and dried by vacuum centrifugation (Speed-Vac, Savant Instruments). The extracts were hydrolysed using 2N HCl at 100 °C for 2 h and dried again under vacuum. Extracts were redissolved in 400 μl water and methanol (2:1, v/v) and subjected to ultrafiltration with a 10 000 mol. wt. cut-off membrane. One hundred μl of the filtrate was sampled and derivatized with phenylisothiocyanate. The amino acid composition of derivatives was determined using high performance liquid chromatography. Amino acids were separated on reverse phase column and detected at 254 nm (Heinrikson and Meredith, 1984). There were two determinations per replicate and two replicates per treatment combination. Data analyses included analysis of variance and mean separation using the LSD test.

RESULTS AND DISCUSSION

Callus culture and establishment of dose-response curves

Embryogenic callus cultures (creamy-white, compact and nodular) were successfully established in mMS agar medium with 3 mg L^{-1} 2,4-D. Cultures were grown sufficiently to supply the mass of callus needed to establish the dose-response curves and to conduct step-wise selection. In the dose-response study, after 17 days of exposure to the herbicide (transfer period 1) there was no significant inhibition of callus relative growth rate (RGR) at any level of imazapyr except for the 100 μM (Table 1). At the second transfer period, RGR's were significantly reduced at the 0.1 and 100 μM imazapyr concentrations. At transfer periods 3, 4, and 5, RGR was reduced at the 0.01, 0.05, 1, 10, and 100 μM except in the cases of 0.1 and 0.01 μM for transfer periods 3 and 4, respectively. Also, the RGR of callus averaged over all transfer periods was significantly reduced compared to the control. During transfer periods 4 and 5, after 65 and 80 days of exposure to the herbicide, respectively, there was a significant near linear growth inhibition with increasing concentrations of imazapyr. Dose response curves at transfers 1 and 4 are illustrated for A-8953 in Fig. 1.

After 65 days in culture (transfer period 4), the 1 μM imazapyr concentration resulted in a decreased RGR most closely approximating 50% of the control, thus suggesting it to be the optimum rate to use in initiating step-wise selection. New embryogenic callus sectors appeared at 0.01, 0.05, 0.1, and 1 μM imazapyr. While the control continued to grow and maintained a healthy appearance, calli cultured at 10 and 100 μM imazapyr showed arrest of growth and discoloration (Fig. 2). Cell

viability tests and microscopic examination after transfer 5 showed that the proportion of viable cells decreased as the concentration of imazapyr increased. Growth was not completely inhibited, with 15% of cells remaining viable at the 100 μM imazapyr concentration (Fig. 3). Callus RGR (between the 4th and 5th transfers) and cell viability were highly positively correlated (r^2 = 0.83).

All tolerant calli failed to regenerate when transferred to shoot induction medium containing their respective imazapyr concentrations. Upon transfer to regeneration medium without imazapyr, cultures from the 1, 0.1, 0.05 and 0.01 μM imazapyr treatments produced plantlets within 6 wk (Fig. 4 and Table 2). calli from the 10 and 100 μM imazapyr treatments turned brown and died within 2 to 3 weeks after being transferred to imazapyr free regeneration medium.

Selection of imazapyr tolerant calli

Step-wise selection was performed by subjecting calli to progressively higher concentrations of imazapyr. Mean callus RGR through 11 transfer periods to fresh media at different concentrations of imazapyr is shown in Tables 3 and 4 for A-8953 and A-8918c, respectively. As the concentration of imazapyr increased, the RGR decreased. The decrease in the number of tolerant calli indicated that greater selection pressure was applied as the concentration of imazapyr increased. During transfer period 8, accessions differed in the number of calli with RGRs exceeding the upper predicted interval (Figs. 5 and 6). A-8918c had more calli exceeding the upper predicted interval than A-8953. During transfer period 8 the number of calli exceeding the control mean for RGR for the 7 and 8 μM concentrations was reduced

relative to previous transfer periods suggesting that near maximum exposure to imazapyr was attained. Consequently, after transfer period 8, imazapyr concentration was stepped down.

Herbicide tolerance in most cases is not from physiological adaptation, but, from gene mutation. This makes the selection of resistant mutant or variant cells or calli easier by measurement of viability and growth. However, the loss of regeneration capability still remains a problem. Calli or cell aggregates represent a heterogenous cell population and cells could be exposed to herbicide concentration gradients in the callus. In order to eliminate some of these problems and to determine accurately the proportion of viable cells in the culture, cell suspension cultures can be utilized for in vitro selection strategies. However, for most species, including OWB, cell suspension cultures have reduced capacity for plant regeneration when compared to callus cultures.

Free amino acid analysis

Valine, leucine, isoleucine, and total free amino acid concentrations differed significantly ($P<0.01$) among callus lines (Table 5). Valine, leucine and total amino acid concentrations of tolerant cell lines were intermediate to those of the control (highest) and sensitive (lowest) cell lines. The isoleucine concentration of two tolerant cell lines was not different from the control while a 3rd tolerant cell line had an isoleucine concentration intermediate to those of the control and sensitive cell lines. Within tolerant lines differences were found only for leucine and isoleucine. The increased concentrations of valine, leucine, and isoleucine in tolerant calli,

relative to sensitive calli, suggests that in vitro selection was successful in selecting cells with greater capability for synthesizing these amino acids in the presence of imazapyr. Shaner and Anderson (1985) found that imazapyr reduced the level of the two biosynthetically related amino acids, valine and leucine in maize (Zea mays) suspension cultures grown in the presence of 1 μM imazapyr. They also reported that the inhibition effect of imazapyr on growth was prevented by supplementing maize suspension culture with 1 mM of valine, leucine, and isoleucine.

A-8953 plants were regenerated from calli subjected to 0.1, 1 and 6 μM imazapyr concentrations. A8918c plants were regenerated from calli subjected to 4, 5, 6, and 8 μM imazapyr concentrations (Table 6). Plants were regenerated from control cultures of both accessions. All the regenerated plants were phenotypically similar to the parents with the exception of fine textured plants regenerated from a callus line exposed to 5 μM imazapyr. Regenerated plants are currently being evaluated for imazapyr resistance.

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Table 1. Least square means of callus relative growth rates of OWB as affected by imazapyr concentration.

Transfer Period	Imazapyr concentration (μM)						
	0	0.01	0.05	0.1	1	10	100
1	0.039	0.039	0.042	0.042	0.040	0.039	0.031**
2	0.029	0.029	0.029	0.022**	0.027	0.030	0.019**
3	0.026	0.022*	0.022*	0.023	0.018**	0.015**	0.011**
4	0.022	0.018	0.014**	0.015**	0.010**	0.007**	0.005**
5	0.020	0.015**	0.016*	0.015*	0.013**	0.010**	0.007**
Mean	0.027	0.025*	0.025*	0.023**	0.022**	0.020**	0.015**

*,** significantly less than the control (0 μM), Prob. diff., P = 0.05 or 0.01, respectively.

Table 2. Number of plants regenerated from callus cultures exposed to imazapyr concentrations during the dose-response study.

Imazapyr concentration	No. of plants regenerated
-- (μM) --	
1	5
0.1	5
0.05	12
0.01	11

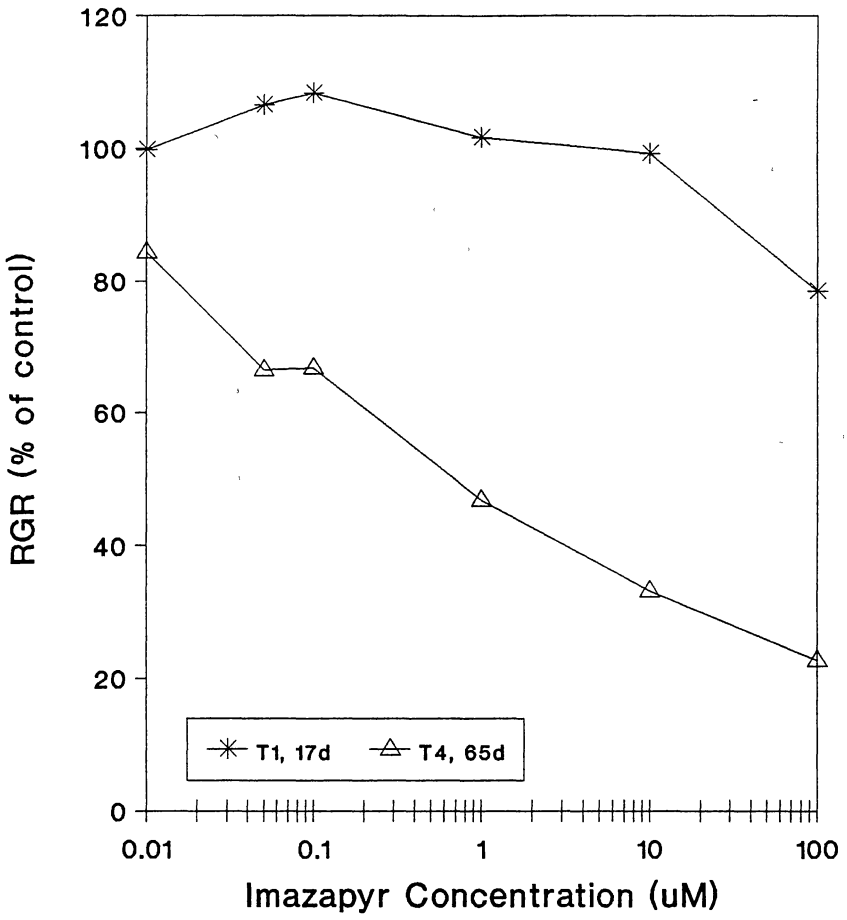

Fig. 1. Relative callus growth rate of OWB (A-8953) for imazapyr tolerance during transfer periods 1 and 4. Each point represents the mean of 7 plates with 4 calli per plate.

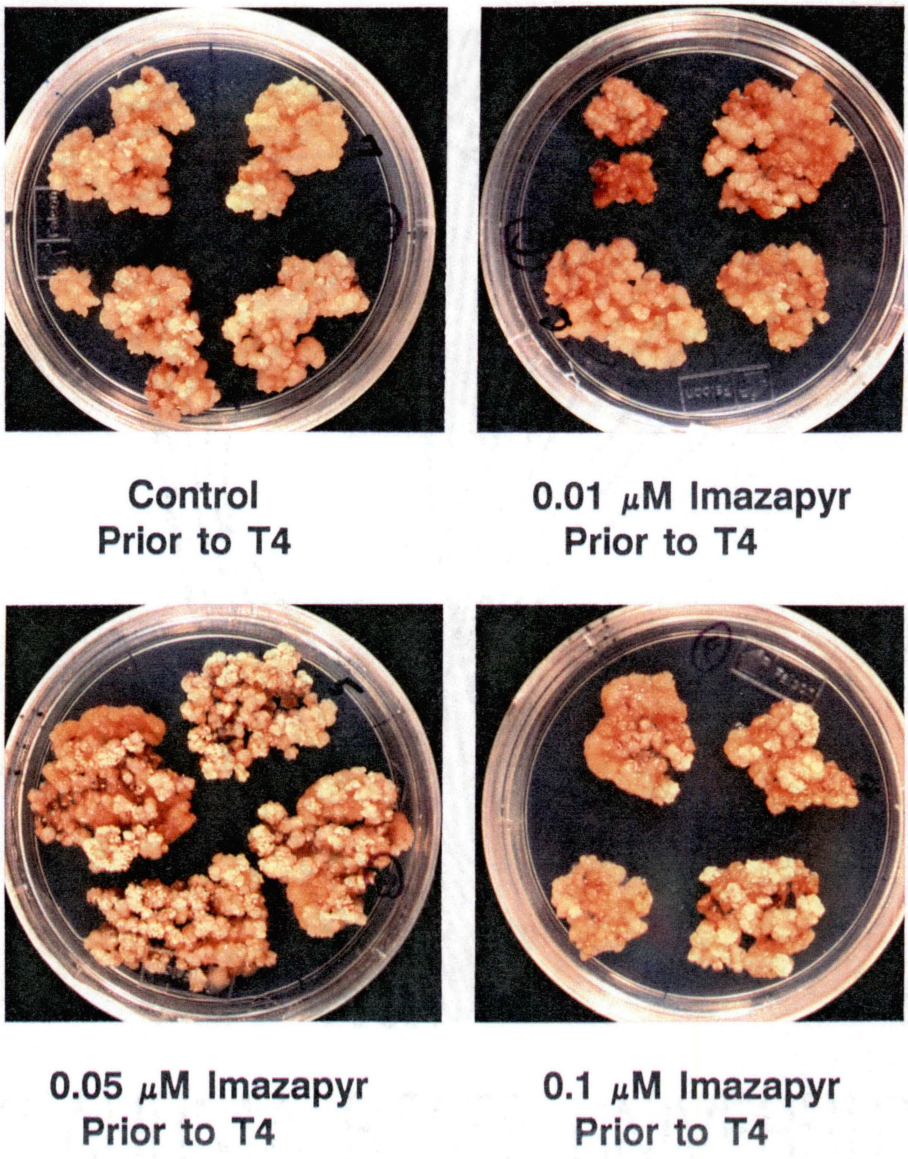

Fig. 2. Callus cultures of accession A-8953 prior to transfer 4.

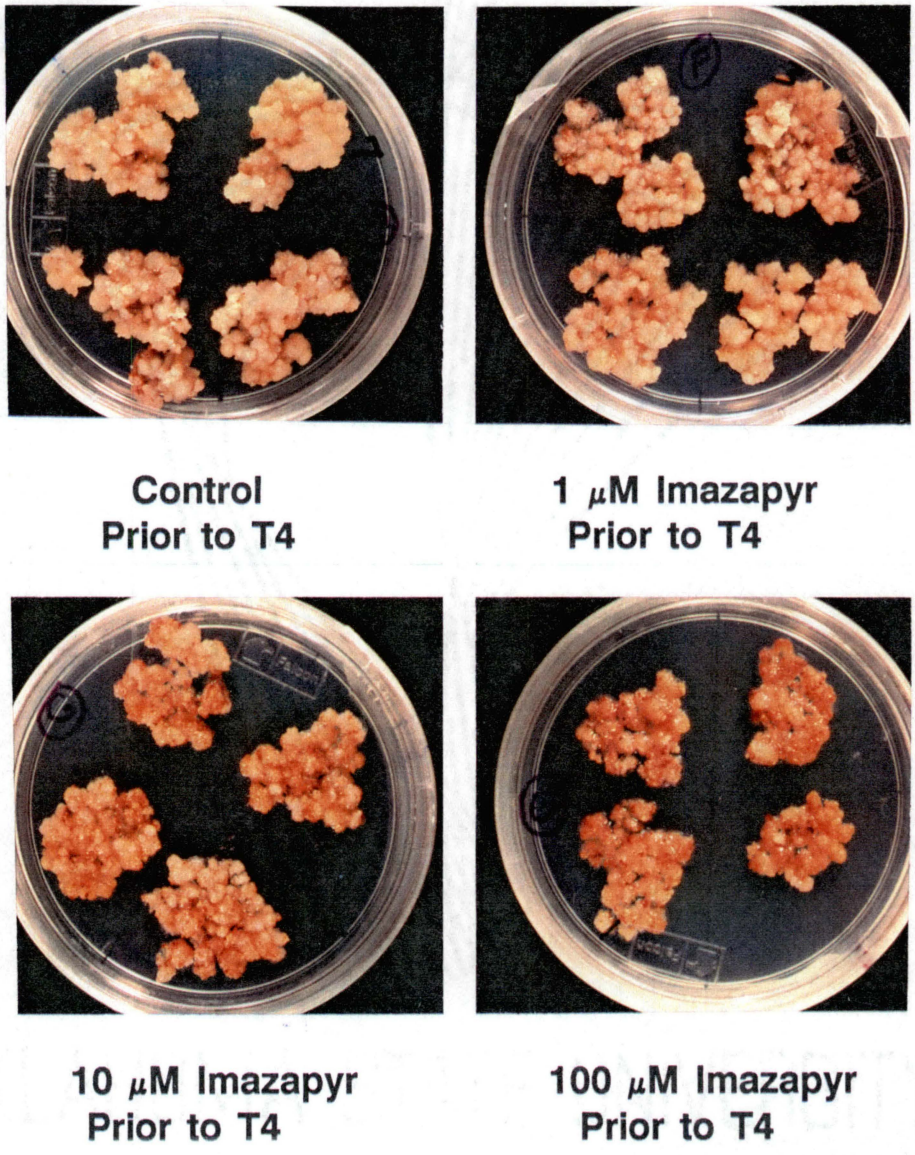

Fig. 2. (Continued).

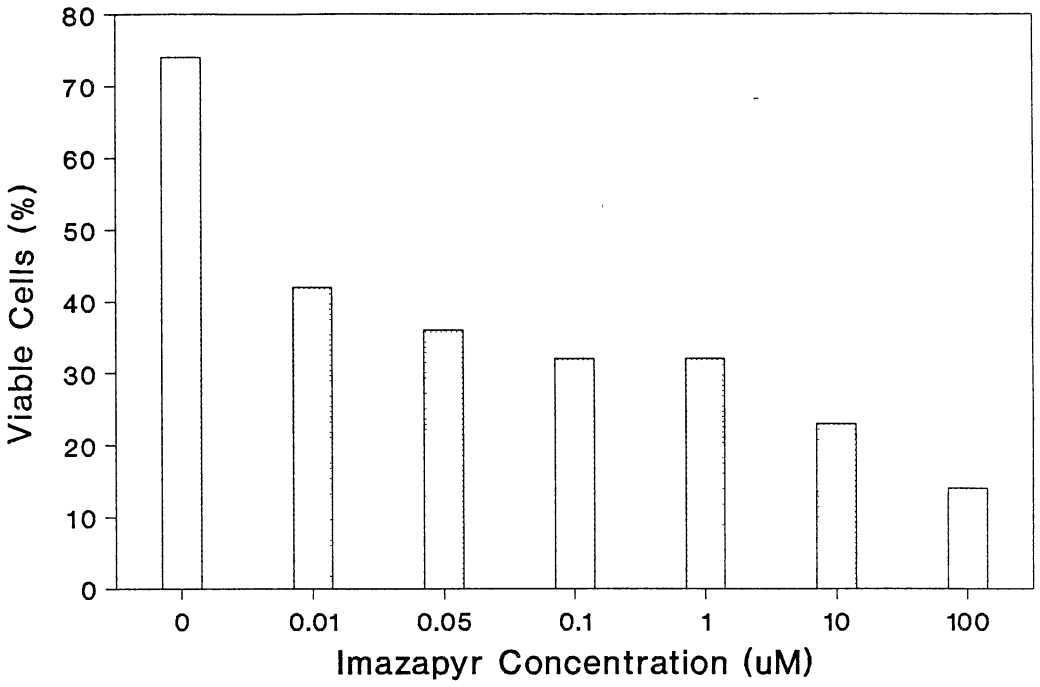

Fig. 3. Imazapyr concentration effects on cell viability of OWB A-8953 callus cultures after transfer period 5 (85 d).

Fig. 4. Plantlets regeneration from selected treatments.

Table 3. Mean callus relative growth rate of OWB accession A-8953 through 11 transfer periods to fresh media with different concentrations of imazapyr.

		Selection Strategy 1		Selection Strategy 2		
Transfer Period	Control RGR[a]	Imaz. Conc.	RGR	Imaz. Conc.	RGR	LSD (0.05)
		(μM)		(μM)		
0	-	0.1	-	1	-	-
1	0.027	1	0.024	2	0.020	0.005
2	0.025	2	0.017	4	0.019	0.007
3	0.020	4	0.016	6	0.008	0.004
4	0.017	6	0.014	6	0.012	0.004
5	0.019	6	0.010	6	0.005	0.003
6	0.016	6	0.011	7	0.009	0.003
7	0.016	7	0.006	8	0.007	0.004
8	0.020	7	0.010	8	0.009	0.006
9	0.014	6	0.005	7	0.008	0.005
10	0.019	6	0.008	7	0.007	0.004
11	0.028	5	0.009	6	0.007	0.005

[a] RGR = Relative growth rate.

Table 4. Mean callus relative growth rate of OWB accession A-8918c through 11 transfer periods to fresh media with different concentrations of imazapyr.

		Selection Strategy 1		Selection Strategy 2		
Transfer Period	Control RGR[a]	Imaz. Conc.	RGR	Imaz. Conc.	RGR	LSD (0.05)
		(μM)		(μM)		
0	-	0.1	-	1	-	-
1	0.041	1	0.040	2	0.040	0.006
2	0.027	2	0.021	4	0.018	0.003
3	0.013	4	0.010	6	0.007	0.002
4	0.008	6	0.006	6	0.005	0.002
5	0.007	6	0.005	6	0.006	0.003
6	0.006	6	0.002	7	0.002	0.004
7	0.011	7	0.007	8	0.008	0.003
8	0.011	7	0.003	8	0.003	0.003
9	0.011	6	0.004	7	0.002	0.003
10	0.015	6	0.004	7	0.006	0.011
11	0.012	5	0.003	6	0.000	0.012

[a] RGR = Relative growth rate.

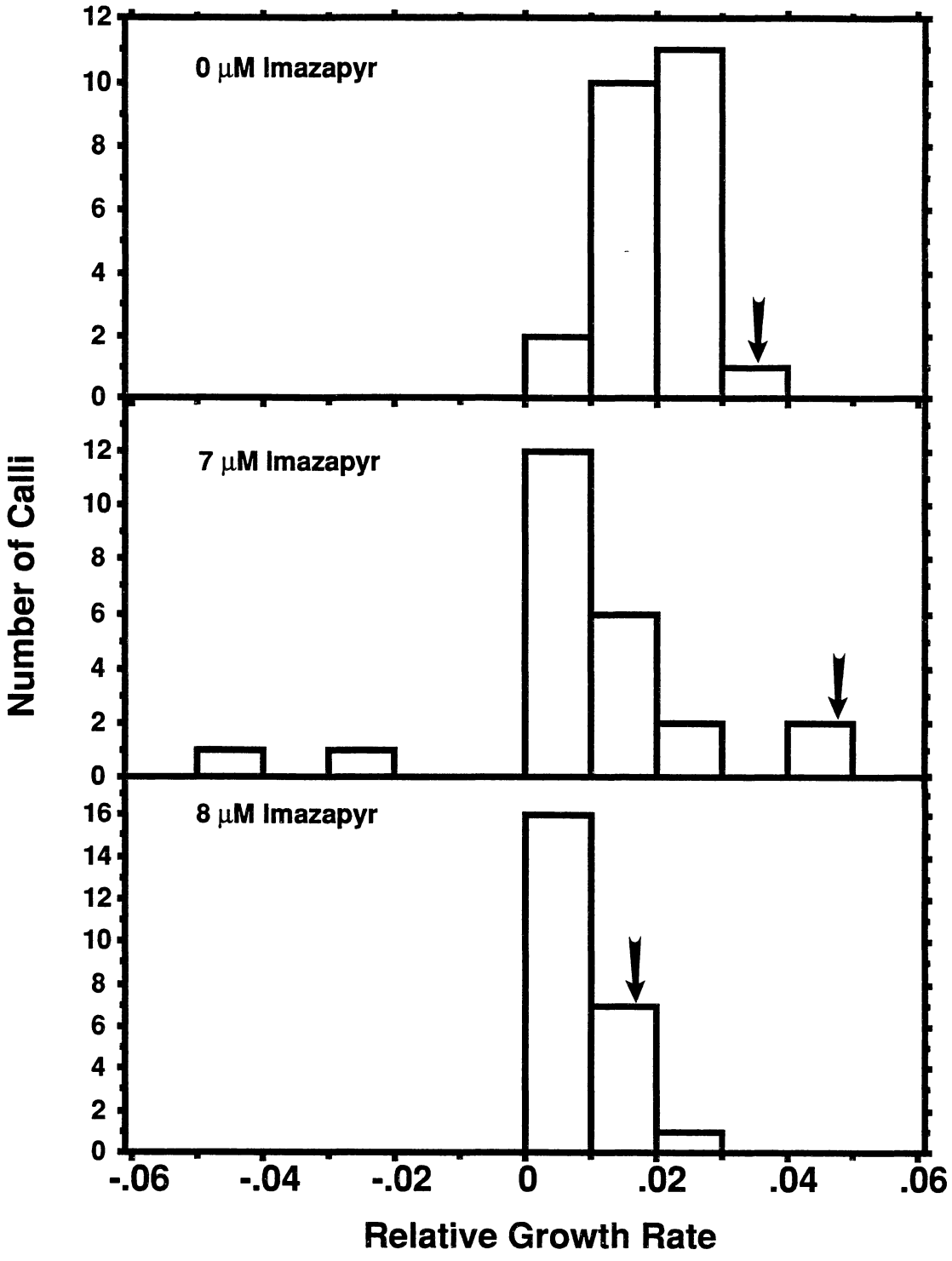

Fig. 5. Histograms of relative growth rates (RGR) during transfer period 8 of A-8953 calli subjected to 0, 7, and 8 μM imazapyr concentrations. Calli with RGR exceeding the upper predicted interval lie to the right of the arrow.

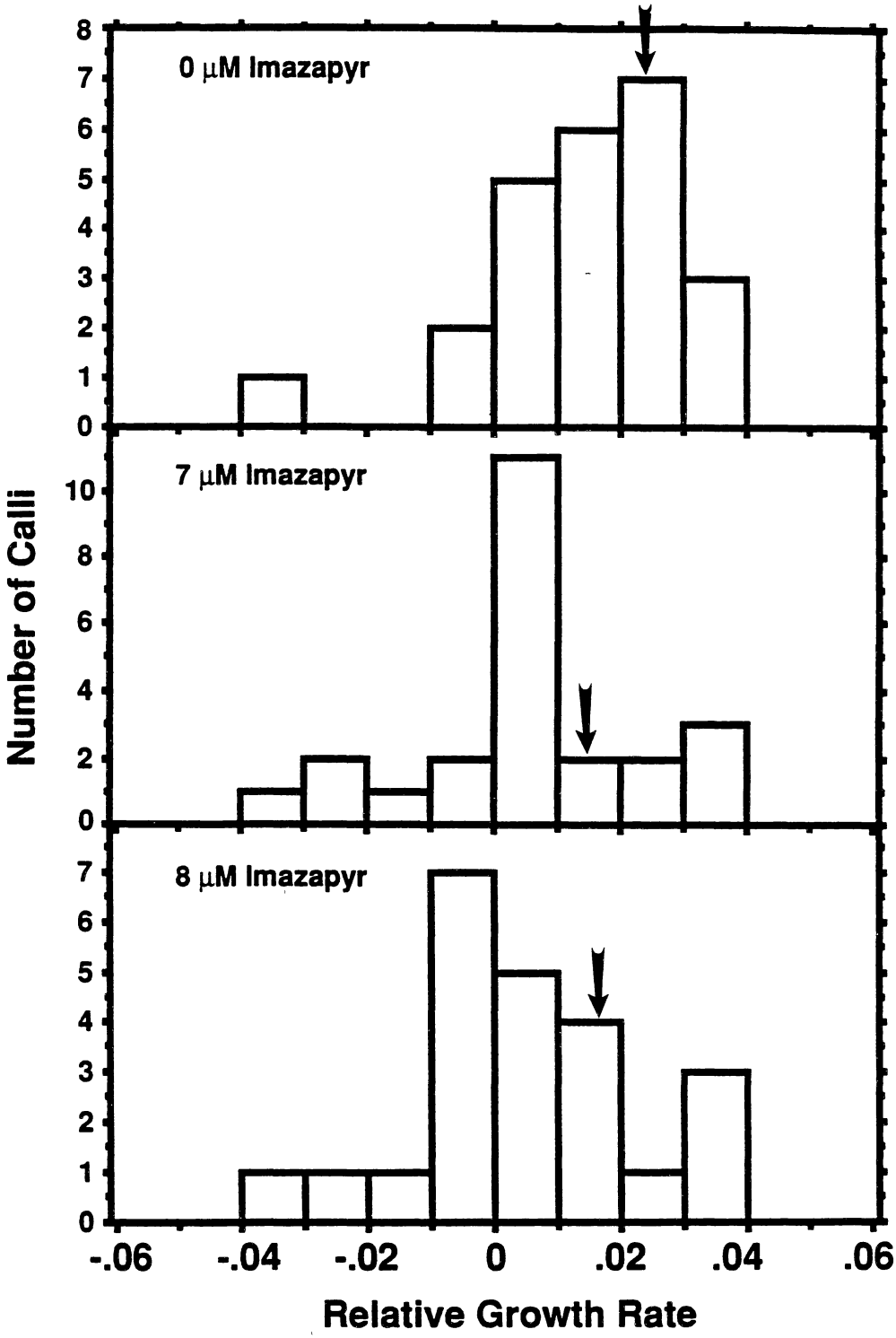

Fig. 6. Histograms of relative growth rates (RGR) during transfer period 8 of A-8918c calli subjected to 0, 7, and 8 μM imazapyr concentrations. Calli with RGR exceeding the upper predicted interval lie to the right of the arrow.

Table 5. Concentrations of valine, leucine and isoleucine (free amino acids) in OWB A-8953 and A-8918c control, tolerant and sensitive calli.

		Amino Acids			
Callus line	Imaz.	VAL	ISE	LEU	Total†
	- μM -	- - - - - - - - pmol/mg - - - - - - - - - - - -			
Control	0	1281.24 a*	439.28 a	723.86 a	28376 a
Tolerant	6	856.62 b	338.74 b	432.73 c	21594 b
Tolerant	5	974.22 b	443.61 a	567.82 bc	21138 b
Tolerant	4	890.62 b	441.72 a	668.84 ab	21927 b
Sensitive	6	460.66 c	166.69 c	252.04 d	7603 c
Sensitive	5	497.48 c	207.13 c	202.35 d	7384 c
Sensitive	4	458.41 c	171.65 c	233.16 d	7384 c

* Within columns, means followed by the same letter are not significantly different as indicated by LSD test.

† Total free pool amino acids.

Table 6. Number of plants regenerated from imazapyr tolerant calli developed during step-wise selection.

Imazapyr concentration	Accession	
	A-8953	A-8918c
-- μM --		
0.1	1	-
1	1	-
4	-	1
5	-	2
6	3	1
8	-	1

APPENDIXES

APPENDIX A

MEAN CALLUS FRESH WEIGHT (FW) PER PETRI DISH AFTER SEVEN WEEKS OF CULTURE IN DARKNESS

Appendix A. Mean callus fresh weight (FW) per petri dish after seven weeks of culture in darkness.

Med	Aux	Con	Accession		
			SS-19	SS-18	A-9959
			g		
mMS	Dic	5	8.808 a*	5.568 a	11.858 a
mMS	Dic	15	5.626 c	4.250 ab	7.802 b
mMS	Dic	30	3.880 d	2.783 b	3.362 c
mMS	2,4-D	5	7.218 b	4.708 ab	5.334 b
mMS	2,4-D	15	2.608 d	2.200 c	5.694 b
mMS	2,4-D	30	0.454 e	1.045 c	4.406 c
N6	Dic	5	0.896 e	0.823 c	1.748 c
N6	Dic	15	0.696 e	0.245 c	0.680 d
N6	Dic	30	0.372 e	0.123 d	0.802 d
N6	2,4-D	5	0.374 e	0.315 d	0.646 d
N6	2,4-D	15	0.158 e	0.123 d	0.172 d
N6	2,4-D	30	0.082 e	0.078 d	0.112 d

Acc = accession; Med = medium; Aux = auxin; Con = concentration.

* Means followed by the same letter are not significantly different at the 5% probability level as tested by LSD.

APPENDIX B

MEAN PERCENTAGE OF EMBRYOGENIC CALLUS (EC) PER PETRI DISH AFTER SEVEN WEEKS OF CULTURE IN DARKNESS

Appendix B. Mean percentage of embryogenic callus (EC) per petri dish after seven weeks of culture in darkness.

Med	Aux	Con	Accession		
			SS-19	SS-18	A-9959
			%		
mMS	Dic	5	0	0	0
mMS	Dic	15	5 b*	0	10 e
mMS	Dic	30	30 a	12.5	55 a
mMS	2,4-D	5	10 b	0	35 bc
mMS	2,4-D	15	25 a	0	10 e
mMS	2,4-D	30	0	0	5 e
N6	Dic	5	5 b	0	10 e
N6	Dic	15	5 b	0	25 cd
N6	Dic	30	30 a	0	45 ab
N6	2,4-D	5	5 b	0	15 de
N6	2,4-D	15	0	0	5 e
N6	2,4-D	30	0	0	0

Acc = accession; Med = medium; Aux = auxin; Con = concentration.

* Means followed by the same letter are not significantly different at the 5% probability level as tested by LSD.

APPENDIX C

MEAN RELATIVE GROWTH RATE (RGR) OF CALLUS PER PETRI DISH FOR THE SECOND GROWTH PERIOD

Appendix C. Mean relative growth rate (RGR) of callus per petri dish for the second growth period.

			Accession		
Med	Aux	Con	SS-19	SS-18	A-9959
mMS	Dic	5	0.025 de*	0.033 c	0.045 b
mMS	Dic	15	0.042 c	0.046 b	0.043 b
mMS	Dic	30	0.045 b	0.051 b	0.046 b
mMS	2,4-D	5	0.049 b	0.047 b	0.041 b
mMS	2,4-D	15	0.052 b	0.050 b	0.050 a
mMS	2,4-D	30	0.061 a	0.062 a	0.058 a
N6	Dic	5	0.025 de	0.019 d	0.025 c
N6	Dic	15	0.024 de	0.024 cd	0.028 c
N6	Dic	30	0.029 d	0.024 cd	0.028 c
N6	2,4-D	5	0.031 d	0.039 bc	0.030 c
N6	2,4-D	15	0.019 ef	0.017 d	0.023 c
N6	2,4-D	30	0.014 f	0.024 cd	0.011 d

Acc = accession; Med = medium; Aux = auxin; Con = concentration.

* Means followed by the same letter are not significantly different at the 5% probability level as tested by LSD.

VITA

Mohamed Chakroun

Candidate for the Degree of

Doctor of Philosophy

Thesis: IN VITRO CULTURE AND SELECTION STRATEGIES FOR SELECTED FORAGE GRASSES

Major Field: Agronomy - Crop Science

Biographical:

Personal Data: Born in Sfax, Tunisia, June 12, 1955, the son of Chedly Chakroun and Nafissa Ghorbel.

Education: Received the "Baccalaureate" of Math and Science from Lycee Hedi Chaker Route de Gabes, Sfax, Tunisia, in June, 1977; the Diplome d'ingenieur with major field Horticulture from Ecole Superieure d'Horticulture de Chott-Mariem, Sousse, Tunisia, in June, 1981; the Master of Science degree in Agronomy from Oklahoma State University, Stillwater, Oklahoma, in December, 1984; and completed the requirements of Doctor of Philosophy degree in Crop Science at Oklahoma State University, Stillwater, Oklahoma, in December, 1991.

Professional Experience: District crop production staff at the Regional Commissariat for Agricultural Development of Sidi Bouzid, Tunisia, October 1981 to June 1982; forage agronomist at the Forage Improvement Laboratory of the National Institute of Agronomic Research of Tunisia, January, 1985 to January, 1988 and teaching assistant, Botany Department, Oklahoma State University, August, 1990 to May, 1991.

Professional Societies: American Society of Agronomy; Crop Science Society of America; and Sigma Xi, The Scientific Research Society.