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CHARACTERIZATION OF BUFFELGRASS (*CENCHRUS CILIARIS* L.) CELL SUSPENSION CULTURES

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SUMMARY

Cell suspension cultures of buffelgrass were established from two types of callus, a friable tan callus and a brown gelatinous callus, using Murashige and Skoog medium containing 13.6 μM 2,4-dichlorophenoxyacetic acid (2,4-D). The friable callus formed a rapidly growing suspension culture, designated BG, which had a doubling time of 2.5 days. The gelatinous callus formed a very slow-growing suspension culture, designated BGG, which had a doubling time of 1 mo. During growth, the medium of the BGG line slowly increased in viscosity, becoming a thickened gel by the end of the subculture period. Both lines had high cell viability. Embryogenesis could be induced in both lines by culturing on charcoal-containing, 2,4-D-free medium. No embryos formed in the absence of charcoal.

Key words: buffelgrass; cell suspension cultures; tissue culture.

INTRODUCTION

Buffelgrass (*Cenchrus ciliaris* L.) is an apomictic, warm-season, perennial grass that has good forage characteristics and excellent drought tolerance. It has potential for rangeland revegetation in the arid regions of the Southwestern United States, but most cultivars lack sufficient winter-hardiness or salt tolerance or both. Little is known regarding the relative contributions of anatomical vs. physiologic adaptations to these stresses. Tissue culture techniques could provide one means to study cellular level stress response if reliable in vitro systems were developed for buffelgrass.

Sankhla and Sankhla (1989) initiated both friable and embryogenic callus lines from immature inflorescences of *Cenchrus ciliaris* (Anjan Dhaman) C.V. 75 using 2,4-dichlorophenoxyacetic acid (2,4-D) supplemented MS medium. No studies describing culture of other explants or initiation of suspension cultures have been reported. It has been suggested that suspension cultures may be preferable to callus cultures for experiments on cellular-level stress responses. The objectives of this study were the establishment and characterization of suspension cultures from the two morphologically distinct buffelgrass callus lines.

MATERIALS AND METHODS

Callus induction. Both friable and gelatinous callus lines were initiated in darkness from shoot apices isolated from etiolated 5-day-old seedlings of a pentaploid buffelgrass accession, PI 409704. The callus induction medium was comprised of MS salts (Murashige and Skoog, 1962) and B5 vitamins (Gamborg et al., 1968) supplemented with 30 g · liter^{-1} sucrose, 13.6 μM 2,4-D, and 7.5 g · liter^{-1} GIBCO (Grand Island, NY) phytagar. Morphologically distinct callus lines were identified, separated from the original explant material, and subcultured at 4-wk intervals.

Suspension culture initiation. Cell suspension cultures were initiated by placing 1 to 2 g of friable (designated BG) or gelatinous (designated BGG) callus into a 125-ml Erlenmeyer flask containing 30 ml of maintenance medium (MS salts and B5 vitamins supplemented with 30 g · liter^{-1} sucrose, and 13.6 μM 2,4-D). Cultures were maintained at 25 ± 2° C on an orbital shaker (130 rpm).

Suspension culture maintenance. Four weeks after the initial inoculation, 25 ml of the BG suspensions were diluted with 25 ml of fresh maintenance medium. The subculture period was gradually reduced to 2-wk intervals and the cell inoculum reduced to 9 ml of suspension diluted with 50 ml of medium.

The BGG suspension culture was initially grown for 5 wk, then 30 ml of cell suspension was inoculated into 20 ml of medium. Thereafter the suspensions were subcultured every 6 wk, and the inoculum was gradually reduced to 25 ml of cell suspension inoculated into 35 ml of medium.

Growth and viability measurements. To determine the optimal subculture inoculum size for each cell line, 0.1, 0.25, 0.4, 0.5, or 1.0 g fresh weight of 14-day-old BG cells or 0.1, 0.25, 0.4, 0.5, 1.0, or 2.0 g fresh weight of 28-day-old BGG cells were transferred to 50 ml of fresh maintenance medium. There were six replicates per treatment. Cells were grown for 2 wk (BG) or 6 wk (BGG) and collected on Whatman no. 1 paper in a Buchner funnel using vacuum filtration. Fresh weight and dry weight of the harvested cells were determined.

BG and BGG growth was measured using 0.5 and 1.0 g fresh weight inoculum, respectively, per 50 ml of maintenance medium with six replicates each. BG cells were harvested at 48-h intervals, over a 2-wk period, and BGG cells were harvested at 1-wk intervals over a 6-wk period. Cell viability was determined using the phenosafranine dye exclusion method (Widholm, 1972). A drop of culture medium containing dye was mixed with a drop of cells on a microscope slide and viewed with a Zeiss microscope. The number of red-staining (dead) and non-staining (viable) cells were counted.

Cell counting. Duplicate flasks of cell suspensions were combined, and the cells collected by vacuum filtration. Three aliquots (0.20 to 0.25 g fresh wt) of cells were suspended in 5 ml of 5% (wt/vol) chromium trioxide, shaken vigorously, and incubated 16 to 18 h at 26° C. To disperse the cells, the suspension was again shaken vigorously, then drawn 6 times through a 23-gauge needle attached to a syringe. After dilution with a seven-fold volume of distilled water, the cells were counted using a Fuchs Rosenthal Ultra Plane counting chamber (0.2 mm depth). Each aliquot of cells was counted 3 times.

BGG cell medium analysis. Aliquots of medium from 8-wk-old cells

TABLE 1

GROWTH AND VIABILITY OF BG AND BGG BUFFELGRASS CELL LINES AT DIFFERENT CELL INOCULA[a]

Initial Fresh Weight, g	Final Fresh Weight, g[b]		Doubling Time, days		Viable Cells, no.[c]	
	BG	BGG	BG	BGG	BG	BGG
0.10	4.25 d	0 d	2.58 a	0 c	94 a	0 b
0.25	10.16 c	0 d	2.62 a	0 c	95 a	0 b
0.40	13.10 b	0 d	2.78 a	0 c	94 a	0 b
0.50	15.93 a	1.20 c	2.80 a	33.25 b	92 a	89 a
1.00	14.01 b	3.10 b	3.67 b	25.73 b	86 b	86 a
2.00	ND	4.71 a	ND	33.99 b	ND	88 a

[a] BG and BGG cells were grown for 2 and 6 wk, respectively, in MS medium containing 13.6 μM 2,4-D.
[b] Means within column followed by same letter do not differ significantly ($P = 0.05$), those followed by a different letter are significantly different, as determined by Duncan's multiple range test; $n = 6$. Means with a value of 0 indicate no cell growth occurred.
[c] Number of viable cells out of 100 cells observed. ND = not done.

were diluted with equal volumes of distilled water (0.5 ml) and filtered through a glass fiber filter to remove cells, broken cells, and debris. Polysaccharides were isolated by a procedure similar to Jones and Morre (1966). Absolute ethanol was added to a concentration of 80%, the precipitate was pelleted at 300 *g* for 15 min, and the supernatant was discarded. After the pelleted precipitate was washed 3 times in 95% ethanol, the precipitate was dried to constant weight. The final product was dissolved in 1 ml of concentrated H_2SO_4. Four milliliters of distilled water were added to each sample and refluxed at 110° C for 1.5 h. Each sample was brought to a final volume of 25 ml. Total glucose equivalents were determined in 1-ml aliquots using the phenol-sulfuric method (Dubois et al., 1956). Protein was determined by the method of Bradford (1976) using bovine serum albumin as the standard.

Growth on semisolid medium. To determine the morphogenetic capacity of the established BG and BGG suspension cultures on gelled medium, 2 ml of cell suspension was transferred to 100 × 20-mm petri dishes containing 25 ml of MS medium gelled with 7.5 g · liter^{-1} phytagar. Media contained either 13.6 μM 2,4-D, no 2,4-D, or no 2,4-D plus 20 g · liter^{-1} acid-washed activated charcoal (Sigma Chemical Company, St. Louis, MO). Each treatment consisted of 10 replicates. Cultures were grown in darkness at 25 ± 2° C for 8 wk.

To determine the frequency of embryo germination and conversion to plants, embryos were transferred to petri dishes containing 25 ml of one-half strength hormone-free MS medium gelled with 7.5 g · liter^{-1} phytagar. Thirty replicate embryos per cell line were cultured. Embryos were incubated for 8 wk at 25 ± 2° C under 18 h of light (125 μmol · m^{-2} · s^{-1}) provided by cool-white fluorescent lights.

The general linear model procedure of SAS was used to analyze the data as completely randomized designs. The means were separated by Duncan's multiple range test (SAS Inst., Inc., 1985). All experiments were conducted at least 3 times.

Results

Viable cell suspensions were initiated from both friable and gelatinous buffelgrass cultures, but the resulting cell lines retained distinct characteristics. The dark-brown BGG cells grew at only one-tenth the rate of the off-white BG cells (Table 1).

Cell inoculum significantly influenced the final fresh weights, doubling time, and cell viability of both cell lines. Maximal growth was obtained with an initial inoculum of 0.5 and 2.0 g fresh weight for the BG and BGG cell lines, respectively. BG cells grew at all inoculum levels tested, whereas the BGG cells failed to grow when inoculum was 0.4 g or less fresh weight. At the highest inoculum level, the BG cells had a slower growth rate and reduced viability. BGG cell doubling time was shortest at 1.0 g fresh weight inoculum. Increasing the inoculum to 2.0 g fresh weight resulted in a longer doubling time but did not affect viability.

Both BG and BGG cell lines exhibited an S-shaped growth curve (Figs. 1 and 2). Fresh weight of the BG cell line increased from mid-cycle through Day 14. For BGG, fresh weight increased after mid-cycle and slowed by Week 6. Maximum dry weights were obtained at Days 12 and 42 for BG and BGG, respectively.

The fresh weight:dry weight ratio over time of both cell lines followed a similar pattern. After an initial decrease in ratio there was a short, stable period, followed by a gradual increase beginning in mid-growth cycle (Figs. 3 and 4). These changes were more pronounced in the BG line, where the ratio dropped by 50% mid-cycle compared to about a 20% decrease in the BGG cells.

At mid-cycle (Day 7 for BG cells and Week 3 for BGG cells), the cell numbers were 12 × 10^6 cells and 23 × 10^6 cells · g^{-1} fresh weight, respectively. By the end of the culture cycle (Day 14 for BG cells and Week 6 for BGG cells), both lines showed a decrease in cell number to 7 and 20 × 10^6 cells · g^{-1} fresh weight, respectively, indicating an increase in cell size. The BGG cells were smaller in size, but showed a similar pattern of cell number (per gram fresh weight) to BG cells during the growth cycles.

The medium in which the BGG cells grew gradually thickened to

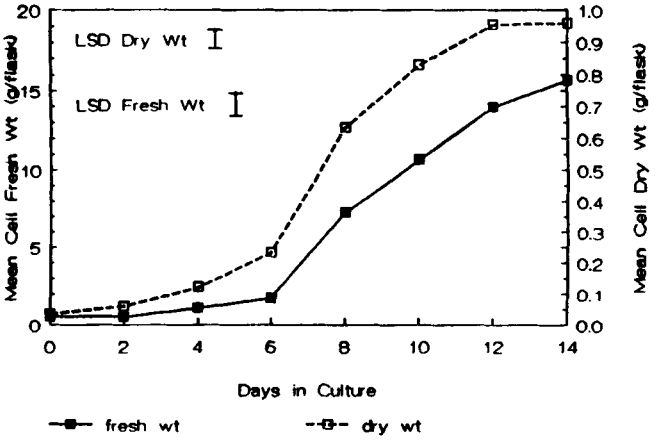

FIG. 1. Growth on a fresh weight and dry weight basis of the BG buffelgrass cell line cultured in liquid MS medium containing 13.6 μM 2,4-D at a cell inoculum of 0.5 g cell fresh weight, over a 2 wk culture period. *Solid line* = fresh weight; *dotted line* = dry weight. *Vertical bars* indicate overall 5% LSD.

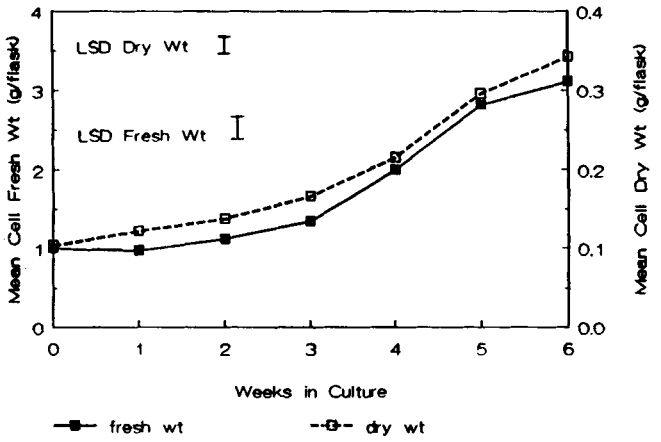

FIG. 2. Growth on a fresh and dry weight basis of the BGG buffelgrass cell line cultured in liquid MS medium containing 13.6 μM 2,4-D at a cell inoculum of 1.0 g cell fresh weight, over a 6-wk culture period. *Solid line* = fresh weight; *dotted line* = dry weight. *Vertical bars* indicate overall 5% LSD.

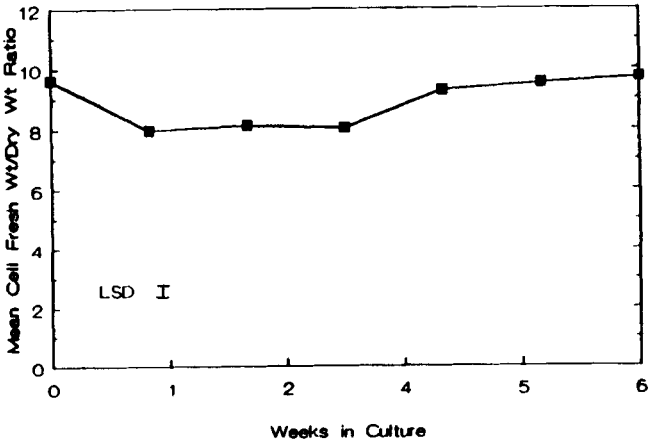

FIG. 4. Cell fresh weight to dry weight ratio of BGG cells cultured at an inoculum of 1.0 g cell fresh weight in MS medium containing 13.6 μM 2,4-D, over a 6-wk culture period. *Vertical bar* indicates overall 5% LSD.

a mucilaginous gel. Compositional analyses revealed that the gelled medium was high in ethanol insoluble polymers (over 2 mg dry weight per ml of medium) and low in protein compared to medium from the BG line. Analysis for glucose equivalents revealed that about one-half of the polymer content was reducing sugars.

Charcoal added to gelled medium promoted embryogenesis in both cell lines compared to cells cultured on gelled medium without charcoal. No embryos formed in the absence of charcoal. BG and BGG produced callus and embryos in 24 and 6 plates with charcoal, respectively, out of a total of 30 plates each. There was a significant ($P < 0.05$) difference in the mean number of embryos produced per gram fresh weight of embryo-producing BG (26.2 embryos) and BGG (11.6 embryos) cell cultures. The rate of somatic embryos conversion to plants was 10 to 12% for both BG and BGG somatic embryos.

On no-charcoal media, with or without 2,4-D, BG produced only callus whereas BGG generated both callus and roots on all such replicates. The BGG cells retained the gelatinous characteristic on all gelled media.

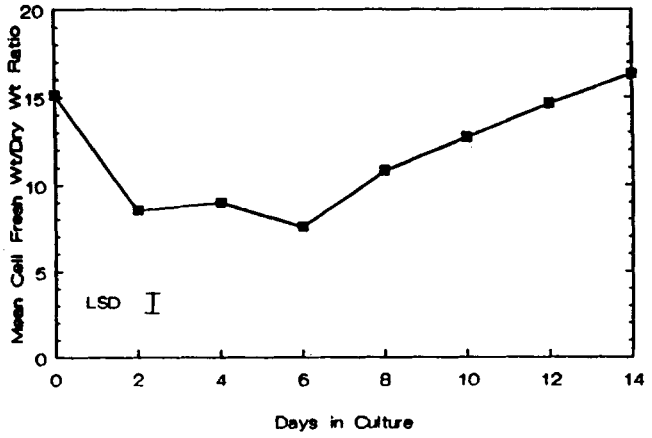

FIG. 3. Cell fresh weight to dry weight ratio of BG cells cultured at an inoculum of 0.5 g cell fresh weight in MS medium containing 13.6 μM 2,4-D, over a 2-wk culture period. *Vertical bar* indicates overall 5% LSD.

DISCUSSION

Two distinct types of callus are routinely derived from seedling apices of buffelgrass on 2,4-D containing media. The first is a typical, rapidly growing off-white friable callus, the second is a slow-growing, gelatinous callus which occurs at a frequency of 10%. Cell suspension cultures were established from the two distinct callus types. These suspensions exhibited distinct and stable growth characteristics for more than 2 yr. BG, which was derived from friable callus cultures, remained comprised of small aggregates of off-white cells. BGG cells retained the dark-brown color and gelatinous nature of the explant callus and were smaller in cell size.

The BG line grew rapidly relative to reports of other grasses. Suspension cultures of wheat species (*Triticum* spp.) and maize (*Zea mays* L.) have doubling times of 7 and 5.5 days, respectively (Schaeffer et al., 1984; Bartkowiak, 1981). The influence of inoculum size on BG and BGG cell growth rates and cell yield is similar to that reported for other suspension cultures. Suspension cells of *Populas alba* have the most rapid initial growth at an inoculation density of 9%, whereas maximum final fresh weight is obtained at 7% inoculum (Park and Son, 1988). The minimum cell density requirement of BGG cells is a characteristic observed in other cell suspension cultures. Street (1977) reported that *Acer pseudoplatonus* cells fail to grow below 9 to 15×10^3 cells/cm^3.

The gelatinous characteristic of the BGG has been reported infrequently in suspension cultures. Wheat suspensions (*T. monococcum*) may become viscous due to the presence of an ethanol-precipitable polysaccharide (Gamborg and Eveleigh, 1968). Pumpkin cell suspension cultures secrete a protein thought to be a chitinase (Esaka et al., 1990). The high polysaccharide content and low protein levels of the BGG medium would suggest the mucilagenous texture was due to the polysaccharide secretion. The roots of many terrestrial plants produce a gelatinous secretion that is composed mainly of polysaccharides (Jones and Morre, 1966; Juniper and

Roberk, 1966; Paull et al., 1975). The gelatinous secretion and root regeneration capacity of the BGG cells may indicate morphogenetic similarities to root tip cells. The reduced growth rate of BGG, compared to BG, may be a result of resources and energy being directed toward the production of high amounts of polysaccharides. The inability to obtain friable suspensions from gelatinous callus cultures is perplexing and may indicate a committed differentiation step. Additional studies need to be completed to ascertain the extent of structural and physiologic similarity between these cell types.

Both cell suspensions demonstrated embryogenic potential only when cultured on charcoal-containing medium. Embryogenic callus cultures could be initiated directly from buffelgrass seedling apices without the use of charcoal (Mark Dahmer, unpublished data). The loss of embryogenic potential with time in culture has been previously reported (Drew, 1979; Zaghmout and Torello, 1988). The beneficial effects of charcoal on restoring embryogenesis in long-term cultures have been ascribed to absorption of 2,4-D and other inhibitors of morphogenesis (Drew, 1979; Fridborg and Eriksson, 1975; Zaghmout and Torello, 1988), which may have been the case with the BG and BGG cell lines. BG and BGG somatic embryos could be germinated and viable plants regenerated.

In conclusion, morphologically stable suspension cultures of buffelgrass were initiated and maintained for more than 2 yr. The cell lines differed in growth rate and appearance and did express some embryogenic potential when cultured with charcoal. This system could be used for studies of stress tolerance in buffelgrass.

Acknowledgements

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