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Cryopreservation of Bermudagrass Germplasm by Encapsulation Dehydration

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ABSTRACT

Genetic conservation of vegetatively-propagated grasses requires intensive care of pot cultures or carefully separated field plots. Even with intensive care, there is high risk of mechanical contamination and loss of plants to biotic and abiotic stresses. Medium- and long-term storage of this germplasm would be more cost effective and provide a backup for field or greenhouse germplasm collections. *Cynodon* spp. (bermudagrass and stargrass) germplasm is typically maintained as growing plants in breeders' collections. The development of a long-term storage protocol for *Cynodon* in liquid nitrogen (LN) could provide a secure backup of these collections. A diverse group of *Cynodon* taxa was evaluated for long-term storage in LN at −196°C. The encapsulation and dehydration (ED) cryopreservation protocol was most effective when combined with a 1- to 4-wk cold-acclimation period and dehydration to 19 to 23% moisture before exposure to LN. Nineteen of the 25 accessions (76%) had >40% regrowth. Thirty shoot tips of each of 25 *Cynodon* accessions are now stored at the National Clonal Germplasm Repository (NCGR), Corvallis, and 50 shoot tips are held at the National Center for Germplasm Resources Preservation (NCGRP) in Fort Collins, CO, as a long-term backup in LN.

Breeders use collections of plant germplasm to develop cultivars with improved characteristics, increased crop yield, and disease or insect resistance. All germplasm requires safe storage because even exotic germplasm without obvious economic merit may contain genes or alleles that may be needed as new disease, insect, environmental, or crop production problems arise (Westwood, 1989). Preservation of clonally propagated grass germplasm in the greenhouse or field incurs risk of contamination from vegetative propagules or seeds.

The grass genus *Cynodon* includes eight species and 10 botanical varieties (Kew Royal Botanic Gardens, 1999) that are phenotypically diverse, and most require vegetative propagation. The National Plant Germplasm Station at Griffin, GA, currently maintains 248 vegetative accessions of 15 *Cynodon* taxa (USDA-ARS, 2005). The predominant *Cynodon* is bermudagrass, which is represented by two main taxa, *C. dactylon* var. *dactylon* (bermudagrass) and *C. transvaalensis* Burtt-Davy (African bermudagrass). Bermudagrass is economically important and used widely for turf and pasture in areas where temperatures remain above 0°C. Many of the collections of these species are maintained by individual breeding programs and are often lost when the breeder leaves the program. Maintenance of bermudagrass germplasm for long periods of time is difficult in the field or greenhouse due to contamination or death, and as a consequence, many of the original clonal stocks of early cultivars of this grass are no longer available (Engelke, 1997).

Alternative forms of storage are available for many types of clonal germplasm. Some clonal crops are kept in slow-growth medium-term storage as in vitro cultures for germplasm conservation (Ashmore, 1997; Benson, 1999; Razdan and Cocking, 1997). In vitro storage of *Zoysia* grass was successful at 21°C for 2 yr with a 16-h photoperiod (Jarret, 1989). *Lolium multiflorum* Lam. can be stored in vitro at 2 to 4°C with continuous cool white light and yearly subculture (Dale, 1980). A broad range of *Cynodon* in vitro germplasm remained healthy in storage at 4°C from 4 mo to >1 yr (Aynalem et al., 2002). However, few long-term management options are available for clonal plant genetic resources. The development of cryopreservation techniques provides the option for long-term backup of active collections that might otherwise be at risk.

Cryopreservation techniques developed during the last 25 yr include controlled freezing, vitrification, ED, dormant bud preservation, and combinations of these protocols and are used in hundreds of species (Benson, 1999; Razdan and Cocking, 1997). Cryopreservation by the ED technique requires dehydration of the encapsulated shoot tips such that meristematic cells have greatly reduced cell water, but do not reach the permanent wilting point, and the cells vitrify on exposure to LN (Benson, 1999). The ability of a particular accession to tolerate reduction of moisture content to 20% can be manipulated through various cultural techniques and pretreatments. For example, cold acclimation (CA) alters membrane composition, thereby increasing dehydration tolerance (Sugawara and Steponkus, 1990) and is known to increase glass formation in woody plant cells (Steponkus et al., 1992), improving survival following exposure to LN. The combination of these factors results in successful cryopreservation of new genera (Reed, 2001).

Cryopreserved shoot tips can be stored for multiple decades without deterioration (Benson, 1999; Razdan and Cocking, 1997). While in vitro systems require some additional input before storage, the ease of recovering and propagating the shoot tips is an advantage, and many accessions can be stored in a small dewar (Reed, 2001). Chang et al. (2000) reported successful cryopreservation of both temperate and subtropical grasses. *Lolium perenne* L. (ryegrass) apices that were cold acclimated (CA) for 4 wk produced 60 to 100% regrowth following cryopreservation by slow freezing or ED tech-

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Abbreviations: CA, cold acclimation or cold acclimated; ED, encapsulation and dehydration; KT, kinetin; LN, liquid nitrogen; MS, Murashige and Skoog; NCGR, National Clonal Germplasm Repository; NCGRP, National Center for Germplasm Resources Preservation.

Table 1. Taxonomy and origin of 25 *Cynodon* accessions cryopreserved by encapsulation dehydration and stored at the National Clonal Germplasm Repository, Corvallis, OR, and the National Center for Germplasm Resources Preservation, Fort Collins, CO.

PI no.	Local no.	Plant name	*Cynodon* taxon and origin
617089	1.001	A-2	*C. dactylon* var. *dactylon*, New Mexico, USA
617090	2.001	A-3	*C. dactylon* var. *dactylon*, New Mexico, USA
617091	3.001	A-4	*C. dactylon* var. *dactylon*, New Mexico, USA
617092	4.001	15-1	*C. dactylon* var. *dactylon*, New Mexico, USA
617093	5.001	AP-1	*C. dactylon* var. *dactylon*, New Mexico, USA
595197	6.001	A9226	*C. aethiopicus* Clayton & J.R. Harlan, Ethiopia
287139	7.001	2-5	*C. barberi* Rang. & Tadul., India
287253	8.001	11-64	*C. barberi*, Sri Lanka
288221	9.001	16-104	*C. dactylon* var. *coursii*, (A. Camus) Harlan & de Wet, Madagascar
289716	10.001	15-89	*C. dactylon* var. *coursii*, Madagascar
289747	11.001	Kweek 21-137	*C. dactylon* var. *polevansii*, (Stent) Harlan & de Wet, S. Africa
291753	13.001	48-313	*C. incompletus* var. *hirsutus*, (Stent) Harlan & de Wet, S. Africa
562690	14.001	'Florico'	*C. nlemfuensis* var. *nlemfuensis*, Clayton & J.R. Harlan, Florida, USA
292570	15.001	74-471	*C. radiatus* Roth, Luxon, the Philippines
292060	16.001		*C. aethiopicus*, Tanzania
223248	18.001		*C. dactylon* var. *afghanicus*, Afghanistan
251809	19.001		*C. dactylon* var. *dactylon*, Italy
291149	21.001		*C. dactylon* var. *elegans*, S. Africa
291150	22.001		*C. dactylon* var. *elegans*, S. Africa
617088	24.001		*C. dactylon* var. *dactylon*, Australia
291189	25.001		*C. incompletus* var. *incompleteus*, S. Africa
617094	27.002	Cultivar no. 2	*C. nlemfuensis* var. *robustus*, Zimbabwe
290874	30.001	'Uganda'	*C. transvaalensis* Burtt Davy, Uganda
617086	33.001	'Tifway'	*Cynodon* hybrid, Georgia, USA
562699	34.001	'Tifton 85'	*Cynodon* hybrid, Georgia, USA

niques. Cold-acclimated *Zoysia matrellas* L. and *Z. japonica* Steud. had >60% regrowth following ED and LN exposure when shoot tips encapsulated in beads were dehydrated to <22% water content. Nonacclimated shoot tips of both genera had little or no regrowth. The goals of this study were to optimize the existing grass cryopreservation protocol for use with *Cynodon* by evaluating the effects of dehydration, CA, and genotype on survival of diverse *Cynodon* taxa.

MATERIALS AND METHODS

Experiments were designed to test the effects of dehydration and CA, and to screen multiple genotypes on viability of shoot tips preserved using ED. General methods used for all three experiments are described first, followed by specific details for each experiment.

Culture Medium

Culture initiation medium was Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) with 3% sucrose, 0.2 mg L^{-1} kinetin (KT), 0.3% agar (Bitek agar, Difco, Detroit, MI), and 0.1% Gelrite (Kelco, San Diego, CA) adjusted to pH 5.8. Multiplication medium was the same as culture initiation medium with 0.5 mg L^{-1} KT and 0.5 mg L^{-1} N^6–benzyladenine (BA). Cryopreservation recovery medium was a softer multiplication medium (agar 0.25% and Gelrite 0.08%) used to aid in rehydrating the beads.

Culture Initiation

Tillers for initiating in vitro cultures were collected from potted greenhouse plants provided by A.A. Baltensperger, C.M. Taliaferro, and the USDA-ARS Plant Germplasm Resource Conservation Unit, Griffin, GA (Table 1). Tillers (2–3 cm) were rinsed with tap water for 30 min, surface sterilized in 15% bleach solution (0.79% sodium hypochlorite) and 0.1 mL L^{-1} of Tween-20 (Sigma Chemical Co., St. Louis, MO) for 25 min, and rinsed three times in sterile water. Shoot tips (≈1.5 mm) were dissected from the tillers and placed in glass tubes (10 by 150 mm) with 5 mL of initiation medium. After 3 wk, shoots were checked for bacterial contaminants and transferred to multiplication medium. Plantlets were multiplied in GA7 Magenta boxes (Magenta Corp., Chicago, IL) with a 4-wk transfer cycle. Growth-room conditions were 25°C with a 16-h light/8-h dark photoperiod (cool white fluorescent illumination, 25 μmol m^{-2} s^{-1}).

Cold Acclimation

Two weeks after transfer to fresh multiplication medium, plantlets were CA in a growth chamber at −1°C 16 h dark/22°C 8 h with light (10 μmol m^{-2} s^{-1}) (Chang and Reed, 1999; Chang et al., 2000; Reed, 1988).

Encapsulation and Dehydration Cryopreservation Procedure

Plantlets were CA before shoot tips (≈1 mm) were dissected from in vitro cultures for cryopreservation. The ED method was originally developed for pear (*Pyrus communis* L.) (Dereuddre et al., 1990) and adapted for grass (Chang et al., 2000). Shoot tips from CA plantlets were encapsulated in alginate beads [3% medium viscosity alginic acid (Sigma Chemical Co., St. Louis, MO) in liquid MS medium with 0.75 *M* sucrose and solidified in MS basal medium with 100 m*M* calcium chloride for 20 min]. The shoot tips in beads were pretreated in liquid MS medium with 0.75 *M* sucrose for 20 h on a rotary shaker at 50 RPM. Shoot tips in beads were removed from the pretreatment medium, blotted on filter paper, and air dried in sterile Petri dishes under laminar flow at 25°C (at about 40% relative humidity) to 20 to 22% moisture content. Dried shoot tips in beads were transferred to 1.2-mL cryovials and plunged directly into LN for at least 10 min. Vials were rewarmed at room temperature for 20 min; shoot tips in beads were rehydrated for 10 min in liquid MS medium, and then transferred to recovery medium in 24-cell plates. Regrowth, assessed as visible new shoot growth, was recorded after 4 wk on the recovery medium. For each replication, five or 10 control shoot tips were encapsulated, dehydrated, and rehydrated, but not exposed to LN.

Experiment 1. Optimal Dehydration

The effect of air dehydration on the moisture content and regrowth of encapsulated shoot tips was studied based on the standard ED procedure outlined above. Shoot tips from 2-wk old *C. transvaalensis* 'Uganda' (Cyn 30.001) plantlets were CA for 4 wk and air-dehydrated for 0, 3, 5, and 7 h under laminar flow after encapsulation. Two replications of 10 beads without shoot tips were used to determine moisture content for each time period. Two replications of 10 control and 20 LN-exposed beads were plated for each time period following the ED protocol. Data were adjusted so that control shoot tip regrowth equaled 100%. Fresh weight was taken at each time period and beads were dried by heating in an oven at 103°C for 16 h for dry weight. Moisture content was determined as the difference between dry weight (DW) and fresh weight (FW) calculated as

$$[(\mathrm{FW} - \mathrm{DW})/\mathrm{FW}] \times 100\%.$$

Data were analyzed by regression.

Experiment 2. Optimal Cold Acclimation

Two-week-old plantlets of *C. dactylon* A-3, *C. radiatus* 74-471, and *C. transvaalensis* Uganda were CA for 0, 1, 2, 3, and 4 wk. Three replications of 20 shoot tips for each treatment were exposed to LN using the ED protocol and 6 h dehydration (20–22% moisture content). Each replicate had five control shoot tips for each treatment. Data were adjusted so that control shoot tip regrowth equaled 100%. Data were analyzed by ANOVA and regression.

Experiment 3. Germplasm Storage and Response to Encapsulation and Dehydration Treatment

Long-term storage of *Cynodon* accessions in LN was accomplished as follows. Each accession was cryopreserved in a single storage experiment that was internally replicated but not repeated. Shoot tips (120 for each accession) were processed for long-term storage by ED with 4-wk CA and 6-h dehydration. Ten controls were dried and rehydrated without LN exposure. For each accession, 110 shoot tips encapsulated in beads were dried, placed in cryovials (10 per vial), and submerged in LN. Of these vials, two were rewarmed to test for viability at NCGR, three were stored at NCGR, and six were shipped to the NCGRP in Ft. Collins, CO, in a LN dry shipper via overnight mail and transferred to long-term LN storage tanks, and one vial was regrown for viability testing. Shoot tips were considered regrown if new shoot growth was observed at 4 wk. Data presented are the mean recovery of the three vials of 10 shoot tips rewarmed from a single storage experiment, two at NCGR and one at NCGRP. Control data (10 shoot tips dried but not exposed to LN) were not replicated.

RESULTS AND DISCUSSION

Optimal Dehydration

Shoot tips responded well to increasing dehydration. Regrowth of 4 wk CA, dehydrated, LN-exposed shoot tips was best at 5 to 7 h drying time, which corresponds to 19 to 23% moisture content (Fig. 1). Hours of dehydration correlated well with both bead moisture content and shoot tip regrowth (Fig. 1). Moisture content of beads declined from 33% for beads dried for 3 h to 19% after 7-h dehydration. Optimum bead moisture content for shoot tips preserved by the ED technique is normally

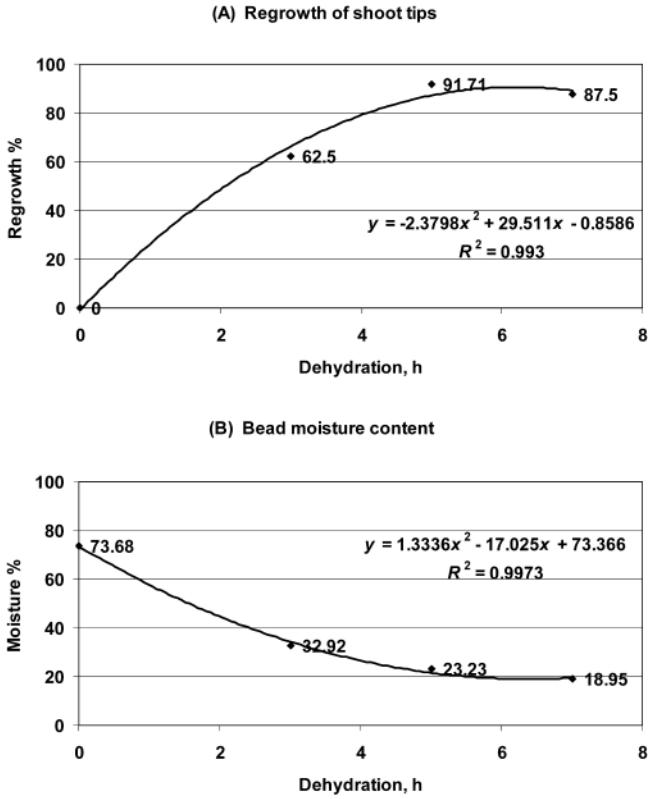

Fig. 1. (A) Regrowth of 4-wk CA *Cynodon transvaalensis* 'Uganda' shoot tips following encapsulation in alginate beads, air drying for 0 to 7 h, and 10 min exposure in liquid N followed by rehydration and plating on recovery medium. Data were adjusted so that control shoot tip regrowth equaled 100%. (B) Bead moisture content at each time period.

about 20% (Chang et al., 2000; Dereuddre et al., 1990). *Lolium perenne*, *Zoysia japonicus*, and *Z. mantrellas* shoot tips dried to 17 to 22% moisture (4–7 h dehydration) and cryopreserved also recovered better than those with <4 h dehydration (Chang et al., 2000). Dehydration tolerance is required for successful cryopreservation by ED because the cells must have no freezable water, thus vitrifying and avoiding ice crystal formation when exposed to LN (Benson, 1999). Scanning differential calorimetry studies show that alginate beads dried to 20% moisture vitrify on exposure to LN and form stable glasses that do not form ice crystals on rewarming (Dumet et al., 2000). This stability is reflected in the high regrowth of viable shoot tips in beads dried to approximately 20% moisture. Alginate-encapsulated pear shoot tips also recovered best at moisture contents near 20% (Scottez et al., 1992). In our study, *Cynodon* cv. Uganda shoot tips with 19 to 23% moisture content had the best regrowth (Fig. 1B).

Cold Acclimation

Cold acclimation was essential for successful cryopreservation of *Cynodon* accessions (Fig. 2). Significant differences in regrowth response by genotype and weeks of CA were noted ($P \leq 0.002$) (Data not shown). All CA shoot tips had significantly more regrowth following LN exposure than the nonacclimated controls. After 1 wk

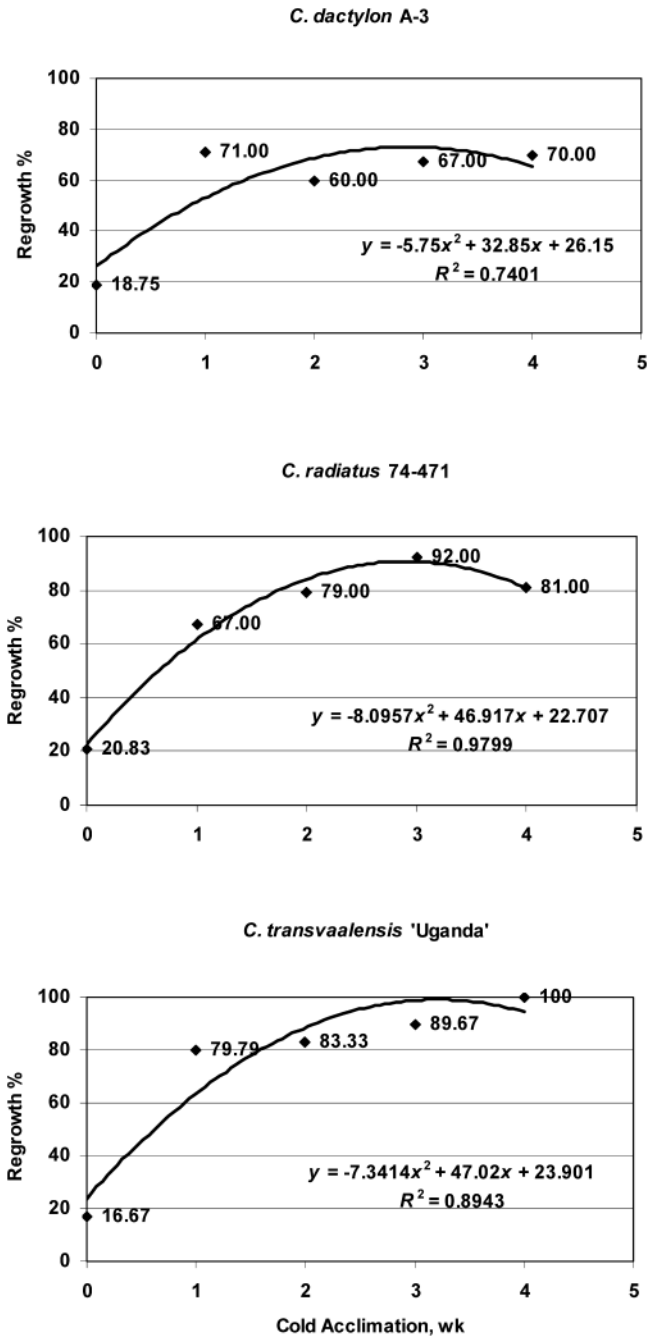

Fig. 2. Response of three *Cynodon* accessions to 0 to 4 wk of cold acclimation followed by 6-h air drying and cryopreservation with the encapsulation and dehydration protocol. Data were adjusted so that control shoot tip regrowth equaled 100%.

of CA, all accessions produced significantly more regrowth ($P \leq 0.001$). Regrowth following 3 or 4 wk of CA was significantly better ($P \leq 0.001$) than with 1 wk of CA for 74-471 and Uganda. Increasing CA from 1 to 4 wk did not further improve regrowth for A-3. Regression analysis also showed better correlations between CA duration and regrowth for 74-471 and Uganda than for A-3. This CA response resembles the results for many other genera tested with this technique. Rubus, Ribes, and Pyrus exhibited low regrowth without CA, and all showed improved regrowth from cryopreservation with appropriate acclimation, but the optimum varied with genotype (Chang and Reed, 1999, 2000; Reed and Yu, 1995; Reed et al., 1998). In cold hardiness tests with pear, shoots survived at temperatures 4°C lower following 1 wk CA and 7°C lower following 2 wk CA compared with non-CA shoots. This amount of CA resulted in improved recovery following cryopreservation (Chang and Reed, 2001). *Lolium* genotypes had improved regrowth after 1 wk of CA and one cultivar showed further improvements after 4 and 8 wk of CA; *Zoysia* had improved regrowth after 8 wk CA (Chang et al., 2000). Low water content results in dehydration damage to cells, often resulting in membrane alterations; however, low moisture content is necessary for successful grass cryopreservation. Cold acclimation treatment not only increased freezing tolerance, but also increased the dehydration tolerance of *Lolium* and *Zoysia* grass shoot tips. Cold acclimated shoot tips dried to ≤20% moisture content retained high viability (Chang et al., 2000) while nonacclimated shoot tips died at bead-moisture contents of 25% or less. Shoot tips of the three *Cynodon* CA for 1 to 4 wk and dehydrated to 19% moisture content all had good to excellent regrowth following LN exposure (Fig. 2).

Germplasm Response to Encapsulation and Dehydration Treatment

Regrowth of cryopreserved *Cynodon* shoot tips ranged from 20 to 87% for the 25 accessions screened (Fig. 3). In most cases the regrowth of the controls and the LN exposed shoot tips was similar, with only one (15-1) exhibiting much less recovery from the cryopreserved shoots. A 40% minimum regrowth standard was established as acceptable for accessions stored in our laboratory (Reed, 2001) and was shown to be valid through statistical modeling (Dussert et al., 2003). Nineteen of the 25 cryopreserved *Cynodon* accessions had >40% regrowth 4 wk after plating. With five vials (10 shoot tips each) stored for each accession, this provides five opportunities to retrieve germplasm for most accessions and two or three for accessions with low test-sample regrowth. The high percentage of regrowth exhibited by these diverse *Cynodon* genotypes indicates the applicability of the ED technique to the genus (Fig. 3).

Cryopreservation is now a viable long-term storage technique for use with *Cynodon*. There are few long-term management options for clonal-plant genetic resources because of the necessity for maintaining accessions as growing plants. In the case of *Cynodon*, the historic loss or mixing of accessions during clonal storage highlights the need for alternative backup storage of the genus. The successful storage of 25 accessions in 14 *Cynodon* taxa (from the National Plant Germplasm System collection of 248 accessions in 15 taxa) demonstrates the potential of this technique as a backup for difficult-to-maintain greenhouse or field collections.

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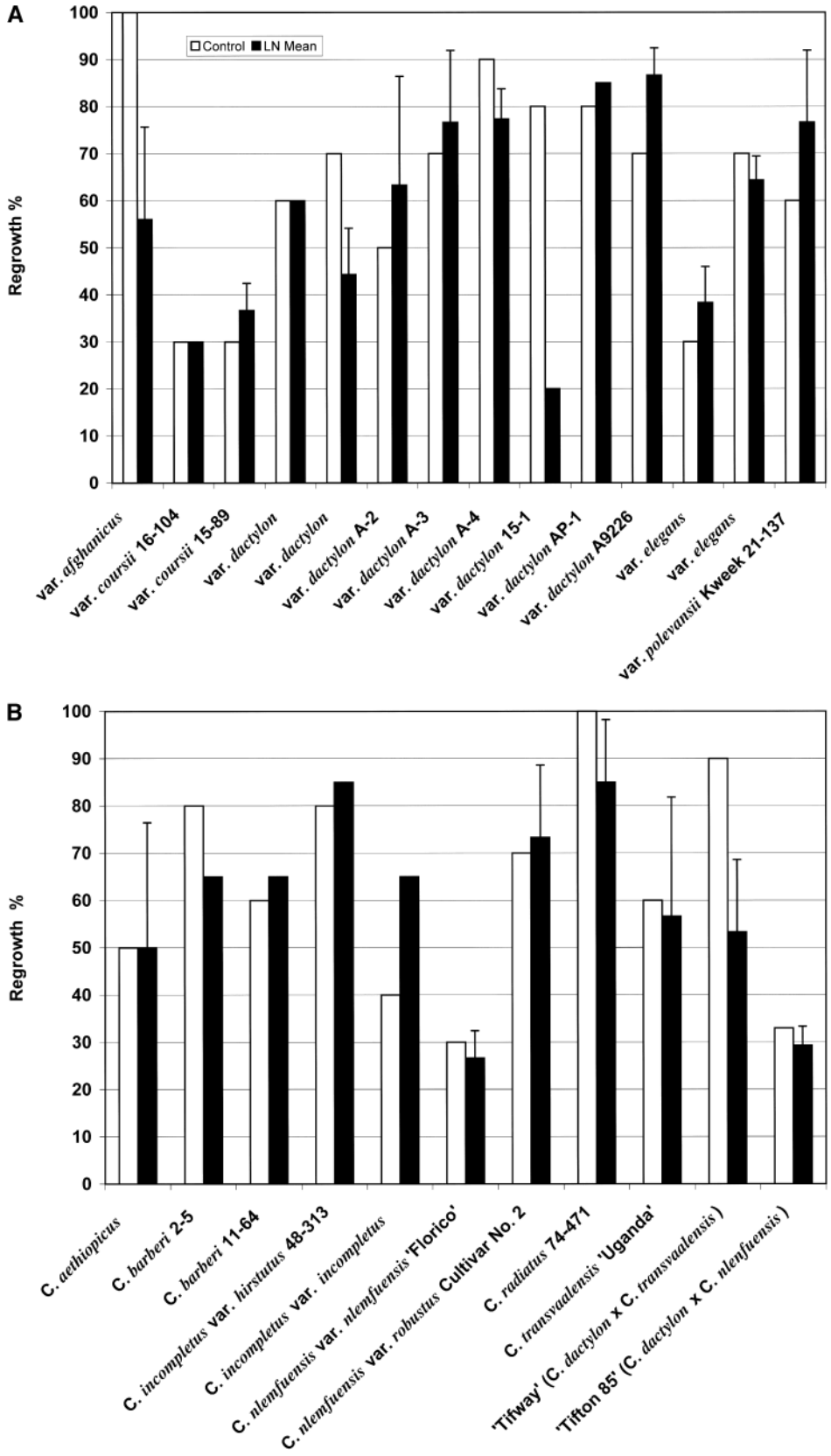

Fig. 3. Mean regrowth of liquid nitrogen (LN) exposed and control shoot tips of (A) *Cynodon dactylon* species and cultivars and (B) *Cynodon* species other than *C. dactylon*, cold acclimation for 4 wk, encapsulated in alginate beads, and dried for 6 h in the laminar flow hood. Shoot tips in beads were exposed to LN for 10 min, rewarmed at room temperature for 20 min, rehydrated for 5 min, and plated on recovery medium. Data presented are the mean recovery of vials from a single storage experiment. Two vials were rewarmed on site at the National Clonal Germplasm Repository (NCGR) while the other was sent by overnight mail to the National Center for Germplasm Resources Preservation (NCGRP) in Ft. Collins, CO, transferred to long-term LN storage tanks, and later rewarmed. Data were taken 4 wk after plating on recovery medium (three replicates of 10, two reps at NCGR, and one rep at NCGRP). Liquid N means without error bars are averages of two vials. Controls were dried and rehydrated but not exposed to LN (one replicate of 10).

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