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Use of artificially-induced freezing temperatures to identify freeze tolerance in above-ground buds of *Saccharum* and *Erianthus* accessions

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Abstract Sugarcane is a crop which is primarily grown between 30°N and 30°S latitude in tropical environments. Small areas of production in sub-tropical regions exist, and there is an increasing desire to produce the crop in colder environments. Cold-tolerant sugarcane is important both to the sub-tropical sugarcane industries and potential biofuels producers who seek to use sugarcane as a feedstock. Selection for this trait under natural conditions is difficult in sugarcane growing regions because damaging freezes are sporadic. The objective of this study was to identify sugarcane accessions for use in introgression breeding which have above-ground buds that are tolerant to freezing conditions. Above-ground (stalk) buds of 63 *Saccharum*, and 4 *Erianthus* accessions were frozen for 6 days at −7 °C, and germinated buds were counted three weeks post-treatment. Accessions which had more bud cold tolerance than the Louisiana commercial cultivar 'L 97-128' were MPTH97-213, SES114, Guangxi87-22 and SES234A. Heritability estimates for percent reduction in bud germination and height of the shoots following freeze treatment was 0.47 and 0.49, respectively. Identified clones will be used in future breeding efforts at the United States Department of Agriculture, Agricultural Research Service, Sugarcane Research Unit in Houma, LA, USA.

Keywords Sugarcane · Cold tolerance · *Saccharum spontaneum*

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Introduction

With escalating oil prices, there is increasing attention being placed on the development of a sustainable biofuels industry. Based on the Energy Independence and Security Act (EISA) of 2007, the U.S. federal government has mandated the annual production of 60.6 billion L of biofuels from cellulosic crops by 2022 (U.S. Department of Energy 2008). Most ethanol produced in the world is derived from sugarcane in Brazil and corn in the United States. The energy output to input ratio of sugarcane in first generation ethanol production is around 8–10 which exceeds the ratio of

1.6 found in corn. It is expected that the ethanol output (L/ha) might increase 40–50% with the development of efficient lignocellulosic conversion (Waclawovsky et al. 2010). While sugarcane has proven to be a successful biofuel feedstock in Brazil, its utility in the United States is limited because most regions of the country are too cold for sustained and economical production of current sugarcane varieties. If sugarcane is to become a viable feedstock alternative to maize in the United States, the region of adaptation will have to be expanded to include areas other than South Louisiana, Florida, South Texas, and Hawaii.

In order to establish a successful sugarcane crop in cold environments, planted sugarcane stalks containing nodal buds (stalk buds) must be able to survive below-freezing soil temperatures. Since intact sugarcane stalks are used for planting, and not all buds germinate simultaneously, selection for cold tolerance of above ground (stalk) buds would help to establish a plant-cane crop in environments which encounter damaging freezes on a routine basis.

Sugarcane is a crop which is primarily grown between 30°N and 30°S latitude in tropical environments and is generally considered a cold sensitive plant (Tai and Lentini 1998). Although it is considered cold sensitive, studies have shown that sugarcane varieties vary in response to cold temperatures, indicating that selection among hybrids could improve tolerance to cold (Legendre et al. 1985; Du et al. 1999). In (1976), Breaux and Irvine demonstrated parent to offspring transmission of cold tolerance in sugarcane. Others have shown that backcrossing sugarcane to *S. officinarum* reduces the probability of recovering cold tolerant progeny (Brandes 1939; Roach 1971). While breeding for cold tolerance is considered the best way to develop varieties adapted to cold environments, heritability estimates for cold tolerance in sugarcane have not been reported outside of studies conducted at the USDA-ARS in Houma, Louisiana (Hale et al. 2016).

In Louisiana, sugarcane is grown on a commercial scale in a temperate environment, and long-term breeding programs have led to the development of varieties capable of surviving fluctuating temperatures and sustaining a profitable sugarcane industry. Because of frequent damaging frosts and freezes in Louisiana, it is one of the few areas where historical emphasis has been placed on breeding for freeze- and frost-tolerant varieties (Eggleston et al. 2004). Introgression of cold tolerance from wild *Saccharum* species and related genera remains a focus of the USDA-ARS Sugarcane Research Unit's sugarcane breeding program.

Wild relatives of sugarcane have been identified in the past as possessing cold tolerance. In 1940, Brandes identified *Saccharum spontaneum* clones that were able to survive 18 days of below-freezing temperatures (Brandes 1940). Cold tolerant hybrids between *Erianthus* and *Saccharum* have also been identified, indicating that *Erianthus* is another possible source of potentially beneficial genes (Ram et al. 2001). *Miscanthus* represents another source of possible freeze tolerance due to its wide geographic distribution and ability to survive at low temperatures. However, variability in *Miscanthus* species was demonstrated in a study conducted in Southern Germany, indicating care must be taken when choosing parental material for cold tolerance from this genus (Clifton-Brown and Lewandowski 2002). Recent studies conducted at the USDA-ARS Sugarcane Research Unit (SRU) have demonstrated a range in cold tolerance among *Saccharum* accessions (Hale et al. 2013a, b). Furthermore, varieties derived from wild *Saccharum* and grown in the temperate environment of Northern Louisiana have significantly less degradation in the juice following a damaging freeze than commercially grown varieties. Broad-sense heritability for late-season cold tolerance in the two-year study was estimated at $H^2 = 0.78$ (Hale et al. 2016).

Because *S. spontaneum* is considered a noxious weed in the United States, screening of this species cannot be conducted under natural field conditions. Therefore, there is a need for an acceptable bioassay to screen *S. spontaneum* clones under artificial conditions.

The objective of this study was to identify clones in the *Saccharum* complex with viable above-ground buds following exposure to freezing temperatures using a bioassay. An additional objective was to calculate broad-sense heritability estimates on the population of early-generation sugarcane under evaluation.

Materials and methods

A total of 63 *Saccharum* and four *Erianthus* clonal accessions were evaluated for survivability of above-ground (stalk) buds following exposure to freezing temperatures. At the time of the study, the USDA-ARS-Sugarcane Research Unit (SRU) in Houma, LA, collection of wild *Saccharum* accessions was comprised of 16 *S. barberi,* seven *S. robustum*, 11 *S. sinense*, nine *S. officinarum*, and 50 *S. spontaneum* clones. In addition, the SRU maintained 13 *Erianthus* clones. The accessions evaluated represented five different *Saccharum* species as well as commercial sugarcane cultivars. Commercial sugarcane cultivars included in the study were 'Ho 95-988', 'HoCP 00-950', 'HoCP 96-540', and 'L 97-128.' Four *Erianthus arundinaceious* accessions along with five *S. barberi,* four *S. officinarum*, three *S. robustum*, five *S. sinense* and 42 *S. spontaneum* were evaluated. Since it is established that the species with the most variability within the *Saccharum* genus is *S. spontaneum* (Mary et al. 2006), more accessions of this species were utilized in the study than the other species under evaluation. Accessions of the remaining species used in this study were selected because of their diversity in Brix levels (% soluble solids), geographic area of collection, and genetic distance. Within each species, an accession was chosen that was relatively high in Brix, and one that was relatively low compared to other members of the species. Brown et al. (2007) described the genetic diversity within the World Collection of Sugarcane maintained at the Subtropical Horticulture Research Station, Miami, FL, USA. An overlap in the Miami and Houma collections allowed selection of accessions that were (1) common between the two collections, (2) genetically distant within the species, and (3) were not outliers (did not group with a different species in principal component analysis). In addition, four commercial Louisiana cultivars were tested.

Screening experiments were conducted in 2007, 2008, and 2009. One nodal bud per clone was planted in a 2.8L pot in the late winter in each of the three years of the investigation. The experimental pots were arranged in a randomized complete block design, with each clone represented in the three replications. In order to contain the clones according to permit restrictions, they were maintained outside on a concrete isolation pad at the USDA-ARS–SRU, and they were subjected to natural variations in temperature. The plants were allowed to grow from the nodal cuttings and produce multiple stalks per pot during the growing season. Since studies have shown that, in some plants, promoters of certain cold-responsive genes are activated in response to low temperatures, the clones were exposed to increasingly colder temperatures as the season progressed (Thomashow 1999). In each of the three years of the study, the plants were exposed to below-freezing temperatures as long as the sub-zero temperatures were not for a prolonged period of time. In the 2009–2010 season, the plants were moved to a heated greenhouse because temperatures dropped to −6 °C for 3 days and remained below zero for 5 days. The greenhouse was set at 15 °C to ensure that the plants did not freeze.

Stalks of all accessions and commercial cultivars were evaluated for the ability of above-ground buds to survive freezing temperatures. Four treatment stalks per replication were cut and transported to the Natural Resources Conservation Service's (NRCS) Golden Meadow Plant Material's Center in Golden Meadow, LA, where they were frozen with the leaf sheaths on for 6 days at −7 °C. Four additional stalks per replication were cut and stored in the dark at 6 °C as a non-frozen control. Following the freeze treatment, for each replication, 12 nodal buds each from the control and treatment groups were planted in Redi-Earth potting media (Sun Gro Horticulture Canada CM Ltd) in 72-cell Speedling® trays (Speedling, Inc. Sun City, FL) and placed in a heated greenhouse at 15 °C. Three weeks after planting, the number of germinated buds was counted and emerged shoots were measured from the soil level to the tallest growing point.

The percent reduction in bud germination and height of treated versus control plants were calculated using the following formula: $[\text{control} - \text{treated}/\text{control}] \times 100$. The calculated reductions were then analyzed for each trait using the PDIFF option (Saxton 1998) of the PROC MIXED procedure in SAS (SAS Institute 2001) at the $\alpha = 0.05$ level, with clones and years as fixed variables and replications as random.

Variance components estimates were estimated using the VarComp procedure (SAS Institute Inc. 1997) using the following model:

$$Y_{ijkl} = \mu + A_j + R(A)_{jk} + G_i + GA_{ij} + GR(A)_{ijk}$$

where Y_{ijk} is the observation for genotype i, in year j, in replication k nested within year; μ is the overall mean; A_j is the effect of the jth year; $R(A)_{jk}$ is the effect of the kth replication nested within the jth year; GA_{ij} is the interaction between the ith genotype and the *j*th year; $GR(A)_{ijk}$ is the interaction effect between the *i*th genotype and the *k*th replication nested within the *j*th year. All the effects in the model, except μ are random with variance σ^2_{G}, σ^2_{A}, σ^2_{GA},, $\sigma^2_{R(A)}$, σ^2_{GA}, and $\sigma^2_{GR(A)}$.

Broad sense heritability estimates were calculated using the formula:

$$H^2 = \sigma^2_G / \left[\sigma^2_G + \left(\sigma^2_{GA}/y\right) + \left(\sigma^2_{GR(A)}/ry\right)\right]$$

Results

Accessions and species under evaluation are shown in Table 1. In the PROC MIXED analysis, the clones were shown to have a significant effect on reduction in germination ($P = 0.0032$), while year ($P = 0.0985$) and year × clone interaction ($P = 0.3802$) did not (Table 2). The sugarcane cultivars 'Ho 95-988', 'HoCP 00-950', 'HoCP 96-540', and 'L 97-128' were not significantly different than one another for percent reduction in germination. 'L 97-128' had the lowest average percent reduction (82.8%) of the commercial cultivars, thus this cultivar was the commercial cultivar to which the accessions were compared. Clones identified as having significantly higher post-freeze germination than the most freeze tolerant commercial cultivar ('L 97-128') were MPTH97-213 and SES234A.

Subsequent analysis of the percent reduction in height of germinated shoots using PROC MIXED showed a significant clonal effect ($P = 0.0012$), but no significant effect was found for year ($P = 0.1667$) or year x clone interaction ($P = 0.4049$). No significant differences were found in the percent reduction in height of the emerged shoots for the tested commercial cultivars, but the average percent reduction was the lowest for 'L 97-128' (62.4%). Therefore, 'L97-128 was the standard against which the accessions were compared. Accessions showing significantly less reduction in height than 'L 97-128' were SES114, Guangxi87-22, and SES234A.

Heritability in the broad sense was estimated at 0.465 for the post-freeze percent reduction in shoot germination and 0.49 for percent reduction in the height of the germinated shoots following the freeze.

Discussion

The ability of above ground buds to germinate following freezing conditions is a beneficial trait when sugarcane is planted in cold environments. It is most likely that the stalks will be cut and planted prior to the onset of a freeze, but the buds must survive underground if the sugarcane is to emerge the following spring. Tolerance of the stalk buds to freezing conditions is expected to increase the post-winter emergence of sugarcane planted in temperate and cold environments.

The height of the emerged shoots was measured to compare the relative rate of emergence between accessions. Early emergence is desirable in sugarcane cultivars because it extends the growing season. Cultivars with early emergence are able to accumulate more mass before harvest, thus increasing yields.

Heritability estimates for the measured traits of percent reduction in germination and percent reduction in shoot height following the freeze are almost identical and indicate that roughly half of the variation seen in the response to freezing temperatures is under genetic control. Additional research should make use of populations developed from known and divergent parents since these estimates are based on measurements of a diverse set of clones with unknown parents.

Limited data is available on the origin of identified cold tolerant clones in this study. SES 114 was collected in 1948 in Andhra Pradesh in India (17°–35′N 81°–15′E). This accession has 64 chromosomes, and has been reported to have moderate resistance to red rot and ratoon stunting disease (RSD), and resistance to smut. The other Indian variety identified as cold tolerant, SES234A, was collected in 1950 in Bihar India in the town of Bhikna Thoree (27°–25′N 83°–55′E). It was reported to be resistant to red rot and smut, but susceptible to RSD (Kandasami et al. 1983). No original collection notes were available for MPTH97-213 or Guangxi87-22; however, the clones were obtained by the USDA from Thailand and Guangxi China, respectively.

One of the accessions identified in this study, SES234A, has several cold tolerant progeny that are currently under investigation in multiple sites across the United States. F_1 progeny from this accession are known to produce ratoon crops in areas as far north as Booneville, Arkansas (35°08′24.33″N 93°55′17.73″W elevation 175 m). These progeny also have

Table 1 Accessions and standard varieties included in a growth chamber assay for cold tolerance

Clone	Species/Genus	Clone	Species/Genus
Ganapathy	*barberi*	IND82-257A	*spontaneum*
Hatuni	*barberi*	IND82-311	*spontaneum*
Newra	*barberi*	MOL1032A	*spontaneum*
Reha	*barberi*	MOL1032B	*spontaneum*
Tereru	*barberi*	MPTH97-200	*spontaneum*
Ho95-988	commercial	MPTH97-204	*spontaneum*
HoCP00-950	commercial	MPTH97-209	*spontaneum*
HoCP96-540	commercial	MPTH97-213	*spontaneum*
L97-128	commercial	MPTH97-216	*spontaneum*
MPTH97-221	*Erianthus*	MPTH97-218	*spontaneum*
MPTH97-260	*Erianthus*	MPTH98-388	*spontaneum*
MPTH98-326	*Erianthus*	PCANOR84-2A	*spontaneum*
SES288	*Erianthus*	PCAV84-12A	*spontaneum*
Fiji1	*officinarum*	PCAV84-12B	*spontaneum*
LA Purple	*officinarum*	PCAV84-12C	*spontaneum*
LA Stripe	*officinarum*	PIN84-1B	*spontaneum*
Muntok Java	*officinarum*	PQ84-3	*spontaneum*
IM72-232	*robustum*	S66-84A	*spontaneum*
IS76-184	*robustum*	S66-84B	*spontaneum*
NG77-159	*robustum*	SES006	*spontaneum*
Chuk Che	*sinense*	SES114	*spontaneum*
Katha	*sinense*	SES147B	*spontaneum*
Tukuyu1	*sinense*	SES189	*spontaneum*
Uba del Natal	*sinense*	SES205A	*spontaneum*
Uba Naquin	*sinense*	SES231	*spontaneum*
Djatiroto	*spontaneum*	SES234A	*spontaneum*
Guangxi86-05	*spontaneum*	SES234B	*spontaneum*
Guangxi87-21	*spontaneum*	SES323A	*spontaneum*
Guangxi87-22	*spontaneum*	SES84-58	*spontaneum*
IMP9089	*spontaneum*	SH249	*spontaneum*
IND81-144	*spontaneum*	Tainan	*spontaneum*
IND81-161	*spontaneum*	US56-13-7	*spontaneum*
IND81-165	*spontaneum*	US56-15-8	*spontaneum*
IND81-80	*spontaneum*		

Accessions include *S. barberi*, commercial sugarcane cultivars, *Erianthus arundinaceious* (Erianthus), *S. officinarum, S. robustum, S. sinense*, and *S. spontaneum*

demonstrated exceptional late-season freeze tolerance, with less degradation in the juice following damaging freezes (Hale et al. 2016). In addition, the leading energycane variety, Ho 02-113, is a progeny of this *S. spontaneum* accession (Hale et al. 2013a, b).

The results from this study show the ability of a subset of the screened cultivars and accessions to survive artificially-induced freezing temperatures. While it is not known how this measurement of freeze tolerance relates to natural freezing, the bioassay identified SES234A which indicates there is a relationship between the bioassay and natural conditions.

Under current commercial production practices, sugarcane seed is cut and planted before the occurrence of a killing freeze. With the pending emergence of a bioenergy industry based on a mandate by the U.S. federal government to significantly increase the amount of biomass-based fuels over the next 20 years, there is a need for the development of high-biomass crops adapted to all areas of the US (U.S. Department

Table 2 F ratio and p values of effects on percent reduction in shoot number and height for genotype, year, and genotype × year interactions

Effect	Shoot number		Height	
	F ratio	P value	F ratio	P value
Clone	1.68	0.0032	1.78	0.0012
Year	4.60	0.0985	2.85	0.1667
Clone × year	1.06	0.3802	1.04	0.4049

of Energy 2008). High-fiber sugarcanes, also known as energy canes, have become a target of research for bioenergy feedstock development for this potential biofuels industry (Bransby et al. 2010). If sugar/energy cane is to become a major component of the U.S. renewable fuels industry, the area of adaption must be increased to colder climates. In the expanded climate zones, viability of above-ground buds following freezing conditions may be of increased concern due to post-freeze deterioration, which can reduce sugar and fiber quality. Deterioration could lead to decreased quantity and quality of feedstock and could impose complications in the processing into biofuels (Eggleston et al. 2004).

An overall level of stress tolerance will be required if energy cane is to become a viable bioenergy feedstock in the U.S. and abroad. With increasing land area devoted to cane production, stress tolerance is becoming an increasing issue as production areas are being expanded to marginal soils (Roka et al. 2010). The selected accessions will be used as parents, and their progeny used to validate the assay under natural conditions, and to breed for sugarcane with enhanced cold tolerance.

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References

Brandes EW (1939) Three generations of cold resistant sugarcane. Sugar Bull 18(4):3–5

Brandes EW (1940) Survival of wild sugarcane buds exposed to below zero (F.) temperatures. Sugar Bull 18(16):3–4

Bransby DI, Allen DJ, Gutterson N, Ikonen G, Richard EP Jr, Rooney W, van Santen E (2010) Engineering advantages, challenges, and status of grass energy crops. In: Mascia PN, Scheffran J, Thomas SR, Widhom JM (eds) Plant Biotechnology for sustainable production of energy and co-products. Springer, Berlin

Breaux RD, Irvine JE (1976) Selection for cold tolerance in sugarcane seedlings from new germplasm. Proc Am Soc Sugar Cane Technol 5:178–181

Brown SJ, Schnell RJ, Power EJ, Douglas SL, Kuhn DN (2007) Analysis of clonal germplasm from five *Saccharum* species: *S. barberi*, *S. robustum*, *S. officinarum*, *S. sinense*, and *S. spontaneum*. A study of inter-and intra-species relationships using microsatellite markers. Genet Resour Crop Evol 54:627–648

Clifton-Brown JC, Lewandowski I (2002) Screening *Miscanthus* genotypes in field trials to optimize biomass yield and quality in Southern Germany. Eur J Agron 16:97–110

Du YC, Nose A, Wasano A (1999) Thermal characteristics of C4 photosynthetic enzymes from leaves of three sugarcane species differing in cold sensitivity. Plant Cell Physiol 40:298–304

Eggleston G, Legendre B, Tew TL (2004) Indicators of freeze-damaged sugarcane varieties which can predict processing problems. Food Chem 87:119–133

Hale AL, Dufrene EO, Tew TL, Pan Y-B, Viator RP, White PM, Veremis JC, White WH, Cobill R, Richard EP, Rukavina H, Grisham MP (2013a) Registration of 'Ho 02-113' Sugarcane. J Plant Regist 7(1):51–57

Hale AL, Viator RP, Veremis JC (2013b) Identification of freeze tolerant *Saccharum spontaneum* accessions through a pot-based study for use in sugarcane germplasm enhancement for adaptation to temperate climates. Biomass Bioenergy 61:53–57

Hale AL, Viator RP, Eggleston G, Hodnett G, Stelly DM, Boykin D, Miller DK (2016) Estimating broad-sense heritability and investigating the mechanism of genetic transmission of cold tolerance using mannitol as a measure of post-freeze juice degradation in sugarcane and energy-cane. J Agric Food Chem 64(8):1657–1663

Kandasami PA, Sreenivasan TV, Ramana Rao TC, Palanchami K, Natarajan BV, Alexander KC, Madhusudana Rao M, Mohan Rao D (1983) In Catalouge on Sugarcane Genetic Resources *1. Saccharum Spontaneum* L. Sugarcane Breeding Institute. Indian Council of Agriculture Research, Coimbatore

Legendre BL, Tsang WS, Clarke MA (1985) Changes in juice composition of sugarcane as affected by post-freeze deterioration in Louisiana. In Proceedings of the 1984 Sugar Proc Res Conf, New Orleans, LA, pp 92–107

Mary S, Nair NV, Chaturvedi PK, Selvi A (2006) Analysis of genetic diversity among *Saccharum spontaneum* L. from four geographical regions of India, using molecular markers. Genet Resour Crop Evol 53(6):1221–1231

Ram B, Sreenivasan TV, Sahi BK, Singh N (2001) Introgression of low temperature tolerance and red rot resistance from *Erianthus* in sugarcane. Euphytica 122:145–153

Roach BT (1971) Nobilization of Sugarcane. Proc Int Soc Sugar Cane Technol 14:206–216

Roka FM, Baucum LE, Rice RW, Alvarez J (2010) Comparing costs and returns for sugarcane production on sand and muck soils of Southern Florida, 2008–2009. J Am Soc Sugar Cane Technol 30:50–66

Saxton AM (1998) A macro for converting mean separation output to letter groupings in Proc Mixed. In Proceedings of 23rd SAS Users Group Intl, SAS Institute, Cary, pp 1243–1246

Tai PYP, Lentini RS (1998) Freeze damage of Florida sugarcane. In: Anderson DL (ed) Sugarcane handbook, Ed. 1. Florida Cooperative Extension Service. University of Florida, Gainesville, p 1

Thomashow MF (1999) Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Ann Rev Plant Physiol Plant Mol Biol 50:571–599

United States Department of Energy (2008) Ethanol Myths and Facts; Department of Energy; Biomass Program, Washington

Waclawovsky AJ, Sato PM, Lembke CG, Moore PH, Souza GM (2010) Sugarcane for bioenergy production: an assessment of yield and regulation of sucrose content. Plant Biotech J 8:263–276