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FINAL REPORT
SRDC PROJECT BS60S
EFFECT OF CULTURE CONDITIONS ON GROWTH AND GENETIC INTEGRITY OF *IN VITRO* SUGARCANE PLANTS

by
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CONTENTS

		Page No
1.0	SUMMARY	1
2.0	OBJECTIVE	1
3.0	BACKGROUND	1
4.0	MATERIALS AND METHODS	2
	4.1 Establishment of *in vitro* plants from apical meristems	2
	4.2 Establishment of embryogenic callus cultures and plant regeneration	3
	4.3 Protoplast callus	3
	4.4 Morphological analysis	3
	4.5 Isozyme analysis	3
	4.5.1 Protein extraction	3
	4.5.2 Electrophoresis	4
	4.6 RAPD analysis	4
	4.6.1 DNA isolation	4
	4.6.2 Polymerase chain reaction	4
5.0	RESULTS AND DISCUSSION	5
	5.1 *In vitro* plants establishment for apical meristems	5
	5.1.1 Morphological analysis	5
	5.1.2 Isozyme analysis	6
	5.1.3 RAPD analysis	7
	5.2 Plant regeneration from embryogenic callus and protoplast-derived callus	8
	5.2.1 Morphological analysis	8
	5.2.2 RAPD analysis	8
6.0	RECOMMENDATIONS	9
7.0	PUBLICATIONS ARISING FROM PROJECT	9
8.0	PUBLICATIONS IN PREPARATION	10
9.0	ACKNOWLEDGMENTS	10
10.0	REFERENCES	10

1.0 SUMMARY

Isozyme analysis using cellulose acetate gel electrophoresis proved to be a reliable method for differentiating sugarcane cultivars. Advantages of this technique over starch and polyacrylamide gels are 1) very small sample volumes are required (3-5 l), analytical runs are quick (30-60 min depending on buffer system), cost of running is reduced by using less stain, and the method for application of samples is semi-automated. Isozymes did not detect any genetic change in sugarcane plants regenerated from apical meristems and stored *in vitro* for up to 12 months.

Random amplified polymorphic DNA (RAPD) analysis using 10-mer oligonucleotide primers efficiently differentiated sugarcane cultivars and proved suitable for detecting gross genetic change such as that which can occur in sugarcane subjected to prolonged tissue culture, for example in protoplast-derived callus. RAPD analysis of sugarcane plants regenerated from apical meristems and embryogenic callus revealed very few polymorphisms, indicating that gross genetic change is infrequent during this tissue culture procedure, although epigenetic effects result in transient morphological changes especially in plants regenerated from embryogenic callus.

These results indicated that *in vitro* culturing and storage from apical meristems or embryogenic callus may not have any effect on the genetic integrity of the plants as tested by isozyme and RAPD analysis. However, more sensitive variations on the RAPD technique may increase the practicality of DNA based screening of regenerated plant lines to reveal somaclonal variants.

2.0 OBJECTIVE

To evaluate the effects of *in vitro* culture conditions on the field growth and genetic integrity of *in vitro* plants.

3.0 BACKGROUND

Genetic variability, termed somaclonal variation, can be generated during tissue culture (Larkin and Scowcroft 1981). The frequency of somaclonal variation may increase with length of time in culture (Scowcroft 1984).

Preserving the genetic integrity of each cultivar is fundamental to the success of long term *in vitro* storage of sugarcane germplasm. In preliminary field trials no differences in morphological characters were detected in plants of four sugarcane cultivars derived from either *in vitro* plants stored for 14 months or from the field, except that the *in vitro* plants had more tillers (P W J Taylor, unpublished). Researchers at CIRAD in France showed that some variation in stalk thickness and tillering occurred in plants that had been in culture at 18°C for up to nine years prior to propagation in the field. However, this variation disappeared after the first vegetative planting in field trials, and so was assumed to be epigenetic (Taylor, 1990).

Biochemical assessment of genetic integrity based on comparison of extracted leaf proteins is more cost and time efficient than field trials. Isozyme analysis using cellulose acetate plate electrophoresis has been used to study genetic diversity in tomato, sunflower and sorghum (Mather *et al.* 1993; Hughes *et al.* 1991). Molecular diagnostic techniques using polymerase chain reaction (PCR) and random amplified polymorphic DNA (RAPD) markers have been used for distinguishing plant species and cultivars, such as the cereals (Ko *et al.* 1994) and *Festuca* (Vallés *et al.* 1993); for genetic mapping (Williams *et al.* 1990; Torres *et al.* 1993; Yu and Pauls 1993); and for analysing the genetic stability of tissue cultured plants (Vallés *et al.* 1993). RAPDs are more specific than isozymes in that they allow analysis of genes rather than gene products.

If permanent changes in leaf protein or DNA profiles occur during *in vitro* culture and storage, then the frequency of such changes may be used to monitor and optimise culture and storage conditions. This paper evaluates the effects of *in vitro* culture conditions on the field growth and genetic integrity of *in vitro* plants using morphological, biochemical and molecular analysis.

4.0 MATERIALS AND METHODS

Two tissue culture systems and three methods of analysis were used to study the genetic stability of *in vitro* plants. Plants were regenerated from apical meristems and embryogenic callus, planted in field trials, and analysed using morphological, biochemical (isozyme) or molecular (RAPD) markers. The genetic stability of protoplast-derived callus was also analysed for comparison. Plants could not be regenerated from this callus thus indicating that some genetic mutations could have taken place during the formation of the callus.

4.1 Establishment of *in vitro* plants from apical meristems

Apical meristems surrounded by two to three whorls of developing leaves were aseptically excised from stalks of six- to eight-month old field grown plants of five genotypically diverse *Saccharum* spp. hybrid cultivars (CL65-279, H50-3511, H73-6110, M1819-63 and TS67-74) and cultured on MS medium as described by Taylor and Dukic (1993). *In vitro* plants were maintained at 18°C for three, six, and 12 months. Four plants of each were established in pots in the glasshouse, then transferred to the field at Meringa Sugar Experiment Station. After 12 months, a three-replicate field trial was planted with plants of the five cultivars originating from the three *in vitro* treatments plus a control treatment of field-grown plants. Setts of field-grown plants were planted adjacent to each sett from plants originating from the *in vitro* treatments. Each replicate consisted of two to four plants from each *in vitro* treatment plus eight to 12 plants from the control treatment of field-grown plants.

4.2 Establishment of embryogenic callus cultures and plant regeneration

Embryogenic callus was initiated from immature leaf explants of sugarcane (*Saccharum* spp. hybrid) cultivar Q63 cultured on MS medium and was selectively subcultured, as described by Taylor *et al.* (1992a). After 4.5 months of culture, embryogenic callus was transferred to solid MS medium without plant growth regulators and incubated under diffuse light with a 12-h photoperiod at 27°C. Individual plantlets were then transferred to rooting medium (MS with 60 g/l sucrose and 1 mg/l NAA; Taylor and Dukic 1993) before transfer to pots for growth in the glasshouse.

4.3 Protoplast callus

Non-morphogenic callus was regenerated from protoplasts isolated from a homogeneous suspension culture of cell line Q63 SP (Taylor *et al.* 1992a; 1992b). This callus was maintained for more than two years by subculture onto fresh medium every three weeks.

4.4 Morphological analysis

The number of stalks were counted for each plant, four to five months after planting a field trial consisting of plants established from apical meristems and stored *in vitro* for various lengths of time. Phenotypic characters such as stalk internode colour; bud groove and shape, width of bud wing; leaf width, length and carriage; and auricle shape were assessed.

4.5 Isozyme analysis

Isozyme analysis was performed on plants established from apical meristems and stored *in vitro* for three, six and 12 months; and on field-grown plants.

4.5.1 Protein extraction

Four to five single bud cuttings, taken from two to three plants from each plot in the three replicate trial of CL65-279, H50-3511, H73-6110, M1819-63 and TS67-74 cultivars, were placed in vermiculite and incubated in a growth chamber in the dark at 29°C. Enzymes were extracted from leaves of young shoots two to four weeks after planting. The extraction buffer used was specifically designed to reduce the effects of phenolic compounds and contained 50 mM Tris-HCl (pH 5.5), 0.1% BSA, 0.5% DTT, 0.1% Triton X-100 and polyvinylpolypyrrolidone (50 mg/mL buffer). Usually, the pH of Tris buffers ranges from 7.0-8.0 however, for sugarcane it was necessary to use a lower pH 5.5, which is the same as the pH of freshly crushed cane juice. Young shoot tissue was homogenised by mechanical grinding with a mortar and pestle at 4°C to avoid enzyme degradation by phenolic compounds. The best ratio of tissue to extraction buffer was 100 mg tissue to 50 µL of buffer. The homogenates were centrifuged for 10 min at 6000 rpm at 4°C. After centrifugation, the supernatant was immediately loaded onto Titan III cellulose acetate plates (Helena Laboratories, Beaumont, Texas) for electrophoresis.

4.5.2 Electrophoresis

Cellulose acetate plates were pre-soaked for 30 min in the same buffer as used in the electrophoresis tank and samples were applied using a 12-sample applicator (Helena Laboratories, Beaumont, Texas). Electrophoresis was carried out at 4°C with either buffer, 75 mM Tris citrate (pH 7.0) for 30 min at 150 V or 50 mM Tris glycine (pH 8.5) for 30 min at 200 V, depending on the particular isozymes to be detected.

Staining methods used for detecting specific isozymes were adapted from Holmes (unpublished data) and Richardson *et a*l. (1986). Prior to staining, 3 mL of 1.5% molten agar (maintained at 60°C) was mixed with the stain solution and poured over the plates. Once the agar had set, the plates was incubated at 37°C until bands appeared. The plates were scored and photographed, before or after the agar and stain mixture was washed off and the plates left to dry. The dried plates retain the banding pattern and were kept as a permanent record. Isozyme assays were repeated to establish reproducibility of results.

4.6 RAPD analysis

RAPD analysis was performed on plants established from apical meristems and stored *in vitro* for three, six and 12 months, and field-grown plants; plants regenerated from embryogenic callus; and protoplast-derived callus.

4.6.1 DNA isolation

DNA was extracted using the modified CTAB method of Graham *et al.* (1994). Samples for DNA extraction were: (1) the leaves of young shoots that were sampled for isozyme analysis from plants of cultivars CL65-279, H50-3511, H73-6110, M1819-63 and TS67-74 in the three replicate field trial; (2) the spindle leaves of each of eight Q63 plants grown in the glasshouse for 12, 16 and 17 months after regeneration from embryogenic callus; and of five field-grown plants of Q63; and (3) protoplast-derived callus of Q63 SP. Genomic DNA was quantified spectrophotometrically (Sambrook *et al.* 1989).

4.6.2 Polymerase chain reaction

A total of 25 arbitrary 10-mer oligonucleotide sequences (Operon Technologies, USA) was screened for amplification of plant genomic DNA fragments. Primers OPB-11, OPC-14, OPC-15, OPD-01 and OPM-01 were used to amplify protoplast-derived genomic DNA. Conditions for DNA amplification were standardised for all primers. Each 25 µl reaction volume contained: 30 ng DNA template, 0.8 U *Taq* DNA polymerase (Boehringer Mannheim Biochemica), 0.24 mM each of dATP, dGTP, dTTP and dCTP (Promega Corporation), 0.2 µM primer and PCR buffer with a final concentration of 10.0 mM Tris-HCl, pH 8.3, 50.0 mM KCl, 3.0 mM $MgCl_2$, 0.1 mg/ml gelatin.

Amplification was performed in a Perkin Elmer 9600 GeneAmp PCR System and was initiated by a denaturation of 1 min at 94°C, followed by 33 cycles of 10 sec each at 94°C, 30 sec at 40°C and 1 min at 72°C. The amplification was completed with one cycle of 5 min at 72°C. Reaction products were resolved by electrophoresis on 1.5% (w/v) agarose gels, stained with ethidium bromide and revealed by UV illumination. PCR reactions were repeated to establish reproducibility of results.

5.0 RESULTS AND DISCUSSION

5.1 *In vitro* plants establishment from apical meristems

A field trial was established with plants of five genotypically diverse *Saccharum* spp. hybrid cultivars that were stored *in vitro* at 18°C for three, six and 12 months; and field-grown plants. Phenotypic, biochemical and molecular characters of cultivars were compared to assess effects of *in vitro* culture conditions on genetic integrity.

5.1.1 Morphological analysis

For each cultivar in the field trial there was no large difference in the number of stalks between treatments four to five months after planting (Table 1) except with H50-3511 which had less stalks per plant in plants originating from a six month *in vitro* treatment. The few plants that germinated in this treatment were stunted and less vigorous than the field-grown plants which may have reflected poor quality planting material. The reason for the poor quality planting material is unknown but may have been due to difficulties in establishing the plants in the glasshouse after *in vitro* culture, however, the same problems were not encounted for plants originating from the 12 month *in vitro* treatment or for plants of other cultivars grown under the same treatment.

Table 1

Number of stalks from plants in a three replicate field trial after in vitro storage for 3, 6 and 12 months. Results are the means . SE for the number of plants shown in brackets.

Cultivar	Number of stalks			
	Field-grown plants	*In vitro* storage time (months)		
		3	6	12
CL65-279	9 . 0.5 (27)	10 . 2.4 (6)	10 . 1.1 (9)	10 . 0.7 (12)
H50-3511	8 . 0.5 (27)	10 . 0.8 (12)	5 . 2.4 (5)	7 . 1.6 (6)
H73-6110	9 . 0.6 (24)	10 . 1.0 (12)	10 . 0.5 (12)	-
M1819-63	7 . 0.5 (30)	9 . 0.8 (12)	8 . 1.4 (9)	9 . 0.5 (12)
TS67-74	10 . 0.4 (36)	8 . 0.8 (12)	8 . 0.6 (12)	8 . 0.7 (12)

There were no differences in stalk internode colour; bud groove and shape, width of bud wing; leaf width, length and carriage; auricle shape. These results indicated that the *in vitro* culture technique had not affected morphological characters.

5.1.2 Isozyme analysis

Fifteen isozymes (Table 2) were developed for use as biochemical markers in sugarcane using cellulose acetate gel electrophoresis with the resolution of over 30 distinct bands showing presence versus absence type of variation. Cellulose acetate electrophoresis proved to be a reliable method for resolving isozymes from sugarcane tissue. Advantages of this technique over starch and polyacrylamide gels are 1) very small sample volumes are required (3-5 l), analytical runs are quick (30-60 min depending on buffer system), cost of running is reduced by using less stain, and the method for application of samples is semi-automated.

Preliminary experiments demonstrated the effectiveness of these isozymes to differentiate polymorphisms between six sugarcane cultivars (Dukic *et al.* 1994). Isozyme analysis of plants of the five cultivars that were stored as *in vitro* plants for three, six and 12 months, and then planted in the field, showed that each cultivar could be distinguished by distinct polymorphisms and there were no major differences in isozymes between replicate plants of each cultivar and between the length of time in *in vitro* storage. Peroxidase with six polymorphic loci (Prx-1, Prx-2, Prx-3, Prx-4, Prx-9 and Prx-10) provided the best discrimination between cultivars. This was due to the number of alleles observed at these loci and their distribution across the examined cultivars.

Table 2

Enzymes and abbreviations used for isozyme analysis in sugarcane

Enzyme	Abbrev.	Enzyme	Abbrev.
Acid phosphatase	ACP	Malate dehydrogenase	MDH
Aconitase	ACON	6 phosphogluconate dehydrogenase	PGD
Adenylate kinase	ADK	Phosphoglucomutase	PGM
Alcohol dehydrogenase	ADH	Phosphoglucose isomerase	PGI
Aspartate aminotransferase	AAT	Peroxidase	PRX
Esterase D	EST-D	Superoxide dismutase	SOD
Hexokinase	HK	Peptidase(A,B,C & D)	PEP
Isocitrate dehydrogenase	IDH		

These results indicated that *in vitro* culturing and storage may not have had an effect on the genetic integrity of the plants as tested by isozyme analysis. However, isozyme analysis may not be specific or sensitive enough to detect some genomic changes that can alter genetic integrity.

5.1.3 RAPD analysis

RAPD analysis of genomic DNA of sugarcane resolved 112 scorable markers, from 13 primers out of 25 primers screened (Table 3). Primers produced between 4 and 15 amplification products, which ranged in size between approximately 400 and 3800 bp. The

primers that did not produce scorable markers either produced faint or non-consistent amplification products, or no amplification product.

RAPD analysis failed to reveal any polymorphism between plants that had been stored *in vitro* for three, six and 12 months; and field-grown plants for each sugarcane cultivar CL65-279, H50-3511, H73-6110, M1819-63 and TS67-74 regenerated from apical meristems.

A selection of six primers (OPB-11, OPC-13, OPC-15, OPC-16, OPD-01, and OPM-05) was sufficient to reveal multiple polymorphisms between all tested cultivars. For example, primers OPC-15 and OPC-16 produced a distinct pattern of amplification products for each cultivar. Consistent banding profiles were obtained for replicates of each cultivar of sugarcane. The level of polymorphisms between the cultivars tested in this study indicates that distinction between any two cultivars of sugarcane should be possible with a small number of appropriate primers. This level of polymorphism is not surprising given that sugarcane cultivars are highly heterozygous, polyploid, interspecific hybrids obtained by outcrossing of diverse parents (Sreenivasan *et al.* 1987).

Table 3

Arbitrary 10-mer primers used in RAPD analysis of sugarcane plants and protoplast-derived callus

Primer[a]	Sequence 5' - 3'	No. of scorable bands	Primer[a]	Sequence 5' - 3'	No. of scorable bands
OPB-02	TGATCCCTGG	4	OPD-05	TGAGCGGACA	5
OPB-03	CATCCCCCTG	0[b]	OPD-08	GTGTGCCCCA	0
OPB-05	TGCGCCCTTC	0[b]	OPE-01	CCCAAGGTCC	0
OPB-06	TGCTCTGCCC	0	OPE-02	GGTGCGGGAA	0
OPB-08	GTCCACACGG	0	OPE-05	TCAGGGAGGT	7
OPB-11	GTAGACCCGT	10	OPE-08	TCACCACGGT	0
OPC-13	AAGCCTCGTC	9	OPM-01	GTTGGTGGCT	8
OPC-14	TGCGTGCTTG	10	OPM-05	GGGAACGTGT	6
OPC-15	GACGGATCAG	15	OPM-06	CTGGGCAACT	12
OPC-16	CACACTCCAG	8	OPM-13	GGTGGTCAAG	4
OPD-01	ACCGCGAAGG	14			

[a] Primers from Operon Technologies, USA.
[b] Amplified product was present but bands were faint and not consistent.

5.2 Plant regeneration from embryogenic callus and protoplast-derived callus

5.2.1 Morphological analysis

All the plants regenerated from embryogenic callus of cultivar Q63 showed some morphological variation compared to the field-grown source plants of the same cultivar. This variation consisted of profuse tillering, twisting of the leaf lamina giving a crinkled appearance and in several plants the spindle leaf was enclosed within an elongated leaf sheath. However, propagation of single-bud cuttings from these plants resulted in plants with typical morphology for cultivar Q63. The morphological changes observed in Q63 plants regenerated from embryogenic callus did not persist through vegetative propagation, indicating that these changes were epigenetic.

5.2.2 RAPD analysis

PCR amplification products for the eight regenerated plants were identical to the field-grown Q63 plants, except with primer OPM-06 where the 950 bp fragment was missing from two of the plants. The polymorphism in these two plants was consistent in three DNA extractions over a five month period and after the plants were vegetatively propagated. Tissue culture conditions are expected to lead to peculiar patterns of gene expression in plant cells, which may cause some transient phenotypic changes in regenerated plants. The uniformity of RAPD patterns in these plants and the parent cultivar is consistent with this explanation.

Taylor *et al.* (1995) also reported the failure of RAPD analysis to reveal any polymorphism in transgenic plants of sugarcane cultivar Pindar regenerated from embryogenic callus after insertion of the *neo* gene. It is thought that somaclonal variation may be less frequent in plants regenerated from young embryogenic cultures than from other tissue culture systems, because the necessity for somatic embryo formation from a single cell selects against many genetically altered cells (Vasil 1988). Chowdhury and Vasil (1993) also found no variation detectable by RFLP analysis in sugarcane plants regenerated from embryogenic cultures. In contrast, plants regenerated from non-embryogenic tissue cultures of *Beta vulgaris* and *Oryza sativa* showed substantial variation by RFLP analysis (Sabir *et al.* 1992, Muller *et al.* 1990).

The single polymorphism revealed in plants of sugarcane cultivar Q63 regenerated from 4.5-month-old embryogenic callus could indicate: (1) a cell line which has undergone a substantial genetic change that does not interfere with regeneration such as loss of a chromosome, (2) a chance coincidence of a minor mutation with an OPM-06 primer site, or (3) an OPM-06 amplicon spanning a highly mutable genomic site. The failure of other primers to reveal the change, and the same polymorphism in two separate regenerants are consistent with the third possibility. This possibility is interesting because the identification of such amplicons would allow the selection of primers with the highest probability of detecting genetically altered lines. It will be necessary to screen more regenerated plants and more primers to test this possibility.

Protoplast-derived callus of Q63 SP cell line showed a high degree of genetic change relative to the source cultivar with polymorphisms being detected by primers OPB-11, OPC-14, OPC-15, OPD-01 and OPM-01. This callus had been in tissue culture for over two years and had lost the ability to differentiate shoots and regenerate plants. It is likely that this cell line has undergone rearrangement, mutation and/or loss of chromosomes (Karp *et al.* 1987).

In this study, effective RAPD primers produced an average of 12 scorable PCR products with an average size of 1.1 Kb. Thus the length of DNA scored for polymorphism per primer averaged 13.2 Kb, or 0.0001% of the typical sugarcane genome size of 1.2 x 10^7 Kb (2C). Even in this portion of the genome, DNA base changes, base substitutions, short deletions or short insertions will not result in detectable polymorphisms unless they occur within a primer binding site. It follows that unless a very large number of primers is used, RAPD analysis will lack the sensitivity to reliably detect small genetic changes due to somaclonal variation.

Recent modifications to the basic RAPD approach may reduce the number of primers required to provide reasonable sensitivity of detection of genetic change. For example, DNA amplification fingerprinting or DAF (Caetano-Anollés *et al.* 1991) uses shorter primers to generate many potential amplicons for comparison, predigestion with a restriction enzyme to eliminate many shared potential amplicons, polyacrylamide gels for improved resolution of bands, and silver staining for increased sensitivity of detection. In another more recent technique known as amplified fragment length polymorphism (AFLP; B Carroll unpublished), specific primer binding sites are produced by ligating adapters with specific sequences to DNA fragments (predigested with restriction enzymes). Generated amplicons are resolved using sequencing gels and sensitivity of detection is improved by using [^{33}P]-end labeled primers. These modifications make the technique somewhat more technically demanding, but the increased sensitivity should make DNA-based screening for somaclonal variants more practical, particularly if combined with primers shown to amplify mutable regions of the plant genome.

6.0 RECOMMENDATIONS

Assess the genetic stability of *in vitro* cultured plants of various species of *Saccharum* and *Erianthus* under various storage conditions. (SRDC Project BS59S addresses this recommendation).

The molecular techniques developed in this project could be used to screen tissue cultured plants transformed with novel genes using the microprojectile technique, for somaclonal variation.

7.0 PUBLICATIONS ARISING FROM PROJECT

Taylor, P W J, Geijskes, J, Ko, H-L, Fraser, T A, Henry, R and Birch, R G (1995). Sensitivity of random amplified polymorphic DNA analysis to detect genetic change in sugarcane during tissue culture. Theoretical and Applied Genetics (in press).

Dukic, S, Taylor, P W J and Mather, P B (1995). Cellulose acetate electrophoresis of

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Dukic, S, Mather, P B and Taylor, P W J (1994). Development of biochemical genetic markers in sugarcane using cellulose-acetate electrophoresis. 4th International Congress of Plant Molecular Biology, Amsterdam, The Netherlands, no. 1867.

8.0 PUBLICATIONS IN PREPARATION

Taylor, P W J and Fraser, T A (1995). Effect of *in vitro* storage conditions on genetic stability of sugarcane.

9.0 ACKNOWLEDGMENTS

The following participated in this project: Snezana Dukic (BSES) developed the isozyme technique for sugarcane, Tracy Fraser (BSES) and Lien Ko (QABC) assisted in the application of the RAPD technique to sugarcane, Warren Owens (BSES) assisted in the planting of the field trial and in the morphological analysis. Nils Berding (BSES), Peter Mather (QUT) and Robert Henry (QABC) also provided guidance in the development of techniques during this project.

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