TRANSCRIPTOMIC ANALYSIS OF SOURCE-SINK DYNAMICS ASSOCIATED WITH SUGAR ACCUMULATION IN SUGARCANE

THESIS
SUBMITTED TO
BABASAHEB BHIMRAO AMBEDKAR UNIVERSITY
LUCKNOW

BABASAHEB
BHIMRAO
AMBEDKAR
UNIVERSITY
प्रज्ञा शील करुणा
ESTABLISHED 1996

FOR THE DEGREE OF
Doctor of Philosophy
IN
BIOTECHNOLOGY

By

KRITI ROOPENDRA
Enrolment no.:236/13

Under the Supervision of

Co-supervisor
Dr. AMARESH CHANDRA
Principal Scientist
Plant Physiology and Biochemistry Division
Indian Institute of Sugarcane Research
Lucknow (U.P.)-226002

Supervisor
Dr. SANGEETA SAXENA
Professor
Department of Biotechnology
Babasaheb Bhimrao Ambedkar University
Lucknow (U.P.)-226025

2019

CERTIFICATE

This is to certify that the thesis titled **'TRANSCRIPTOMIC ANALYSIS OF SOURCE-SINK DYNAMICS ASSOCIATED WITH SUGAR ACCUMULATION IN SUGARCANE'** submitted by **Ms. Kriti Roopendra** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other University.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow, satisfies all the requirements as stipulated in the *Doctor of Philosophy (Ph.D.) regulation-1999 as amended in 2008/2010/2013* and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

Co-Supervisor

Sangeeta

Supervisor

Date: 18/1/2019

Head of the Department

Scanned by CamScanner

DECLARATION

I, **Kriti Roopendra** hereby declare that the thesis titled **'TRANSCRIPTOMIC ANALYSIS OF SOURCE-SINK DYNAMICS ASSOCIATED WITH SUGAR ACCUMULATION IN SUGARCANE'** is an authentic research work carried out by me under the guidance of Dr. Sangeeta Saxena, Professor, Department of Biotechnology, Babasaheb Bhimrao Ambedkar University (A Central University), Lucknow and Dr. Amaresh Chandra, Principal Scientist, ICAR-Indian Institute of Sugarcane Research, Lucknow. The research work is original, and no part of this work has been submitted for any other degree or diploma.

I also declare that the thesis is essentially free from all kinds of plagiarism.

All the above given information is true to the best of my knowledge.

Date: 18|1|2019

Submitted by

Kriti

(Kriti Roopendra)
Enrolment No. 236/13
Department of Biotechnology,
BBAU, Lucknow (U.P.) -226025

Scanned by CamScanner

ACKNOWLEDGEMENTS

I am grateful to God for blessing me with the opportunity of pursuing and successfully accomplishing my research work, for award of the valuable Ph.D. degree. It was my great fortune to have worked under the dynamic supervision of Dr. Amaresh Chandra, Principal Scientist, ICAR-IISR, Lucknow and Dr. Sangeeta Saxena, Professor, Department of Biotechnology, BBAU, Lucknow. Their able guidance, painstaking instructions, co-operation and encouragement at every step, inspired me to 'strive for better' throughout this journey. My research work could take its present shape only due to their valuable suggestions and mentoring.

I am also thankful to the former Director, Dr. S. Solomon and now Director, Dr. A.D. Pathak, IISR, Lucknow, for allowing me the opportunity to work in this esteemed institute and utilize the various facilities. I am also grateful to Dr. D.R. Modi, Head, Department of Biotechnology, BBAU, for encouragement and provision of all necessary facilities. I am thankful to Dr. M.Y. Khan, Dr. G. Sunil Babu, Dr. M. Sharma, Dr. Y. Akhter for their valuable suggestions during work-progress presentations, which channelized my work in the right direction. I also wish to thank Mr. Deep and office staff of BBAU, who helped me with all the office-related paper work. I am also grateful to Dr. R. Jain and Dr. A.K. Shrivastava for their valuable teachings.

I avail this opportunity to especially thank two of my now good friends, my senior, Dr. Amita Sharma and my labmate, Ms. Indu Verma, who have been pillars of support in my research pursuit. From molecular biology skills to biochemical expertise, learning from them and with them, made this research journey more interesting and joyful. I was also lucky to have found true well-wishers and good friends in Ms. Kavita, Ms. Pramila Tripathi, Ms. Priyanka Varun, whose constant support and advice helped me throughout. I also acknowledge the valuable contribution of the lab assistant, Ravi, in on-field jobs and during sample processing. The assistance lent by the technical support staff, Mr. R.K. Singh and Ms. M. Nigam, has also greatly helped in the successful completion of my research work.

I am also grateful to CSIR, New Delhi for lending financial support to my research pursuit through the CSIR-NET fellowship.

I also take this opportunity to express my heartfelt gratitude to my parents who have always stood by me through thick and thin. I will always be indebted to them for all that they have done for me and for the way they have unconditionally supported and guided me throughout. I am also especially thankful to my supportive and understanding husband who patiently accommodated my preoccupation due to work. His encouraging ways and his confidence in me, have reassured me at difficult times.

Kriti

Kriti Roopendra

Scanned by CamScanner

TABLE OF CONTENTS

CERTIFICATE i

CANDIDATE'S DECLARATION ii

ACKNOWLEDGEMENTS iii

TABLE OF CONTENTS iv-vi

ABBREVIATIONS vii-ix

LIST OF TABLES x

LIST OF FIGURES xi-xiv

Chapter 1- Introduction 1-6

Chapter 2- Review of Literature 7-37

- 2.1 Sugarcane: general overview 7
- 2.2 Source-sink concept 8
- 2.3 Sink strength and sink demand 10
- 2.4 Primary sucrose metabolizing enzymes 13
  - 2.4.1 Invertases 13
  - 2.4.2 Sucrose phosphate synthase 20
  - 2.4.3 Sucrose synthase 22
- 2.5 Source to sink transport of sucrose 24
  - 2.5.1 Photosynthetic carbon assimilation 25
  - 2.5.2 Phloem loading 26
  - 2.5.3 Phloem unloading 28
- 2.6 Sucrose cycling and accumulation 30
- 2.7 Gibberellins as modulator of sink demand 32
- 2.8 Molecular approaches to improve sucrose accumulation 34

Chapter 3- Materials and Methods 38-49

- 3.1 Plant material 38
- 3.2 Treatments and sampling 38
  - 3.2.1 Gibberellin spray 38
  - 3.2.2 Partial shading 40
- 3.3 Morphological analysis 41

- 3.3.1 Measurement of morphological parameters 41
- 3.3.2 Scanning electron microscope (SEM) study 41

3.4 Biochemical parameters 42
- 3.4.1 Chlorophyll content 42
- 3.4.2 Nitrate reductase activity 42
- 3.4.3 Quantification of reducing sugar and sucrose 42
- 3.4.4 Brix value 43
- 3.4.5 Pol% (or juice sucrose%) 43
- 3.4.6 Enzyme assay 43

3.5 Molecular analysis 45
- 3.5.1 Total RNA isolation 45
- 3.5.2 End-point PCR analysis 46
- 3.5.3 Real-time PCR analysis 46
- 3.6 Statistical analysis 47
- 3.7 Transcriptomic analysis 47
  - 3.7.1 Transcriptome sequencing 47
  - 3.7.2 *De novo* assembly of transcriptome 47
  - 3.7.3 Differential expression analysis 48
  - 3.7.4 Functional classification and annotation 48
  - 3.7.5 Validation of results by qRT-PCR analysis 49

Chapter 4- Results and Discussion 50-128

4.1 Gibberellin treatment 52
- 4.1.1 Morphological study 52
  - 4.1.1.1 Measurement of morphological parameters 52
  - 4.1.1.2 Scanning electron microscope (SEM) analysis 54
- 4.1.2 Biochemical analysis 57
  - 4.1.2.1 Estimation of chlorophyll content 57
  - 4.1.2.2 Estimation of nitrate reductase activity 59
  - 4.1.2.3 Estimation of sugar and related parameters 60
  - 4.1.2.4 Quantification of enzyme activity 66
- 4.1.3 Molecular analysis 70
  - 4.1.3.1 End-point PCR expression analysis 70

4.1.3.2 Real-time PCR analysis 79
4.1.4 Transcriptomic analysis 87
4.1.4.1 Transcriptome sequencing and assembly 87
4.1.4.2 Functional classification and annotation 92
4.1.4.3 Real-time PCR validation 102
4.2 Partial shading 115
4.2.1 Biochemical analysis 115
4.2.1.1 Estimation of chlorophyll content 115
4.2.1.2 Estimation of NR activity 117
4.2.1.3 Sugar analysis 118
4.2.2 Molecular analysis 122
4.2.2.1 End-point PCR expression analysis 122
4.2.2.2 Real-time estimation of SPS expression 127

Summary and Conclusions **129-135**
References **136-159**
Annexures **160-162**
Publications

ABBREVIATIONS

ATP	adenosine triphosphate
B	bottom portion
BLAST	Basic Local Alignment Search Tool
BSA	bovine serum albumin
ºC	degrees Celsius
CAM	crassulacean acid metabolism
cDNA	complementary DNA
chl	chlorophyll
CO_2	carbon dioxide
C_t	cycle threshold
$CuSO_4.5H_2O$	copper sulphate pentahydrate
CWI	cell wall invertase
DAP	days after planting
DAS	days after (GA_3) spraying/shading
DEPC	diethyl pyrocarbonate
DMSO	dimethyl sulphoxide
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol-bis(β-aminoethyl ether)-NNN'N'-tetraacetic acid
EtBr	ethidium bromide
E-value	expect value
F 1,6P	fructose-1,6-bisphosphate
F2,6P	fructose-2,6-bisphosphate
F6P	fructose-6-phosphate
FBPase	fructose-1,6-bisphosphatase
g	gram
GA	Gibberellin
GA_3	Gibberellic acid
GB	gigabyte
GO	Gene Ontology

HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
H_2SO_4	sulphuric acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
K_2HPO_4	dipotassium hydrogen phosphate
KH_2PO_4	potassium dihydrogen phosphate
$KNaC_4H_4O_6.4H_2O$	potassium sodium tartrate
KNO_2	potassium nitrite
KNO_3	potassium nitrate
KOH	potassium hydroxide
kV	kilo Volt
L1	source leaves
L2	sink leaf
LTM	last transverse mark leaf
M	molar/ middle portion
mg	milligram
$MgCl_2$	magnesium chloride
min.	minute/minimum
ml	millilitre
mM	milli molar
mRNA	messenger RNA
msl	mean sea level
MST	monosaccharide transporter
N	normal
Na_2CO_3	sodium carbonate
$NaHCO_3$	sodium bicarbonate
NaOH	sodium hydroxide
Na_2SO_4	sodium sulphate
NCBI	National Center for Biotechnology Information
NDD	N-(1-naphthyl)-ethylene diamine dihydrochloride
ng	nanogram
NI	neutral invertase
nm	nanometre

NR	nitrate reductase
OAA	oxaloacetic acid
O.D.	optical density
PEPC	phopshoenolpyruvate carboxylase
Phyre2	Protein homology/analogy recognition engine
pmf	proton motive force
ppm	parts per million
psi	pounds per square inch
RIN	RNA integrity number
RNA	ribonucleic acid
RPKM	reads per kilobase of transcript per million mapped reads
rpm	revolutions per minute
RQ	relative quantification
RS	reducing sugar
RT-PCR	reverse transcription-polymerase chain reaction
Rubisco	ribulose-1,5-bisphosphate carboxylase
SAI	soluble acid invertase
SE-CCC	sieve element/companion cell complexes
SEM	scanning electron microscope
SPS	sucrose phosphate synthase
SuSy	sucrose synthase
SUT	sucrose transporter
T	top portion
UDP	uridine diphosphate
w/v	weight/volume
µg	microgram
µl	microlitre

LIST OF TABLES

Table no.	Table title	Page no.
1	Effect of GA_3 application (35 ppm) on morphological parameters	53
2	Difference in length of corresponding internodal samples of control and GA_3 treated canes	55
3	Amount of chlorophyll a and b in control and GA_3 treated LTM leaf	58
4	*De novo* assembly statistics for C_T-G_T	90
5	Annotation summary of total transcripts	92
6	Transcript sequences selected for real-time primer designing for validation	102
7	List of designed real-time primers for validation	104
8	Chlorophyll a and b content in non-shaded and shaded leaves	116

LIST OF FIGURES

Figure no.	Figure legend	Page no.
1	Key molecular players of sucrose metabolism pathway operating in (a) source and (b) sink	18
2	Source-sink communication depicting fate of sucrose	24
3	Application of GA_3 in the form of spray using battery-operated automatic sprayer	39
4	Partial shading done by enclosing all leaves, except the LTM leaf, in 90% shade cloth, for varying periods of time	40
5	(A) Crop planted in the plantation area of IISR at the end of February, using three-bud setts (B) Post germination	50
6	(A) Tillering phase (B) Grand growth phase	51
7	Visible differences between CoJ64 and BO91 in October, at the start of maturation phase	51
8	Difference in height between control and GA_3 treated (A) BO91 (B) CoJ64 canes	54
9	Difference in internodal length of control and GA_3 treated canes of (A) BO91 and (B) CoJ64	55
10A	Difference in cell length seen in (a) control and (b) GA_3 sprayed internodal samples of BO91 cane, as observed through SEM	56
10B	Difference in cell length seen in (a) control and (b) GA_3 sprayed internodal samples of CoJ64 cane, as observed through SEM	56
11	Total chlorophyll content in LTM leaves of control and GA_3 sprayed (a) BO91 (b) CoJ64 canes	58
12	Nitrate reductase activity in LTM leaves of control and GA_3 treated (a) BO91 (b) CoJ64 canes	59
13	(a) Reducing sugar% (b) sucrose% (c) brix% (d) pol% recorded in various internodal samples of control and GA_3 sprayed (50ppm) BO91 canes	61
14	(a) (b) Reducing sugar% (c) (d) sucrose% recorded in the bottom, middle and top internodal samples of control and GA_3 treated (35ppm) BO91 and CoJ64 canes, respectively	62
15	(a) (b) Brix% (c) (d) Pol% recorded in the bottom, middle and top internodal samples of control and GA_3 treated (35ppm) BO91 and CoJ64 canes, respectively	63
16	(a) (b) Reducing sugar% (c) (d) sucrose recorded in LTM leaf samples of control and GA_3 treated BO91 and CoJ64 canes, respectively	64
17	Specific invertase activity in (a) (b) internodal samples and (c) (d) LTM leaf of control and GA_3 treated BO91 and CoJ64 canes, respectively	68
18	Specific SPS activity in (a) (b) internodal samples and (c) (d) LTM leaf of control and GA_3 treated BO91 and CoJ64 canes, respectively	69
19	(a) Total RNA in normalized concentration visualized on 1% agarose gel (b) Sugar metabolizing enzymes viz. SAI (Soluble Acid Invertase) (c) CWI (Cell Wall Invertase) (d) NI (Neutral Invertase) (e) SuSy (Sucrose Synthase) exhibiting differential expression in internodal samples of control and GA_3 sprayed cane (50ppm spray) (f) 25SrRNA gene primer	73

	employed to validate RNA normalization	
20	(a) Normalized concentration of total RNA in (A) top and (B) middle internodal samples of BO91 (b) Differential expression of sugar related genes SAI (c) CWI (d) NI (e) SuSy in (A) top and (B) middle segment of control and GA_3 treated BO91cane (35ppm spray) (f) 25SrRNA gene primer employed to validate RNA normalization	73
21	(a) Normalized concentration of total RNA of (A) top and (B) middle internodal samples of CoJ64 (b) Differential expression of sugar related genes SAI (c) CWI (d) NI (e) SuSy in (A) top and (B) middle segment of control and GA_3 treated CoJ64 cane (35ppm spray) (f) 25SrRNA gene primer employed to validate RNA normalization	74
22	(a) Agarose gel depicting total RNA of (A) BO91 and (B) CoJ64 LTM leaves in normalized concentration (b) Differential expression of SAI (Soluble Acid Invertase) (c) FBPase (Fructose-1,6-bisphosphatase) (d) PEPC (Phospho enol pyruvtae carboxylase) (e) sucrose transporter (SUT) gene in control and GA_3 treated LTM leaves of (A) BO91 and (B) CoJ64 (f) 25SrRNA gene primer employed to validate RNA normalization	78
23	Quantification of difference in expression ($\log_{10}$RQ) of SAI, CWI, SuSy, SPS in control and GA_3 sprayed samples from top portion of (A) BO91 (B) CoJ64 canes	81
24	Quantification of difference in expression ($\log_{10}$RQ) of SAI, CWI, SuSy, SPS in control and GA_3 sprayed samples from mid portion of (A) BO91 (B) CoJ64 canes	84
25	Quantification of difference in expression ($\log_{10}$RQ) of SPS in control and GA_3 sprayed LTM leaf samples of (A) BO91 (B) CoJ64 canes	87
26	Quality values of all bases at each position in the 'base quality check' using fastQC for top internodal sample of (a) control cane (C_T) (b) GA_3 treated cane (G_T)	88
27	Venn diagram depicting the number of transcripts expressing exclusively in C_T or G_T and in both samples	89
28A	Heat map representing expression profile of upregulated differentially expressed transcripts between C_T and G_T	90
28B	Heat map representing expression profile of downregulated differentially expressed transcripts between C_T and G_T	91
29	Screening for 558 differentially expressed transcripts between C_T and G_T	91
30	Workflow for identification and annotation of differentially expressed transcripts	92
31	Chart depicting length distribution of sequences	94
32	E-Value distribution chart depicting the distribution of E-values for all top BLAST hits	94
33	Blast hit distribution chart depicting the distribution of number of hits for the blasted sequences	94
34	Sequence similarity distribution chart depicting the distribution of all calculated sequence similarities (in percentages)	95
35	Species distribution chart displaying different species to which most Blast hits were aligned	95
36	Top-hit species distribution chart displaying different species to which all	95

	top Blast hits were aligned	
37	IPS domains distribution chart showing the number of sequences that belong to a particular IPS domain	96
38	IPS repeats distribution chart depicting the number of sequences that belong to a particular IPS repeat	97
39	IPS sites distribution chart representing the number of sequences that belong to a particular IPS site	97
40	GO mapping distribution chart shows the distribution of GO terms assigned to each sequence by GO mapping	97
41	EC distribution chart denoting Evidence Codes associated to Blast hits	98
42	EC distribution chart denoting the distribution of Evidence Codes for the functional terms obtained by mapping	98
43	The chart gives the distribution of number of annotations (GO terms) retrieved from the different source databases	98
44	Annotation distribution chart shows the distribution of GO terms assigned to each sequence by GO annotation	99
45	WEGO plot showing functional distribution of differentially expressing transcripts under the biological process (BP) category	100
46	WEGO plot showing functional distribution of differentially expressing transcripts under the cellular component (CC) category	100
47	WEGO plot showing functional distribution of differentially expressing transcripts under the molecular function (MF) category	100
48	Chart depicting enzyme annotation of the sequences with GO annotation by KEGG	101
49	KEGG classification of differentially expressed transcripts	101
50	Checking of designed primers for amplification	106
51	Real-time validation using designed primers	112
52	Secondary structure analysis of uncharacterized protein ycf39 predicted 45% of the amino acid sequence to consist of α-helices and 12% as β-strands	113
53	The 3-D model predicted for mRNA of uncharacterized protein ycf39 of *Sorghum bicolor*, based on sequence identity with template	114
54	Total chlorophyll content in shaded source leaves (L1), non-shaded LTM leaf (L2) and shaded sink leaves (L3) of control and shaded (a) BO91 and (b) CoJ64 canes respectively	116
55	Drying symptoms in shaded leaves and non-shaded LTM leaf (L2) of (A) BO91 (B) CoJ64, after 13/12 days of shading, respectively	117
56	Nitrate reductase activity in shaded source leaves (L1), non-shaded LTM leaf (L2) and shaded sink leaves (L3) of control and shaded (a) BO91 and (b)CoJ64 canes, respectively	118
57	(a) (b) Reducing sugar% (c) (d) sucrose% in internodal samples of control and shaded BO91 and CoJ64 canes respectively	121
58	(a) (b) Brix% (c) (d) Pol% in internodal samples of control and shaded BO91 and CoJ64 canes, respectively	121
59	(a) (b) Reducing sugar% (c) (d) sucrose% in shaded source leaves (L1), non-shaded LTM leaf (L2) and shaded sink leaves (L3) of control and shaded BO91 and CoJ64 canes, respectively	122

60	(a) Agarose gel depicting total RNA of (A)BO91 (B) CoJ64 in normalized concentration (b) Differential expression analysis of SAI (c) CWI (d) NI (e) SuSy gene in stalk samples of control and shaded cane over the shading period in October (f) RNA normalization verified using 25S gene primer as internal control	125
61	(a) Agarose gel depicting total RNA of shaded and non-shaded leaves of (A) BO91 and (B)CoJ64 cane in normalized concentration (b) Differential expression of SAI (c) CWI (d) PEPC (e) SUT gene in shaded and non-shaded leaves of (A) BO91 and (B) CoJ64 (f) RNA normalization verified using 25S gene primer as internal control	126
62	Quantitative analysis of differential expression ($\log_{10}$RQ) of SPS in shaded source leaves (L1), non-shaded LTM leaf (L2) and shaded sink leaves (L3) of control and shaded (a)BO91 and (b)CoJ64 canes, respectively	127
63	Nutshell diagram of some molecular determinants of high sucrose accumulation in culm	134

Chapter 1

Introduction

INTRODUCTION

Sugarcane (*Saccharum* spp. hybrids) is a C_4 crop plant belonging to *Poaceae* (grass family), grown in both tropical and subtropical regions throughout the world (Lunn and Furbank, 1999). The crop is spread over an estimated area of 26.52 million hectares across the world, producing an estimated 1877.10 million tonnes of sugarcane, accounting for a cane productivity of 70.77 tonnes/hectare (sugarcane.dac.gov.in/statisticsAPY). Since sugarcane stalk hoards the high economic value product, sucrose, it serves as primary source for production of more than 80% of sucrose produced worldwide (Zhang *et al.*, 2018). In India alone, sugarcane is grown across 5.06 million hectare area producing an average of 341.2 million tonnes of cane per year and productivity of 67.43 tonnes/hectare. The Indian sugar industry is currently ranked 2nd in the world, producing approximately 27.25 million tonnes of sugar per season, which is nearly 15.8% of the total sugar production of the world (172.36 million tonnes) (sugarcane.dac.gov.in/statisticsAPY). Additionally, sugarcane processing generates useful by-products like molasses, fibre, fertilizer and ethanol for fuel.

The communication between the photosynthetic apparatus (source) and the carbon storage tissues (sinks) regulates biomass accumulation in plants by way of a complex feedback process (Ho, 1988). Source and sink work in coordination to control plant growth and development at enzymic and gene level (Rolland *et al.*, 2006). Increasing evidence from several crop species, has highlighted the significant role of major transport sugars, sucrose and hexoses (glucose and fructose), in exercising control over the source-sink interaction (Pego *et al.*, 2000; Rolland *et al.*, 2006; McCormick *et al.*, 2006, 2008c). Sugarcane has evolved to possess an extraordinary source-sink arrangement wherein the stalk functions as a sucrose reservoir or 'sink' and the photosynthesizing leaves form the 'source' of photassimilate (Alexander, 1973; Ho, 1988). Sugarcane utilizes its growing stalk (a vegetative organ) to store assimilate (sucrose) at exceptionally high concentration and this distinguishes it from other cultivated grasses, that usually accumulate their reserve products within seeds (Moore and Maretzki, 1996). A demand and supply chain operates between the source and sink; wherein the sink generates a sucrose demand for supporting plant's growth, differentiation and developmental processes and/or storage (Hawker, 1985) while the leaves synthesize the photoassimilate, furnishing sucrose to meet this sink demand (Hussain *et al.*, 2004).

As in most plants, in sugarcane too, photosynthetically synthesized sucrose is translocated through the phloem (Hatch and Glasziou, 1964). With increasing sucrose pile up in the storage tissues, the translocated sucrose is imported against the concentration gradient, powered by energy from respiration (Bieleski, 1960). As plant matures, changes in sink activity and utilization of photoassimilate viz. concurrent partitioning for cell growth, metabolism, respiration and/or accumulation for storage, cause consequent changes in sink demand and the rate of phloem loading is altered to accommodate the change (Lalonde *et al.*, 2003). Thus, the synchronization between the rate of photosynthesis, rate of photosynthate export and culm requirement, determines the pace at which sucrose is synthesized in the source leaves (Roitsch, 1999; Watt *et al.*, 2005).

Several studies in the past have established the gradual rise in sucrose concentration with advancing maturity of the culm while a comparable decrease occurs in the glucose and fructose concentrations (Fernandes and Benda, 1985). The sucrose concentration in the stalk is regulated by a continuous cycle of sucrose degradation and synthesis (Chandra *et al*, 2011). The phloem-transported sucrose makes entry into the culm parenchyma either symplastically, apoplastically or via both (Moore, 1995). The sucrose unloaded from the phloem into the stem parenchyma is either cleaved by sucrose synthase (SuSy) or by the different invertases present in discrete compartments and operating at specific pH. Cell wall invertase (CWI) breaks down sucrose in the apoplastic space, thus providing monosaccharides to be transported across the plasma membrane, neutral invertase (NI) hydrolyses sucrose in the cytoplasm while soluble acid invertase (SAI) functions in the vacuole (Glasziou and Gayler, 1972). The hexoses thus produced by hydrolysis of sucrose, are channelized into various metabolic processes whilst the surplus sugar (if any) is routed back into sucrose formation by sucrose phosphate synthase (SPS) and/or SuSy and immobilized in sugarcane internodes (vacuoles) (Bieleski, 1960; Batta and Singh, 1986; Whittaker and Botha, 1997).

Maturing sugarcane is found to display a characteristic sucrose gradient with low sucrose concentrations in the younger, upper internodes and a rise in sucrose levels with increasing maturity of culm, bottom down. However, this gradient is more prominent in growing cane and gets fairly evened out as the cane matures and accumulates more and more sucrose (Glasziou and Gayler, 1972; Hawker, 1985). The variation in sucrose level observed across varieties can be attributed to various genetic factors viz. the degree of maturation of cane and physiological capacity of the culm to hoard sucrose, which perhaps limit and control sucrose accumulation in cane. The average range of sucrose content in sugarcane has been experimentally determined to be 500 to 560 mg g^{-1} (which is approximated to account to 50-60% and 12–16% of dry and fresh weight of cane, respectively) (Moore, 1995; Inman-Bamber *et al.*, 2008). Nonetheless, the culm is estimated to be able to accumulate a physiological maxim of as much as 30% of its fresh weight as sucrose (Bull and Glasziou, 1963). Also, Berding (1997) has experimentally reported sucrose content to be as high as 600 mg g^{-1} (on dry matter basis) in whole sugarcane stalks. These data demonstrate that the current yield attained (350-400 mg g^{-1} dry matter) by various commercially cultivated sugarcane varieties is far lower than that attainable (Moore *et al.*, 1997; Jackson, 2005). Hence, the biophysiological ceiling of sucrose content in sugarcane still offers scope for expansion, to accommodate more sucrose (Moore *et al.*, 1997; Bull and Glasziou, 1963).

The sugar yield of commercial sugarcane varieties has been found to have stagnated since the 1970s. A number of aspects viz. genetic complexity of crop, environmental factors, availability of limited genetic resources to design breeding and improvement programmes (Roach, 1989; Lakshmanan *et al.*, 2005; Chandra *et al.*, 2011), impede any significant increase in sucrose accumulation in the developed varieties. Even though conventional breeding efforts have led to introduction of many newer varieties, however, a mere 1-1.5% annual gain in sucrose yield has been obtained (Jackson, 2005; Waclawovsky *et al.*, 2010). In most sugarcane yield-improvement programmes over the world, enhanced sugar yield has mainly been realized through increase in cane yield, and not as much by increase in sucrose concentration in culm (Moore, 2005; Jackson, 2005). Since the field-to-mill processing of cane involves huge production costs, hence improving sucrose productivity of cane is perhaps a much better proposition than resorting to increasing cane number, for better sucrose yield (Jackson *et al.*, 2000; Grof and Campbell, 2005). Thus, enhancement of sucrose productivity of cane by maximal exploitation of the sucrose accumulation capacity of sugarcane should be the key aim of all cane improvement efforts.

Taking trail from the fact that sugarcane culms are capable of accommodating more sucrose than achieved till date, a number of research endeavours worldwide have explored at molecular level, ways to manipulate the basic sucrose accumulation process and improve sucrose content therein. However, achieving the larger target of improving sucrose accumulation is possible only by first taking small steps towards gaining a detailed insight into the synthesis and processing of sucrose and role of source-sink communication in determining sucrose content of culm (Rae *et al.*, 2005a; Bihmidine *et al.*, 2013). In the past too, some studies have attempted to explore the source-sink concept in sugarcane (Marcelis, 1996; Pammenter and Allison, 2002; McCormick *et al.*, 2006). Though most reports have affirmed the dominance of sink in influencing various metabolic processes (Chiou and Bush, 1998; McCormick *et al.*, 2009), no discrete inference has yet been drawn to help improve agronomic sucrose yield. In support, sink demand has been reported to influence the rate of assimilate production such that photosynthetic rate increases with rise in sucrose demand from culm (Roitsch, 1999; Watt *et al.*, 2005). Also, the export of photosynthates from mature leaves has been reported to be governed by the demand of sink tissue. Hence it can be inferred that maximum photosynthetic capacity of leaves is indeed flexible and is regulated at sink level (Lalonde *et al.*, 2003).

A possible strategy to improve sucrose accumulation in the culm can be by devising ways to perturb source-sink communication so as to augment the sink demand. The heightened sink demand will in turn stimulate increased production of photosynthate at the source and enhance translocation to the sink (Grof and Campbell, 2005). In accordance, experimental pursuits designed to perturb the source to sink ratio in sugarcane (viz. partial shading, defoliation), have been found to replicate the condition of high sink demand. Defoliation exhibited a consequent influence on carbon partitioning and sugar levels of source and sink tissues, corroborating escalation in photosynthetic activity of the remaining leaves to meet up the amplified culm requirement (McCormick *et al.*, 2009). Partial shading treatment too deduced a valid correlation between photosynthetic activity of the single non-covered leaf and sucrose need of the immature culm (McCormick *et al.*, 2006), perhaps causing better assimilate production and leading to improved sucrose accumulation in the culm.

These findings make evident the ability of source to adapt to variation in sink demand and point to disruption of source-sink communication as a credible option to enhance sucrose yield of cane.

Gibberellins (GAs) are one such alternative that alter source-sink relationship by promoting plant growth, thereby aiding sink formation and improving sink's capacity to accommodate sucrose (Zhang *et al.*, 2007). Studies have shown that exogenously applied gibberellins bring about increase in internodal length by promoting elongation and division of cells, thereby increasing sink size (Iqbal *et al.*, 2011). The boosted sink demand may perhaps affect source-sink communication by establishing a steeper sucrose gradient between sink and source, thus facilitating better transport of photoassimilate (Cole *et al.*, 1972). Since the phloem is not saturated, and is capable of facilitating increased export of the photosynthate supply, this would in turn promote photosynthesis to increase assimilate concentration in the cytosol of leaf mesophyll cells. Gibberellin action can hence be envisaged to promote sucrose synthesis and subsequent sucrose metabolism in the source-sink system. Thus, gibberellin spray can perhaps be employed for improving sucrose accumulating capacity of sugarcane.

Objectives of the study:

- To develop a biophysiological insight into the source (leaf)-sink (culm) communication of sugarcane, leading to sucrose accumulation in the culm.
- To study the effects of modulation of source-sink dynamics, on sucrose accumulation in the culm.
- To analyse changes in expression of genes, in response to source-sink perturbation and identify novel genes.

The current study was aimed at analysing the source-sink relationship that exists in sugarcane, in the light of a supply-demand concept. Manipulation strategies viz. gibberellin spray and partial leaf shading, were employed and their effect on source-sink interaction was weighed by way of increase in sink demand and consequential increase in flow of assimilate to the sink. Making a valid comparison between control and treated canes, we attempted at analysing various determinants, at morphological, biochemical and molecular level, which work in correspondence to bring about differential sucrose storage under perturbed source-sink communication. Thus, utilizing the knowledge from past pursuits, study of modulation of key factors viz. various sugar related parameters and sucrose synthesizing and metabolizing enzymes that regulate the sucrose accumulation process, can give an insight into the physiological and molecular events underlying the source-sink relationship, in turn paving the way for improved sucrose productivity of sugarcane. Also, a transcriptome level analysis has lent light on the changes at expression level, as a consequence of manipulated sink demand (under the effect of GA_3), thus adding a novel dimension to the concept of sucrose accumulation.

Chapter 2

Review of Literature

REVIEW OF LITERATURE

2.1 Sugarcane: general overview

Sugarcane, which is counted among the world's most important cash crops, belongs to *Saccharum* genus and is member of the 'tall grasses' family (*Poaceae*). Being a C_4 plant, carbon fixation in it occurs via the C_4 type photosynthetic pathway, wherein the photosynthetic reactions are segmented among two distinct kinds of cells viz. mesophyll cells and bundle sheath cells. The characteristic arrangement of distal mesophyll cells and proximal bundle sheath cells is referred to as Krantz anatomy (Haberlandt, 1884). CO_2 is initially fixed in the mesophyll cells, which is then transported to bundle sheath cells. This cyclical reaction effectively suppresses photorespiration by concentrating CO_2 in the bundle sheath cells where photosynthesis occurs exclusively, via the photosynthetic carbon reduction (PCR) cycle (Hatch and Slack, 1966), accounting for its photosynthetic efficiency. Thus, C_4 plants exhibit a complex adaptation of photosynthetic mechanism in C_3 plants, since CO_2 fixation and sucrose synthesis involves the co-ordination between mesophyll and bundle sheath cells rather than just between chloroplast and cytosol (Lunn and Furbank, 1997). The sugarcane culm is said to elongate throughout its life span, with internodes developing in succession and their flanking leaves developing sequentially, in chronological order. As the cane matures, the older leaves gradually senesce and dry up but remain attached to the stem while newer, metabolically active leaves arise and expand to photosynthesize (Pammenter and Allison, 2002).

In most plants, photoassimilate is transported in the form of sucrose (Lalonde *et al.*, 1999), providing the driving force to phloem transport (Taiz and Zeiger, 1991). Thus, it acts as a determinant of translocation and storage and also exercises control over plant growth, metabolism and development. Sucrose has been largely employed and the molecule of choice among most plants, mainly due to its non-reducing nature. Also, much research has demonstrated sucrose and derived metabolites to work at gene level to control cellular metabolism (Huber and Huber, 1996). Sucrose accumulates to high concentrations in the sugarcane culm and hence forms the basis of all research directed towards manipulation of sucrose accumulation.

Sugarcane begins to accumulate sucrose in the elongation phase itself, though majority of the sucrose accumulation occurs during maturation, when the cane has ceased to elongate (Lingle, 1999). This is because the transported sucrose is initially used up for plant growth and development of leaves, stem and roots. Later, during maturation phase, when most internodes have adequately elongated, the imported sucrose, in excess of that required for growth and maintenance, is mainly focussed to storage (Hoffmann-Thoma *et al.*, 1996; Slewinski, 2012). Sugarcane culm has been reported to accumulate up to 62% and 27% of its dry and fresh weight, respectively, as sucrose (Bull and Glasziou, 1963; Sachdeva *et al.*, 2011). While the immature, upper internodes display a purity ratio (defined as the ratio between sucrose and total sugar) as low as 50%, the older, mature internodes exhibit purity ratio of more than 90% during the maturation phase (Welbaum and Meinzer, 1990; Moore, 1995). The level of sucrose accumulation demonstrated by the cane determines the sugar recovery percentage (Batta *et al.*, 2011).

2.2 Source-sink concept

A composite source-sink communication model can be said to operate in all higher plants in which the photosynthetic apparatus or leaves form the carbon 'source' and the non-photosynthetic storage organs form the carbon-utilizing 'sinks', consuming the photoassimilate for various metabolic processes viz. growth, respiration and storage (McCormick *et al.*, 2006). Sugarcane is considered a unique source-sink system in that it stores assimilate in the form of osmotically active, non-reducing solute sucrose while many other plants stock up the insoluble starch. Also, sugarcane characteristically hoards large volumes of sucrose in the parenchyma tissue of the growing stalk, rather than any storage organ (Alexander, 1973; Ho, 1988). Within the leaf, once the biosynthesis of sucrose has occurred in the cytosol, it can be channelized to get piled up in the vacuole or be transported via phloem (Kingston-Smith *et al.*, 1998). Majorly, source activity is co-ordinated to metabolic activities of the sink and to the requirements of the plant as a whole (Paul and Foyer, 2001). With progressing plant growth and development, priority shifts among different sinks for allocation of photoassimilate. Study on tomato has demonstrated a shift in preference for assimilate distribution with growing maturity, such that it is directed to leaves in young plants while to expanding fruits in older plants (Ho, 1988). Likewise, the elongating culm of sugarcane forms a priority sink for sucrose accumulation, stimulating preferential distribution of assimilate even in the event of decreased assimilate supply (Pammenter and Allison, 2002).

Phloem transport which delivers the photoassimilate and inorganic nutrients from the source (net assimilate exporter) to the sink (net assimilate importer), forms the means of communication between them (Lalonde *et al.*, 2003). The mass flow hypothesis proposed by Münch and followers (Münch, 1930) explains sap movement in the phloem as mass-flow from source (which is maintained at higher hydrostatic pressure by means of osmosis) to sink (at relatively lower pressure). As per this model, high sugar concentrations inside phloem cells of source organs, cause water to be drawn into them, under the effect of water potential gradient, thus, in turn, causing their turgor pressure to be raised. From the phloem, the sugars are unloaded down the water potential gradient into sink organs, causing a resultant decrease in turgor pressure. Consequently, under the effect of this hydrostatic pressure difference, bulk flow occurs between source and sink via the phloem channel. Thus, each time the sugar concentration at particular sink varies due to utilization of sugars, the sugar concentration in phloem is also modified, thus creating a high pressure difference that stimulates rapid phloem sap movement and sugar movement into the phloem ensues (Watt *et al.*, 2014).

In addition to factors like span and period of maturation phase (Moore *et al.*, 1997), many physiological aspects can ascertain level of sucrose accumulation in sugarcane stalk viz. leaf photosynthetic rates and carbon partitioning into various metabolic and developmental processes, rate of phloem loading for transport and rate of import into the sink (Lalonde *et al.*, 2003) and consumption in the culm w.r.t. partitioning into various processes viz. growth, respiration, remobilization (Uys *et al.*, 2007). Thus, the sugar-governed interaction of source and sink tissues is perhaps the primary factor determining amount of sucrose stored in the stalk and consequently crop yield (McCormick *et al.*, 2006).

Boussingault (1868) pointed that accumulation of assimilate in leaves regulates photosynthetic rate wherein accumulation of end products occurs as a difference between biosynthesized product and photoassimilate consumption in various growth and metabolic processes of the plant. Additionally, photosynthetic rates of sugarcane leaves have been found to bear a correlation with age of the plant such that leaves of younger plants displayed significantly higher assimilation rates as compared to older canes (Hartt and Burr, 1967). These studies justify the concept of end-product repression of photosynthesis in terms of sucrose accumulation (Hartt, 1963). Source–sink communication has been explored and manoeuvred by employing a variety of innovative methods viz. girdling, partial shading, defoliation (Urban *et al.*, 2004, McComick *et al.*, 2006). By way of girdling, a whole ring of phloem was excised from around a vegetative axis, such that it hindered carbon metabolism and transport, thereby preventing basipetal transport of assimilates through the phloem (Roper and Williams, 1989, Li *et al.*, 2003). The study helped deduce that disrupting the communication of surplus assimilate between source and sink tissues, can bring about improved assimilate production and consequently, lead to improved sucrose accumulation in sink.

In sugarcane, an association has been demonstrated between the processes of sucrose production in the source and hoarding in the sink in various studies (Hartt, 1963). Though the source (leaves) have been known to be actively involved in carbon fixation and carbohydrate metabolism, only recently the sink (culm) tissue which was earlier visualized as a static storage vessel, has been highlighted as a dynamic system with variable storage capacity (Grof and Campbell, 2005). Various approaches can be employed to improve assimilate production viz. improving light harvesting capacity of source, either by raising the number of leaves or by boosting the area of individual leaves, thereby enhancing the assimilation efficiency of the plant (Raines, 2011). The flip side approach could be by enhancing sink capacity by way of increase in cell number, size or improvement of sucrose hoarding ability of cane (Herbers and Sonnewald, 1998). Thus, sink demand could act as a key regulatory parameter for determining sucrose transport to the storage parenchyma, and hence, influence crop yield (Patrick, 1997).

2.3 Sink strength and sink demand

Sugar concentration is an ever changing index, since it co-ordinates with growth and development of plant, and accordingly modifies (Smeekens, 2000). Sink strength can be described as the intrinsic ability of a non-photosynthetic sink to preferentially draw photoassimilates towards itself. Sink strength is said to be genotypically determined and is largely governed by the sink size and sink activity (Herbers and Sonnewald, 1998).

The dynamics of the source-sink relationship in sugarcane can be better explained as a supply–demand concept. In sugarcane, the culm acts as a large reservoir or sink for photoassimilate (Marcelis, 1996) and changes in its sucrose accumulating activity due to instantaneous sucrose consumption for growth and respiration and/or hoarding for storage, may result in adjustments in the sucrose need of the sink (Watt *et al.*, 2014). Demand for sucrose is generally found to decrease with increasing maturation of the culm. The net sucrose concentration in the phloem is influenced by the gross photoassimilate translocation from source to sink viz. the difference between rate of sucrose export from source and the rate of sucrose import into sink (Chiou and Bush, 1998, Vaughn *et al.*, 2002). The source activity viz. rate of photosynthesis must be complementary to the carbon need of the sink i.e. sink demand. Hence, to avoid an oversupply or undersupply of photoassimilate, the source activity modulates in response to the phloem-communicated changes in sink demand and to phloem loading by and large (Herold, 1980; Roitsch, 1999; Lalonde *et al.*, 2003). Photoassimilate can be exported from both, chloroplasts and the vacuole, when photosynthetic production of assimilate is unable to meet existing sink demand (Ludewig and Flügge, 2013).

Thus, improving sink capacity and sink demand is perhaps a viable alternative for manipulation of sucrose accumulation in the culm. Increasing culm (sink) size, by bringing about an increase in cell count and/or an increase in cell dimensions can be a possible strategy, thereby providing greater room for unloading and storage of sugars in the parenchyma cells. This possibly also explains the research efforts that produced thicker stems through breeding in sugarcane and sweet sorghum, consequently leading to increased sucrose concentration in both (Glassop *et al.*, 2007).

Various studies like partial leaf shading, defoliation and many transgenic studies lend support to the proposition that sugar accumulation in sugarcane can be furthered. Also, the inherent capacity of leaves to supply assimilate to the culm, seems expandable under increased assimilate requirement (McCormick *et al.*, 2009). The disproportionate distribution of source and sink brought about by such treatments, causes a variation in both soluble (sucrose, glucose and fructose) and insoluble carbohydrates in leaves. Results of leaf shading studies carried out by McCormick *et al.* (2006) have revealed that increase in sucrose demand of sink causes an increase in rates of photosynthesis and a subsequent rise in sucrose export from leaves. This indicates the role of sink demand in regulating photosynthetic rates and consequently sucrose accumulation in the stalk of sugarcane (Watt *et al.*, 2005).

Also, the sugarcane plant exhibits an instinctive inclination towards sucrose accumulation, compromising on structural growth, under such conditions of decreased source leaf quantum (through shading or defoliation) (Pammenter and Allison, 2002). Observations from partial defoliation studies have demonstrated that culm sucrose concentrations are not affected by such treatment, hinting at the ability of sugarcane leaves to adjust their photosynthetic capacity as per the culm demand (Gutiérrez-Miceli *et al.*, 2004). Hence, sink demand can be inferred to more strongly govern the sucrose concentration in phloem than the photoassimilate content of other leaf tissues (Chiou and Bush, 1998).

Low carbon requirement of the sink prevents unloading from the phloem, resulting in sucrose accumulation in the source phloem, which in turn slows down phloem loading. This in turn causes hoarding of photoassimilate in the mesophyll and a decline in A (photosynthetic rate) in response to the feedback, thus rationalizing regulation of A by sink demand (Chiou and Bush, 1998). Many studies including those in species like maize (Jeannette *et al.*, 2000), have described this correlation between decreased sink demand and consequent build up of sugar in leaves, causing downregulation of A (photosynthetic rate). This concept of 'sink limitation of photosynthesis' i.e. limitation of photosynthetic rate under the effect of decrease in carbon demand from the sink (Paul and Pellny, 2003), also implies that preservation of sink demand to draw photoassimilate from source would uphold photosynthesis (McCormick *et al.*, 2006).

The heaping of sugar in phloem due to the sink demand constraint (Vaughn *et al.*, 2002) can be imitated by way of exogenous feeding of sugars to a particular plant organ as in studies by Iglesias *et al.* (2002) and cold-girdling treatments by McCormick *et al.* (2008b), leading to decrease in chlorophyll content and photosynthetic rate due to down regulation of photosynthetic enzymes (Krapp and Stitt, 1995) and sugar transporters in turn (Chiou and Bush, 1998). Basu *et al.* (1999) demonstrated in a similar approach that when the sink quantum of the plants was artificially manipulated by excision of tubers, accumulation of sugar occurred in the leaves with a significant decrease in maximum photosynthetic rate (A_{max}), electron transport rate (ETR) and quantum yield (F_v/ F_m). Various pursuits in plants with differing source:sink ratios viz. by way of defruition, have depicted the same rise in carbohydrate content of leaves and decrease in photosynthesis in response to the altered sink demand, denoting that the operating sink demand influences the sugar content of leaves and dominates photosynthesis in plants.

2.4 Primary sucrose metabolizing enzymes

Various sucrose synthesising and degrading enzymes operate as part of the sucrose metabolism pathway, determining sucrose concentration in the sugarcane culm. Initial research suggested the prime role of invertases in controlling growth and culm sucrose concentration (Gayler and Glasziou, 1972), while it was later suggested that in addition to the key invertases, other enzymes viz. sucrose synthase (SuSy) (Geigenberger and Stitt, 1991; Goldner *et al.*, 1991), sucrose phosphate synthase (SPS) (Botha and Black, 2000) and the interplay of many other sucrose metabolizing enzymes governs sucrose accumulation in sugarcane (Veith and Komor, 1993; Pan *et al.*, 2009; Tana *et al.*, 2014)

Past efforts hold evidence that the sugarcane stem has the capacity to amass greater sugar levels than achieved yet. In order to explore such possibilities through approaches like metabolic engineering, it is important to develop a thorough understanding of the mechanism of sugar metabolism and transport in sugarcane and the factors that regulate and determine sink strength (Patrick *et al.*, 2013). Many studies have attempted manipulation of the operating sucrose metabolizing pathway and the enzymes engaged therein so as to interpret their significance and regulatory role (Grof and Campbell, 2005). Earlier efforts to enhance sucrose content in sugarcane have employed transgenics to target a sucrose processing enzyme; however these explorations have met with little success (Bihmidine *et al.*, 2013).

2.4.1 Invertases

Sucrose transported from source to the culm, is degraded into glucose and fructose in an irreversible reaction catalysed by invertase (Sturm, 1999), utilizing ATP. Primarily, traits like solubility and pH optima differentiate the three different isoforms of invertase which are known to hydrolyse sucrose at different subcellular locations. Three soluble invertases have been reported to be present in barley leaves (Obenland *et al.*, 1993). The action of invertases resulting in change in sucrose/hexose content and consequent signalling from the generated sugar sensors, has been reported to affect gene expression at various levels (Koch, 1996; Sturm and Tang, 1999).

Studies undertaken to decipher the significance of invertases through molecular manipulation, have emphasized their role in source-sink system (Verma *et al.*, 2011; Chandra *et al.*, 2012; Tana *et al.*, 2014). Invertases can be said to have a two sided role, wherein on one hand, they partake in sucrose metabolism by cleaving sucrose into glucose and fructose for consumption, and on the other hand, contribute to construction of strong sinks to invite sucrose since the generated hexoses serve as signal molecules that control processes like cell cycle and cell division (Gonzalez and Cejudo, 2007; Bihmidine *et al.*, 2013). Ma *et al.* (2000) employed a yeast invertase gene (suc 2), whose expression was governed by the constitutive ubiquitin promoter of maize. This study explored the role of invertase in sugarcane callus. It was found that heightened invertase levels in the cytosol led to a drop in sucrose accumulation in the cells while those that exhibited high apoplastic invertase levels, displayed no significant change. Also, with decrease in soluble acid and neutral invertases, the sucrose content of juice in young cane has been found to increase, thereby defining an inverse relationship between them (Venkataramana *et al.*, 1991). A chemical ripener employed in sugarcane, glyphosate, utilizes this principle and inhibits the elongation of immature internodes, bringing about a concurrent decrease in acid invertase activity leading to high sucrose accumulation (Su *et al.*, 1992). Invertases are reported to generate twice the number of hexoses as compard to those by sucrose synthase and hence boast of greater capacity to produce hexose signals (Black *et al.*, 1987).

2.4.1.1 Soluble acid invertase (SAI)

In sugarcane, soluble acid invertase (SAI) is considered the key invertase which breaks down sucrose into hexose sugars, glucose and fructose. They are known to function at an acidic pH optimum of 4.4 and are localised in the vacuole (Sturm, 1999) (hence also referred to as vacuolar acid invertase). Primarily, actively growing tissues viz. cell cultures and young, immature internodes are known to exhibit high SAI activity (Hatch and Glasziou, 1963). The escalation in SAI activity in correspondence with developmental stage depicts the increase in need for hexoses during development and cell elongation and the build up of increased demand for sucrose i.e. sink demand in the developing tissues (Figure 1). In one study, SAI and CWI activities were stifled in tomato plants by employing a SAI encoding gene (whose expression was under the influence of CaMV 35S promoter). Fruits thus obtained were 30% smaller than the control, undisturbed ones (Ohyama *et al.*, 1995; Klann *et al.*, 1996). This demonstrates the role of this enzyme in ascertaining sink strength and photoassimilate movement. SAI has also been found to display the capacity to remobilize stored sucrose in case of requirement for growth or to withstand stress (Grof and Campbell, 2005).

During growth phase, the high SAI activity supports culm growth by hydrolysing sucrose to produce hexose sugars to be used up by the growing internodes (Kaur *et al.*, 2002). Thus, SAI can be said to be instrumental in sugar signalling and in promoting import of sucrose into sink tissues (Sturm and Tang, 1999), by encouraging sucrose hydrolysis, especially during sink growth and cell wall expansion (Ricardo and ap Rees, 1970; Tymowska-Lalanne and Kreis, 1998). Many studies associated with SAI activity in maturing internodes have been carried out in sugarcane (Glasziou and Bull, 1965; Lingle, 1999) demonstrating the role of SAI in aiding sucrose accumulation by generation of sink demand (Batta *et al.*, 2007). Overall, SAI activity in the entire culm exhibits an inverse relation to sucrose content, increasing within individual internodes, bottom up, with decreasing sucrose concentration. Thus, SAI activity is most prominent in the top, immature internodes of sugarcane, that have minimal sucrose content, while least SAI activity has been recorded in the greater sucrose containing mature internodes (McCormick *et al.*, 2006). Thus, during ripening, sucrose content in maturing internodes as well as the sucrose-to-total sugar ratio are inversely related with SAI activity.

Verma *et al.* (2011) performed a comparative study in the internodal samples over different stages of development, between changes in activity of SAI enzyme and its transcript expression levels, among sugarcane varieties with contrasting sucrose hoarding capacity. With increasing age, over the course of development, SAI activity and its transcript levels were found to decrease in both the cultivars. SAI activity was inferred to be directly related to hexose sugar content of immature and mature internodes and inversely related to sucrose content. Thus, as discussed earlier, here too, highest SAI activities were recorded in the developmentally immature internodes with low sucrose content while the lowest SAI activity was associated with mature internodes exhibiting high quantity of sucrose. SAI activity is evidently linked with maturity and has been observed to drop when internode growth ends but resurfaces in case of recommencement of growth (Ho, 1988).

Sucrose concentration in the sink can be asserted to be governed by activity of sucrose metabolizing enzymes like SAI. Different sugarcane varieties display diverse sucrose hoarding capability, with those that store high levels of sucrose, generally exhibiting low SAI activity during maturation (Zhu *et al.*, 1997). The early maturing cultivars that accumulate high sucrose levels are reported to display characteristic early decay of SAI activity, thereby facilitating high sucrose accumulation in their culms (Sehtiya *et al.*, 1991; Sehtiya and Dendsay, 2000; Dendsay *et al.*, 1995). Hence, analysis of characteristics like level of SAI activity exhibited by young, growing internodes, extent of sucrose pile up in the fully grown internodes, and the coordination between beginning of maturation (sucrose accumulation) and receding of SAI activity, can help distinguish between genotypes differing in level of sucrose storage. Zhu *et al.* (1997) demonstrated through a study conducted on offspring of cross between sugarcane varieties with contrasting sucrose hoarding capacity, that sucrose accumulation was inhibited in culm displaying SAI activity above a level, thus verifying the role of SAI in governing sucrose accumulation in the sugarcane stalk.

SAI is known to possess a number of gene isoforms, which differ in their expression w.r.t different developmental stages and tissues. However, the exact function of the various isoforms has not yet been deciphered. A certain SAI isoform *Ivr1* found in maize, is upregulated in response to reduction in sugar. High expression of another isoform, *Ivr2*, is noticed in case of plentiful sugar supply (Koch, 1996). Chandra *et al.* (2014) determined the foremost nucleotide sequence of SAI, exclusively for *Saccharum spontaneum* SES34, one of the ancestors of the current *Saccharum* hybrid. Additionally, the sequence for SAI gene was deciphered for *Saccharum* spp. hybrids CoJ64 and for *Saccharum officinarum* 28NG210, respectively. Phylogeny analysis of these sequences revealed close homology with the SAI sequences derived from various *Saccharum* cultivars, while they were found to be relatively distantly related to SAI sequence data corresponding to sorghum and maize, validating genic level conservation of SAI sequence.

2.4.1.2 Cell wall invertase (CWI)

CWI being ionically tethered to the cell wall, is an insoluble invertase. Like SAI, CWI too works in an acidic environment with optimum pH of 3.8 (Albertson *et al.*, 2001).

CWI is the only invertase that carries out sucrose hydrolysis in extracellular (apoplasic) space. The hexoses generated by CWI action on sucrose are sent across the parenchyma plasma membrane by active transport (Grof and Campbell, 2005). CWI facilitated sucrose hydrolysis causes increase in sink strength and sink demand providing a concentration slope from source to sink for continuous sucrose transport (Figure 1). Thus, CWI is a major participant in apoplasmic phloem unloading and facilitates uptake of sucrose in the sink (Roitsch *et al.*, 2003; Koch, 2004; Chandra *et al.*, 2015), in turn increasing the amount of sucrose accumulated (Ma *et al.*, 2000). Affirming the role of cell wall invertase in regulating sucrose flux from the phloem to growing tissues (Hatch and Glasziou, 1963), Godt and Roitsch (2006) have demonstrated that CWI partakes in supplying hexoses to young tap root tissues of sugarbeet through apoplasmic route.

Acid invertases viz. SAI and CWI have perhaps been proposed to govern amassing of sucrose by the maturing culm and also to bring about breakdown of stored sucrose after harvest (Lontom *et al.*, 2008). However, in contrast to SAI activity which is known to be highest in the most immature internode and decreases with increasing maturity, CWI is seen to be more active in mature internodes. After harvest, the activity of SAI in the immature internode increased with time in storage whereas that of CWI decreased in both immature and mature internodes. Cheng *et al.* (1996b) studied a maize mutant (*miniature1*) whose endosperm specific CWI had been eliminated. It showed a small seed phenotype confirming CWI function in sugar partitioning. In a similar pursuit, antisense silencing of CWI inhibited formation of tap root in carrot (Tang *et al.*, 1999). In either studies, normal development was achieved by supply of hexoses through apoplasmic channel.

Various CWI isoforms showing highly tissue-specific expression patterns, have been identified (Roitsch *et al.*, 2003). Four isoforms isolated in maize (*Incw1*, *Incw2*, *Incw3* and *Incw4*) differ from each other in their location of expression (Qin *et al.*, 2004) with *Incw3* and *Incw4* exhibiting low expression in grain (Kim *et al.*, 2000b). CWI activity has been inferred to be regulated by various factors viz. tissue type (Cheng *et al.*, 1999) while Roitsch *et al.* (2003) indicated phytohormones'-stimulated promotion of CWI activity. Roitsch *et al.* (1995) established the role of sugars in modulating CWI expression and activity, using photoautotrophic suspension cultures of *Chenopodium rubrum* (L.). Greater CWI enzyme activity and expression was observed in the presence of sucrose and glucose. Also, many CWI isoforms have been found to display elevated transcript expression in the presence of glucose in plants like tobacco (Krausgrill *et al.*, 1998) and in tomato, in response to sucrose (Godt and Roitsch, 1997).

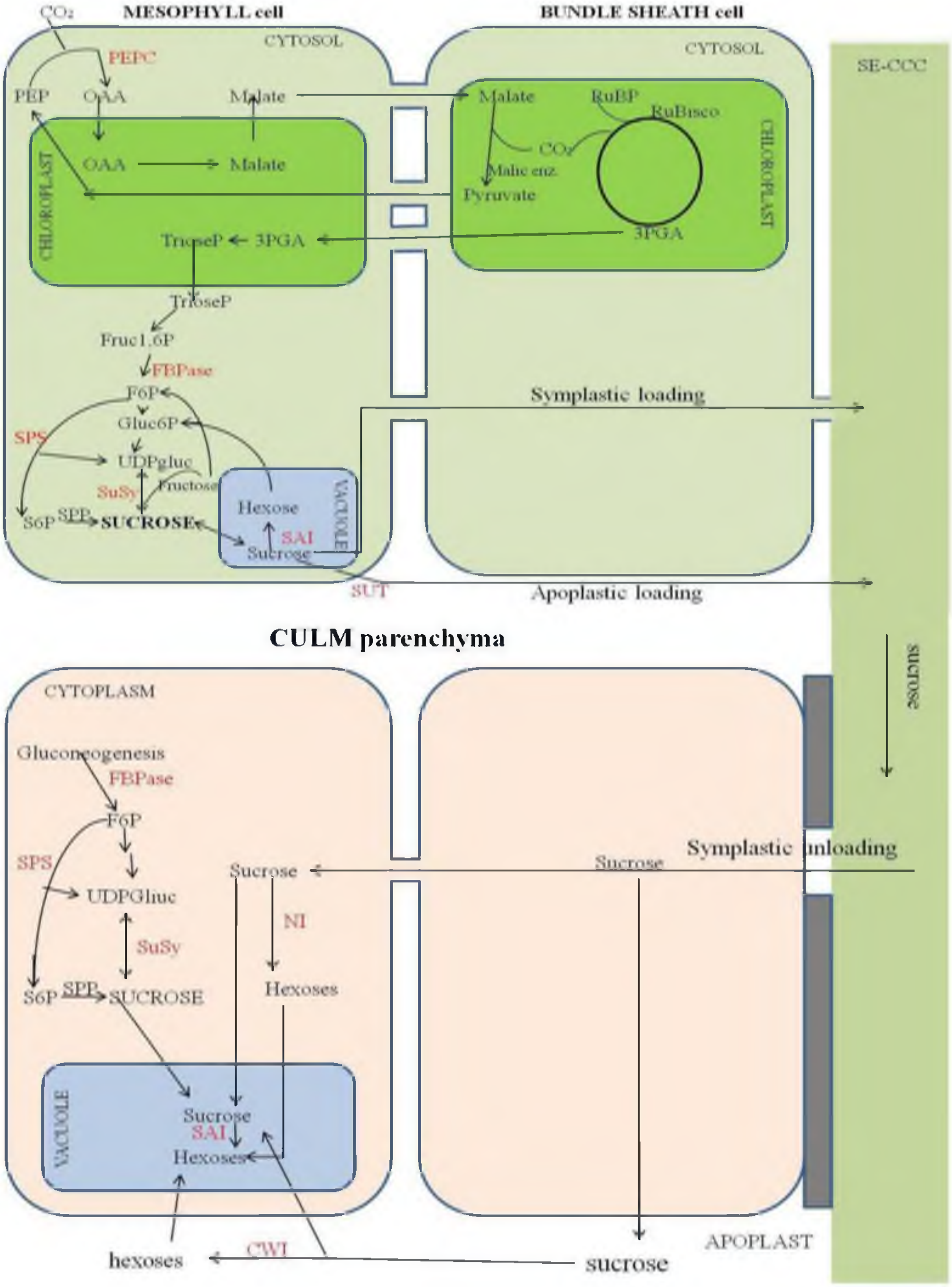


Figure 1: Key molecular players of sucrose metabolism pathway operating in (a) source and (b) sink (adapted from Watt *et al.*, 2014)

2.4.1.3 Neutral invertase (NI)

Neutral invertase (NI) is a soluble, cytosolic invertase operating at neutral pH, with a pH optima of 7.0. NI activity was first remarked in sugarcane by Hatch and Glasziou (1963). Study of certain sequences supposed as neutral/alkaline invertases, belonging to rice and *Arabidopsis*, categorized them into a-type neutral and b-type alkaline invertases, wherein the neutral invertases were intended for the mitochondria and chloroplast (Ji *et al*., 2005). The role of NI was initially uncertain and it was mainly seen as a 'maintenance' enzyme (Batta and Singh, 1986) and its activity was mainly linked to local environmental and cyclic factors. However, the NI enzyme has now been characterized in a number of species (Chen and Black, 1992; Lee and Sturm, 1996; Vorster and Botha, 1998; Rose and Botha, 2000) and its function better deciphered. NI can broadly be said to be associated with active growth, as evident from the link between NI activity and hexoses accumulated in ripe fruits of *Lycopersicon* species (Balibrea *et al*., 2006). Also, upregulated NI expression observed in developing carrot tissues or high NI activity seen in developing seed of lupin, all support the proposition (Gonzalez and Cejudo, 2007).

Various endeavours to determine role of NI in sugarcane have indicated that NI may be involved in facilitating pile up of sucrose in sugarcane during maturation (Batta and Singh, 1986; Bosch *et al*., 2004; Mao *et al*., 2006) and in providing hexoses to young culm tissue via sucrose hydrolysis (Gayler and Glasziou, 1972) (Figure 1). NI was reported to be highly expressed in the maturing culm by Rose and Botha (2000), wherein sucrose was low and hexoses were high, with a gradual decline in activity as the culm matured. On similar lines, glucose or fructose content of internodal tissues was stated to be directly related to NI protein content by Bosch *et al*. (2004) while low NI content was observed in internodal tissues with high sucrose content. The NI transcript has been found to exhibit expression in all sugarcane tissues viz. leaves, culm and root of sugarcane, albeit at relatively low levels. The young developing tissues displayed high NI transcript expression while lower expression was observed in the low sucrose containing mature tissues, affirming inverse relationship between sucrose content and NI transcript expression. Also, Rossouw *et al*. (2010) reported high sucrose concentration but reduced respiration in transgenic sugarcane with down regulated NI expression. Sturm *et al*. (1999) displayed similar distribution of NI in carrot, with the young and developing tissues possessing high NI content.

Contrastingly, NI has also been suggested to bring about sucrose degradation in mature tissues, when VAI and SuSy exhibit low activity (Winter and Huber, 2000). In general, NI activity is known to recede with advancing maturity of culm. However, maturing tissue continue to exhibit substantial activity hydrolysing ~10% of the stored sucrose content (Sacher *et al.* 1963).

Since neutral invertase activity has largely been seen to be associated with actively growing tissues, it may be inferred to play a part in stimulating cell division and cell expansion. Also, high NI activity can perhaps be said to promote sink activity and increase sink strength, also mediated by increased invertase activity, thus promoting phloem unloading. Since growth and development involves network of signals including phytohormones like GAs, NI expression can perhaps be associated to it. In one study, NI and VAI expression was explored in sugar beet plants, to ascertain developmental and GA-induced effect on their petioles. The juvenile plants displayed similar NI and SAI activities (Gonzalez *et al.*, 2005), verifying that both kinds of invertases play part during growth of petioles, refuting the presumption that neutral invertases come in play only in the absence of other invertases and not in co-ordination (Winter and Huber, 2000).

2.4.2 Sucrose phosphate synthase (SPS)

SPS is known to be the prime enzyme that brings about synthesis of photosynthetically produced sucrose (Stitt and Quick, 1989). The formation of sucrose-6-phosphate using UDP-glucose and fructose-6-phosphate, the penultimate step in sucrose production process, is mainly catalysed by SPS. The sucrose-6-phosphate thus produced is then converted to sucrose in a final reaction catalysed by sucrose phosphate phosphatase (SPP). Since sucrose production occurs in large proportions, the SPS catalysed reaction is largely irreversible, thereby leaving little scope for sucrose phosphate (Krause and Stitt, 1992). Thus, SPS exercises a major control over the flux of carbon into sucrose formation (Fernie *et al.*, 2002).

The expression of SPS is regulated by developmental, environmental and nutritional signals and, at least in some cases, at the translational level (Winter and Huber, 2000). A number of studies have validated the significance of SPS in facilitating sucrose accumulation (Figure 1). Botha and Black (2000) have suggested that SPS activity and amount of sucrose are interlinked. The play of SPS activity in ascertaining sucrose accumulation in culm has been affirmed in a study employing hybrid offspring of a cross between *Saccharum* species with contrasting sucrose amassing capabilities (Louisiana Purple *Saccharum officinarum* L. as female parent and *Saccharum robustum* Brandes and Jesw. ex Grassl. Molokai 5829 as male parent), wherein SPS activity was seen to facilitate high sucrose content (Zhu *et al.*, 1997). In a transgenic pursuit involving tomato plants bearing maize SPS gene, recorded up to twelve-fold increase in SPS activity in leaves of even successor plants. Higher dry weight was observed in the SPS-transformed plants, with the fruits ripening sooner than those of non-transformed plants denoting the role of SPS in promoting carbon partitioning. The transformed plants yielded fruits which contained substantially higher level of sugar than those from non-transformed ones (Laporte *et al.*, 1997).

The association of leaf SPS activity to leaf sucrose content has been explored in many sugarcane varieties by Grof *et al.* (1998). In one study, transgenic tomato plants inserted with maize SPS were found to display up to six times escalation in SPS activity of leaves and escalated photosynthetic rates and sucrose synthesis, as compared to that in control plants (Figure 1). Baxter *et al.* (2003) explored the significance of SPS in tobacco (*Nicotiana tabacum*) by employing a SPS gene from maize. The SPS gene was found to overexpress in all leaves with upto 10 times greater expression in the youngest and oldest leaves and 2-3 fold higher SPS activity in source leaves as compared to that in control. A marked increase in photosynthesis was evident in the oldest leaves, in response to overexpression of SPS. The study validated the role of SPS in promoting photoassimilate production, especially in mature leaves, thus supporting the whole plant's development. However, some have reported no increase in sucrose content by constitutive expression of SPS gene in sugarcane, perhaps pointing to SPP as limiting factor. Hence, it has been proposed that both enzymes (SPS and SPP) work in synchronization and hence must be upregulated simultaneously in order to bring about a change in sucrose content (Vickers *et al.*, 2005).

Grof *et al.* (2006) reported five of the SPS gene families in sugarcane namely SPS A, B, C, D1 and D2, which exhibited differential expression over the various developmental phases, in the different stem and leaf tissue samples of sugarcane. The genes of type A SPS family, showed least expression in leaves while in the culm, the SPS expression was reported to increase from top down. The expression of members of type B and type C of SPS gene families showed significant expression in both immature and mature leaves. Comparable expression in all tissues was displayed by SPS gene members of the two subfamilies D1 and D2 of type D family. SPS gene families A and D have been found to be highly expressed in both, the growing and the fully-grown internodes of sugarcane. In congruence with the inherent characteristic of SPS, expression of the SPS type A family, displayed a pattern in keeping with the internodal sucrose concentration. In maize, both light and developmental stage, were seen to influence SPS expression (Cheng *et al.*, 1996a; Sugiharto *et al.*, 1997) Also, SPS enzyme activity in many species, has been suggested to be regulated by the action of multiple serine protein kinases and phosphatases (Lunn and Furbank, 1997; Lunn and MacRae, 2003).

2.4.3 Sucrose synthase (SuSy)

SuSy catalyses a two-way reaction, wherein it is involved in the cleavage of sucrose in one direction (sucrose+UDP to fructose+ UDP-glucose) and in the other, it partakes in anabolic processes for sucrose synthesis, requiring UDP-glucose as substrate (Hawker, 1985; Huber and Akazawa, 1986). The catabolic reaction of SuSy is favoured in certain instances, since it is said to expend half the energy in comparison to the sucrolytic reaction involving invertases (Black *et al.*, 1987). However, invertase mediated sucrose cleavage produces twice as many hexoses, as compared to the SuSy reaction, thus generating fewer hexose-based signals (Wobus and Weber, 1999; Koch, 2004). Since such signals are considered harmful for growing and maturing tissues, SuSy can be reasoned to be favoured in such developmental phases (King *et al.*, 1997; Wobus and Weber, 1999). Results from experimentations involving feeding of labelled sugars, evidently support the participation of SPS and SuSy in sucrose production (Geigenberger and Stitt, 1991). The deviation in sucrose supply and the demand for assimilate to carry out various processes is coped with, by the co-ordinated action of the sucrolytic pathways mediated by invertases together with sucrose synthesis pathways involving SPS and SuSy (Geigenberger *et al.*, 1997).

A number of studies vouch for the role of SuSy in carbon partitioning in many species including tomato, cotton and sugarcane (D'Aoust *et al.*, 1999; Botha and Black, 2000; Ruan *et al.*, 2003). Various pursuits have explored the functional facets of SuSy w.r.t. facilitation of the association between source and sink (Zrenner *et al.*, 1995) and many others, namely, role in production of starch (Nguyen-Quoc and Foyer, 2001), build-up of cell wall (Chourey *et al.*, 1998). The unique ability of SuSy of functioning under low oxygen conditions prevalent in maturing sinks, makes it a particularly important enzyme (Zeng *et al.*, 1999). This is because, the consequent production of UDP-Glc will help preserve adenylate in comparison to modus operandi of invertases, which utilizes ATP to produce hexoses. Thus, the sucrolytic role of SuSy may be even more vital during sucrose accumulation, when a hypoxic condition is maintained in the sucrose-amassing mature internodes of sugarcane.

Activty of SuSy has been correlated with growth in many species and hence it can also be considered a measure of sink strength (Farrar, 1993) (Figure 1). During the elongation phase, the peak activity of both SuSy and acid invertase denotes the participation of both enzymes in internodal elongation (Lingle, 1999). An association between the sucrolytic trait of SuSy and internodal elongation has been drawn by Lingle and Smith (1991), in sugarcane. The internodes that are in the phase of elongation, have been found to exhibit higher SuSy activity as compared to internodes those that are fully elongated and matured (Buczynski *et al.*, 1993). The importance of SuSy in sucrose unloading has also been demonstrated in many studies (Hänggi and Fleming, 2001) and thus, tissues importing sucrose viz. mature internodes of sugarcane, can be reasoned to have high SuSy activity (Xu *et al.*, 1989; Martin *et al.*, 1993). It has been suggested that SuSy perhaps carries out sucrose production in younger internodes since its sucrose synthesizing activity was found to be inversely related to stem maturity (Goldner *et al.* 1991; Botha and Black, 2000; Schafer *et al.* 2004).

Wächter *et al.* (2003) have identified SuSy to be present close to the sieve tube elements' plasma membrane and a phloem-specific ATPase that supports sucrose transport and compartmentalization. Three isoforms of the SuSy gene have been identified in monocot species and two in dicot species (Fu *et al.*, 1995; Huang *et al.*, 1996). These genes are predicted to perform metabolic function, exhibit differential expression w.r.t. time and space (Koch, 2004), tissue type and the carbohydrate metabolic state (Winter and Huber, 2000). Carbohydrate-limiting conditions (0.2% compared to the required 2% glucose) are suggested to stimulate the increased expression of SuSy isoform *Sh1*, which also displays significant increase in expression and enzyme activity under low oxygen conditions(i.e. 3% oxygen) (Zeng *et al.*, 1998). On the other hand, high glucose concentrations induce the expression of *sus1* (Hellman *et al.*, 2000).

2.5 Source to sink transport of sucrose

Past efforts have largely explored the sucrose synthesis pathway (Sachdeva *et al.*, 2011) lending light on factors affecting the sucrose synthesis pathway and the effect of alterations therein. Sucrose accumulation in the sugarcane culm occurs as an outcome of co-ordination between numerous factors like photosynthesis, interplay of sucrose metabolizing enzymes, carbon distribution within the leaf, sucrose translocation and storage (Figure 2).

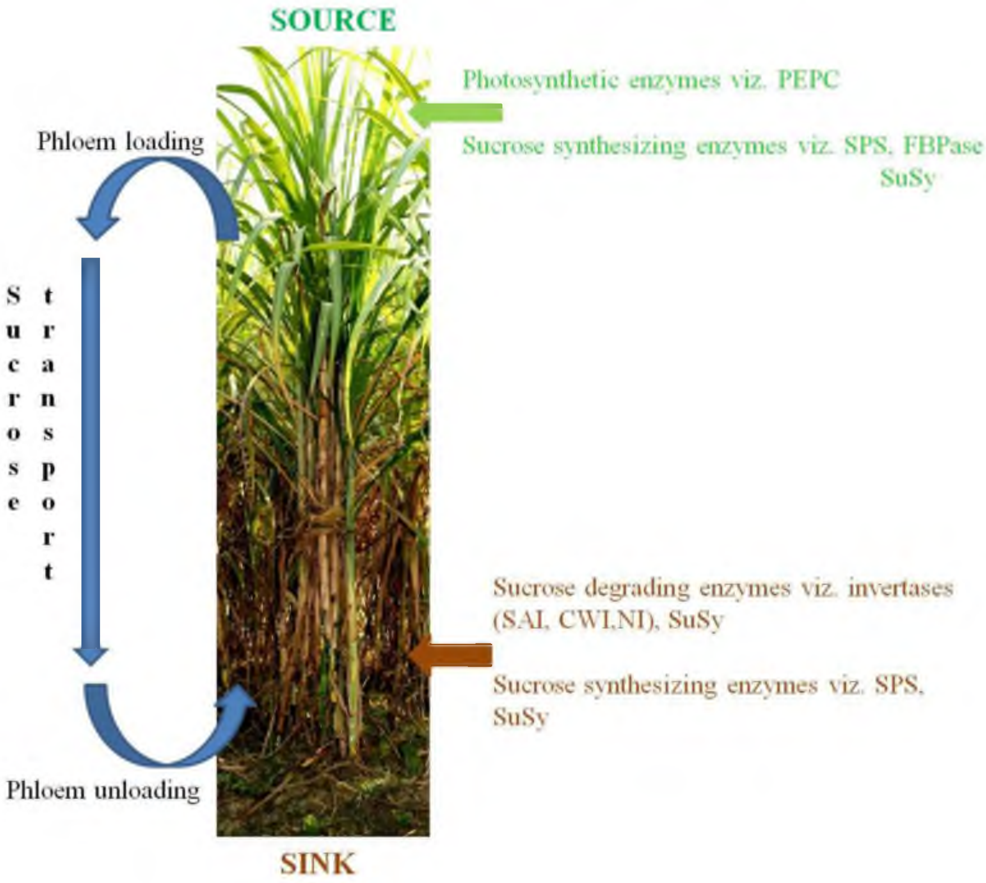


Figure 2: Source-sink communication depicting fate of sucrose

The sink demand mainly depends upon supply from source, since the genes ascertaining sink strength function on the basis of their response to sugar (Koch, 1996; Ramon *et al.*, 2008; McCormick *et al.*, 2008c; Tiessen and Padilla-Chacon, 2013). Such genes whose expression is governed by sugars are involved in a variety of functions in the sucrose metabolism pathway viz. sucrose hydrolysis, sugar export/import, sucrose accumulation and storage (Li *et al.*, 2002; Ramon *et al.*, 2008). Thus, in order to manipulate and improve the sucrose accumulating ability of the sink, it is vital to understand the factors that affect sink strength at molecular level. For instance, a move towards understanding carbon distribution in sugarcane, has been the isolation and characterization of the molecular factors/ genes governing sucrose translocation viz. genes belonging to sugar transporter families, those involved in import and export from phloem and genes of the sucrose metabolism pathway (Carson and Botha, 2002; Grivet and Arruda, 2002; Casu *et al.*, 2005; Rae *et al.*, 2005a).

2.5.1 Photosynthetic carbon assimilation

Carbon assimilation occurs via the C_4 pathway, whereby CO_2 is initially fixed in the leaf by exterior mesophyll cells and then transported to internalised bundle sheath (BS) cells. The key feature of C_4 photosynthesis is the compartmentalisation of activities, typically into the two specialised cells. Concluding from the site of SPS activity in other C_4 species (Lunn and Furbank, 1997), the mesophyll cells in leaves can be inferred as the location of sucrose synthesis in sugarcane too. From its production site, sucrose adopts a symplastic route, travelling via plasmodesmata, reaching the BS cells and then from there into the vascular parenchyma cells in order to be translocated (Russin *et al.*, 1996). In sugarcane, photosynthesis and photoassimilate production largely occurs in response to sugar signalling, involving the combined sensing of both symplastic and apoplastic sugar concentrations and hence is governed by the rate of sucrose loading and export into the phloem.

All C_4 plants employ phopshoenolpyruvate carboxylase (PEPC) at the very start, to fix HCO_3^- into the dicarboxylic acid, oxaloacetic acid (OAA) (hence the name, C_4). Those using PEPC as the carboxylase are categorized to follow either crassulacean acid metabolism (CAM) pathway or the C_4 pathway. This is determined by whether expression of PEPC and RuBisCO is distinguished temporally or spatially. The activity of PEPC in C_4 pathway is regulated by various metabolic factors, including feedback inhibition triggered by malate, activation brought about by glucose-6-phoshate and a highly complex, light-dependent reversible phosphorylation process (Bakrim *et al.*, 1993). Many pursuits have aimed to manoeuvre the level of PEPC (Jeanneau *et al.*, 2002) and Rodriguez-Penagos and Munoz-Clares (1999) correlated PEPC activity to the control of carbon flux in maize, grown under water restrained conditions. Also, there have been many attempts to introduce C_4 PEPC into several C_3 species, including rice and tobacco (Kogami *et al.*, 1994; Agarie *et al.*, 2002).

The primary CO_2-fixing enzyme of C_4 pathway, PEPC (Figure 1), occurs in the cytosol of mesophyll cells. OAA, generated by fixing of CO_2, is then converted into malate or aspartate, and subsequently reaches the bundle sheath cells. Decarboxylation of these C_4 acids results in CO_2, and carbon fixation is catalysed by Rubisco. C_4 photosynthesis is regulated at the level of metabolite transfers, by exercising control over cell-specific transporters viz. dicarboxylic acid transporters and symplastic transport (Edwards *et al.*, 2001). A number of factors have been devised to facilitate carbon metabolism in leaves of C_4 plants; for instance, mesophyll cells being the site of sucrose synthesis, house the genes related to sucrose biosynthesis (Lunn and Furbank, 1999). However, the factors that regulate the rate of assimilation in C_4 plants, such as sugarcane, are yet to be completely elucidated.

Fructose-1,6-bisphosphatase (FBPase) and SPS are among the major factors that influence the sucrose synthesis process (Stitt and Quick, 1989). During the pathway, fructose-1,6-bisphosphate (F1,6P) is converted to fructose-6-phosphate (F6P) by either the cytosolic FBPase or pyrophosphate-dependent phosphofructokinase (PFP). Of the two, FBPase acts as a more vital control point in the sucrose production pathway under the influence of light (Stitt *et al.*, 1987; Quick and Schaffer, 1996). The activity of cytosolic FBPase, which catalyses the prime irreversible step of the sucrose synthesis pathway (Figure 1), is under allosteric control and is hampered by decrease in the ratio of F1,6P to Pi and/or level of AMP (Stitt *et al.*, 1982) which in turn is influenced by the concentration of fructose 2, 6-bisphosphate (F2,6P) (Stitt, 1990).

2.5.2 Phloem loading

Sugar transport from source to sink occurs via the phloem. The other tissues utilize the translocated sugar to sustain their maintenance and growth processes. The cells around the phloem conduit are thought to store sugars and polysaccharides as reserve, in their vacuoles, to make certain that there is no shortage of supply even when source is inoperative. This storage ability has been particularly well-developed in sugarcane, by employing approaches like breeding and selection, to enable the culm parenchyma to store large amounts of sucrose (Jackson, 2005). Loading of sucrose from source leaf into the phloem can occur via active or passive transport. The active transport route involves energy-fuelled, transporter protein-mediated, transmembrane movement of sucrose through the apoplastic space (called apoplastic loading). Symplastic loading, on the other hand, is the passive transfer of sucrose through symplasm (via plasmodesmata) into the phloem, referred to as passive because the transport does not occur against a concentration gradient (Komor, 2000; Slewinski and Braun, 2010). Symplastic phloem loading has been reported in plants like squash and melon. Plant species that exclusively display apoplastic loading viz. potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum* L.), do so, in the absence of symplastic connection with the phloem such that export and import of sucrose into the phloem and apoplastic space, occurs by means of transmembrane protein transporters (Kühn and Grof, 2010). Since sugarcane leaves possess a transport system wherein the SE-CCC is not symplastically connected to the neighbouring tissues (Robinson-Beers and Evert, 1991), Rae *et al.* (2005a) have rightly suggested the loading into SE-CCC to occur apoplastically. Sucrose movement occurs from the leaf into phloem and through the phloem, its transport occurs along concentration gradients towards sink tissues (van Bel, 2003; Rae *et al.*, 2005a).

The rate of export of sucrose from the leaf is governed by the rate of sucrose synthesis and accumulation in the source leaf (Ho and Thornley, 1978). Since triose phosphate used in sucrose synthesis is supplied from the chloroplast, hence rate of photosynthesis can be said to affect the rate of sucrose synthesis (Stitt, 1986; Battistelli *et al.*, 1991) and consequently rate of sucrose transport (Rocher *et al.*, 1989). Certain proton symporters positioned on the plasma membranes of sieve element/companion cell complexes (SE-CCC) facilitate loading of sucrose and perhaps certain amino acids, from source leaf apoplasms into the minor veins (Lalonde *et al.*, 2003).

Sucrose transporters are active transporter complexes that transport sucrose across membranes in response to source-sink imbalance. The H+/sucrose-symporter (SUT) is one of the most well-studied in the sucrose transporter family (Lalonde *et al.*, 2004; Kühn and Grof, 2010; Ainsworth and Bush, 2011). It provides for ATP energy-driven loading of sucrose from apoplasm into the SE-CCC (Lalonde *et al.*, 1999) (Figure 1). Sucrose symporters are commonly found to be colocalized with H^+-ATPases on plasma membranes of SE-CCC (Bouché-Pillon *et al.*, 1994) that provide the driving force (pmf) to aid symport.

Chiou and Bush (1998) reasoned that sucrose pile up in leaves perhaps inhibited SUT activity and prevented sucrose loading in turn. They demonstrated this by externally feeding sucrose into the transpiration stream of cut sugarbeet leaves, thus mimicking the condition of low sink demand. The elevated sucrose level thus developed in the vascular tissue was found to reduce transporter activity, perhaps to slow down phloem loading. In turn, increased sugar levels were recorded in mesophyll cells which caused photosynthesis to reduce. Increased sink demand would perhaps lead to the reverse sequence of events. Ainsworth and Bush (2011) have also corroborated the correlation between sugar export in leaves and sink demand. Hence, sucrose transport through phloem can be thought as a vital manipulation target for enhancing sucrose production and improving plant productivity as a whole.

SUT1 has been reported to facilitate sucrose export into the phloem of leaves in maize (Slewinski *et al.*, 2009). Though it is known to express in stems too (Aoki *et al.*, 1999), the exact role of SUT in stem has not yet been declared. However, Rae *et al.* (2005b) targeted the orthologous SUT1 protein (ShSUT1) in sugarcane and by raising a peptide antibody against it, asserted the presence of ShSUT1 in the mestome sheath and vascular parenchyma cells of stalk and leaf samples. This proposes that this SUT1 protein perhaps has a role other than phloem loading too. It has been further suggested that ShSUTI probably plays a part in retrieving sucrose leaked from transport along the SE-CC or in acquisition of translocated sucrose into sink (Patrick, 1990; Rae *et al.*, 2005b). In a study by Leggewie *et al.* (2003), the upregulated expression of a spinach SUT in potato plant, produced tubers with higher sugar content and lower amino acid levels, than control, though not much difference was observed in the biomass. However, due to dearth of knowledge w.r.t transporters known so far, the area must be explored further before these membrane proteins can be employed for improving sucrose accumulating capacity of sugarcane.

2.5.3 Phloem unloading

It has been indicated that the presence of sucrose is not restricted to the vacuole but is also present in the symplast and apoplast in significant amount (Hawker, 1985; Welbaum and Meinzer, 1990). Depending on the route adopted, phloem unloading into the sink tissues can be said to occur via symplastic and apoplastic channel. In the developing sinks of various plant species viz. potato stolons (Viola *et al.*, 2001) and sugarcane culms (Jacobsen *et al.*, 1992), phloem unloading is said to occur symplastically at first and an apoplastic step is to be interposed later (Lalonde *et al.*, 2003).

The report stating presence of invertase activity in the apoplast and detection of hexose transport in the storage tissue, can be interwoven to conclude that sucrose imported into the apoplast, is hydrolysed by invertase to yield hexoses which are subsequently transported into storage cells, wherein they are utilized to resynthesize sucrose (Patrick, 1997). Vacuole is deduced as a major sucrose storage site since it occupies a large volume of the storage parenchyma in the sugarcane culm. Storage of sucrose in the vacuole, helps provide room for inviting more sucrose by maintaining low sucrose concentrations in the cytoplasm, thus facilitating continued movement of sucrose along the gradient.

The presence of abundant plasmodesmata connections within the vascular bundle vouches for symplastic movement of sucrose from SE-CCC to the parenchyma (Lingle, 1989; Walsh *et al.* 2005). Welbaum *et al.* (1992) have affirmed the absence of apoplastic movement by studying the sap in the xylem (adjoining phloem apoplast) of sugarcane stems, which displayed no sucrose content. As sucrose concentration builds up in the maturing stalk, turgor pressure drives the transport of some sucrose to the apoplast of parenchyma cells in order to maintain homeostasis. Thus, apoplast furnishes additional storage space for sucrose accumulation in the stem parenchyma and aids in enhancing sink strength. Studies have reported nearly 400–700mM sucrose concentration to have been attained in apoplast, as a result of this leakage (Welbaum and Meinzer, 1990; Welbaum *et al.*, 1992). Thus, in order to prevent the development of high turgor pressure, the sucrose in the symplasm must be distributed to the apoplasm, other than storage in the vacuole of parenchyma (Rae *et al.*, 2005b).

The presence of high sucrose content in the apoplast, perhaps indicates that sucrose unloading into the apoplasm of sink tissues, occurs via a specific mechanism. Several studies have indicated that sucrose transporters mediate apoplastic unloading of sucrose into the sink (Lalonde *et al.*, 1999). Carpaneto *et al.* (2005) inferred from their studies on expression of maize sucrose transporter (ZmSUTI) in *Xenopus laevis*

(Daudin) oocytes, that SUT facilitates transport of sucrose reversibly with the direction of transport being governed by the existing pH and sucrose gradient. Thus, evidence suggests that SUTs aid sucrose loading at the source and unloading at the sink.

Cell wall-bound invertase (CWI) is stated to be a prominent factor regulating the apoplastic unloading pathway (Figure 1). As per its invertase nature, it irreversibly hydrolyzes the sucrose unloaded in sink, into hexoses. The influx of these generated hexoses, is in turn, facilitated by monosaccharide-H^+ symporters (MSTs). Since CWI activity is affected by various factors like glucose, phytohormones and stress, hence CWI can be seen to play a vital role in adjusting sink strength in response to different stimuli (Roitsch *et al.*, 2003). Phloem unloading in sugarcane culm can be affirmed to occur via apoplastic route due to the identification of CWI activity in the culm tissues (Glasziou and Gayler, 1972). Also, sucrose supplied to sugarcane cell suspension, was observed to be utilised as glucose, perhaps under the action of CWI, thus confirming the presence of an apoplastic unloading step (Komor *et al.*, 1981). On the other hand, labeling studies have demonstrated sucrose being directly taken up by slices of sugarcane culm. These studies lend evidence for transport mechanisms mediated by MST as well as sucrose transporter, to be operating (Lingle, 1989).

Since CWI activity and hexose quantum have been observed to decrease in maturing culm (Albertson *et al.* 2001), it may be derived that the prevalence of MST-mediated apoplastic unloading decreases with increasing maturity. The vascular bundles in maturing culm, have been reported to get enveloped by cells that face lignification and suberization over time (Jacobsen *et al.* 1992), separating the stem parenchyma from the phloem. This barrier thus developed, perhaps hinders apoplastic unloading, due to which, resorting to the symplastic route becomes inevitable in sugarcane.

2.6 Sucrose cycling and accumulation

After being transported via phloem and unloaded into the culm parenchyma, the sucrose is hydrolysed by the three different invertases (depending on location and operational pH) and sucrose synthase. The hexoses are utilized to regenerate sucrose by the action of SPS and SuSy (Hatch *et al.*, 1963; Hatch, 1964) (Figure 2), which is subsequently stored in the vacuole. Hence, invertases furnish hexoses to support various metabolic processes in the growing sugarcane stem and hence aid in the development of sink strength. However, as the cane ripens and elongation phase recedes, the sink tissue displays lower metabolic activity and hence lesser proportion of the translocated sucrose is degraded during its transfer and more and more of it is stored in the vacuoles of stem parenchyma (Tarpley *et al.*, 1996; Tarpley and Vietor, 2007). Defying the increasing sucrose pile up in storage tissue, sucrose hoarding in sugarcane, is fuelled by respiration, to occur opposed to the concentration slope (Burg and Bieleski, 1962). Glasziou and Gayler (1972) first postulated the process of sucrose amassing in sugarcane, as a model.

High sucrose concentrations hoard in the mature sugarcane culm because sucrose supply from the source, surpasses culm need or demand. Generally, the activity of one enzyme will predominate over another, depending on whether the tissue is operating in the direction of net sucrose synthesis or sucrolysis (Rae *et al.*, 2005a). Thus, the net accumulation of sucrose is determined by the cycle of degradation–synthesis of sucrose operating in the sink (Batta and Singh, 1986; Chandra *et al.*, 2011). The 'futile' cycling of carbon occurs between concurrent degradation of sucrose into hexoses and resynthesis of sucrose from these hexoses, in the sink tissue, as reported in many including sugarcane (Geigenberger and Stitt, 1991; Whittaker and Botha, 1997; Nguyen-Quoc and Foyer, 2001; van der Merwe *et al.*, 2010). This 'futile cycling' though still prevalent, decreases with tissue maturity (Bindon and Botha, 2002; Uys *et al.*, 2007). In sugarcane, sucrose accumulation brings about an alteration in carbon allocation, it decreasing towards respiration (Whittaker and Botha, 1997). Hence, if the concentration of substrate viz. hexose phosphate, is increased, it may encourage greater sucrose synthesis and consequently higher sucrose accumulation (van der Merwe *et al.*, 2010).

During maturation, mainly SAI and CWI have been known to regulate sucrose accumulation in sugarcane. The SAI activity is said to be highest in the youngest internode, its level decreasing with increase in maturity of the internode. The ratio of SPS/SAI varies with the maturity of the culm, and thus governs the rate of sucrose hoarding and in turn, the final sucrose level in culm. Sucrose biosynthesis in source leaves is mainly catalysed by SPS, which is suggested to work together with SPP, by forming a complex *in vivo*. In addition, SPS also partakes in the series of sucrose hydrolysis and resynthesis reactions that occur in sink tissues (Rae *et al.*, 2005a).

As sucrose accumulates in the maturing culm, the physiological maximum of sucrose concentration, has been indicated to be governed by the feedback sent by signalling molecules like hexoses, to delimit sucrose accumulation (Bindon and Botha, 2002). Several reports have evidenced the major control that sink activity exercises on leaf assimilation via the inhibitory feedback communicated by sugars. Hence, an approach to improve sugar yield would be to disrupt the negative signalling between source and sink tissues, which in turn shall improve photoassimilate production at source (McCormick *et al.*, 2009). In similar light, the negative regulation exercised by sucrose pile up, on SPS activity, has been proposed as a possible factor controlling sucrose production (Kortschak *et al.*, 1965). Thus, exploring SPS activity in detail could also yield a promising target for manipulating sucrose productivity.

2.7 Gibberellins as modulator of sink demand

The first reference of gibberellins was given by in 1898, when the development of tall spindly plants in rice was first noted and termed 'bakanae disease' (or foolish seedling) (Arteca, 1996). In 1935, 'gibberellin' (GA) was isolated by Yabuta, as a crystalline substance, from *Gibberella fujikuroi*, the disease causing fungus (Moore, 1989). Gibberellins (GAs) are plant growth regulators which are basically tetracyclic diterpenes, known to play part in a number of plant processes viz. leaf expansion, stem elongation and many others (Yamaguchi, 2008). GA_3 is the most important GA from among 136 naturally occurring GAs in plants. It is an economically important secondary metabolite formed as the end product of the GA pathway (Takahashi *et al.*, 1986). In many species viz. *Brassica campestris* (Pressman and Shaked, 1991), GA has been reported to increase shoot length by enhancing its rate of elongation.

GAs alter the source–sink relations by stimulating photosynthetic activity on one hand and developing sink strength and sink demand, on the other, in turn improving plant yield (Kozlowska *et al.*, 2007). Gibberellins are known to influence the leaf-area and light capturing capacity of leaves, thus improving the photosynthetic efficiency of plants. Exogenous application of GA_3 has been shown to increase source photosynthetic rate by stimulating photosynthetic enzymes viz. enzymes of dark reaction (Khan *et al.*, 2007). GA treated plants exhibit increased carbon assimilation, as qualified by more soluble carbohydrate observed in them than in controls. The competence of GA in promoting photosynthesis is affirmed by the rise in glucose content observed on GA application (Brian *et al.*, 2006). SPS activity in soybean and spinach leaves has been proposed to be regulated by GA. GA perhaps facilitates sucrose synthesis in leaf by stimulating the two major enzymes of sucrose synthesis i.e. fructose-1,6-biphosphatase and sucrose phosphate synthase (Iqbal *et al.*, 2011). GA is also said to aid phloem loading by influencing cell turgor and apoplast pH (Zamski and Schaffer, 1996).

Numerous studies carried out in plants viz. *Avena* (Kaufman *et al.*, 1973), *Phaseolus vulgaris* (Morris and Arthur, 1985) and *Pisum sativum* (Wu *et al.*, 1993), have demonstrated that externally applied GA causes an escalation in activity of acid invertases, in immature, growing plant tissues. GA promotes apoplastic unloading in elongating internodes by enhancing invertase activity. This causes increased hydrolysis of sucrose to hexose, providing a slope driven by concentration difference, thereby encouraging unloading of sucrose into the sink (Cole *et al.*, 1972). In particular, GAs stimulate the expression of extracellular invertase, which controls assimilate translocation. This increased partitioning of photosynthates to sink, is utilized in causing growth of sink tissues, thereby building sink strength (Khan *et al.*, 2007). Thus, GA improves sink strength by affecting sucrose metabolism. Additionally, GA increases sink potential by promoting cell division or enlargement that determines sink size. This results in elevated sink demand due to more space for assimilate deposition and dry matter accumulation. GA application is suggested to affect the expression and activity of various sucrose metabolizing enzymes, which in turn affect sink demand (McCormick *et al.*, 2006). Hence, gibberellins can be explored as a viable option to enhance sucrose accumulation in the sugarcane culm and consequently cane yield as a whole.

GA has been reported to promote nutrient transport and improve nutrient-use efficiency in plants (Khan *et al.*, 2005). Elevation in activities of various enzymes viz. carbonic anhydrase (CA) and nitrate reductase (NR), under the influence of GA, has been reported viz. in mustard (Khan *et al.*, 1996). The higher NR activity vouches improvement in N-use efficiency under GA action. It has been interpreted by Khan *et al.* (2002) that GA_3 application brings about increase in shoot growth, causing greater utilization of nitrogen and improved photosynthesis, which in turn stimulates N uptake. Xu *et al.* (2014) have also explored the effect of GA on plants' response to various environmental factors.

Uniconazol, a substance that works antagonistic to GA_3 (Izumi *et al.*, 1984; Kim *et al.*, 1988), is an inhibitor of gibberellin biosynthesis and other GA_3-mediated metabolic processes. It is a growth retardant and has been found to hamper leaf growth, though its inhibitory effect was nullified by exogenous GA treatment, highlighting the importance of endogenous gibberellins in controlling photoassimilate metabolism in leaf. Uniconazol treatment has been reported to impede both, phloem export from the leaf and import of translocate into the sink, though, unloading into the root remained unperturbed. Uniconazol has been stated to deter stem growth and reduce invertase activity, as a result. Since invertase hydrolyses translocated sucrose to make hexoses available to cells of growing sink (McCollum *et al.*, 1988; Ricardo and ap Rees, 1970), uniconazol-induced reduction in invertase, perhaps affects sink activity. The reduction in the rate of sucrolysis may in turn hinder the import of sucrose, thus checking phloem unloading.

2.8 Molecular approaches to improve sucrose accumulation

Sugarcane is an aneuploid allopolyploid, with modern-day cultivars derived from a succession of interspecific crosses particularly between *S. officinarum* (2n=8x=80) and *S. spontaneum* (2n=5x=40 to 16x=128) (D'Hont *et al.*, 1998). The genetic complexity of this crop makes its improvement through conventional breeding alone, a challenging task (Dunckelman and Legendre, 1982). The total genome size of sugarcane has been reported to be nearly 10 Gb, while the monoploid genome size is said to vary between 800-900 Mb (D'Hont and Glaszmann, 2001). Recently, Garsmeur *et al.* (2018) have assembled the first sugarcane BAC-based monoploid genome reference sequence.

Even though genetic maps were postulated for classical varieties as well as many modern cultivars (Grivet *et al.*, 1996), none were all inclusive, and only represented about two-thirds of the genome, due to large ploidy and genome size (Dufour *et al.*, 1997). Gene discovery in sugarcane, especially those particularly related to sucrose metabolism and accumulation, has also been fairly limited. In the past, expression analysis employing EST data has led to large scale identification of genes involved in various pathways, to determine response of tissues or whole organisms and in the discovery of novel genes. In a significant effort, Brazilian project SUCEST generated an information bulk of EST sequences from eleven sugarcane tissues under varied conditions, derived from nearly 26 libraries (Vettore *et al.*, 2003). Employing cDNAs drawn from this compilation, genes including those related to sugar signalling, expressing under different conditions, were determined (Felix *et al.*, 2009; Papini-Terzi *et al.*, 2009).

Past works have pointed at the expandable capacity of sugarcane culm to accumulate more sucrose than achieved yet (Grof and Campbell, 2001), and efforts to obtain this ceiling solely by conventional breeding has been futile. Hence, employing molecular level approaches to decipher the processes involved in sucrose hoarding and the factors affecting therein, would help design alternative approaches to improve sucrose concentration in sugarcane culm.

Transgenic efforts to attain better sucrose hoarding in sugarcane have mostly involved manipulation of expression of single genes encoding vital sucrose metabolizing enzymes. Ma *et al.* (2000) reported transgenic manipulation employing an invertase derived from yeast, aimed to the apoplast, cytosol or vacuole compartments. However, such attempts were not met with much success since the transgenic plants developed, did not exhibit remarkable increase in sucrose hoarding (Botha *et al.*, 2001; Rossouw *et al.*, 2007; Groenewald and Botha, 2007; van der Merwe *et al.*, 2010). Thus, thorough exploration of the various mechanisms known to control sugar metabolism in various plants (Paul and Pellny, 2003; Rolland *et al.*, 2006), and those suggested in sugarcane, in specific (McCormick *et al.*, 2006), should precede transgenic endeavours employing genes as single or in tandem. The co-ordinated manipulation of a variety of enzymes can perhaps bring about improvement in sucrose accumulation (Mao *et al.*, 2006).

Sugars have long been established as sensor molecules controlling photosynthesis and other metabolic processes, however much molecular level information about the sensing and signalling mechanisms of sugars, remain to be delved into (Rolland *et al.*, 2002; Gibson, 2005). During movement of sucrose from photosynthetic cells in sugarcane leaves, to the phloem, regulatory signalling operates at several points (McCormick *et al.*, 2009). As discussed earlier, loading of sucrose into the phloem occurs actively (Rae *et al.*, 2005a) and hence sucrose symporters, such as SUT1 and SUT-like homologues (Kühn and Grof, 2010) can perhaps serve as vital signals for presence of sugar outside the cell (Lalonde *et al.*, 1999). Additionally, several enzymes essential to photosynthetic rates in sugarcane leaves, can act as intracellular sugar sensor in the cytosol (Moore *et al.*, 2003). The operation of several sugar sensing systems viz. for glucose, sucrose and trehalose, has been discerned. Hexokinase (HXK) is an important enzyme influencing photosynthetic rate and it modulates expression as per glucose, thus functioning as a glucose sensor. In addition, sucrose non-fermenting 1-related protein kinases also participate in sugar signalling (Rolland *et al.*, 2002; Felix *et al.*, 2009). Though most regulatory mechanisms operating in C_4 plants are common with those existing in C_3 plants (Sheen, 2001), many aspects of regulation exercised by sink on source still need to be elucidated in C_4 species like sugarcane. A number of studies have suggested the significance of trehalose 6-phosphate (T6P), in controlling carbohydrate processing (Ramon and Rolland, 2007). Through microarray analysis of sugarcane leaf samples, McCormick *et al.* (2008c) also ascertained the role of T6P as an important signalling molecule and identified many other molecules viz. TPP, that showed potential of influencing sucrose accumulation. On doubling of leaf sugar concentration, maximum photosynthetic rate was found to decrease by 80% while nearly 5-fold escalation was recorded in the expression of various trans-membrane sugar transport associated genes.

Furthermore, some studies have examined the sugarcane metabolome (Bosch *et al.*, 2003). Also, several molecular markers for improved sucrose hoarding in the stalk, have been identified, and presently employed in various improvement strategies to enhance sucrose yield (Snyman *et al.*, 2008). Papini-Terzi *et al.* (2009) identified differentially expressing genes among genotypes differing in terms of Brix (sugar), through a cDNA microarray study. The selected genes corresponded with protein kinases and transcription factors, indicating that such genes may be instrumental in governing sucrose accumulation.

The widespread approaches adopted so far and the persistent endeavour in this direction, have as yet failed to realize the goal of improving the culm sucrose content. The availability of limited genetic resources for sugarcane, has compelled the use of sorghum which is the closest relative of sugarcane, as reference, for identification of gene members by PCR cloning. In the face of such crisis, a number of studies (Varshney *et al.*, 2009; Edwards and Batley, 2010) vouch for the utility of the latest well-equipped DNA sequencing technologies viz. Next-generation DNA sequencing

(NGS), to decipher complex crop genomes at molecular level. The huge bulk of data generated can be analysed and assigned functional annotation using various bioinformatic tools available (Lee *et al.*, 2012). Certain research pursuits have attempted at identifying sections of the genome precisely associated with and detemining stalk's sucrose content (Ritter *et al.*, 2008). Calviño *et al.* (2009) carried out transcriptional profiling of stem tissues of sweet and grain sorghum in order to identify differentially expressing genes. The study helped sieve out several genes related to sucrose amassing in sweet sorghum.

Due to the genetic complexity of sugarcane and the high costs involved in exploring its large genome, whole genomic sequencing of sugarcane has been elusive. However, using productive sequencing technologies, such as Roche/454 and Illumina/Solexa, it has now become possible to obtain even transcriptomic level information in minimal cost and time (Mardis, 2008). Transcriptome sequencing is a competent method for transcript level expression analysis, especially of complex genomes and for furnishing functional annotation of differentially expressing genes, which may be associated with vital agronomic traits and depict allelic variation, to be used as potential targets for conventional breeding (Wolf, 2013). In one pursuit, Solexa technology was employed to study sugarcane infected with *Sporisorium scitaminea*, which brought to light 2015 genes thought to be involved in pathogen response (Wu *et al.*, 2013). *De novo* assembly by Illumina RNA-seq platform and transcriptome annotation was utilized to generate database of six sugarcane genotypes (Cardoso-Silva *et al.*, 2014). Also, in a recent attempt, Wu *et al.* (2018) carried out transcriptome-level differential expression analysis in the leaves of *Saccharum spontaneum*, in response to drought stress, thereby identifying key related genes. In a similar study, Yu *et al.* (2016) examined differentially expressed genes in response to salt stress, thus elucidating the molecular basis of development of salt tolerance. Thus, updating our current knowledge on similar lines, by employing advanced approaches viz. transcriptomic analysis, may help to analyse and interpret factors affecting sucrose accumulation in sugarcane.

Chapter 3

Materials and Methods

MATERIALS AND METHODS

3.1 Plant material

As per standard agricultural practices in India, sugarcane planting done in February (spring) or October (autumn), supports high cane yield, with the crop's life-cycle generally spanning 360 and 420 days, respectively (Kapur *et al.*, 2011; Rai *et al.*, 2017). The present pursuit used canes from two plantations/growth cycles, with planting done in the last week of February (hence called spring planting), in 2014 and 2015, respectively. The crop was planted in plantation areas of ICAR-Indian Institute of Sugarcane Research (IISR) (26.78° N, 80.99° E, 111 msl) Lucknow, India, using three-bud setts (cut cane pieces containing three axillary buds, one at each node). The study was carried out by utilizing two sugarcane varieties with distinct sucrose amassing capacities viz. CoJ64: a variety reported to mature relatively early and record high peak sucrose values and BO91: known to mature relatively late and store comparatively lower concentration of sucrose. This endeavour was intended at exploring the difference in their responses to different treatments and also assessing the scope of manipulation of their sucrose accumulating capacity. The setts were duly pre-treated with Chloropyriphos (1.5ml/litre) and bavastine (2g/litre) to prevent infestation by pests. The pre-treated setts were laid in furrows (sprinkled with adequate fertilizer viz. urea, DAP and MOP), with nearly 250 setts per 50 m row.

3.2 Treatments and sampling

The study was carried out over the entire duration of maturation starting from October, when ambient temperature lowered to 14^{o}-20^{o}C (min.) and 28^{o}-32^{o}C (max.), since cooler temperatures divert photoassimilate from growth to promoting sucrose accumulation. Here, source-sink communication was perturbed by means of two different treatments viz. application of gibberellins and partial leaf shading, and the changes produced therein, were gauged especially w.r.t. sucrose accumulation.

3.2.1 Gibberellin spray

In the 2014 plantation, 50ppm gibberellic acid (GA_3) was exogenously applied in the form of spray, to canes of BO91 variety, in the month of September. A battery-operated automatic sprayer was employed for the purpose, in order to facilitate uniform coverage of the spray liquid over the entire plant (Figure 3). Samples collected on the day of spraying (0 DAS) were used as start point reference. Sampling was done from non-treated (control) and GA_3 treated BO91 canes, at monthly intervals, from October to January (i.e for four months after spraying).

Figure 3: Application of GA_3 in the form of spray using battery-operated automatic sprayer

Canes of both varieties (viz. BO91 and CoJ64) of 2015 plantation, were subjected to exogenous application (35ppm spray) of gibberellic acid (GA_3), applied thrice, at monthly intervals, during the grand growth phase (July–September). On the last day of spraying in September (0 DAS), a non-sprayed cane was harvested, to be used as 0^{th} day control. GA_3 sprayed and non-sprayed samples were sampled during the entire span of maturation i.e. from October to January for CoJ64 and October to March for BO91. Three canes of comparable height and girth were sampled as biological replicates, for each sample type viz. control (non-sprayed) and GA_3 sprayed, of both varieties, BO91 and CoJ64. Each cane was equally divided into bottom, middle and top segments. Tissue sampled from specific (middle and top) segments of each cane was utilized for RNA isolation while sugar and other related parameters were estimated in juice extracted from various segments of cane. As for sampling from source, the most recently opened and metabolically active LTM leaf (distinguishable as the one which displays last transverse mark) was used for RNA extraction as well as assessment of sugar and other biochemical parameters.

3.2.2 Partial shading

Another experiment was set up by utilizing the partial leaf shading technique to disrupt source-sink communication and evaluate changes therein. All leaves excluding the photosynthetically most active LTM leaf, were covered with 90% shade cloth for variable days, for a span of 12/13 days, in October (Figure 4). Consequently, in this duration, the exposed LTM leaf functioned as the sole light-harvesting source. Sampling was carried out in three biological replications, using canes of both varieties, of comparable height and girth, reared under partially shaded and normal conditions. The non-shaded cane sampled at the start of shading period (i.e. 0 day after shading (DAS)) was taken as control. The canes subjected to partial shading were subsequently sampled at varied times (viz. on 2, 5 and 13 DAS for BO91 and 3, 6, 12 DAS for CoJ64) over the period of shading.

Figure 4: Partial shading done by enclosing all leaves, except the LTM leaf, in 90% shade cloth, for varying periods of time

RNA was isolated from tissue of each of the three segments of cane and juice from the respective cane segments was utilized for sugar analysis. Also, sampling was done from source (unopened leaf tuft), LTM and sink leaves (the two leaves just below LTM leaf, attached to the sink) of control and shaded canes, in order to isolate RNA and determine sugar content and other related parameters.

3.3 Morphological analysis

3.3.1 Measurement of morphological parameters

5 on-field canes were randomly selected from each of the two treatment groups: control and GA_3 (35ppm) treated, of both varieties, BO91 and CoJ64, and were profiled for various morphological parameters viz. total cane height, LTM leaf length, LTM leaf width, in order to ascertain the changes under the effect of GA_3 treatment.

3.3.2 Scanning electron microscope (SEM) study

Three control and GA_3 treated canes each, from the two varieties, BO91 and CoJ64, were profiled for internodal length and girth, at weekly interval, for a month, after the last 35ppm GA_3 spray. Corresponding internodes which showed maximum length difference among control and GA_3 treated canes were identified. Tissue was sampled from these internodes, precisely from in between the rind and pith and was examined using scanning electron microscope (SEM).

The tissue samples were fixed by using 5% w/v glutaraldehyde in 0.05M sodium cacodylate buffer for 4 hours' incubation at pH=7.0 (Busse *et al.*, 2005). Fresh fixative was replaced after 2 hours. The samples were rinsed with buffer and subjected to serial dehydration in ethanol. They were incubated for half an hour each in increasing concentration of ethanol viz. 30%, 50%, 70%, 90%, at 4°C, in order to remove water. The samples were critical point dried (at 31.5°C and 1100 psi) and mounted onto aluminium stubs. They were then coated with palladium by sputter-coater to make the sample conductive. The specimens were then examined for distinctly visible differences using JEOL 6490LV scanning electron microscope with an accelerating voltage of 15kV.

3.4 Biochemical parameters

3.4.1 Chlorophyll content

50mg sample from leaf was cut up and immersed in 5ml DMSO to be incubated in dark, overnight. The absorbance of the solution was then read at 645nm and 663nm, and the values for each sample were recorded in triplicate for confidence. Using these observations, amount of Chlorophyll a, Chlorophyll b and total chlorophyll was computed using Arnon's method (Arnon, 1949).

3.4.2 Nitrate reductase activity

It was determined vide an *in vivo* assay (Nicholas *et al.*, 1976), in which uniformly cut leaf segments (200mg) were incubated in potassium phosphate buffer (0.005M, pH=7.2) (Annexure I(A)) with KNO_3 (0.1M) and propanol (0.1%), in the dark, for 1 hour. (The vials were filled to the brim with sample solution, so as to maintain total anaerobic condition). 1ml of 1% sulfanilamide in HCl and 1ml of 0.02% NDD were used as colorigenic mixture with 100µl of the assay solution and the final solution developed a light pink colour, asserting conversion of nitrate to nitrite in the presence of nitrate reductase. The nitrite content of each sample was determined by recording absorbance of the assay solution at 540nm, in triplicate. The enzyme activity was calculated in terms of µmole min^{-1}/g fresh wt., taking KNO_2 as reference.

3.4.3 Quantification of reducing sugar and sucrose

In order to ascertain reducing sugar (RS) content in standardized dilution (1:10) of juice extracted from the different cane portions, and in leaf extract (500mg ground in 2ml water), Nelson's method was used (Nelson, 1944). The aliquot was taken in a Folin glass tube and 1ml of alkaline copper tartrate solution (Annexure II(A)) was added to it. This sample solution was subjected to boiling in a thermostatic water bath for 10 min. and then brought down to ambient temperature. Finally, 1ml of arseno-molybdate reagent (Annexure II(B)) was added, and the final volume made up to 12.5ml with water. Total sugar content was estimated in serially diluted juice sample, by incorporating a sucrose hydrolysis step mediated by 0.1N HCl, at the beginning of the above mentioned procedure. The solution was then mixed by inverting and the absorbance of the coloured solution hence obtained, was taken in triplicate, at 540nm. The standard curve of glucose was used as reference in order to compute the final sugar values. The amount of sucrose was calculated as the difference between total and reducing sugar content.

3.4.4 Brix value

Brix value is determined using a refractometer. It represents the entire soluble solid matter present in sample, and is expressed as percentage (Papini Terzi *et al.*, 2009). Though it takes into account sugars as well as non-sugars, its value primarily denotes sucrose content in the sample. Ideally, sample exhibits lower brix when immature, the value increasing with maturity status of sample. Hence, a narrow range of brix from bottom to top of cane indicates that the cane has ripened while a wide difference suggests that the cane is yet to mature. Brix of juice samples from various cane portions was ascertained by digital refractometer (PAL-1, Atago, Tokyo), with all observations made in triplicates.

3.4.5 Pol% (or juice sucrose%)

Pol% or polarization value gives the estimate of actual sucrose/cane sugar present in the juice. It is ascertained using a polarimeter, hence termed pol% (http://sugarcanecrops.com/agronomic_practices/harvesting_management/). Adapting from Horne's method (Horne, 1903), approximately 150ml of sieved juice from sample cane was thoroughly mixed with 2g of anhydrous lead acetate for clarification. This mixture was then allowed to stand for 5 min. and later filtered using Whatman no.1 filter paper, to remove any precipitate. Polarization reading of each clarified juice sample was determined in Autopol 880 automatic saccharimeter (Rudolph research analytical).

3.4.6 Enzyme assay

3.4.6.1 Enzyme extraction

For enzyme extraction, the stalk/leaf samples were ground in chilled extraction buffer which consisted of 50mM HEPES buffer (pH=7.5), 12mM $MgCl_2$, 1mM EDTA, 1mM EGTA, 10mM DTT, 2mM benzamidine, 2mM N-aminocapronate and 10mM diethyldithiocarbamate (Zhu *et al.*, 1997). The slurry was subjected to centrifugation in a refrigerated centrifuge ($4^{\circ}C$) at 10,000 rpm for 10 min., to obtain the enzyme extract as supernatant. The enzyme extract stored at $4^{\circ}C$, was used for ascertaining protein content and enzyme activity. The amount of protein in the enzyme extract was estimated using the Lowry method (1951) (Annexure III) by taking BSA as the reference protein.

3.4.6.2 Assessment of invertase activity

In order to ascertain invertase activity (Zhu *et al.*, 1997), 250µl of the enzyme extract when added to 2ml of 0.1M citrate buffer (pH=5.2) (Annexure I(B)) and 1ml of 2% sucrose solution (substrate for invertase activity) initiated invertase action. On the other hand, the control reaction mixture containing 250µl in 3ml citrate buffer (and no sucrose), was boiled in water bath for 30 min., in order to denature any endogenous enzyme and eliminate any possibility of hydrolysis of sucrose. Both the reaction mixes were then incubated at 37°C for 2 hours, providing ideal reaction condition for invertase activity. Thereafter, the reaction mixes were subjected to 10 min. boiling in order to cease all reaction. Final hexose content in the experimental and control reaction mixes was estimated using Nelson's method (1944), by measuring absorbance at 540nm. All observations were made in triplicates for each of the samples. The difference in absorbance denoted invertase action. The specific invertase activity was calculated in terms of unit/ mg protein with one unit=0.1 O.D.

3.4.6.3 Estimation of sucrose phosphate synthase (SPS) activity

SPS activity was assessed by way of synthesis of sucrose by SPS at 37°C and pH=7.5 (Zhu *et al.*, 1997). In the experimental mix, 12.5µl enzyme extract plus 12.5µl assay mixture (consisting of 100mM HEPES buffer (pH=7.5), 20mM glucose-6-phosphate, 4mM fructose-6-phosphate, 3mM UDP-glucose, 5mM $MgCl_2$ and 1mM EDTA) was used. Since SPS brings about the formation of sucrose-6-phosphate using UDP-glucose and fructose-6-phosphate, UDP-glucose was excluded in the control mix in order to inhibit SPS reaction. Optimum reaction conditions were provided by incubating the reaction mixes at 37°C for 1 hour, which were later subjected to 3min. boiling to cease reaction completely. Anthrone method was used to determine the sucrose produced by SPS activity (van Handel, 1968) involving adding of 20µl of 30% KOH to 20µl of the reaction mix, in a separate vial, and then subjected to boiling for 10 min. After its temperature came down to normal, 5µl of this reaction mixture was incubated with 2ml of newly made anthrone reagent (Annexure II(C)) at 37°C for 20 min. Absorbance was measured at 650nm and the difference in absorbance between experimental and control mixes, represented net sucrose produced and hence depicted SPS action. The specific SPS activity was calculated in terms of unit/ mg protein with one unit=0.1 O.D.

3.5 Molecular analysis

3.5.1 Total RNA isolation

Tissue from specific segments of culm and leaf tissues, of control and GA_3 treated canes as well as shaded canes, of BO91 and CoJ64 variety was used as starting material. 0.1g sample tissue was pulverized into a smooth powder using mortar and pestle, with the help of liquid nitrogen, and the powdered sample was then suspended in Trizol reagent (Invitrogen, USA). The slurry was subjected to centrifugation under refrigerated condition (4°C) at 15,000 rpm for 15 min., thus obtaining supernatant which was removed into another tube. 330μl chloroform:isoamyl alcohol (24:1) was pipetted into the supernatant. The mix was shaken thoroughly and then given an incubation time of 2 min. at room temperature. The mix was then centrifuged at 12,000 rpm for 20 min. at 4°C. From among the phases separated, the upper phase (containing the RNA) was pipetted out into yet another tube. 0.5ml of 100% isopropanol was then added to it, lightly mixed and set aside for 10 min. incubation. The transparent pellet obtained on centrifuging the mix (12,000 rpm for 10 min. at 4°C), was preserved while the supernatant was disposed off. The pellet was subjected to two subsequent washes with 75% ethanol (7500 rpm for 10 min. at 4°C). The vial containing the pellet was kept open to dry and then dissolved in DEPC water and stored at -80°C).

Good RNA quality was made certain by verifying band quality on 1% agarose gel. Also, the quantity of RNA was ascertained and its purity ratios were checked using Quawell UV-visible spectrophotometer (nanodrop). Normalization was done by drawing consensus from the gel and nanodrop observations and the RNA concentration of all samples in each sample set were equalized. DNase I (Thermo Scientific, USA) was used to make the RNA samples free from any potential DNA contamination.

3.5.2 End-point PCR analysis

The influence of perturbation of source-sink dynamics was ascertained at molecular level, by analysing differential expression of gene-specific primers (Annexure IV(A)) for various sucrose metabolism-related genes (viz. Soluble acid invertase (SAI), Cell wall invertase (CWI), Neutral invertase (NI), Sucrose synthase (SuSy), Phosphoenolpyruvate carboxylase (PEPC), Sucrose transporter (SUT)) in the stalk and leaf samples, derived from GA_3 treatment and partial shading studies. 25S rRNA gene primer was employed as reference in order to validate the normalization of RNA.

The coding DNA was synthesized by one-step RT-PCR kit (Qiagen, India), subjecting the RNA samples to a composite reaction set up comprising of both, reverse transcription for synthesis of cDNA coupled with PCR reaction, carried out in PTC 200 thermal cycler (MJ Research/BioRad, USA). The reaction mix consisted of 200ng RNA plus 2.5µl reaction buffer, 0.5µl dNTP mix, 2.5µl Q buffer, 1.5µl primer mix (F/R) and 0.5µl enzyme mix. The specific conditions employed to facilitate the semi qRT-PCR were: 50°C for 30 min., 95°C for 15 min., 40 cyclic reactions of 94°C for 1 min, 60°/58°C for 1 min. and 72°C for 1 min. followed by 72°C for 10 min. The amplified product was observed on 1.6% agarose gel as distinct band (due to EtBr), in a gel imaging system (Alpha Innotech, USA).

3.5.3 Real-time PCR analysis

The qRT-PCR reaction was preceded by synthesis of cDNA. 2µg of RNA sample was used as starting material, primed with 1 µl oligo-dT by incubating at 65° C for 5 min., and by using 1µl RevertAid H minus Reverse Transcriptase (Thermo Scientific) plus 2µl dNTP mix, 1µl Ribolock, and 4µl reaction buffer, cDNA synthesis was conducted. The difference in expression of various genes, under controlled and treated conditions, was precisely quantified using real-time PCR, by employing gene-specific primers viz. SAI, CWI, SuSy, SPS and 25S rRNA as calibrator) (Annexure IV(B)) (Chandra *et al.*, 2015).

Real-time PCR reaction was executed on Step One Real-Time PCR system (Applied Biosystems, USA), employing 48-well plates. The reaction mix per sample consisted of 5µl cDNA, 1µl primer mix (F/R), 10µl SYBR Green PCR Master mix (Applied

Biosystems, USA) and 4µl water. The reaction was set up in triplicate for each sample. The cycling conditions used were: 50°C for 2 min. and 95°C for 10 min. as holding stage, 40 cyclic reactions of 95°C for 15 secs. and 60° C for 1 min. A melt curve step of 95°C for 15 secs., 60°C for 1 min. and 95°C for 15 secs. was also run. The qRT-PCR analysis was conducted by the relative quantification method wherein expression was determined relative to control sample, using cycle threshold values (C_t), and expressed in terms of RQ values ($=2^{-\Delta\Delta C_t}$), which are a measure of fold change in expression.

3.6 Statistical analysis

The results derived from GA_3 treatment and partial shading study, were noted as the mean of three reproductions each, obtained from three distinct canes (biological replicates) for each sample. The results were recorded in terms of mean of three biological replicates ± standard deviation. The magnitude of distinction between the values corresponding to control and GA_3 treated/ shaded plants was statistically corroborated using two-sided Student's t-test. The significance of deduced differences between control and treatment-derived values was ascribed as $P \leq 0.05$ (*), $P \leq 0.01$(**), and $P \leq 0.001$ (***) where * represents level of significance.

3.7 Transcriptomic analysis

3.7.1 Transcriptome sequencing

RNA isolated from culm tissue of top segments of control and GA_3 treated canes (C_T and G_T respectively), in October (30 days after last GA_3 spray), was used as sample. The two RNA samples were checked for quantity and quality (RNA QC analysis) on Agilents 2100 BioAnalyser using RNA Nano Chip. Poly (A) mRNA was retrieved from total RNA and was used for cDNA synthesis. Paired-end transcriptome sequencing was conducted on Illumina HiSeq™ 2500 which yielded two sequenced libraries.

3.7.2 *De novo* assembly of transcriptome

RNA sequencing yielded raw reads which were refined by trimming adapters and low-quality sequences were done away with (Fast QC analysis), in order to get absolute reads. These were then subjected to *de novo* assembling by the Trinity pipeline (Grabherr *et al.*, 2011). The assembly program utilized the paired-end information to compile the short reads of a particular length and form longer contiguous sequences (contigs). The contigs from different transcripts were mapped, and based on their overlapping regions, transcript sequences were generated. After mapping, read counts corresponding to a particular transcript signified transcript abundance (Wolf, 2013).

Greater number of reads may perhaps get mapped to a longer transcript as compared to that to an equally expressed shorter transcript. Hence, in order to negate the impact of disparity in gene lengths, normalization criteria viz. RPKM was employed (Mortazavi *et al.*, 2008)

3.7.3 Differential expression analysis

Fold-change i.e. the ratio of normalized read count values, was determined to detect the level of differential expression among the samples. In order to sieve out transcripts exhibiting significant difference in expression, between control and GA_3 treated samples, we employed a cut-off of at least two fold change with significance $P<=0.01$.

3.7.4 Functional classification and annotation

Functional annotation of the assembled transcripts was brought about by aligning them to publicly available databases viz. NCBI (https://www.ncbi.nlm.nih.gov), Uniprot (https://www.uniprot.org), Gene Ontology (GO) (http://wego.genomics.org.cn/cgi-bin/wego/index.pl), and KEGG (http://www.genome.jp/kegg/kegg2.html), thereby assigning them to different functional categories.

Blast2GO (https://www.blast2go.com) was employed for the functional annotation of transcripts showing significant differential expression. Blast2GO (Conesa and Götz, 2008) is a bioinformatics tool that facilitates functional analysis of genome scale sequences. It makes use of the BLAST program to retrieve information about sequences that share homology with the input sequences. Here, NCBI Blast was used to determine homologous sequences to the query set (BLASTx analysis was performed with E-value of 10^{-3} as threshold and using nr reference database). InterPro identifies protein domains and families. The software also provides a user-friendly interface for Gene Ontology annotation. KEGG enrichment analysis revealed the functional categorization and distribution of transcripts into various metabolic pathways.

3.7.5 Validation of results by qRT-PCR analysis

Various screening criteria were adopted to shortlist the transcripts differentially responding to GA_3. In order to verify the credibility of the RNA-seq data, the expression quantum of the shortlisted transcripts was ascertained by real-time PCR. Employing primers designed for the selected transcripts, using the IDT primer designing tool (https://eu.idtdna.com), a comparison was made between their expression in control and GA_3 treated samples. The designed primers were verified for amplification by end-point PCR and checking the desired product on 1.6% agarose gel. 25S rRNA was employed as normalizer gene for assessing expression of all selected transcripts. qRT-PCR was carried out in three technical replicates by using SYBR Green. The optimal annealing temperature for each primer pair was ascertained by testing one of the sample's expression at different annealing temperatures (viz. 58°, 60° and 62°C). Primer specificity was confirmed by employing a negative control for each primer and by melt curve analysis.

Two uncharacterized proteins shortlisted among the transcripts were further characterized using the Phyre2 tool (https://www.sbg.bio.ic.acc.uk>~phyre), to carry out its structural analysis and predict a possible 3-D model for the protein on the basis of its similarity to other known protein structures (Kelley *et al.*, 2015).

Chapter 4

Results and Discussion

RESULTS AND DISCUSSION

The crop was planted in 4 rows of 50m each, at the end of February, using three-bud setts and 35-40% germination was recorded 45-50 days after planting (DAP) (Figure 5). Thereafter, the tillering phase, wherein additional shoots emerge from the base of initial culm, lasted upto 90 days (i.e. till mid July). The grand growth phase of around 75-90 days ensued, in which the structural growth and development of the culms occurred (Figure 6). Minimal pest infestation was observed due to pre-treatment of setts and also time to time spraying of standard pesticides. At this stage, one half of the standing canes of both varieties, BO91 and CoJ64, were sprayed with GA_3 solution. The maturation phase soon followed (210 DAP), marking the beginning of effective sucrose accumulation in the culms. The phase spanned about 4 months in case of the early maturing CoJ64 and 6 months in case of late maturing BO91 (Figure 7). The plantation yielded a population of about 1,25,000 plants/ hectare. Each plant on an average weighed 600 to 800g, thus registering a sugarcane yield of 75-85 tonnes/ hectare. During the maturation phase, the top of the culm showed minimal expansion by way of development of new internodes, and hence the cane exhibited nominal growth during this phase.

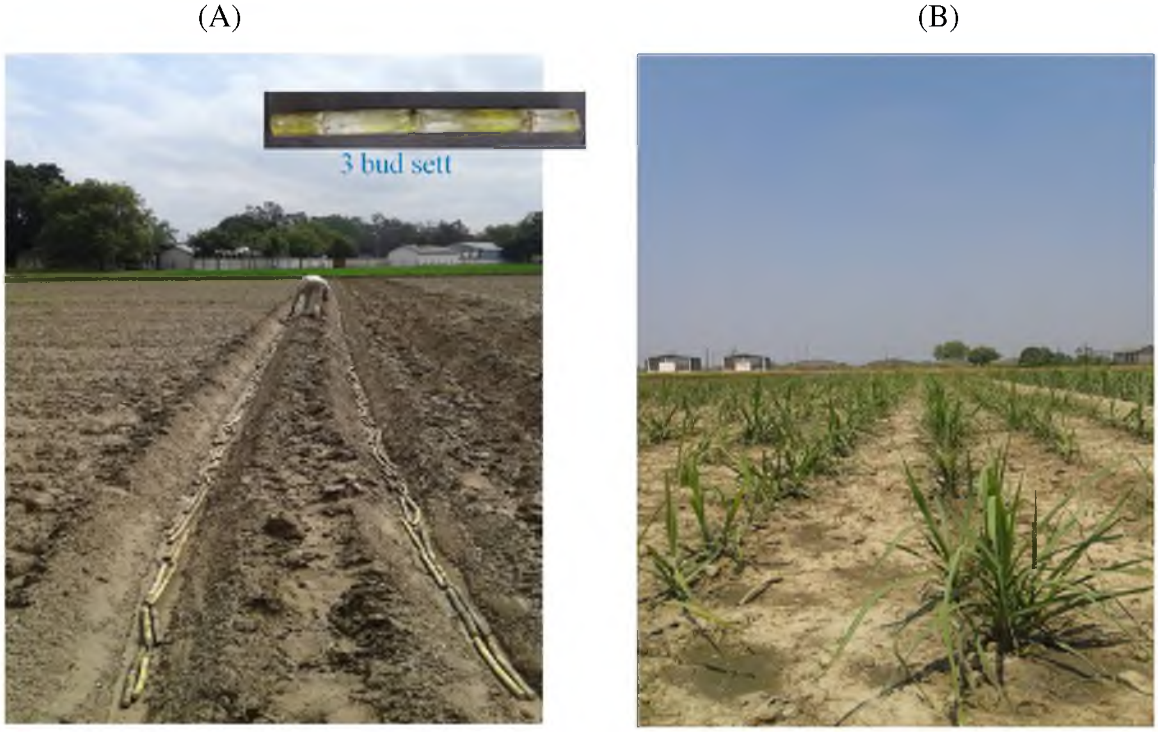


Figure 5: (A) Crop planted in the plantation area of IISR at the end of February, using three-bud setts (inset) (B) Post germination

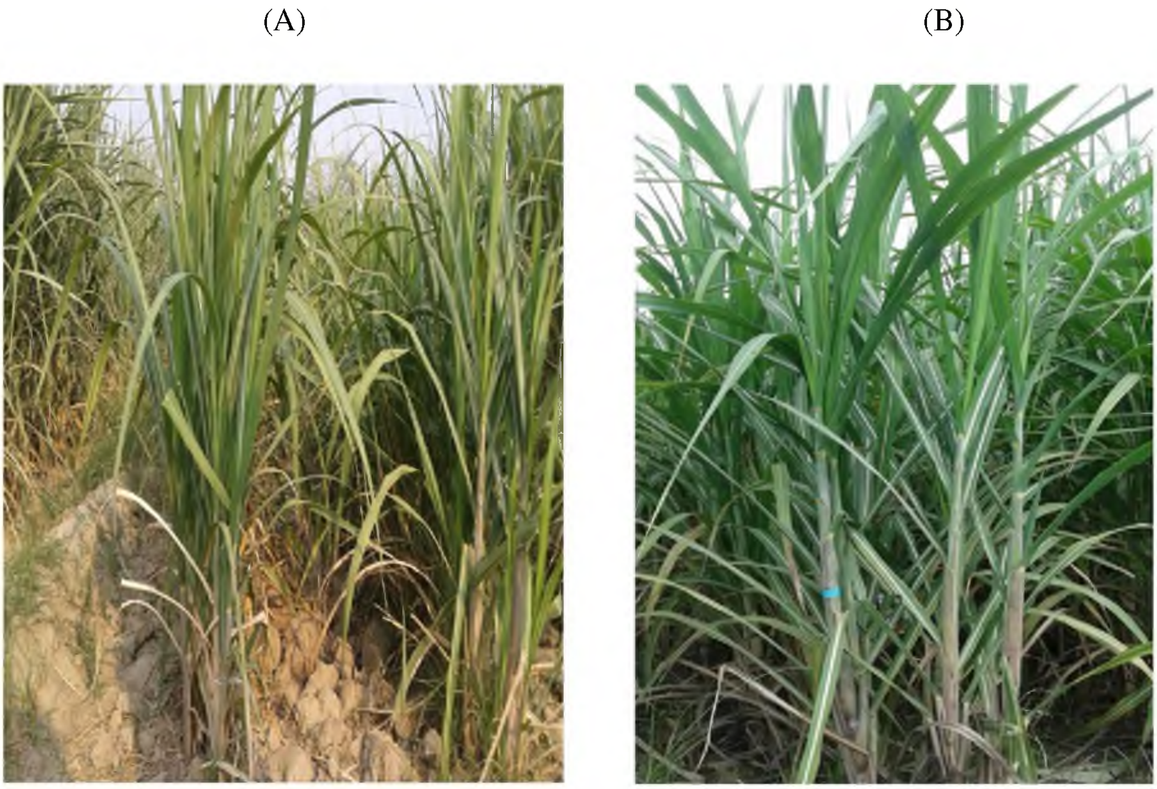


Figure 6: (A) Tillering phase (B) Grand growth phase

Figure 7: Visible differences between CoJ64 and BO91 in October, at the start of maturation phase

4.1 Gibberellin treatment

4.1.1 Morphological study

The role of gibberellins (GAs) viz. GA_3 has long been established as growth hormones, known for bringing about morphological changes in plants viz. increase in height of plant. Studies have shown that exogenously applied gibberellins can bring about internodal elongation by either propping up cell division to increase cell number or causing cell enlargement and thereby increasing cell size (Iqbal *et al.*, 2011). Thus, in sugarcane, in addition to the regular growth capacity of the culm, exogenous application of GA can be expected to prolong the state of growth and immaturity in the culm.

4.1.1.1 Measurement of morphological parameters

Data from the control and GA_3 treated canes profiled in 5 biological replicates for various morphological parameters viz. total cane height, LTM leaf length, LTM leaf width, over the entire maturation phase, validated the role of GA_3 in affecting plant morphology. Records represented as mean of three biological replicates ± standard deviation, for each sampling group, are charted in Table 1. As expected, a large, visibly distinguishable difference in height was recorded among control and GA_3 treated canes of both varieties (Figure 8). Hence, this accounts for the role of GA_3 in improving the dimension of sink and thereby its capacity for over-all greater accumulation of sucrose. Also, LTM leaves of GA_3 treated canes were notably longer than the control ones especially in the growth phase (when GA_3 was sprayed in one-month intervals) and in early maturation phase. This can be extrapolated to the need for increased photosynthesis and assimilation in order to meet the augmented sink demand under the effect of GA_3.

Table 1: Effect of GA$_3$ application on morphological parameters

Time of observation	Total cane height (cm)				LTM leaf length (cm)				LTM leaf width (cm)			
	BO91cane		CoJ64cane		BO91 leaf		CoJ64 leaf		BO91 leaf		CoJ64 leaf	
	control	GA$_3$ sprayed	control	GA$_3$ sprayed	control	GA$_3$ sprayed	control	GA$_3$ sprayed	control	GA$_3$ sprayed	control	GA$_3$ sprayed
July (just before 1st 35 ppm GA$_3$spray)	65±0.82	62.5±1.08	56.6±0.93	55.3±0.9	116.6±0.24	115.5±0.42	114.2±0.54	112.6±0.29	4.4±0.06	4.4±0.02	3.75±0.03	3.75±0.06
August (before 2nd 35 ppm GA$_3$spray)	116±1.34	140±1.2 ***	107±1.60	147±0.70 ***	125±0.82	138±0.62 **	130±0.98	138±1.02 **	3.9±0.09	3.7±0.04 **	3.4±0.11	3.3±0.09*
September (before 3rd 35 ppm GA$_3$spray)	169±1.72	190±1.07 ***	145±1.22	188±0.89 ***	142±1.34	148±0.82 **	144±0.89	152±1.22 **	3.8±0.03	3.6±0.12 ***	3.8±0.16	3.5±0.08 ***
October (30DAS)	235±2.02	252±1.23 ***	184±1.1	206±1.68 ***	146±0.45	151±0.86**	115±0.92	129±1.37 ***	4.0±0.05	3.7±0.03 ***	3.5±0.11	3.3±0.03 **
November (60 DAS)	242±1.83	260±1.97 ***	196±2.12	229±1.8 ***	147±0.87	145±0.77 *	106±0.28	108±0.79 ***	4.0±0.09	3.8±0.16 ***	3.1±0.14	3.1±0.07
December (90 DAS)	250±2.02	266±2.30 ***	200±1.87	254±1.92 ***	133±0.44	133±0.63	88±0.81	91±0.48 **	3.6±0.08	3.5±0.08 ***	2.9±0.13	3±0.06 *
January (120 DAS)	256±1.86	272±1.52 ***	202±1.58	263±2.16 ***	125±0.87	122±0.56 *	80.5±0.63	85±0.71 *	3.5±0.15	3.3±0.09 ***	2.7±0.04	2.9±0.07 *
Feb (150DAS)	259.5±1.77	279±2.05 ***	--	--	112±0.72	123±0.52 ***	--	--	3.4±0.12	3.2±0.08 ***	--	--
March (180 DAS)	261±1.48	282±1.82 ***	--	--	90±1.01	98±0.82 **	--	--	3.1±0.05	3.0±0.06 ***	--	--

± standard deviation of mean among three biological replicates DAS- days after spraying

* significance of difference between treatments

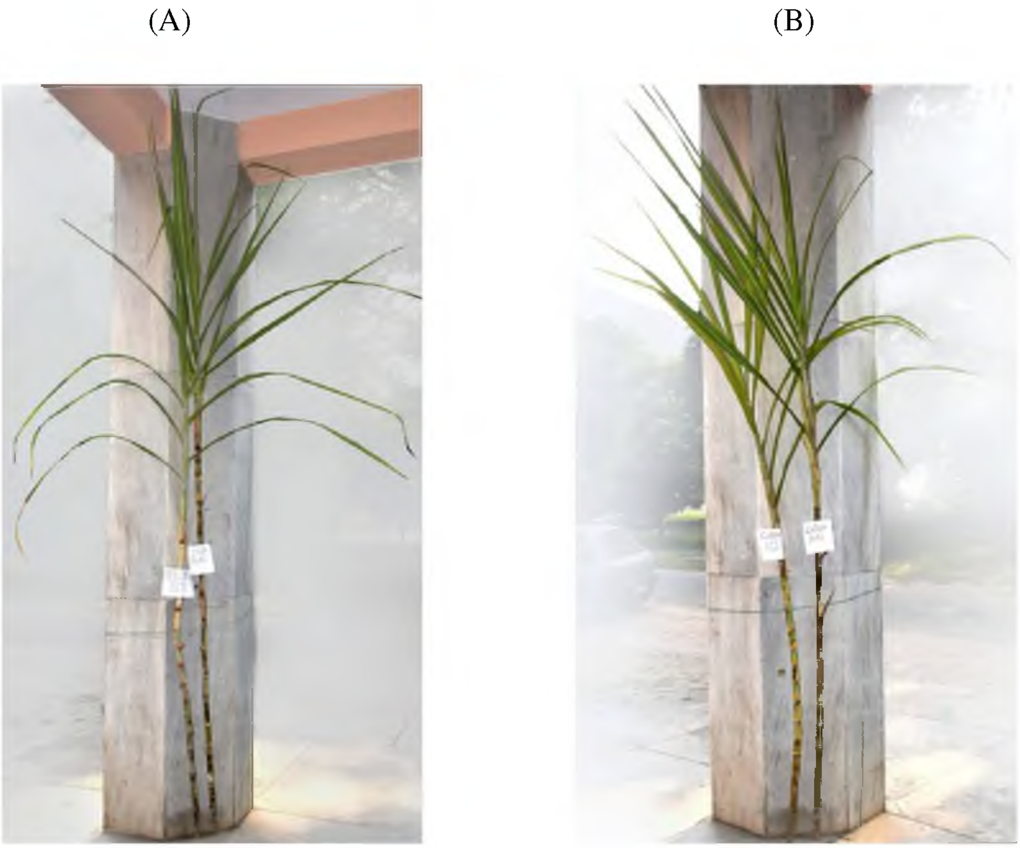


Figure 8: Difference in height between control and GA_3 treated (A) BO91 (B) CoJ64 canes

4.1.1.2 Scanning electron microscope (SEM) analysis

In the first month after last spray (i.e. in October), weekly profile of internodal length and girth was generated in 3 biological replicates of each sampling group, in order to deduce a pattern among them. The internodes were numbered bottom up and the corresponding internodes of control and GA_3 sprayed cane of each variety were compared for internodal length and girth. The middle portion of the control and GA_3 treated canes, consistently showed greatest difference in length among corresponding internodes of all biological replicates. Based on consistency in records among biological replicates, one representative cane was selected from each sampling group. The internodes of control and GA_3 treated canes, especially internode no. 11-13 and no. 10-12 of CoJ64 and BO91 respectively, exhibited a distinct difference in length (Table 2). The striking difference in internodal length of control and GA_3 sprayed samples, corroborate the enhanced size and potential of sink under the effect of GA_3. Greatest difference in internodal length between control and their corresponding samples from GA_3 sprayed canes, was seen in internode no.10 of BO91 and no.11 of

CoJ64 (Figure 9). SEM was employed to closely ascertain the basis of increase in internodal length under the effect of GA$_3$. SEM visualization of the internodal tissue revealed distinctly greater cell size in GA$_3$ treated samples as compared to control. Although an increase in cell number cannot be warranted from this analysis, the significant increase in cell size detected in samples of both BO91 (~19.2% bigger) and CoJ64 (~ 42.3% bigger) (Figure 10 A,B), asserts the augmentation in size of sink and consequent rise in sucrose accumulation capacity of sink, under the effect of GA$_3$.

Table 2: Difference in length of corresponding internodal samples of control and GA$_3$ treated canes

Internode no.	**Internodal length/girth (cm)**		**Internode no.**	**Internodal length/girth (cm)**	
	BO91 cane			**CoJ64 cane**	
	Control	**GA$_3$ sprayed**		**Control**	**GA$_3$ sprayed**
9	12.2	13.6	**10**	11.0	9.9
	2	2.1		2.2	2.65
10	11.2	15.6	**11**	10.2	13.0
	1.9	2.1		2.3	2.5
11	11.0	15.0	**12**	11.3	13.7
	1.9	2.1		2.3	2.5
12	10.1	13.3	**13**	9.7	12.0
	2	2		2.5	2.5
13	9.6	10.7	**14**	9.0	11.0
	2	2.1		2.5	2.5

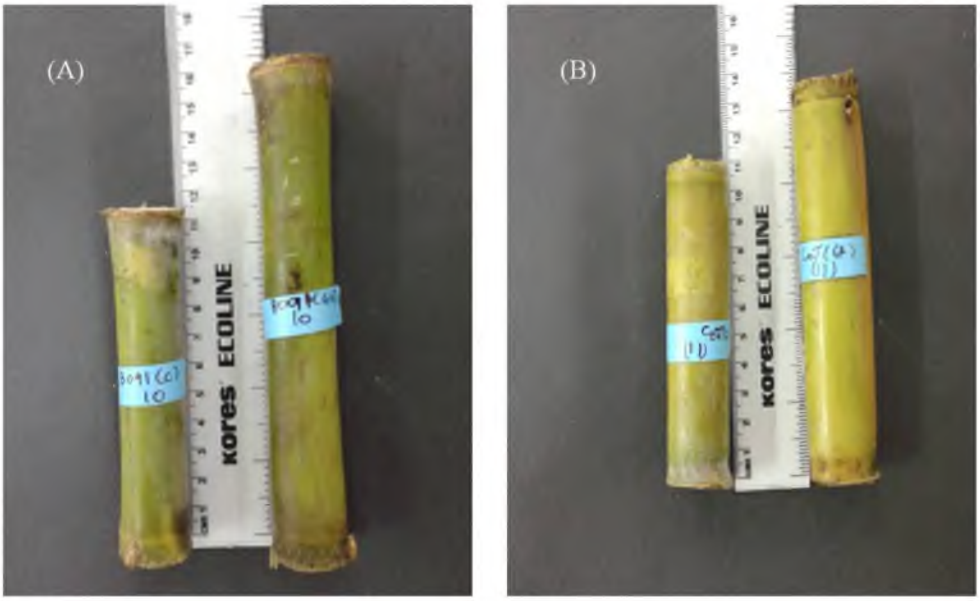
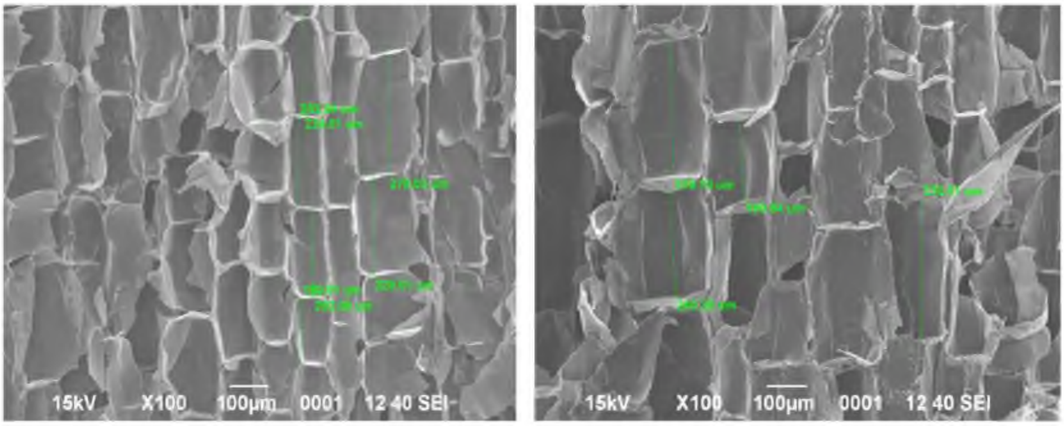
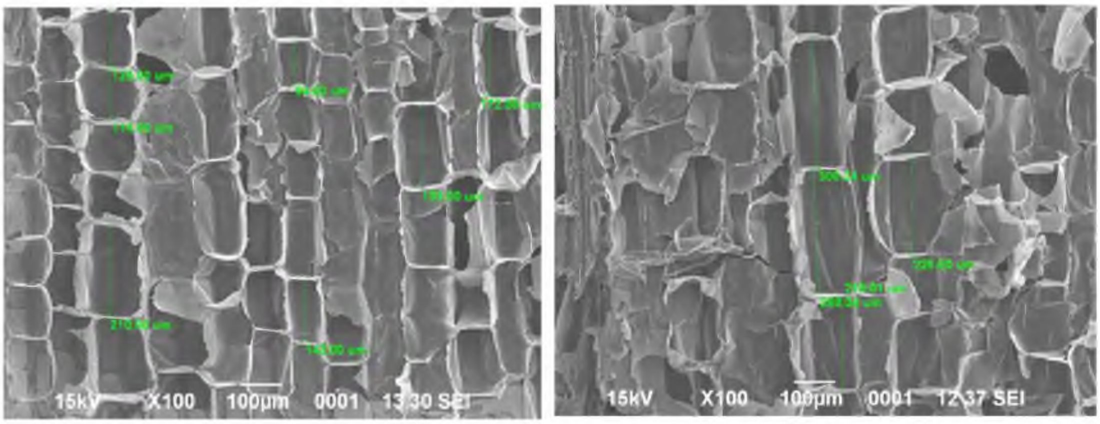


Figure 9: Difference in internodal length of control and GA$_3$ treated canes of (A) BO91 and (B) CoJ64



(a) (b)

Figure 10A: Difference in cell length seen in (a) control and (b) GA_3 sprayed internodal samples of BO91 cane, as observed through SEM



(a) (b)

Figure 10B: Difference in cell length seen in (a) control and (b) GA_3 sprayed internodal samples of CoJ64 cane, as observed through SEM

By common convention, maximum growth and vigorous metabolic activity is seen in the top segment of cane. However, in the current pursuit, the internodal samples of middle portion, displayed most conspicuous difference in length among control and GA_3 sprayed plants. It can perhaps be rationaled that during the intermittent GA_3 spray, the now middle internodal portion must have then been the actively growing top region. Hence it must have perhaps been most affected by the freshly sprayed GA_3 (during growth phase of July to September), accounting for extraordinary length displayed by this region. The portion of cane which now forms the top segment was only developing then and thus may have not been significantly affected by the GA_3 treatment.

4.1.2 Biochemical analysis

4.1.2.1 Estimation of chlorophyll content

Photosynthetic rate is reported to be directly related to chlorophyll content, thus in turn improving the biomass production (Araus *et. al.*, 1997; Thomas *et. al.*, 2005). Therefore, understanding the effect of GA on chlorophyll content would contribute to better understanding of its role in affecting photosynthetic activity (Pandey and Singh, 2010). In the present study, total chlorophyll content was found to be greater in GA_3 sprayed LTM leaves as compared to that in control (Figure 11). The photosynthetic activity of source is reported to be largely dictated by sink. Thus, the greater chlorophyll content observed in GA_3 sprayed leaf could be reasoned to be due to increased photosynthesis for production of more photoassimilate, required to meet the higher sink demand in GA_3 treated canes. Also, photosynthetic activity has been indicated to decrease with increasing maturity of sugarcane culm (McCormick *et al.*, 2006). Many works have reported higher photosynthetic rate in younger plants as compared to that displayed by leaves of older sugarcane plants (Hartt and Burr, 1967; Bull and Tovey, 1974). Perhaps the maturity-associated accumulation of sucrose triggers end-product inhibition causing decline in photosynthetic rate (Goldschmidt and Huber, 1992; McCormick *et al.*, 2008d). Sugar heaping in leaves, as in sugar feeding and cold-girdling treatments, has also been shown to cause a decline in chlorophyll content and photosynthetic rates. In concert, in case of both BO91 and CoJ64, gradual decline in total chlorophyll content was recorded over time. Increased photosynthesis (also qualified by increase in reducing sugar in GA_3 treated leaf samples of this study) due to better redistribution of photoassimilates, can ultimately bring about better yield. In general, chlorophyll content was found to be higher in BO91 as compared to CoJ64, which is also supported by the report that photosynthetic activity is generally higher in lower sucrose accumulating varieties than higher sucrose accumulating ones.

Light is mainly utilized by chlorophyll a and b present in leaves of higher plants and hence they are the main pigments involved in photosynthesis in the chloroplasts (Pan and Dong, 1995). Chlorophyll a mainly reflects green-yellow light (and absorbs the rest), contributing to the green appearance of leaves (https://en.m.wikipedia.org). Broadly, greater amount of chlorophyll a was noted in BO91 LTM leaves, justifying their visually greener appearance as compared to those of CoJ64. Significant levels of chlorophyll a were seen in both BO91 and CoJ64 at 0 DAS, which gradually lowered as cane matured (Table 3). Chlorophyll b is said to be the chlorophyll that facilitates photosynthesis by harvesting light. In both the varieties, the GA_3 treated leaves exhibited evidently greater measure of chlorophyll b, denoting greater absorption of light energy for photosynthesis. The drastic rise observed in chlorophyll a of BO91 LTM leaf, can be correlated to the high RS values observed at 60 DAS.

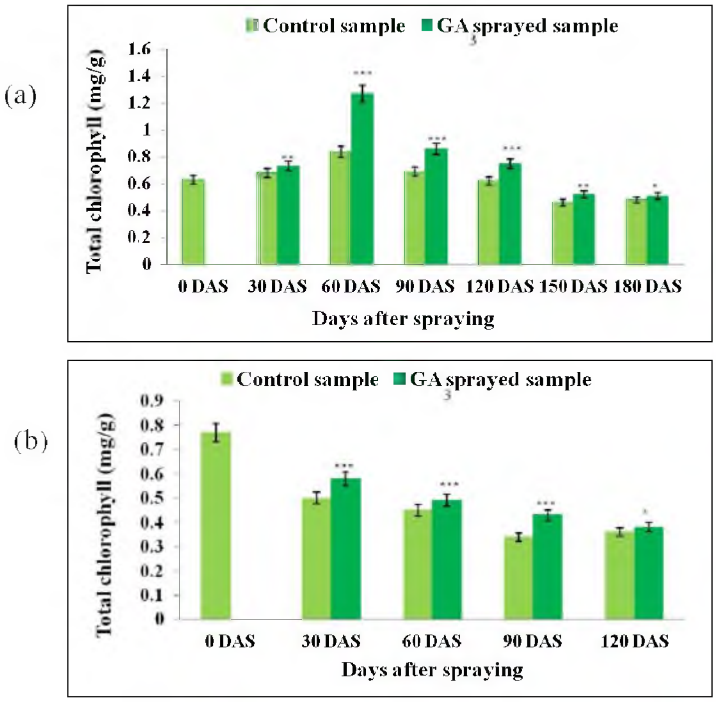


Figure 11: Total chlorophyll content in LTM leaves of control and GA_3 sprayed (a) BO91 (b) CoJ64 canes

Table 3: Amount of chlorophyll a and b in control and GA_3 treated LTM leaf

Days after spraying	BO91 LTM leaf				CoJ64 LTM leaf			
	Control		GA_3 sprayed		Control		GA_3 sprayed	
	Chl a	Chl b	Chl a	Chl b	Chl a	Chl b	Chl a	Chl b
0 DAS	0.49±0.04	0.14±0.02	---	---	0.6±0.01	0.17±0.02	---	---
30 DAS	0.52±0.03	0.16±0.01	0.54±0.04 *	0.19±0.06 **	0.32±0.07	0.18±0.03	0.34±0.03 *	0.24±0.01 **
60 DAS	0.6±0.01	0.21±0.04	0.99±0.05 ***	0.28±006 **	0.34±0.03	0.11±0.01	0.35±0.04 ***	0.14±0.06 **
90 DAS	0.61±0.01	0.08±0.03	0.63±0.08 **	0.23±0.05 ***	0.31±0.03	0.03±0.02	0.35±0.04 **	0.08±0.01 **
120 DAS	0.48±0.09	0.14±0.02	0.65±0.07 ***	0.10±0.04 **	0.28±0.06	0.09±0.07	0.33±0.01 **	0.05±0.03 **
150 DAS	0.33±0.07	0.12±0.05	0.34±0.01 *	0.18±0.09 ***	---	---	---	---
180 DAS	0.34±0.03	0.15±0.05	0.4±0.01 **	0.11±0.08 **	---	---	---	---

± standard deviation of mean among three biological replicates
* significance of difference between treatments

4.1.2.2 Estimation of nitrate reductase activity

Nitrate in leaves is reduced to ammonia by a series of enzymes where nitrate reductase catalyses the first reaction (Shaner and Boyer, 1976). Metabolic factors like nitrogen and Pi have been shown to affect photosynthetic rates (Paul and Driscoll, 1997). Various processes influencing the association between source and sink viz. N-use efficiency of plants, has been indicated to be impacted by GA (Khan, 1996). Following GA application, the GA-enhanced shoot growth perhaps causes more consumption of nutrients and hence results in increase in uptake and utilization of soil nitrogen (N) (Khan *et al.*, 2002). Increased N utilization by plants perhaps also leads to higher nitrate reductase (NR) activity. For example, in mustard (Khan *et al.*, 2005), GA_3 (in association with N treatment) has been found to mediate an increase in NR activity and photosynthesis. Enhancement of NR activity by GA_3 or GA_3+cytokinin has also been described by Roth-Bejerano and Lips (1970) in tobacco leaves. Thus, as expected, the NR activity was found to be higher in LTM leaf of GA_3 sprayed cane, as compared to control, especially up to 2 months after spraying. The drop in NR activity at 90 DAS and 60 DAS, in case of BO91 and CoJ64 respectively, can perhaps be linked to the decrease in effect of GA_3 and consequent decrease in N utilization by the plant. Hence, the difference in NR activity between control and GA_3 sprayed LTM leaves gradually levelled thereafter (Figure 12).

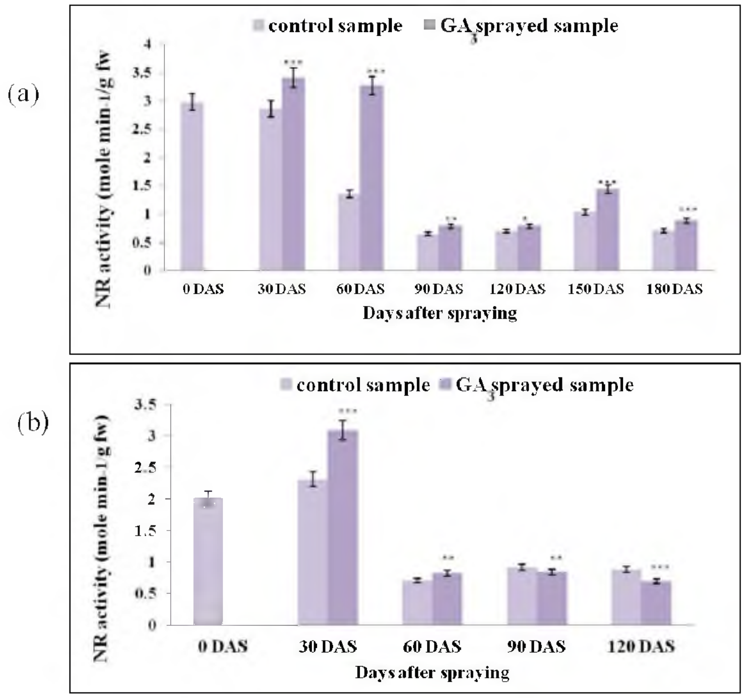


Figure 12: Nitrate reductase activity in LTM leaves of control and GA_3 treated (a) BO91 (b) CoJ64 canes

4.1.2.3 Estimation of sugar and related parameters

In the present pursuit, the sugar pattern in control and GA_3 treated canes was tracked by employing basic sugar related parameters viz. reducing sugar%, sucrose%, brix%, pol%, to gauge the effect of gibberellin spray on source-sink communication and eventually sucrose accumulation.

4.1.2.3.1 Analysis for the 50 ppm GA_3 spray study

During the 2014 study, the sugar pattern in the cane samples conformed to the regular pattern reported in classical studies. The reducing sugar (RS) content steadily decreased while the sucrose%, brix% and pol% values exhibited a gradual rise, demonstrating the growing maturation of canes, over time. However, a remarkable difference was observed between the RS content of ,especially the top internodal GA_3 sprayed (9.51%), and control cane sample (3.43%) (Figure 13 (a)), a month after the 50ppm GA_3 spray. In accordance, lesser sucrose% (Figure 13 (b)), brix% (Figure 13 (c)) and pol% values (Figure 13(d)) were observed, particularly in the upper internodal samples of GA_3 sprayed canes, compared to control. This drastic swell in RS content can be reasoned to be due to heightened action of invertase in the culm tissue, under the effect of gibberellins, causing hydrolysis of sucrose, and contributing to increase in sink strength in turn (Iqbal *et al.*, 2011).

Noticeably greater RS content was observed in the upper GA_3 treated internodes compared to the control ones, even 60 DAS. Parallely increased sucrose accumulation was seen corroborating the increase in sink potential in the GA_3 sprayed plants. The RS content suddenly dropped at 90 DAS, in both, control and GA_3 treated canes (Figure 13 (a)), probably due to dimmed effect of gibberellins and dormant plant activity under the very low prevailing temperature. Correspondingly, escalation in sucrose%, brix% and pol% values was obtained in control and GA_3 sprayed canes (Figure 13(b), (c), (d)), conforming to the fact that low ambient temperature favours sucrose accumulation. At 120 DAS, prominent increase was particularly observed in sucrose content of the GA_3 treated canes (25.7-33.03%) as compared to control ones (20.81-24.64%). This study affirmed that GA_3 spray perhaps improves the sink's potential to attract assimilates by developing higher sink demand due to better sink strength, compared to control. However, this GA_3 treatment was seen to cause spindling and splitting of cane culm and hence standardization of GA_3 concentration was needed.

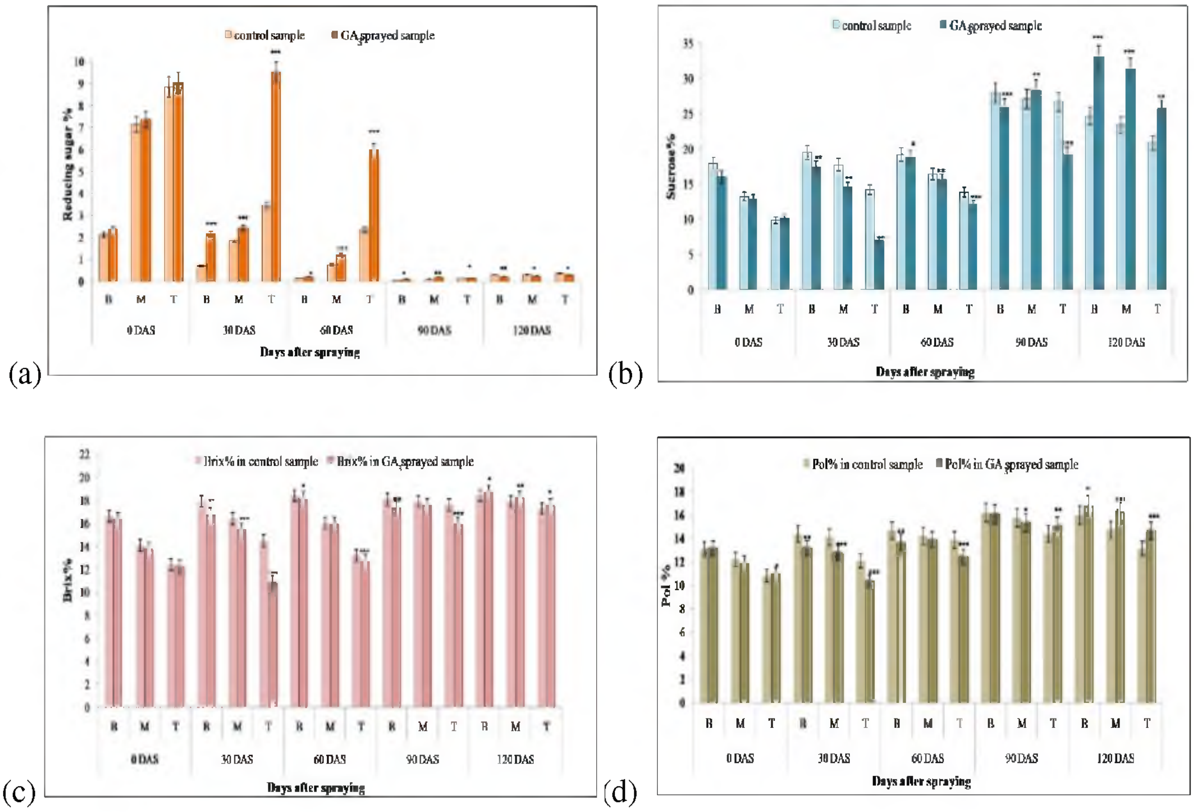


Figure 13: (a) Reducing sugar% (b) sucrose% (c) brix% (d) pol% recorded in various internodal samples (B:bottom, M:middle and T:top) of control and GA_3 sprayed (50ppm) BO91 canes

4.1.2.3.2 Analysis for the 35 ppm GA_3 spray study

Three consecutive GA_3 sprays of 35 ppm each, were applied to the 2015 plantation and no splitting or spindling of canes was observed, thus optimizing the concentration for GA_3 treatment. A month past the last spray (30 DAS), a notable difference was recorded especially between the reducing sugar content of control (4.01%) and GA_3 treated (9.23%) top internodal samples of BO91 (Figure 14 (a),(b)) while those in CoJ64 too, displayed a lesser yet significant difference (2.46% in control and 6.85% in GA_3 treated one). Comparable results were obtained in the 50 ppm study too, thus confirming that the drastic rise seen in RS% values was indeed due to stimulation of invertase activity in sink tissues, by gibberellins, causing increased conversion of sucrose into hexoses. This in turn increased sink strength and sink demand. Higher RS% values were also obtained in GA_3 treated middle and bottom internodes of BO91 and CoJ64 compared to control, though the difference was slighter. Correspondingly, low sucrose concentration was observed, especially in the GA_3 treated top internode (8.02% in BO91; 10.98% in CoJ64) (Figure 14 (c), (d)) validating sucrose hydrolysis under GA_3 action. This pattern was in congruence with that noted in the 2014 (50 ppm) study, thus establishing increase in sink strength under GA_3 action. Similarly, a substantial difference was seen in the brix% values (Figure 15 (a),(b)), particularly in top internodal samples of control (14.8) and GA_3 treated (10.7) BO91 canes while those in CoJ64 samples, did not exhibit considerable difference. The pol% values also displayed a pattern analogous with the sucrose% pattern of both, BO91 and CoJ64 canes (Figure 15 (c),(d)). Thus, the dip in sucrose content of the sink facilitates increased assimilate uptake (van Bel, 2003) due to heightened sink demand.

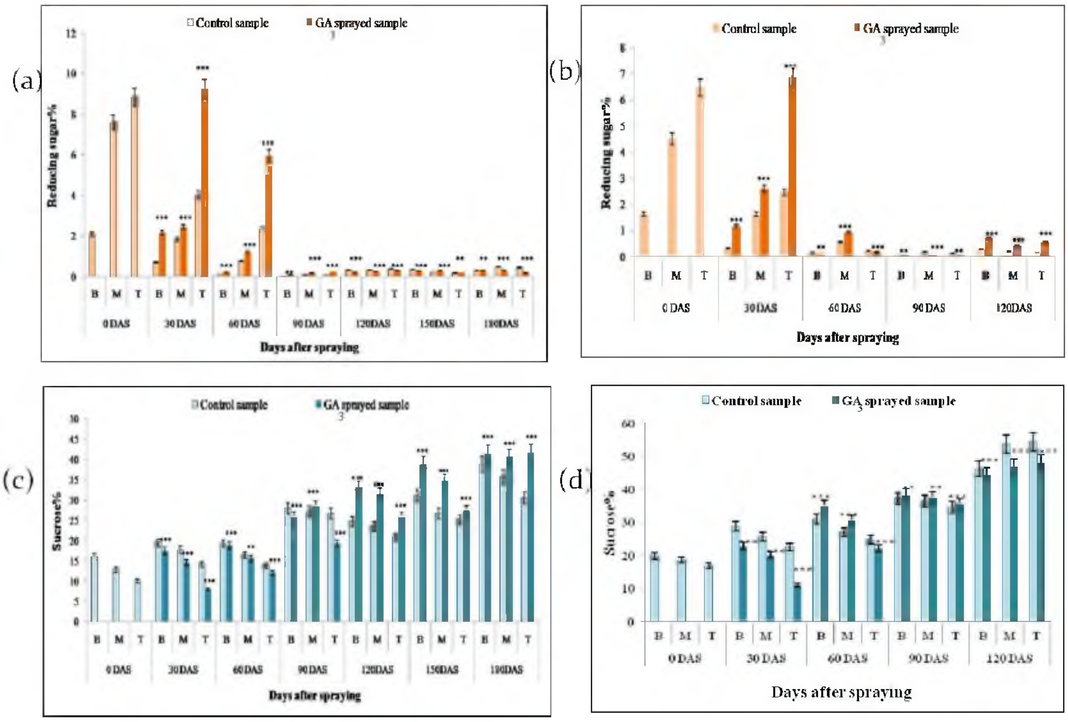


Figure 14: (a) (b) Reducing sugar% (c) (d) sucrose% recorded in the bottom, middle and top internodal samples of control and GA_3 treated (35ppm) BO91 and CoJ64 canes, respectively

In an earlier study, McCormick *et al.* (2006) have shown leaf hexose concentration to be inversely related to photosynthetic rate and developed sink demand. The LTM leaf of GA_3 treated BO91 cane showed lower RS content (0.96%) in comparison to that of control (1.18%) (Figure 16 (a)). The lower RS content perhaps points to employment of RS in sucrose synthesis to answer the GA_3-induced sink demand. Concomitantly, sucrose% value of the GA_3 treated leaf was found to be higher (0.56%) than that in control cane. This further affirmed the conversion of RS into sucrose, asserting that GA_3 treatment perhaps stimulates assimilate transport, and phloem loading in turn. The LTM leaf of control cane displayed low sucrose value, perhaps denoting that sucrose is being loaded into the phloem at a rate higher than its production in the source. However, CoJ64 exhibited a converse pattern, with the GA_3 treated leaf displaying greater RS content (1.65%) than control (1.16%). This may be reasoned as a consequence of the high photosynthetic rate stimulated in answer to the already developed high sink demand in early maturing CoJ64.

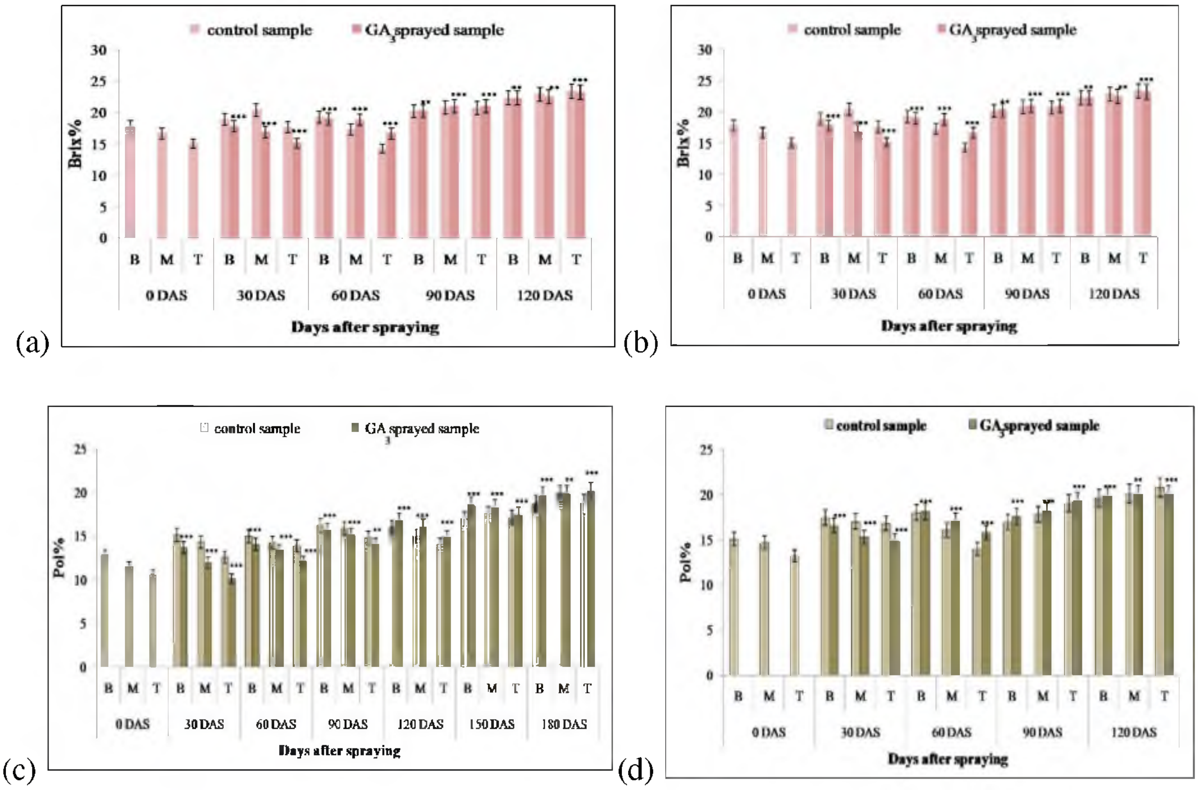


Figure 15: (a) (b) Brix% (c) (d) Pol% recorded in the bottom, middle and top internodal samples of control and GA_3 treated (35ppm) BO91 and CoJ64 canes, respectively

Even 60 DAS, the GA_3 treated canes displayed significantly greater RS% values, compared to the control canes. This GA_3 effect was more pronounced in BO91, possibly due to prolonged state of immaturity induced by GA_3 in the late maturing BO91. The upper internodes of GA_3 treated BO91 cane still displayed RS% value as high as 5.97% compared to 2.34% in control (Figure 14 (a)), perhaps still denoting high sink strength and sink demand.

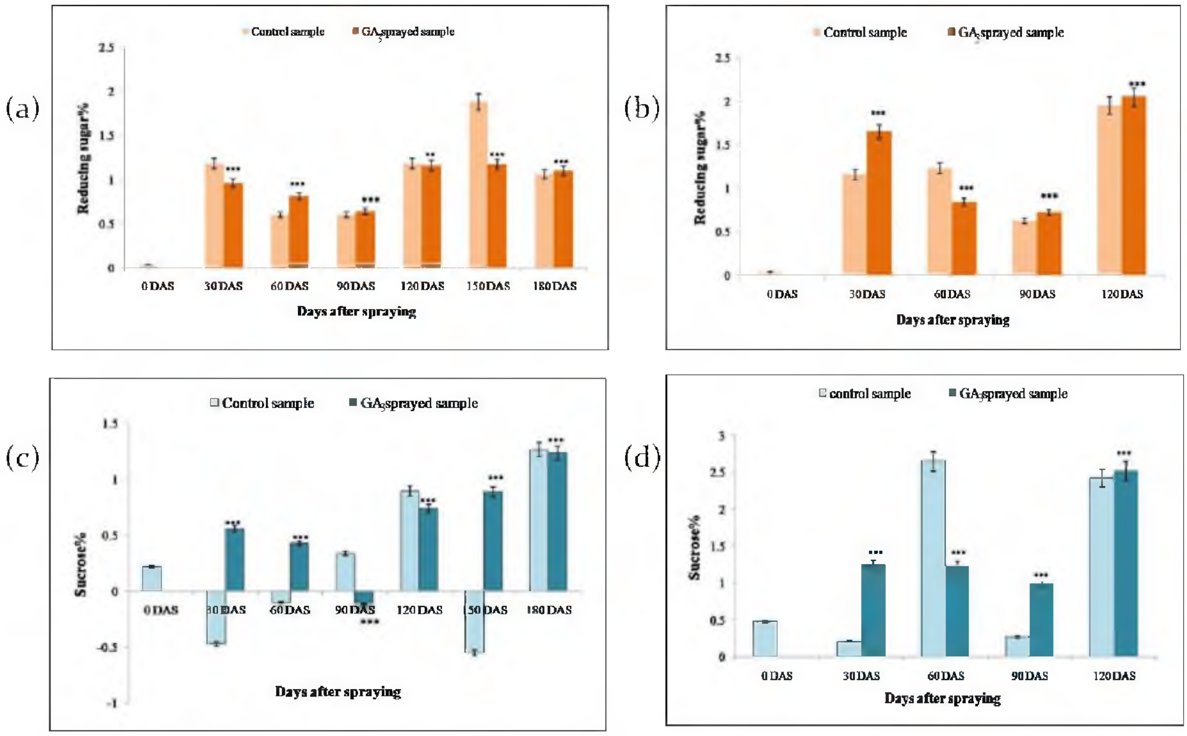


Figure 16: (a) (b) Reducing sugar% (c) (d) sucrose recorded in LTM leaf samples of control and GA_3 treated BO91 and CoJ64 canes, respectively

However, a far lesser variation was seen in the RS content of middle internodal samples of control and GA_3 treated canes. In contrast, not much significant difference in RS value was recorded between control and GA_3 treated samples of CoJ64 depicting comparable sink strength. This implies gradual maturation of sink perhaps because the GA_3 treatment was effective for a shorter span in the early maturing CoJ64. Additionally, higher sucrose content was particularly noted in middle and top internodal samples of GA_3 treated plants in comparison to those of control CoJ64 plants (Figure 14 (d)). In concurrence with sucrose pattern, the Brix% and pol% values were also higher in the GA_3 treated samples than control. Hence, this asserts the ability of GA treatment in bringing about greater sucrose accumulation by improving the cane's sink strength and sink potential. Conversely, lower sucrose values were observed in GA_3 treated BO91 samples, when weighed against control, which in consistence with the augmented RS values seen, perhaps denote the existing high sink strength and sink demand. Concurrently, utilization of RS in generating sucrose caused its level to drop in control and GA_3 treated leaves. As reasoned earlier too, for early maturing CoJ64 at 30 DAS, the GA_3 treatment possibly stimulated higher photosynthetic rate in BO91 treated leaf, accounting for greater RS% value, at

60 as well as 90 DAS. On the other hand, GA_3 sprayed leaf displayed lower RS (0.84%) and sucrose values (1.23%) than the corresponding values (1.23%; 2.65%) in leaf sample from control CoJ64 cane. This denotes the enhanced translation of RS to sucrose, and increased phloem loading in response to sink demand under GA_3 effect.

Both varieties exhibited an abrupt fall in RS levels and an escalation in sucrose content, at 90 DAS (Figure 14), possibly due to low ambient temperature which promotes sucrose accumulation and also the receding effect of GA_3. The GA_3 treated samples showed considerably reduced RS values (0.04%-0.11%) and complementarily augmented sucrose content (30.96%-38.32%) in comparison to that in control cane samples (0.08%-0.18%; 30.23%-37.37%), thus depicting more sucrose accumulation as a consequence of the GA_3 spray. The high brix% and pol% values obtained, also validated the elevated sucrose content in the CoJ64 canes. On the contrary, BO91 being a late maturing variety, exhibited extended effect of GA_3 treatment displaying greater RS% values and lower sucrose content than control canes even at 90 DAS, pointing to the still high sucrose demand. In resemblance to the pattern seen in CoJ64 at 60 DAS, the GA_3 treated leaves of BO91 exhibited lower RS and higher sucrose content compared to those of control, justifying the channelization of reducing sugars to facilitate sucrose production in order to meet up the still prevalent sink demand.

At 120 DAS, the GA_3 treated CoJ64 cane displayed lower sucrose and higher RS values compared to control cane. Rise in RS perhaps demonstrates onset of inversion in the GA_3 cane. It may possibly be reasoned that by this time sucrose sufficiency was signalled in the GA_3 treated cane (i.e. earlier as compared to control cane), thus causing decreased sucrose transport to GA_3 treated sink. Simultaneously, the GA_3 sprayed leaf of CoJ64 exhibited greater RS and sucrose content than that in control (Figure 16). This heaping of RS and sucrose perhaps depicts lesser formation of sucrose from RS and lesser sucrose export from leaf, thereby affirming decrease in demand from sink and diminished phloem loading in turn. A study on coffee leaves has evidenced the role of soluble sugars in mediating feedback from sink for down regulation of photosynthetic rate (Franck *et al.*, 2006). On the other hand, in case of BO91, the RS content in GA_3 treated canes was lesser and sucrose content higher than control cane, denoting active and elevated sucrose accumulation due to GA_3-stimulated sink demand. The GA_3 treated leaf exhibited lower sucrose value than control (Figure 14 (d), depicting greater sucrose transport to meet heightened sink demand. Even at 150 DAS, the GA_3 treated samples were found to have relatively lesser RS and higher sucrose levels compared to control, pointing to more active sucrose formation in source to fulfil the still prevailing sink demand.

With progressing maturity, the sucrose content steadily increased while the reducing sugar content dipped over time. The brix% and pol% profile validated the high sucrose levels recorded in GA_3 treated BO91 cane. The peak sucrose values attained by the GA_3 sprayed BO91 culm (40.54%-41.6%) were strikingly higher than those of control cane (30.44%-38.8%) (Figure 14 (d)). Hence, this analysis of sugar profile over the maturity phase emphatically asserted the role of GA_3 in improving sucrose hoarding in the culm by developing better sink strength and encouraging greater sucrose transport in turn (Iqbal *et al.*, 2011).

4.1.2.4 Quantification of enzyme activity

Invertases are considered to play prime role in controlling sucrose accumulation in sugarcane (Hatch *et al.*, 1963; Hawker and Hatch, 1965). It is now established that SAI activity is generally elevated in actively growing tissues viz. immature internodes in case of sugarcane (Gayler and Glasziou, 1972) gradually diminishing with progressing maturity. Decline in SAI activity governs the quantity and span of sucrose accumulation in the individual internodes and the culm as a whole. SAI activity above a critical threshold is said to prevent hoarding of high concentrations of sucrose. Lower sucrose-storing genotypes perhaps generally exhibit SAI activity beyond this limit, accounting for the low sucrose accumulation in them. Reiterating, the invertase activity in the low sucrose accumulating variety BO91 employed in this study too, was found to be noticeably higher than that in CoJ64.

However, sucrose accumulation in the culm cannot be thought to be solely governed by SAI activity. Studies have proposed an interplay of SPS and SAI activity (provided it is less than the critical limit) in determining level of sucrose hoarding among various varieties in the population (Zhu *et al.*, 1997). The net rate of sucrose accumulation can be visualized as the difference between the rate of sucrose formation and sucrose breakdown. Though no confident correlation has been deduced between SPS activity and sucrose accumulation in different internodes, the difference in SPS and SAI action has been demonstrated to strongly determine sucrose concentration.

In our current pursuit, the invertase activity obtained was largely in accordance with the RS level pattern in the culm internodes, justifying the hydrolysis of translocated sucrose into hexoses. Overall, invertase activity displayed the expected pattern of being higher in the top (immature) internodes with a decreasing gradient towards the bottom (mature) region; the gradient gradually evened out with advancing maturity. At 0 DAS, the already lower invertase activity observed in CoJ64 as compared to BO91, affirms the early setting-in of maturation in the early maturing variety CoJ64. 30 DAS, the higher invertase activity observed in the internodes of GA_3 treated cane, more so in BO91 (Figure 17), depicts the higher sink demand developed compared to control cane. The effect of GA_3 spray was more pronounced up to 30 DAS, wherein invertase activity was highest, especially in top internodes, after which the invertase activity gradually decreased over time depicting the ripening of the culm, allowing more and more sucrose accumulation. At this time, SPS activity also boosted up, perhaps to resynthesize sucrose for storage. However, at the end of maturation phase, far lesser invertase activity was recorded in the culm samples of GA_3 treated BO91 as compared to that of CoJ64, validating the higher sucrose level observed. Although decreasing with time, the significantly high SPS activity observed in BO91 (between 30 and 120 DAS) and CoJ64 (between 30 and 90 DAS) declare the resynthesis of sucrose from the hydrolysed hexoses of translocated sucrose.

Photosynthesis in sugarcane ultimately results in the synthesis of sucrose which is partly exported to sink whereas part of it gets channelled into leaf vacuole where SAI degrades it into hexoses. These are then moved back into the cytosol where they enter into a synthesis-degradation cycle viz. formation of sucrose and/or utilization by the leaf for its own metabolism (Huber, 1989). This explains the low RS levels observed in the LTM leaves in September (0 DAS) inspite of significant invertase activity wherein the RS generated may perhaps have been utilized more and more in growth and development of the leaves. This is further verified by the corresponding low SPS activity recorded. Hence, little sucrose production occurred when SPS activity was less and invertase activity was high.

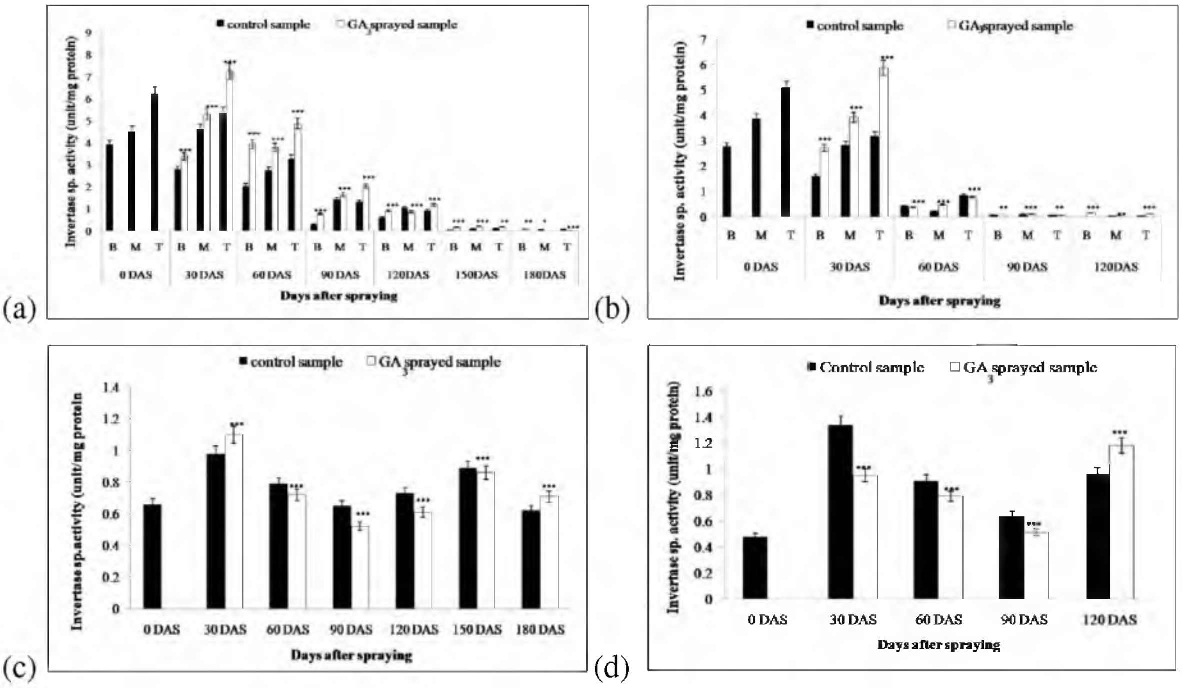


Figure 17: Specific invertase activity in (a) (b) internodal samples and (c) (d) LTM leaf of control and GA_3 treated BO91 and CoJ64 canes, respectively

At 30 DAS, evidently more RS got converted to sucrose in GA_3 sprayed LTM leaves, hence greater SPS activity was observed, compared to control. Greater sucrose formation may be linked to the developing high sucrose demand in sink. Since the sink tissue is in early maturation phase, it is possibly only beginning to hoard sucrose, justifying the high sucrose level available in leaf, for translocation. With progressing maturation, the invertase activity gradually diminished, more so in GA_3 sprayed leaves, possibly to keep hydrolysis to a bare minimum and facilitate more and more conversion of RS into sucrose. This is also confirmed by the rise in SPS activity observed in the LTM leaves (Figure 18). Enhanced SPS activity in GA_3 sprayed leaves, compared to control ones, can logically be associated to more sucrose synthesis to answer the greater sink demand. The reappearance of high invertase activity and low SPS activity at the end of maturation phase, in both BO91 and CoJ64 leaves, explains the high RS levels and sucrose heaping in the leaves. This could perhaps be extrapolated to be an indication of end product inhibition working in the leaves.

Stitt *et al.*(1987) have reasoned that in the case of phloem loading capacity being unable to match up to the rate of production of assimilate, the consequential sucrose hoarding in leaf may alter SPS activation state and the pile up of fructose 2,6-

bisphosphate that occurs as a result, can regulate sucrose metabolism in the leaf. In a comparative study between high and low sucrose accumulating progenies, it was revealed that they differed in the final sucrose concentration reached in older internodes and also in the pattern of sucrose accumulation along the stalk. The onset of sucrose accumulation in high sucrose accumulating clones, was reported to occur three to four internodes earlier than that in low sucrose accumulating ones. The early decline in invertase activity and early rise in SPS activity as observed in CoJ64 cane, compared to the pattern in BO91, further lends basis to this assertion. Thus, the measured enzyme activities could aptly explain and relate to the sugar analysis data.

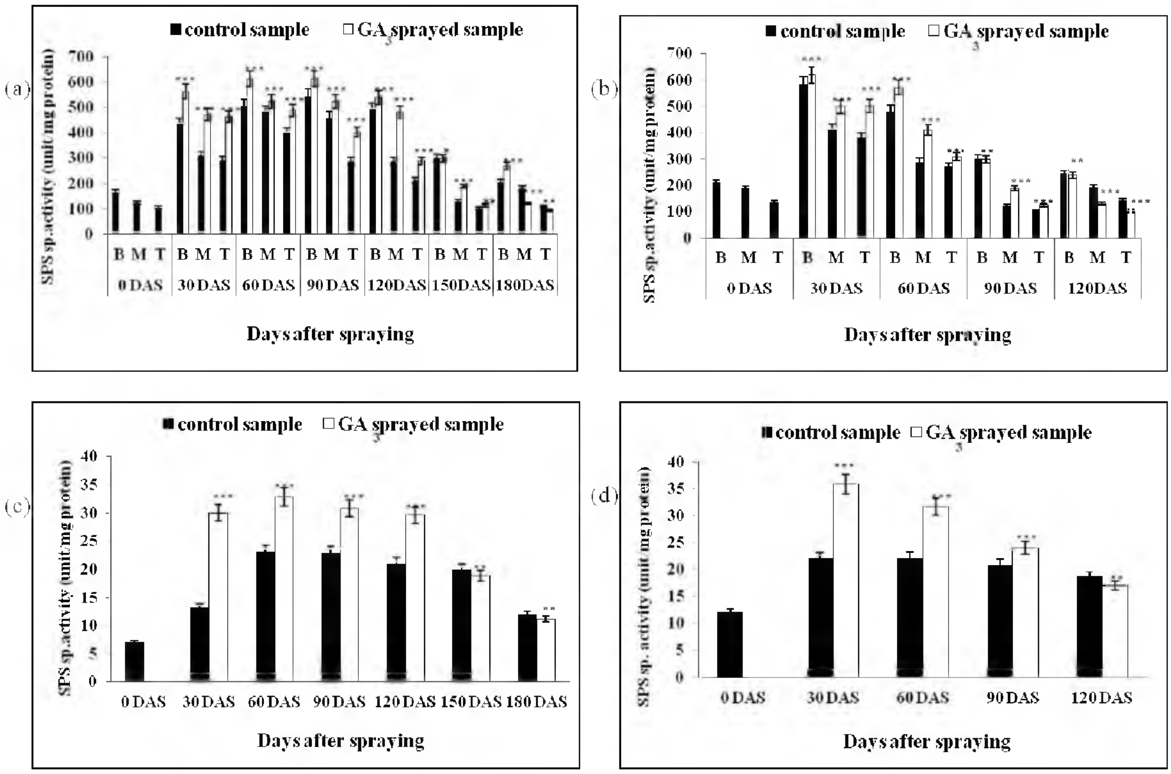


Figure 18: Specific SPS activity in (a) (b) internodal samples and (c) (d) LTM leaf of control and GA_3 treated BO91 and CoJ64 canes, respectively

4.1.3 Molecular analysis

4.1.3.1 End-point PCR expression analysis

This effort aimed at ascertaining the effect of gibberellin spray on sucrose metabolism at molecular level, by analysing the modification in expression of various sucrose metabolizing enzymes. Many studies have demonstrated gibberellins to bring about escalation in acid invertase activity as in oats (Kaufman *et al.*, 1973), bean (Morris and Arthur, 1985) and pea (Wu *et al.*, 1993).

4.1.3.1.1 SAI expression

Soluble acid invertase (SAI) is one of the primary sucrose hydrolysing enzymes, known to house in the vacuole particularly of elongating internodes, bringing about the breakdown of sucrose into hexose sugars. Hence, hexose concentrations have been found to escalate as a manifestation of heightened acid invertase activity in response to gibberellins (Iqbal *et al.*, 2011) (as also noted in earlier sections). SAI is said to synchronize with internodal growth i.e. it is mainly active during growth and dormant otherwise (Ho, 1988). The common convention that SAI is most active in immature tissues explains why lower sucrose content occurs in younger internodes that possess highly active SAI while maturing internodes with less active SAI accumulate high sucrose (Verma *et al.*, 2011).

In the present endeavour, either of the 50 ppm (Figure 19) and 35 ppm GA_3 spray (Figure 20 and 21) studies showed similar SAI expression. The expression of SAI transcript was significant in the immature (top) internodal tissues of both, control and GA_3 sprayed canes at the beginning. However, significantly greater SAI expression was recorded, especially in the GA_3 treated top internodes, at 30 DAS. Since internodal growth is facilitated by reducing sugars generated by the breakdown of imported sucrose (Kaur *et al.*, 2002), SAI is perceived to be dominant in actively growing tissues (Zhu *et al.*, 1997). Hence, the high SAI expression justified the prominently high concentration of reducing sugars observed in GA_3 sprayed canes, in the earlier section. Invertase activity is found to share an inverse relation with age of tissue, with maximum activity recorded in the most immature tissue and declining with advancing maturity (Jacobsen *et al.*, 1992). Accordingly, dimmer SAI transcript expression was detected in the middle internodes as compared to that in top. Since gibberellins are said to induce cell elongation/division in the target tissue, they perhaps prolong its state of immaturity, causing delayed onset of maturity. This was supported by the fact that SAI expression was prominently higher in the 'still growing' GA_3 sprayed internodes of both BO91 and CoJ64.

At 60 DAS, the GA_3 treated top internodal sample exhibited slightly stronger SAI expression, validating higher sink strength to draw assimilate, under the effect of GA_3. SAI expression then gradually faded over the time, as the effect of spraying waned and as maturation set in. SAI expression was visible in the BO91 top internodes, upto 60 DAS in the 50ppm study and upto 120 DAS in case of 35 ppm study, the GA_3 treated top internode still showing noticeably higher expression. However, the expression in the middle internodes became inconspicuous beyond 90 DAS. Evidently, the top CoJ64 samples displayed SAI expression up to 90 DAS while it faded out in the middle ones, after 30 DAS. Both reducing sugars and sucrose showed similar values in the control and sprayed canes, in and around 90 DAS. However, beyond 150 DAS, as maturation phase neared completion, the GA_3 sprayed samples exhibited almost nil expression and noticeably greater accumulation of sucrose. This clearly exhibits how gibberellin spray has brought about better assimilate deposition through manipulation of invertase activity.

In the 35 ppm study, BO91 LTM leaf exhibited significant SAI expression in September too, compared to succeeding months, also qualified by the recorded invertase activity. At 30 DAS, the high SAI expression depicts high rate of hydrolysis of the sucrose being synthesized in response to the high sink demand. Conversely, the lower SAI expression levels observed in GA_3 treated LTM at 60 DAS in case of BO91 and 30 DAS in CoJ64 (Figure 22), inspite of the corresponding high RS values perhaps illustrate the elevated photosynthetic rate to meet up the sucrose demand cultivated in sink. The lower SAI expression and parallely lower RS levels in GA_3 treated LTM, as compared to control, at 90 and 120 DAS in BO91 and 60 DAS in CoJ64, perhaps denote promotion in the direction of sucrose synthesis and translocation, also verified by a drop in leaf sucrose content. SAI expression gradually dimmed in both control and GA_3 treated LTM, marking complete maturation and saturation of the respective culm sinks. However, SAI expression was found to resurface at 180 DAS in case of BO91 and 120 DAS in CoJ64, in both control and

GA_3 treated LTM leaves qualified by high RS values, signifying increased inversion of sucrose.

4.1.3.1.2 CWI expression

Occurring in conjunction with the cell wall, CWI (cell wall invertase) forms the insoluble form of acidic invertase, and is unique in hydrolysing sucrose in extracellular (apoplastic) space. The active role of CWI in phloem unloading and participation in assimilate uptake in sink tissues, makes CWI another key sucrose metabolizing enzyme (Chandra *et al.*, 2015). Also, higher CWI activity in the sink tissue results in greater hydrolysis of unloaded sucrose, in turn increasing the capacity of sink to draw assimilate and promoting greater sucrose import (Ma *et al.*, 2000). In the 50 ppm study, CWI expression was found to be nearly absent in the early months of study perhaps due to delayed onset of maturity (Figure 19). CWI expression was very faint at 30 DAS and was found to surface 60 DAS, perhaps when sucrose accumulation became predominant, with comparable expression in control and GA_3 sprayed canes. However, 120 DAS, differential CWI expression was evident among control and GA_3 sprayed canes. The top intermodal sample of GA_3 sprayed plant still exhibited substantial CWI expression while it dimmed in control sample, pointing to more viable sucrose accumulation in GA_3 sprayed sample. This, in congruence with biochemical data, clearly indicates that gibberellin spray perhaps, can cause higher sucrose hoarding in the sugarcane stalk.

However, in the 35 ppm study, CWI expression, though faint, was visible in both, BO91 and CoJ64 samples, even at 0 DAS. Raised CWI expression levels were observed at 30 DAS, it being greater in GA_3 treated samples as compared to control, both in case of BO91 and CoJ64 (Figure 20 and 21). Notable expression was seen in top and middle internodal samples of both varieties up to 120 DAS. More prominent CWI transcript level was visible in the GA_3 sprayed top and middle internodes of BO91, at 120 DAS, beyond which the expression diminished. However, in case of CoJ64 top internode, CWI expression was fairly reduced in GA_3 treated sample and still noticeable in control, possibly marking commencement of inversion.

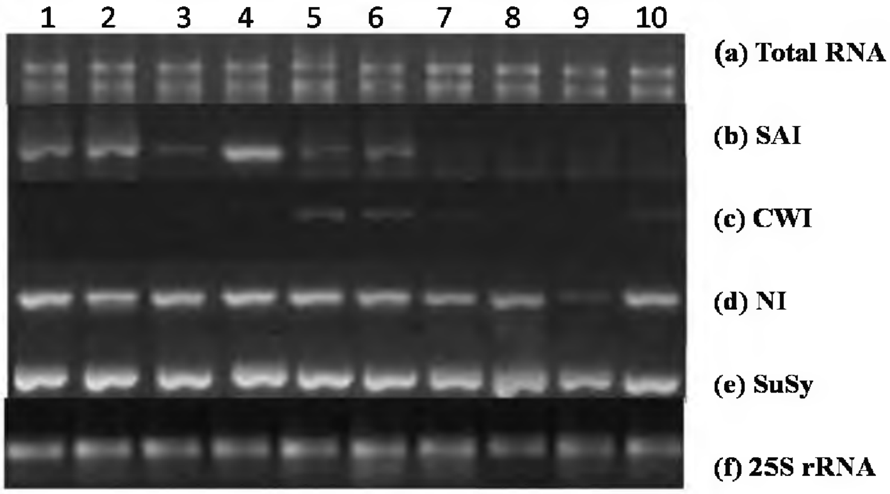


Figure 19: (a) Total RNA in normalized concentration visualized on 1% agarose gel **(b)** Sugar metabolizing enzymes viz. SAI (Soluble Acid Invertase) **(c)** CWI (Cell Wall Invertase) **(d)** NI (Neutral Invertase) **(e)** SuSy (Sucrose Synthase) exhibiting differential expression in internodal samples of control and GA_3 sprayed cane (50ppm spray) **(f)** 25SrRNA gene primer employed to validate RNA normalization **Lanes 1-2:** top internodal samples from control and GA_3 treated cane at 0 DAS, respectively;

Lanes 3-4: top internodal samples from control and GA_3 treated cane at 30 DAS, respectively**; Lanes 5-6:** top internodal samples from control and GA_3 treated cane at 60 DAS, respectively; **Lanes 7-8:** top internodal samples from control and GA_3 treated cane at 90 DAS, respectively; **Lanes 9-10:** top internodal samples from control and GA_3 treated cane at 120 DAS, respectively.

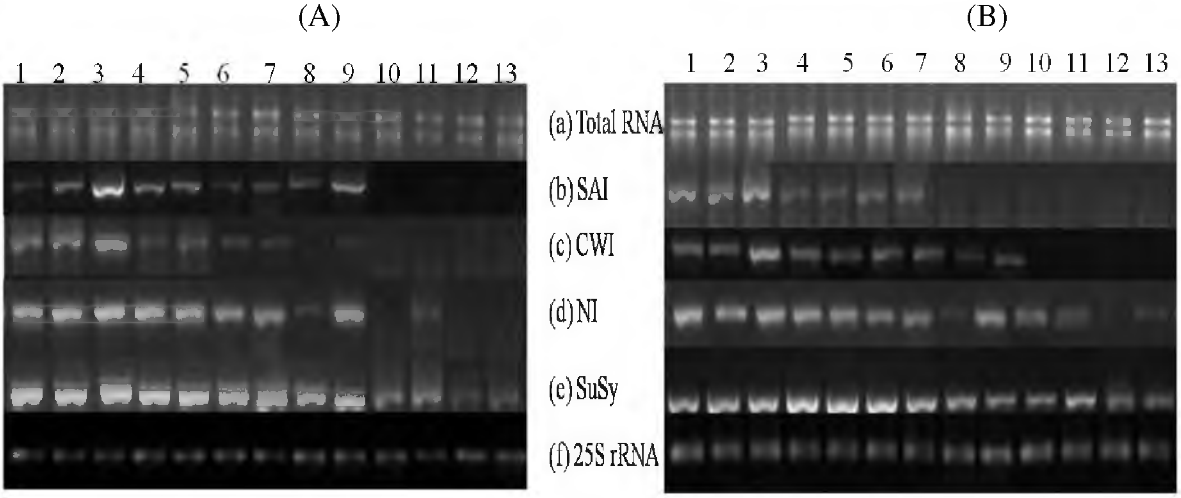


Figure 20: (a) Normalized concentration of total RNA in (A) top and (B) middle internodal samples of BO91 (b) Differential expression of sugar related genes SAI (c) CWI (d) NI (e) SuSy in (A) top and (B) middle segment of control and GA_3 treated BO91cane (35ppm spray) (f) 25SrRNA gene primer employed to validate RNA normalization

Lane 1: top (A) and middle (B) internode of control cane at 0 DAS; Lanes 2-3: top (A) and middle (B) internodes of control and GA_3 treated cane at 30 DAS, respectively; Lanes 4-5: top (A) and middle (B) internodes of control and GA_3 treated cane on 60 DAS, respectively; Lanes 6-7: top (A) and middle (B) internodes of control and GA_3 treated cane on 90 DAS, respectively; Lanes 8-9: top (A) and middle (B) internodes of control and GA_3 treated cane on 120 DAS, respectively; Lanes 10-11: top (A) and middle (B) internodes of control and GA_3 treated cane on 150 DAS, respectively; Lanes 12-13: top (A) and middle (B) internodes of control and GA_3 treated cane on 180 DAS, respectively

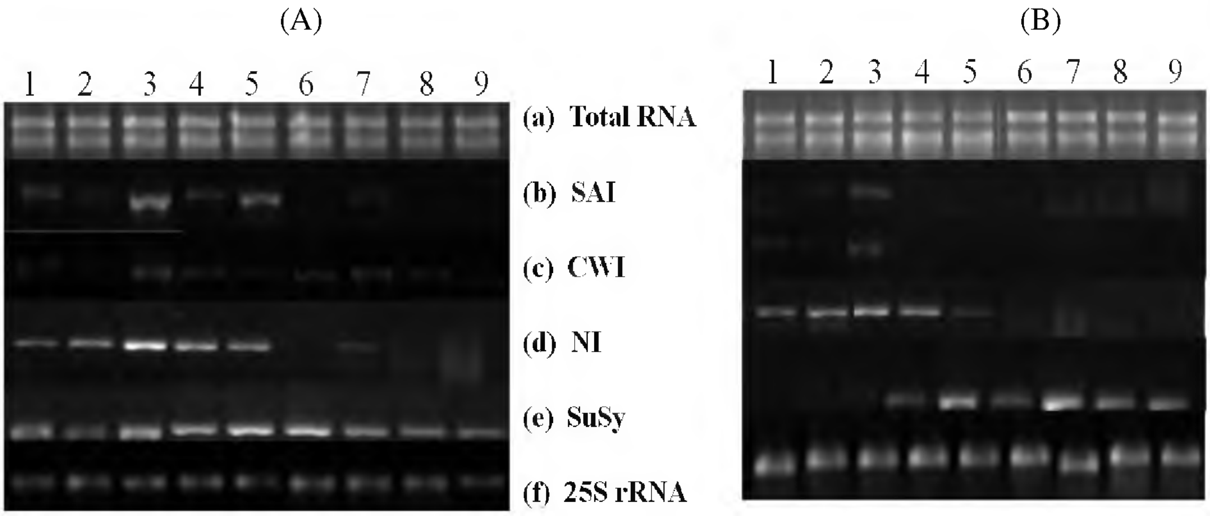


Figure 21: (a) Normalized concentration of total RNA of (A) top and (B) middle internodal samples of CoJ64 (b) Differential expression of sugar related genes SAI (c) CWI (d) NI (e) SuSy in (A) top and (B) middle segment of control and GA_3 treated CoJ64 cane (35ppm spray) (f) 25SrRNA gene primer employed to validate RNA normalization

Lane 1: top (A) and middle (B) internode of control cane at 0 DAS; Lanes 2-3: top (A) and middle (B) internodes of control and GA_3 treated cane at 30 DAS, respectively; Lanes 4-5: top (A) and middle (B) internodes of control and GA_3 treated cane on 60 DAS, respectively; Lanes 6-7: top (A) and middle (B) internodes of control and GA_3 treated cane on 90 DAS, respectively; Lanes 8-9: top (A) and middle (B) internodes of control and GA_3 treated cane on 120 DAS, respectively; Lanes 10-11: top (A) and middle (B) internodes of control and GA_3 treated cane on 150 DAS, respectively; Lanes 12-13: top (A) and middle (B) internodes of control and GA_3 treated cane on 180 DAS, respectively

4.1.3.1.3 NI expression

Neutral invertase (NI) facilitates breakdown of sucrose in the cytosol and hence is referred to as a soluble invertase (Batta and Singh, 1986). Studies have shown that NI transcript expression is correlated to level of maturity of culm, with the expression diffusing with advancing maturity of culm (Rose and Botha, 2000). As also reported by Bosch *et al.* (2004), elevated NI expression in the immature internodal tissues of sugarcane while fainter expression in the mature ones. Since high NI expression has been noted in immature tissues with high reducing sugar and low sucrose levels, a direct relation can be said to exist between sugar content and NI expression, hinting at the role of NI in furnishing hexoses to immature tissues. In concurrence, significantly high NI expression was recorded in early phase of both (50 ppm and 35 ppm spray) studies (Figure 19 and 20, 21). At 30 DAS, the canes had only begun to mature and hence equivalent NI expression was seen in top and middle internodes of control and GA_3 treated canes. The NI transcript expression began diffusing beyond 90 DAS, depicting the progressing maturation of the cane.

Even at 120 DAS, top and middle internodal samples of the late maturing BO91cane exhibited substantial NI transcript level. However, the NI expression was significantly greater in the GA_3 treated samples than control due to prolonged immaturity under the effect of GA_3. NI is known to furnish hexoses thereby improving strength of culm sink (Quick and Schaffer, 1996) to attract more assimilate. Thus, the higher NI expression in GA_3 treated samples perhaps points to better sink strength and hence better sucrose accumulating potential. Contrastingly, in the early maturing CoJ64 cane, NI expression weakened beyond 90 DAS and was negligible by 120 DAS. Some studies have reported a substantial amount of NI activity in mature tissues of sugarcane suggesting its role in influencing sucrose hoarding (Bosch *et al.*, 2004). This perhaps justifies the faint expression visible in later phase of maturity. Quite expectedly, NI expression became insignificant by the end of the study, both in CoJ64 and BO91, acknowledging its role primarily in building the sink strength.

4.1.3.1.4 SuSy expression

As reviewed in earlier section, SuSy facilitates a two-way reaction (Geigenberger and Stitt, 1993), wherein it partakes in synthesis of sucrose in one direction while in the other, facilitates breakdown of sucrose in the sucrose-stocked sink (Black *et al.*, 1987). Due to its hydrolyzing nature, SuSy readily furnishes hexoses to promote growth in immature internodes, which in turn invites sucrose unloading through build up of high sink strength (Chandra *et al.*, 2015). Since the cane was only beginning to mature in the early phase of both studies (50ppm and 35ppm spray), the control and

GA_3 treated samples of both cane varieties, displayed comparable expression (Figure 19, 20, 21). At 60 and 90 DAS, the middle internodes of BO91 and CoJ64 exhibited distinctly greater SuSy expression in GA_3 sprayed sample, pointing to higher sink strength, for accumulating more assimilate. However, the SuSy expression gradually dimmed 120 DAS and beyond, marking the progressing maturation and reducing sink strength. Dimmer yet distinct SuSy expression was observed in the later phase of maturity, perhaps suggesting the role of SuSy in sucrose synthesis.

4.1.3.1.5 FBPase expression

Fructose 1, 6-bisphosphatase (FBPase) is a vital enzyme involved in sucrose synthesis, in conjunction with other enzymes. Specifically, it facilitates the synthesis of fructose-6-phosphate, consequently yielding UDP-glucose, one of the essential participants in formation of sucrose (Grof and Campbell, 2005). The LTM leaves of both BO91 and CoJ64 exhibited noticeable FBPase expression corroborating its active participation in formation of sucrose in the source. At 30 and 60 DAS, particularly prominent FBPase expression was noted in GA_3 sprayed LTM leaves of both varieties, more pronounced in sample of BO91 (Figure 22). As maturation progressed, FBPase expression was quite predictably found to reduce. Although weakened, yet greater FBPase expression was observed in GA_3 treated LTM leaves even at the later phase of maturity, especially in BO91. This can perhaps be extrapolated to prolonged supply of sucrose to GA_3 treated BO91 cane, thus justifying the high final sucrose concentrations recorded.

4.1.3.1.6 PEPC expression

Species like sugarcane, employing the C_4 pathway, use PEPC as a carboxylase option to initially fix carbon into oxaloacetic acid. Thus, PEPC is reported to have a stimulatory effect on photosynthesis in C_4 plants (Lian *et al.*, 2014). The role of gibberellins in facilitating photosynthetic activity is well documented, such that the increased photoassimilate produced may be utilized by sink (Iqbal *et al.*, 2011). Analysing the source-sink relation in many plants, including sugarcane, has led to the deduction that photosynthetic activity of leaves bear a direct relation with sucrose demand of sinks such that diminished sink demand causes a drop in photosynthetic assimilation rate (Franck *et al.*, 2006). Conversely, workers like McCormick *et al.*

(2006) have demonstrated a significant rise in photosynthesis and improved carboxylation efficiency to cater heightened demand in culm.

The sugar data which revealed remarkably high RS in GA_3 treated culm perhaps denoted GA_3-induced higher sink strength and resultant higher sink demand. GA_3 treated leaf of CoJ64 and BO91 also exhibited elevated RS levels at 30 DAS and 60 DAS, respectively (Figure 22), perhaps depicting higher photosynthetic activity. Correspondingly, the higher PEPC expression observed in GA_3 treated leaf corroborates the higher photosynthetic rate developed in answer to the high sink demand and sink strength. In sugarcane, maturity status of the culm has been stated to influence photosynthetic rate of source (Hartt and Burr, 1967; Sweetlove *et al.*, 1998; McCormick *et al.*, 2008d). The hoarding of sucrose in the mature culm perhaps causes the sink to gradually saturate and sink demand to reduce, thus in turn causing a drop in source photosynthetic rate. This elucidates that the declining RS content recorded in leaf samples of BO91 and CoJ64, in the later phase of study, probably signified the drop in photosynthetic rate, as also validated by the fading PEPC expression. Also, the sucrose pile up (observed in earlier section) in the LTM leaves possibly ensue end product inhibition leading to decline in photosynthetic rate (Hartt, 1963; Goldschmidt and Huber, 1992). In a study involving cold girdling of petioles to impede sucrose transport away from the source, Krapp *et al.* (1993) reported repression of photosynthesis genes due to accumulation of sucrose.

4.1.3.1.7 SUT expression

Sucrose synthesized in the leaf is loaded into the phloem for export to sink, via the process of phloem loading mediated by membrane transporters (Rae *et al.*, 2005a). Among the sucrose transporters known so far, sucrose-H^+ symporter (SUT) is amongst the most distinguished, and mediates phloem loading from the apoplasm (Ainsworth and Bush, 2011). The fact that SUT mainly occurs in the phloem and that it is largely active during the sucrose accumulation period, affirms the function of sucrose transporter in mediating sucrose loading from apoplasm into phloem (Lalonde *et al.*, 1999). At 30 DAS, the faint SUT expression obtained in BO91 GA_3 treated leaf, perhaps points to lower sucrose export initially, also validated by the elevated sucrose content seen (Figure 22). The heightened expression of SUT observed in GA_3 sprayed samples at 30 DAS in CoJ64 and 60 DAS in BO91 is validated by the elevated sucrose levels detected in GA_3 treated leaf of CoJ64, over control. The raised SUT expression seen in GA_3 treated leaves of BO91 and CoJ64, 120 and 90 DAS respectively, pointed to increased transportation in response to sink demand. This is justified by the larger drop in sucrose levels recorded in the GA_3 treated samples. The low ambient temperature at this time of the study, may have also stimulated increased transportation as cold temperature promotes sucrose accumulation. The considerable SUT expression noted especially in GA_3 treated CoJ64 and BO91 LTM leaf, even in the later phase of the study (at 120 DAS and at 150 DAS respectively), denoted the still active sucrose export. At the conclusion of maturation phase, the faded SUT transcript expression in the samples of both varieties represented the weakened sucrose transportation perhaps because of decreased sink demand in the event of saturation of culm sink. Various studies viz. sugar feeding and cold-girdling, have given an account of such inhibition of sugar transporter activity (Chiou and Bush, 1998).

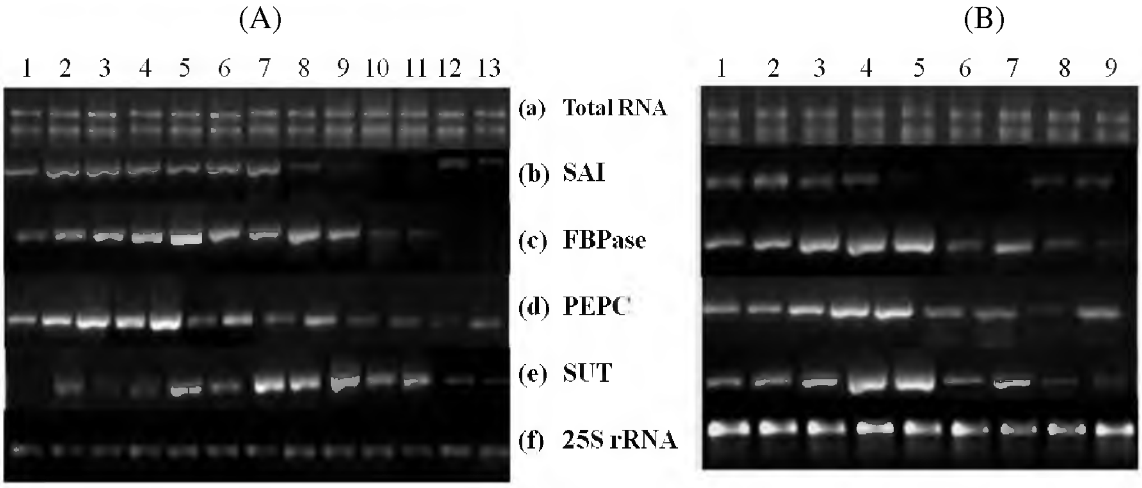


Figure 22: **(a)** Agarose gel depicting total RNA of (A) BO91 and (B) CoJ64 LTM leaves in normalized concentration **(b)** Differential expression of SAI (Soluble Acid Invertase) (c) FBPase (Fructose-1,6-bisphosphatase) (d) PEPC (Phospho enol pyruvtae carboxylase) (e) sucrose transporter (SUT) gene in control and GA_3 treated LTM leaves of (A) BO91 and (B) CoJ64 **(f)** 25SrRNA gene primer employed to validate RNA normalization

Lanes 1: LTM leaf sample from control cane at 0 DAS; **Lanes 2-3:** LTM leaf sample from control and GA_3 treated cane at 30 DAS**;** **Lanes 4-5:** LTM leaf sample from control and GA_3 treated cane at 60 DAS; **Lanes 6-7:** LTM leaf sample from control and GA_3 treated cane at 90 DAS; **Lanes 8-9:** LTM leaf sample from control and GA_3 treated cane at 120 DAS; **Lanes 10-11:** LTM leaf sample from control and GA_3 treated cane at 150 DAS; **Lanes 12-13:** LTM leaf sample from control and GA_3 treated cane at 180 DAS

4.1.3.2 Real-time PCR analysis

Different sugarcane varieties display varied ability to accumulate sucrose depending on several morphological and enzymatic factors (Hatch and Glasziou, 1963) viz. sucrose synthesizing and degrading enzymes. In the present endeavour, the modification in gene expression of a variety of sucrose degrading/synthesizing enzymes (viz. SAI, CWI, SPS, SuSy) under the effect of GA_3 treatment, was determined in middle and top internodal samples from control and GA_3 treated BO91 and CoJ64 canes over the maturation phase (at 30, 120 and 180 DAS) using quantitative real-time PCR. The results were expressed w.r.t. initial control sample (0 DAS), in terms of $\log_{10}$ RQ values (where $RQ=2^{-\Delta\Delta C_t}$), denoting fold change. Nil amplification in the negative control (i.e. without template) and single peak obtained in melt curve analysis verified the specificity of primer for the sample (template).

4.1.3.2.1 In terms of SAI expression

SAI activity has generally been found to be heightened in the actively growing tissues, for example, immature internodes of sugarcane (Zhu *et al.*, 1997). Also, GA treatment has been found to bring about an escalation in acid invertase activity (Iqbal *et al.*, 2011), thereby causing increased hexose production by hydrolysis of imported sucrose. 30 DAS (as maturation phase set in), augmented SAI expression was noted in control and GA_3 treated samples of both BO91 and CoJ64, when compared with 0th day (September) control. This augmented SAI activity observed in GA_3 treated samples may perhaps be reasoned to be due to maintenance of state of growth/ immaturity in the culm, by GA_3. 30 DAS, the 4.6 fold greater SAI expression exhibited by the GA_3 treated top internodal sample of BO91, in comparison to the 1.8 fold in control, relative to 0th day control (Figure 23). The remarkably higher SAI expression displayed by GA_3 treated samples, more so in BO91, perhaps depicts greater cleavage of transported sucrose (affirmed by the rise in RS content reported in sugar data) to support internodal growth (Hatch and Glasziou, 1963) and greater sink strength and sink potential to draw assimilate, under the effect of GA_3 treatment. Conversely, CoJ64 being an early maturing variety had comparatively matured by 30 DAS, thus perhaps SAI expression was found to be only 1.15 fold greater in the GA_3 treated top internodal sample, while control sample exhibited 0.25 fold higher expression than September (0 DAS) control. Since the escalation in SAI transcript level under the effect of GA_3, was much more substantial in BO91 as compared to CoJ64, it also points to greater sucrose transport to meet the higher sink demand and gives good reason for the higher final peak sucrose values recorded in GA_3 treated BO91.

The middle internodal sample of GA_3 treated BO91 cane exhibited the expected higher SAI expression (Figure 24). However, that of CoJ64 displayed reduced SAI expression in comparison to control, perhaps because, by this time, the almost mature middle internode was less affected by the GA_3 treatment. High sugar accumulating, early maturing varieties are said to accumulate high sucrose concentrations due to characteristic early suppression of SAI activity (Dendsay *et al.*, 1995). This justifies the lower SAI transcript expression observed in CoJ64, known to mature early and stock high sucrose levels. SAI activity is said to perhaps govern the occurrence and level of sucrose accumulation by way of a threshold, beyond which high sucrose accumulation is prevented (Zhu *et al.*, 1997). Since the internodal samples of BO91 (especially GA_3 treated) exhibited more substantial SAI gene expression, perhaps greater than threshold, this accounts for the restricted sucrose accumulation obtained.

While SAI furnishes hexose sugars to growing immature tissues, it also participates in sucrose unloading by bringing about hydrolysis of imported sucrose. However, this activity is reported to diminish as the culm matures and ages (Glasziou and Bull, 1965). With advancing maturation, by 120 DAS, SAI expression prominently dipped in control and GA_3 treated internodal samples of BO91. Nevertheless, the SAI expression was a noticeable 0.5 times greater than September control in the still maturing BO91 top internodal sample (Figure 23). The dip in SAI expression, with a downregulation of nearly 0.58 fold in top internodal control sample and 1.3 fold in GA_3 treated one, compared to September control, conformed to the fact that CoJ64 being an early maturing variety, nearly totally matured by 120 DAS. The greater downregulation in GA_3 treated sample perhaps denotes saturation of sink, perhaps avoiding any further sucrose accumulation. At 180 DAS, SAI expression vividly showed signs of complete maturity through prominent 3.8 fold downregulation in GA_3 treated top internode of BO91 and 2.2 fold in the control ones.

Both SAI and CWI are presumed to impact sucrose accumulation in maturing sugarcane culm; with them being most active in the immature internodes and their activity decreasing with increasing maturity of cane (Lontom *et al.*, 2008). The middle internodes of BO91 had relatively matured even more than the top ones by January (120 DAS), hence displaying a 0.63 fold downregulation of SAI expression in control sample and an even greater 0.92 fold in GA_3 treated sample, relative to the September control. Since CoJ64 has reached peak maturity by this time, the middle internodal sample of GA_3 treated cane showed down regulated SAI expression. However, the corresponding control sample exhibited a 0.46 fold increase in SAI expression, perhaps depicting the commencement of inversion in the fully matured middle portion (Figure 24).

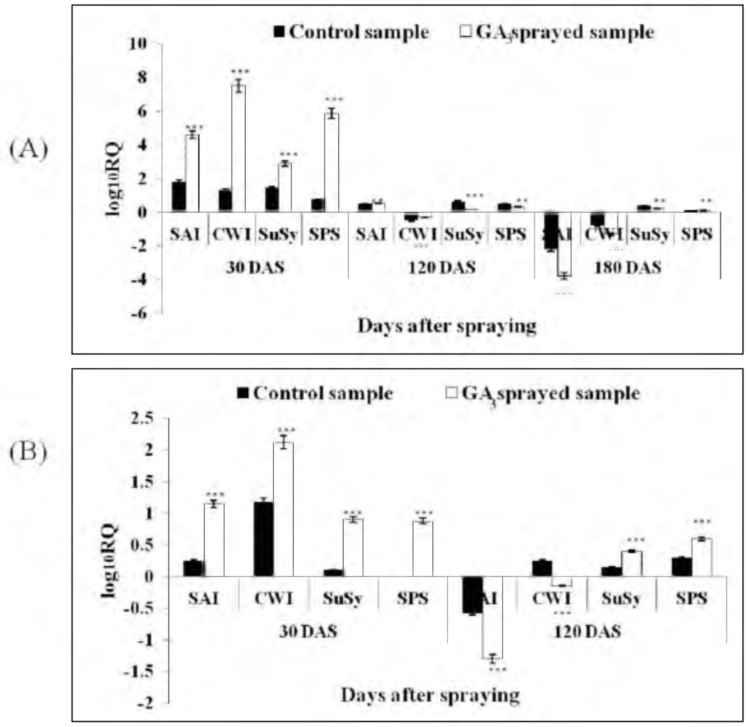


Figure 23: Quantification of difference in expression ($\log_{10}RQ$) of SAI, CWI, SuSy, SPS in control and GA_3 sprayed samples from top portion of (A) BO91 (B) CoJ64 canes

4.1.3.2.2 In terms of CWI expression

In mature tissue, CWI plays a primary role in phloem unloading via apoplasm, hydrolysing imported sucrose, thereby furnishing hexoses (Hawker and Hatch, 1965) increasing the sink strength and sucrose transport in turn (Ma *et al.*, 2000). The existence of apoplastic path as a valid route for phloem unloading is verified by the presence of CWI activity in sugarcane stalk (Albertson *et al.*, 2001). Wu *et al.* (1993) have demonstrated escalation in CWI gene expression in response to GA_3 treatment in pea shoot, through a transcriptional level study. Coherently, distinctly heightened CWI expression (7.5 fold) was recorded in the GA_3 treated top internodal samples while a much lesser ~1.25 fold elevation was visible in non-treated samples, relative to September control, at the beginning of maturation phase, 30 DAS (Figure 23). Parallely, control and GA_3 treated samples from top segment of CoJ64 canes also exhibited augmented CWI transcript level, relative to September control. The higher (~2.15 fold) CWI expression in GA_3 treated sample, compared to the ~1.25 fold in control, gives evidence for increased unloading into the sink, due to heightened sucrose requirement under the influence of GA_3. Escalated CWI expression levels especially in GA_3 treated sample of BO91, perhaps indicates greater scope for apoplastic unloading, in turn pointing to greater sucrose accumulation potential. On similar lines, augmented CWI expression was exhibited in middle internodal samples of BO91, more so in the GA_3 treated one. On the other hand, ~0.2 fold downregulated

CWI expression was obtained in the middle internodal sample of control cane while a greater, ~0.2 fold upregulated expression in corresponding GA_3 treated sample. The higher CWI expression affirms better sink strength to invite more assimilate by facilitating greater sucrose unloading.

The activity of acid invertases viz. SAI and CWI is particularly high in growing internodes, and begins to recede with progressing maturation of the culm (Albertson *et al.*, 2001). In congruence, at 120 DAS, the top internodal samples of BO91 displayed a substantial drop in CWI expression, with the GA_3 treated sample displaying lesser downregulation (greater expression), thereby encouraging greater sucrose translocation by aiding greater sucrose unloading. Concurrently, since CoJ64 had almost completely matured by this time, the GA_3 treated top internodal sample, showed downregulated expression, perhaps affirming culmination of maturation phase and denying any further apoplastic unloading of sucrose. Yet, upregulated CWI expression observed in control, probably marks the commencement of inversion. However, in the peak maturity phase (180 DAS), the top intermodal samples of BO91 showed visible downregulation of CWI expression, with the GA_3 treated sample exhibiting greater downregulation.

By January (120 DAS), the early maturing CoJ64 had gained absolute maturity and thus, both control and GA_3 treated middle internodal samples displayed downregulation of CWI expression (Figure 24). The 0.9 fold downregulation of CWI expression in the GA_3 treated sample and correspondingly 0.15 fold in control one, possibly denotes earlier attainment of maturity and greater saturation of sink. Contrastingly, the middle internodal samples of the late maturing BO91 exhibited substantial CWI expression even at 120 DAS. The control sample showed fair (0.5 fold) up regulation of CWI expression, relative to September control (Figure 24), representing the possibility for sucrose unloading whereas the GA_3 sprayed sample exhibited a mere 0.15 fold higher CWI expression. This indicates comparatively smaller requisite for sucrose translocation in the high sucrose bearing middle cane portion (as evident from sugar data too). The greater downregulation observed in the GA_3 treated middle intermodal sample in comparison to control, depicted greater saturation of culm perhaps due to greater GA_3-induced sucrose accumulation.

4.1.3.2.3 In terms of SuSy expression

Sucrose synthase catalyses a two-way reaction, wherein, on one hand it promotes the hydrolysis of imported sucrose into hexoses, while on the other, it facilitates sucrose synthesis using UDP-glucose as substrate (Hawker, 1985; Huber and Akazawa, 1986). At 30 DAS, the top internodal samples of control canes exhibited significant SuSy expression, ~1.5 fold greater in case of late maturing BO91 and ~0.075 fold higher in CoJ64 sample (Figure 23), relative to September control. SuSy is said to be particularly higher in growing internodes than in fully mature tissue (Lingle and Smith, 1991). In congruence, ~3 fold greater SuSy transcript quantum was recorded in the top intermodal sample of GA_3 treated BO91 cane and 0.9 fold higher in the corresponding CoJ64 sample, due to relative state of immaturity maintained by GA_3 treatment. This can be extrapolated to higher sink strength (affirmed by the high RS values, low sucrose values) suggesting better sucrose accumulation under the effect of GA_3.

Likewise, in the case of middle internodal sample of late maturing BO91, the GA_3 sprayed sample displayed 0.65 fold higher SuSy expression while the control one exhibited a lower expression (downregulation of ~0.7 fold) (Figure 24), validating the role of GA_3 in preserving immaturity of tissue for longer duration. Contrastingly, 0.35 fold downregulated SuSy expression was noted in the middle segment of GA_3 treated cane, perhaps as a consequence of the early maturing nature of CoJ64. With progressing maturity, the SuSy expression substantially decreased such that at 120 DAS, the top internodal GA$_3$ treated sample displayed low expression perhaps suggesting saturation of sink due to ample sucrose hoarding. Conversely, a significant rise in SuSy expression of control and GA$_3$ treated middle internodal samples of early maturing CoJ64 was probably due to onset of inversion. At 180 DAS, low SuSy expression seen in top and downregulated expression in middle internodes of BO91 canes, validate attainment of peak maturity.

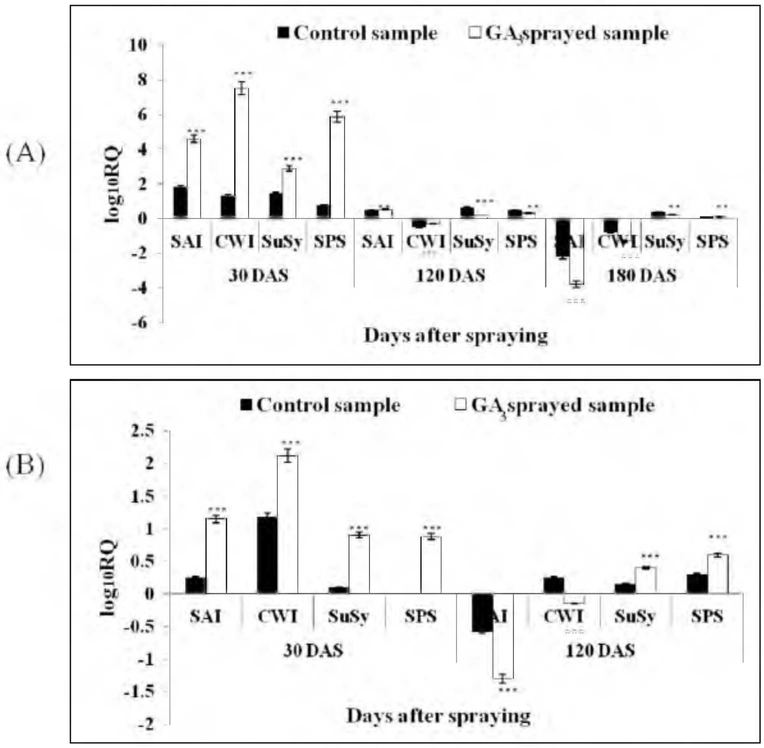


Figure 24: Quantification of difference in expression (log$_{10}$RQ) of SAI, CWI, SuSy, SPS in control and GA$_3$ sprayed samples from mid portion of (A) BO91 (B) CoJ64 canes

4.1.3.2.4 In terms of SPS expression

SPS is the primary sucrose synthesizing enzyme functioning in leaves as well as non-photosynthetic tissues viz. sink (by way resynthesizing sucrose from hexoses) (Huber and Huber, 1996). At the beginning of maturation phase (30 DAS), particularly elevated SPS expression was observed in top internodal samples of BO91 and CoJ64, more so in GA$_3$ treated samples. The heightened SPS expression in GA$_3$ treated sink confirms the augmented synthesis of sucrose from hexoses. This adds evidence to the idea of increased apoplastic uptake of sucrose under the effect of GA$_3$, its subsequent hydrolysis into hexoses (confirmed by rise in RS) which are subsequently resynthesized into sucrose by SPS (affirmed by net rise in sucrose levels). Additionally, the potential of sink to attract assimilate has been found to be altered under the effect of elevated SPS activity. Lending support, Nguyen-Quoc et al. (1999) reported a rise in sucrose unloading due to SPS overexpression in tomato fruit. In congruence, greater SPS transcript level was displayed by top internodal samples of GA_3 sprayed canes of both varieties, ~6 fold in case of BO91 and ~0.85 fold in CoJ64, relative to September control (Figure 23). Hence, these results corroborate greater sucrose synthesis in answer to greater sink demand developed under GA_3 effect. Similarly, the GA_3 treated middle internode of late maturing BO91 displayed a 0.3 fold increased SPS expression (Figure 24). However, the middle internode of the early maturing CoJ64 had relatively matured by this time, hence control and even GA_3 treated sample exhibited a downregulation of ~0.6 and 0.7 fold, respectively. Since the top internodes may have become more mature by 120 DAS, decreased SPS expression was recorded in GA_3 treated as well as non-treated samples of BO91 and CoJ64. The lesser yet significant SPS expression seen denotes the existing sucrose requirement in the top internodes. At this time, the GA_3 treated middle internodes of both BO91 and CoJ64 showed particularly downregulated expression, suggesting adequate sucrose accumulation in the sink. On the other hand, upregulated SPS expression was obtained in the middle internodal control samples of both BO91 and CoJ64, perhaps hinting at active sucrose synthesis now. Past studies have reported conflicting SPS behaviour where workers like Zhu *et al.* (1997); Grof *et al.* (2007) have stated that immature internodes exhibit greater SPS activity while elevated SPS activity has been described in mature, older internodes by Botha and Black (2000). Overall, GA_3 treatment was found to stimulate sucrose synthesis in the early maturation phase itself, by developing high sink strength and sink demand while SPS-mediated sucrose production in control samples was seen to accelerate in the later phase of study.

GA_3 treatment has been said to promote SPS activity thus escalating synthesis of sucrose and its loading and transport in turn (Iqbal *et al.*, 2011). As concluded from the sugar data earlier, increased sucrose hydrolysis occurs due to stimulation of invertase activity by GA_3. As a consequence, the sink strength and sink demand increases, thus promoting sucrose transport. A significant 2 fold greater SPS expression was noted in GA_3 treated LTM leaf in answer to the GA_3-induced heightened sink demand. Reasonably, mere ~0.6 fold upregulation in SPS expression was seen in control sample due to comparatively lesser sink demand (Figure 25). GA_3 treated leaf of CoJ64 showed a similar rise in SPS expression at 30 DAS. However, this augmented expression was seen to wither by 120 DAS, as the cane attained peak maturity. This observation being in correspondence with sucrose data, affirms the increased sucrose synthesis to fulfil the sucrose demand of maturing sink.

Photosynthetic rate has been found to be related to maturity of plant such that leaves of immature plants photosynthesize more than those in mature plants, indicating end-product signalling (Hartt, 1963). On similar lines, Kortschak *et al.* (1965) suggested sucrose mediated suppression of SPS activity. In accordance, SPS transcript level was notably lesser in January (120 DAS) as compared to that 30 DAS. Both, exogenously supplied sugars (Hesse *et al.*, 1995) and manoeuvring of endogenous sugars (Müller-Röber *et al.*, 1992) has been described to affect SPS expression. Thus the endogenous build up of hexose sugars due to increased photosynthesis in GA_3 treated leaves, may have stimulated increased SPS expression, 30 DAS, both in BO91 and CoJ64. At 120 DAS, the LTM leaf of still maturing BO91 displayed heightened SPS expression viz. single fold up regulation in control and ~2 fold greater expression in the GA_3 treated leaf. This perhaps implies greater formation of sucrose to provide for the effective sucrose demand, more so in the GA_3 sprayed cane. At 180 DAS, SPS expression dropped in LTM leaf, much in congruence with enzyme activity data. SPS mRNA level has been reported to increase on provision of glucose to excised sugarbeet or potato leaves while exogenous sucrose inhibited the expression (Hesse et al., 1995). On similar lines, downregulation of SPS expression as observed in LTM leaf of BO91 and CoJ64 at 180 DAS and 120 DAS respectively, can perhaps be correlated to end product (sucrose) repression of SPS expression. Thus, the relative amount of sucrose accumulated by the sugarcane plant perhaps works by way of end point repression of photosynthesis. When the rate of assimilate production exceeds rat of export, the consequent sucrose pile up in the leaf adjusts SPS activity accordingly, causing a feedback reduction in leaf sucrose metabolism (Hartt, 1963; Stitt *et al.*, 1987). This elucidates the diminished SPS expression seen in control and GA_3 treated LTM leaves of both, CoJ64 and BO91, perhaps in reaction to the sufficiency signalled by sucrose-stocked mature culm.

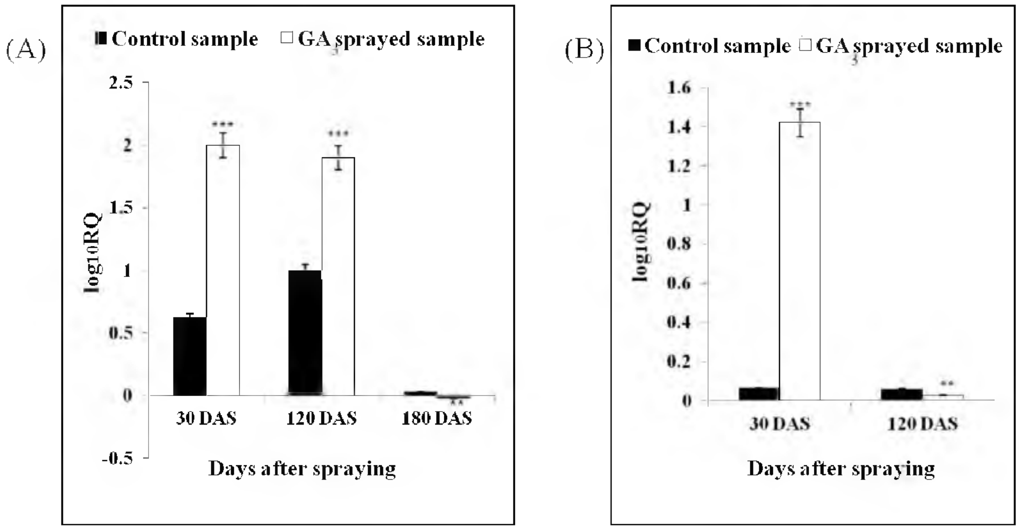


Figure 25: Quantification of difference in expression ($\log_{10}RQ$) of SPS in control and GA_3 sprayed LTM leaf samples of (A) BO91 (B) CoJ64 canes

4.1.4 Transcriptomic analysis

Gibberellins are known to favourably affect growth and many other physiological and metabolic factors and thus can be extrapolated to affect sucrose accumulation, as seen in this study too. Thus, a transcript level functional analysis of differential expression can be anticipated to weave in information about many yet unknown factors viz. those involved in carbohydrate metabolism, sugar-based feedback signals limiting photosynthetic rates, that may be regulating sucrose accumulation under the effect of GA induced perturbation. Many earlier endeavours (Feng *et al.*, 2012; Cardoso-Silva *et al.*, 2014; Huang *et al.*, 2016) have demonstrated the advantage of RNA-seq analysis in generating transcriptomic data even without a reference genome, as is the case with sugarcane. Here, transcriptome level analysis was carried out on control and GA_3 treated internodal samples of BO91 since it displayed a more significant difference in sucrose accumulation, compared to CoJ64, in response to GA_3 (as per morphological, biochemical and molecular level data recorded).

4.1.4.1 Transcriptome sequencing and assembly

Total RNA isolated from top internodes of control and GA_3 treated canes, 30DAS, was used as starting material. A quality check (total RNA QC analysis) was made for quantity and quality on Agilents 2100 BioAnalyser using RNA Nano Chip. C_T (RNA sample from top segment of control cane) was found to have a concentration of 48 ng/ul with rRNA ratio (25S/18S) of 2.0 and RIN of 8.3. G_T (RNA sample from top segment of GA_3 treated cane) displayed a concentration of 54 ng/ul with rRNA ratio (25S/18S) of 2.2 and RIN of 7.8. Since the RNA met the conventional quality standards, it could be used to carry out further analysis. Thus, two sequencing (cDNA) Illumina libraries were synthesized from top internodal samples of non-treated and GA_3 treated cane viz. C_T and G_T, respectively, for RNA-Seq study. The two libraries generated 16 GB data each from paired end reads. The two libraries produced raw reads with average read length of 100. A read count of 11465230 was recorded for C_T and 17254100 for G_T , with GC percentage of 49 and 50 each. The raw reads were subjected to base quality check using the fastQC tool. No raw data trimming was needed since all reads lay within the acceptable range (i.e. within the green region) and had a Phred score of ≥ 30 (Figure 26).

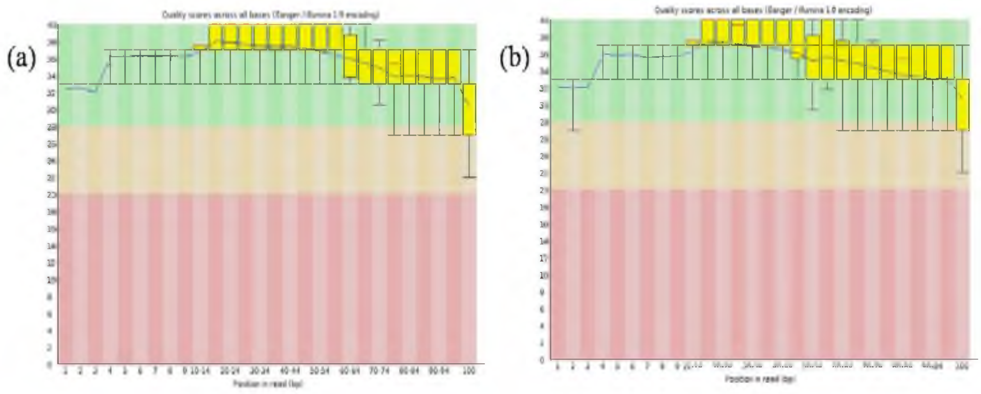


Figure 26: Quality values of all bases at each position in the 'base quality check' using fastQC for top internodal sample of (a) control cane (C_T) (b) GA_3 treated cane (G_T)

Since the sugarcane genome remains largely elusive till date, the Trinity pipeline (Grabherr *et al.*, 2011) was employed to assemble the reads into transcripts, *de novo*. The short reads from libraries, when subjected to assembling, yielded 165410 assembled transcripts, displaying an N50 length of 1003 bp. These were assembled into 120013 genes (Table 4). Based on read count, out of the 165410 assembled transcripts, 25053 transcripts were expressed exclusively in the top internodal sample of control cane (C_T) while 27031 were unique to the GA_3 treated sample (G_T) and the remaining 113326 were found to express in both control and GA_3 treated samples (Figure 27). Normalized gene expression levels were reported as RPKM values.

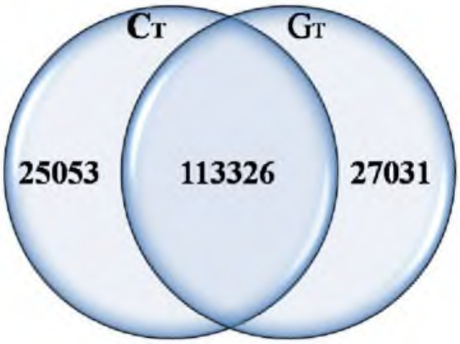


Figure 27: Venn diagram depicting the number of transcripts expressing exclusively in C_T or G_T and in both samples

Read counts represent the number of reads mapping to a transcript that has been assembled *de novo*. Transcript abundance or quantification denotes the number of reads that correspond to each kind of transcript and hence can be inferred as a measure of absolute expression (Wolf, 2013). Here, read count values per transcript ranged from 0.01 to as high as 1138709 in control and 2660327 in GA_3 treated sample, wherein most transcripts were upregulated in G_t. The differential response of samples to GA_3 treatment was further explored by generating heat map. The heat map represents the expression profiles of overlapping transcripts among top internodes of control and GA_3 treated cane (Figure 28). The 165410 transcripts were sorted on the basis of read count, such that the read count for a particular transcript be no less than 100 in one and a minimum of 1000 in the other sample. None of the exclusively expressing transcripts faired in this screening i.e. remarkably all sole expressing transcripts possessed very low read count (<100) and were thus negated. A large chunk of the transcripts were thus kept out leaving us with 11466 transcripts. 9756 transcripts exhibited substantial read count (>100) in both samples but failed to show a good differential expression between C_t and G_t, .and were thus excluded. Thus, a total of 1709 transcripts were found to exhibit considerable differential read count among the samples. Filtering on the basis of difference in read count, 558 transcripts were found to display a significant read count difference of twice or more among the samples. Out of these 558 transcripts, 513 were upregulated in the GA_3 treated sample while 45 were downregulated (Figure 29). Figure 30 depicts the workflow employed for identification and annotation of differentially expressed transcripts.

Table 4: *De novo* assembly statistics for C_T-G_T

Total genes	120013
Total transcript s/contigs	165410
Percent GC	49
N10	1912
N20	1626
N30	1377
N40	1198
N50	1003
Contigs >= 100 bp	165410
Contigs >= 500 bp	89066
Contigs >= 1000bp	39723
Contigs >= 10000bp	2
Contigs >= 1 Mbp	0
Median contig length	450
Average contig	609.8
Total assembled bases	100867018

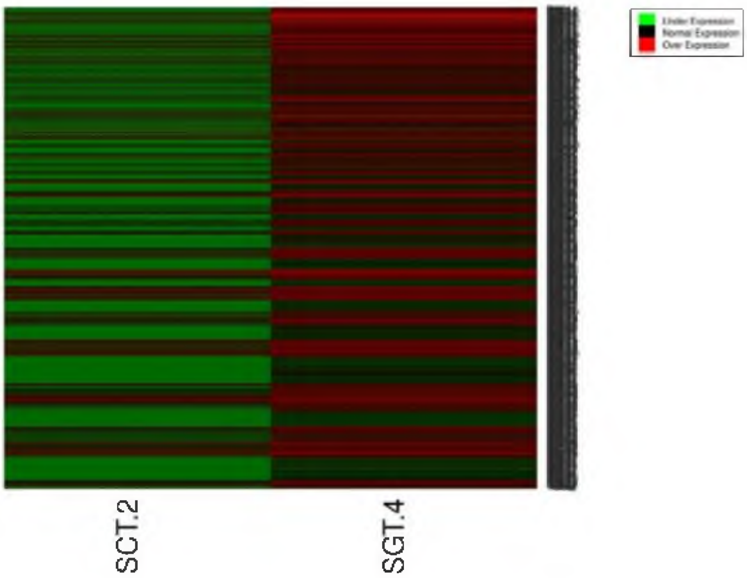


Figure 28A: Heat map representing expression profile of upregulated differentially expressed transcripts between C_T and G_T

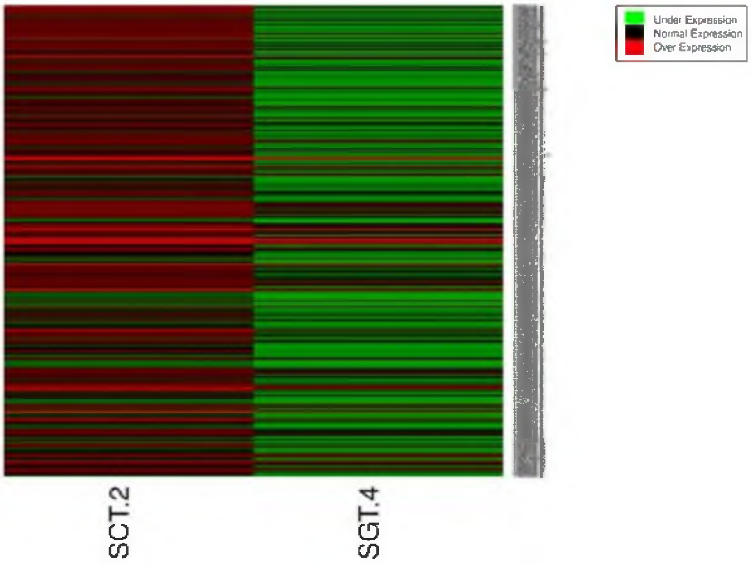


Figure 28B: Heat map representing expression profile of downregulated differentially expressed transcripts between C_T and G_T

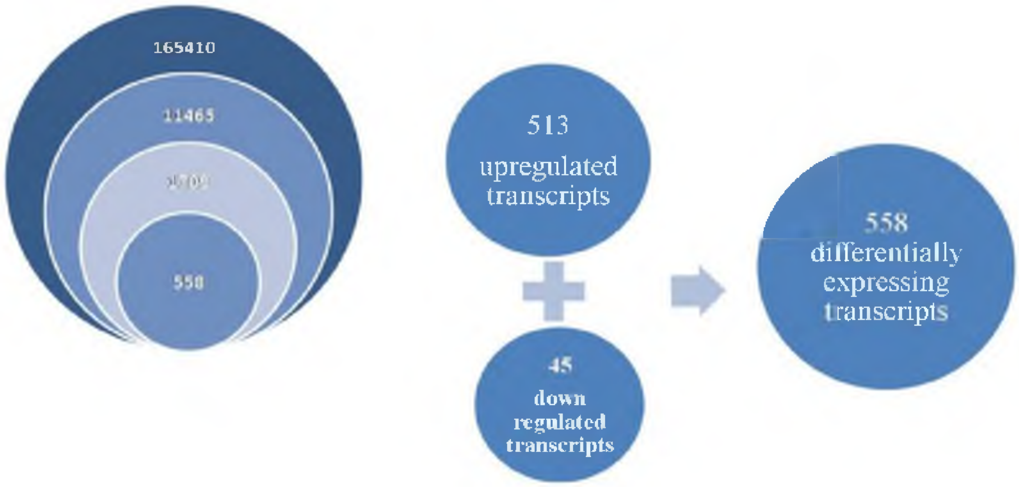


Figure 29: Screening for 558 differentially expressed transcripts between C_T and G_T

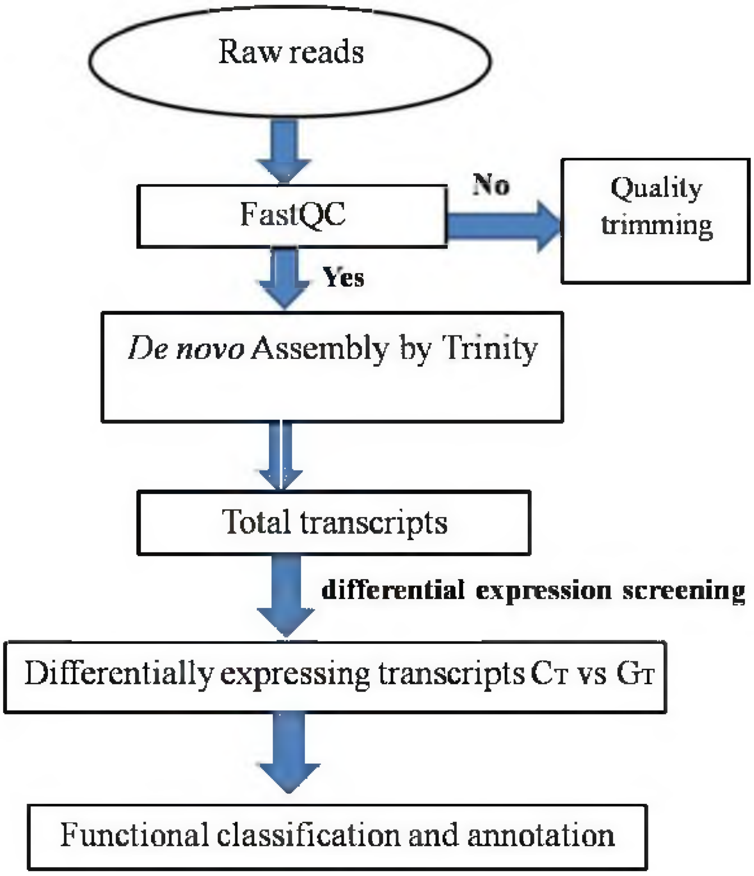


Figure 30: Workflow for identification and annotation of differentially expressed transcripts

4.1.4.2 Functional classification and annotation

Automated functional annotation aligned the total 165410 transcripts to various public databases. 72521 transcripts substantially matched to entries in the NCBI database and 53392 in the UniProt database. In all, 69273 transcripts were annotated by GO (Table 5).

Table 5: Annotation summary of total transcripts

Total transcripts	165410
Annotated by NCBI	72521
Annotated by Uniprot	53392
Annotated by KEGG	22007
Annotated GO biological process	17215
Annotated GO molecular process	32602
Annotated GO cellular process	19456

The thus identified 558 differentially expressed transcripts were annotated by utilizing Blast2GO (Conesa and Götz, 2008), which assigned a function to each of them based on homology and carried out their gene ontology analysis. Figure 31 depicts the length distribution of the transcript sequences wherein average sequence length was found to be 1119 bp. They were blasted using NCBI Blast (blastx) against the nr database, with E-value hit filter of 1.0E-6. Figure 32 shows chart depicting range of E-values for all top BLAST hits. As evident from the chart, most hits displayed an E-value of around 1.0E-180, demonstrating the accuracy and success of the alignment for the selected transcripts. Most query transcript sequences could retrieve a minimum of 20 blast hits (Figure 33), suggesting that the transcripts exhibited good homology with sequences in the reference database. 85.3% (i.e. 476 transcripts) of the 558 transcripts were successfully annotated due to significant homology with a known gene/protein. 14.2% (79 transcripts) were either labelled hypothetical or uncharacterized or had a putative or predicted function while 3 of the transcripts showed no significant hit and hence could not be assigned any function. Figure 34 depicts the accuracy of alignment in terms of percentage of sequence similarity displayed by transcripts to the reference sequence database. As illustrated in the chart, majority of the hits displayed a similarity-length/homology of >90% i.e. >90% identity between query (transcript) sequence and database hit. Thus the alignment can be said to have been successful in fishing out high homology matches for many transcripts. Among the annotated transcripts, maximum representation was under transcription factors followed by those that showed homology to various proteins related to sugar metabolism, plant hormone metabolism, cell division, respiration, protein degradation and ubiquitination and also some transporter proteins. Overall, maximum number of transcripts (more than 1400) were found to display homology with *Zea mays* and then *Sorghum bicolor* (Figure 35). However, the top hits of most query transcripts shared closest homology with *Sorghum bicolor*, followed by *Zea mays*, *Sertaria italica* and *Saccharum* hybrid cultivar R570 (Figure 36).

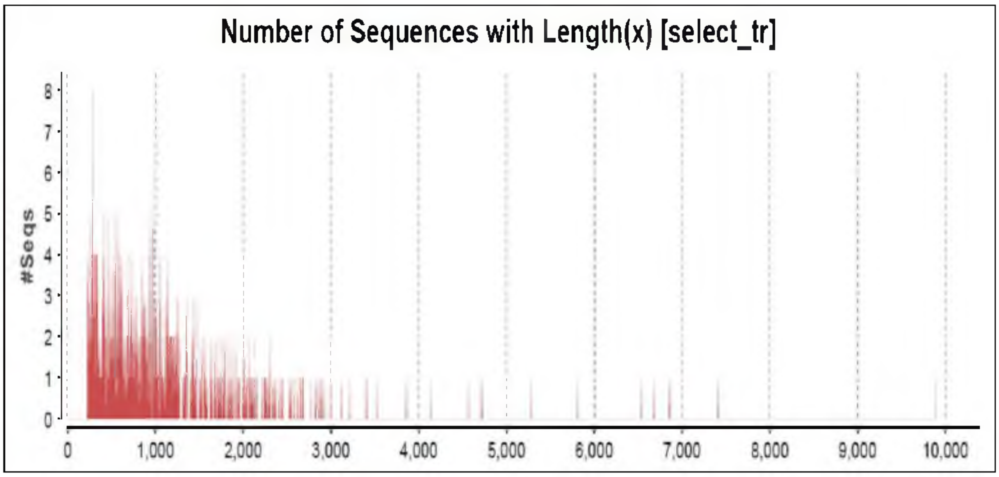


Figure 31: Chart depicting length distribution of sequences

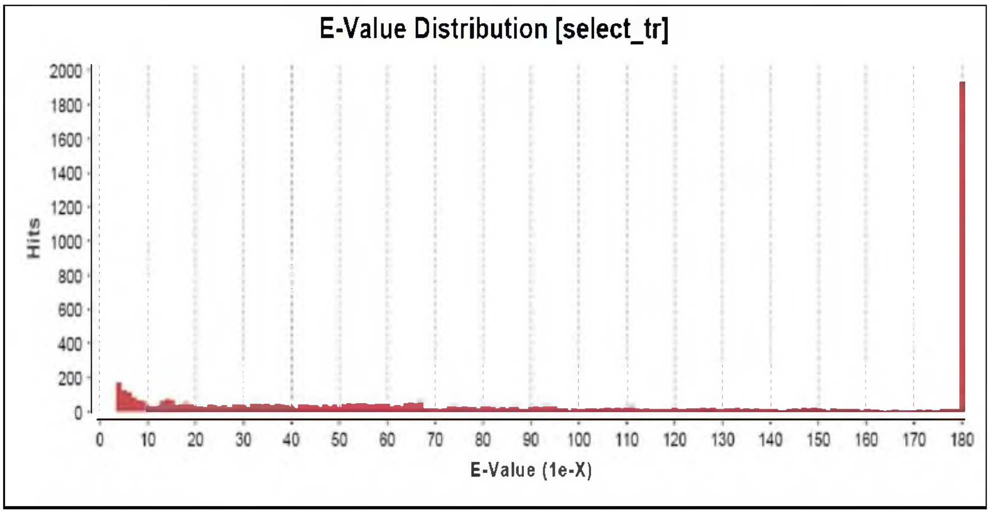


Figure 32: E-Value distribution chart depicting the distribution of E-values for all top BLAST hits

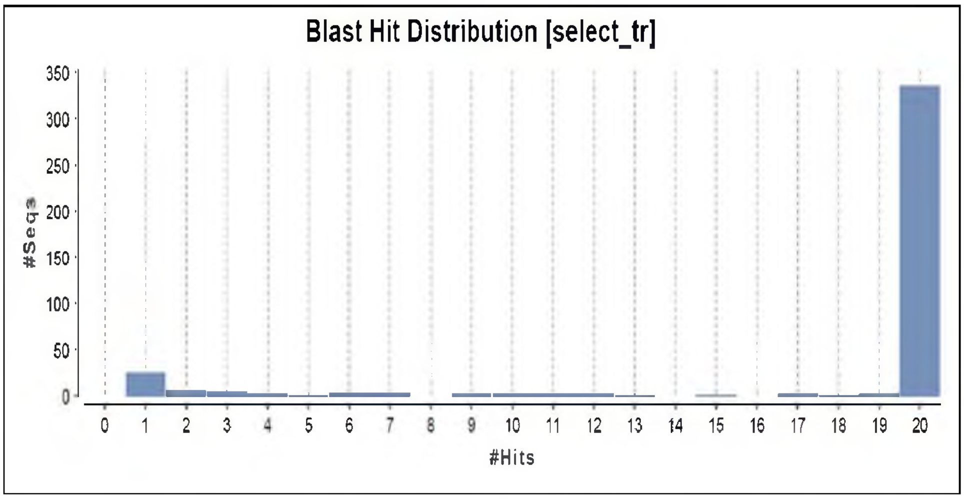


Figure 33: Blast hit distribution chart depicting the distribution of number of hits for the blasted sequences

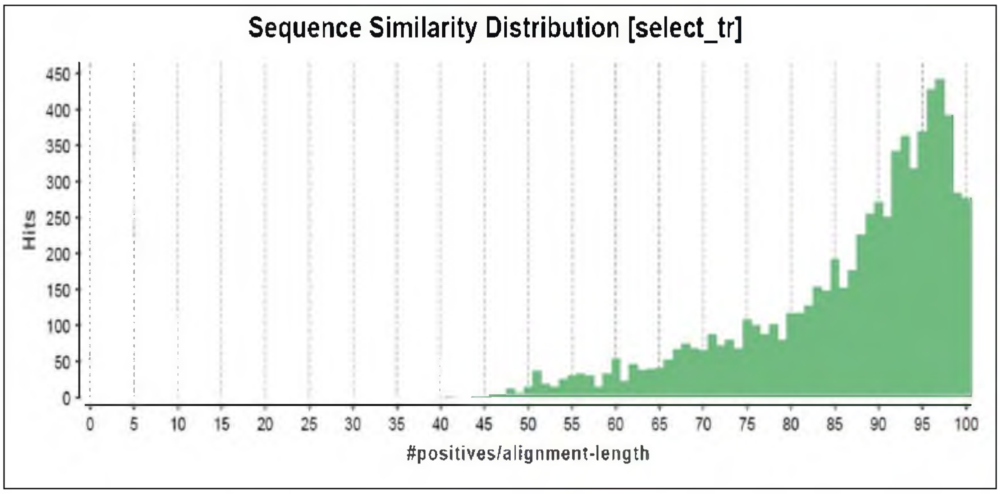


Figure 34: Sequence similarity distribution chart depicting the distribution of all calculated sequence similarities (in percentages)

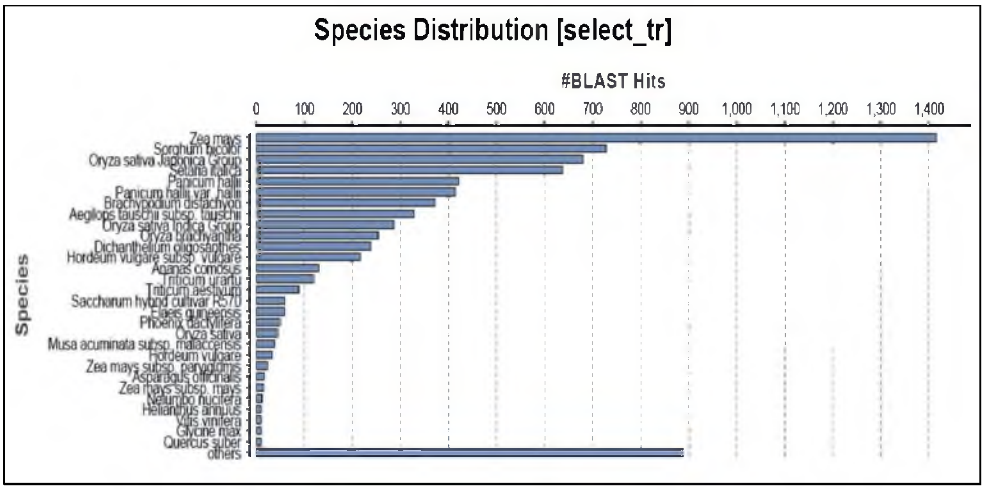


Figure 35: Species distribution chart displaying different species to which most Blast hits were aligned

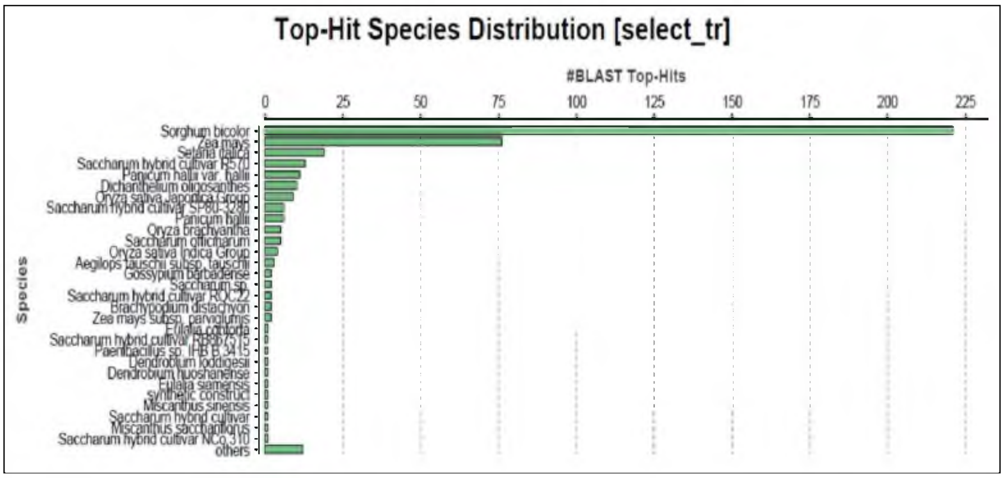


Figure 36: Top-hit species distribution chart displaying different species to which all top Blast hits were aligned

InterPro Scan (IPS) in BLAST2GO provides information about domain/motifs in a particular query sequence. Figure 37 shows the number of sequences that possessed a particular IPS domain wherein protein kinase domain, AAA$^+$ ATPase domain, ATPase AAA type core, GTP-binding transcription factor were highly represented. According to IPS repeats distribution chart (Figure 38) about 5 sequences had pentatricopeptide repeats while 3 had leucine rich repeats. One sequence had a twin-arginine translocation conserved site and one had a helix-turn-helix motif (Figure 39). IPS GO terms are fused with the already existing GO annotation of corresponding sequences, based on the BLAST results.

GO mapping was performed in order to retrieve the total GO terms linked with top hits obtained by the BLAST search. GO Mapping Distribution chart (Figure 40) represents the amount of Gene Ontology terms assigned to each sequence by GO Mapping. Distribution of the Evidence Codes associated to Blast hits i.e. the already obtained GO pool, as well as to functional terms obtained during mapping illustrates that maximum number were inferred from electronic annotation (IEA) while some belonged to the IBA (inferred from biological aspect of ancestor) category (Figure 41 and 42). As per statistics (Figure 43), maximum number of annotations (GO terms) were derived from the Uniprot database after Interpro.

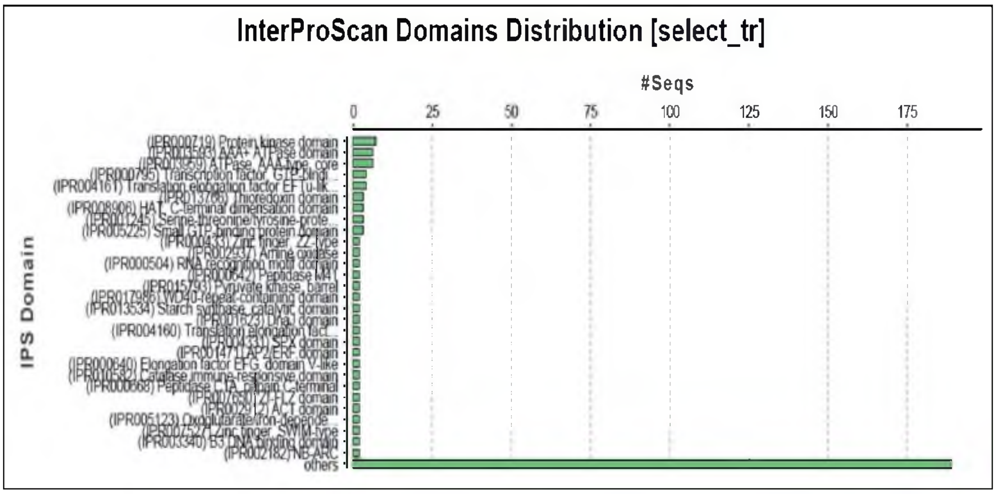


Figure 37: IPS domains distribution chart showing the number of sequences that belong to a particular IPS domain

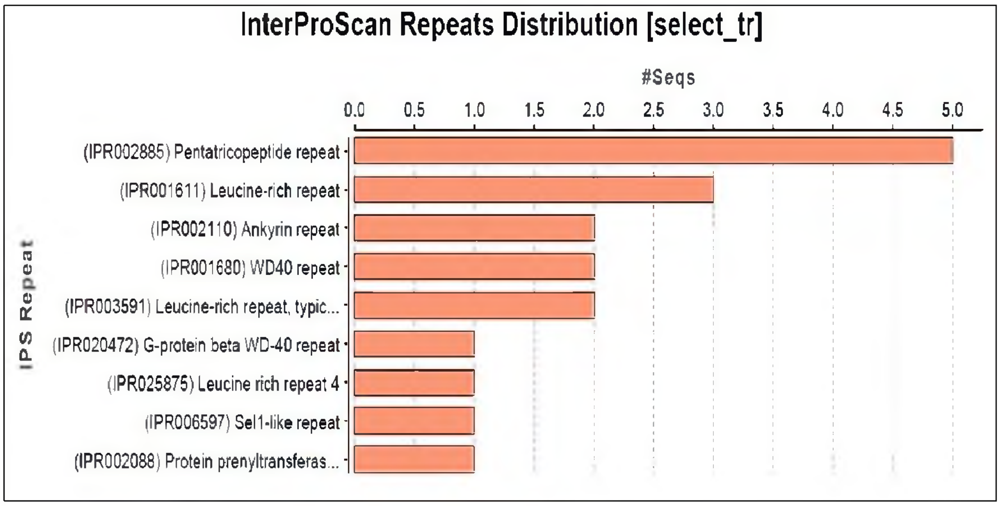


Figure 38: IPS repeats distribution chart depicting the number of sequences that belong to a particular IPS repeat

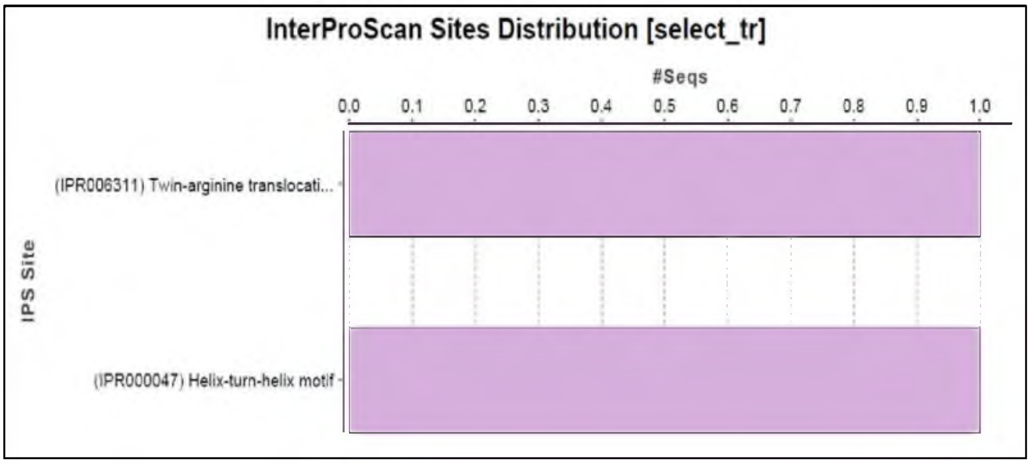


Figure 39: IPS sites distribution chart representing the number of sequences that belong to a particular IPS site

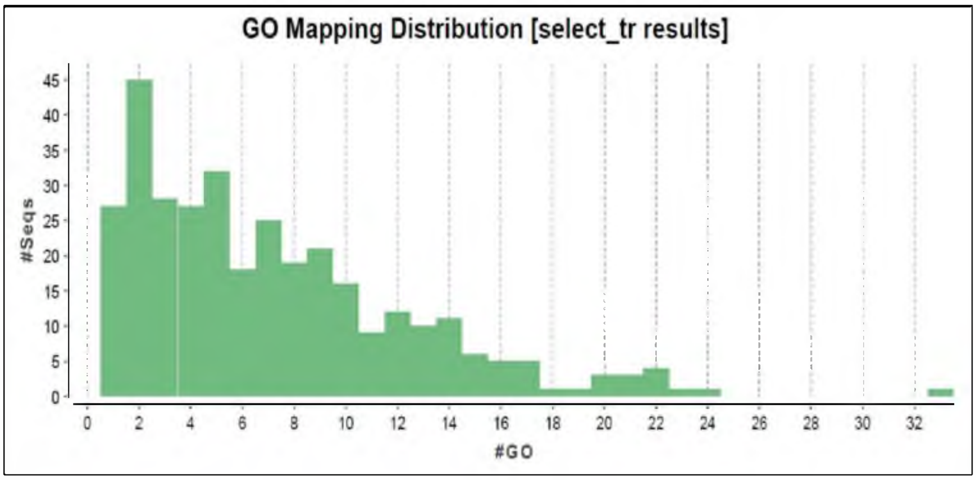


Figure 40: GO mapping distribution chart shows the distribution of GO terms assigned to each sequence by GO mapping

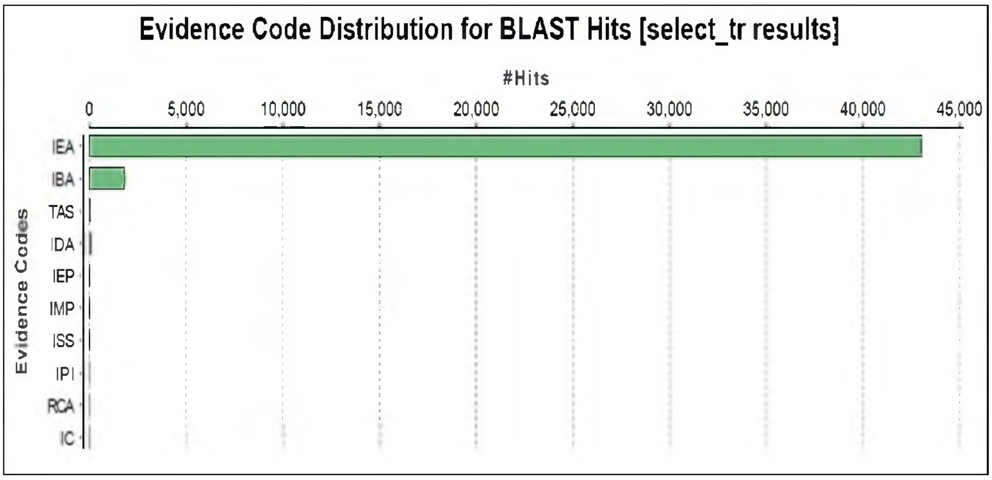


Figure 41: EC distribution chart denoting Evidence Codes associated to Blast hits

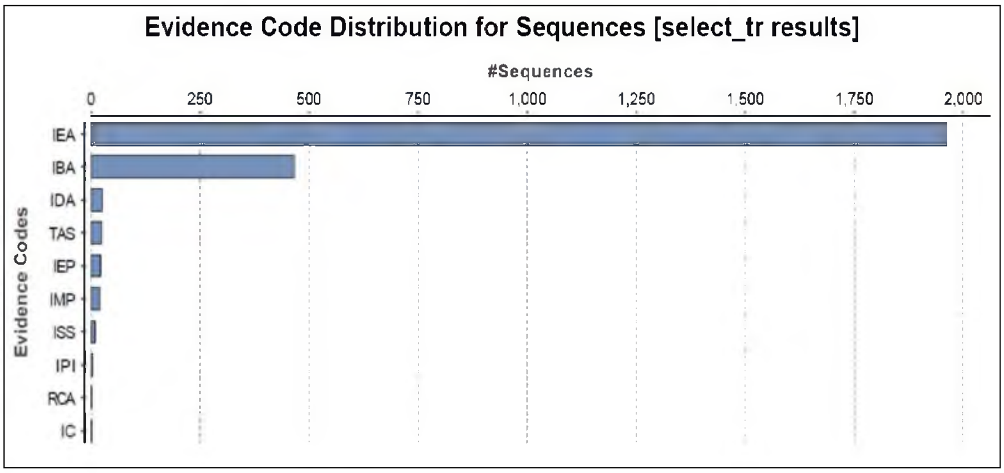


Figure 42: EC distribution chart denoting the distribution of Evidence Codes for the functional terms obtained by mapping

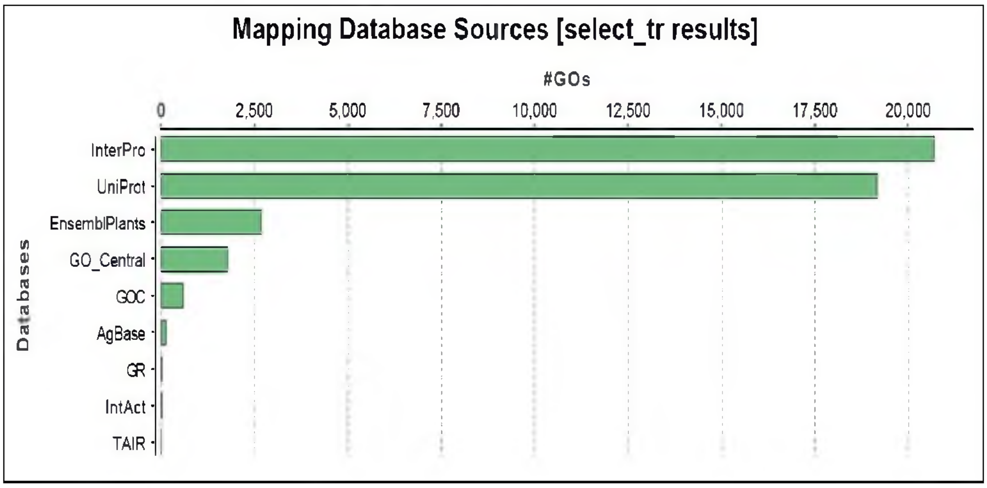


Figure 43: The chart gives the distribution of number of annotations (GO terms) retrieved from the different source databases

The 558 differentially expressed transcripts were then subjected to GO annotation, wherein GO terms were selected from the GO pool obtained by mapping step and were assigned to the query transcript sequences (Figure 44). GO annotation categorized the transcripts into different ontologies: biological process (BP), cellular component (CC) and molecular function (MF). The number/percentage of transcripts belonging to each ontology can be represented through WEGO plots where each plot depicts the proportion of genes belonging to different functional groups under a particular ontology. The 558 differentially expressed transcripts were observed under 46 biological process (BP) GO terms, wherein maximum number of trancripts mainly fitted under the heads of metabolic process (GO:0008152), cellular process (GO:0009987), organic substance metabolic process (GO: 0071704), nitrogen compound metabolic process (GO:0006807) (Figure 45). 25 GO terms expressed the functional distribution of differentially expressing transcripts under the cellular component (CC) category, wherein cell part (GO:0044464), cell (GO:0005623), membrane (GO:0016020), intracellular (GO:0005622), intracellular part (GO:0044424) were over represented than others (Figure 46). The differentially expressed transcripts exhibited significant expression under 27 GO terms of the molecular function (MF) category of which particularly high depiction was seen in functions like catalytic activity (GO:0003824), binding (GO:0005488), heterocyclic compound binding (GO:1901363), organic cyclic compound binding (GO:0097159), ion binding (GO:0043167) (Figure 47).

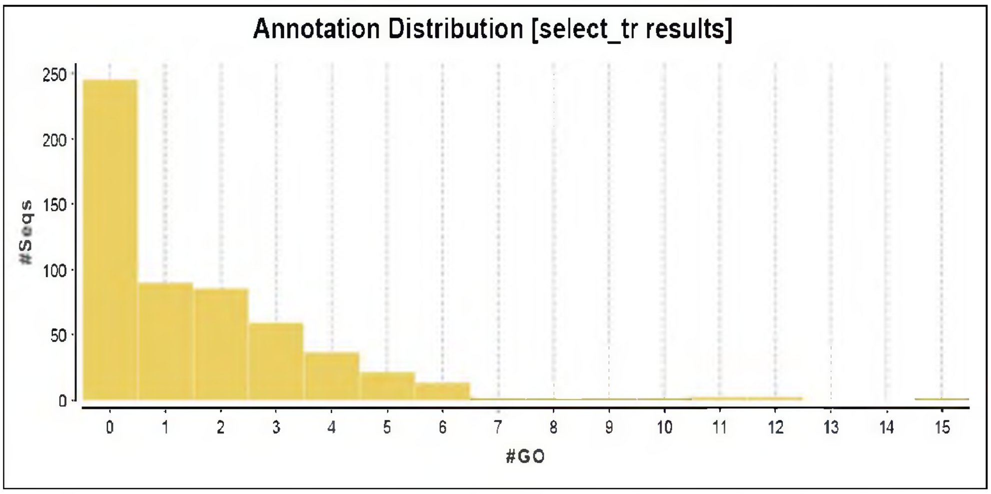


Figure 44: Annotation distribution chart shows the distribution of GO terms assigned to each sequence by GO annotation

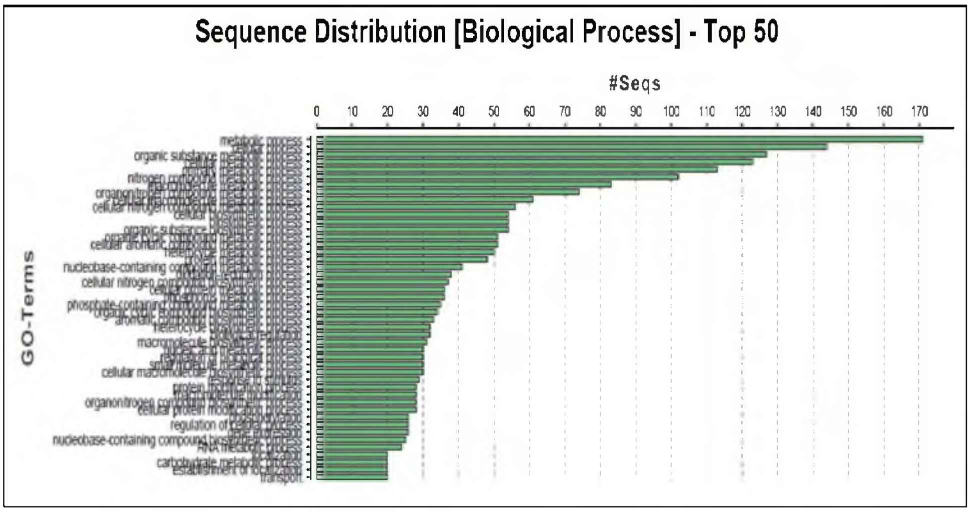


Figure 45: WEGO plot showing functional distribution of differentially expressing transcripts under the biological process (BP) category

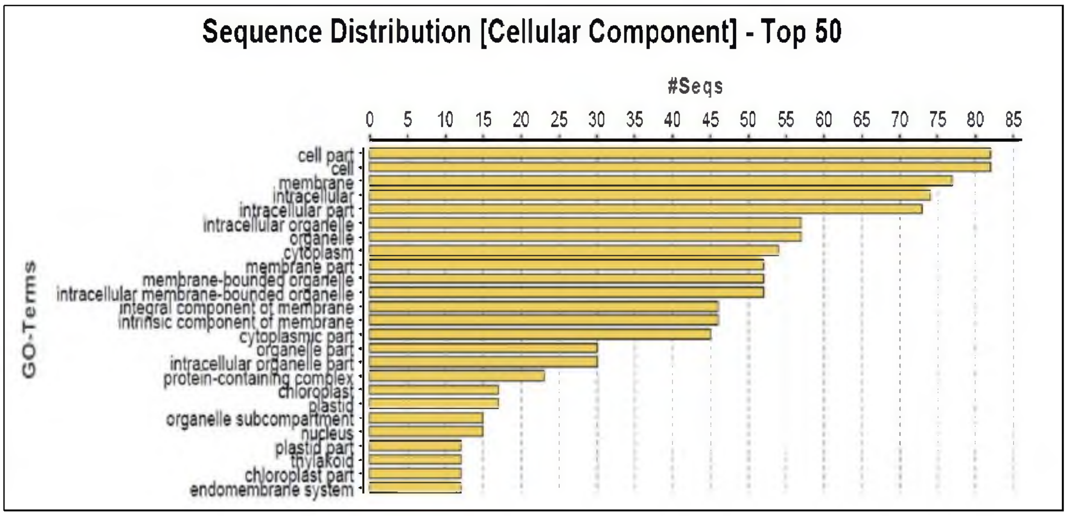


Figure 46: WEGO plot showing functional distribution of differentially expressing transcripts under the cellular component (CC) category

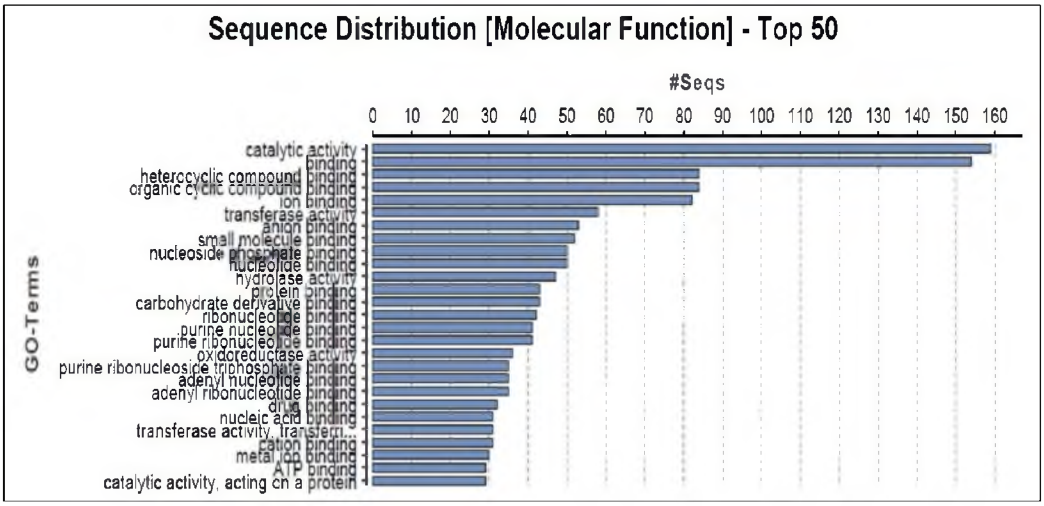


Figure 47: WEGO plot showing functional distribution of differentially expressing transcripts under the molecular function (MF) category

Enzyme annotation of the sequences with GO annotation was done by employing KEGG analysis (Figure 48). Also, KEGG (enrichment) analysis revealed that the 558 differentially expressed transcripts were associated with several metabolic pathways. Significant number of transcripts were found to be component of DNA replication, amino-acyl tRNA biosynthesis, plant hormone signal transduction, starch and sucrose metabolism, plant hormone signal transduction (Figure 49). A fair share of the differentially expressed transcripts being related to plant hormone signalling corroborates the effect of gibberellin intervention in the plant.

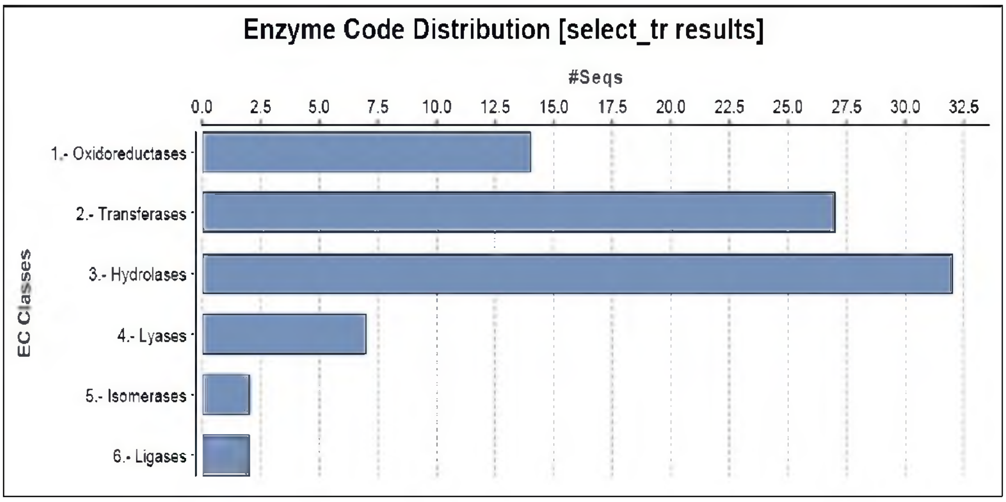


Figure 48: Chart depicting enzyme annotation of the sequences with GO annotation by KEGG

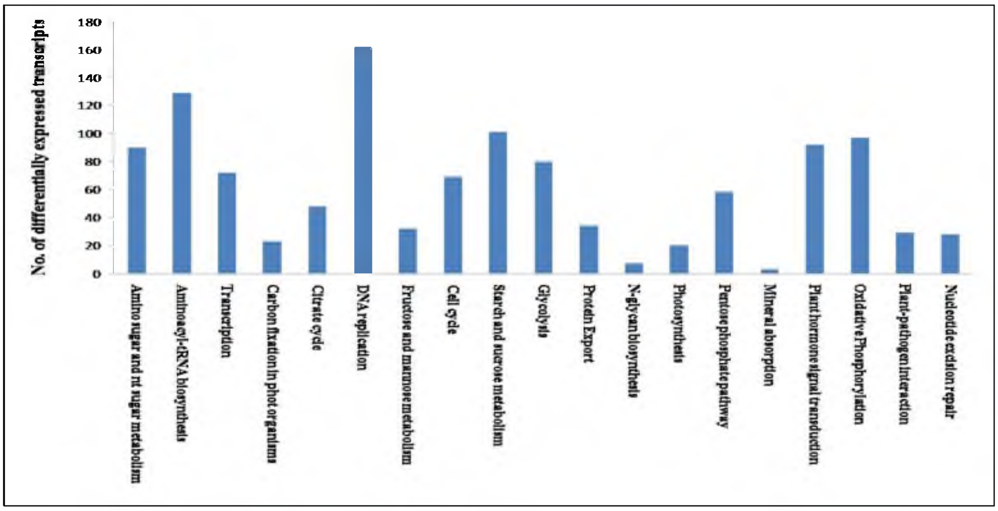


Figure 49: KEGG classification of differentially expressed transcripts

4.1.4.3 Real-time PCR validation

The 558 differentially expressed transcripts identified, were screened on the basis of function, % similarity (homology with gene/protein of sugarcane or other close homolog plants like Sorghum, maize, that share close ancestry) and E-value of BLAST2GO (Blastx) annotation. The sieving yielded 26 transcripts (Table 6) out of which 4 were transcription/ translation factors, 5 were proteins related to sugar metabolism and transportation, 4 were uncharacterized, 1 hypothetical and others with varied valid annotation. Real-time primers were designed against these 26 transcripts by employing the IDT primer designing tool (https://eu.idtdna.com) (Table 7). Out of the 26 primers synthesized against shortlisted transcripts, 19 primers showed amplification on being tested by PCR (Figure 50) but 4 of them gave faint or multiple bands and were thus excluded. Hence, 15 of these primers were used to validate the expression of differentially expressing transcripts between control and GA_3 treated top internodal samples obtained from RNA-seq data. Thus, the expression profile of the selected transcripts was analysed by employing the designed primers, using three replicates of qRT-PCR measurements, over different intervals after GA_3 treatment (viz. 30, 120, 180 DAS). However, two transcripts viz. TR55393|c2_g2_i10 homologous to *Zea mays* clone 394766 hypothetical protein mRNA and TR11991|c0_g1_i1 predicted homologous to *Sorghum bicolor* novel plant SNARE 12 mRNA, failed to show significant differential expression and hence were not relevant for further discussion. Real-time validation by employing such primers may also help in determining transcripts involved in promotion of sucrose accumulation and in molecular mechanisms underlying differential response to gibberellin treatment, thus finding novel transcripts therein.

Table 6: Transcript sequences selected for real-time primer designing for validation

S.No.	Transcript id	Annotation	E-value	Similarity mean
1	TR55854|c0_g1_i1	*Sorghum bicolor* probable sugar phosphate/ phosphate translocator At1g06470	0	95%
2	TR85315|c1_g3_i2	PREDICTED: *Sorghum bicolor* UTP--glucose-1-phosphate uridylyltransferase (LOC8077233), mRNA	0	95%
3	TR3728|c0_g1_i1	*Saccharum* hybrid cultivar GT28 sucrose transporter protein (ERD6) gene, complete cds	0	99%
4	TR12123|c0_g1_i1	*Sorghum bicolor* WAT1-related protein At3g30340	0	90%
5	TR80763|c2_g1_i6	*Sorghum bicolor* MYR1	0	86%

6	TR28230|c1_g1_i1	*Sorghum bicolor* V-type proton ATPase subunit G1	0	89%
7	TR70154|c2_g1_i1	PREDICTED: *Sorghum bicolor* U1 snRNP-associated protein usp106 (LOC8072437), transcript variant X1, mRNA	2.00E-39	99%
8	TR29189|c0_g1_i1	*Sorghum bicolor* uncharacterized LOC8066524 (LOC8066524), transcript variant X2, mRNA	8.00E-58	95%
9	TR4797|c0_g1_i1	PREDICTED: *Sorghum bicolor* protein GIGANTEA (LOC110433900), transcript variant X2, mRNA	0	98%
10	TR70550|c0_g2_i1	*Sorghum bicolor* transcription repressor OFP8	2.00E-128	94%
11	TR43564|c0_g1_i1	*Saccharum* hybrid cultivar putative C_4 phosphoenolpyruvate carboxylase mRNA, complete cds	0	99%
12	TR57278|c0_g1_i1	PREDICTED: *Sorghum bicolor* uncharacterized LOC8080981 (LOC8080981), transcript variant X2, mRNA	8.00E-18	98%
13	TR20346|c4_g1_i2	*Saccharum officinarum* elongation factor mRNA, complete cds	0	97%
14	TR55393|c2_g2_i10	*Zea mays* clone 394766 hypothetical protein mRNA, complete cds	2.00E-54	93%
15	TR70064|c0_g1_i1	PREDICTED:*Sorghum bicolor* scarecrow-like protein 34 (LOC8068115), mRNA	2.00E-74	90%
16	TR11991|c0_g1_i1	PREDICTED: *Sorghum bicolor* novel plant SNARE 12 (LOC8063535), mRNA	0	94%
17	TR44279|c4_g3_i1	PREDICTED: *Sorghum bicolor* cytochrome b-c1 complex subunit 8 (LOC8060490), mRNA	6.00E-69	97%
18	TR75913|c0_g1_i1	PREDICTED: *Sorghum bicolor* uncharacterized protein ycf39 (LOC8058013), mRNA	0	97%
19	TR28023|c0_g1_i1	PREDICTED: *Sorghum bicolor* probable glycosyltransferase At5g03795 (LOC8072350), transcript variant X3, mRNA	0	97%
20	TR84318|c0_g1_i1	*Saccharum* hybrid cultivar H65-7052 mRNA for fructose-1,6-bisphosphatase	0	99%
21	TR42380|c0_g1_i1	*Sorghum bicolor* phosphoglycerate kinase, cytosolic	0	96%
22	TR30583|c0_g1_i1	PREDICTED: *Sorghum bicolor* uncharacterized LOC8060147 (LOC8060147), mRNA	4.00E-21	98%
23	TR83507|c0_g1_i3	PREDICTED: *Sorghum bicolor* uncharacterized LOC8069918 (LOC8069918), mRNA	5.00E-20	100%
24	TR74986|c0_g1_i1	PREDICTED: *Sorghum bicolor* uncharacterized LOC8065126 (LOC8065126), mRNA	2.42E-15	96%
25	TR80785|c2_g1_i1	*Saccharum* hybrid cultivar ROC22 translation initiation factor5A (elF5A) mRNA, complete cds	0	98%
26	TR22147|c1_g1_i6	PREDICTED: *Sorghum bicolor* ER membrane protein complex subunit 6 (LOC8063068), mRNA	2.00E-29	95%

Table 7: List of designed real-time primers for validation

S.No.	Transcript id	Primer sequence (5'→3')	T_m(°C)	GC%	Product length	Primer fate
1	TR55854|c0_g1_i1	GTAACCAAAGGATACCCCACG TACACCGAAACAGTCTACAGC	62.3 61.7	52.4 47.6	86	Showed amplification /Taken
2	TR85315|c1_g3_i2	TTAATGTTCCCCGCTCAAGG AGGTCCAAGCTCAATCGAAG	62.2 61.9	50 50	149	Taken
3	TR3728|c0_g1_i1	ATGAAATGCCTAGAGATACCGC CCCCAACCCTATTCCGAATATC	62.1 62	45.5 50	79	Taken
4	TR12123|c0_g1_i1	AGCACAACTGAAGACGGAC GAGATCTTATTGCCGGGACTG	61.9 62.1	52.6 52.4	76	Taken
5	TR80763|c2_g1_i6	TGCTTCTGATCACTACCTGTTG CAGAGCGAGAGTACATTCCTG	62.1 61.6	45.5 52.4	114	Taken
6	TR28230|c1_g1_i1	TGTTGCTGTCTCTTCCTCAAG TTCCAGAGAAAGGTTGCAGAG	62 62.1	47.6 47.6	75	Taken
7	TR70154|c2_g1_i1	AGTAGAAGTAAATGTACCCAGATCG TTATGATTCCAGAAGCCACC	61.8 62.2	40 47.6	86	Double band
8	TR29189|c0_g1_i1	ACAATAGGATGGAAGCTGGC AGAAGCCCATTAAGAGACACAG	62.1 61.9	50 45.5	87	Taken
9	TR4797|c0_g1_i1	GTAGCACGAAGTAGAAGGTCAG TGGATTGCTAGAGACGATGAAC	61.8 61.9	50 45.5	149	No amplification
10	TR70550|c0_g2_i1	TGGATTGCTAGAGACGATGAAC GTCACCGTCACCTGCTC	62.7 61.6	61.1 64.7	131	Faint band
11	TR43564|c0_g1_i1	TTCTGTTCTTGGATGGGTGG AGCTCAAACATCAGGTCTTCG	61.9 62.3	50 47.6	137	No amplification
12	TR57278|c0_g1_i1	AATAGATAATGGACACAGCCGAG TGATCTGCAAGGCTCTGATG	62.1 62	43.5 50	146	Double band
13	TR20346|c4_g1_i2	ATCTGAAGCGTGGGTATGTG GTGTGAGGTGTGGCAGTC	61.9 62.2	50 61.1	146	Double band
14	TR55393|c2_g2_i10	TGGCCTAGCACATACACAAC AGGGATAGAGGCAGAGGAAG	62.1 62.1	50 55	141	Amplified but not taken

15	TR70064|c0_g1_i1	AGGACTGATTTCGGAATGCC TGAGGAACATTGGTGAGTGG	62.3 61.8	50 50	82	Taken
16	TR11991|c0_g1_i1	CCGCCCATTCTTTCCCTATTG ACAGACAACCAGAAAGCCAG	63 62.3	52.4 50	103	Amplified but not taken
17	TR44279|c4_g3_i1	GCGAGGGATTTCAAACCAAC TGAACTCCGATGTATATTGAATTACTTG	61.7 62	50 42.1	109	Taken
18	TR75913|c0_g1_i1	CAAGCCAGAGACCATACCAG GAATTCCCATAGCCTTTGCAC	61.8 61.6	55 47.6	123	Taken
19	TR28023|c0_g1_i1	ACCCTGCTAAACCAAACTGAG GAGTCGATACATAACCCTGCAC	62.4 62.2	47.6 50	118	Taken
20	TR84318|c0_g1_i1	CGTAGCTGCCGAGGAATATC AGCTCCGTGTCCTTTATGAAG	61.8 61.9	55 47.6	131	Taken
21	TR42380|c0_g1_i1	GCCATCACAACTTCAACTCC TCACCCCAAAGTACAGCTTG	61.1 62.1	50 50	73	No amplification
22	TR30583|c0_g1_i1	TCTGACATTAGTGAGTTGCAGC TGAATTCGTGTCCTTGGCC	62.6 62.4	45.5 52.6	111	No amplification
23	TR83507|c0_g1_i3	ATGGGAGAAAGATGGTTGGG CTGTTTATGCTGGTGTTTCTTCT	61.8 61.5	50 39.1	149	No amplification
24	TR74986|c0_g1_i1	CTGGAGCTGGCATTGATAATC CTCGTCCTCTTCTTGGTGTTG	60.9 62.3	47.6 52.4	109	No amplification
25	TR80785|c2_g1_i1	AAGGATTTGCTACCGAGACG CAATCCCTGCACTTGTGTTTG	61.8 62.2	50 47.6	78	Taken
26	TR22147|c1_g1_i6	TTGATCTCTGGACGCACATAAG GACTGTCAACTCGTGTAACCC	62.1 62.3	45.5 52.4	137	No amplification

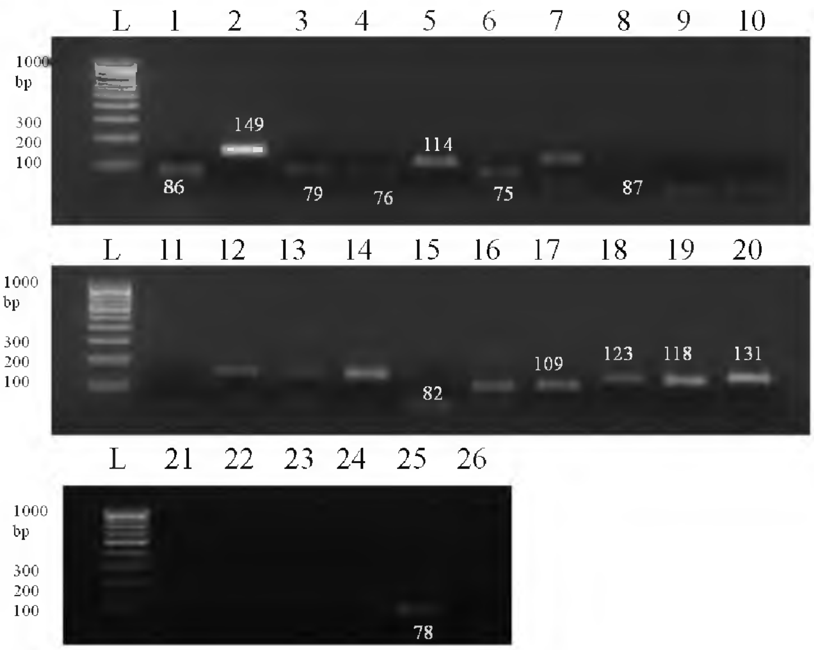


Figure 50: Checking of designed primers for amplification
L-100bp ladder

In this screening, we identified a transcript showing 95% homology with a probable sugar phosphate/phosphate translocator At1g06470 of *Sorghum bicolor.* This sugar translocator (STr) has been found to be involved in carbohydrate transport, more precisely in transmembrane transport of UDP-glucose. Since UDP-glucose is the immediate breakdown product of sucrose viz. by SuSy, the presence of this STr can easily be reasoned in the sucrose transport in sugarcane. Real-time analysis with the corresponding primer showed significant differential expression between control and GA_3 treated samples over time. At early stage of maturation (30 DAS), the STr exhibited upregulated expression in GA_3 treated sample and downregulated in control (Figure 51 (a)). This could perhaps be due to more prevalent sugar transport in the GA_3 treated sample in answer to the high sucrose demand. Thus, the high expression of such translocator at early stage perhaps proves an advantage for higher sucrose content recorded later in GA_3 treated cane. 120 DAS, the STr expression heightened in both control and GA_3 treated sample (higher) depicting the ongoing vigorous sugar transport in both. However, quite aptly, at 180 DAS there was a dip in Str expression in both samples probably due to the receding sugar transport due to sink saturation. Overall, the differential gene expression observed validate the role of this gene product as a sugar translocator and suggest that it perhaps plays a vital role in sugar transport even in response to GA_3, in sugarcane.

UTP-glucose-1-phosphate uridylyltransferase converts glucose 1-phosphate to UDP-glucose. UDP-glucose is one of the chief substrates for SPS activity and hence it is essential for the synthesis of sucrose; with additional role in formation of starch, cell wall and callose deposition (https://www.uniprot.org). Thus, a transcript showing 95% homology with a *Sorghum bicolor* UTP-glucose-1-phosphate uridylyltransferase could be a relevant pick for our analysis. Here, higher transcript expression was observed in GA_3 treated sample, at 30 DAS (Figure 51 (b)), perhaps indicating the prevalence of this enzyme for converting the large amount of glucose in GA_3 treated sinks into UDP-glucose, to be fed into the SPS pathway. Thus, the temporal expression of this enzyme too warrants the heightened expression of SPS and greater synthesis of sucrose in GA_3 treated sample. Also, correlating with the increased expression of SuSy in this phase, it too may be utilizing part of the UDP-glucose to synthesize sucrose. However, 60 DAS, analogous to SPS behaviour, the enzyme expression dropped in GA_3 treated sample as compared to control, perhaps due to relative sucrose sufficiency in the GA_3 treated one. SuSy being available as another alternative for generation of UDP-glucose may be reasoned to compensate the decline.

A transcript displayed as high as 99% homology with *Saccharum* hybrid cultivar GT28 sucrose transporter protein (ERD6) gene, (complete cds). Even though it showed high similarity to sugarcane protein itself, the transcript exhibited a differential yet downregulated expression (compared to September control) throughout the maturation phase (Figure 51(c)). On the contrary, the RNA seq results had shown an upregulation in GA_3 treated samples. It can be predicted that this sucrose transporter perhaps functioned well only very early in maturation phase viz. September, also affirmed by the very faint band observed in the gel. However, its less downregulation in GA_3 treated sample in the early maturation phase, can be said to point to better transportation of translocated sucrose, compared to that in control.

A transcript showing 90% homology with WAT1-related protein of *Sorghum bicolour* was reported to express more in GA_3 treated sample, according to RNA-seq analysis data. When quantified molecularly, the results were in congruence with the RNA seq reults. WAT-1 is considered to be a transmembrane transport facilitator, and has particularly been reported for vacuolar auxin transport (https://www.uniprot.org). It can thus be predicted to facilitate transport of a plant hormone like GA_3 too, thus justifying its higher expression in GA_3 treated sample at 30 DAS (Figure 51 (d)). Quite expectedly, its expression went down with passing time, perhaps due to waning effect of GA_3 and in the absence of any fresh GA_3 spray done.

Another transcript with 95% homology to *Sorghum bicolour* MYR1 displayed higher expression in GA_3 treated sample as compared to control. MYR1 is known to be a myb-related protein and is a member of the Myb transcription factors, which contain the conserved MYB DNA-binding domain. In *Arabidopsis*, over-expression of this transcription factor has been reported to produce GA-deficiency like symptoms which were recovered by application of gibberellic acid (GA_3) (Zhao *et al.*, 2011). It perhaps suppresses reactions stimulated in respone to gibberellic acid and alters levels of bioactive GA. Thus, MYR1 can be reasoned to be upregulated (Figure 51 (e)) in order to repress any responses to the exogenous GA_3 spray in the plant and probably also affect the biosynthesis of endogenous GA.

V-ATPases (vacuolar-type H+ ATPase) operate to pump protons across intracellular and plasma membranes of many kinds of cells, by utilizing energy generated from ATP hydrolysis, thus creating an acidic environment in many intracellular organelles and creating an electrochemical H+-gradient which serves as driving force for transport of various ions and metabolites. A transcript with 89% homology to *Sorghum bicolor* V-type proton ATPase, subunit G1, showed higher expression in GA_3 treated sample as compared to control, perhaps denoting higher rate of transport of metabolites under the effect of GA_3. Earlier, Cooley *et al.* (1999) too have reported increased amounts of subunit A and B proteins and high transcript level of subunit C in gibberellin-deficient tomato mutants, in response to exogenous application of gibberellins. Precisely, H^+-ATPases are said to be located alongside sucrose symporters to facilitate sucrose transport and sucrose accumulation, by way of preserving a proton motive force (Rae *et al.*, 2009; Ludewig and Flügge, 2013). Here too, V-ATPase expression was inferred to be escalated initially by GA_3 treatment (Figure 51(f)) with the expression gradually levelling with progressing maturation and at 180 DAS, GA_3 treated samples exhibited a downregulated expression. Since the expression pattern of V-ATPase transcript was in congruence with that seen for SPS (in earlier analysis), it perhaps points to its role in facilitating sucrose hoarding in maturing culm.

Also, V-ATPase action is believed to be vital to plant's response to stresses viz. water stress where maintenance of ion and metabolite concentrations in the vacuole is required. As per RNA-seq data, three drought stress related transcripts were also found to be upregulated in our GA_3 treated sample (though excluded from primer synthesis). This can be extrapolated to propose that exogenous GA_3 application possibly arouses osmotic stress in the plant, which in turn promotes greater sucrose accumulation in the long run.

Scarecrow (SCR) is a transcription factor of the GRAS family which is known to influence plant development. Based on the presence of VHIID domain and high sequence homology to the carboxyl terminus in SCR (Pysh *et al.*, 1999; Bolle, 2004), some gene members of the GRAS family with yet unknown function, have been called SCLs (SCR-LIKE). Scarecrow-like 3 (SCL3) is a GRAS transcription regulator that functions as a positive regulator of gibberellin (GA) signalling. SCL3 and transcriptional factor DELLA antagonize work antagonistically to control both downstream GA responses and upstream GA biosynthetic genes. Both coregulators, DELLA and SCL3 use intermediate domain proteins (IDDs) as transcriptional scaffolds for DNA binding (Hirsch and Oldroyd, 2009). Since our transcript was 90% homologous to scarecrow-like protein 34 (an allele of SCL3), (mRNA) of *Sorghum bicolor*, it can be predicted to exhibit a similar behaviour. As anticipated, the GA_3 treated sample exhibited a much higher transcript expression (compared to control), in response to the GA_3 spray. Quite likely, the expression was gradually lost with fading effect of GA_3 (Figure 51 (h)).

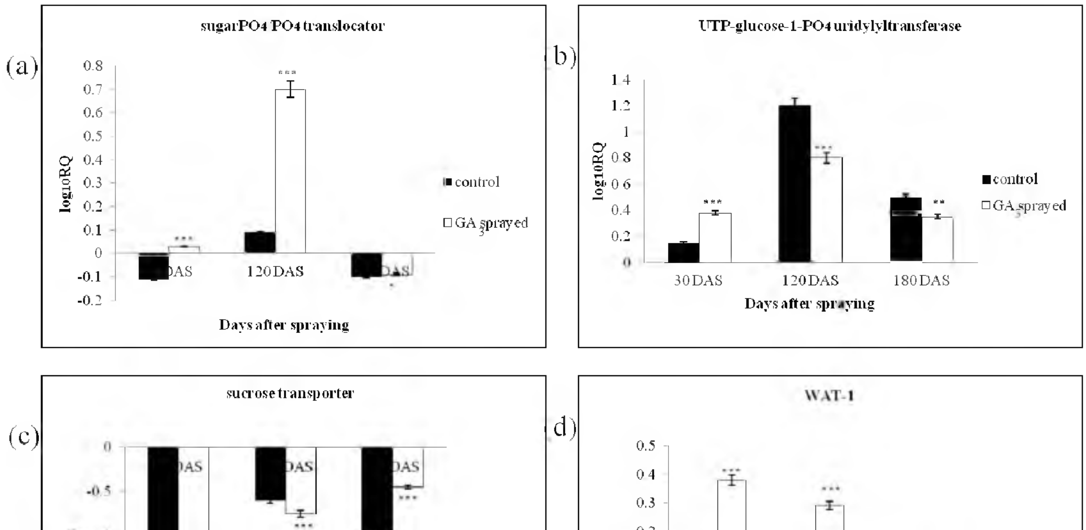
Yet another transcript displayed 97% homology with *Sorghum bicolor* cytochrome b-c1 complex subunit 8. This subunit is part of the mitochondrial respiratory chain and along with cytochrome b, binds to ubiquinone (https://www.uniprot.org). Higher transcript expression in GA_3 treated sample points to heightened respiration which can perhaps be traced back to higher metabolic activity in GA_3 treated samples. Quite logically, the transcript expression declined over time (Figure 51 (i)) justifying the drop in metabolic activity with progressing maturation. This is also supported by the heightened glucose levels observed biochemically which perhaps serve as a ready substrate for respiration.

In general, glycosyltransferases (GTFs) are known to aid formation of glycosidic linkages by facilitating the transfer of saccharides from an activated nucleotide sugar to a nucleophilic glycosyl acceptor molecule producing a carbohydrate, glycoside, oligosaccharide, or a polysaccharide (https://en.m.wikipedia.org). A transcript was predicted as 97% homologous to mRNA of a probable glycosyltransferase of *Sorghum bicolor.* Transcript expression increased in the maturing internodal samples of both control and GA_3 sprayed canes, over time (Figure 51 (k)). The expression was stronger in GA_3 treated sample pointing to greater carbohydrate formation.

Fructose-1,6-bisphosphatase (FBPase) facilitates sucrose formation through the irreversible conversion of fructose-1,6-bisphosphate to fructose-6-phosphate and inorganic phosphate. Photosynthetic tissues of plants are said to have two isozymes, one cytosolic and the other chloroplastic. In non-photosynthetic tissues, it catalyses the same reaction as part of gluconeogenesis and thus can be said to furnish fructose-6-phosphate. This product can perhaps be extrapolated to partake in resynthesis of sucrose by SPS in the culm's storage tissues. A screened transcript with 99% homology to a sequence of cytosolic fructose-1,6-bisphosphatase for *Saccharum* hybrid cultivar H65-7052 mRNA, displayed differential expression among control and GA_3 treated samples over time. FBPase was evidently less expressed initially perhaps because more and more glucose was being metabolized and utilized in various metabolic processes during the sink building (growth) phase. 30 DAS, both control and GA_3 treated samples exhibited increased FBPase expression (Figure 51(l)) probably because gluconeogenesis comes into play. Since glucose supply exceeds sink demand, part of pyruvate generated from glucose metabolism is possibly channelized into gluconeogenesis wherein fructose-6-phosphate synthesised may in turn be directed to sucrose resynthesis by SPS. As maturation progressed, FBPase expression declined perhaps due to end product repression caused by sucrose accumulation. However, this could be one justification and does not exclude the possibility of some other possible route for generation of fructose 1,6-bisphosphate.

The greater upregulation of a transcript in GA_3 treated sample, showing 98% homology to translation initiation factor5A (eIF5A) mRNA (complete cds) of *Saccharum* hybrid cultivar ROC22, warrants its role in regulation of protein synthesis in response to GA_3 application. eIF-5A is stated to work as a bimodular protein, capable of binding to both RNA and proteins, though its specific function in protein biosynthesis is yet to be deciphered (Feng *et al.*, 2007). However, it has been reported to affect plant growth and development in *Arabidopsis*, by altering cell processes. The declining expression of elF5A in GA_3 sprayed sample with the waning effect of GA_3 during the maturation phase, further establishes the positive association between them (Figure 51 (m)).

A transcript exhibited 95% homology to an uncharacterized LOC8066524 transcript variant X2 (mRNA) of *Sorghum bicolor*. The transcript displayed marked downregulated expression, in both control and GA_3 treated samples, more so in the GA_3 treated one. Though lesser relative to September control, the transcript expression increased in the GA_3 treated sample and decreased in the control sample, with progressing maturation (Figure 51 (g)). This differential expression pattern corroborates a possible role of this transcript in influencing sucrose accumulation. Another transcript showed 97% homology with a PREDICTED: *Sorghum bicolor* uncharacterized protein ycf39 (LOC8058013) mRNA. Upregulated differential transcript expression was observed in the samples, higher in the GA_3 treated one (Figure 51 (j)). Also, the expression level increased with progressing maturation. Hence, the transcript can be deduced to respond to GA_3 treatment, though it would be difficult to assign it a definite function without detailed functional analysis.



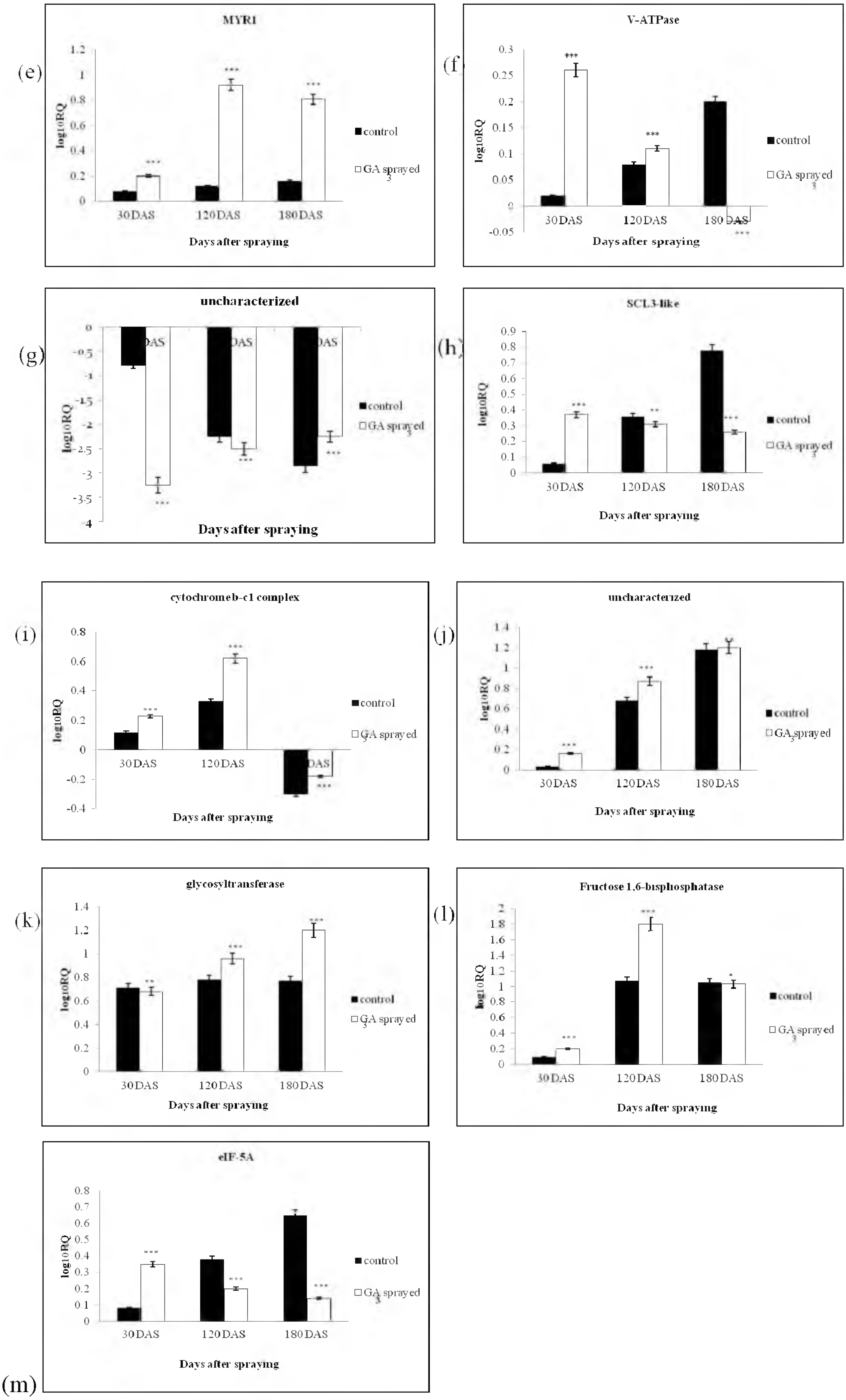
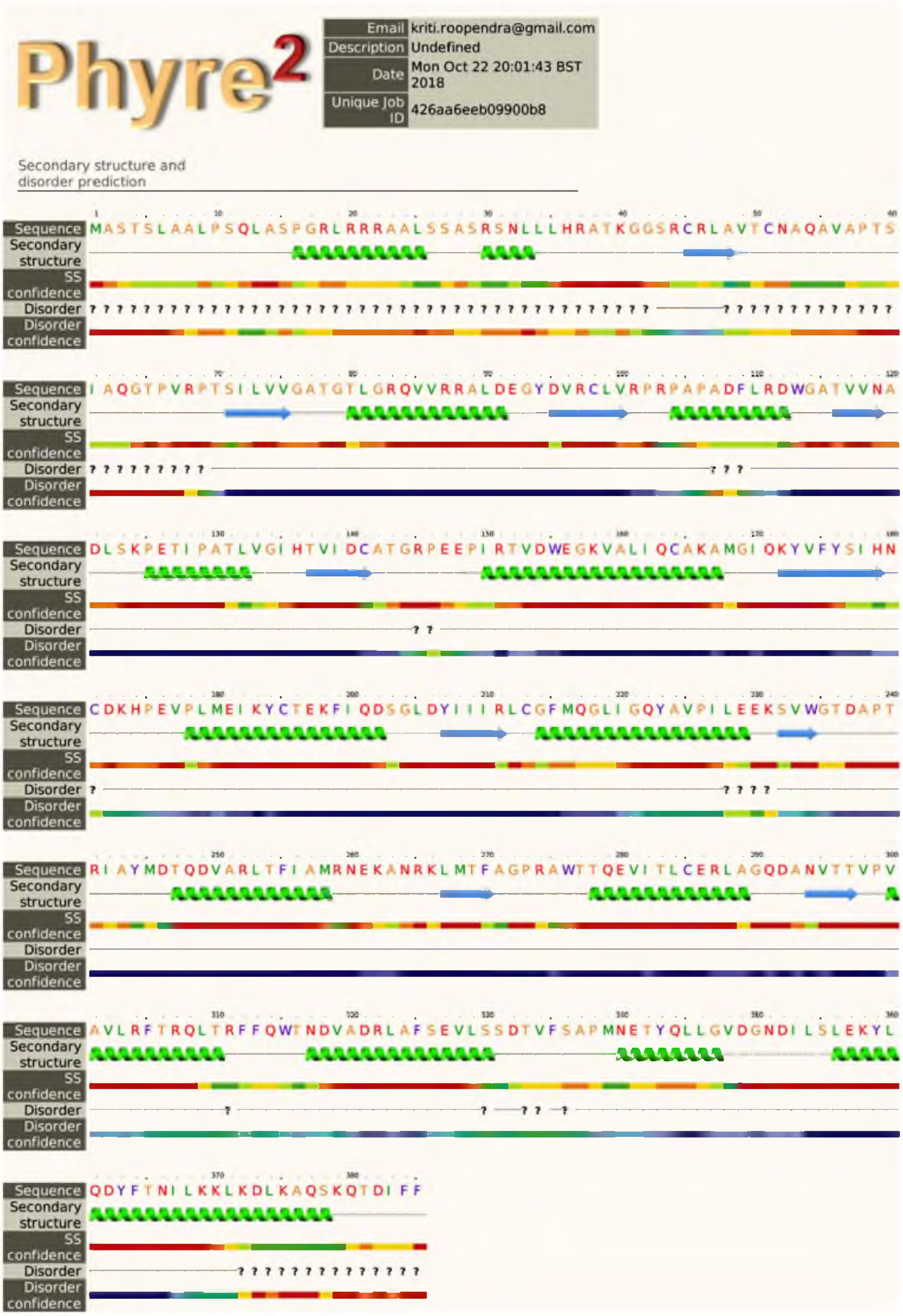


Figure 51: Real-time validation using designed primers

The two uncharacterized transcripts were further characterized using the Phyre2 tool (Protein homology/analogy recognition engine). Secondary structure analysis utilizing their respective amino acid sequences predicted α-helices and β-strands (Figure 52).



Email	kriti.roopendra@gmail.com
Description	Undefined
Date	Mon Oct 22 20:01:43 BST 2018
Unique Job ID	426aa6eeb09900b8

Secondary structure and disorder prediction

Sequence MASTSLAALPSQLASPGRLRRRAALSSASRSNLLLHRATKGGSRCRLAVTCNAQAVAPTS
Sequence IAQGTPVRPTSILVVGATGTLGRQVVRRALDEGYDVRCLVRPRPAPADFLRDWGATVVNA
Sequence DLSKPETIPATLVGIHTVIDCATGRPEEPIRTVDWEGKVALIQCAKAMGIQKYVFYSIHN
Sequence CDKHPEVPLMEIKYCTEKFIQDSGLDYIIIRLCGFMQGLIGQYAVPILEEKSVWGTDAPT
Sequence RIAYMDTQDVARLTFIAMRNEKANRKLMTFAGPRAWTTQEVITLCERLAGQDANVTTVPV
Sequence AVLRFTRQLTRFFQWTNDVADRLAFSEVLSSDTVFSAPMNETYQLLGVDGNDILSLEKYL
Sequence QDYFTNILKKLKDLKAQSKQTDIFF

Figure 52: Secondary structure analysis of uncharacterized protein ycf39 predicted 45% of the amino acid sequence to consist of α-helices and 12% as β-strands

Various domains identified based on sequence matching, yielded possible 3-D models for the two transcripts. 31% (coverage) of the sequence for transcript sharing homology with uncharacterized LOC8066524 transcript variant X2 of *Sorghum bicolor*, displayed identity with a template of retrovirus capsid protein, N-terminal core domain, but at a low (25.9%) confidence level. Additionally, 77% (295 residues) of the transcript sequence found homologous to mRNA of uncharacterized protein ycf39, was modelled to template of an hydrolase, with 100% confidence level (Figure 53). This transcript if further explored, may perhaps prove to be a novel stimulant of sucrose accumulation process.

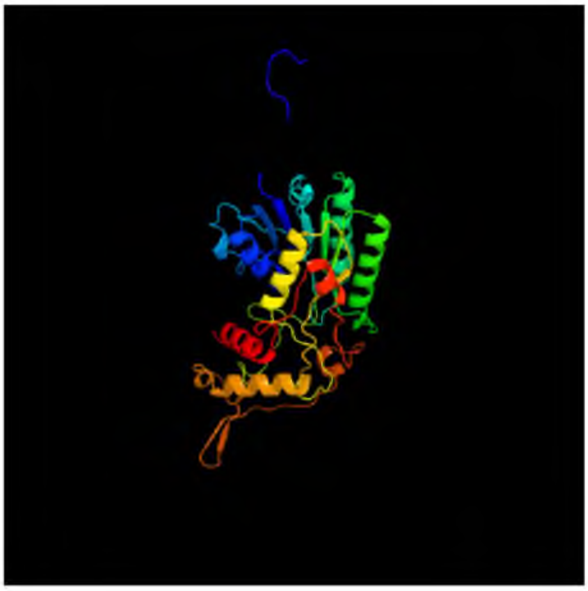


Figure 53: The 3-D model predicted for mRNA of uncharacterized protein ycf39 of *Sorghum bicolor*, based on sequence identity with template

The expression behaviour detected through qRT-PCR analysis was in concurrence with the RNA-seq results for most transcripts, validating the reliability of the transcriptomic analysis' results. The single peak obtained for all primers in the melt curve analysis, validate the precision of primer designing tool. The data generated in this study has supplemented the existing sugarcane transcriptome resource and may be utilized for further deliberation and exploration of functional genomics of sugarcane. The study sheds light on transcripts which are particularly differentially expressed under conditions of GA_3 perturbed source-sink communication. The results may help elucidate additional factors/ genes that bring about better sink strength to facilitate sucrose accumulation, specifically in sugarcane.

4.2 Partial shading

We employed partial shading in another attempt to explore the effect of perturbation of source-sink communication on sugar metabolism and source-sink dynamics. Partial shading treatment was employed to mirror the case of elevated sink strength and sink demand by manipulating the proportion of sink to source. All leaves (source leaves (L1) and sink leaves (L3)) were practically converted into 'sinks' by covering them with 90% shade cloth while the metabolically most active LTM leaf (L2) was kept uncovered, as the sole photosynthesizing 'source'. Since in doing so, the plant sink size was increased, therefore a consequent increase in sink strength and sink demand was quite explicable.

4.2.1 Biochemical analysis

4.2.1.1 Estimation of chlorophyll content

At the beginning of the shading experiment, highest total chlorophyll content was recorded in the sink leaves, followed by source leaves and LTM leaf, in both varieties. In congruence with the GA_3 study, greater chlorophyll was found in the leaves of BO91as compared to CoJ64 (Figure 54). Chlorophyll a formed the larger share of the total chlorophyll, with BO91 leaves exhibiting greater amount of chlorophyll a, accounting for the deeper green colour (Table 8). Both, chlorophyll a and b levels dropped in the source and sink leaves denoting loss of photosynthetic ability as they adopted the sink property. Paul and Foyer (2001) have reported that leaves of plants growing in shaded environment, adapt to the prevalent dim light conditions by modifying the chlorophyll composition so as to enhance the light-use efficiency, thereby reducing the rate of photosynthesis. The decline was noticeably greater in the sink leaves, perhaps explaining their brown and dry appearance at the end of shading period (Figure 55). On the other hand, the boost in chlorophyll content observed in non-shaded LTM leaf of both varieties, demonstrates the groundwork for improving their photosynthetic capacity. In an earlier study too, an escalation in photosynthetic activity and electron transport rate of the solitary exposed leaf, has been shown in response to the heightened sucrose demand created by a similar partial shading experiment (McCormick *et al.*, 2006).

This is also validated by the increased PEPC expression observed. It is noteworthy that in LTM leaves of both varieties, chlorophyll b content dropped soon after shading and resurfaced to levels comparable to those at start, by the 12th/13th day of shading. This too reaffirms the cane's adaptation to the perturbation caused by shading.

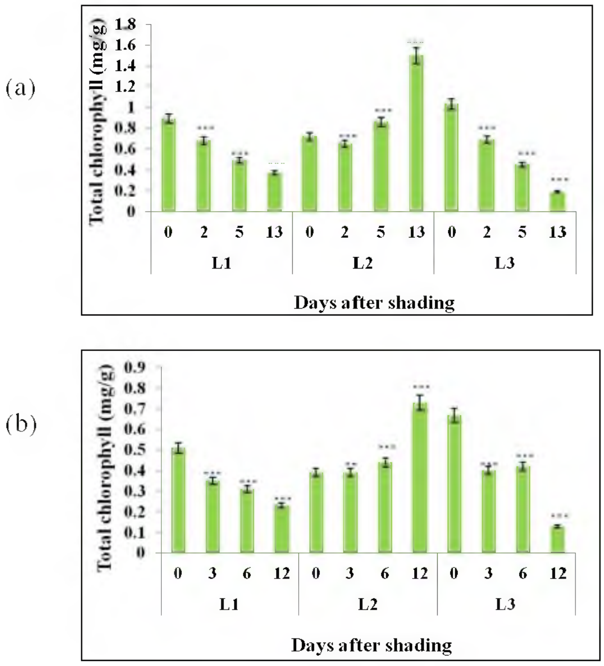


Figure 54: Total chlorophyll content in shaded source leaves (L1), non-shaded LTM leaf (L2) and shaded sink leaves (L3) of control and shaded (a) BO91 and (b) CoJ64 canes respectively

Table 8: Chlorophyll a and b content in non-shaded and shaded leaves

Days after shading	BO91					
	Source leaves (L1)		LTM leaf (L2)		Sink leaf (L3)	
	Chl a	Chl b	Chl a	Chl b	Chl a	Chl b
0	0.53±0.04	0.35±0.03	0.41±0.02	0.31±0.01	0.68±0.05	0.34±0.08
2	0.42±0.02	0.26±0.02	0.48±0.09	0.18±0.05	0.48±0.03	0.21±0.07
5	0.29±0.02	0.20±0.05	0.61±0.07	0.25±0.05	0.32±0.04	0.13±0.02
13	0.24±0.09	0.13±0.08	1.13±0.01	0.37±0.02	0.16±0.03	0.03±0.07
CoJ64						
0	0.38±0.04	0.13±0.05	0.23±0.04	0.16±0.07	0.49±0.06	0.28±0.03
3	0.25±0.06	0.10±0.04	0.28±0.06	0.11±0.02	0.32±0.01	0.08±0.02
6	0.25±0.01	0.06±0.03	0.35±0.04	0.09±0.04	0.36±0.06	0.06±0.06
12	0.19±0.02	0.04±0.02	0.54±0.04	0.19±0.05	0.12±0.06	0.03±0.01

± standard deviation of mean among three biological replicates
* significance of difference between treatments

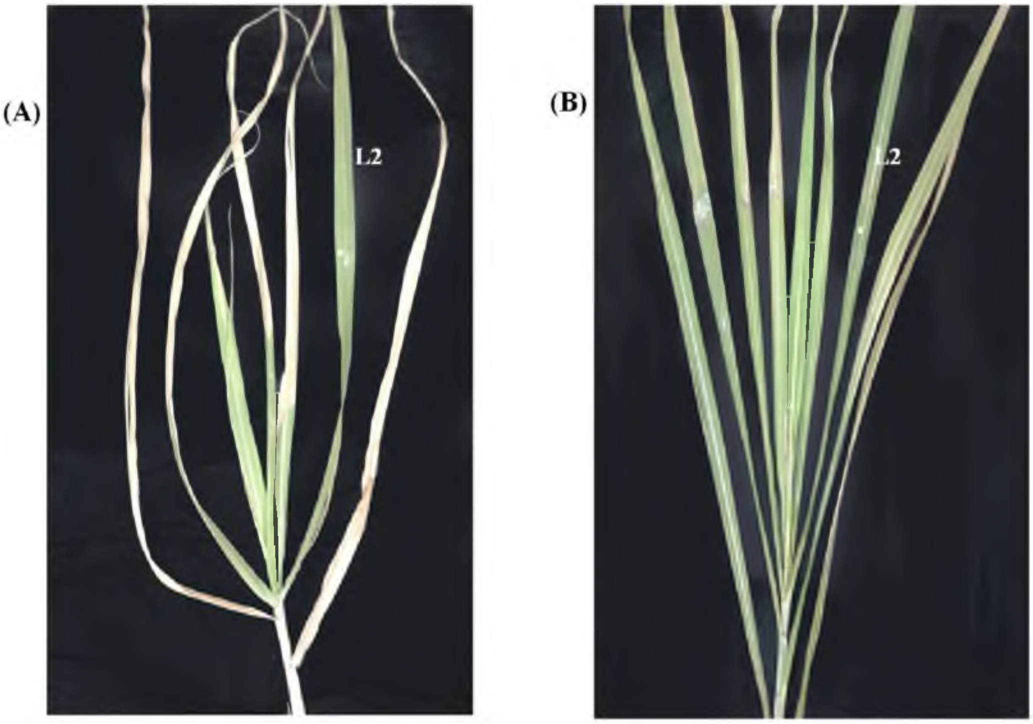


Figure 55: Drying symptoms in shaded leaves and non-shaded LTM leaf (L2) of (A) BO91 (B) CoJ64, after 13/12 days of shading, respectively

4.2.1.2 Estimation of NR activity

NR activity of plants is known to be positively influenced by light with both, intensity and span of light exposure regulating the amount of enzyme (Hageman *et al.*, 1961; Nicholas *et al.*, 1976). NR activity has been reported to vary during the day as per light conditions (Harper and Hageman, 1972), with upsurge in activity during illumination and reduced activity during a dark period (Travis *et al.*, 1970). Conclusively, shading has been reported to decrease NR activity (Hageman *et al.*, 1961). In our present pursuit too, the source and sink leaves of both varieties, exhibited a drop in NR activity soon after shading (Figure 56). This was apparently because light was nearly completely blocked for the ‘covered’ leaves, thus hindering the stimulation of NR. According to Beevers *et al.* (1965), light increases NO_3- uptake by leaves, causing elevated intracellular [NO_3^-] for induction. In concert, the non-shaded LTM leaf displayed significantly greater NR activity as compared to the shaded leaves. Also, the LTM leaf demonstrated a gradual increase in NR activity over the span of shading, perhaps due to increased uptake of nitrate by it, to support increased sink size. However, a drop in NR activity was observed in the non-shaded LTM by the 12th/13th day of shading, probably due to plant’s natural ability to cope, leading to gradual stabilization of N metabolism. Since the canes were subjected to one-time shading, the decline in NR activity may also be due to gradual senescence of the aged LTM leaf.

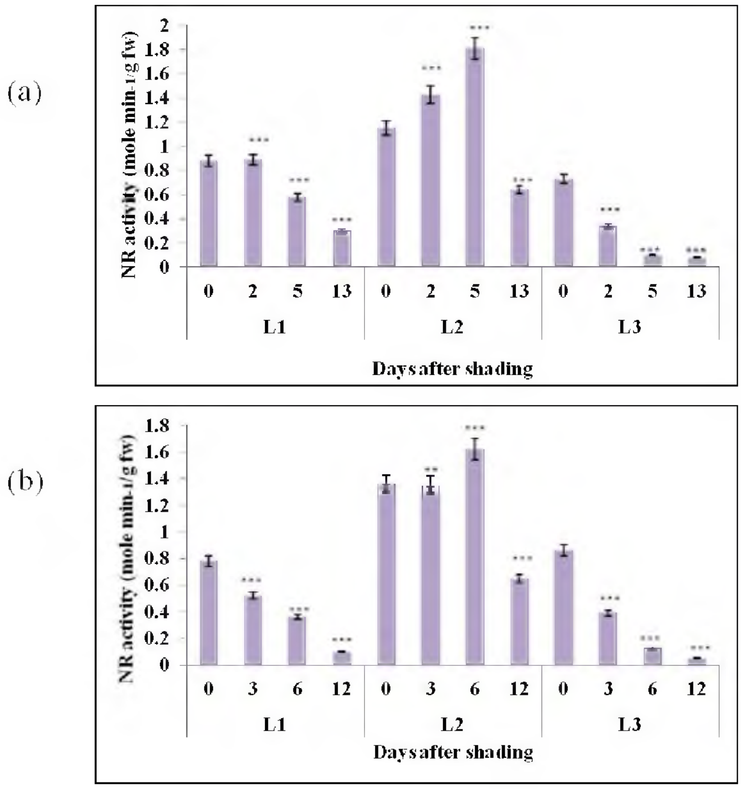


Figure 56: Nitrate reductase activity in shaded source leaves (L1), non-shaded LTM leaf (L2) and shaded sink leaves (L3) of control and shaded (a) BO91 and (b)CoJ64 canes, respectively

4.2.1.3 Sugar analysis

In October, at the onset of maturity, sugar analysis of the samples revealed the conventional pattern in control (non-shaded) canes of both BO91 and CoJ64. The reducing sugar (RS) content increased bottom up while sucrose content increased top down i.e. with increase in level of maturity of the internodal sample. The RS levels were lower and sucrose levels were higher in CoJ64 canes as compared to BO91, it being an early maturing variety. RS level was found to go up significantly on 2nd day in case of BO91 (7.63%) and 3rd day in CoJ64 (5.52%), particularly in the top internodal samples from shaded canes of both varieties. The augmentation in reducing sugar level (Figure 57) can be extrapolated to signify increase in sink strength (Iqbal *et al.*, 2011) as also seen in the case of GA_3 treatment. In an earlier attempt, McCormick *et al.* (2006) have also reported such a surge of hexoses, under the effect of shading treatment. This can be reasoned to be due to increase in invertase action on translocated sucrose to generate hexoses to meet up the high sink demand. Thus, shading too favours increase in sink strength, displaying biochemical changes similar to those observed in case of GA_3 treatment. Parallely, sucrose level went up on 2nd day of shading, in all regions of the shaded BO91 cane (Figure 57), probably due to greater unloading of sucrose to meet up the heightened sucrose demand in sink. On 5th day of shading, a drastic decline in sucrose% was recorded in the BO91 cane, probably due to breakdown of sucrose largely into hexoses (supported by a corresponding gain in RS level) to support the heightened sink strength. This is in concert with the observations of McCormick *et al.* (2006) who have also documented a decrease in sucrose content and rise in hexose content of partially shaded immature culm representing the developed high sink demand.

The Brix% and pol% values (indicative of sucrose content) exhibited a pattern abiding by the sucrose content pattern (Figure 58). On the other hand, sucrose% displayed a downward trend in the CoJ64 shaded cane, over the 3rd and 6th day, possibly due to hydrolysis of the culm sucrose to furnish hexoses in order to meet the high sink demand. This proposition perhaps points to the utilization of already stored sucrose in the early maturing CoJ64 (reflective in the high sucrose and brix values) and hence a slower and weaker stimulation of sucrose translocation in response to sink demand. At 12th and 13th day of shading, high sucrose% and brix%, pol% values observed in the shaded canes of both varieties, signify the pile up of sucrose supply due to increased sucrose translocation. However, the lowering of RS% and regain of sucrose levels also imply that the canes began to get accustomed to the perturbation by shading. The heighetened sucrose levels seen in culm subjected to partial defoliation study, vouch the ability of sugarcane leaves to augment their supplies, as an adaptation to inadequate source material, in order to meet out the sink demand (Pammenter and Allison, 2002).

A drastic depletion in leaf sucrose level was noted on the 2nd day of shading, perhaps in response to the sudden increase in sink demand. Reducing sugar content in LTM leaf of BO91 shaded cane exhibited a downward trend (Figure 59) over the period of shading. Since the LTM leaf is the photosynthetically active leaf here, this decline in hexose levels depicts the increase in channelization of hexoses into sucrose production. This is affirmed by the drastic rise in sucrose% especially on the 5th day in BO91 shaded cane, representing the escalation in sucrose production and need for phloem loading to meet up the growing sink demand. The decrease in sucrose content by 13th day of shading demonstrates draining of synthesized sucrose to quench the culm demand. On the other hand, the non-shaded LTM leaf of shaded CoJ64 displayed a slightly different response to the perturbation. RS levels piled up in the LTM leaf upto 6th day after shading (Figure 59) while the sink demand was mainly being fulfilled by hydrolysis of the culm's sucrose reserve in the early maturing CoJ64. Sucrose levels increased over the period of shading with particularly heightened sucrose concentration noted on 6th day of shading, perhaps in response to the communicated sink demand. The photosynthetically synthesized hexoses were perhaps actively translocated as sucrose thereafter, accounting for the dip in RS% and sucrose% observed on 12th day of shading.

The covered source and sink leaves in both varieties exhibited a similar RS pattern wherein the RS% drastically dipped after shading, as observed on the 2nd/3rd day after shading (Figure 59). This was perhaps because the 'till now' photosynthesizing leaves were converted into sucrose amassing sinks as a result of partial shading. As discussed earlier too, the leaves of shaded plants perhaps adjust their light-utilization capability, and in turn lower their photosynthetic activity to adapt to prevalent light conditions (Paul and Foyer, 2001). On the 5th/6th day of shading, a rise in RS levels was exhibited by both types of 'converted' sinks (viz. source and sink leaves) in both varieties affirming their 'switch' into sinks. The increase in RS% denotes the boosted sink strength and decreased tendency for biosynthesis of sucrose. By 13th day of shading, the RS level and sink strength gradually normalized. Sucrose levels exhibited a decay over the period of shading in the 'source leaf-derived sink' and 'sink leaf-derived sink' of both varieties (Figure 59), possibly due to hydrolysis of the sucrose present, thereby contributing to the heightened sink strength.

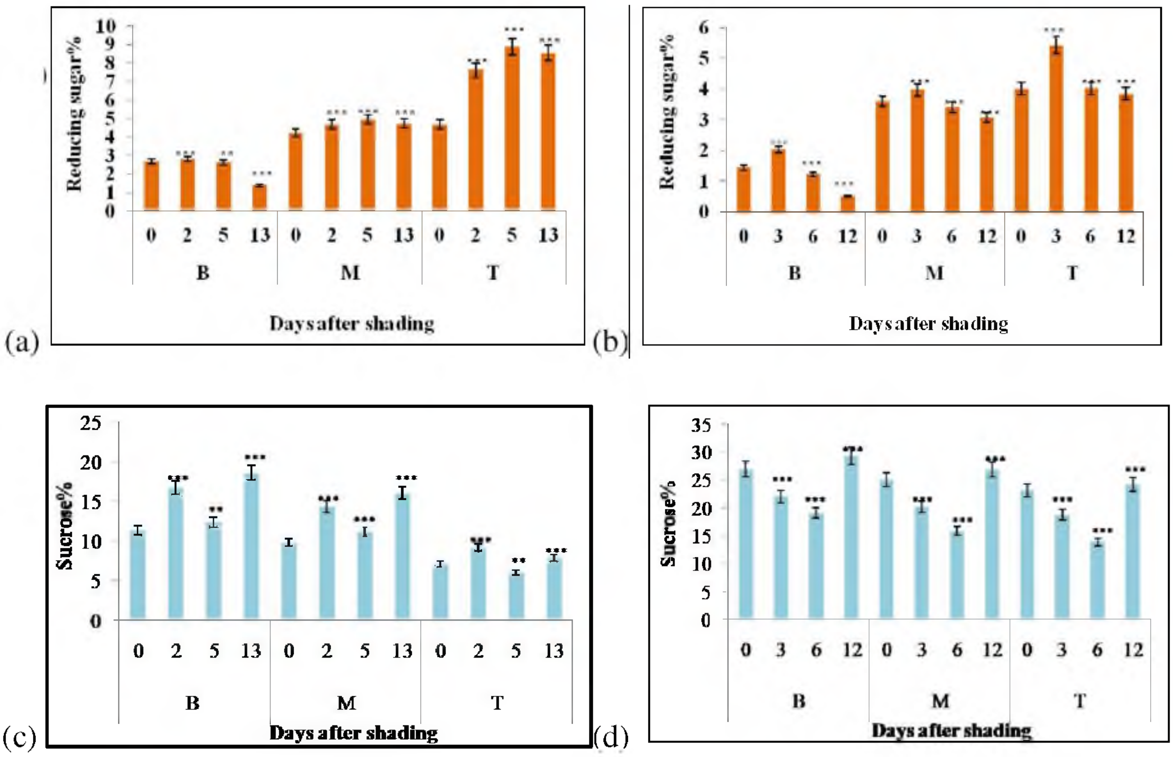


Figure 57: (a) (b) Reducing sugar% (c) (d) sucrose% in internodal samples of control and shaded BO91 and CoJ64 canes respectively

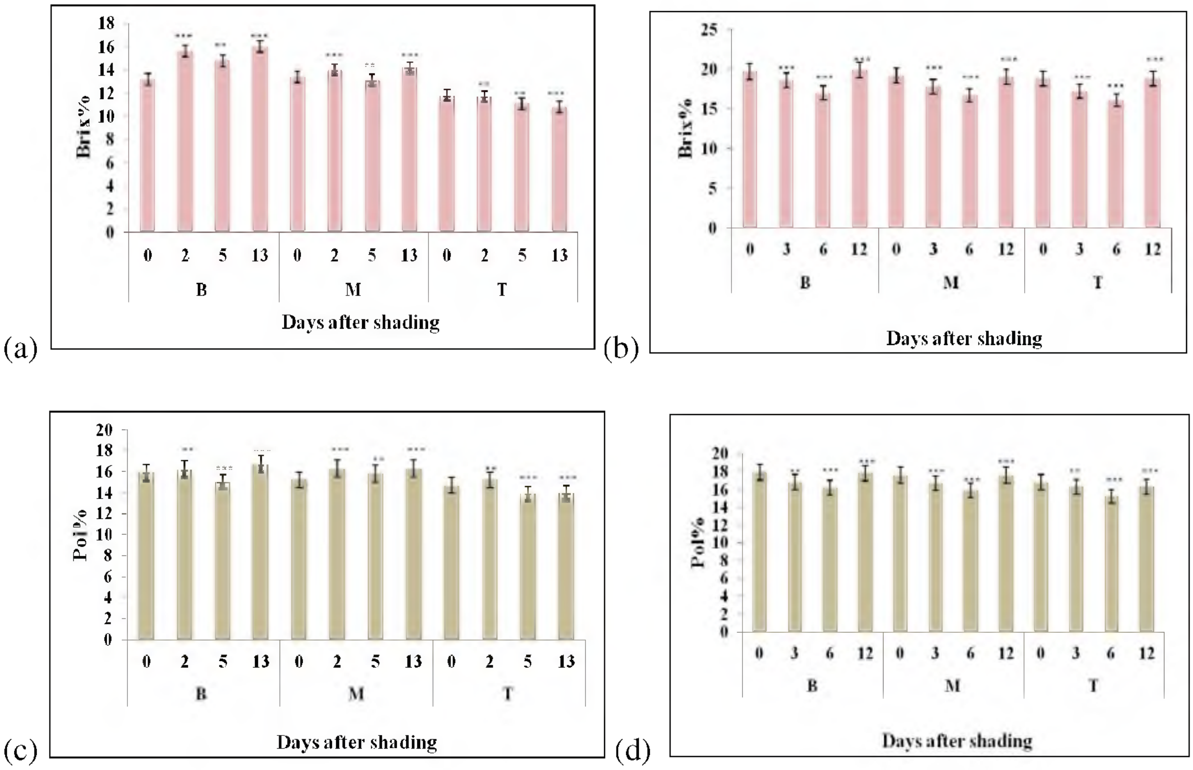


Figure 58: (a) (b) Brix% (c) (d) Pol% in internodal samples of control and shaded BO91 and CoJ64 canes, respectively

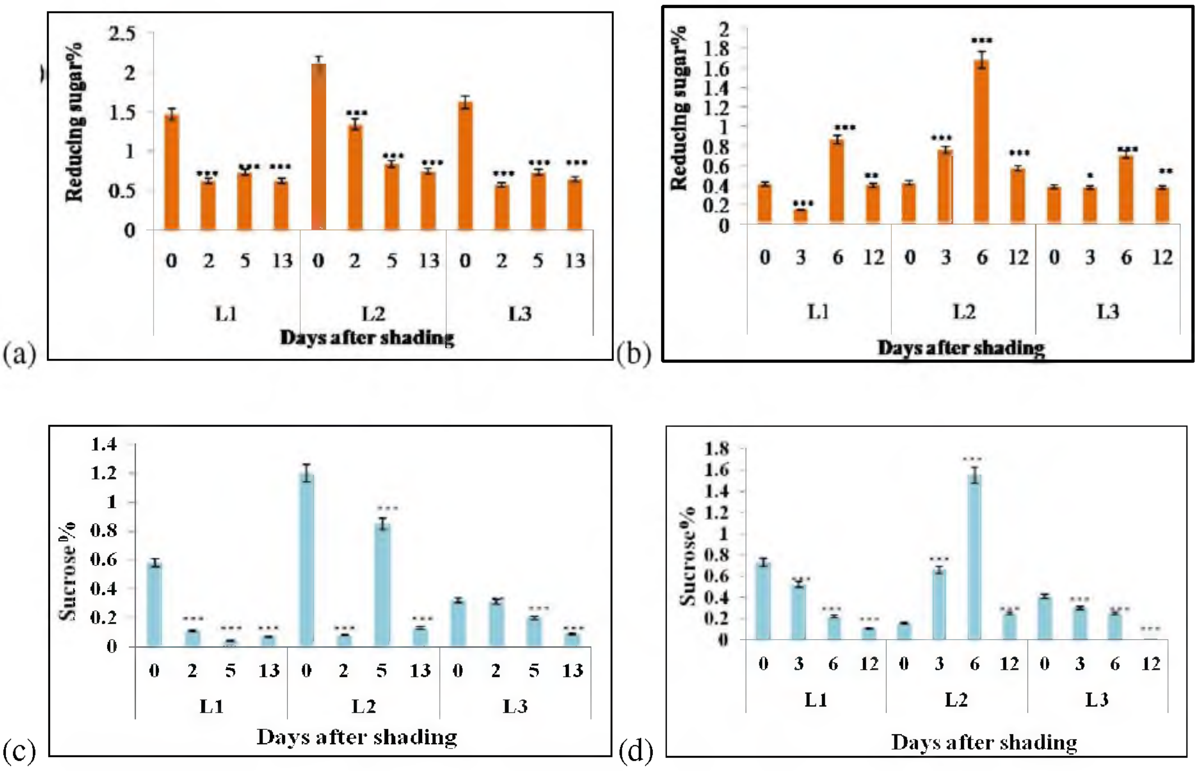


Figure 59: (a) (b) Reducing sugar% (c) (d) sucrose% in shaded source leaves (L1), non-shaded LTM leaf (L2) and shaded sink leaves (L3) of control and shaded BO91 and CoJ64 canes, respectively

4.2.2 Molecular analysis

4.2.2.1 End-point PCR expression analysis

4.2.2.1.1 SAI expression

SAI characteristically hydrolyses sucrose, thereby furnishing hexose sugars especially to growing tissues. SAI activity is found to be higher in immature internodes of sugarcane, its level gradually decreasing with increase in maturity (Lontom *et al.*, 2008). This experimental pursuit was undertaken in October, at the onset of maturity, and hence the control samples of both varieties exhibited high SAI expression. The escalation in RS levels points to increased ability of sink to attract sucrose. This is further justified by the elevated SAI transcript expression observed on 2nd day in BO91 and 3rd day in CoJ64, especially in the internodal samples of top segment of shaded cane (Figure 60), in keeping with the elevated RS values recorded. Substantially high SAI transcript level was even seen on 5th and 6th day of shading, in BO91 and CoJ64 shaded canes respectively, signifying the high rate of sucrose hydrolysis brought about by SAI to support the high sink demand. However, significant SAI expression was seen on 12th/13th day of shading, even after sucrose levels were regained. This may be because the cane was only beginning to mature in October and hence the SAI level did not totally recede then.

SAI transcript level was noted in the exposed LTM leaves and 'converted' sinks of both BO91 and CoJ64. A prominent decline in SAI expression was noted in the BO91 LTM leaf (Figure 61), over the period of shading, depicting the falling RS levels and increased channelization into sucrose synthesis to cater to the high sink demand. The weakened SAI expression seen in CoJ64 LTM leaf justifies the pile up of hexoses, when rate of sucrose synthesis was low (as discussed earlier too). Contrarily, the 'converted' sinks, exhibited a rise in SAI expression over the period of shading. This pattern was in congruence with that perceived in culm sinks. Thus, here too, the boost in SAI expression can be deduced to point to the increasing sink strength and sink demand of 'leaf-derived sinks'. However, SAI expression was almost negligible in the 'sink leaf-sink' of both varieties, perhaps due to decline in metabolic activity of the shaded leaves and a consequent drying up.

4.2.2.1.2 CWI expression

CWI, being cell wall-bound, is involved in the breakdown of sucrose in extracellular space and hence is a major player regulating phloem unloading by sustaining a constant gradient for sucrose flux between source and sink (Roitsch *et al.*, 2003). Noticeable CWI expression was recorded in all internodal samples, due to the ongoing maturation and the active sucrose translocation and unloading promoted by CWI. Prominent CWI expression was visible on the 2nd/5th day and 3rd/6th day of shading in shaded BO91and CoJ64 cane respectively (Figure 60). This elevated CWI expression in the culm tissue depicts the rise in phloem unloading and translocation to answer the elevated demand for sucrose in sink (Ma *et al.*, 2000; Koch, 2004). 12/13 days after shading, the CWI transcript level dropped but was still substantial, denoting the still ongoing need for translocation and phloem unloading. In the non-shaded LTM leaves, CWI expression was found to gradually fade (Figure 61), perhaps due to priority routing of hexoses into sucrose production and augmented phloem loading and translocation. On the flip side, the leaf sinks showed an upregulation in CWI expression especially towards the end of shading period i.e. 12th/13th day. This may perhaps be to help metabolize the inflow of sucrose in the event of developed sink strength.

4.2.2.1.3 NI expression

The soluble invertase, neutral invertase (NI) is engaged in sucrose hydrolysis in the cytosol and is known to influence sucrose pile up in sugarcane stalk (Rose and Botha, 2000). It has been reported to primarily supply hexoses in younger internodes. Similar to SAI behaviour, NI expression has been shown to predominate in relatively immature culm tissue with low sucrose and high hexose concentrations, diminishing with progressing maturation (Bosch *et al.*, 2004). As evident from the high RS levels, high sink strength and sink demand developed after 2/3 days of shading in BO91 and CoJ64 respectively. Thus, the heightened NI expression observed in both varieties even on 5th and 6th day of shading (Figure 60), especially in the top internodal samples validate the dominant need for increased translocation and phloem unloading. NI expression faded on the 12th/13th day of shading as the sugar metabolism in the cane recouped.

4.2.2.1.4 SuSy expression

Sucrose synthase (SuSy) is another sucrose hydrolyzing enzyme that plays a major role in regulating hexose level in growing plant sink tissues (Geigenberger and Stitt, 1993). SuSy is also thought to be reflective of sink strength in maturing sink tissue (Sung *et al.*, 1989). Since the canes were subjected to shading treatment at the onset of maturity, prominent SuSy expression was observed throughout. Peak SuSy transcript level was observed on the 2nd and 3rd day of shading in BO91 and CoJ64, depicting increased sucrose hydrolysis and augmented sink strength (Figure 60). Conspicuous but lesser expression was observed on 5th and 6th day pointing to relatively answered sink demand. By the end of shading period, the SuSy expression had fairly leveled in both varieties, suggesting that the sink strength gradually normalized.

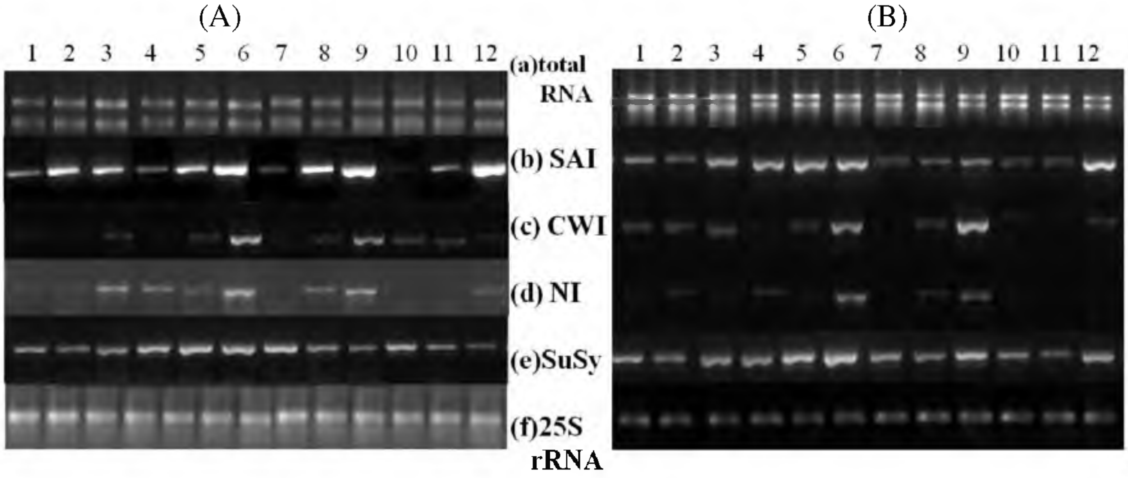


Figure 60: (a) Agarose gel depicting total RNA of (A)BO91 (B) CoJ64 in normalized concentration **(b)** Differential expression analysis of SAI **(c)** CWI **(d)** NI **(e)** SuSy gene in stalk samples of control and shaded cane over the shading period in October **(f)** RNA normalization verified using 25S gene primer as internal control

Lanes 1-3: internodes aof bottom, mid and top portions of control cane on 0th day; **Lanes 4-6:** internodes of bottom, mid and top portions of shaded cane on 2nd /3rd day of shading **Lanes 7-9:** internodes of bottom, mid and top portions of shaded cane on 5th /6th day of shading; **Lanes 10-12:** internodes of bottom, mid and top portions of shaded cane on 13th /12th day of shading

4.2.2.1.5 PEPC expression

Since phopshoenolpyruvate carboxylase (PEPC) is a key performer in C_4 photosynthesis, its high expression recorded in all leaves of both varieties, at the beginning of the shading treatment, validate ‘normal’ leaf role at the start. As the source leaves got converted into sinks, the PEPC expression gradually dimmed (Figure 61), denoting marked decrease in their photosynthetic potential. Then again, PEPC expression receded in sink leaves soon after shading and was completely lost beyond 2nd/3rd day of shading in case of BO91 and CoJ64 shaded cane, respectively. This demonstrates the complete loss of photosynthetic ability and senescence in the sink leaf-derived sinks, also affirmed by their conspicuous browning and drying observed at the end of shading period. On the other hand, high PEPC expression was observed in the LTM leaves of both varieties, even more heightened by the 5th/6th day of shading, probably to cater to the increased sink demand. This validates the role of LTM leaf as the sole photosynthesizing ‘source’ once the source and sink leaves are converted to sinks.

4.2.2.1.6 SUT expression

The LTM leaves of both varieties exhibited a justified level of sucrose transporter transcript in both varieties (Figure 61), at this stage, when cane was beginning to mature and accumulate sucrose. The SUT expression distinctly picked up after shading. This further explains the drop in sucrose levels on the 2nd day of shading in BO91 shaded cane. Since sucrose initially piled up in the LTM leaf of CoJ64 shaded cane, SUT expression was found to pick up more in the latter phase.

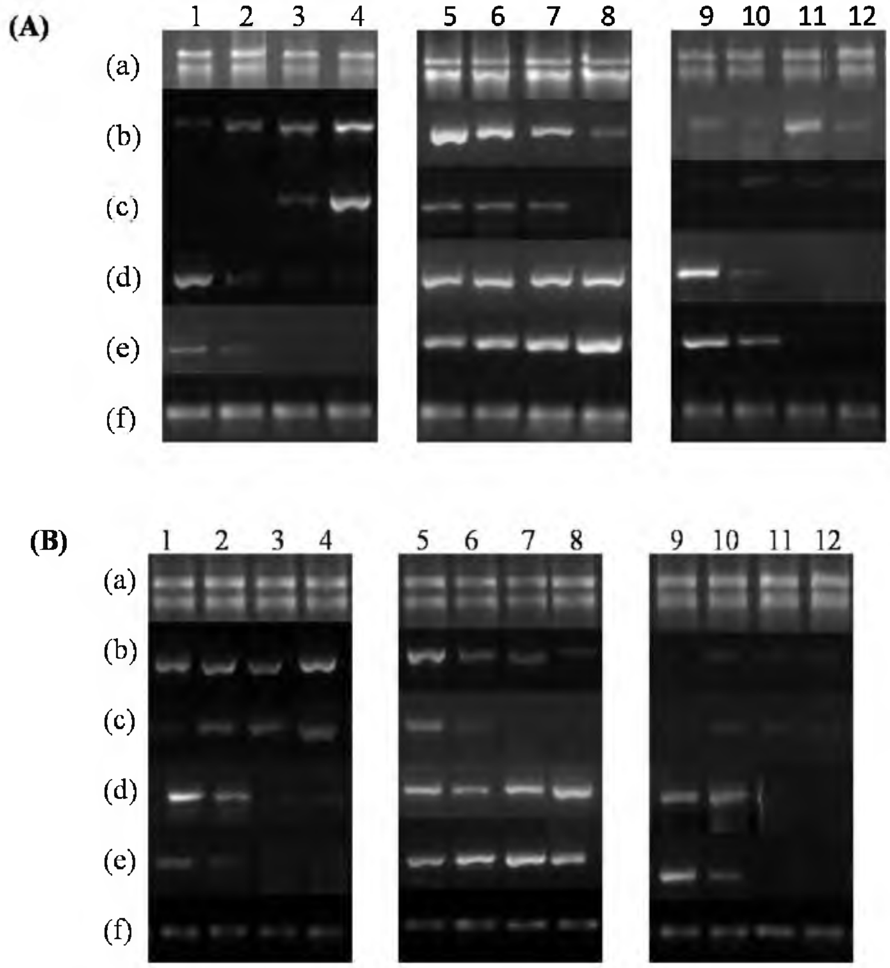


Figure 61: **(a)** Agarose gel depicting total RNA of shaded and non-shaded leaves of (A) BO91 and (B)CoJ64 cane in normalized concentration **(b)** Differential expression of SAI **(c)** CWI **(d)** PEPC **(e)** SUT gene in shaded and non-shaded leaves of (A) BO91 and (B) CoJ64 **(f)** RNA normalization verified using 25S gene primer as internal control

Lanes 1-4: shaded source leaves (L1) on 0th,2nd/3rd, 5th/6th, 13th/12th day after shading
Lanes 5-8: non-shaded LTM leaf (L2) on 0th,2nd/3rd, 5th/6th, 13th/12th day after shading
Lanes 9-12: shaded sink leaves (L3) on 0th,2nd/3rd, 5th/6th, 13th/12th day after shading

4.2.2.2 Real-time estimation of SPS expression

Taking the 0th day source leaf (L1) as reference sample, SPS expression was quantified in the three different leaf sample sets (viz. L1, L2, L3) over the period of shading, SPS being the chief sucrose synthesizing enzyme was aptly found to be upregulated in the non-shaded LTM of both varieties, over the period of shading (Figure 62). The SPS expression peaked on 2nd day of shading (1.83 fold as compared to reference) in case of BO91, demonstrating the immediate response to communicated increase in sink demand. In case of CoJ64, SPS transcript level heightened around the 6th day of shading (1.12 fold), indicating that the reserve culm and leaf sucrose were initially utilized to feed the augmented sink demand. On 0th day, the sink leaf displayed an upregulated SPS expression, compared to source leaf reference, possibly denoting its role in providing sucrose to culm. In general, the source and sink leaf 'converted' sinks displayed a downregulated SPS expression accounting for the gradual loss of sucrose biosynthesis' function.

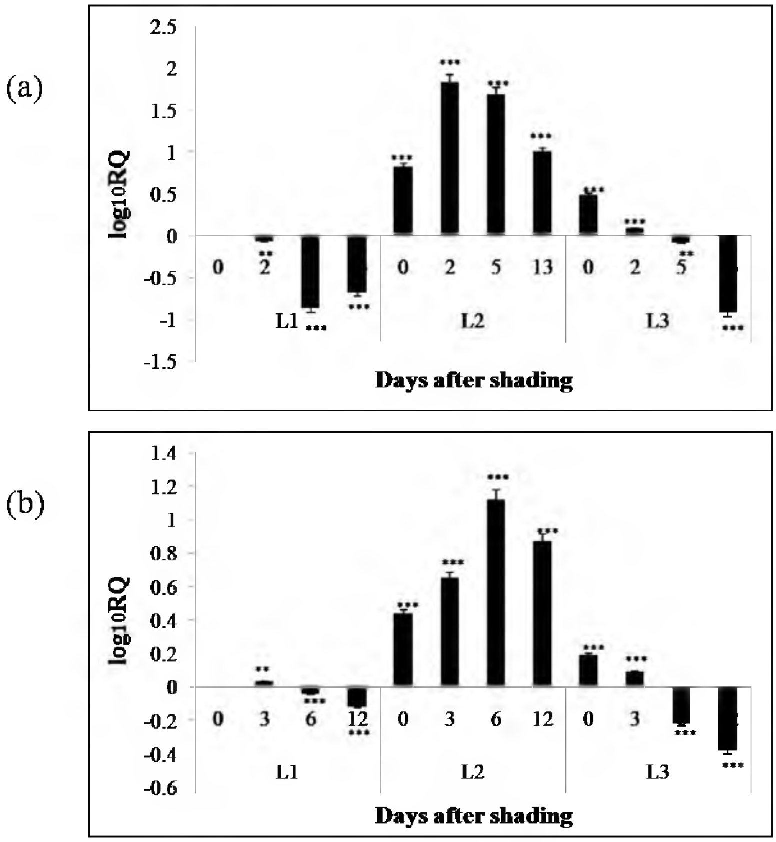


Figure 62: Quantitative analysis of differential expression ($\log_{10}RQ$) of SPS in shaded source leaves (L1), non-shaded LTM leaf (L2) and shaded sink leaves (L3) of control and shaded (a)BO91 and (b)CoJ64 canes, respectively

Thus, the above results potray the interplay of various sucrose metabolizing enzymes in determining sink strength, regulating photosynthesis, phloem loading/ unloading in response to perturbation of source-sink communication. The sink strength stabilized by the end of 2 weeks (shading period), which depicts that the culm sink in association with the source, gradually gets accustomed to the prevalent perturbed conditions by manipulating the source-sink network accordingly. However, the over dried sink leaves and withered leaf tuft observed at the end of shading period, indicate that it would be difficult for the shaded cane to sustain for long under shading treatment in spite of the adaptation.

Summary and Conclusions

SUMMARY AND CONCLUSIONS

In most higher plants, the photosynthetically active leaves serve as ‘source’ for carbon and energy. Particularly in sugarcane, the culm serves as reservoir or sink for the photoassimilate that is transported as sucrose from source to the sink. The transported sucrose is either immediately utilized for growth, developmental and metabolic processes or immobilized for storage. Such consumption of sucrose causes a high sink demand to develop, thus resulting in the extraordinary levels of sucrose observed in sugarcane. Hence, the communication between source and sink can be visualized in the light of a supply-demand concept wherein the source leaves synthesize and ‘supply’ sucrose in response to the sucrose ‘demand’ that is generated in the sink. The crop yield can perhaps be enhanced by either improving source capacity (to produce photoassimilate) or by increasing consumption of photoassimilates by sink (thereby enhancing sink demand). However, since the inflow of sucrose in sugarcane is primarily governed at the level of sink, sucrose accumulation in the culm is perhaps mainly sink-regulated.

Sugar-sensitive systems are said to operate in source leaves causing leaf photosynthetic rate to be modified by sugar molecules viz. sucrose and hexose, indicating their key role in altering the carbon distribution between source and sink. The activity of various sugar metabolizing enzymes and expression of related gene transcripts, is perhaps adapted according to the sink demand. Hence, studying the modulation of expression of sucrose metabolism-related genes under conditions of modified sink demand may help better decipher the source-sink relationship and consequently sucrose accumulation in the culm. Some earlier efforts made in this direction have brought to light variation in the expression of some carbohydrate metabolism-related genes in answer to source/sink manipulation in sugarcane. Defoliation study in sugarcane pointed to escalation in photosynthetic rate of the sole source leaf in response to drop in sucrose level in culm, emphasizing that photosynthesis is regulated at the level of sink. However, the studies so far have largely lacked in providing a molecular basis to the complex mechanism underlying source-sink dynamics. This necessitates the scrutiny of sucrose metabolism (synthesis and storage) at source-sink level, in order to better understand sucrose accumulation in sugarcane.

An array of factors governs sucrose synthesis and transport leading to sucrose accumulation in the sugarcane culm. Several works (as discussed in review of literature) have brought to light genes/enzymes involved in sugar synthesis and metabolism, those that facilitate effective sucrose transport and that govern partitioning of sugar into subcellular compartments. Exploring the interplay of all such factors through perturbation of source-sink communication and the study of consequential changes therein, at physiological, biochemical and molecular level, over the sucrose accumulation phase, will facilitate better understanding of the source-sink dynamics that underlies sucrose accumulation process.

The present work provides a more holistic picture about the interplay of factors (morphological, biochemical and molecular) that determine sucrose accumulation, providing connecting links to earlier studies. To the best of my knowledge, this is a first-of-its-kind work where sink behaviour has been tracked and analysed in relation to source activity under the effect of gibberellic acid (GA_3) treatment. Gibberellins have long been known to encourage elongation and division of cells, thus providing more room for assimilate accumulation, thereby improving sink's capacity to accommodate sucrose. In the present pursuit, GA_3 spray was employed to delay saturation of sink by enhancing sink strength and sink demand, perhaps, thereby facilitating greater sucrose accumulation. A comparative was drawn between difference in responses of a high sugar (CoJ64) and a low sugar accumulating variety (BO91) to elucidate the factors that facilitate better sucrose accumulation. The differential pattern of various morphological (viz. plant height, internode length, cell size) and biochemical (RS, sucrose, pol%, brix%, NR, chlorophyll, invertase and SPS activity) parameters, in control (untreated) and treated canes was analysed in response to such manipulation of sink capacity. Also, the difference in expression of various sucrose metabolizing enzymes *viz.* soluble acid invertase (SAI), cell wall invertase (CWI), neutral invertase (NI), sucrose synthase (SuSy) and sucrose phosphate synthase (SPS), which are considered to play important role in sucrose accumulation in sugarcane, was analysed. Additionally, expression of other related molecular determinants (viz. PEPC, SUT), was explored under controlled and GA_3-induced perturbation to ascertain the effect of GA_3 on sucrose metabolism and transport, at molecular level.

SEM visualization revealed larger cell size in the GA_3 treated internodal samples of both varieties, as compared to that in control. This indicates the role of GA_3 in enhancing sink size and sink capacity, providing more room for sucrose accumulation. In general, greater chlorophyll content was observed in the GA_3 treated leaf defending the role of GA_3 in facilitating higher photosynthetic rate to meet higher sink demand developed under GA_3 effect. Higher NR activity was seen in the GA_3 sprayed leaf during initial phase pointing to increased N uptake and utilization, in order to support increased sink demand under GA_3 effect.

The GA_3 treatment produced most conspicuous change, 30 days after spraying (30 DAS), wherein the reducing sugar (RS) level significantly escalated, especially in the upper internodes of GA_3 treated canes. This perhaps points to increased sink strength and in turn better sink demand to attract more assimilate. The difference was more drastic in case of BO91, than in CoJ64, suggesting more scope for GA_3-induced manipulation of sink capacity, perhaps due to its late maturing nature. The sharp rise in RS levels and corresponding fall in sucrose content was validated by a parallel higher invertase activity observed in GA_3 treated samples. Higher invertase activity in agreement with higher RS values observed in BO91 can be extrapolated to better sink strength, as compared to CoJ64. Concurrently, lower RS and greater sucrose levels were noted in the GA_3 treated LTM leaf, indicating greater conversion of RS to sucrose, to answer the high sink demand developed. These observations can be extrapolated to interpret that GA_3 perhaps maintains a prolonged state of immaturity in the culm tissue, thereby resulting in better sink strength. Noticeably higher RS values were observed up to 60 DAS, particularly in BO91, beyond which the sucrose levels rose with analogously high brix%, pol% values. Even as the GA_3 effect gradually waned and maturation advanced, the GA_3 treated canes exhibited evidently higher sucrose levels, substantiating the envisaged increase in sink capacity. Higher SPS activity was observed in the GA_3 treated internodal samples of CoJ64 and BO91, up to 60 and 120 DAS respectively, In congruence with RS values, invertase activity declined over time. As a result, higher final sucrose concentrations were obtained in GA_3 treated BO91, affirming the possibility that GA_3 treatment can bring about improvement in sucrose accumulation in culm.

The end-point and real time-PCR based differential expression data was in congruence with these biochemical findings. Evidently higher expression of SAI was obtained especially 30 DAS, pointing to relative immaturity or delayed onset of maturity due to GA_3 effect. The higher CWI expression obtained in GA_3 treated plants, perhaps signifies higher apoplastic unloading of sucrose, thereby affirming greater sucrose uptake under GA_3 influence. The particularly higher SPS expression observed in GA_3 treated BO91 cane, compared to control, perhaps explains its high sucrose yield at the end of study. Correspondingly, heightened SUT expression and coherent dip in sucrose levels observed in GA_3 treated LTM leaf, also corroborate increased sucrose transportation. GA_3 treated LTM leaf also displayed higher PEPC expression, than in control, in congruence with high RS levels recorded, validating greater photosynthetic rate in response to high sink demand. GA_3 was also found to stimulate marked rise in SPS expression in GA_3 treated LTM leaf depicting greater sucrose synthesis in response to the higher sink demand. At the end of maturation phase, inhibition of phloem loading due to decreased sink demand perhaps signalled sucrose sufficiency, causing diminished SPS expression in leaf.

BO91 displayed a more promising response to GA_3 treatment, with final peak sucrose values as high as (40.54%-41.6%) recorded in GA_3 treated BO91 canes, compared to (30.44%-38.8%) in control ones. In view of the above analysis, the transcriptome sequencing and analysis of control and GA_3 treated top internodal samples (C_T and G_T respectively) was carried out *de novo*. The transcripts derived from C_T and G_T, were screened on the basis of read count and those exhibiting substantial read count difference, of two fold and more, were sieved out to obtain 558 transcripts. BLAST2GO was employed to determine the functional annotation of these transcripts. BLASTing revealed that top hits of most transcripts shared homology with *Sorghum bicolor*, followed by *Zea mays*, *Sertaria italica* and *Saccharum* hybrid cultivar R570. From among these, 26 differentially expressing transcripts were selected on the basis of assigned function and homology. Real-time primers were designed using the online IDT tool, in order to validate the RNA-seq results. Out of the 26, 13 primers were employed to ascertain their differential expression among C_T and G_T, over the maturation phase. In this pursuit, a valid transcript showing 95% homology with a probable sugar phosphate/phosphate translocator At1g06470 of *Sorghum bicolor*, was identified. Its augmented expression in the GA_3 treated sample affirmed the active role of this transcript in sugar transport. The quest also fished out transcripts homologous to vital genes from close homologs viz. mRNA of fructose-1,6-bisphosphatase of *Saccharum* hybrid cultivar H65-7052, UTP-glucose-1-phosphate uridylyltransferase of *Sorghum bicolor,* cytochrome b-c1 complex subunit of *Sorghum bicolor*, WAT1-related protein of *Sorghum bicolor*, MYR1 of *Sorghum bicolor*, translation initiation factor5A (elF5A) mRNA (complete cds) of *Saccharum* hybrid cultivar ROC22, scarecrow-like protein of *Sorghum bicolor*, V-ATPases (vacuolar-type H+ ATPase) of *Sorghum bicolor*, glycosyltransferase. Most transcripts exhibited expression in keeping with the RNA-seq results except one displaying homology to a sucrose transporter protein of *Saccharum* hybrid cultivar GT28 which showed downregulated expression throughout the maturation phase. A transcript was also found to display 97% homology with mRNA for a predicted uncharacterized protein ycf39 (LOC8058013) of *Sorghum bicolor*.

Overall, the key outcome of this study was that GA_3-treated BO91 cane displayed significantly higher final sucrose concentration, compared to control cane. Though, similar observation was also made in case of GA_3-treated CoJ64, however the high sucrose levels could not be sustained perhaps due to onset of inversion in the early maturing variety.

The key findings that can be concluded from this study are:

- GA_3 treatment induced an increase in cell size denoting increase in sink size and sink capacity to accommodate more sucrose
- GA_3 treatment perhaps prolonged the state of immaturity in cane thereby stimulating invertase (SAI) activity and consequently reducing sugar content of the culm, thereby depicting increase in sink strength and heightened sink demand to attract more assimilate
- The escalated expression of various genes viz. CWI (pointing to apoplastic unloading), SPS (bringing about sucrose synthesis), PEPC (depicting photosynthetic rate), SUT (involved in sucrose transportation), all lent support to greater sucrose accumulation in GA_3 treated cane
- Chlorophyll content increased in response to GA_3 treatment to support increased photosynthesis for production of more photoassimilate to meet the high sink demand; also qualified by increase in reducing sugar in GA_3 treated leaf samples
- The RS, sucrose values validated a prolonged state of immaturity induced by GA_3 in the late maturing BO91(higher RS even at 60 DAS) while GA_3 treatment was effective for a shorter span in the early maturing CoJ64, thus corroborating the higher sucrose accumulated in GA_3 treated BO91

- Transcriptomic analysis identified several transcripts exhibiting significant differential expression under the effect of GA_3 spray. Transcripts identified as homologous to sugar phosphate/phosphate translocator, UTP-glucose-1-phosphate uridylyltransferase, V-ATPases (vacuolar-type H+ ATPase) were found to be perhaps correlated to the high sucrose content observed in GA_3 treated cane. A differentially expressing transcript found homologous to mRNA of an uncharacterized protein and characterized as hydrolase (using online resource) may prove to be novel stimulant of sucrose accumulation process.

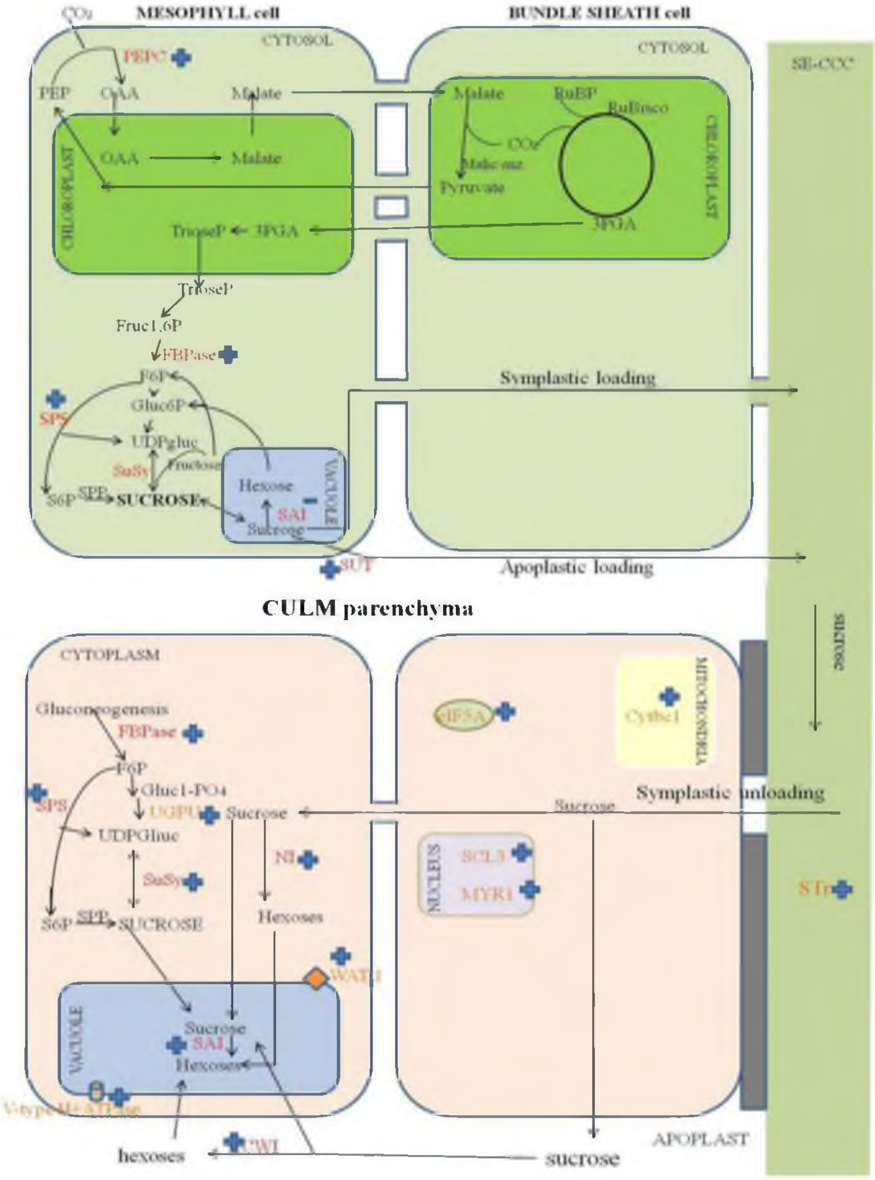


Figure 63: Nutshell diagram of some molecular determinants of high sucrose accumulation in culm (as learnt by comparison of control and GA_3 treated samples, a month after GA_3 spray) (adapted from Watt *et al.*, 2014)

The well-known determinants conventionally used in studies include SAI, CWI, NI, SuSy, SPS, FBPase, SUT, PEPC while the novel stimulants ascertained in the transcriptomic study include sucrose translocator (STr) , UTP-glucose-1-PO_4 uridylyltransferase (UGPU), cytochrome b-c1 complex subunit (cytbc1), WAT1, V-type H^+ ATPase, transcription factors MYR1and SCL3, elongation factor eIF5A

\+ denotes up regulated expression and - denotes down regulated expression

Since upregulated expression of various genes viz. SAI, CWI, SPS, as a consequence of GA_3 treatment, especially during early maturation phase has been found to augment sucrose accumulation, these may be employed as molecular determinants of sucrose yield of new varieties. Also, these known factors can perhaps be exploited to manipulate sucrose accumulation at molecular level. The transcripts identified to exhibit significant differential expression under GA_3 treatment perhaps influence source-sink communication and dynamics and can thus be utilized (in transgenic research) as novel factors to improve sucrose content of sugarcane. Further investigation about the localization of expression and the activity of these identified transcripts may aid in developing a more lucid model of the sucrose accumulation pathway from source to sink and the check points involved therein. This in turn may help in building strategies for improving sucrose concentration of the culm.

References

REFERENCES

Agarie S, Miura A, Sumikura R, Tsukamoto S *et al.* (2002) Overexpression of C_4 PEPC caused O_2-insensitive photosynthesis in transgenic rice plants. *Plant Science*, 162: 257–265.

Ainsworth EA, Bush DR (2011) Carbohydrate export from the leaf: a highly regulated process and target to enhance photosynthesis and productivity. *Plant Physiology*, 155:64–69.

Albertson PL, Peters KF, Grof CPL (2001) An improved method for measurement of cell wall invertase activity in sugarcane tissue. *Australian Journal of Plant Physiology*, 28:323-328.

Alexander AG (1973) Sugarcane Physiology: A comprehensive study of the *Saccharum* source-to-sink system. Elsevier, Amsterdam and New York.

Aoki N, Hirose T, Takahashi S, Ono K *et al.* (1999) Molecular cloning and expression analysis of a gene for a sucrose transporter in maize (*Zea mays* L.). *Plant Cell Physiology*, 40:1072–1078.

Araus JI, Bort J, CecCadelli S, Grando S (1997) Relationship between leaf structure and Carbon isotope discrimination in field grown barley. *Plant Physiology and Biochemistry*, 35:533–541.

Arnon DI (1949) Copper enzymes in isolated chloroplasts. Polyphenoloxidase in *Beta vulgaris. Plant Physiology*, 24:1–15.

Arteca RN (1996) Plant Growth Substances: Principles and Applications. Chapman and Hall Inc., New York

Bakrim N, Prioul J-L., Deleens E, Rocher J-P *et al.* (1993) Regulatory phosphorylation of C_4 phospho*enol*pyruvate carboxylase. *Plant Physiology*, 101:891–897.

Balibrea ME, Martínez-Andújar C, Cuartero J, Bolarín MC *et al.* (2006) The high fruit soluble sugar content in wild *Lycopersicon* species and their hybrids with cultivars depends on sucrose import during ripening rather than on sucrose metabolism. *Functional Plant Biology*, 33(3):279-288.

Basu PS, Sharma A, Garg ID, Sukumaran NP (1999) Tuber sink modifies photosynthetic response in potato under water stress. *Environmental and Experimental Botany*, 42: 25–29.

Batta SK, Benkush, Sital JS, Mann APS (2007) Internodal soluble invertase isoenzymes in relation to sucrose storage and maturity behaviour in sugarcane cultivars. *SugarTech*, 9(1): 43-50.

Batta SK, Deeksha, Thind KS, Singh Pritpal *et al.* (2011) Variability in activity of sucrose metabolizing enzymes in relation to sucrose accumulation among parents and their progenies of sugarcane. *SugarTech* 13(2): 114-122.

Batta SK, Singh R (1986) Sucrose metabolism in sugarcane grown under varying climatic conditions: synthesis and storage of sucrose in relation to the activities of sucrose synthase, sucrose phosphate synthase and invertase. *Phytochemistry,* 25: 2431–2437.

Battistelli A, Adcock MD, Leegood RC (1991) The relationship between the activation state of sucrose-phosphate synthase and the rate of CO_2 assimilation in spinach leaves. *Planta,* 183: 620-622.

Baxter CJ, Foyer CH, Turner J, Rolfe SA *et al.* (2003) Elevated sucrose-phosphate synthase activity in transgenic tobacco sustains photosynthesis in older leaves and alters development. *Journal of Experimental Botany*, 54(389):1813-1820.

Beevers L, Schrader LE, Flesher D, Hageman RH (1965) The role of light and nitrate on the induction of nitrate reductase in radish cotyledons and maize seedlings. *Plant Physiology*, 40:691-698.

Berding N (1997) Clonal improvement of sugarcane based on selection for moisture content: fact or fiction. *Proceedings of the Australian Society of Sugarcane Technologists*, 19:245-253.

Bieleski RL (1960) The physiology of sugarcane III. Characteristics of sugar uptake in slices of mature and immature storage tissue. *Australian Journal of Biological Sciences,* 13:203-220.

Bihmidine S, Hunterlll CT, Johns CE, Koch KE *et al.* (2013) Regulation of assimilate import into sink organs: update on molecular drivers of sink strength. *Frontiers in Plant Science*, 4(177):1-15.

Bindon KA, Botha FC (2002) Carbon allocation to the insoluble fraction, respiration and triose–phosphate cycling in the sugarcane culm. *Physiologia Plantarum*, 116:12–19.

Black CC, Mustardy L, Sung SS, Kormanik PP *et al.* (1987) Regulation and roles for alternative pathways of hexose metabolism in plants. *Physiologia Plantarum,* 69(2):387-394.

Bolle C (2004) The role of GRAS proteins in plant signal transduction and development. *Planta*, 218 (5): 683-692.

Bosch S, Grof CPL, Botha FC (2004) Expression of neutral invertase in sugarcane. *Plant Science*, 166: 1125–1133.

Bosch S, Rohwer JM, Botha FC (2003) The sugarcane metabolome. *Proceedings of the South African Sugarcane Technologists Associaton*, 7:129-133.

Botha FC, Black KG (2000) Sucrose phosphate synthase and sucrose synthase activity during maturation of internodal tissue in sugarcane. *Australian Journal of Plant Physiology*, 27: 81–85.

Botha FC, Sawyer BJB, Birch RG (2001) Sucrose metabolism in the culm of transgenic sugarcane with reduced soluble acid invertase activity. *Proceedings of the International Society of Sugarcane Technologists*, 24: 588-591.

Bouché-Pillon S, Fleurat-Lessard P, Fromont J-C, Serrano R *et al.* (1994) Immunolocalisation of the plasma membrane H+-ATPase in minor veins of *Vicia faba* in relation to phloem loading. *Plant Physiology*, 105: 691–697.

Boussingault JB (1868) Agronomie, Chemie Agricole et Physiologie, 2nd edn. Mallet Bachelier, Paris, France

Brian PW, Elson GW, Hemming HG, Radley M (2006) The plant-growth-promoting properties of gibberellic acid, a metabolic product of the fungus *Gibberella fujikuroi. Journal of the Science of Food and Agriculture*, 5:602–612.

Buczynski SR, Thom M, Chourey P, Maretzki A (1993) Tissue Distribution and Characterization of Sucrose Synthase Isozymes in Sugarcane. *Journal of Plant Physiology*, 142(6):641-646.

Bull TA, Glasziou KT (1963) The evolutionary significance of sugar accumulation in *Saccharum. Australian Journal of Biological Sciences*, 16: 737–742.

Bull TA, Tovey DA (1974) Aspects of modelling sugarcane growth by computer simulation. *Proceedings of the International society for sugarcane technologists*, 165:1021-1032.

Burg SP, Bieleski RL (1962) The physiology of sugarcane V. Kinetics of sugar accumulation. *Australian Journal of Biological Sciences*, 15: 429–444.

Busse JS, Cabanas MF, Stiimart DP (2005) Developmental anatomy of adventitious shoot formation on Snapdragon (*Antirrhinum majus* L.) hypocotyls *in vitro. Journal of the American Society for Horticultural Science*, 130(2):147–151.

Calviño M, Miclaus M, Bruggmann R, Messing J (2009) Molecular markers for sweet sorghum based on microarray expression data. *Rice*, 2:129–142.

Cardoso-Silva CB, Costa EA, Mancini MC, Balsalobre TWA *et al.* (2014) *De novo* assembly and transcriptome analysis of contrasting sugarcane varieties. PLoSOne, 9: e88462.

Carpaneto A, Geiger D, Bamberg E *et al.* (2005) Phloem-localized, proton-coupled sucrose carrier ZmSUT1 mediates sucrose efflux under the control of the sucrose gradient and the proton motive force. *The Journal of Biological Chemistry*, 280:21437-21443.

Carson DL, Botha FC (2002) Genes expressed in sugarcane maturing intermodal tissue. *Plant Cell Reports*, 20: 1075–1081.

Casu RE, Manners JM, Bonnett GD, Jackson PA *et al.* (2005) Genomics approaches for the identification of genes determining important traits in sugarcane. *Field Crops Research*, 92:137–147.

Chandra A, Jain R, Rai RK, Solomon S (2011) Revisiting the source–sink paradigm in sugarcane. *Current Science*, 100(7): 978-980.

Chandra A, Jain R, Solomon S (2012) Complexities of invertase controlling sucrose accumulation and retention in sugarcane. *Current Science*, 102(6): 857–866.

Chandra A, Roopendra K, Sharma A, Jain R, Solomon S (2014) Partial gene sequence of soluble acid invertase gene from *Saccharum spontaneum*: A first report. *National Academy Science Letters*, 37(4):317-323.

Chandra A, Verma PK, Islam MN,Grisham MP, Jain R, Sharma A, Roopendra K, Singh K, Singh P, Verma I, Solomon S (2015) Expression analysis of genes associated with sucrose accumulation in sugarcane (*Saccharum* spp. hybrids) varieties differing in content and time of peak sucrose storage. *Plant Biology*, 17:608-617.

Chen JQ, Black CC (1992) Biochemical and immunological properties of alkaline invertase isolated from sprouting soybean hypocotyls. *Archives of Biochemistry and Biophysics*, 295:61–69.

Cheng WH, Burrus M, Audran J-C (1999) Sugars modulate an unusual mode of control of the cell-wall invertase gene (Incw1) through its 3’ untranslated region in a cell suspension culture of maize. *Proceedings of the National Academy of Sciences (USA)* 96: 10512–10517.

Cheng WH, Im KH, Chourey PS (1996a) Sucrose-phosphate synthase expression at the cell and tissue level is coordinated with sucrose sink-to-source transition in maize leaf. *Plant Physiology*, 111: 1021–1029.

Cheng WH, Talliercio EW, Chourey PS (1996b) The *miniature1* seed locus of maize encodes a cell wall invertase required for normal development of endosperm and maternal cells in the pedicel. *The Plant Cell*, 8: 971–983.

Chiou TJ, Bush DR (1998) Sucrose is a signal molecule in assimilate partitioning. *Proceedings of the National Academy of Science* (USA), 95:4784–4788.

Chourey PS, Taliercio EW, Carlson SJ, Ruan YL (1998) Genetic evidence that the two isoenzymes of sucrose synthase present in developing maize endosperm are critical, one for cell wall integrity and the other for starch biosynthesis. *Molecular and General Genetics*, 259:88–96.

Cole DF, Dobrenz AK, Massengale MA (1972) Effect of gibberellic acid on alfalfa (*Medicago sativa* L.). *Crop Science*, 12:674–676.

Conesa A, Götz S (2008) Blast2GO: A Comprehensive Suite for Functional Analysis in Plant Genomics. *International Journal of Plant Genomics*, 2008:619832, 1-12.

Cooley MB, Yang H, Dahal P, Mella RA *et al.* (1999) Vacuolar H^+-ATPase is expressed in response to gibberellin during tomato seed germination. *Plant Physiology*, 121:1339-1348.

D'Aoust MA, Yelle S, Nguyen-Quoc B (1999) Antisense inhibition of tomato fruit sucrose synthase decreases fruit setting and the sucrose unloading capacity of young fruit. *The Plant Cell*, 11: 2407–2418.

Dendsay JPS, Singh P, Dhawan AK, Sehti'.u HL (1995) Activities of internodal invertases during maturation of sugarcane stalks. *Sugarcane*, 6:17-19.

D'Hont A, Glaszmann JC (2001) Sugarcane genome analysis with molecular markers: A first decade of research. *Proceedings of the International Society of Sugarcane Technologists*, 24:556-559.

D'Hont A, Ison D, Alix K, Roux C *et al.* (1998) Determination of basic chromosome numbers in the genus *Saccharum* by physical mapping of ribosomal RNA genes. *Genome*, 41: 221–225.

Dufour P, Deu M, Grivet L, D'Hont A *et al.* (1997) Construction of a composite sorghum genome map and comparison with sugarcane, a related complex polyploidy. *Theoretical and Applied Genetics,* 94: 409–418.

Dunckelman PH, Legendre BL (1982) Guide to sugarcane breeding in the temperate zone. *Agricultural reviews and manuals*; ARM-S-United States Dept. of Agriculture, Science and Education Administration, Agricultural Research, Southern Region

Edwards D, Batley J (2010) Plant genome sequencing: applications for crop improvement. *Plant Biotechnology Journal*, 7:1–8.

Edwards GE, Furbank RT, Hatch MD, Osmond CB (2001) What does it take to be C_4? Lesson from the evolution of C_4 photosynthesis. *Plant Physiology* 125: 46–49.

Farrar JF (1993) Sink strength: What is it and how do we measure it? Introduction. *Plant, Cell & Environment*, 16(9):1015-1016.

Felix JM, Papini-Terzi FS, Rocha FR, Vêncio RZN *et al.* (2009) Expression Profile of Signal Transduction Components in a Sugarcane Population Segregating for Sugar Content. *Tropical Plant Biology*, 2:98–109.

Feng C, Chen M, Xu CJ, Bai L *et al.* (2012) Transcriptomic analysis of Chinese bayberry (*Myrica rubra*) fruit development and ripening using RNA-seq. *BMC Genomics*, 13:19

Feng H., Chen Q., Feng J., Zhang J. *et al.* (2007) Functional characterization of the Arabidopsis eukaryotic translation initiation factor 5A-2 that plays a crucial role in plant growth and development by regulating cell division, cell growth, and cell death. *Plant Physiology*, 144:1531-1545.

Fernandes AN, Benda GTA (1985) Distribution patterns of brix and fibre in the primary stalk of sugar cane. *Sugar Cane*, 5: 8-13.

Fernie JF, Willmitzer L, Trethewey RN (2002) Sucrose to starch: a transition in molecular plant physiology. *Trends in Plant Science*, 7: 35–41.

Franck N, Vaast P, Genard M, Dauzat J (2006) Soluble sugars mediate sink feedback down-regulation of leaf photosynthesis in field grown *Coffea arabica*. *Tree Physiology*, 26: 517–525.

Fu H, Kim S-Y, Park (1995) A potato Sus3 sucrose synthase gene contains a contextdependent 3’ element and a leader intron with both positive and negative tissue-specific effects. *The Plant Cell*, 7: 1395–1403.

Garsmeur O, Droc G, Antonise R, Grimwood J *et al.* (2018) A mosaic monoploid reference sequence for the highly complex genome of sugarcane. *Nature communications*, 9:2638.

Gayler KR, Glasziou KT (1972) Physiological Functions of Acid and Neutral Invertases in Growth and Sugar Storage in Sugar Cane. *Physiologia Plantarum*, 27(1):25-31.

Geigenberger P, Reimholz R, Geiger M, Merlo L *et al.* (1997) Regulation of sucrose and starch synthesis in potato tubers in response to short-term water deficit. *Planta*, 201: 502–518.

Geigenberger P, Stitt M (1991) A ‘futile’ cycle of sucrose synthesis and degradation is involved in regulating partitioning between sucrose, starch and respiration in cotyledons of germinating *Ricinus communis* L. seedlings when phloem transport is inhibited. *Planta*, 185(1):81-90.

Geigenberger P, Stitt M (1993) Sucrose synthase catalyses a readily reversible reaction *in vivo* in developing potato tubers and other plant tissues. *Planta*, 189: 329–339.

Gibson SI (2005) Control of plant development and gene expression by sugar signaling. *Current Opinion in Plant Biology*, 8: 93–102.

Glassop D, Roessner U, Bacic A, Bonnett GD (2007) Changes in the sugarcane metabolome with stem development. Are they related to sucrose accumulation? *Plant Cell Physiology*, 48, 573–584.

Glasziou KT, Bull TA (1965) The relation between total invertase activity and internode expansion in sugarcane stalks. *Proceedings of the International Society of Sugar Cane Technologists*, 12: 575–581.

Glasziou KT, Gayler KR (1972) Storage of sugars in stalks of sugarcane. *Botanical Reviews*, 38: 471–490.

Godt D, Roitsch T (2006) The developmental and organ specific expression of sucrose cleaving enzymes in sugarbeet suggests a transition between apoplasmic and symplasmic phloem unloading in the tap roots. *Plant Physiology and Biochemistry*, 44:656–665.

Godt DE, Roitsch T (1997) Differential regulation of a tomato invertase gene family suggests an important function of extracellular isoenzymes in establishing and maintaining sink metabolism. *Plant Physiology*, 115: 273–282.

Goldner W, Thom M, Maretzki A (1991) Sucrose metabolism in sugarcane cell suspension cultures. *Plant Science*, 73(2):143-147.

Goldschmidt EE, Huber SC (1992) Regulation of photosynthesis by end-product accumulation in leaves of plants storing starch, sucrose and hexose sugars. *Plant Physiology*, 99:1443-1448.

Gonza´lez M, Cejudo FJ (2007) Gibberellin-regulated expression of neutral and vacuolar invertase genes in petioles of sugar beet plants. *Plant Science*, 172: 839–846.

Gonza´lez M, Roitsch T, Cejudo FJ (2005) Circadian and developmental regulation of vacuolar invertase expression in petioles of sugar beet plant. *Planta*, 221: 386–395.

Grabherr MG, Haas BJ, Yassour M, Levin JZ *et al.* (2011) Full-length transcriptome assembly from RNA-Seq data without a reference genome. *Nature Biotech*, 29:644-652.

Grivet L, Arruda P (2002) Sugarcane genomics: depicting the complex genome of an important tropical crop. *Current Opinion in Plant Biology*, 5:122-127.

Grivet L, D'Hont A, Roques D, Feldmann P *et al.* (1996) RFLP mapping in cultivated sugarcane (*Saccharum* spp.): Genome organization in a highly polyploid and aneuploid interspecific hybrid. *Genetics*, 142: 987–1000.

Groenewald J-H, Botha FC (2007) Down regulation of pyrophosphate: fructose 6-phosphate 1-phosphotransferase (PFP) activity in sugarcane enhances sucrose accumulation in immature internodes. *Transgenic Research*, 17:85–92.

Grof CPL, Albertson PL, Bursle J, Perroux JM *et al.* (2007) Sucrose-phosphate synthase, a biochemical marker of high sucrose accumulation in sugarcane. *Crop Science*, 47:1530–1539.

Grof CPL, Campbell JA (2001) Sugarcane sucrose metabolism: scope for molecular manipulation. *Australian Journal of Plant Physiology*, 28(1):1-12.

Grof CPL, Campbell JA (2005) Molecular manipulation of sucrose metabolism in sugarcane.

In: Solomon S, Grewal SS, Li Yang-rui, Magarey RC, Rao GP (eds.) Sugarcane: Production management and agro-industrial imperatives, pp: 507-521, International Book Distributing Company .

Grof CPL, Knight DP, McNeil SD, Lunn JE *et al.* (1998) A modified assay method shows leaf sucrose-phosphate synthase activity is correlated with leaf sucrose content across a range of sugarcane varieties. *Australian Journal of Plant Physiology*, 25(4):499-502.

Grof CPL, So CTE, Perroux JM, Bonnett GD *et al.* (2006) The five families of sucrose-phosphate synthase genes in *Saccharum* spp. are differentially expressed in leaves and stem. *Functional Plant Biology*, 33: 605–610.

Gutiérrez-Miceli FA, Morales-Torres R, Espinosa-Castañeda JY, Rincón-Rosales R *et al.* (2004) Effects of parital defoliation on sucrose accumulation, enzyme activity and agronomic parameters in sugar cane (*Saccharum* spp.). *Journal of Agronomy and Crop Science*, 190: 256–261.

Haberlandt G (1884) *Physiological Plant Anatomy*. London: MacMillan

Hageman RH, Flesher D, Gitter A (1961) Diurnal variations and other light effects influencing the activity of nitrate reductase and nitrogen metabolism in corn. *Crop Science*, 1:201-204.

Hänggi E, Fleming AJ (2001) Sucrose synthase expression pattern in young maize leaves: implications for phloem transport. *Planta*, 214: 326–329.

Harper JE, Hageman RH (1972) Canopy and seasonal profiles of nitrate reductase in soybeans (*Glycine max* L. Meff.). *Plant Physiology*, 49: 146-154.

Hartt CE (1963) Translocation as a factor in photosynthesis. *Die Naturwissenschaften*, 50:666-667.

Hartt CE, Burr GO (1967) Factors affecting photosynthesis in sugarcane. *Proceedings of the International society for sugarcane technologists,* 12:590-609.

Hatch MD (1964) Sugar accumulation by sugarcane storage tissue: the role of sucrose phosphate. *Biochemical Journal*, 93: 521–526.

Hatch MD, Glasziou KT (1963) Sugar Accumulation Cycle in Sugar Cane. II. Relationship of Invertase Activity to Sugar Content and Growth Rate in Storage Tissue of Plants Grown in Controlled Environments. *Plant Physiology*, 38(3): 344–348.

Hatch MD, Glasziou KT (1964) Direct evidence for translocation of sucrose in sugar cane leaves and stems. *Plant Physiology*, 39: 180–184.

Hatch MD, Sacher JA, Glasziou KT (1963) Sugar accumulation cycle in sugarcane. I. Studies on enzymes of the cycle. *Plant Physiology,* 38: 338–343.

Hatch MD, Slack CR (1966) Photosynthesis in sugar cane leaves. A new carboxylation reaction and the pathway of sugar formation. *Biochemical Journal,* 101:102–111.

Hawker JS (1985) Sucrose In: Dey, P.M. and R.A. Dixon (eds.) *Biochemistry of storage carbohydrates in green plants*, pp: 1–51, Academic Press, Orlando. F.L

Hawker JS, Hatch MD (1965) Mechanism of sugar storage by mature stem tissue of sugarcane. *Physiologia Plantarum*, 18: 444–453.

Hellman H, Barker L, Funk D, Frommer (2000) The regulation of assimilate allocation and transport. *Australian Journal of Plant Physiology*, 27: 583–594.

Herbers K, Sonnewald U (1998) Molecular determinants of sink strength. *Current Opinion in Plant Biology*, 1:207–216.

Herold A (1980) Regulation of photosynthesis by sink activity-the missing link. *New Phytologist*, 86:131-144.

Hesse H, Sonnewald U, Willmitzer L (1995) Cloning and expression analysis of sucrose phosphate synthase from sugarbeet (*Beta vulgaris* L.). *Molecular and General Genetics,* 247(4):515-520.

Hirsch S, Oldroyd GED (2009) GRAS-domain transcription factors that regulate plant development. *Plant Signal Behaviour*, 4(8): 698–700.

Ho LC (1988) Metabolism and compartmentation of imported sugars in sink organs in relation to sink strength. *Annual Review of Plant Physiology and Plant Molecular Biology*, 39: 355–378.

Ho LC, Thornley JHM (1978) Energy Requirements for Assimilate Translocation from Mature Tomato Leaves. *Annals of Botany*, 42(178):481-483.

Hoffmann-Thoma G, Hinkel K, Nicolay P, Willenbrink J (1996) Sucrose accumulation in sweet sorghum stem internodes in relation to growth. *Physiologia Plantarum,* 97:277–284.

Horne (1903) Journal of the American chemical society In: Spencer GL and Meade GP (1959) Cane Sugar Handbook, pp. 390, John Wiley and Sons Inc. Newyork, Chapman and Hall ltd. London

Huang D, Gao Y, Gui Y, Chen Z *et al.* (2016) Transcriptome of High-Sucrose Sugarcane Variety GT35, 18(5):520-528.

Huang JW, Chen JT, Yu WP, Shyur LG *et al.* (1996) Complete structures of three rice sucrose synthase isogenes and differential regulation of their expression. *Bioscience, Biotechnology, and Biochemist*, 60: 233–239.

Huber SC (1989) Biochemical mechanism for regulation of sucrose accumulation in leaves during photosynthesis. *Plant Physiology*, 91: 656–662.

Huber SC, Akazawa T (1986) A novel sucrose synthase pathway for sucrose degradation in cultured sycamore cells. *Plant Physiology*, 81:1008-1013.

Huber SC, Huber JL (1996) Role and regultion of Sucrose Phosphate Synthase in higher plants. *Annual Review of Plant Physiology and Plant Molecular Biology*, 47: 431-444.

Hussain A, Khan ZI, Ghafoor MY, Ashraf M *et al.* (2004) Sugarcane, sugar metabolism and some abiotic stresses. *International Journal of Agriculture and Biology*, 6(4):732-742.

Iglesias DJ, Lliso I, Tadeo FR, Talon M (2002) Regulation of photosynthesis through source: sink imbalance in citrus is mediated by carbohydrate content in leaves. *Physiologia Plantarum*. 116:563–572.

Inman-Bamber NG, Bonnett GD, Spillman MF, Hewitt ML *et al.* (2008) Increasing sucrose accumulation in sugarcane by manipulating leaf extension and photosynthesis with irrigation. *Australian Journal of Agricultural Research*, 59:13-26.

Iqbal N, Nazar R, Khan MIR, Masood A *et al.* (2011) Role of gibberellins in regulation of source-sink relations under optimal and limiting environmental conditions. *Current Science*, 100: 998-1007.

Izumi K, Yamaguchi I, Wada A, Oshio H *et al.* (1984) Effect of a new plant growth retardant (E)- 1-(4-chlorophenyl)- 4,4-dimethyl-2-(1,2,4-triazol-1-yl)- l-pentane-3-ol(S-3307) on the growth and gibberellin content of rice plant. *Plant Cell Physiology*, 25: 611-617.

Jackson PA (2005) Breeding for improved sugar content in sugarcane. *Field Crops Research*, 92: 277–290.

Jackson PA, Bonnett G, Chudleigh P, Hogarth M *et al.* (2000) The relative importance of cane yield and traits affecting CCS in sugarcane varieties. *Proceedings of the Australian Society of Sugarcane Technologists*, 22:23-29.

Jacobsen KR, Fisher DG, Maretzki A, Moore PH (1992) Developmental changes in the anatomy of the sugarcane stem in relation to phloem unloading and sucrose storage. *Botanica Acta*, 105: 70–80.

Jeanneau M, Vidal J, Gousset-Dupont A, Lebouteiller B *et al.* (2002) Manipulating PEPC levels in plants. *Journal of Experimental Botany*, 53: 1837–1845.

Jeannette E, Reyss A, Gregoery N, Gantet P *et al.* (2000) Carbohydrate metabolism in heat-girdled maize source leaf. *Plant Cell Environment*, 23:61–69.

Ji X, Ende WV, Laere AV, Cheng S *et al.* (2005) Structure, Evolution, and Expression of the Two Invertase Gene Families of Rice. *Journal of Molecular Evolution*, 60:615–634.

Kapur R, Duttamajumder SK, Rao KK (2011) A breeder's perspective on the tiller dynamics in sugarcane. *Current Science*, 100(2):183–189.

Kaufman PB, Ghosheh NS, LaCroix JD, Soni SL *et al.* (1973) Regulation of invertase levels in *Avena* stem segments by gibberellic acid, sucrose, glucose, and fructose. *Plant Physiology*, 52:221–228.

Kaur S, Batta SK, Sital JS, Sharma KP *et al.* (2002) Partial purification and properties of soluble invertase isoforms from sugarcane storage tissue. *Indian sugar*, 51(2): 851-857.

Kelley LA, Mezulis S, Yates CM, Wass MN *et al.* (2015) The Phyre2 web portal for protein modeling, prediction and analysis. *Nature Protocols*, 10:845–858.

Khan NA (1996) Effect of gibberellic acid on carbonic anhydrase, photosynthesis, growth and yield of mustard. *Biologia Plantarum*, 38:145–147.

Khan NA, Ansari HR, Mobin M (1996) Effect of gibberellic acid and nitrogen on carbonic anhydrase activity and mustard biomass. *Biologia Plantarum*, 38:601–603.

Khan NA, Mir R, Khan M, Javid S *et al.* (2002) Effects of gibberellic acid spray on nitrogen yield efficiency of mustard grown with different nitrogen levels. *Plant Growth Regulation*, 38:243–247.

Khan NA, Mobin M, Samiullah (2005) The influence of gibberellic acid and sulphur fertilization rate on growth and S-use efficiency of mustard (*Brassica juncea* L.). *Plant Soil*, 270:269–274.

Khan NA, Singh S, Nazar R, Lone PM (2007) The source– sink relationship in mustard. *Asian and Australasian Journal of Plant Science and Biotechnology*, 1:10–18.

Kim HY, Watanabe H, Abe T, Suzuki Y (1988) Interaction of the growth retardant Uniconazol (S-07) and GA_3 on the development and flowering of Zinnia under different day lengths. *Chemical Regulation in Plants,* 23: 83-88.

Kim J-Y, Mahé A, Guy S, Brangeon J *et al.* (2000b) Characterisation of two members of the maize gene family, *Incw3* and *Incw4*, encoding cell wall invertases. *Gene*, 245: 89–102.

King SP, Lunn JE, Furbank RT (1997) Carbohydrate content and enzyme metabolism in developing canola siliques. *Plant Physiology*, 114: 153–160.

Kingston-Smith AH, Galtier N, Pollock CJ, Foyer CH (1998) Soluble acid invertase activity in leaves is independent of species differences in leaf carbohydrates, diurnal sugar profiles and paths of phloem loading. *New Phytologist*, 139:283-292.

Klann EM, Hall B, Bennett AB (1996) Antisense Acid Invertase (TIV1) Gene Alters Soluble Sugar Composition and Size in Transgenic Tomato Fruit. *Plant Physiology,* 112:1321-1330.

Koch K (2004) Sucrose metabolism: regulatory mechanisms and pivotal roles in sugar sensing and plant development. *Current Opinion in Plant Biology,* 7:235–246.

Koch KE (1996) Carbohydrate modulated gene expression in plants. *Annual review of Plant Physiology and Plant Molecular Biology*, 47:509-540.

Kogami M, Shono M, Koike T, Yanigisawa S *et al.* (1994) Molecular and physiological evaluation of transgenic tobacco plants expressing a maize phospho*enol*pyruvate carboxylase gene under the control of the cauliflower mosaic virus 35S promoter. *Transgenic Research*, 3:287–296.

Komor E (2000) Source physiology and assimilate transport: the interaction of sucrose metabolism, starch storage and phloem export in source leaves and the effects on sugar status in phloem. *Australian Journal of Plant Physiology*, 27: 497–505.

Komor E, Thom M, Maretski A (1981) The mechanism of sugar uptake by sugarcane suspension cells. *Planta*, 153:181–192.

Kortschak HP, Hartt CE, Burr GO (1965) Carbon dioxide fixation in sugarcane leaves. *Plant Physiology*, 40:209-213.

Kozłowska M, Rybus-Zając M, Stachowiak J, Janowska B (2007) Changes in carbohydrate contents of *Zantedeschia* leaves under gibberellin-stimulated flowering. *Acta Physiologiae Plantarum*, 29:27–32.

Krapp A, Hofmann B, Schafer C *et al.* (1993) Regulation of the expression of rbcS and other photosynthetic genes by carbohydrates: a mechanism for the 'sink regulation' of photosynthesis? *The Plant Journal*, 6:817-828.

Krapp A, Stitt M (1995) An evaluation of direct and indirect mechanisms for the 'sink-regulation' of photosynthesis in spinach: changes in gas exchange, carbohydrates, metabolites, enzyme activities and steady-state transcript levels after cold-girdling source leaves. *Planta*, 195(3): 313-323.

Krause K, Stitt M (1992) Sucrose-6-phosphate levels in spinach leaves and their effects on sucrose-phosphate synthase. *Phytochemistry*, 31(4): 1143-1146.

Krausgrill S, Greiner S, Koster U, Vogel R *et al.* (1998) In transformed tobacco cells the apoplasmic invertase inhibitor operates as a regulatory switch of cell wall invertase. *The Plant Journal*, 13: 275–280.

Kühn C, Grof C (2010) Sucrose transporters of higher plants. *Current Opinion in Plant Biology,* 13:1–11.

Lakshmanan P, Geijskes RJ, Aitken KS, Grof CLP (2005) Sugarcane biotechnology: the challenges and opportunities. *In Vitro Cellular and Developmental Biology*, 41: 345–363.

Lalonde S, Boles E, Hellmann H, Barker L *et al.* (1999) The dual function of sugar carriers: transport and sugar sensing. *The Plant Cell*, 11: 707–726.

Lalonde S, Tegeder M, Throne-Holst M *et al.* (2003) Phloem loading and unloading of sugars and amino acids. *Plant Cell Environment*, 26:37-56.

Lalonde S, Wipf D, Frommer WB (2004) Transport mechanisms for organic forms of carbon and nitrogen between source and sink. *Annual Review of Plant Biology,* 55:341–372.

Laporte MM, Galagan JA, Shapiro JA, Boersig MR *et al.* (1997) Sucrose phosphate synthase activity and yield analysis of tomato plants transformed with maize sucrose phosphate synthase. *Planta*, 203(2):253-259.

Lee HC, Lai K, Lorenc MT, Imelfort M (2012) Bioinformatics tools and databases for analysis of next-generation sequence data. *Briefings in Functional Genomics*, 2(1):12-24.

Lee HS, Sturm A (1996) Purification and characterization of neutral and alkaline invertase from carrot. *Plant Physiology*, 112: 1513–1522.

Leggewie G, Kolbe A, Lemoine R, Roessner U *et al.* (2003) Overexpression of the sucrose transporter SoSUT1 in potato results in alterations in leaf carbon partitioning and in tuber metabolism but has little impact on tuber morphology. *Planta*, 217: 158–167.

Li C-Y, Wiess D, Goldschmidt EE (2003) Girdling affects carbohydrate-related gene expression of leaves, bark and roots of alternate-bearing citrus trees. *Annals of Botany*, 92:137–143.

Li X, Xing J, Gianfagna TJ, Janes HW (2002) Sucrose regulation of ADP-glucose pyrophosphorylase subunit genes transcript levels in leaves and fruits. *Plant Science*, 162: 239–244.

Lian L, Wang X, Zhu Y *et al.* (2014) Physiological and photosynthetic characteristics of indica Hang2 expressing the sugarcane PEPC gene. *Molecular Biology Reports*, 41:2189–2197.

Lingle SE (1989) Evidence for the Uptake of Sucrose Intact into Sugarcane Internodes. *Plant Physiology*, 90:6-8.

Lingle SE (1999) Sugar metabolism during growth and development in sugarcane internodes. *Crop Science*, 39: 480–486.

Lingle SE, Smith RC (1991) Sucrose Metabolism Related to Growth and Ripening in Sugarcane Internodes. *Crop Science*, 31(1):172-177.

Lontom W, Kosittrakun M, Lingle SE (2008) Relationship of acid invertase activities to sugar content in sugarcane internodes during ripening and after harvest. *Thai Journal of Agricultural Science*, 41:143-151.

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the Folin phenol reagent. *The Journal of Biological Chemistry*, 193:265–275.

Ludewig F, Flügge U (2013) Role of metabolite transporters in source-sink carbon allocation. *Frontiers in Plant Science*, 4(231):1-16.

Lunn JE, Furbank RT (1997) Localisation of sucrose-phosphate synthase and starch in leaves of C_4 plants. *Planta*, 202(1):106-111.

Lunn JE, Furbank RT (1999) Sucrose biosynthesis in C_4 plants. *New Phytologist*, 143:221–237.

Lunn JE, MacRae E (2003) New complexities in the synthesis of sucrose. *Current Opinion in Plant Biology*, 6:208–214.

Ma H, Albert HA, Paull R, Moore PH (2000) Metabolic engineering of invertase activities in different subcellular compartments affects sucrose accumulation in sugarcane. *Australian Journal of Plant Physiology*, 27:1021–1030.

Mao L, Que F, Wang G (2006) Sugar metabolism and involvement of enzymes in sugarcane (*Saccharum officinarum* L.) stems during storage. *Food Chemistry*, 98(2):338-342.

Marcelis LF (1996) Sink strength as a determinant of dry matter partitioning in the whole plant. *Journal of Experimental Botany*, 47:1281–1291.

Mardis ER (2008) The impact of next-generation sequencing technology on genetics. *Trends in Genetics*. 24(3):133-141.

Martin T, Frommer WB, Salanoubat M, Willmitzer L (1993) Expression of an Arabidopsis sucrose synthase gene indicates a role in metabolization of sucrose both during phloem loading and sink organs. *The Plant Journal*, 4(2):367-377.

McCollum TG, Huber DJ, Cantliffe DJ (1988) Soluble sugar accumulation and activity of related enzymes during muskmelon fruit development. *Journal of the American Society for Horticultural Science*, 111: 399-403.

McCormick AJ, Cramer MD, Watt DA (2006) Sink strength regulates photosynthesis in sugarcane. *New phytologist*, 171: 759-770.

McCormick AJ, Cramer MD, Watt DA (2008b) Differential Expression of Genes in the Leaves of Sugarcane in Response to Sugar Accumulation. *Tropical Plant Biology*, 1:142-148.

McCormick AJ, Cramer MD, Watt DA (2008c) Regulation of photosynthesis by sugars in sugarcane leaves. *Journal of Plant Physiology*, 165(17):1817-1829.

McCormick AJ, Cramer MD, Watt DA (2008d) Changes in Photosynthetic Rates and Gene Expression of Leaves during a Source–Sink Perturbation in Sugarcane. *Annals of Botany*, 101:89–102.

McCormick AJ, Watt DA, Cramer MD (2009) Supply and demand: sink regulation of sugar accumulation in sugarcane. *Journal of Experimental Botany*, 60(2): 357–364.

Moore B, Zhou L, Rolland F, Hall Q *et al.* (2003) Role of *Arabidopsis* glucose sensor HXK1 in nutrient, light, and hormonal signaling. *Science,* 300: 332–336.

Moore PH (1995) Temporal and spatial regulation of sucrose metabolism in the sugarcane stem. *Australian Journal of Plant Physiology*, 22: 661–679.

Moore PH (2005) Integration of sucrose accumulation processesacross hierarchial scales: towards developing an understanding of the gene-to-crop continuum. *Field Crops Research*, 92:119-135.

Moore PH, Botha FC, Furbank RT, Grof CPL (1997) Potential for overcoming physio-biochemical limits to sucrose accumulation. In: Keating BA, Wilson JR, eds. Intensive sugarcane production: meeting the challenges beyond 2000, pp.:141–155, Wallingford: CAB International.

Moore PH, Maretzki E (1996) Sugarcane. In: Zamski E, Schaffer AA (eds) Photoassimilate distribution in plants and crops: source-sink relationships, pp.:643-669, Marcel Dekker Inc., New York, Basel, Hong Kong.

Moore TC (1989) Biochemistry and Physiology of Plant Hormones. Springer-Verlag Inc, New York

Morris DA, Arthur ED (1985) Effects of gibberellic acid on patterns of carbohydrate distribution and acid invertase activity in *Phaseolus vulgaris*. *Physiologia Plantarum*, 65:257–262.

Mortazavi A, Williams BA, McCue K, Schaeffer L *et al.* (2008) Mapping and quantifying mammalian transcriptomes by RNA-Seq. *Nature Methods*, 5:621-628.

Muller-Rober B, Sonnewald U, Willmitzer L (1992) Inhibition of the ADP-glucose pyrophosphorylase in transgenic potatoes leads to sugar-storing tubers and influences tuber formation and expression of tuber storage protein genes. *The EMBO Journal*, 11(4):1229-1238.

Münch E (1930) Die Stoffbewegunen in der Pflanze. Verlag von Gustav Fischer, Jena, Germany

Nelson N (1944) A photometric adaptation of Somogyi method for the determination of glucose. *Journal of Biological Chemistry,* 153:375-380.

Nguyen-Quoc B, Foyer CH (2001) A role for “futile cycles” involving invertase and sucrose synthase metabolism of tomato fruit. *Journal of Experimental Botany*, 52: 881–889.

Nguyen-Quoc B, N’Tchobo H, Foyer CH, Yelle S (1999) Over-expression of sucrose phosphate synthase increases sucrose unloading in transformed tomato fruit. *Journal of Experimental Botany,* 50:785–791.

Nicholas JC, Harper JE, Hageman RH (1976) Nitrate Reductase Activity in Soybeans (*Glycine max* [L.J Merr.): Effects of light and temperature. *Plant Physiology*, 58:731-735.

Obenland DM, Simmen U, Boller T, Wiemken A (1993) Purification and Characterization of Three Soluble Invertases from Barley *(Hordeum vulgare* 1.) Leaves. *Plant Physiology*, 101: 1331-1339.

Ohyama A, Ito H, Sato T, Nishimura S *et al.* (1995) Suppression of Acid Invertase Activity by Antisense RNA Modifies the Sugar Composition of Tomato Fruit. *Plant and Cell Physiology*, 36(2):369-376.

Pammenter NW, Allison JCS (2002) Effects of treatments potentially influencing the supply of assimilate on its partitioning in sugarcane. *Journal of Experimental Botany*, 53:123–129.

Pan R, Dong Y (1995) Photosynthesis and respiration In: Pan R, Dong Y, Plant Physiology, pp.: 67-143, 3rd Ed. *Higher educational press,* Beijing.

Pan Y, Luo H, Li Y (2009) Soluble acid invertase and sucrose phosphate synthase: key enzymes in regulating sucrose accumulation in sugarcane stalks. *Sugar Tech*, 11(1):28-33.

Pandey RM, Singh R (2010) Genetic studies for biochemical and quantitative characters in grain amaranth(*Amaranthus hypochondriacus* L.). *PlantOmics Journal*, 3(4):129-134.

Papini-Terzi FS, Rocha FR, Vêncio RZN, Felix JM (2009) Sugarcane genes associated with sucrose content. *BMC Genomics*, 10:120

Patrick J (1997) Phloem unloading: sieve element unloading and post-sieve element transport. *Annual Review of Plant Physiology and Plant Molecular Biology,* 48:191–222.

Patrick JW (1990) Sieve element unloading: cellular pathway, mechanisms and control. *Physiologia Plantarum.*78:298-308.

Patrick JW, Botha FC, Birch RG (2013) Metabolic engineering of sugars and simple sugar derivatives in plants. *Plant Biotechnology Journal,* 11:142–156.

Paul MJ, Driscoll SP (1997) Sugar repression of photosynthesis: the role of carbohydrates in signalling nitrogen deficiency through source:sink imbalance. *Plant, Cell and Environment*, 20:110-116.

Paul MJ, Foyer CH (2001) Sink regulation of photosynthesis. *Journal of Experimental Botany*, 52:1383–1400.

Paul MJ, Pellny TK (2003) Carbon metabolite feedback regulation of leaf photosynthesis and development. *Journal of Experimental Botany*, 54:539–547.

Pego JV, Kortsee AJ, Huijser C, Smeekens SCM (2000) Photosynthesis, sugars and the regulation of gene expression. *Journal of Experimental Botany*, 51: 407–416.

Pressman E, Shaked R (1991) Regulation of stem elongation in Chinese cabbage by inflorescence removal and application of growth regulators. *Journal of Plant Growth Reguation*, 10:225–228.

Pysh LD, Wysocka Diller J, Camilleri C, Bouchez D *et al.* (1999) The GRAS family in Arabidopsis: sequence characterization and basic expression analysis of the scarecrow like genes. *The Plant Journal,* 18(1): 111-119.

Qin L, Trouverie J, Chateau-Joubert S, Simond-Côte E *et al.* (2004) Involvement of the *Ivr2*-invertase in the perianth during maize kernel development under water stress. *Plant Science*, 166: 371–379.

Quick WP, Schaffer AA (1996) Sucrose metabolism in sources and sinks In: Zamski E, Schafer AA (eds), Photoassimilate Distribution in Plants and Crops Source-Sink Relationships, Marcel Dekker Inc. NewYork, USA.

Rae AL, Grof CPL, Casu RE *et al.* (2005a) Sucrose accumulation in the sugarcane stem: pathways and control points for transport and compartmentation. *Field Crops Research*, 92:159-163.

Rae AL, Jackson MA, Nguyen CH, Bonnett GD (2009) Functional specialization of vacuoles in sugarcane leaf and stem. *Tropical Plant Biology*, 2(1):13—22.

Rae AL, Perroux J, Grof CPL (2005b) Sucrose partitioning between vascular bundles and storage parenchyma in the sugarcane stem: a potential role for the ShSUT1 sucrose transporter. *Planta,* 220: 817–825.

Rai RK, Tripathi N, Gautam D, Singh P (2017) Exogenous Application of *Ethrel* and Gibberellic Acid Stimulates Physiological Growth of Late Planted Sugarcane with Short Growth Period in Sub-tropical India. *Journal of Plant growth regulation*, 36(2):472-486.

Raines CA (2011) Increasing photosynthetic carbon assimilation in C_3 plants to improve crop yield: current and future strategies. *Plant Physiology,* 155:36–42.

Ramon M, Rolland F (2007) Plant development: introducing trehalose metabolism. *Trends in Plant Science*, 12(5):185-188.

Ramon M, Rolland F, Sheen J (2008) Sugar sensing and signaling. *Arabidopsis Book* 6:e0117

Ricardo CPP, ap Rees T (1970) Invertase activity during the development of carrot roots. *Phytochemistry*, 9: 239-247.

Ritter K, Jordan D, Chapman S, Godwin I *et al.* (2008) Identification of QTL for sugar-related traits in a sweetx grain sorghum (*Sorghum bicolor* L. Moench) recombinant in bred population. *Molecular breeding,* 22:367–384.

Roach BT (1989) Origin and improvement of the genetic base of sugarcane. *Proceedings of Australian Society of Sugar Cane Technologists*, 11:34–47.

Robinson-Beers K, Evert RF (1991) Ultrastructure of and plasmodesmatal frequency in mature leaves of sugarcane. *Planta*, 184:291–306.

RocherJ-P, Prioul SL, Leehamy A, Reyss A *et al.* (1989) Genetic variability in carbon fixation, sucrose-P synthase and ADP-glucose pyrophosphorylase in maize plants of differing growth rate. *Plant Physiology*, 89:416–420.

Rodriguez-Penagos JL, Munoz-Clares R (1999) Response of phospho*enol*pyruvate carboxylase from maize leaves to moderate water deficit. *Journal of Plant Physiology*, 155: 631–638.

Roitsch T (1999) Source-sink regulation by sugar and stress. *Current Opinion in Plant Biology*, 2:198-206.

Roitsch T, Balibrea ME, Hofmann M, Proels R (2003) Extracellular invertase: key metabolic enzyme and PR protein. *Journal of Experimental Botany,* 54:513–524.

Roitsch T, Bittner M, Godt DE (1995) Induction of apoplastic invertase of *Chenopodium rubrum* by *D*-glucose and a glucose analog and tissue-specific expression suggest a role in sink-source regulation. *Plant Physiology,* 108: 285–294.

Rolland F, Baena-Gonzalez E, Sheen J (2006) Sugar sensing and signaling in plants: conserved and novel mechanisms. *Annual Review of Plant Biology*, 57:675–709.

Rolland F, Moore R, Sheen J (2002) Sugar sensing and signaling in plants. *The Plant Cell,* 14:185–205.

Roper TR, Williams L (1989) Net CO_2 assimilation and carbohydrate partitioning of grapevine leaves in response to trunk girdling and gibberellic acid application. *Plant Physiology*, 89:1136–1140.

Rose S, Botha FC (2000) Distribution patterns of neutral invertase and sugar content in sugarcane internodal tissues. *Plant Physiology and Biochemistry,* 38: 819–824.

Rossouw D, Bosch S, Kossmann J, Botha FC *et al.* (2007) Downregulation of neutral invertase activity in sugarcane cell suspension cultures leads to a reduction in respiration and growth and an increase in sucrose accumulation. *Functional Plant Biology*, 34:490–498.

Rossouw D, Kossmann J, Botha FC, Groenewald J (2010) Reduced neutral invertase activity in the culm tissues of transgenic sugarcane plants results in a decrease in respiration and sucrose cycling and an increase in the sucrose to hexose ratio. *Functional Plant Biology,* 37:22–31.

Roth-Bejerano N, Lips SH (1970) Seasonal Characteristics of the Daily Fluctuation in Nitrate Reductase Activity. *Physiologia Plantarum*, 23(3):530-535.

Ruan YL, Llewellyn DJ, Furbank RT (2003) Suppression of sucrose synthase gene expression respresses cotton fiber initiation, elongation and seed development. *The Plant Cell,* 15: 952–964.

Russin WA, Evert RF, Vanderveer TD *et al.* (1996) Modification of a specific class of plasmodesmata and loss of sucrose export ability in the sucrose export defective1 maize mutant. *The Plant Cell*, 8:4645-4658.

Sachdeva M, Bhatia S, Batta SK (2011) Sucrose accumulation in sugarcane: a potential target for crop improvement. *Acta Physiologiae Plantarum*, 33:1571–1583.

Sacher JA, Hatch MD, Glasziou KT (1963) Sugar accumulation cycle in sugar cane III. Physical and metabolic aspects of cycle in immature storage tissues. *Plant Physiology*, 39: 348–354.

Schäfer WE, Rohwer JM, Botha FC (2004) Protein-level expression and localization of sucrose synthase in the sugarcane culm. *Physiologia Plantarum*, 121:187–195.

Sehtiya HL, Dendsay JPS (2000) Sucrose uptake and accumulation by internode tissue of high and low sugar cultivars of sugarcane from exogenously supplied sucrose medium. *Indian Journal of Plant Physiology*, 5(3):223-227.

Sehtiya HL, Dendsay JPS, Dhawan AK (1991) Intemodal invertases and stalk maturity in sugarcane. *Journal of Agricultural Science*, 166:239-243.

Shaner DL, Boyer JS (1976) Nitrate Reductase Activity in Maize (*Zea mays* L.) Leaves: Regulation by nitrate flux. *Plant Physiology*, 58:499-504.

Sheen J (2001) Signal transduction in maize and Arabidopsis mesophyll protoplasts. *Plant Physiology,* 127: 1466–1475.

Slewinski TL (2012) Non-structural carbohydrate partitioning in grass stems: a target to increase yield stability, stress tolerance, and biofuel production. *Journal of Experimental Botany,* 63:4647–4670.

Slewinski TL, Braun DM (2010) Current perspectives on the regulation of whole-plant carbohydrate partitioning. *Plant Science*, 178: 341-349.

Slewinski TL, Meeley R, Braun DM (2009) *Sucrose transporter 1* functions in phloem loading in maize leaves. *Journal of Experimental Botany*, 60:881-892.

Smeekens S (2000) Sugar-induced signal transduction in plants. *Annual Review of Plant Physiology and Plant Molecular Biology,* 51:49–81.

Snyman SJ, Baker C, Huckett BI, McFarlane SA *et al.* (2008) South African Sugarcane Research Institute: embracing biotechnology for crop improvement research. *Sugar Tech*, 10: 1–13.

Stitt M (1986) Regulation of sucrose synthesis: integration, adaptation and limits In: Cronshaw J (ed), *Proceedings of the 3rd International Congress of Phloem Transport*, Asilomar, California. Alan R. Liss, New York.

Stitt M (1990) Fructose 2,6-bisphosphate as a regulatory molecule in plants. *Annual Review of Plant Physiology and Plant Molecular Biology,* 41: 153–185.

Stitt M, Huber S, Kerr P (1987) Control of photosynthetic sucrose formation In: Hatch MD, Boardman NK (eds.) The Biochemistry of Plants, Photosynthesis, Vol. 10: pp.:327-409, New York: Academic Press

Stitt M, Mieskes G, Söling H-D, Heldt HW (1982) On a possible role of fructose 2,6-bisphosphate in regulating photosynthetic metabolism in leaves. *FEBS Letters,* 145: 217–222.

Stitt M, Quick WP (1989) Photosynthetic carbon partitioning: its regulation and possibilities for manipulation. *Physiologia Plantarum,* 77:633–641.

Sturm A (1999) Invertases: Primary Structures, Functions, and Roles in Plant Development and Sucrose Partitioning. *Plant Physiology,* 121:1-7.

Sturm A, Hess D, Lee H, Lienhard S (1999) Neutral invertase is a novel type of sucrose-cleaving enzyme. *Physiologia Plantarum*, 107(2):159-165.

Sturm A, Tang GQ (1999) The sucrose-cleaving enzymes of plants are crucial for development, growth and carbon partitioning. *Trends in Plant Science*, 4:401–407.

Su LY, Cruz AD, Moore PH, Maretzki A (1992) The Relationship of Glyphosate Treatment to Sugar Metabolism in Sugarcane: New Physiological Insights. 142(2):168-173.

Sugiharto B, Sakakibara H, Sumadi, Sugiyama T (1997) Differential expression of two genes for sucrose-phosphate synthase in sugarcane: molecular cloning of the cDNAs and comparative analysis of gene expression. *Plant Cell Physiology,* 38: 961–965.

Sung SS, Xu D, Black CC (1989) Identification of Actively Filling Sucrose Sinks. *Plant Physiology*, 89:1117-1121.

Sweetlove LJ, Kossmann J, Riesmeier JW *et al.* (1998) The control of the source to sink carbon flux during tuber development in potato. *The Plant journal*, 15:697-706.

Taiz L, Zeiger E (1991) Phloem translocation. In: Beard-Brady E, Donohoe L, Funston J (eds.) Plant Physiology, pp.: 145–175, The Benjamin/Cummings Publishing Company, Inc., Redwood City, California.

Takahashi N, Yamaguchi I, Yamane H (1986) Gibberellins In: Takahashi N (ed.), Chemistry of Plant Hormones, pp.: 57–151, CRC Press, Boca Raton.

Tana B, Chanprame S, Tienseree N, Tadakittisarn S (2014) Relationship Between Invertase Enzyme Activities and Sucrose Accumulation in Sugarcane (*Saccharum* spp.) *Kasetsart Journal (Natural Science)*, 48:869-879.

Tang GQ, Luscher M, Sturm A (1999) Antisense repression of vacuolar and cell wall invertase in transgenic carrot alters early development and sucrose partitioning. *The Plant Cell*, 11:177–189.

Tarpley L, Vietor DM (2007) Compartmentation of sucrose during radial transfer in mature sorghum culm. *BMC Plant Biology,* 7:33.

Tarpley L, Vietor DM, Miller FR (1996) Metabolism of sucrose during storage in intact sorghum stalk. *International Journal of Plant Science,* 157:159–163.

Thomas JA, Jeffrey AC, Atsuko K, David MK (2005) Regulating the proton budget of higher plant photosynthesis. *Proceedings of National Academy of Sciences USA*, 102: 9709–9713.

Tiessen A, Padilla-Chacon D (2013) Subcellular compartmentation of sugar signalling: links among carbon cellular status, route of sucrolysis, sink-source allocation, and metabolic partitioning. *Frontiers in Plant Science,* 3:306.

Travis RL, Huffaker RC, Key JL (1970) Light-induced development of polyribosomes and the induction of nitrate reductase in corn leaves. *Plant Physiology*, 46:800-805.

Tymowska LZ, Kreis M (1998) The plant invertases: Physiology biochemistry and molecular biology. *Advances in Botanical Research*, 28:71–117.

Urban L, Lechaudel M, Lu P (2004) Effect of fruit load and girdling on leaf photosynthesis in *Mangifera indica* L. *Journal of Expeimental Botany*, 44:2075–2085.

Uys L, Botha FC, Hofmeyr JS, Rohwer JM (2007) Kinetic model of sucrose accumulation in maturing sugarcane culm tissue. *Phytochemistry,* 68:2375–2392.

van Bel AJE (2003) The phloem, a miracle of ingenuity. *Plant, Cell and Environment*, 26:125–149.

van der Merwe MJ, Groenewald J-H, Stitt M, Kossmann J *et al.* (2010) Downregulation of pyrophosphate: D-fructose-6-phosphate1-phosphotransferase activity in sugarcane culms enhances sucrose accumulation due to elevated hexose-phosphate levels. *Planta*, 231:595–608.

van Handel E (1968) Direct microdetermination of sucrose. *Analytical Biochemistry*, 22: 280-283.

Varshney RK, Nayak SN, May GD, Jackson SA (2009) Next-generation sequencing technologies and their implications for crop genetics and breeding. *Trends in Biotechnology*, 27(9):522-530.

Vaughn MW, Harrington GN, Bush DR (2002) Sucrose-mediated transcriptional regulation of sucrose symporter activity in the phloem. *Proceedings of National Academy of Sciences* USA, 99:10876–10880.

Veith R, Komor E (1993) Regulation of growth, sucrose storage and ion content in sugarcane cells, measured with suspension cells in continuous culture grown under nitrogen, phosphorus or carbon limitation. *Journal of Plant physiology*, 142(4): 414-424.

Venkataramana S, Naidu KM, Singh S (1991) Invertases and growth factors dependent sucrose accumulation in sugarcane. *Plant Science*, 74: 65–72.

Verma AK, Upadhyay SK, Srivastava MK, Verma PC (2011) Transcript expression and soluble acid invertase activity during sucrose accumulation in sugarcane. *Acta Physiologia Plantarum*, 33:1749–1757.

Vettore L, da Silva FR, Kemper ER, Souza GM *et al.* (2003) Analysis and Functional Annotation of an Expressed Sequence Tag Collection for Tropical Crop Sugarcane. *Genome Research*, 13:2725–2735.

Vickers JE, Grof CPL, Bonnett GD, Jackson PA *et al.* (2005) Overexpression of polyphenol oxidase in transgenic sugarcane results in darker juice and raw sugar. *Crop Science,* 45:354–362.

Viola R, Roberts AG, Haupt S *et al.* (2001) Tuberization in potato involves a switch from apoplasmic to symplasmic phloem unloading. *The Plant Cell*, 13:385-398.

Vorster DJ, Botha FC (1998) Partial purification and characterization of sugarcane neutral invertase. *Phytochemistry,* 49: 651–655.

Wächter R, Langhans M, Aloni R, Götz S (2003) Vascularization, high-volume solution flow, and localized roles for enzymes of sucrose metabolism during tumorigenesis by *Agrobacterium tumefaciens*. *Plant Physiology,* 133:1024–1037.

Waclawovsky AJ, Sato PM, Lembke CG, Moore PH *et al.* (2010) Sugarcane for bioenergy production : an assessment of yield and regulation of sucrose content. *Plant Biotechnology Journal*, 8(3):263-276.

Walsh KB, Sky RC, Brown SM (2005) The anatomy of the pathway of sucrose unloading within the sugarcane stalk. *Functional Plant Biology,* 32: 367–374.

Watt DA, McCormick AJ, Cramer MD (2014) Source and sink physiology In: Moore PH, Botha FC (eds.) *Sugarcane: Physiology, Biochemistry and Functional Biology*, first edition, pp.:483-520, John Wiley and Sons, Inc.

Watt DA, McCormick AJ, Govender C, Carson DL *et al.* (2005) Increasing the utility of genomics in unravelling sucrose accumulation. *Field Crops Research*, 92(2-3):149-158.

Welbaum G, Meinzer F, Grayson R, Thornham K (1992) Evidence for the consequences of a barrier to solute diffusion between the apoplast and vascular bundles in sugarcane stalk tissue. *Functional Plant Biology,* 19:611–623.

Welbaum GE, Meinzer FC (1990) Compartmentation of solutes and water in developing sugarcane stalk tissue. *Plant Physiology,* 93:1147–1153.

Whittaker A, Botha FC (1997) Carbon partitioning during sucrose accumulation in sugarcane internodal tissue. *Plant Physiology,* 115:1651–1659.

Winter H, Huber SC (2000) Regulation of sucrose metabolism in higher plants: localisation and regulation of activity of key enzymes. *Critical Reviews in Plant Sciences,* 19: 31–67.

Wobus U, Weber H (1999) Sugars as signal molecules in plant seed development. *Journal of Biological Chemistry,* 390: 937–944.

Wolf JBW (2013) Principles of transcriptome analsis and gene expression quantification: an RNA-seq tutorial. *Molecular Ecology Resources*, 13:559-572.

Wu KC, Wei L, Huang C-M, Wei Y-W, *et al.* (2018) Transcriptome reveals differentially expressed genes in *Saccharum spontaneum* GX83-10 leaf under drought stress. *Sugar Tech* /doi.org/10.1007/s12355-018-0608-0

Wu L, Mitchell JP, Cohn NS, Kaufman PB (1993) Gibberellin (GA_3) enhances cell wall invertase activity and mRNA levels in elongating dwarf pea (*Pisum sativum*) shoots. *International Journal of Plant Science*, 154:280–289.

Wu Q, Xu L, Guo J, SuY *et al.* (2013) Transcriptome Profile Analysis of Sugarcane Responses to *Sporisorium scitaminea* Infection Using Solexa Sequencing Technology. *BioMed Research International,*298920:1-9.

Xu D, Sung SS, Loboda T, Kormanik PP *et al.* (1989) Characterization of Sucrolysis via the Uridine Diphosphate and Pyrophosphate-Dependent Sucrose Synthase Pathway. *Plant Physiology*, 90:635-642.

Xu H, Liu Q, Yao T, Fu X (2014) Shedding light on integrative GA signalling. *Current Opinion in Plant Biology*, 21: 89–95.

Yabuta T (1935) Biochemistry of the "bakanae" fungus of rice. Agr. Hortic., 10:17-22.

Yamaguchi S (2008) Gibberellin Metabolism and its Regulation. *Annual Review of Plant Biology*, 59:225–251.

Yu C, Xu S, Yin Y (2016) Transcriptome analysis of the Taxodium 'Zhongshanshan 405' roots in response to salinity stress. *Plant Physiology and Biochemistry*. 100:156-165.

Zamski E, Schaffer AA (1996) *Photoassimilate Distribution in Plants and Crops: Source–Sink Relationships*, CRC Press, Boca Raton.

Zeng Y, Wu Y, Avigne WT, Koch KE (1998) Differential regulation of sugar-sensitive sucrose synthases by hypoxia and anoxia indicate complementary transcriptional and posttranslational responses. *Plant Physiology,* 116:1573–1583.

Zeng Y, Wu Y, Avigne WT, Koch KE (1999) Rapid repression of maize invertases by low oxygen. Invertase/sucrose synthase balance, sugar signaling potential, and seedling survival. *Plant Journal*, 121: 599–608.

Zhang C, Tanabe K, Tamura F, Itai A *et al.* (2007) Roles of gibberellins in increasing sink demand in Japanese pear fruit during rapid fruit growth. *Plant Growth Regulation*, 52:161–172.

Zhang J, Zhang X, Tang H, Zhang Q (2018) Allele-defined genome of the autopolyploid sugarcane *Saccharum spontaneum* L.. *Nature Genetics***,** 50:1565–1573.

Zhao C, Hanada A, Yamaguchi S, Kamiya Y *et al.* (2011) The Arabidopsis Myb genes MYR1 and MYR2 are redundant negative regulators of flowering time under decreased light intensity. *Plant Journal: for cell and molecular biology*, 66(3):502-515.

Zhu YJ, Komor E, Moore PH (1997) Sucrose accumulation in the sugarcane stem is regulated by the difference between the activities of soluble acid invertase and sucrose phosphate synthase. *Plant Physiology*, 115:609-616.

Zrenner R, Salanbout M, Willmitzer L, Sonnewald U (1995) Evidence of the crucial role of sucrose synthase for sink strength using transgenic potato plants (*Solanum tuberosum* L.). *Plant Journal,* 7: 97–107.

Annexures

ANNEXURES

Annexure I

(A) Preparation of 1M potassium phosphate buffer stock (pH=7.2)

71.7ml of 1M K_2HPO_4 + 28.3ml of 1M KH_2PO_4

(B) Preparation of 0.1 M citrate buffer (pH=5.2)

180ml of 0.1M citric acid + 320ml 0.1M sodium citrate +500ml distilled water

Annexure II

(A) Preparation of alkaline copper tartrate solution

Solution 1: 90g of Na_2SO_4 boiled in 250ml distilled water

Solution 2: 8g of $NaHCO_3$ in 12.5ml distilled water

Solution 3: Mix of solution1 and 2

Solution 4: 6g of $KNaC_4H_4O_6.4H_2O$ + 12g Na_2CO_3 in 100ml distilled water

Solution 5: 2g of $CuSO_4.5H_2O$ in 12.5ml distilled water

Solution 6: Solution5 added dropwise to solution4

Final reagent: Solution6 mixed with solution3 and the final volume made up to 500ml

(B) Preparation of arseno-molybdate reagent

Solution 1: 25g ammonium molybdate in 250ml distilled water

Solution 2: 3g disodium arsenate in 25ml distilled water

Solution 3: Mix of solution 1 and 2

Final reagent: 21ml H_2SO_4 added dropwise to solution3 and the final volume made up to 500ml

(C) Preparation of anthrone reagent

150mg of anthrone added to 76ml of H_2SO_4 and 30 ml of distilled water

Annexure III

Lowry's method:

Reagent 1: 0.4g of NaOH and 2g of Na_2CO_3 in 100ml distilled water

Reagent 2: 1g of potassium sodium tartrate ($KNaC_4H_4O_6.4H_2O$) and 0.5g of $CuSO_4.5H_2O$ in 100ml distilled water.

Reagent 3: Reagent A+ Reagent B (50: 1)

5ml of reagent3 was added to 20µl aliquot and incubated at room temperature for 10min. 0.5ml of Folin-ciocalteau reagent added to the solution and incubated in dark for 30min. O.D. of the solution was measured at 660nm.

Annexure IV

(A) Oligonucleotide primers used for semi qRT-PCR analysis

Target gene	**Forward primer (5'-3')**	**Reverse primer (3'-5')**
25S	GCAGCCAAGCGTTCATAGC	CCTATTGGTGGGTGAACAATCC
Soluble acid invertase (SAI)	GCAGCGCACGGGATTCCACT	GCCAGGTGTTGTCCGACGGG
Cell wall invertase (CWI)	CTCAAGAACAGCCTCGACCT	AGGTTTGGGTTCAGAGACGA
Neutral invertase (NI)	TGTGAACAATGGATCAAAA	GCTGGGTAGCATATCTTGAG
Sucrose synthase (SuSy)	CAGCATAGCTTGTTTAAGTAATAC	CTGGTGATGATGAAATCAGTGTG
Phospho enol pyruvate carboxylase (PEPC)	ATCAAGGAGAAACTGGATG	TCAGGAAAGAACTAGACTGC
Sucrose transporter (SUT 1)	GTGCTCATCTGCATTGCTGT	CTTGTGCCAATTGTTTGTGG
Fructose-1,6-bisphosphatase (FBPase)	AAAAACTGGGATGGGCCTAC	GATCTCCTCCACGTCATCGT

(B) Oligonucleotide primers used for qRT-PCR analysis

Target gene	**Forward primer (5’-3’)**	**Reverse primer (3’-5’)**
25S	GCAGCCAAGCGTTCATAGC	CCTATTGGTGGGTGAACAATCC
Soluble acid invertase (SAI)	CAGAGGAACTGGATGAACGA	CCGCTTGAAATGTCAATGTC
Cell wall invertase (CWI)	TCTGTACAAGCCAACCTTCG	CCGCTTGAAATGTCAATGTC
Sucrose synthase (SuSy)	GGCTGTTGCCTGATGCTGTT	TGCTCGGTTCCAATGACCTT
Sucrose phosphate synthase (SPS)	CCCGAACATTGCAAGAATTA	CTCCGCTCCTCTCTGTTACC

Publications

Author's personal copy

3 Biotech (2018) 8:418
https://doi.org/10.1007/s13205-018-1429-2

ORIGINAL ARTICLE

Gibberellin-induced perturbation of source–sink communication promotes sucrose accumulation in sugarcane

K. Roopendra[1,2] · **A. Sharma**[1] · **A. Chandra**[1] · **S. Saxena**[2]

Received: 15 May 2018 / Accepted: 10 September 2018
© Springer-Verlag GmbH Germany, part of Springer Nature 2018

Abstract

Constant removal of sugars from the site of synthesis (i.e., leaves), in response to elevated sink (culm) demand, may perhaps prevent damping of photosynthesis, by sugar, and hence promote further sucrose accumulation in the culm. In this study, gibberellic acid (GA_3) induced nearly 42.3% enlargement in cell size and about 39.3% increase in internodal length (sink capacity), 177% escalation in reducing sugar level (sink strength), amplified the expression of sucrose-metabolizing enzymes (sink demand), viz., 7.5-fold for SAI, 4.5-fold for CWI, sixfold for SPS, all demonstrating facilitation of augmented sucrose accumulation in the culm. The GA_3-treated BO 91 cane (late maturing sugarcane variety) exhibited an elevated final sucrose concentration (40.54–41.6%) as compared to control (30.44–38.8%). The GA_3-sprayed cane of early maturing Co J64 also showed such a boost, but it was lost by the end of maturity, perhaps due to inversion and/or the less effective GA_3 treatment. Thus, results demonstrated the role of GA_3 in augmenting sucrose content of cane culm, possibly by influencing source–sink dynamics in sugarcane.

Keywords Source–sink · Sugarcane · Gibberellin · Sucrose · Gene expression · qRT-PCR

Introduction

Sugarcane houses a unique source–sink system wherein the mature culm serves as a large sucrose reservoir called the 'sink' and the leaves play the 'source' of photosynthetic sugars (Watt et al. 2014). The supply of photoassimilate works on a demand and supply basis wherein sucrose demand for growth and differentiation during plant development originates in the sink tissues and sucrose synthesis to meet this demand, occurs in the leaves of plants (Hussain et al. 2004). Changes in sink activity as plants grow and mature, result in change in the demand for photosynthate (viz., immediate utilization for growth and respiration or for storage too). The rate of phloem loading is accordingly adjusted, to accommodate the changing sink demand (Lalonde et al. 2003). Thus, the rate of sucrose synthesis in the source is governed by the rate of photosynthesis and also the rate of export from leaves (Battistelli et al. 1991).

Sucrose accumulation in sugarcane culm occurs against the concentration gradient powered by respiration-generated energy (Bieleski 1960). Sucrose has been reported to be present in the vacuole as well as the apoplastic space and symplast, in similar concentrations (Welbaum and Meinzer 1990). As the culm matures, sucrose concentration along the entire stalk increases, while the proportion of glucose and fructose decreases (Lontom et al. 2008). Hence, a sucrose gradient exists in the culm, with low levels in young internodes to more than 200 g kg^{-1} fresh weight in the mature internodes (Glasziou and Gayler 1972).

The quantum of sucrose accumulation is regulated by various enzymes involved in its synthesis and breakdown (Moore 1995), viz., invertases and synthases. SPS (sucrose phosphate synthase) is a key sucrose-synthesizing enzyme which regulates photosynthetic sucrose synthesis (Stitt et al. 1988). Photosynthetically produced sucrose present in the source mesophyll cells is either loaded symplastically via bundle sheath cells or exported to apoplasm and loaded into SE-CCC (sieve element-companion cell complex) by a proton-sucrose symporter (Riesmeier et al. 1994). Upon transportation and arrival of sucrose in the culm/stem, it

---

✉ A. Chandra
amaresh_chandra@rediffmail.com

[1] Division of Plant Physiology and Biochemistry, ICAR-Indian Institute of Sugarcane Research, Lucknow 226002, India

[2] Department of Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow 226025, India

Published online: 18 September 2018

Sugar Tech
https://doi.org/10.1007/s12355-018-0665-4

SHORT COMMUNICATION

Increase in Sink Demand in Response to Perturbed Source–Sink Communication by Partial Shading in Sugarcane

K. Roopendra[1,2] · A. Chandra[1] · S. Saxena[2]

Received: 19 April 2018 / Accepted: 25 August 2018
© Society for Sugar Research and Promotion 2018

Abstract The sugarcane culm serves as 'sink' to store photosynthates and the photosynthesizing leaves, as its 'source.' In the present pursuit, partial shading of the source leaf was employed to improve the sink's strength. An increase in reducing sugar%, over time, and a consequent decline in sucrose% and brix% were noted, in the internodal samples of shaded cane, in contrast to those of control cane. On the other hand, reducing sugar level in the non-shaded leaf decreased over the period of shading. The sugar dynamics observed between source and sink perhaps validates growing sink demand and innate capacity of leaves for adapting to it. Molecular level analyses w.r.t. sucrose metabolizing enzymes also showed congruent results. Constant removal of sugars from the site of synthesis, in response to elevated sink demand, perhaps prevents suppression of photosynthesis, by sugar. Thus, exploring the disturbance of source–sink communication caused by partial shading and the consequential changes therein, will aid in enhanced understanding of the source and sink dynamics.

Keywords Sink strength · Source–sink · Sugar · Sugarcane · RT-PCR

Sugarcane is a C_4 plant, belonging to Poaceae (grass family), grown in tropical as well as subtropical regions. It is a vital cash crop credited with ~ 70% of the sugar produced globally (Gayler and Glasziou 1972). Sugarcane is predicted to have an accumulation capacity up to 500–560 mg g^{-1} dry matter. However, the current commercial cultivars yield much lesser viz. 350–400 mg g^{-1} dry matter, which is still much lesser than the predicted capacity of sugarcane culm tissue (Jackson 2005) indicating significant room for raising the threshold of sucrose content in the stalk. Unlike other grasses, sugarcane stores assimilate (sucrose) at exceptionally high concentration in the growing stalk (culm), forming 'sink.' Thus, sugarcane exhibits a unique source–sink system wherein the sugar synthesizing leaves form the 'source,' furnishing assimilate supply to meet the 'sink demand' for sugars, for supporting various plant processes (growth, respiration and storage) (Chandra et al. 2011). Accumulation of sugar in the culm starts alongside its elongation and as the culm matures, sucrose content of the culm builds up and the level of glucose and fructose concentration goes down (Fernandes and Benda 1985). Ideally, since sugarcane stalk matures from bottom upwards, higher sucrose concentration occurs in the mature older internodes down below and lesser accumulation in the upper younger internodes; the gradient getting fairly levelled as the cane matures.

The amount of sucrose in the culm is primarily governed by the cycle of degradation–synthesis of sucrose. The activity of various sugar metabolizing enzymes (viz. invertases and sucrose synthesizing) and related gene/transcripts' expression, is perhaps altered by sink demand (Rolland et al. 2002), to manipulate uptake and storage of sucrose in culm parenchyma. Since, till date, productivity of sugarcane has mainly been improved, only by increasing cane yield (Chandra et al. 2011), there is an urgent need to decipher the mechanism of sucrose accumulation in sugarcane and devise alternative means to improve its sucrose productivity.

✉ A. Chandra
amaresh_chandra@rediffmail.com

[1] Division of Plant Physiology and Biochemistry, ICAR-Indian Institute of Sugarcane Research, Lucknow 226002, India

[2] Department of Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow 226025, India

Published online: 08 September 2018

Vegetos
An International Journal of Plant Research & Biotechnology

Roopendra K. *et al.*
Vegetos 31(4):75-81, 2018
Doi:10.5958/2229-4473.2018.00097.6

Research Article A SOCIETY FOR PLANT RESEARCH PUBLICATION

Influence of Gibberellins on sink strength and Expression of Genes associated with Sucrose accumulation in Sugarcane (*Saccharum* spp. Hybrids)

Roopendra K.[1,2], A. Chandra[1*], I. Verma[1] and S. Saxena[2]

Abstract

In sugarcane, stem 'sinks' store photosynthates as soluble disaccharide, sucrose, and share a closely co-ordinated relationship with 'source' leaves. Phytohormones like gibberellins (GA) have long been known to promote rapid elongation and division of cells. The current study has ascertained the effects of gibberellins on sucrose metabolism and consequently sucrose accumulation in the sugarcane culm. Since brix%, pol%, sucrose% and reducing sugar% are basic and mandatory parameters in assessing the sugar content of cane, these have been closely observed, and their pattern tracked and analysed. The effect of GA_3 was clearly visible, 30 days after spraying (30 DAS), with a drastic increase in reducing sugar (RS) level, especially in the upper internodes, pointing to increase in sink potential. Even as the GA effect waned 60 DAS, the GA_3 sprayed canes still showed prominently higher RS% values and complementarily, lesser sucrose%, brix%, pol% values, as compared to control canes. Also, 120 DAS, notably higher sucrose levels were observed in the GA_3 sprayed culms. These biochemical findings have been further explored and correlated with end-point and qRT-PCR based data exhibiting differential expression of various sucrose metabolizing genes *viz*. the three invertases: soluble acid invertase (SAI), cell wall invertase (CWI), neutral invertase (NI) and also sucrose synthase (SuSy). Visibly higher expression of SAI and modified expression of other genes in GA_3 treated plants, can perhaps be interpreted as a consequence of increase in sink strength caused by gibberellin treatment. This in turn, extrapolates to better assimilate uptake and hence, the obtained results affirm the role of GA_3 in facilitating better sucrose accumulation.

Keywords
Sugarcane, sink strength, gibberellins, sucrose, semi qRT-PCR

Introduction

Most plants, including sugarcane, fix carbon through photosynthesis, with phloem acting as the channel to transport sucrose (Hatch and Glasziou 1964). The translocated sucrose is delivered into the culm parenchyma either through the symplasm, the apoplasm, or both. Soon after reaching the stem, the sucrose may be broken down into hexoses by the action of either of the three common invertase isoforms: soluble acid invertase (SAI; present in young culm vacuoles, pH optimum 4.4), cell wall bound acid invertase (CWI; working at a pH optimum of 3.8), and cytosolic neutral invertase (NI; with a pH optimum 7.0) and sucrose synthase (SuSy) (Glasziou and Gayler 1972). In the parenchyma cells, the hexoses derived from breakdown of translocated sucrose are either utilized in various biochemical pathways, or channelised into sucrose synthesis by sucrose phosphate synthase (SPS) (Hatch 1964). Since SPS is the primary sucrose synthesizing enzyme (Stitt *et al* 1988), it perhaps governs the allocation of carbon to sucrose synthesis (Huber and Huber 1996) and hence plays an important role in the transport of photosynthate from source (Jang and Sheen 1994). Sucrose synthase (SuSy) is also known to partake in synthesis of sucrose (Goldner *et al* 1991), though it is mainly known to be involved in sucrose breakdown. Thus, sucrose in the culm storage tissue results from an ever continuous cycle of break down and resynthesis (Whittaker and Botha 1997; Hussain *et al.* 2004; Rossouw *et al.* 2010).

By general convention, the photosynthetic apparatus (leaves) are termed as 'source' and the carbon storage tissues are referred to as 'sink' (Ho 1988). It is believed that 'source activity' results in synthesis of sugars in leaf tissue, to meet the 'sink demand' for sugars, in order to support various plant processes. Sugarcane can be said to be a model source-sink system, constituted of the photosynthesizing leaves which form the source and the growing culm which serves as the major storage tissue i.e. sink (Chandra *et al.* 2011). Culm sucrose content is affected by period of plant development; sucrose content being lesser during rapid growth, and increasing manifold, once maturation phase sets in. It is well established that a sucrose gradient exists in sugarcane, wherein the young internodes at the top accumulate much lesser sucrose than the bottom, older internodes, reported to hoard more than 200 gkg^{-1} fresh weight (Glasziou and Gayler 1972; Hawker 1985). A steep sugar gradient is evident during growth phase, which gradually levels as the cane matures.

Sugarcane is estimated to have physiological limit of accumulating upto 600mgg^{-1} sucrose (dry matter) in a given volume of cane stalk (Inman-Bamber *et al.* 2008). Reports suggest that current commercial cultivars' yields (350-400 mgg^{-1} dry matter) are still much lesser (Jackson 2005), disclosing the possibility of greater sucrose accumulation in the culm. Past efforts have been

---

Corresponding author: Division of Plant Physiology and Biochemistry, ICAR-Indian Institute of Sugarcane Research, Lucknow-226002, India

Email: amaresh_chandra@rediffmail.com

Received:05.09.2018 Revised:12.10.2018 Published: 31.12.2018

All articles published in Vegetos: International Journal of Plant Research & Biotechnology are the property of SPR, and is protected by copyright laws. Copyright © 2018, SPR, All Rights Reserved.