"INVESTIGATIONS ON THE INTERPLAY OF SUCROSE PHOSPHATE SYNTHASE, SUCROSE SYNTHASE AND INVERTASE(S) IN RELATION TO SUCROSE ACCUMULATION IN SUGARCANE"

by

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CANDIDATE'S DECLARATION

I hereby declare that this thesis or part there of has not been submitted by me or other person to any other University or Institute for a Degree or Diploma

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The assistance and help received during the course of this investigation have been acknowledged.

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ABBREVIATIONS

Symbol		Reference
ATP	:	Adenosine -triphosphate
Bp	:	base pairs
BSA	:	bovine serum albumin
^{o}C	:	degree centigrade
^{14}C	:	radio–labeled carbon
CCS	:	commercial cane sugar
CWI	:	cell wall–bound invertase
VAI	:	Vacuolar soluble acid invertase
AI	:	acid invertase
ddH_2O	:	double distilled water
DTT	:	1, 4–dithiothreitol
DNA	:	deoxyribonucleic acid
EDTA	:	ethylene diaminetetra acetic acid
FW	:	fresh weight
g	:	Gram
x g	:	gravitational force
H	:	Hour
kDa	:	kilo Dalton
L	:	Liter
M	:	Molar
mM	:	millimolar
Min	:	Minute
NAD^{+}	:	Nicotinamide adenine dinucleotide (oxidized)
NI	:	neutral invertase (β- fructofuranosidase)
ηm	:	Nanometer
OD	:	optical density/Absorbance

PCR	:	polymerase chain reaction
PVP	:	Polyvinylpyrrolidine
rpm	:	Revolution per minute
Sec	:	Seconds
SAI	:	soluble acid invertase
SPS	:	sucrose – phosphate synthase (EC 2.4.1.14)
SSf	:	sucrose synthase forward, functioning to
	:	synthesize sucrose
SSr	:	sucrose synthase reverse, functioning to cleave sucrose
SuSy	:	sucrose synthase (EC 2.4.1.13)
Tris	:	2 – amino – 2- (hydroxymethyl)–1,3 – propanediol
UDP	:	Uridine - diphosphate
UDP- Glc	:	Uridine – diphosphate glucose
V	:	Volume
W	:	Weight
Suc	:	Sucrose
Fru	:	Fructose
SPP	:	Sucrose phosphatase
Suc 6- P	:	Sucrose 6–phosphate
MAP	:	Months after planting
GM	:	Genetically modified
Non GM	:	Non genetically modified
qRTPCR	:	Quantitative real time polymerase chain reaction

ABSTRACT

INVESTIGATIONS ON THE INTERPLAY OF SUCROSE PHOSPHATE SYNTHASE, SUCROSE SYNTHASE AND INVERTASE(S) IN RELATION TO SUCROSE ACCUMULATION IN SUGARCANE

by

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A candidate for the award of the degree of
DOCTOR OF PHILOSOPHY (AGRICULTURE)
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Sugarcane varieties differing in sucrose content *viz.* CoM 0254 and Co 94012 (high sucrose), CoM 0265 (medium sucrose) and Co 62175 (low sucrose) were planted on the P.G. farm of M.P.K.V. Rahuri during 2011-12 and 2012-2013. Additionally, some crosses of sugarcane cultivars were developed to improve the sucrose content of sugarcane variety CoM 0265, a widely cultivated sugarcane variety occupying an area of over 80-85 % in the state and which is salt tolerant. Another known salt tolerant variety Co-740 was also used in the crossing programme with an object to improve salt tolerance of high sucrose sugarcane clones. Two high sucrose sugarcane cultivars *viz.* CoM 0254 and CoC 671 were used for crossing.The progeny of such crosses were planted on the research farm of C.S.R.S. Padegaon by following the recommended agronomic practices.The plant samples (internodes) of high and low sucrose sugarcane cultivars as as well as the progenies of the crosses exhibiting high sucrose and low sucrose were collected at monthly intervals after 240, 270, 300, 330, 360 and

390 DAP to analyze the expression and activity profiles of the sucrose metabolizing enzymes.

The stem was further divided into two equal portions *viz.* top and bottom representing the immature and mature storage tissues respectively and was immediately frozen in liquid nitrogen to stop metabolic activity and to avoid diurnal variation in enzyme activity and sugar levels during different developmental stages. The level of sucrose increased steadily during development and the bottom portion of the developing cane recorded much higher increase in sucrose in high sugar cultivars probably suggesting high rate of sucrose synthesis and translocation to sink organ. The top i.e. immature portion of the developing canes recorded very high level of hexose pool at the early stage of development and which subsequently declined during maturity. Both the top and bottom portion of the canes of the low sugar sugarcane variety Co 62175 recorded 2-3 fold higher hexose content at all the developmental stages.

Invert ratio (hexose : sucrose) declined gradually with age and was about 50 % less in the bottom internodes. The maximum sucrose phosphate synthase (SPS) and sucrose synthase (SuSy) activity was recorded in the 30-60 % ammonium sulphate fraction and the activity in this fraction was used for comparative evalution of the activity profile. The SPS activity increased gradually during cane maturation in both top (immatured) and bottom (matured) portion of high and low sucrose sugarcane varieties recording an increase of about 63-65 %. At maturity, i.e. 390 DAP the sucrose phosphate synthase (SPS) activity in the top portion of high sugar sugarcane variety CoM 0254 was almost 2.6 fold higher than the low sugar sugarcane variety Co 62175. However, at maturity, the bottom portion

of high sugar sugarcane variety recorded an increase of 1.3 fold over low sugar sugarcane variety. The activity profile of sucrose synthase (SuSy) in both high and low sucrose sugarcane cultivars declined during maturation and the activity was significantly lower in the mature internodes as compared to immature internodes. At the full mature stage, i.e. 390 DAP the activity of SuSy was higher in low sugar sugarcane variety Co 62175 both in top and bottom internodes.

The activity profile of invertase(s) *viz.* SAI, NI and CWI exhibited a definite trend in the high and low sugar accumulating sugarcane cultivars at different development stages. In high sugar accumulating cultivrs, the activity of SAI was comparatively much less both in top and bottom internodes and at maturity differences in the activity was significant both in the top and bottom portion. At maturity i.e. 390 DAP, the SAI activity in the top portion of high sugar accumulating sugarcane variety CoM 0254 was 70 ηmoles glucose formed mg^{-1} protein min^{-1} as against the activity of 200 ηmoles glucose formed mg^{-1} protein min^{-1} in top portion of low sugar accumulating variety Co 62175.

The activity of neutral invertase increased during cane maturation and was positively correlated with sucrose content. The mature internode of low sugar sugarcane variety although recorded low NI activity in the early stage of development, the difference was less pronounced at maturity.

However, the mature internodes of low sugar sugarcane variety during maturation and was higher in both high sugar sugarcane varieties. The mature internodes of both high and low

sugar sugarcane parents recorded high CWI activity as compared to top portion.

The high and low sugar progenies of the crosses when evaluated for the activity profile of the sucrose metabolizing enzymes also exhibited similar trend in mature (bottom) and immature (top) internodes during development. These results could highlight the validation of activity profile of sucrose metabolizing enzymes and the sugar accumulation ability of sugarcane clones.

The primers NKS-9, NKS-31, STMS-34, UGSM-359, UGSM-458, GUSM-550, UGSM-632 and UGSM-644 have been reported to be linked with sucrose content amplified genomic DNA with a specific amplicon size in all high sugar sugarcane parents and progenies clones exhibiting high sucrose content. On the contray, primers NKS-5, NKS-45 and NKS-46 amplified genomic DNA with specific amlicon size in low sugar sugarcane parents and clones.

The exression profile of SPS, SuSy and SAI recorded higher expression of SPS in high sugar sugarcane progeny whereas, the expression of SuSy and SAI was much higher in low sugar sugarcane progeny as evident from semiquantitative gene expressin analysis.The activity profile of SPS, SuSy and SAI at different developmental stages corresponded well with the differential expression profile as evident from qRTPCR suggesting the major regulation at the transcriptional level.

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1. INTRODUCTION

Sugarcane (*Saccharum* spp. complex) is a commercially important crop grown in more than 100 countries and accounts for approximately 70 % of the world sugar production. In India, it is grown on an area of 5064 thousand ha with a production of 338.9 million tons with an overall yield of 66.9 t/ha. In 1920s, with the advent of new inter specific hybrids (*S. officinarum* x *S. spontaneum*) the sugarcane production worldwide was revolutionized, particularly in India. These intersecific hybrids made a breakthrough in increasing the yield as well as disease resistance. The modern sugarcane varieties which are mainly derived from interspecific crosses between the noble cane *S. officinarum* (2n= 80) and the wild species *S. spontaneum* (2n = 40-128), possessed complex genome associated with polyploidy/ aneuploid nature with variation in chromosome number.

Sucrose is the prime product of sugarcane hence continued research is needed to improve its level in cane stalk. In India a distinct difference in sucrose content (pol % cane) is observed in tropical and subtropical varieties which ultimately lead to variation in sucrose recovery. The sucrose content and recovery of Indian varieties show distinct variation compared to the varieties of Brazil, China and Australia. In order to understand these variations, concerted efforts are needed to better understand the sucrose metabolism, transport and source – sink interactions that regulate sucrose accumulation which ultimately provide details about the processes governing overall stalk sucrose concentrations.

With increasing population pressure and industrialization, agricultural land is diminishing in India and

therefore chances for increasing area even for cultivation of cash crop like sugarcane needs better quality land and high and extensive irrigation both in tropical and subtropical areas which is quite remote. At the global level, contrary to the demand of high sucrose containing genotypes, sugarcane improvement especially sucrose content during the last 50 years have been largely through the increase of cane yield rather than sucrose content per unit mass (Jackson, 2005). These situations warrant vertical improvement in cane yield vis-a-vis sucrose content per unit area as the only option left to the Indian sugar industry. Development of improved sugarcane varieties efficient in sucrose accumulation has become the key component of all the advanced sugarcane breeding programmers (Lingle *et al.*, 2009).

It has been established that, mature sugarcane has the capacity to store up to 25 % sucrose on a fresh weight basis under favourable conditions, but in practical sense it has never been achieved. Some of high sugar accumulating early maturing variety like CoJ 64 accumulates 18-20 % (fresh wt. basis) sucrose in its mature stalk. A positive relationship between free space in the internode parenchyma tissue and pol %, support the role of free space in sucrose accumulation and possibly this can be used as a physiological indicator for sucrose yield.

Three invertase(s) namely soluble acid (SAI), cell wall bound (CWAI) and neutral (NI) along with sucrose synthase (SS) and sucrose phosphate synthase (SPS) play important role in sucrose metabolism in sugarcane. In general, SAI activity decline with ageing coupled with rapid rise in sucrose/reducing ratio indicated better sink strength (Batta *et al.*, 2007). Sucrose

phosphate synthase the pivotal sucrose syhthesizing enzyme needs to be addressed along with soluble acid invertase as the difference in the activities of these two enzymes reflect the sucrose accumulation in sugarcane. Different isoforms of SPS perform tissue specific manner and require strict control of promoter for efficient and selective expression. However, it has been also emphasized that alone SPS for that matter any specific enzyme alone can not make significant impact on sucrose accumulation. It is therefore, important to target many different enzymes simultaneously genes for the sucrose improvement. The advances in research on sugarcane have indicated that attributes like delayed leaf senescence, increased sucrose loading rates in source tissues, high photosynthetic activity (electron transport rate) and higher activity of cell wall bound acid invertase (CWAI) are also found to be associated with the high total sugar phenotype of a sugarcane line having ability to accumulate higher level of sucrose in the culm. In many countries, including India in the last 50 years the improvement of sugarcane has been primarily attempted from the angle of cane yield rather than sucrose productivity. In view of the limited area, growing domestic demand for white sugar and necessity of extra ethanol production for blending it is imperative to augment productivity per se.

The lack of progress in improving stalk sucrose content in sugarcane has been due to narrow genetic pool used in commercial breeding program. Enhanced sugar accumulation in sugar booster line (transgenic bearing SI gene) was accompanied with increased rate of photosynthesis, electron transport reflecting photosynthetic efficiency, sugar transport and most importantly

sink strength further augmented the impact of physiological research in improving the stalk sucrose content (Wu and Birch 2007). Biochemical markers especially associated with high sucrose phenotype that can be measured at early developmental stages would have better utility. Among the category of synthesis and cleavage enzyme of sucrose, sucrose phosphate synthase (SPS) has been reported as a useful marker when measured in the uppermost internodes of young plants which have grown past the elongation phase (possessing six internodes) (Grof *et al.*, 2007).

In culm tissues where sucrose content was low and hexose contents were high, neutral invertase transcript level was higher than in those dedicated to sucrose storage (Bosch *et al.*, 2004). In last five years a fresh re- look has been noticed and the research has been mainly focused on source- sink relationships so as to increase the sucrose content by elucidating the genes and enzymes in sugarcane leaves and stalk that were responsive to changes in the ratio of source sink. Two school of thoughts are clearly visible and interesting information pertaining to source – sink relationships have been generated to increase sucrose content in the stalk. One school suggests pursued research and development to be continued by targeting leaf extension rate, photosynthesis, water regimes and temperature management vis a vis their manipulation in improving sucrose content (Inman–Bamber *et al.*, 2011) while other group thinks of targeting more precise research on regulation of expression of gene(s) associated with carbohydrate metabolism vis a vis overall plant physiological response that might detect mechanism mediating the relationship between source and sink tissues (McCormick *et al.*, 2008, 2009).

Transporters of the plasma membrane appear to play an important role not only in phloem loading and unloading, but also in transferring sucrose between apoplastic and symplastic compartments (Chen *et al.,* 2012). Thousands of transcript sequences from maturing sugarcane culms reveled that transcripts for sugar metabolizing enzymes are relatively rare in maturing culms while transcripts for sugar transporters are very abundant (Casu *et al.,* 2003) mRNAs for ShSUT1 transport gene of a sugarcane hybrid were abundant in both source leaves and sink stems actively accumulating sucrose. The presence of these ShSUT1 transcripts at the periphery of the vascular parenchyma and sheath cells, instead of in the phloem itself, is consistent with a role other than that of direct phloem loading.

Consensus among different research groups about the relationship between the sucrose level and the activities of enzymes contributing to sucrose accumulation in the internodes is still wanting. However, the molecular breeding work with designed primer pairs of SAI, SS and SPS genes are being utilized in advance sugarcane breeding aimed at improving sugar accumulation characteristic of sugarcane plants (Chandra *et al.,* 2010). The qRT-PCR analysis has indicated differential expression of SS and SAI gene in response to foliar application of enzyme effectors on sucrose metabolizing enzymes (Jain *et al.*, 2013). The advance technologies like RNAi and transformation are being attempted to improve sucrose content. An increase in the hexose-phosphate concentrations resulting from a restriction in the conversion of hexose phosphates to triose phosphate has been reported to derive sucrose synthesis in young internodes (Merwe *et al.,* 2010).

The GM and non GM approach are being attempted to improve the sucrose content and identification of key regulatory enzyme(s) and validation of markers linked with sucrose accumulation is much needed.

The present investigation entitled, "Investigations on the interplay of SPS, SuSy and Invertase in relation to sucrose accumulation in sugarcane" has therefore been proposed to study variability on sucrose metabolizing enzymes in relation to sucrose accumulation in contrasting parents and progenies of crosses involving high and low sugar parents during cane development with the following major objectives :

1. To evaluate the developmental changes of sucrose metabolizing enzymes, *viz.* sucrose phosphate synthase (SPS), sucrose synthase (SuSy) and invertases in high and low sucrose accumulating cultivars and progenies of crosses with high and low sucrose content.
2. To analyze progenies of crosses with high and low sucrose using informative SSR primers and analyzing SPS and SAI activities.
3. To examine the DNA polymorphism in sugarcane genotypes by RAPD, ISSR analysis and by using SSR primers reported to be linked with higher sucrose content.
4. To analyze the expression level of SPS, SS and SAI during cane development in high and low sugar sugarcane cultivars.

2. REVIEW OF LITERATURE

Sugarcane being a C_4 plant is one of the most efficient crop in converting solar energy into chemical energy. Typically about 35 to 40 % of sugar fixed during photosynthesis is consumed by the living cell to provide energy to cell growth, division, differentiation, nutrient up take and maintenance during development. Some portion remains as metabolic intermediate and remaining can be stored as such in vacuoles or fixed in polymers. During the sugarcane maturation the fate of assimilated carbon shift from insoluble and respiratory component to sucrose an osmotically active storage solute. In sugarcane, sucrose storage occurs in stock (culm) parenchyma cells which can reach exceptionally high up to 650 mM concentrations (Welbaum and Meinzer, 1990) or 80 % of stem fresh weight in commercial sugarcane varieties. During sugarcane maturation, sucrose can be rapidly broken down for respiration and then resynthesised in sink which maintain dynamic balance between storage and respiration and other uses. The extent of partioning in sucrose degradation and resynthesis cycle depends on developmental changes, in capacities of different sink, feedback from sink photosynthetic rates and levels of sucrose supplied from source leaves.

Despite of advances in understanding the importances of sucrose metabolizing enzymes in sucrose accumulation and use of certain hormones or specific chemicals to regulate the activities of enzymes, the exact coordination in understanding the biochemical processes of carbon partioning is far from fully understood. In sugarcane, sucrose phosphate synthase (SPS)

activity has been correlated with sucrose content in diverse genotypes, however, over expression of sucrose phosphate synthase alone in transgenic sugarcane plants has not led to improve the sucrose yield. It has also been reported that, transcripts for sugar metabolizing enzymes are relatively rare in maturing culms while transcripts for sugar transporters are vary abundant. Besides synthesis, genes encoding invertases have been suggested as a key regulator for sucrose accumulation and effects to down regulate the activities of these invertases (acid and neutral invertases) improved sucrose content in the immature and mature culms but this benefit was out weighted by a sever reduction in plant vigor. Cell wall invertase is often considered as a gateway for the entry of sucrose into the cells and increased in cell wall invertsae activity was associated with higher sucrose content in sugarcane.

Exploitation of genetic variability for sucrose accumulation is very important for developing high sugar producing genotypes. Hence, an understanding of the pattern of variability for expression of key metabolic enzymes in relation to sucrose accumulation among genotypes would be important in assisting sugarcane breeding programme. The literature pertaining to the aspects of sugar metabolism, loading, unloading, transport and manipulation of key enzymes involved either through spray of hormones or activators or through biotechnological means has been reviewed under the appropriate heading in this chapter.

2.1 The sucrose–sink system in sugarcane

Sugarcane, primarily used for sugar production, has a unique source- sink system. It's stem sink store photosynthate as soluble disaccharide, sucrose which can reach up to 18 % of stem

fresh weight in commercial sugarcane varieties (Inman-Bamber *et al.*, 2011). In contrast, most other plant stems store carbon as insoluble polysaccharides such as starch or cellulose with a low concentration of sucrose. Another distinctive feature of sugarcane is that sucrose storage occurs in the stalk (culm) parenchyma cells and not in terminal sink organs such as tubers, grains or fleshy fruits. Sugarcane accumulate sucrose both inside and outside the cells in the symplast and apoplast, respectively (Welbaum and Meinzer, 1990).

During development, sucrose synthesized in photosynthetic sugarcane leaves is translocated *via* phloem to stem internodes including both immature stem internodes, the meristematic growth sink and mature internodes, the storage sink. During maturation of commercial sugarcane cultivars, the leaves photosynthetic activity decreases significantly, as culm sucrose content increases (McCormick *et al.,* 2008, 2009). Sucrose can be rapidly broken down for respiration and then resynthesized in sinks, which allows for a dynamic balance between storage and respiration or other uses (Wendler *et al.*, 1990). During the sucrose degradation and re- synthesis cycle, carbon is partitioned into other competing metabolic sink including respiratory pathways, cycling through hexose pools, organic acids and amino acids, proteins and cell walls (Botha *et al.*, 1996).

2.2 Sucrose accumulation and transportation in sugarcane

The biochemical basis for the regulation of sucrose accumulation in sugarcane has been investigated by several workers (Whittaker and Botha, 1997; Zhu *et al.,* 1997). Primary

sucrose metabolism is governed by several enzymes such as sucrose phosphate synthase (SPS), sucrose synthase (SS) and invertases (Botha and Black, 2000 and Schafer *et al.*, 2005). Sucrose can be synthesized only in the cytosol by SPS or SS activity. It is distributed to various degrees between the apoplast, the cytosol and the vacuole of the storage parenchyma. The hydrolytic and or cleavage activities of an invertases and sucrose synthase in all these subcellular compartments could therefore, exert an influence on sucrose metabolism, translocation and storage (Lee and Vattuone, 1996).

Sugarcane culms deposit sucrose in both the stem parenchyma cell vacuole and apoplast surrounding these cells. Parenchyma cells in mature sugarcane stalks can accumulate sucrose to levels having an osmotic potential - 2.2 MPa (Welbaum and Meinzer, 1990). Sugarcane ripening is associated with an increase in sucrose concentration in mature stalk tissue and its accumulation is probably associated with the balance between the breakdown and synthesis reactions.

Sucrose is transferred from phloem to storage cells in the culm, probably through the symplast and apoplast (Rae *et al.*, 2005a) and predominantly the symplast in mature internoeds (Patrick, 1997). The subsequent compartmentation of sugars between the apoplast, cytosol and vacuole is an important feature of storage in sugarcane stem's parenchyma cells. Sucrose unloading from phloem into the apoplast can follow two paths to vacuoles of parenchyma cells. In one path, sucrose is transported directly into parenchyma cells by sucrose transporters of the plasma membrane then into the vacuole, mostly under low turgor

conditions. In the other path, sucrose in the apoplast is hydrolyzed by apoplastic acid invertase into glucose and fructose (Fig. 1). Their function may contribute to a biochemical barrier that inhibit sucrose apoplastic back-flow out of tissues and also aid retrieval of sucrose released to the apoplast (Rae *et al.,* 2005a). The ShSUT1 gene product may have a role in the partitioning of sucrose between vascular tissue and storage sites in sugarcane stem parenchyma cells (Reinders *et al.* 2006).

2.3 Enzymes involved in sucrose metabolism

2.3.1 Sucrose phosphate synthase

During the sucrose synthesis in higher plants, sucrose phosphate synthase (SPS; UDP–glucose: D-fructose–6 phosphate 2–α-D- glucosyltransferase, EC 2.4.1.14) catalyzes the reaction:

UDP- glucose + Fru – 6 – Ph ↔ sucrose- 6 - Ph + UDP + H^+

This enzyme was soluble in cytoplasm and catalyzes freely reversible reactions (Huber and Huber, 1996).

Sucrose phosphate synthase (SPS) is a key enzyme for sucrose synthesis in leaves, and is also expressed in non-photosynthetic tissues which controls the fluxs of carbon into sucrose (Huber and Huber, 1996; Im, 2004) and is the major exporter of photosynthate from source to sink tissues (Jang and Sheen, 1994). SPS is regulated by three distinct mechanisms: i) gene expression (Huber and Huber, 1996), ii) allosteric control by Glu-6-P (activators) and inorganic phosphate (inhibitor) (Doehlert and Huber, 1985) and iii) covalent modification *via* reversible phosphorylation (Huber and Huber, 1996).

Chau *et al.* (2008) reported that sucrose phosphate synthase catalyzes the first step in the pathway of sucrose

synthesis by transferring a glycosyl group from an activated donor sugar such as uridine diphosphate glucose (UDP-Glu), to a sugar acceptor D-fructose 6- phosphate (F6P), resulting in the formation of UDP and D-sucrose-6-phosphate (S6P). The reversible reaction is followed by an irreversible reaction by sucrose phosphate phosphatase (SPP), where sucrose-6-phosphate is dephosphorylated to sucrose, concluding the sucrose biosynthesis pathway.

Huber *et al.* (1985) reported that, sucrose phosphate synthase activity contributes to the control of C-flux into sucrose and can be taken as an indicator of the capacity for sucrose synthesis.

Stitt and Quick (1989) also reported that, a key enzyme which has been identified as potentially rate limiting to the formation of sucrose in the leaf is sucrose phosphate synthase.

Sugiharto *et al.* (1995) found that sucrose levels in sugarcane leaf were increased by about 2.5 fold by nitrate or ammonium ions. The increase in sucrose was correlated with increase in sucrose phosphate synthase activity. These increase in sucrose phosphate synthase activity lead to increased accumulation of sucrose in leaf blades.

Zhu *et al.* (1997) studied the genotypes in a segregating F1- population and reported that, sucrose content was strongly correlated to the difference between SPS and acid invertase activity and sucrose accumulation is only possible when SPS activity exceeds that of acid invertase. He also reported that, SPS is negatively correlated with sucrose content in cane, which is in contrast with Lingle's (1999) findings.

Lingle (1998) studied the internodes of seven commercial cultivars prior to elongation and sampled at intervals from July until December during two growing season and reported that the activities of sucrose metabolizing enzymes determind the rate of total sugar accumulation in internodal tissues of sugarcane during development. He also reported that the activity of sucrose phosphate synthase increased during development. Sucrose content of mature internodes were not correlated with such enzyme activities.

Stitt *et al.* (1987) reported that, sucrose synthesis in the sink tissues occurs exclusively in the cytosolic compartment of the cell via two alternative routes. The first, catalyzed by the enzyme sucrose phosphate synthase, utilizes fructose–6-phosphate and UDP-glucose to synthesize sucrose-6-phospate, which is converted to sucrose by the abundant enzyme sucrose phosphatase (EC 3.1.3.24.) Sucrose content during internode development can be correlated with the difference between SPS and invertase activities (Zhu *et al.*, 1997). SPS is regarded as the predominant source synthesizing enzyme in sink tissues (Batta and Singh, 1986; Wendler *et al.*, 1990 and Botha and Black, 2000).

Botha and Black (2000) measured the sucrose accumulation rates, sucrose phosphate synthase activity in the intermodal tissue from a sugarcane (*Saccharum* species hybrids) variety N 19 and reported that, the sucrose accumulation rate sharply increased between internodes 3 to 11. In the older internodes SPS activity was at least three times higher than the SuSy activity. A highly significant positive correlation was found between SPS activity and sucrose content. This clearly indicated

that SPS is the major contributor of sucrose synthesis activity in the culm of sugarcane.

In young internodes, sucrose phosphate synthase contribute to sucrose synthesis. However, SPS activity becomes gradually more important in sucrose synthesis as the internodes get older, exceeding sucrose synthase more than three fold.

Lunn and MacRae (2003) reported that, SPS enzyme itself is allosterically activated by glucose 6-phosphate, light and osmotic stress and inhibited by inorganic phosphate and this activation is brought about by changes in the phosphorylation state of several serine residues in the protein. SPS catalyse the synthesis of sucrose 6–phosphate (Suc6P) which is then hydrolyzed to sucrose by sucrose phosphatase (SPP). Bosch *et al.* (2004) also reported that SPS was the key regulators for sugar accumulation in sugarcane stem storage parenchyma.

Gutierrez-Miceli *et al.* (2005) studied the activities of sucrose phosphate synthase in callus culture of four Mexican sugarcane cultivars (*Saccharum spp.*) with a different capacity to accumulate sucrose in stem parenchyma cells and reported that sucrose accumulation in callus was positively correlated to the activity of SPS activity. Luo (2006) reported that the enzyme activity of SPS gradually increased with stalk development and decreased with maturity in sugarcane.

Grof *et al.* (2007) examined the sucrose phosphate synthase activity in four high and four low CCS clones from an initial cross between *S. officinarum* and commercial cultivar Q-165. Subsequently, SPS activity was measured in clones derived from a backcross of the progeny to another commercial cultivar Mida and

reported that SPS was significantly higher in the upper internode (1 to 3) of high CCS clones as compared to low CCS clones in both the populations, suggesting that this enzyme may have a key role in establishing metabolic and developmental processes necessary for high sucrose accumulation during stem growth and maturation. He also noticed higher SPS activity in immature internodes than in mature internodes.

Botha and Black (2000) reported that, SPS activity was higher in mature internodes than in immature internodes. The sucrose/hexose ratio provides a clear indicator of early maturing clones which on final harvest possess higher CCS and sucrose concentration. He also reported SPS to be the key enzyme in the metabolic compartment of storage tissues. He also reported that due to complexity of the sugarcane genome, in particular its large ploidy nature, DNA marker technology has got its own limitations. Under such situation, biochemical markers especially associated with high sucrose phenotype can be measured at early developmental stage would have better utility. Among the category of the synthesis and cleavage enzymes of sucrose, sucrose phosphate synthase (SPS) has been reported as useful marker when measured in the uppermost internodes of young plants which has grown past the elongation phase. Pan *et al.* (2007) reported that SPS enzymes made great contribution to sucrose accumulation in sugarcane internodes and was positively correlated with sucrose content in cane respectively.

Pan *et.al.* (2009) studied the relative importance of sucrose phosphate synthase (SPS) in regulation of sucrose accumulation during the growth and development of internodes in

two sugarcane cultivars i.e. ROC20 and RB72-454 from internodes 2 to 15 and reported that, the sucrose content was positively correlated with the activity of SPS which was the key enzymes in regulating sucrose accumulation during the growth and development of the internodes of sugarcane stalk.

Nookaraju *et al.* (2010) reported that, SPS catalyze the synthesis of sucrose 6-phosphate from UDP-glucose and D-fructose 6-phosphate. It increase the rate of sucrose accumulation in tissues by maintaining the hexose gradient between the apoplast and cytosol.

Verma *et al.* (2010, 2011) studied the functional analysis of sucrose phosphate synthase activity as well as transcript expression by using semi – quantitative RT-PCR at different developmental stages in two early maturing, high sugar accumulating cultivars (CoS 96268 and CoS 95255) and two late maturing, low sucrose accumulating cultivars (CoS 97264 and CoSe 9423) and reported that high sugar cultivars showed increased transcript expression.

Kale *et al.* (2012) studied the sucrose metabolizing enzymes in leaf blade, sprouted leaf and stem part of CoM 0265 sugarcane variety and reported that the sucrose phosphate synthase activity was maximum in leaf blade at 10 and 11 months after planting (MAP). At 12 MAP, SPS activity was maximum in sprouted leaf than leaf blade. In leaf blade SPS activity increased at 10 and 11 MAP and at 12 MAP the activity was decreased.

Dongliang *et al.* (2012) reported that sucrose phosphate synthase is the key enzyme that control the sucrose biosynthesis in plants. There are at least three SPS families in higher plants,

named A, B and C respectively, but there are at least five SPS families, named A, B, C, D and E, respectively, in the monocotyledonous plants from Poaceae.

Ramalakshmi *et al.* (2012) studied the expression profiling of sucrose genes in *Saccharum*, sorghum and their hybrids and reported that, the expression of SPS was high in the first three months, low during four to six months thereafter remained high from seven to twelve months in high sucrose varieties of sugarcane. The trend was reversed in low sucrose sugarcane varieties which showed a low expression of SPS during first three months followed by high expression during four to six months and thereafter low expression during seven to twelve months. High expression of SPS was associated with high sucrose accumulation in high sucrose sugarcane varieties, while low expression of these genes was associated with low sucrose varieties.

Prathima *et al.* (2012) studied the changes in transcript expression and enzymatic activities of key genes of sucrose metabolism during stem development of high sugar (CoC 671 and Co 99004) and low sugar genotypes (MS 6847 and Co 62175) at three different critical stages of cane development at 90, 180 and 360 days after planting (DAP) and reported that, the specific activity of sucrose phosphate suynthase was significantly high in high sugar genotypes (45.58 ± 0.429 µmol g^{-1} protein min^{-1}) as compared to low sugar genotypes (32.75 ± 20.3 µmol g^{-1} protein min^{-1}). There was gradual increase in sucrose phosphate synthase activity from top to middle internodes and reaches maximum at bottom internodes of both high and low sugar genotypes. The highest mean sucrose concentration was observed in bottom

internodes (247.94± 1.459 mg g^{-1} fresh weight) at 360 DAP in high sugar genotypes as compared to low sugar genotypes (174.12± 0.572 mg g^{-1} fresh weight). Sucrose concentration significantly increased from top internodes to middle and bottom internodes in all the genotypes. Low sugar genotypes had higher concentration of total hexoses as compared to high sugar genotypes. There were significant decreases in the total hexoses concentration when the crop reached matured stage (360 DAP). Decreasing trend in total hexoses concentration from top internodes to bottom internodes was found to be correlated with the increased sucrose accumulation in mature internodes.

Joshi *et al.* (2013) studied the activity of enzyme involved in synthesis and degradation of sucrose in popular sugarcane varieties Co 86032 and CoG 93076 during the maturation phase from 9th to 13th month after planting and reported that, in both the varieties maximum sucrose phosphate synthase activity in top internodes correlated to high sucrose accumulation.

2.3.2 Sucrose synthase

Sucrose synthase (SuSy, UDP glucose: D fructose 2-α-D- glucosyltransferase (EC 2.4.1.13) catalyse the reversible reaction,

Sucrose + UDP ↔ Fructose + UDP - glucose

Sucrose synthase enzyme was discovered by Leloir and co- workers in 1955 (Cardini *et al.,* 1955) which is a bidirectional enzyme capable of both cleaving and synthesizing sucrose. The reaction catalyzed by sucrose synthase operating in the direction to synthesize of sucrose is termed as SSf whereas the reaction

operating in the direction of sucrose cleavage is termed as SSr. SuSy is largely localized in the companion cells of vascular bundles (Rouhier and Usuda, 2001) and may function to fuel respiration to satisfy the high ATP demand of the plasma membrane H^+-translocating ATPase (H^+/ATPase) (Nolte and Koch, 1993). The H^+/ATPase in companion cells is especially important in maintaining an H^+ gradient between the apoplast and the cytosol for the plasmalemma sucrose/H^+ symport system. In sugarcane, though the role of the SuSy is still a matter of debate, significant levels of SuSy activity in sucrose accumulating internodes have been reported.

Copeland (1990) reported that, sucrose synthase is a glycosyl transferase which converts sucrose in the presence of uridine diphosphate (UDP) into UDP-glucose and fructose. Sucrose synthase enzyme is implicated in a wide variety of processes including cellulose synthesis, phloem transport, storage carbohydrate accumulation and stress response mechanism (Dejardin *et al.,* 1999). Sugarcane is unique because it stores its food not in the form of glucose but in the form of sucrose. Sucrose synthase (SuSy) plays a central role in carbohydrate metabolism in general, and sucrose accumulation in particular in all plant species. It belongs to a family of invertase which are specialized in hydrolyzing sucrose into glucose and fructose. SuSy catalyzes the reversible cleavage of sucrose and UDP (Uracil diphosphate) to UDP glucose and fructose (Schafer *et al.,* 2004 and Winter and Huber, 2000). The cleaved product, UDP-glucose, acts as the substrate for cellulose and callose synthesis (Amor *et al.*, 1995). In monocotyledonous species like sugarcane, SuSy is encoded by two

differentially expressed nonallelic loci Sus1 and Sus2 (Winter and Huber, 2000). In sugarcane, SuSy is associated exclusively with vascular bundles where sucrose accumulation/degradation activity occurs (Buczynski *et al.,* 1993). SuSy activity was thought to be associated with starch synthesis, cell wall synthesis and overall sink strength. Frydman and Hassid (1963) reported the discovery of sucrose synthase activity in sugarcane leaf extracts. It was first believed that, this was the only sucrose forming enzyme operative since the replacement of fructose with fructose -6- phosphate terminated all sucrose forming activity. However, use of isolated chloroplasts as enzyme source revealed that both fructose and fructose–6- phosphate served as glycosyl accepters from UDP-glucose.

Hatch and Glasziou (1963) isolated sucrose synthase from both immature and mature storage tissue of sugarcane. Optimum pH of sucrose synthase reaction in internodal storage tissue was 8.2 compared to 7.4 for wheat germ enzyme and 8.0 for the sucrose forming reaction in sugarcane leaf homogenate. Sucrose synthase was also reported in sugarcane leaf chloroplasts (Haq and Hassid, 1965).

Hawker and Hatch (1965) initially suggested a vascular localization for sucrose synthase in sugarcane internodes despite nonvascular storage parenchyma making up approximately 70 % of their isolated transport tissues.

Patil and Joshi (1972) reorted that pH optimum of enzyme for sucrose synthesis ranges between 7.5 and 8.0 and sucrose cleavage between 6.5 and 7.0. Divalent cations particularly Mg^{2+} have stimulatory effect on the activity of this enzyme when

measured in synthesis direction and inhibitory effect when measured in cleavage direction.

Giaquinta (1983) suggested that, in the phloem, sucrose is translocated by a mass flow to sink tissues, where it is cleaved by sucrose synthase to produce UDPG and fructose which was then used for other biosynthetic pathways as energy and source of structural components.

Hawker (1985) and Hawker *et al.* (1991) reported that, upon arrival in the stem, sucrose can be catabolized by sucrose synthase. Sucrose synthase activity is low in storage parenchyma cell but relatively higher in vascular strands from both immature and mature tissue.

Claussen *et al.* (1985) reported that, sucrose synthase was more active on a fresh weight basis in midribs than in laminae of eggplants leaves but not of sugarcane or maize. Black *et al.* (1987) reported that, SuSy was involved in the breakdown of sucrose. SuSy requires only half the net energy of the sucrose metabolic pathway catalyzed by invertase. Wendler *et al.* (1990) using sugarcane cell suspension cultures reported that sucrose synthase activity was 10-15 fold higher than sucrose phosphate synthase activity at all stages.

Goldner *et al.* (1991) reported that, SuSy is involved in sucrose synthesis but the equilibrium is usually in the direction of degradation. Lingle and Smith (1991) reported that sucrose accumulation is related to carbon partitioning and sucrose synthase may be determinant of sink strength or carbon partitioning in sugarcane internodes. In sugarcane, though the role of SuSy is still a matter of debate, significant levels of SuSy activity

in sucrose accumulating internodes have been reported. Botha *et al.* (1996) found that, SuSy activity remained constant as internode matured, showing only a minor increase towards the more matured internodes. However, no significant correlations between SuSy activity and sucrose accumulation rate have been found.

Buczynski *et al.* (1993) reported that, very high SuSy activity is present in sink tissues which includes both sucrose accumulating (culm) and non–accumulating tissue, such as the leaf roll and roots of sugarcane (activities in sucrose synthesis direction), varying from about 450 nmol min^{-1} mg $protein^{-1}$ in the apex to 1600 nmol min^{-1} mg $protein^{-1}$ in internode. The SuSy activity in the leaf roll tissues is also very high, about 1400 nmol min^{-1} mg $protein^{-1}$. Lingle and Irvine (1994) correlated high levels of sucrose synthase activity with an increase in sucrose accumulation rate and plant ripening. Zrenner *et al.* (1995) reported that, sucrose can be cleaved to form fructose as well as glucose or UDP-glucose by two different enzymes, invertase and sucrose synthase.

Zhu *et al.* (1997) and Botha and Black (2000) reported no correlation between sucrose synthase activity and sucrose accumulation. Black *et al.* (1995) and Sung *et al.* (1999) reported that SuSy activity is an indicatior of sink strength. It is evident that SuSy is not as good an indicator of a sink for sucrose accumulation. The higher specific activity of SuSy in the young internodes, suggest that, it may be an indicator of a sink for sucrose import for respiration and other biosynthetic activities. SuSy catalyzes the reversible conversion of UDP-glucose and fructose to sucrose and UDP. Substrate concentration in most tissues where SuSy is found causes the enzyme to function in the

sucrose breakdown direction (Xu *et al.,* 1989 and Amor *et al.*, 1995). The ratio of maximum sucrose breakdown to synthesis activity in sugarcane was found to increase with internode maturity, from internode 3 to internode 9. There is a ppositive correlation between the sucrose breakdown activity of SuSy and total sugar content (Schafer *et al.,* 2004).

Lingle (1996) determined the sugar accumulation rate and sucrose synthase activity in early ripening and late ripening cultivars. The internodes with highest rates of total sugar accumulation had higher activity of sucrose synthase in cleavage direction than internodes with low sugar accumulation rates suggesting that sucrose synthase plays a role in sugar import into storage tissues. Zhu *et al.* (1997) indicated no correlation between sucrose synthase activity and sucrose accumulation.

Lingle (1997) examined growth, sugar accumulation and sucrolytic activities of sucrose synthase during internode development in two sugarcane cultivars and reported that enzyme activities between the cultivars were not significantly different. Sucrose synthase activity appeared to be related to total sugar (glucose + fructose + sucrose) accumulation rate. Sturm *et al.* (1999) reported that, utilization of sucrose as a source of carbon and energy depends on its cleavage into hexoses and in plants sucrose synthase catalyzes this reaction. Sucrose synthase is glycosyl transferases that not only converts sucrose into UDPG and fructose in the presence of UDP, but also catalyze sucrose synthesis in a reversible manner, although it is usually believed that it is primarily involved in sucrose breakdown. Sucrose

synthase is a cytoplasmic enzyme and in most plants two closely related isoforms have been identified.

Botha and Black (2000) studied the sucrose accumulation rate and sucrose synthase activity in internodal tissues from a sugarcane variety N 19 and reported no significant correlation between sucrose synthase activity and sucrose content. Carlson *et al.* (2002) reported that, the enzyme sucrose synthase catalyze the reversible cleavage of sucrose in presence of UDP to form UDP-glucose and fructose. Sucrose is the primary form of assimilated carbon within plants, transported from source tissues to sink tissues. The cleave of sucrose at the sink tissues by SuSy provides the precursors subsequently used for a diverse array of biosynthetic pathway, including the synthesis of cell wall polysaccharides and starch. In the sink storage tissues such as developing seed or tubers, the SuSy pathway of sucrose cleavage is usually associated with starch biosynthesis.

Schafer *et al.* (2004) evaluated the expression and localization of SuSy in young (internode 3) to mature (internode 9) internodes of sugarcane variety N19. Enzyme activity in the top and bottom as well as the peripheral and core parts of the internodes suggested that SuSy is present ubiquitously but that the levels can differ significantly in different parts of the internodes with maturity. Koch (2004) reported that the channeling of sucrose into sink metabolism requires the cleavage of sucrose by sucrose synthase. The former hydrolyses of sucrose into fructose and UDP–glucose whereas the latter converts sucrose and UDP into fructose and UDP-glucose. Both enzymes are crucial for plant growth, development and carbon partitioning in sink tissues.

Schafer *et al.* (2004) reported that the sucrose synthase activity is associated with phloem tissues and has been implicated in sucrose breakdown for the generation of UDPG and possible preservation of energy *via* UDP activation of hexose moiety (Huber and Akazawa, 1986). SuSy activity is also associated with carbon sink activation (Zrenner *et al.,* 1995). In sugarcane, SuSy is thought to favour the generation of UDPG from sucrose for the formation of cell wall material and starch, However, it has also been associated with internode elongation (Lingle and Smith, 1991 and Buczynski *et al.,* 1993) and could be involved in sucrose synthesis in young internodes (Goldner *et al.*, 1991 and Schafer *et al.,* 2004).

Sucrose synthase isoforms exhibit spatial and temporal expression patterns and are differentially regulated at transcriptional and translational levels (Dejardin *et al.*, 1999). Expression may also vary according to tissue type and carbohydrate metabolic state (Winter and Huber, 2000). Sugarcane has two SS isoforms, which are differentially expressed during development (Lingle and Dyer, 2001 and Schafer *et al.*, 2004). When sucrose concentrations are low in immature plants, CitSUS1 and SS1 isoforms are most abundant, however these isoforms decline when sucrose is high. As sugarcane plants mature (attain high sucrose in internodes), sucrose synthase activity increases in the break-down direction (Schafer *et al.,* 2004, 2005). Hence, sucrose synthase activity could lay a dual role by providing hexoses to actively growing internodes and creating a strong sink for unloading sucrose into mature internodes.

Verma *et al.* (2010) studied the functional analysis of sucrose synthase in two early maturing, high sucrose accumulating sugarcane cultivars (CoS 96268 and CoS 95255) and two late maturing, low sucrose accumulating cultivars (CoS 97264 and CoSe 92423) from 270 to 390 DAP and reported that a negative correlation of sucrose synthase with sucrose and a positive correlation with hexoses was established in both high and low sucrose cultivars. Sucrose synthase was expressed at high levels in low sucrose accumulating cultivars and was responsible for the high hexose concentrations in the respective tissues.

Batta *et al.* (2011) studied the variability in sucrose metabolizing enzymes in relation to sucrose accumulation in stem tissues of parents and a set of ten progenies of the cross Co 89003 (early maturing) x CoSe 92423 (late maturing) during development and reported that, sucrose content increased from stem elongation stage to maturity stage in both parents and progenies. Sucrose concentrations of the progenies were intermediate between those of the parents at stem elongation stage. The reducing sugar content, i.e. glucose plus fructose continuously decreased with advancement of the growth of the stem in all genotypes. Specific activity of sucrose synthase and sucrose phosphate synthase peaked at ripening stage and declined thereafter in each of the parents and their progenies.

2.3.3. Invertase

Invertases (β-fructosidase, β-fructofuranosidase) have been suggested to be key regulators for sucrose accumulation in sugarcane stem parenchyma (Hatch and Glasziou, 1963; Sacher *et al.*, 1963; Gayler and Glasziou, 1972 and Zhu *et al.*, 1997) and

involved in the hydrolysis of sucrose into glucose and fructose to provide cells with fuel for respiration and with carbon and energy for the synthesis of numerous different compounds (Ma *et al.,* 2000). The hydrolytic and or cleavage activities of the invertase in all subcellular compartments exert an influence on sucrose metabolism, translocation and storage (Lee and Vattuone, 1996). Invertase catalyze the irreversible hydrolysis of sucrose to glucose and fructose.

Sucrose + H_2O ---› Glu + Fru

Studies of the distribution of these enzyme have suggested that, high invertase activity occurs in tissue in which active cell elongation is occurring (Sung *et al.*, 1998).

Several different physiological functions have been proposed for invertase i.e. to provide growing tissues with hexoses as a source of energy (Rees, 1974), to generate a sucrose concentration gradient and to partition sucrose between source and sink tissues as well as to aid sucrose transport (Eschrich, 1980). Invertase hydrolyse sucrose to glucose and fructose and play a key role in the control of metabolic fluxes, sucrose partitioning and ultimately plant development and crop productivity (Sonnewald *et al.*, 1991; Sturm, 1999 and Tang *et al.*, 1999) and the hexoses thus generated increase the turgor pressure of cells, suggesting their role in cell elongation and plant growth.

Glasziou (1960) and Sacher *et al.* (1963) studied the relationship between acid invertase activity and sucrose storage in young internodes suggesting that sucrose uptake from the apoplast was dependent upon its hydrolysis prior to transfer to the storage compartment of the parenchymatous tissues. Sucrose can be

hydrolyzed in three distinct compartments: cell wall, metabolic compartment (cytoplasm) and the storage compartment (vacuole), through the action of invertases. Plant invertases are divided into two groups defined by pH optima.

Three classes of invertases can be distinguished based on their pH optima and sub cellular localization. Acid invertase are optimally active between pH 5.0 and 5.5 and exist in a soluble vacuole form (Sacher *et al.,* 1963) as well as insoluble form bound to cell wall (Hatch and Glasziou, 1963). These two sub classes of acid invertases are not present in significant amounts in the mature internodes of sugarcane resulting in neutral invertase (NI, EC 3.2.1.26) being the predominant sucrose hydrolyzing activity in these tissues.

Invertases are the key enzymes involved in sucrose metabolism in sugarcane and highly correlated with sucrose and reducing sugar content during plant growth. They cleave sucrose to glucose and fructose which are classified by stability, cellular location and pH optimum. Based on their sub cellular localization, pH optima, solubility and isoelectric point, three different types of invertase isoenzymes could be distinguished: vacuolar soluble acid invertase (VAI), cell wall– bound invertase (CWI), and cytoplasmic neutral invertase (NI) (Quick and Schaffer, 1996 and Link *et al.*, 2004).

2.3.3.1 Soluble acid invertase

Soluble acid invertase (SAI, EC 3.2.1.26) activity is reported to be high in the apoplast and vacuoles of young, actively growing internodes and almost absent from the mature internodes which are optimally active between pH 5.0 and 5.5 and exist in a

soluble vacuole form. The vacuolar acid invertase has been implicated in the mobilization of stored photosynthate to provide substrate necessary for the growth of immature, expanding tissues in sugarcane (Sacher *et al.,* 1963). The developmental variation in SAI activity reflects both the demand of hexoses during cell elongation and the greater requirement for metabolic substrates in developing cells. SAI is also believed to mediate remobilization of sucrose from storage for growth, or to maintain cellular processes during periods of stress. Transgenic sugarcane cells in which soluble acid invertase activities in different sub cellular compartments were lowered and increased sucrose accumulation (Ma *et al.,* 2000). There is a strong indication that genetically modified sugarcane with reduced acid invertase might increase sucrose accumulation.

Hatch and Glasziou (1963) and Glasziou and Bull (1995) reported that there is an inverse correlation between increasing sucrose and soluble acid invertase activity in maturing sugarcane. Soluble acid invertase activity plays a key role in internode elongation and that elongation rate was directly correlated with soluble acid invertase. The activity of SAI is usually high in tissues that are rapidly growing such as cell and tissue cultures root apices and immature stem internodes. During internode growth and development, SAI activity is decreased by more than two orders of magnitude (Hatch and Glasziou, 1963). SAI activity occurs primarily in the vacuoles of storage parenchyma cells, but it is also reported to occur in the apoplastic cell wall space either as a soluble enzyme or bound to the cell wall fraction (Hawker *et al.,* 1991).

Hawker and Hatch (1965) reported that, soluble acid invertase, occurring in the vacuole and apoplastic space of elongating internodes disappeared when internode growth ceased and reappeared when growth resumed. The vacuolar form appeared to be involved with regulation of turgor and the internal sugar pools; the apoplastic form appeared to be the major controller for dry matter import accompanying cell extension growth. He also reported that mature sucrose storing internodes of sugarcane contain negligible soluble acid invertase activity than immature internodes, which do not accumulate sucrose. Ricardo and Rees (1970) reported that, tissue that accumulate sucrose have been characterized by a loss of acid invertase activity and the loss of acid invertase activity seems to be a prerequisite for sucrose accumulation.

Glasziou and Gayler (1972), Batta and Singh (1986) and Zhu *et al.* (2000) reported that in sugarcane stem tissue, soluble acid invertase is partitioned between large fraction, which occurs in the vacuole, and a smaller fraction, which occurs in the apoplastic space. The apoplastic soluble acid invertase is postulated to control the flow of sucrose from conducting tissue to young growing cells, the vacuolar soluble acid invertase is concerned with the return of sucrose from storage compartment i.e. vacuole.

Glasziou and Gayler (1972) reported that immature (i.e. stem top) storage tissues where cell elongation is predominant, the sucrose stored in the vacuole is rapidly hydrolyzed by soluble acid invertase and the resulting hexoses return to the metabolic compartment by diffusion movement. Escherich (1980) suggested that, vacuolar acid invertase cleaves sucrose when there is a high

demand for sucrose hydrolysis (e.g. cell expansion). Cells containing high acid invertase can not store sucrose in the vacuole and thus in these cells the need for sucrose hydrolysis may be met by neutral/alkaline invertases.

Batta and Singh (1986) studied the activities of soluble invertase in leaf blade and soluble and wall bound invertase in stem tissue of sugarcane at different stages of cane growth and reported that, soluble invertase in leaf blade was most active at tillering stage. In stem tissues soluble acid invertase was highest at stem elongation stage and then declined. The activities of soluble neutral invertase and wall bound acid invertase increased with the age of cane.

Miron and Schaffer (1991) reported that sucrose accumulating storage tissues were characterized by a metabolic transition during development. During the initial growth phase, soluble acid invertase activity was relatively high, which declined concomitantly with sucrose accumulation. Acid invertase activities were high in apoplast and vacuoles of young, actively growing internodes while remaining absent from the mature internodes. Sehtiya *et al.* (1991) emphasized that the acid invertase activity was higher in immature internodes than in mature internodes.

Goldner *et al.* (1991) studied the enzymes of sucrose metabolism in cell suspension cultures of sugarcane and reported that the activity of soluble acid invertase (pH optima 5.5) was highest at beginning of cultural cycle followed by sharp decline during sucrose accumulation.

Venkataramana *et al.* (1991) reported the changes in activities of invertases in relation to sucrose accumulation in

sugarcane. Immature stem contained both acid and neutral invertases. An increased in acid invertase activity up to grand growth phase and a sharp decline thereafter indicated early onset of maturity. Generally acid invertase was very active in immature stem tissue whereas neutral invetase was relatively high during 240-300 days but decreased thereafter considerably. Thus acid invertase was involved in cane tissue formation while neutral invertase balanced the sugar content in storage parenchyma of stem tissues. The SAI activity shows marked seasonal variation being high when growth is rapid and low otherwise. SAI activities are usually high in storage tissues that are rapidly growing during internode growth and development (Lontom *et al.,* 2008). Hawker *et al*. (1991) reported that soluble acid invertase actively occurs mostly in the vacuole of storage parenchyma cells and a few in the apoplastic cell wall space either as a soluble enzyme or bound to the cell wall fraction.

Sehtiya *et al*. (1991), Sehtiya and Dendsay (2000) and Dendsay *et al*. (1995) reported that, sugarcane cultivars vary in their potential of sucrose accumulation. Vacuolar invertase activity is responsible for high sucrose accumulation in internode tissue of high sugar and early maturing cultivars sugarcane storage tissue accumulating sugar against a concentration gradient using energy provided by respiration (Bieleski, 1960). This is accompanied by a continuous cleavage and synthesis of sucrose during accumulation of sucrose in storage tissue (Hatch and Glaszious, 1963; Batta and Singh, 1986 and Whittaker and Botha, 1997).

Dendsay *et al*. (1992) used the sugarcane hybrids CO-64, CO-7717, COS-767 CO-1148 and CO-12 to investigate the

relationship between internodal elongation, sucrose accumulation and the invertase activity and reported that maximum invertase activity coincided with the period of fastest growth and the activity in elongated internodes was four to six times lower than that in elongating internodes. Furthemore, the level and timing of sucrose accumulation in the whole stalk and within individual internodes has been correlated with the down regulation of soluble acid invertase activity (Zhu *et al.*, 1997).

Dali *et al.* (1992) reported that acid invertase catalyze the conversion of sucrose to glucose and fructose. It is one of the most important enzymes involved in sucrose metabolism in fruits. Sucrose hydrolysis by acid invertase may determine the rate and extent of sucrose storage in tomato fruits. Venkataramana and Naidu (1993) studied the invertase sucrose relationship in young and mature stem of sugarcane. In sugarcane, a high soluble acid invertase in young stem was associated with low sucrose concentration in juice and as the cane matured, the soluble acid invertase decline with concomitant increase in sucrose. Sucrose concentration in juice was negatively correlated with soluble acid invertase.

Zhu *et al.* (1996, 1997) studied the relationship of sucrose metabolism enzymes with sucrose storage in a high sucrose clone and a low sucrose storing clone of sugarcane on a whole stalk basis and reported that there was an inverse relationship between the soluble acid invertase (SAI) activity and the concentration of sucrose. He was also reported that, during stem elongation, rapid cell expansion occurs; the accumulated sucrose is rapidly hydrolyzed by high activity of vacuolar acid

invertase leading to free movement of hexoses to cytoplasm for their utilization in growth processes. During maturity stage, there is marked decline in vacuolar acid invertase and the cytoplasmic neutral invertase becomes predominant which governs the accumulation of sucrose in the vacuole at this stage. The level and timing of sucrose accumulation in the whole stalk and within individual internodes was correlated with the down regulation of soluble acid invertase (SAI) activity above which high concentration of sucrose did not accumulate. This low level of soluble acid invertase activity was always exceeded in the internodes of the lower sucrose storing genotypes.

Lingle (1997) reported that, the level and timing of sucrose accumulation in the whole stalk and within individual internodes was correlated with the down regulation of acid invertase activity. High acid invertase activity appeared to prevent most but not all sucrose accumulation. He also reported that, the high soluble acid invertase activity at late grand growth stage (165 DAP) may be associated with cellular metabolism for internodal expansion and not with accumulation of sucrose.

Terauchi *et al.* (1999) characterized the early maturing sugarcane variety with high sugar content in relation to growth and invertase activities. The acid invertase activity, a negative factor for sucrose accumulation, decreased in cultivar NiF_4. The decline in acid invertase activity contributed significantly to the increase in sucrose concentration in this variety through inhabitation of stalk elongation. The activity of soluble acid invertse was relatively higher in cultivar NiF_4 than F_{172}. Thus the level of soluble acid invertase activity caused varietal difference in sucrose

concentration. The soluble neutral invertase activity, a positive factor for sucrose accumulation was higher in NiF_4 than F_{172}. Thus the soluble neutral invertase activity contributed to the sucrose accumulation through high flux of sugars in NiF_4 cultivar.

Sturm (1999) and Grof and Campbell (2001) reported that, soluble acid invertase (SAI) is localized in the vacuole and play an important role in the remobilization of stored sucrose from the vacuole (Sacher *et al.,* 1963) and is believed to be important in the regulation of hexose levels in certain tissues (Singh and Kanwar,1991). Sturm (1999) reviewed the role of invertase in plant development and sucrose partitioning. The invertase existed in several isoforms with different biochemical properties and subcellular locations. The genes for acid invertase isozymes are regulated developmentally and by sugar, wounding and pathogen. He also reported that, most plant species contain at least two isoforms of vacuolar invertase, which accumulate as soluble proteins (soluble acid invertase) in the lumen of this acidic compartment. Several isoforms of extracellular invertase (cell wall invertase) that are ionically bound to the cell wall. Vacuolar and cell wall invertases share some biochemical properties e.g. they cleave sucrose most efficiently between pH 4.5 and 5.0 and attack the disaccharide from the fructose residue. Plants have at least two isoforms of cytoplasmic invertase with pH optima for sucrose cleavage in the neutral and slightly alkaline range. Neutral and alkaline invertases are less well characterized but in contrast to the acid invertases, these enzymes are to be sucrose specific.

Cheng *et al.* (1999) reported that, in plant sucrose is predominant sugar of transport between a source tissue

(autotrophic) and heterotrophic sink organs such as developing seeds and tubers. Enzyme invertase provide an energy point for sucrose into cellular metabolism and the cell wall invertase in the apoplast is the first enzyme to commit sucrose carbon into downstream metabolism inside the cell. Invertase plays a crucial role in the control of metabolic fluxes, down stream sucrose partitioning and ultimately plant development and crop productivity.

Zhu *et al.* (2000) reported that SAI concentration is high in meristimetic tissues but decreases rapidly during growth and development of internode. Therefore, sugarcane varieties that are of low sucrose level retain high levels of SAI. He also reported that the hydrolytic activity of soluble acid invertase is strongly correlated to sucrose accumulation in sugarcane. Plants exhibiting SAI activity above a low threshold level do not accumulate high concentration of sucrose. A high rate of soluble acid invertase activity has been measured in developing internodes of sugarcane where it is thought to have a role in control of turgor. As the sugarcane stalk matures, reduced invertase activity may be important for the accumulation of sucrose. Correlations between low activity of SAI and high sucrose accumulation have certainly been demonstrated in some sugarcane clones.

Hongmei *et al.* (2000) reported that suppression of SAI gene by RNAi technology has resulted in increased sucrose content in sugarcane confirming the role of vascular acid invertase in the control of sucrose accumulation. Ma *et al.* (2000) revealed that SAI had a negative correlation and was important in the accumulation of sucrose. Hocine *et al.* (2000) reported that, invertase catalytic

activity was dependent on sucrose concentration which decreased markedly with increasing sucrose concentration. Invertase exhibited only hydrolytic activity producing exclusively fructose and glucose from sucrose.

Hongmei *et al.* (2000) reported that increased invertase activities in the cell wall and in the cytosol cause a decrease in the sucrose content in the sugarcane callus cells. They also reported that, SAI activity can suppresed by introducing an anti–SAI construct into sugarcane cells and then determined the resulting sucrose level. A sugarcane soluble acid invertase cDNA, scinvm, was isolated from Molokai 5829 (*Saccharum robustum*), a low sucrose accumulating variety. A protein of the cDNA was placed under control of the maize Ubi-1 promoter in the antisense orientation and this construct was transformed into sugarcane embryogenic calluses derived from H62–4671, a high sucrose accumulating cultivar. The antisense gene suppressed acid invertase activity up to 50 % in the soluble fraction and 25-30 % in the cell wall fraction in the cultured cells. In transgenic cells, the sucrose level was increased about two fold. These result indicated that SAI activity is indeed a limiting factor in the sucrose accumulation process of sugarcane.

Albertson *et al.* (2001) isolated different isoforms of invertase from sugarcane and reported that soluble acid invertases (pH optimum 4.5) were linked to growth and differentiation. He was also developed extraction and assay method for determination of soluble and cell wall invertase activity in sugarcane (*Saccharum* spp.) leaves and stem and reported that, soluble acid invertase activity was greatly reduced in mature tissue extracts.

Botha *et al.* (2001) reported that in sugarcane, a reduction in AI activity had no effect on sucrose accumulation. Kubo *et al.* (2001) reported that decreasing in SAI activity correlates with ripening in sink tissues and the level of sucrose may be regulated through the modulation of SAI activity and the synthesis reaction of sucrose synthase. SAI may play a role in the remobilization of stored sucrose from the vacuole (Sacher *et al.*, 1963) and is also believed to regulate hexose levels in certain tissues. Correlation of sucrose with SAI activity was significantly negative and positive with SPS and SS activity. The level and timing of sucrose accumulation in the whole stalk and within individual internodes was correlated with the down regulation of SAI activity (Zhu *et al.*, 1997).

Rohwer and Botha (2001) reported that, when SAI activity is high, the sucrose content is always low, but when SAI activity is low, the sucrose content is can either be low or high can fit a hyperbolic function. The net sucrose accumulation in the storage tissue of sugaracne is determined by futile cycling operative in storage tissue involving continuous synthesis and breakdown of sucrose where SS, SPS and soluble invertases played the key role. Gutierrez *et al.* (2002) reported that sucrose accumulation in sugarcane stem is regulated by the difference between the activities of SAI and SPS.

Yau and Simon (2003) reported that sucrose is the major end product of leaf photosynthesis and is a major sugar transported in the phloem of most higher plants which can not used directly for most metabolic processes and must be cleaved into hexoses by invertase or sucrose synthase before use. Invertase

is present in most plant tissues and irreversibly catalyzes the breakdown of disaccharide sucrose into fructose and glucose. Isoforms of invertase are characterized and classified according to pH potima (acid, neutral and alkaline), subcellular locations (vacuole or cell wall). Acid invertase has a pH optimum between 3 and 5, whereas neutral and alkaline invertase have pH optima of about 7 and 8 respectively (Lee and Sturm, 1996). Acid invertase are divided into vacuolar or soluble and extracellular or insoluble forms involved in phloem unloading, control of sugar type in storage organs, normal development of endosperm and response to pathogen infection or wounding.

Sachdeva *et al.* (2003b) reported that, soluble invertase *viz.*vacuolar acid invertase and cytolasmic neutral invertase are the key metabolic regulators for sucrose cycling and accumulation in sugarcane stem (Rae *et al.,* 2005a; Batta *et al.*, 2008 and Pan *et al.*, 2009), which in turn determine the maturity behavior in sugarcane. Soluble invertase occurs in multiple forms with different pH optima and subcellular localizations.

Hothorn *et al.* (2004) reported that plant acid invertase catalyze the hydrolytic cleavage of the transport sugar sucrose which is the major transport form of carbohydrates in higher plants. Sucrose is exported from the source tissues (leaves) and transported *via* the phloem to different sink tissues (root, stem, reproductive organs and vegetative storage organs). Cell in the target tissue may take up sucrose symplastically or apolastically, and the sucrose can be hydrolyzed by invertase subsequently. These enzymes reside in the vacuole and the extracellular space, where invertase activity facilitates long range carbohydrate

transport by creating sucrose concentration gradients. Sucrose and its hydrolysis products glucose and fructose provide growing tissues with energy and can serve as signals regulating gene - expression. Plant invertase is highly glycosylated proteins that are found in the extracellular acidic compartments (cell wall and vacuole). In plant cell, invertase metabolism operates in the cell wall compartments where the pH ranges between 4 and 6. Cell wall invertase activity is strongly pH dependent with a maximum activity at about pH 4.0.

Thangavelu and Rao (2004) reported that the activity of soluble acid invertase was much higher in the top than the mid and bottom portions at the stem elongation stage. With the advancement of cane growth, this activity fell but more drastically in the top part of the stem. Batta *et al.* (2007) reported that, sugarcane cultivars vary in their potential of sucrose accumulation. The rapid decline in the activity of SAI with the maturity of the cane correlated with the high level of sucrose in early maturing varieties of sugarcane. The reduction of SAI activity was dramatic in early maturing and high sucrose storing cultivars, whereas the decline in was lower in late maturing and low sucrose storing cultivars (Sachdeva *et al.,* 2003b and Terauchi *et al.*, 1999). However, low activity of SAI is not sufficient to account for high sucrose accumulation in high sucrose storing genotypes. The major difference in sucrose accumulation was attributed to the difference between activity of SAI and SPS, provided the SAI activity is below the critical threshold concentration (Zhu *et al.*, 1997).

Batta *et al.* (2007b) reported that, the rapid fall in the activity of SAI with the age of cane has also been related to early

maturation in sugarcane under sub- tropical conditions of Punjab, India. Batta *et al.* (2008) examined the metabolic changes in the contents of sucrose and hexoses in relation to the activities of the principal enzymes involved in the accumulation of sucrose in internodal storage tissues in four sugarcane genotypes differing in maturity behaviour *viz.,* Sel. 69/01, Sel. 943/98 (early maturing) and CoS 8436 and Sel. 17/00 (mid late maturing) at different physiological stages of crop growth and reported that in storage tissues, the higher rate of decline in the activity of soluble acid invertase (pH 5.5) with age of cane resulted in faster rate of sucrose accumulation in early maturing sugarcane genotypes and was related to higher sink strength of these genotypes. Unlike soluble acid invertase, the activity of soluble neutral invertase increased with cane maturation in each of these genotypes. The wall bound acid invertase (pH 5.5) showed maximum activity at stem elongation stage.

Lontom *et al. (*2008) studied the activities of SAI in selected immature and mature internodes during ripening and during post harvest storage at 18^{o}C in two sugarcane varieties HoCP 96-540 and L 97-128 and reported that, during ripening, the activity of SAI was highest in the youngest internode and it decreased with internode age. Sucrose content during ripening was negatively correlated with SAI activity in both cultivars. Verma *et al.* (2011) studied the transcript expression and soluble acid invertase activity during sucrose accumulation levels in the immature and mature internodal tissues of sugarcane cultivars CoS 96268, CoS 95255 (high sugar accumulating) and CoS 972654, CoSe 94423 (low sucrose accumulating) during the 240-

390 DAP stages (grand growth to ripening stage) and reported that, in high and low sucrose- accumulating cultivars, sugars, (i.e. sucrose and hexoses) were accumulated differently in immature and mature internodal tissues. Higher concentration of sucrose was found in the differential tissues of high sucrose accumulating cultivars than in low sucrose accumulating cultivars from 240 to 390 DAP. In both high and low sucrose accumulating cultivars, hexose sugars decreased with advancement of developmental stages. The accumulation of sucrose was found to be inversely correlated with the SAI activity. The SAI activity was higher in immature internodes as compared to mature internodes. However, sugar accumulation was higher in mature internodes. There was a negative correlation between SAI activity and sucrose concentration, whereas, a positive correlation with hexose sugar concentration was observed in both immature and mature internodal tissues. Batta *et al.* (2011) reported that, specific activity of soluble acid invertase in stem was highest at the stem elongation stage and decline thereafter.

2.3.3.2 Soluble netural invertase

The soluble neutral/alkaline invertases (SNI, EC 3.2.1.26) are found in the cytosol and considered as 'maintenance' enzymes involved in sucrose degradation when the activities of acid invertase and sucrose synthase are low (Winter and Hubber, 2000). Sucrose is belived to be hydrolysed in the mature storage parenchyma tissue by netural invertase which is present at low levels in very young tissue and at greater levels in older tissues (Hatch and Glasziou, 1963 and Batta and Singh, 1986). Neutral invertase (SNI) regulates sucrose movement from the vascular to

storage tissues in mature internodes or it is involved in the turnover of hexoses in mature tissues. The neutral invertase increased during maturation and appeared to be involved in controlling sugar flux in the mature storage tissue. Neutral invertase is also known as alkaline or cytoplasmic invertase because of their pH–optima (between 6.8 and 8.0).

Glasziou and Gayler (1972) reported that neutral invertase occurring in cytoplasm is active at a low level in very young tissue and at greater levels in older tissue. He was also reported that, at each stage of cane development, the activity of soluble neutral invertase was higher in the mature portion of the stem tissue. This cytolasmic neutral invertase may be involved in regulation of sugar flux in mature stem tissue. The involvement of neutral invertase in sucrose turnover may require the quantification of flux in sugarcane stem tissue. The neutral invertase may play a key role in control of hexose concentrations in the cytosol of sugarcane stem cells, thus affecting the control over the expression sugar responsive genes.

Vorster and Botha (1998) reported that, SNI play a key role in controlling the hexose concentration in the cytosol of the stem cells, hence affecting the expression of sugar responsive genes though the exact role of SNI is not known as yet. Bosch *et al.* (2004) isolated and characterize the SNI gene and analyze transcription pattern in different tissues by comparison to sucrose, glucose and fructose levels. The SNI cDNA was isolated using the *L. temulentum* clone as a probe and then subsequently identified. It showed 53 % homology on an amino acid level compared to published sequences of neutral invertases from *D. carota* (Sturm,

1999) and *L. temulentum* (Gallagher *et al.,* 1998). SNI transcript and protein levels were higher in the younger internodes with low sucrose concentration than in the mature tissues, suggesting that SNI could supply young tissues with hexose. He was also reorted that, in culm tissues where sucrose content was low and hexose contet were high, neutral invertase transcript level was high than in those tissues dedicated to sucrose storage.

Neutral invertase is involved in sucrose degradation when the activities of VAI and SS are low (Winter and Hubber, 2000), involved in the response of plants to environmental factors, such as wounding and infection (Sturm and Chrispeels, 1990).

Sehtiya *et al.* (1991) reported that the the neutral invertase activity was higher in mature internodes than in immature internodes, in agreement with the result of Verma *et al.* (2010). Singh and Kanwar (1991) reported that neutral invetase (NI) activity (expressed on fresh weight basis) increases with internode maturity and correlate positively with sucrose concentration in intermodal tissues. Goldner *et al.* (1991) studied the enzymes of sucrose metabolism in cell suspension cultures of sugarcane and reported that the activity of the neutral invertase activity (pH optima 7.0) was lower than acid invertase activity and decreased throughout the cultural cycle.

Dendasy *et al.* (1995) reported that, both acid and neutral invertase seem to be involved in internode elongation/expansion activities in immature internodes, but the high activity of neutral invertase on a fresh weight basis in the maturing internodes suggest an additional role in these tissues. Early maturing sugarcane varieties, which show higher sucrose

accumulation than the late varieties, exhibited an early decline in invertase levels, with low NI activity levels being maintained throughout the maturation phases. In low sucrose–storing varieties, however, NI remained fairly active during the late maturity months. Hexose levels have been shown to correlate positively with NI and not AI levels in mature sugarcane stem tissue (Gayler and Glasziou, 1972 and Rose and Botha, 2000).

Singh and Kanwar (1991) argued that, invertase activities are not significantly correlated with hexoses levels but positively correlated with sucrose concentration in the sugarcane stem tissue. Both acid and neutral invertase seem to play an important role in sugarcane by regulating the net concentration of sucrose in internodal tissue and NI is likely to be partially responsible for the cycle of degradation and synthesis of sucrose that has been found in sugarcane suspension cells (Wendler *et al.*, 1990), in the immature sugarcane stem (Sacher *et al.,* 1963) and in sugarcane tissue discs (Bindon and Botha, 2002) or that, it regulates sucrose movement from vascular to storage tissue in mature internodes (Hatch and Glasziou, 1963).

Lingle (1997) reported that neutral invertase activity was highly variable among internodes of same age and between sampling dates. Vorster and Botha (1998) reported that, sugarcane neutral invertase partially purified from a mature sugarcane stem tissue were found to be non- glycosylated and they are exhibited catalytic activity in various forms such as monomer, dimmer and tetramer but most of the activity eluted as a monomer of native MW 60 kDa. The enzyme displayed a typical hyperbolic saturation kinetic for sucrose hydrolysis. It has a Km of 9.8 mM for sucrose

and a pH optima of 7.2. The end product of soluble neutral invertase inhibited its activity and fructose was stronger inhibitor than glucose. Soluble neutral invertase is significantly inhibited by $HgCl_2$, $AgNO_3$, $ZnCl_2$, $CuSO_4$ and $CoCl_2$ but not by $CaCl_2$, $MgCl_2$ and $MnCl_2$. He was also reported that, in sugarcane NI activity was detected in the 60, 120 and 240 kDa forms and located in the cytosolic compartment where it functions optimally at pH 7.0. NI activity (on a per mg protein basis) increase up to the fifth internode before declining as the internodes mature further.

Gallagher and Pollock (1998) reported that a gene encoding an enzyme with neutral/alkaline invertase activity has been cloned for the first time from *Lolium temulentum.* Vorster and Botha (1999) showed a relationship between extractable invertase activities and sucrose accumulation in the sugarcane stalk and *in vivo* invertase mediated sucrose hydrolysis to determine the significance of invertase in sucrose utilization and turnover and they found that sugarcane neutral invertase had a higher specific activity than SAI (apoplastic and vacuolar) in the sucrose accumulating region of the sugarcane stem. The optimum pH of sugarcane neutral invertase was 7.2. He was also reported that, the SNI protein has a molecular weight between 58 and 66 kDa and subunit aggregation as well as biochemical properties, including pH optima ranging between 7.0 and 7.5. He also showed a relationship between extractable invertase activities and sucrose accumulation in the sugarcane stalk and *in vivo* invertase mediated sucrose hydrolysis to determine the significance of invertase in sucrose utilization and turnover. They also found that sugarcane neutral invertase had higher specific activity than SAI (apoplastic and

vacuolar) in the sucrose accumulating region of the sugarcane stem.

Gayler and Glasziou (1972) and Bosch *et al.* (2004) reported that, neutral invertase regulates sucrose movement from vascular to storage tissues in mature internodes or that it is involved in the turnover of hexoses in mature tissues. Rose and Botha (2000) studied the neutral invertase and sugar from the developing clum tissues of sugarcane according to developing stages (internode 3, 6 and 9) and anatomical differentiation (enriching for elongation, vascular or storage tissues) and reported that the lowest sucrose content was found in the core of bottom of each of the internodes. The ratio between the two hexoses, glucose and fructose and sucrose was the highest in the young internodes and was also significantly different between the top and bottom parts of the young internodes. He was also reported that the relationship between extractable invertase activities and sucrose accumulation in sugarcane stalk, and *in vivo* invertase mediated sucrose hydrolysis to determine the significance of invertase in sucrose utilization and turnover. *In vitro* activities were determined by assaying the soluble acid, cell wall bound acid and neutral invertase from internodes 3-10 in mature sugarcane plants. Extractable activities were verified by imunoblotting. Sugarcane neutral invertase had a higher specific activity than soluble acid invertase (apoplastic and vacuolar) in the sucrose accumulation region of the sugarcane stem. He was also reported a significant variation in NI expression and sucrose content within sugarcane internodal tissue but concluded that NI activity definitely dose not increase with maturation. A weak inverse correlation was however,

found between NI activity and sucrose especially in the bottom part of the internode where active growth occurs.

Albertson *et al.* (2001) reported that, neutral invetrtases (pH optima 7.3) appeared to have a housekeeping role in maintaining hexose concentrations within the cytosol. Rohwer and Botha (2001) reported that, the total as well as specific activity of soluble natural invertase was relatively lower in ratoon crop as compared to plant crop at each stage of crop development. Cytoplasmic neutral invertase has been postulated to regulate sugar flux in mature storage tissue. Reduction in cytoplasmic neutral invertase level, exhibiting numerically largest control coefficient in futile cycle of sucrose, has also been suggested being a promising for higher sucrose accumulation in storage tissue. He was also reported a theoretical kinetic model of sucrose accumulation in sugarcane and identified NI as a possible key regulatory point in the control of flux in the 'futile cycle' between sucrose and the monosaccharide pool, predicted that as much as 22 % of sucrose that is synthesized in sucrose accumulating tissues is broken down again by invertase. This suggests that NI activity could be a key determinant of the sucrose accumulating capacity of sugarcane clum.

Thangavelu and Rao (2004) reported that, the activity of soluble neutral invertase was, however, higher in the bottom than the mid and top portions and markedly increased in the bottom part of the mature cane. Rossouw *et al.* (2007) reported that, in sugarcane, down regulation of neutral invertase in cell suspension cultures led to enhanced sucrose levels that diminished with

maturation. Batta *et al.* (2011) reported that, neutral invertase activity increased with the cane maturation.

2.3.3.3 Cell wall invertase

Cell wall bound invertase (CWI, EC 3.2.1.26) is active in both young and older internoodes and may control the flow of sucrose from conducting tissue to young actively growing cells (Hatch and Glasziou, 1963). In mature tissue, lacking a measurable soluble acid invertase, there was a cell wall bound acid invertase which functioned in cleaving sucrose in the apoplastic space to control dry matter import for sucrose storage (Hawker and Hatch, 1965).

Escherich (1980) suggested that, the cell wall invertase is thought to play a role in assimilate uptake into some sink tissues by establishing steep concentration gradient of sucrose from source to sink. He was also reported that, cell wall invertase also referred as extracellular, apoplasmic, periplasmic or free space invertase are characterized by a low pH optimum (pH 3.5-5.0), a high isoelectric point and are ionically bound to the cell wall. CWIs cleave sucrose leaked or transported by an assumed efflux sucrose transporter from the sieve elements of the phloem into the apoplast. The cleavage products are then transported into the sink cell by hexose transporters.

Batta and Singh (1986) reported that, the activity of wall - bound invertase (pH 4.0) during stem elongation stage was low in each portion of the stem and increased with the maturity of the cane. In the top part of the cane, the activity of this enzyme was much higher throughout the period of cane development.

Batta *et al.* (1995a) compared the activities of invertase in an early maturing (CoJ 64) and late maturing (Co 1148) cultivars of sugarcane at different stages of cane growth and reported that, the activities of cell wall bound acid invertase in stem were found to be higher in CoJ 64 than Co 1148 invertase in stem concomitant with fast accumulation of sucrose in this tissue.

Lingle (1998) reported that, cell wall invertase is often considered as a gateway for the entry of sucrose into the cells of juvenile tissues that have an apoplastic path of phloem loading. Increase in cell wall invertase activity are associated with higher sucrose content in sugarcane. Vorster and Botha (1999) reported that, cell wall bound acid invertase was present in significant quantities in both immature and mature tissues.

Albertson *et al.* (2001) reported that, CWI is localized in the apoplast, ionically linked to the cell wall. In sugarcane internodes, CWI probably controls the flow of sucrose from the conducting tissues to the young growing parenchyma cells.

Roitsch *et al.* (2000) noted that, cell wall bound invertase were specifically expressed under conditions that required a high carbohydrate supply to sink tissues. Substrate and reaction product of invertase are not only nutrients, but also signal molecules like hormones and in combination with hormones and other stimuli, they can regulate many aspects of plant development from gene expression to long distance nutrient allocation.

An increase in CWI activity with interode age has been reported by Vorster and Botha (1999) and Lingle (2004). Hussain *et al.* (2004) studied the CWI activity using a pellet mix procedure and pH optima ranged between pH 3.2 and 3.6 in sugarcane tissues of

varying ages and reported that, the CWI activity was reduced in old leaves and activity from stem extracts remained constant irrespective of tissue age.

Thangavelu and Rao (2004) reported that, the activity of the wall buond invertase (pH 4.0) during the stem elongation stage was low in each portion of the stem and increased with the maturity of the cane. In the top part of the cane the activity of this enzyme was much higher throughout the period of cane development.

Lingle (2004) studied CWI in sugarcane in detail and reported that increased CWI activity, sucrose and sucrose to total sugar ratio with an increase in internode age. Higher activity of cell wall acid invertase in high sugar genotypes enhances sucrose unloading into the internode tissue. He was also reported that, the internodes of the high sucrose storing genotypes appears to be metabolically more active than those of the low sucrose genotypes, thus the cell wall invertase gene may be a good candidate for improving sucrose accumulation in sugarcane. Similar results have also been reported by Botha *et al.* (1996). A strong correlation between the rate of cell extension and CWI activity has been reported, as this enzyme is the gateway for the entry of sucrose into the cell in juvenile tissues. It plays a key role in phloem unloading and assimilate uptake, specifically in sink tissues where an apoplastic step is involved (Koch, 2004). In sugarcane internodes, CWI probably controls the flow of sucrose from the conducting tissue to the young growing parenchyma cells.

Joshi *et al.* (2012) studied the CWI activity during maturity and cloning of CWI gene in sugarcane variety Co 86032

during maturity stages from 9th to 13th months of planting at top (immature), middle (maturing) and bottom (matured) portion of cane and reported that, CWI activity was high at immature top position and gradually reduces towards the mature bottom position of the cane. The CWI activity gradually decreased from 9th to 13th MAP.CWI was involved in the apoplastic and symplastic sucrose translocation between source and sink tissues and its activity reduced with maturity. CWI activity was high in immature internodes. Since the photosynthate supply from sink tissue reduced with maturity, the CWI activity also got reduced. The positive relationship between sucrose accumulation and CWI in the matured portion of the cane suggests that CWI finds a major role in increasing the sucrose concentration by allocating most of the photosynthate produced to the storage tissues (cane).

Chandra *et al.* (2012) reported the greater cell wall invertase activity in high sugar genotypes may operate by balancing sucrose unloading into the internode tissue.

2.3.4 Invert Ratio

Sugarcane crop remains at peak maturity for a short period only and then it starts to deteriorate when sucrose splits to glucose and fructose. In the early maturing varieties the rate of accumulation is faster during the early period of growth and peak maturity is attained earlier than the late maturing varieties (Singh and Venkatramana, 1987). Parthasarathi and Vijayasarathi (1958) reported that fructose/glucose ratio might serve as an index of maturity. Sayed *et al.* (1972) considered fructose/glucose ratio to be a reliable index for cane maturity.

Batta *et al.* (2002) reported that the higher rate of decline in the activity of soluble acid invertase (pH 5.5) with age and high rate of hexose: sucrose ratio in stem was an index of early maturity.

Thangavelu and Rao (2004) studied the invert ratio in juice of top and bottom portion of 30 clones at 9,10,11 and 12 months and reported that invert ratio in juice had significant positive correlation with reducing sugars, glucose , fructose and negative associations with brix, sucrose, purity, CCS %, hand refractometer brix, F/G ratio, HR T/B ratio in top and bottom portion cane juice at 9,10,11 and 12 months.

The only sugars constituting the sugar pool of the stem were sucrose, glucose and fructose where sucrose was a predominant sugar at each stage of cane development in both parents and their progenies. The sum of glucose and fructose constituted the reducing sugar pool. Higher concentrations of reducing sugars in immature stem tissue has physiological significance as these hexoses are utilized for suppling energy for cell division and cell expansion during internodal elongation. During later stage of cane growth, as internodal elongation ceases, a fall in the level of reducing sugars has been observes (Batta *et al.,* 2008).

2.3.5. Biotechnological interventions for improvement of sucrose content

Sugarcane is a major source of food and fuel worldwide and sucrsoe content is a highly desirable trait in sugarcane as the worldwide demand for cost effective biofuels sugars. Sugarcane cultivars differ in their capacity to accumulate sucrose and

breeding programme routinely perform crosses to identify genotypes able to produce more sucrose. Traditional breeding can be improved considerably if the breeder have biotechnological tools available, such as genes that could be used as markers in the selection of genotypes. Biotechnology has the potential to improve economically important traits in sugarcane as well as diversify sugarcane beyond traditional application such as sucrose production.

In the field of biotechnology, due to the importance as sucrose yielding crop sugarcane has been targeted by the novel gene manipulation technique to unravel the complexities of the metabolism of sucrose and releated compounds and also to increase sucrose synthesis/storage capacity *in vivo*. There are no commercial transgenic sugarcane cultivars. Sucrose content in mature internodes reach around 20 % of the culm dry weight. Sugarcane is capable of increasing sucrose upto 25 % more than what is currently available, we predict a great benefit if can successfully target sucrose metabolism genes for increased accumulation (Grof and Campbell, 2001).

Hongmei *et al.* (2000) reported suppression of SAI gene by RNAi technology which resulted in increased sucrose content in sugarcane confirming the role of vacuolar soluble acid invertase in the control of sucrose accumulation.

Papini-Terzi *et al.* (2005) studied thirty genotypes having different Brix (sugar) levels and identified genes differentially expressed in internodes using cDNA microarrays. These genes were compared to existing gene expression data for sugarcane plants subjected to diverse stress and hormone treatments and reported

that, the comparisons revealed a strong overlap between the drought and sucrose content datasets and a limited overlap with ABA signaling. Genes associated with sucrose content were extensively validated by qRTPCR, highlighting several protein kinases and transcription factors that are likely to be regulators of sucrose accumulation. They also reported that, aquaporins as well as lignin biosynthesis and cell wall metabolism genes are strongly related to sucrose accumulation.

Wu and Birch (2007) reported that the bacterial gene namely sucrose isomerase has been tailored for vacuolar compartmetation resulted in sugarcane lines with remarkable increases in total stored sugar levels.

Hongmei *et al.* (2000) created transgenic sugarcane (*Saccharum officinarum*) to express altered invertase isoform activity to elucidate the role(s) of invertase in the sucrose accumulation process. A sugarcane soluble acid invertase cDNA (SCINVm, AF0 62734) in the antisense orientation was used to decrease invertase activity. The *Saccharomyces cerevisiae* invertase gene (SUC2) fused with appropriate targeting elements was used to increase invertase activity in the apoplast, cytoplasm and vacuole. A callus/liquid culture system was established to evaluate changes in invertase activity and sucrose concentration in the transgenic lines and reported that, increased invertase activity in the apoplast led to rapid hydrolysis of sucrose and rapid increase of hexose in the medium. The cellular hexose content increased dramatically and the sucrose level decreased. Cells with higher cytoplasmic invertase activity did not show a significant changes in the sugar composition in the medium, but did significantly reduce the

sucrose content in the cells.Transformation with the sugarcane antisense acid invertase gene produce a cell line with moderate inhibition of soluble acid invertase activity and a 2-fold increase in sucrose accumulation. Overall, intracellular and extracellular sugar composition was very sensitive to the changes in invertase activity. Lowering acid invertase activity increased sucrose accumulation.

Ma *et al.* (2000) reported that the manipulation of the expression of the invertase gene(s) to increase sucrose accumulation in the storage organs such as the stem internodes of sugarcane has been suggested as a good approach to increase sucrose content in sugarcane.

Pinto *et al.* (2009) reported QTLs associated with sucrose content in sugarcane. Expressed sequenced tags (ESTs) derived from cDNA libraries provide an invaluable resource to study genes in non-sequenced complex organism like sugarcane (Watt *et al.*, 2010) help to identify genes encoding enzymes, transcription factors and protein kinases involved in determining agronomically important traits,. These ESTs were used to develop custom cDNA microarrays which are used to study gene expression associated with specific metabolic pathways. The Affymetrix Sugarcane Genome Gene Chip was used to study sucrose metabolism and transport in sugarcane (Casu *et al.*, 2003). This has proven to be an invaluable resource in studing the pathways of gene expression involved in sugar accumulation as well as sugar sensing and signaling in mediating the source-sink relationship in sugarcane (Casu *et al.*, 2004; McCormick *et al.*, 2006 and McCormick *et al.*, 2009).

Increasing sucrose stored plays a crucial role in yield improvement in sugarcane. The areas that are of importance for increasing sucrose accumulation in sugarcane are the rate of photosynthesis, enzymes involved in sucrose synthesis pathways, rate of phloem loading and transport to the ripening stalk, carbon partitioning within the stem, vacuole and also rate of sucrose remobilization to support vegetative growth (Grof and Campbell, 2001 and Govender, 2008).

The vacuole of the parenchyma cells within these tissues was targeted as a possible site for genetic manipulation to increase sucrose accumulation (Rowher and Botha, 2001). The potential ability to store sucrose within this organelle has been determined to be more than twice that of current commercial cultivars (Wu and Birch, 2010). H^+/sucrose transporters situated on the tonoplast membrane of the vacuole rely on a proton gradient between the cytosol and lumen to pump sucrose into lumen. A tonoplast H^+/PPase (V-PPase) has been identified to be important in driving this gradient (Maeshima, 2000 and Ferjami *et al.*, 2011) and increased expression has been correlated with increased sucrose accumulation in the sugarcane culm. The proton gradient generated from increased V-PPase activity would increase the amount of sucrose transported into and stored within the vacuole. Increased activity of this protein may increase sucrose for different reason. The protein will decrease PPi concentration within the cytosol. Lowered PPi should decrease PFP activity and as antisense PFP sugarcane plants accumulate more sucrose should increase the amount that accumulates.

Significant progress in biotechnological front especially generation of ESTs and molecular markers and functional analysis of genes involved in sucrose metabolism have generated fresh and new platform to deliver a better understanding about the differential sucrose accumulation. With the latest knowledge generation through and genetic resources created, biotechnology will certainly influence at a greater level in understanding the physiology of sucrose accumulation especially source-sink relationship and miRNAs development and its utility in controlling the sucrose in sugarcane stalk.

2.3.6 Expression profiling of genes involved in sucrose metabolism

In sugarcane, sucrose metabolism is the most studied physiological precesses. The ability to accumulate sucrose in storage parenchyma is the net result of sucrose synthesis and breakdown. The important enzymes involved in sucrose metabolism include sucrose phosphate suynthase (SPS), sucrose synthase (SuSy) and invertases. SPS, SuSy and invertases have been proposed as key regulators for the accumulation of sucrose in sugarcane stem storage parenchyma (Zhu *et al.* 1997). Understanding differences in the expression of genes related directly or indirectly to sucrose accumulation in different *Saccharum* spp. is an important step for improvement of sucrose content (Casu *et al.,* 2003). The expression profiling of genes could help in the identification of genes involved in the regulation of sucrose metabolism and provide valuable target genes for increasing the sucrose content of sugarcane through the genetic manipulation.

Prathima *et al.* (2012) studied the expression profiling of genes involved in sucrose metabolism in different *Saccharum* spp. and commercial hybrids and reported that, the transcript level of SPS was higher in *Saccharum* spp. than in the commercial sugarcane hybrids. The expression of SPS was found to be higher in high sugar commercial sugarcane hybrids than in low sugar commercial sugarcane hybrids. In contrast, all the *Saccharum* spp. shared low transcript levels of SPS in the high sugar clones when compared to the low sugar clones. Expression of SPS was observed in all the species of *Saccharum* although no differentiation could be derived between the high and low sugar clones. However, in the commercial sugarcane hybrids, there was clear differentiation in the transcript levels between the high and low sugar genotypes. Among the *Saccharum* spp. high transcript levels of SAI were observed in low sugar *S. robustum* clone and in both high and low sugar *S. officinarum* clones. Expression of SuSy was uniform both in *Saccharum* spp. and commercial sugarcane hynbrids.

Casu *et al.* (2007) analysed the sugarcane culm extensively at the gene expression levels releated to sucrose metabolism and reported that gene expression releated to sucrose metabolism has been shown to decrease during culm maturation and genes releated to cellulose, lignin and cell wall metabolism to be modulated during internode metabolism.

Verma *et al.* (2010) studied the transcript expression levels to verify the changes in sucrose phosphate synthase and sucrose synthase enzyme activity in two early maturing, high sugar accumulating and two late maturing, low sugar accumulating sugarcane cultivars using semi- quantitative RT-PCR at different

developmental stages and reported that, high sugar cultivars showed increased transcript expression and enzyme activity of SPS compared to low sugar cultivars at all developmental stages whereas, sucrose synthase activity was higher in immature internodes than in mature internodes in all cultivars; sucrose synthase transcript expression showed a similar pattern. He also reported that, SAI did not show gradual decline in the expression of transcript as the cane matured.

2.3.7 Impact of exogenously applied enzymes effecter on sucrose metabolizing enzymes

Improvement in cane productivity and sugar recovery is the major concern of cane growing countries. In order to obviate climatic and physio – biochemical constraints, the concept of uniform cane ripening and quality sustenance through sugar enhancing chemicals has been successfully exploited on commercial scale in many cane growing countries since 1920s (Gilbert, 2002). The ripners such as ethrel, glyphosate analogue, Fusilade super, Gallant super, Polaris are being used in large scale in sugarcane plantation (Morgan *et al.*, 2007).

Patil and Joshi (1972) reported that, in sugarcane leaves, the highest sucrose synthase activity was observed when both Mg and Mn were present in the metabolic environment.

Solomon *et al.* (1997) studied the efficacy of ethrel, glyphosate and dinitrocifrol (DNC) in improving sucrose content during early season reported that, the foliar application of DNC showed relatively higher sucrose phosphate synthase enzymes compared to control in sugarcane varieties. The upper portion of the stalk showed higher acid invertase than lower joints and there

was marked suppression in acid invertse activity in DNS treated canes. Folier treatment with formulation containing glyphosate–isopropylamine salt showed appreciable decline in soluble acid invertse in the internodal tissues. This formulation showed considerable improvement in sucrose content in low sugar variety BO 91 and increased in the CCS % in the ranged between 2 and 4 units.

Vorster and Botha (1999) reported inhibitory effects of Hg on the activity of sugarcane neutral invertase. Among the different metal ions tested, manganese chloride strongly inhibited the activity of all soluble acid isoforms and thus may be utilized to induce early maturity in sugarcane (Sachdeva *et al.*, 2003).

Jain *et al.* (2013) studied the effect of different enzyme effectors *viz.* divalent cations and plant growth regulators (PGR) on sucrose content and sucrose metabolizing enzymes *viz.* sucrose phosphate synthase, sucrose synthase and soluble acid invertase throughout cane ripening in low sugar genotypes BO 91 and reported that, foliar application of these enzyme effectors improved sucrose content and CCS %. Quantitative activity of soluble acid invertase enzyme declined, while sucrose phosphate synthase and sucrose synthase activity increased in the apical portion of the cane stalk by chemical treatments indicating their regulatory effect on sucrose metabolizing enzymes. The qRT-PCR analysis indicated differential expression of sucrose synthase and soluble acid invertase gene in response to foliar application of enzyme effectors. Effect of PGR chemicals was not consistent in terms of sucrose accumulation. The reducing sugar content decreased while sucrose to reducing sugar ratio increased due to chemical treatment

indicating effectiveness of divalent cations in improving sucrose accumulation during the early crushing season particularly in low sucrose accumulating genotypes of sugarcane.

Guimaraes *et al.* (2005) studied the effect of chemical ripener trinexapac- ethyl on growth, sucrose accumulation and soluble and cell wall bound invertase activity in sugarcane variety SP81-3250 and reported that these ripner suppresses vegetative growth and favors sucrose accumulation in sugarcane stem by inhibiting the biosynthesis of GA_1 (Gibberelic acid isoform1) from GA_{20}. Trinexapac-ethyl significantly reduced the activity of soluble acid invertase in the youngest internodes (top) but no effect was found in older tissues. These results corroborate previous result of Albertson *et al.* (2001) and Batta *et al.* (2002).

3. MATERIAL AND METHODS

The present investigation entitled, "Investigations on the interplay of SPS, SuSy and invertase(s) in relation to sucrose accumulation in sugarcane" was carried out at the Department of Biochemistry, Mahatma Phule Krishi Vidyapeeth, Rahuri, Dist. Ahmednagar during 2011-13.

The details of materials used and methods adopted in the present studies mentioned under following subheading. The details of sugarcane varieties used for analysis are given in Table 1.

Table 1. Pedigree of cultivars

	CoM 0254	Co 94012	Co 62175	CoM 0265	CoC 671	Co 740
Year		2004	1976	2006	1994	1956
Parents	Co 86002 (GC)	Somaclone CoC 671	Co 951 x Co 419	Co 87044 (GC)	Q 63 x Co 775	P 3247 x P 4775
Maturity	Early	Early	Midlate	Midlate	Early	Late
CCS %	14.26	11.86	9.00	11.61	14.43	9.52
Cane yieldt/ha	146.00	52.88	175.00	144.00	111.00	115.00

3.1 Material

Four sugarcane varieties differing in sucrose content *viz.* CoM0254 and Co 94012 (high sucrose), CoM 0265 (medium sugar) and Co 62175 (low sugar) were planted on the P.G. farm of M.P.K.V., Rahuri during 2011-12 and 2012-2013. Simultaneously, some crosses of sugarcane cultivars were also effected to improve the sucrose content of CoM 0265, a widely cultivated sugarcane variety occupying an area of more than 85 % in the state and which is salt tolerant by making crosses with the high sucrose sugarcane varieties CoM 0254 and CoC 671. Another sugarcane variety Co 740 was also used for effecting the crosses. The progenies of such

crosses were evaluated for sucrose content and the high and low sucrose progenies were planted on the research farm of C.S.R.S., Padegaon by following the recommended agronomic practices. The plant samples were collected after 240, 270, 300, 330, 360 and 390 DAP at monthly intervals. The stem was further divided into two equal portions *viz.* top and bottom representing the immature and mature storage tissues, respectively and were immediately frozen in liquid nitrogen to stop metabolic activity and to avoid diurnal variation in enzyme activity and sugar levels.

Brix and sucrose in cane juice was determined by using a "Brix Hydrometer" and Saccharimeter according to the A.O.A.C. 1995. Sugar recovery percentage was calculated according to the following equation as described by Yadav and Sharma (1980).

Sugar recovery % = [(Sucrose – (Brix - Sucrose) x 0.4) x 0.74)].

3.2 Methods

3.2.1 Assay of Sucrose synthase and sucrose phosphate synthase

The activity of both sucrose synthase (SuSy) and sucrose phosphate synthase (SPS) were assayed as per the modified method of Hawker (1967).

Extraction of enzyme

The top and bottom portion of the stalk of both the high and low sucrose varieties and the progenies of the crosses were homogenized and extracted in a minimum volume of extraction buffer containing 0.1 M Tris-HCl buffer (pH, 7.6) containing 0.3 M Mannitol, 0.01 M $MgCl_2$, 0.02 M EDTA, 0.02 M cystein-HCl, 0.02 M sodium diethyl–dithiocarbamate (DIECA) and 1 % Triton-X100 and the homogenate was filtered through two layers of cheese cloth and

centrifuged at 15,000 x g for 10 min. To the known volume of supernatant ammonium sulphate and different fraction 0- 30 %, 30-60 % and 60–80 % were tested for activity of sucrose phosphate synthase (SPS) and sucrose synthase (SuSy). The fraction 30–60 % giving maximum activity was used for comparying the activity profile of these enzymes. The 30–60 % saturation fraction was kept for 4h and centrifuged at 20,000 x g for 15 min. The supernatant was discarded and the precipitate was dissolved in minimum volume of extraction buffer and used for assay of sucrose synthase and sucrose phosphate synthase (Fig. 2).

Enzyme Assay

The reaction mixture for sucrose synthase contained 125 µl 0.015 M UDPG, 125 µl 0.05 M fructose, 700 µl 0.2 M Tris-HCl buffer (pH 8.2) containing 0.025 M $MgSO_4$ and 50 µl of enzyme preparation in total volume of 1.0 ml.

Reaction mixture for sucrose phosphate synthase contained 125 µl 0.015 M UDPG, 125 µl 0.05M fructose–6-phosphate, 700 µl 0.2 M Tris- HCl buffer (pH 7.4) containing 0.025 M $MgSO_4$ and 0.4 M NaF (as phosphatase inhibitor) and 50 µl enzyme preparation in a total volume of 1.0 ml.

3.2.2 Estimation of Sucrose

Sucrose was determined as per the method of Roe (1934) with some modifications.

Reagents

1. Resorcinol solution: 0.1 % (w/v) resorcinol in glacial acetic acid containing 0.25 g thiourea.
2. 6 % KOH solution : 6g KOH in 100 ml distilled water.
3. 75 % (v/v) H_2SO_4

Procedure

The reaction mixture of both sucrose phosphate synthase and sucrose synthase were incubated at 37^{0}C for 30 min and subsequently the tubes were kept in boiling water bath for 10 min and cooled. After cooling the tubes, 0.5 ml 6 % KOH was added and again kept in boiling water bath for 20 min. The cooled test extract was then used for sucrose estimation. To suitable aliquots of the test extract, 1ml resorcinol solution and 3 ml 75 % H_2SO_4 were added and then incubated at 80^{o}C for 10 min. The intensity of pink colour was measured at 490 nm by preparing the control and expressed the enzyme activity as µmoles of sucrose formed mg^{-1} protein min^{-1} and the concentration of sucrose was calculated from the standard curve prepared by using sucrose standard (10-100 µg ml^{-1}).

3.2.3 Invertase

Soluble acid invertase, neutral invertase and cell wall invertase from both top and bottom portion of sugarcane storage tissues were extracted by the method of Vattuone *et al*. (1981).

3.2.3.1 Soluble acid invertase and neutral invertase

Extraction of enzyme

Both top and bottom portion of canes were homogenized and extracted in minimum volume of 50 mM sodium phosphate buffer (pH 7.5) containing 1 mM β-mercaptoethanol and 5 µM $MnS0_4$ and filtered the content through two layers of cheese cloth and centrifuged the homogenate at 10,000 x g for 10 min. The supernatant and pellet were carefully separated. To the measured volume of the supernatant solid ammonium sulphate was added to get 80 % saturation and kept for 4 h. after which the contents were

centrifuged at 10,000 x g for 10 min. The supernatant was discarded and the pellet was dissolved in minimum volume of 50 mM sodium phosphate buffer (pH 7.0) and used for determination of soluble acid invertase and neutral invertase activities (Fig. 3).

3.2.3.2 Cell wall invertase

Both top and bottom portion of canes were homogenized and extracted in minimum volume of 50 mM sodium phosphate buffer (pH 7.5) containing 1 mM β-mercaptoethanol and 5 µM $MnS0_4$, filtered through two layers of cheese cloth and centrifuged the homogenate at 10,000 x g for 10 min. The supernatant and pellet were carefully separated. The supernatant was discarded and the pellet was suspended in 5 ml of 50 mM sodium acetate buffer (pH 5.5) containing 1 mM β-mercaptoethanol. The homogenate was frozen and thawed and centrifuged at 10,000 x g for 10 min. The supernatant was discarded and the pellet was resuspended in 5 ml of 50 mM sodium acetate buffer, centrifuged for 10 min. at 10,000 x g and repeated this procedure for two to three times before used for assay of cell wall invertase (CWI) activity.

Enzyme assay

Soluble acid invertase and cell wall invertase activity was assayed by adding 50 µl enzyme to 750 µl of 50 mM sodium acetate buffer (pH 5.5). The enzyme reaction was started by addition of 0.2 ml 0.5 M sucrose solution and the reaction was terminated after 30 min by adding 1ml of alkaline copper reagent and kept the mixture exactly for 20 min. in boiling water bath. The tubes were cooled under running tap water or using ice and the reducing sugar produced was assayed by the method of Nelson

(1944). The activity of the enzyme was expressed as ηmole glucose formed mg^{-1} protein min^{-1}.

The neutral invertase activity was assayed similar to that of SAI except that the reaction was conducted at pH 7.5.

3.2.4 Estimation reducing sugars

The reducing sugar content was estimated by method of Nelson (1944).

Reagents

1. Reagent A : Anhydrous sodium carbonate (25 g), potassium sodium tartarate (25 g), sodium bicarbonate (20 g) and anhydrous sodium sulphate (200 g) were dissolved in distilled water making final volume to one liter.
2. Reagent B : Copper sulphate (15 g) was dissolved in 100 ml distilled water containing 2-3 drops of conc. H_2SO_4.
3. Reagent C : It was freshly prepared by mixing reagent A and reagent B in the ratio of 25:1 (v/v).
4. Reagent D : Twenty five g ammonium molybdate $(NH_4)_6 Mo_7O_{24}, 4H_2O$ were dissolved in 450 ml distilled water and 21 ml conc. H_2SO_4 were added to this slowly with stirring three g of sodium arsenate ($NaHA_5O_4$, $7H_2O$) was dissolved in 25 ml of distilled water separately and this solution was added drop wise to ammonium molybdate solution. The resulting solution was then incubated for 24 h at 37°C and stored in an amber coloured bottle.

Procedure

To one ml extract, 1 ml of reagent C was added. The contents of tubes were heated in a boiling water bath for 20 min. After cooling the tubes to room temperature, 1 ml of reagent D was

added and mixed thoroughly by shaking the tubes on a stirrer. After making the volume to 10ml with distilled water, the intensity of blue color was measured at 520 nm against blank. The concentration of reducing sugars was calculated from the standard curve using glucose as standards.

3.2.5 Estimation of soluble protein

The protein content in all enzyme extract was estimated by method of Lowry *et al.* (1951) as described under,

Reagents:

1. Reagent A : 2 % Na_2CO_3 in 0.1 N NaOH
2. Reagent B : 0.5 % $CuSo_4$, 5 H_2O in 1 % solution of sodium potassium tartarate.
3. Reagent C : 50 ml reagent A was mixed with 1ml reagent B just before use.
4. Reagent D : Folin- Ciocalteau phenol reagent (FCR) was diluted two folds to make it 1N.

Procedure :

To 50 µl test sample of protein in a test tube 950 µl distilled water was added and made the volume to 1 ml. The contents were mixed well by adding 5 ml reagent C and allowed to stand for 10min at room temperature. After 10 min. 0.5 ml reagent D was added and allowed to stand for 30 min. The intensity of blue colour was read at 660 nm against the blank. The amount of protein in sample was calculated from standard curve prepared with bovine serum albumin (BSA).

3.2.6 Isolation of genomic DNA from sugarcane

The isolation of genomic DNA from fresh young seedlings of different sugarcane cultivars and their crosses was

carried out by a modified cetyltrimethylammonium bromide (CTAB) method described by Keim *et al.* (1988)

Reagents :

1. CTAB buffer :1 M Tris (pH.8.0), 5 M NaCl, 0.5 M EDTA, 20 g CTAB, PVP 5000 µl and 40 g β-Mercaptoethonol.
2. Chloroform: isoamyl alcohol : (24:1)
3. Ethanol : 70 % (v/v)
4. TE (10/1) buffer: 10 mM Tris-HCl, 1 mM EDTA (pH 8.0)
5. Ammonium acetate : 7.5 M

Procedure

1. About 0.5 g of fresh young leaves of different sugarcane cultivars and progenies of crosses were taken and cut into small pieces (about 10 mm^2) with blade and powdered in liquid nitrogen (N_2) with prechilled mortar and pestle.
2. The powder was homogenized in prechilled 1.5 ml CTAB buffer with mortar and pestle and transferred in 2 ml tubes.
3. The tubes were incubated for 60 min at 65^0C in a thermostatic water bath.
4. The contents in the tubes were mixed after every 15 min by inversion during incubation.
5. After incubation the tubes were allowed to cool at room temperature.
6. An equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added and mixed gently by inversion. The tubes were then centrifuged at 10,000 x g for 10 min at 4^oC in a high speed refrigerated centrifuge (Kubota 6500, Japan).
7. Then aqueous phase was carefully recovered and transferred to a fresh tube.

8. An equal volume of isopropanol was added into the contents of fresh tube. The tube was gently vortexed and incubated at -20^{0}C for overnight for precipitation of DNA.
9. The tubes were centrifuged at 12,000 x g for 10 min.
1. 500 µl of ice-cold 70 % (v/v) ethanol was added into the tubes and add 50 µl of 7.5 M ammonium acetate. Kept it for precipitation for 1 h at 20^{0}C. Collected the pellets carefully and washed with 70 % ethanol. The DNA pellet was air dried till the last traces of ethanol was evaporated.
2. The pellet was resuspended in suitable volume of TE (10/1) buffer.

3.2.6.1 Purification of genomic DNA

Reagents

1. Sodium acetate (pH.4.8): 3 M
2. Ribonuclease A (10 mg/ml)
3. Proteinase K
4. Isopropanol
5. Ethanol : 70 % (v/v)

Procedure

1. For purification, 500 µl of DNA sample was taken in a fresh eppendorf tube.
2. To this 10 µl of RNase A (10 mg/ml) was added and incubated at 37^{0}C for 1 h with occasional gentle shaking.
3. After incubation 20 µl of Proteinase K and 100 µl of 3 M sodium acetate (pH 4.8) was added and again incubated for 60^{0}C for 1 h.
4. After incubation equal volume of C:I was added.

5. The tubes were then centrifuged at 12,000 rpm for 10 min at 4^0C
6. Supernatant was removed and transferred into another fresh eppendorf tube.
7. To the supernatant equal volume of isopropanol was added.
8. The tubes were chilled at -20^0 C for 1 h/overnight.
9. Then the tubes were centrifuged at 12,000 rpm for 10 min at 4^0C.
10. Supernatant was decanted carefully and DNA pellets were washed with 70 % ice cold ethanol and air dried.
11. Purified DNA was then dissolved in suitable aliquot of TE buffer.

3.2.6.2 DNA quantification and purity analysis

Reagents

1. TBE buffer (10 X) : 1 M Tris Base, 830 mM boric acid in 10 mM EDTA, pH 8.0
2. TBE buffer (1 X): The 10 X buffer was diluted to 100 ml with milli Q water
3. Ethidium bromide : 10 mg/ml
4. Tracking dye : 1 % (w/v) BPB + 20 % (w/v) Ficoll + 10 mM EDTA

Concentrations of purified DNA were measured using (Nanodrop, ND- 1000 UV visible spectrophotometer USA) at 260 and 280 nm wavelength. The ratio of absorbance 260/280 was calculated. Five µl of all DNA extracts were electrophoresed (Bio Rad sub cell model 96 USA) on 0.8 % (w/v) agarose gel containing 0.5 µg/ml ethidium bromide at 6V/cm in TBE buffer. After electrophoresis the band intensity of genomic DNA was visulized on

gel documentation unit (Image Quant LAS 4000 mini) and compared to that of standard uncut λ DNA. These gels also provided a visual measure of the purity and integrity of the DNA.

3.2.6.3. DNA amplification by RAPD primers

Twelve primers (markers) of random sequence (Operon Technologies, Inc., Alameda, California, USA) (Table 2) were used for amplification of genomic DNA of the four varieties of sugarcane. PCR amplification was done in oil free thermal cycler (Eppendorf, Mastercycle gradient, Germany) following the PCR profile of 94^{o}C for 5 min. (initial denaturation) followed by 35 amplification cycles of 1 minute denaturation at 94^{o}C, 45 sec. annealing at 35^{o}C and elongation or extension at 72^{o}C 90 sec. After the last cycle, a final step of 7 min. at 72^{o}C was added to allow the complete extension of all amplified fragments. After completion of the cycling programme, the reaction was held at 4^{o}C. PCR amplification was carried out with 2 µl Buffer E (10X) with 15 mM $MgCl_2$, 0.5 µl 2.5 mM dNTPs, 50 ηg template DNA, 0.5 µl Taq DNA polymerase (3U/µl) (Bangalore Genei Pvt. Ltd., India), suitable amount (9.5 µl) of sterile deionized distilled water and 2 µl of RAPD primer from 10 µM working solution. PCR products from each samples were confirmed by running on 1.2 % agarose gel containing 6 µl ethidium bromide (10 mg/ml) in 100 ml 1 X TBE buffer at 120 V for 1.5 h. Loading dye (2.5 µl) was added to the PCR products and loaded in to the wells. A molecular weight marker, *Eco* R I/Hind *III double* digest was also loaded on either side of the gel.

Table 2. Sequences of the 12 decamer random primers used for RAPD analysis

Sr.No.	Random primers	Sequence of Primers (5' – 3')
1.	OPB 03	CATCCCCCTG
2.	OPB 05	TGCGCCCTTC
3.	OPB 07	GGTGACGCAG
4.	OPB 10	CTGCTGGGAC
5.	OPB 13	TTCCCCCGCT
6.	OPB 14	TCCGCTCTGG
7.	OPB 15	GGAGGGTGTT
8.	OPB 16	TTTGCCCGGA
9.	OPB 17	AGGGAACGAC
10.	OPQ 10	TGTGCCCGAA
11.	OPQ 11	TCTCCGCAAC
12.	OPQ 12	AGTAGGGCAC

3.2.6.4. DNA amplification by ISSR primers

PCR amplification was performed with twelve ISSR primers obtained from Banglore Ge Nei™ were used for the four varieties of sugarcane (Table 3). PCR amplification was done in oil free thermal cycler (Eppendorf, Mastercycle gradient, Germany) following the PCR profile of 94°C for 5 min. (initial denaturation) followed by 40 amplification cycles of 45 sec. denaturation at 94°C, 45 sec. followed by annealing temperature (T_a) for 1 min and elongation or extension at 72°C 90 sec. After the last cycle, a final step of 10 min. at 72°C was added to allow the complete extension of all amplified fragments. After completion of the cycling programme, the reaction was held at 4°C. The PCR condition particularly annealing temperature (varying from 42.4°C to 47.4°C)

for each primer was standardized. PCR amplification was carried out with 2 µl Buffer E (10X) with 15 mM $MgCl_2$, 0.5 µl 2.5 mM dNTPs, 50 ηg template DNA, 0.5 µl Taq DNA polymerase (3U/µl) (Bangalore Genei Pvt. Ltd., India), suitable amount (9.5 µl) of sterile deionized distilled water and 2 µl of ISSR primer from 10 µM working solution. PCR products from each samples were confirmed by running on 1.2 % agarose gel containing 6 µl ethidium bromide (10 mg/ml) in 100 ml 1 X TBE buffer at 120 V for 1.5 h. Loading dye (2.5 µl) was added to the PCR products and loaded in to the wells. A molecular weight marker, *Eco* R I/Hind *III* double digest was also loaded on the either side of the gel.

Table 3. Sequences of the 12 ISSR primers used for ISSR analysis

Sr.No.	Random primers	Sequence of Primers (5'–3')
1.	UBC 807	AGAGAGAGAGAGAGAGT
2.	UBC 812	GAGAGAGAGAGAGAGAA
3.	UBC 813	CTCTCTCTCTCTCTCTT
4.	UBC 814	CTCTCTCTCTCTCTCTA
5.	UBC 815	CTCTCTCTCTCTCTCTG
6.	UBC 816	CACACACACACACACAT
7.	UBC 817	CACACACACACACACAA
8.	UBC 818	CACACACACACACACAG
9.	UBC 820	GTGTGTGTGTGTGTGTC
10.	UBC 821	GTGTGTGTGTGTGTGTT
11.	UBC 822	TCTCTCTCTCTCTCTCA
12.	UBC 823	TCTCTCTCTCTCTCTCC

3.2.6.5. DNA amplification by SSR primers

The sequences of oligonucleotide of eleven SSR primers were synthesized from Banglore Ge Nei™ and screened for polymorphism in three cultivated high and low sugar commercial varieties and the high and low sucrose progenies of the crosses *viz.* CoM 0265 x CoC 671, CoM 0265 x CoM 0254 and Co 740 x CoC

671. Primer sequences, annealing temperature are described in Table 4.

Table 4. Sequences of the SSR primers

Sr. No	Primer Name	Primer sequences	T_a (°C)
1.	NKS 9	F : CTTTCAGTGGCCATCTCCAT	58
		R : GAATGCGCAGGGATAGGATA	
2.	NKS 5	F : ATAGCTCCCACACCAAATGC	54
		R: TTGGCAAAATTGACCCAAAT	
3.	NKS 45	F : GTCGGTCGTGAGAAGGAAAG	58
		R : CACGTATAAAGGCCCTGTGG	
4.	NKS 46	F : ACAATAACCCCGCAGACATC	58
		R : TAATGCGTCATTTGGAGCAG	
5.	NKS 31	F : AACCACCACTCATCGTCCTC	58
		R : CACCGAGTTCCCATTGTTCT	
6.	STMS 34	F : CGTCTTGTGGATTGGATTGG	58
		R : TGGATTGCTCAGGTGTTTCA	
7.	UGSM359	F : TGGTAACCCTAGGCAGGTGA	55
		R : GTGCACCAGATTTGGATGGT	
8.	UGSM 550	F : AGACCAACTCGAGAGATGAGCAC	53
		R: ACAAGCCAATATACCTCTGTAGTC	
9.	UGSM 458	F : GTAGCACTAACAATAGCAGTTCAT	55
		R : TTTGTGTAGATGTTTAATTCGTTT	
10.	UGSM 632	F : CGTTCGTCTCTCTCTCCTC	53
		R : ATTTACAGGTCATCCCAAAC	
11.	UGSM 644	F : TCCACAAACAGAAACAGTCC	53
		R : CTACCGTGAGAAGCACCA	

PCR amplification was performed with USB inter-simple sequence repeat primers obtained from Operon Technologies, Inc., Alameda USA. Amplification was performed in a 0.2 ml PCR tubes having 20 µl reaction volume as described by Singh *et al.* (2011) with some modification. PCR reaction was carried out in a total of 20 µl volume containing 50 ηg templet DNA, 2.0 µl of each forward and reverse primer. The detailed composition mixture for PCR was given below:

Sr. No.	Constituents	Stock concentration	Volume of PCR reaction mixture per tube (20µl)
1.	Taq buffer E (Tris with 15 mM $MgCl_2$)	10 X	2.0 µl
2.	Taq DNA polymerase	3U/ µl	0.5 µl
3.	dNTPs	10 mM	1.0 µl
4.	Primer	10 uM	F- 2 .0 µl R- 2.0 µl
5.	Template DNA	25 ηg/ µl	2 µl
6.	Sterile distilled water	--	10.50 µl
7.	Total volume	--	20 µl

Procedure

The 20 µl reaction mixture was gently vortexed and spinned down. The DNA amplification was carried out on a thermal cycler (Eppendorf, Mastercycle gradient, Germany) with the following conditions: initial denaturation at 94^0 C for 5 min. followed by 35 amplification cycles. Each amplification cycle was initially at 94^0C for 1 min followed by annealing temperature as per the TM values of primers for 45 sec and primer extension at 72^0 C for 1.5 min. A final extension at 72^0 C for 7 min. was given at the end of the cycles and the samples were held at 4^0 C till retrieval. The PCR conditions particularly the annealing temperature of each primer was standardized. The amplified product was stored at 4^0C. The amplified products were separated by 7.5 % denaturating polyacrylamide gel electrophoresis in 1 x TBE buffer. The size of the amplified fragments was estimated using 100 and 500 bp DNA ladder. Amplified bands were visualized after staining with 0.5 µg/ml ethidium bromide. Gel photographs were taken under UV light in GelDoc system (Image Quant Las 4000 mini).

3.2.6.6 Separation of amplified PCR products on polyacrylamide gel electrophoresis (PAGE)

Reagents

1. Acrylamide bisacrylamide solution

   Acrylamide : 30.0 g

   Bisacrylamide : 0.8 g

   Volume : 100.0ml

   Filtered the solution through Whatman No.1 filter paper and stored at 4°C in brown bottle.

2. TEMED – Used as supplied.

3. Ammonium persulphate solution (APS) (10 %) in distilled water APS 0.5 g

   Volume : 5.0 ml

   Prepared fresh before use.

4. Stacking gel buffer (0.5 M)

   Tris (amino- methane) : 6.0 g

   pH : 6.8

   Volume : 100 ml

   Added 6.0 g Tris in 50 ml of water, adjusted pH to 6.8 by conc. HCl, the final volume was made upto 100 ml.

5. Resolving gel buffer (3 M)

   Tris (amino- methane): 36.3 g

   pH : 8.8

   Volume : 1000 ml

   Added 36.3 g Tris in 50 ml of water, added conc. HCl slowly and mixed the contents till pH 8.8 and final volume was made to 100 ml.

6. Reservior buffer

Tris	:	3.0 g
Glycine	:	14.4 g
pH	:	8.3
Volume	:	1L

Composition of gel for native PAGE

Stock solution	**Stacking gel (ml) 5 %**	**Resolving gel (ml) 7.5 %**
Acrylamide- bis-acrylamide	1.7 ml	7.5 ml
Stacking gel buffer (pH 6.8)	2.50 ml	--
Resolving gel buffer (pH 8.8)	--	4.69 ml
APS (10 %)	0.1 ml	0.37 ml
TEMED	0.02 ml	0.03 ml
Distilled waster	5.68	17.41 ml
Total	10 ml	30 ml

Procedure

1. Carried out electrophoresis on slab gels apparatus.
2. Washed the glass plates with chromic acid, rinsed with distilled water and dried them in oven. Wiped the plates with an ethanol swab, air- dried and assemble in the gel casting assembly.
3. Sealed the sides of the plates by tygon rubbing clap to make a mould and poured the desired resolving gel solution.
4. Gently overlaid a layer of water using a syringe.
5. Waited for about 30 min to polymrerize the gel which was indicated by a sharp interface between water and gel.
6. Removed the water, poured the stacking gel solution and inserted the comb immediately.

7. After polymerization, the comb and tygon tubing was removed and fixed the gel plates to the polyectrophoretic apparatus. The PCR products were resolved on a 7.5 % non-denaturing polyacrylamide gel using 1 x TBE buffer. Loaded 20 µl PCR products in each well and carried out electrophoresis at a constant current of 10 mA for first 30min followed by 20 mA constant current till the tracking dye reached cm away from the lower end of the gel and stained in Ethidium bromide. The gel was visualized under UV transilluminater on gel documentation system (Image Quant LAS 4000 mini) and image was captured.

3.2.7 RNA Isolation

Material

1. Sugarcane leaf sample
2. Qiagen RNesay Plant Mini Kit (Cat # 74903)

Principle

The RNeasy Plant Mini Kit provided QIshredder column for homogenization and filtration of viscous plant or fungal lysates by microcentrifugation in combination with RNeasy Mini sin column for RNA purification. RNeasy technology simplifies total RNA isolation by combining the stringency of guanidine-isothiocyanate lysis with the speed and purity of silica-membrane purification. RNeasy Kits provided the highest quality RNA with minimum copurification of DNA, the residual amounts of DNA remaining can be removed using convenient on-column DNase treatment during the RNeasy procedure.

Samples were first lysed and then homogenized in the QI Ashredder columns. Ethanol was added to the lysate to provide

ideal binding conditions. The lysate was then loaded onto the RNeasy silica membrane. RNA binds, and all contaminants are efficiently washed away. Pure, concentrated RNA was eluted in water.

Procedure

1. One hundred mg of leaf sample was taken.
2. Immidiately placed the weighted tissue in liquid nitrogen and ground thoroughly with a mortar and pestle. The tissue powder and liquid nitrogen was decanted into an RNase- free, liuid nitrogen cooled, 2 ml microcentrifuge tube. Allowed the liquid nitrogen to evaporate without allowing the tissue to thaw. Proceeded immediately to step 3.
3. Added 450 µl Buffer RLT to a maximum of 100 mg tissue powder and vortexed vigorously.
4. Transferred the lysate to a QIA shredder spin column (lilac) placed in a 2 ml collection tube and centrifuged for 2 min at 12000 rpm speed. Carefully transfered the supernatant of the flow-through to a new micro centrifuge tube without disturbing the cell–debris pellet in the collection tube. Used only this supernatant in subsequent steps.
5. Added 0.5 volume of ethanol (96-100 %) to the cleared lysate, and mixed immediately by pipetting.
6. Transferred the sample (650 µl), including any precipitate that may have formed, to an RNeasy spin column placed in 2 ml collection tube. Closed the lid gently, and centrifuged for 1min at 10000 rpm. Discarded the flow-through.
7. Added 700 µl Buffer RW1 to the RNeasy spin column. Closed the lid gently, and centrifuged for 1 min at 10000 rpm to

wash the spin column membrane. Discarded the flow-through.

8. Added 500 µl Buffer RPE to the RNeasy spin column. Closed the lid gently, and centrifuged for 1 min at 1000 rpm to wash the spin column membrane. Discarded the flow- through and repeated the same step once more.
9. Placed the RNeasy spin column in a new 1.5 ml collection tube. Added 50 µl RNase–free water directly to the spin column membrane. Closed the lid gently and centrifuged for 1 min at 10,000 rpm to elute the RNA.

The contamination of DNA was removed by using RNAse free DNase (Fermentas). The purified RNA was stored at -40^{o}C for further analysis.

3.2.7.1 Qualitative Assessment of RNA by Agarose gel electrophoresis

Material required

1. Agarose (1 % w/v) gel in 0.5 X TBE buffer spiked with Ethidium bromide at the final concentration 5 µg/ml.
2. Running buffer (0.5 X TBE).
3. RNA Loading dye.

Method

Two microlitre of each RNA sample was loaded in Agarose gel and electrophoresed for 30 min. at 5V/cm (Plate 11).

3.2.7. 2 Quantification of total RNA

Total RNA from both samples was quantified using Qubit® RNA BR (Broad–Rang) Assay Kit with the Qubit® 2.0 Fluorometer (Life Technology, USA), which provides an accurate

and selective method for the quantification of high abundance RNA samples.

Material

1. 1.5 ml Plastic tubes (supplied with the kit for mixing the Qubit™ working solution).
2. Qubit assay tubes (500 µl),
3. Qubit RNA reagent and buffer
4. RNA standard

Method

1. The required number of assay tubes were taken and labeled with the sample name.
2. The Qubit working solution was diluted for the preparation of Qubit RNA reagent 1:200 in Qubit RNA buffer supplied in kit.
3. 198 µl Qubit working solution was distributed in 8 different tubes for test samples and 190 µl for working standard solution.
4. Two µl RNA sample was added in respective tube and 10 µl standard were distributed in two tubes labeled as Std 1 Std 2.
5. All tubes were incubated in dark for 15 min and reading was recorded.

Table 5. Concentration of RNA (µg/mL)

Sr. No	Sample Name	RNA (µl)	Qubit working solution (µl)	Final volume (µl)	Final total RNA concentration (µg/ml)
1.	CoM 0265x CoM 0254 (HS)	2	198	200	774
2.	CoM 0265x CoM 0254 (LS)	2	198	200	766

3.2.7.3 Expression analysis of SPS, SS and SAI gene by Semi quantitative RT-PCR

Requirements:

1. Primers (Enlisted in table below
2. PCR reagents: Superscript One – Step RT-PCR System with Platinum Taq Polymerase from Life Technologies.

Table 6. Sequences of SPS, SS, and SAI gene specific primers

Primer Name	Primer sequences	Primer annealing Temp.(c)
SAI	F : GTGCTCATCTGCATTGCTGT	45
	R : CTTGTGCCAATTGTTGTGG	
SPS	F : GGTGGTCAGGTGAAATATGTTG	45
	R : CGTTGAGTGCCCCAGACAG	
SS	F: CAGCATACTTGTTTAAGTAATAC	45
	R : CTGGTGATGATGAAATCAGTGTG	

Method

1. Following semi quantitative PCR was performed for all three samples using gene specific primer pair. Total 3 One step RT-PCR reactions were performed. Final volume of each reaction was 50.0 µl.
2. The reaction mixture was performed for all samples and added into 200 µL PCR tubes.
3. One microgram total RNA was added as template for the amplification in each tube after complete addition of master reaction mix into each tube.

Table 7. PCR reaction mixture for semiquantitative RT-PCR

Materials	Volume (µl)	Final Concentration
Total RNA	10.00 (diluted to each 1 µg)	(1.0 microgram)
2X reaction mixture	25.0	1 X
Forward primer	1.0	0.2 µM
Reverse Primer	1.0	0.2 µM
RT/Platinum Taq Mix	1.0	-
Nuclease	12.0	-

One negative control reaction per gene was set which included nuclease free water as template to check the false positive results.

4. Thermal cycling conditions used for PCR amplification as m entioned in the table below.

Table 8. Thermal cycling conditions for semiquantitative RT-PCR

Tage	Temprature (°C)	Time (min: sec)	Cycles
Initial Reverse Transcription	50	30:00	
Initial denaturation	95	15:00	
Denaturation	94	1:00	25 Cycles
Annealing	45	1:00	
Extension	72	1:00	
Final extension	72	10:00	
Hold	4	Until use	

3.3 Statistical analysis

The data on biochemical constituent were analysed by using factorial randomized block design (Panse and Sukatme, 1985).

4. RESULTS AND DISCUSSION

The primary use of sugarcane is to produce sucrose and nearly 76 % of the world's sugar supply is derived from sugarcane. The record sucrose yields are more or less 60 % of the theoretical maximum and there is considerable potential for sucrose accumulation in sugarcane of the physio-biochemical limits can be identified and modified. Attempts have been made through traditional breeding methods has resulted in improved plants, however the selection procedure is very long and arduous. Identification of molecular markers closely linked to the sucrose content will certainly case the selection process and accelerate the efforts through breeding. Despite extensive studies on sucrose accumulation in sugarcane, the biochemical processes controlling the yield of sucrose remain poorly understood. The genes encoding the key enzymes are being cloned and used to transform plants to modify enzyme activity, however the results are not very encouraging as the control in major metabolic pathways seems to be shared.

An increase in sucrose content is likely be achieved by better understanding of the enzyme activity profile of sucrose metabolizing enzymes during cane development. An identification of key regulator enzyme and molecular marker associated with this important QTL and validation of such linkage can definately help in selecting the parents and for early selection of the progenies of the crosses. The present investigation is an attempt in this direction. The high and low sucrose parents and the high and low sucrose progenies of the crosses have been evaluated for major sucrose metabolizing enzymes during development in relation to

sucrose accumulation and to identify validate the expression profile. The reported SSR markers linked with high sucrose content have been used to confirm and validate the linkage. The results obtained are discused in the light of the available literature in this chapter.

4.1 Brix©, sucrose and CCS content of varieties

The Brix©, sucrose and CCS % of sugarcane varieties at different developmental stages is presented in Table 9. It is evident from the table that the mean brix© of CoM 0254 over the developmental stages was maximum i.e. 19.8 followed by 16.8 in Co 94012 and the least mean brix© reading of 13.7 was recorded in Co 62175. The varietial mean brix© reading increased from 14.5 at 240 DAP to 18.8 at 390 DAP. The high sugar sugarcane variety CoM 0254 recorded maximum brix© reading of 17.4 at the early stage of cane development i.e. 240 DAP as against 11.0 brix© in Co 62175 at 240 DAP. The interaction effects of variety and development stage were significant indicating the contribution of both genotypes and the developmental stage in influencing the brix©. The increase in the brix© reading in CoM 0254 from 240 DAP to 390 DAP was 28 % whereas, the increase in the brix© reading of the low sugar variety Co 62175 from 240 DAP to 390 DAP was almost 50 %.

The mean sucrose % of CoM 0254 over the developmental stages was maximum i.e. 18.2 % followed by 15.4 % in Co 94012 and the least mean sucrose % i.e. 10.7 % was recorded in Co 62175. The varietal mean sucrose % increased from 13.0 % at 240 DAP to 16.4 % at 390 DAP. The high sugar sugarcane variety CoM 0254 recorded maximum sucrose (16.0 %)

Table 9. Brix, sucrose and CCS at different developmental stages of high and low sugar varieties

DAP/ Variety	Brix ©				Sucrose %				CCS %			
	CoM 0254	**Co 94012**	**Co 62175**	**Mean**	**CoM 0254**	**Co 94012**	**Co 62175**	**Mean**	**CoM 0254**	**Co 94012**	**Co 62175**	**Mean**
240	17.4	15.0	11.0	14.5	16.0	14.5	8.6	13.0	11.4	10.3	5.7	9.1
270	18.0	16.3	12.2	15.5	17.5	15.0	9.4	14.0	12.8	10.7	6.1	9.7
300	19.7	17.2	13.8	16.9	18.3	15.6	10.2	14.7	13.1	11.1	6.5	10.2
360	21.5	17.6	15.0	18.0	19.0	15.9	12.2	15.7	13.3	11.3	8.2	10.9
390	22.3	17.7	16.5	18.8	20.0	16.1	13.0	16.4	14.1	12.4	8.5	11.7
Mean	19.8	16.8	13.7		18.2	15.4	10.7		12.9	11.1	7.0	

		SE±	CD at 5%		SE±	CD at 5%		SE±	CD at 5%
	Variety	0.13	0.36	Variety	0.08	0.23	Variety	0.07	0.21
	DAP	0.16	0.47	DAP	0.11	0.31	DAP	0.09	0.27
	Variety x DAP	0.28	0.81	Variety x DAP	0.18	0.53	Variety x DAP	0.16	0.48

at the early stage of cane development i.e. 240 DAP as against 8.6 % in low sugar variety Co 62175 at 240 DAP. The interaction effect of variety and developmental stages were significant indicating the contribution of both genotypes and the developmental stage influencing the sucrose content. The increase in the sucrose % in high sugar variety CoM 0254 from 240 DAP to 390 DAP was 25 % whereas, the increase in the sucrose % of low sugar variety Co 62175 from 240 DAP to 390 DAP was 51 %.

The CCS % which is calculated based on brix and sucrose also revealed similar pattern during cane maturation in high and low sugar genotypes. The high sugar variety CoM 0254 recorded maximum CCS % (11.4 %) at early stage of cane development at 240 DAP as against low sugar variety (5.7 %) at 240 DAP. The increase in the CCS % in CoM 0254 from 240 DAP to 390 DAP was 23.68 % whereas, the increase in the CCS % of low sugar variety Co 62175 from 240 DAP to 390 DAP was 49.12 %.

McCormick *et al.*(2008, 2009) reported that changes in internodal sugar concentrations depended on internode maturity. Immature internodes had low sucrose concentrations compared to mature internodes

Batta *et al.* (2011) reported that the sucrose content increased from stem elongation stage to maturity stage in both parents and progenies.

Verma *et al.* (2011) reported that sucrose was the major sugar in different developmental stages and increased gradually from 240 to 390 DAP in both high and low sugar cultivars. A higher concentration of sucrose was found in the different internodal

tissues of high sucrose accumulating cultivars in comparison to the low sucrose accumulating cultivars from 240 to 390 DAP.

Our results are in agreement with these reports on sucrose accumulation at different developmental stages and in different internodal tissues of both high and low sugar accumulating cultivars.

4.2 Sucrose content of varieties

Sucrose content at different developmental stages in top and bottom portion of high and low sugar varieties is presented in Table 10. It is revealed that, the sucrose content gradually increased during the developmental stages from 240 DAP to 390 DAP. In high sugar variety, the sucrose content of the top portion increased from 160 mg g^{-1} fr. wt. to 205 mg g^{-1} fr. wt. in CoM 0254 and from 145 mg g^{-1} fr. wt. to 163 mg g^{-1} fr. wt. in Co 94012. The level of sucrose was much less in low sugar variety and which increased from 86 mg g^{-1} fr. wt. at 240 DAP to 136 mg g^{-1} fr. wt. at 390 DAP in Co 62175. The mean sucrose content in the top portion of the high sugar variety CoM 0254 was 185.50 mg g^{-1} fr. wt. in CoM 0254 and 155.67 mg g^{-1} fr. wt. in Co 94012, whereas the mean sucrose content in the top portion of the low sugar variety Co 62175 was 111.67 mg g^{-1} fr. wt. When the bottom portion of the cane was analyzed for sucrose content, similar trend of increase in sucrose content was noticed. The mean sucrose content of the high sugar sugarcane varieties was 203.17 and 170.67 mg g^{-1} fr. wt. in CoM 0254 and Co 94012, respectively.

The sucrose content of the bottom portion of low sugar variety Co 62175 although showed increase in sucrose content during the developmental stages but was less as compared to high

Table 10. Sucrose content at different developmental stages in top and bottom portion of high and low sugar varieties

DAP/ Variety	Sucrose (mg g^{-1} fresh wt.)							
	TOP				Bottom			
	CoM 0254	Co 94012	Co 62175	Mean	CoM 0254	Co 94012	Co 62175	Mean
240	160.00	145.00	86.00	130.33	174.00	150.00	110.00	144.67
270	175.00	150.00	94.00	139.67	180.00	163.00	122.00	155.00
300	183.00	156.00	102.00	147.00	197.00	172.00	138.00	169.00
330	190.00	159.00	122.00	157.00	215.00	176.00	150.00	180.33
360	200.00	161.00	130.00	163.67	223.00	180.00	165.00	189.33
390	205.00	163.00	136.00	168.00	230.00	183.00	167.00	193.33
Mean	185.50	155.67	111.67	150.94	203.17	170.67	142.00	171.94

		SE±	CD at 5%		SE±	CD at 5%
	Variety	1.680	4.828	Variety	1.269	3.659
	DAP	2.376	6.828	DAP	1.794	5.174
	Variety x DAP	4.115	11.826	Variety x DAP	3.107	8.962

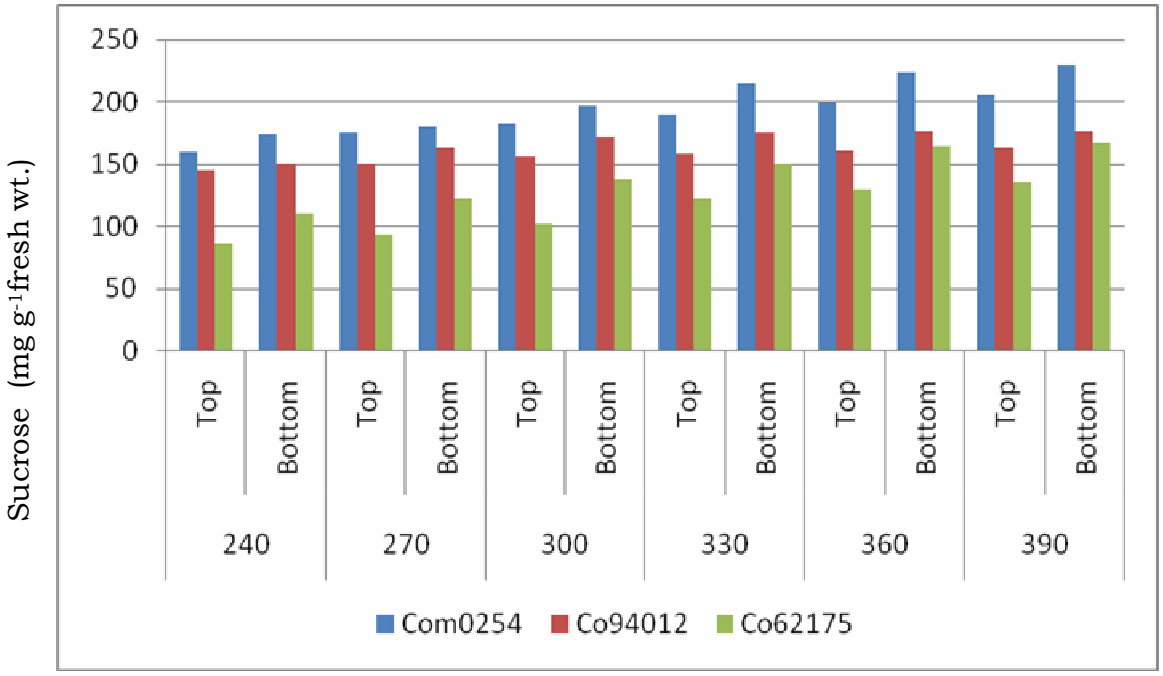

Fig. 4. Sucrose content at different developmental stages in top and bottom portion of high and low sugar varieties

sugar varieties with the mean sucrose content of 142.00 mg g^{-1} fr. wt.

Analysis and comparison of the sucrose content of the top and the bottom portion of the cane at different developmental stages revealed a much higher sucrose gradient in the top and bottom portion of low sugarcane variety. The sucrose content of Co 62175 in the top portion at 240 DAP was 86 mg g^{-1} fr.wt. and at the same stage, bottom portion recorded sucrose content of 110 mg g^{-1} fr.wt. On the contrary, in high sugar sugarcane variety CoM 0254, the level of sucrose in the top portion was 160 mg g^{-1} fr.wt. and at the same developmental stage, the level of sucrose in the bottom portion of cane was 174 mg g^{-1} fr.wt. This difference in the gradient of sucrose in the top and bottom portion suggests the difference in the source strength (activities of sucrose synthesizing enzymes) in the high and low sugar sugarcane varieties. The data of sucrose content showed significant varietal differences as well as the differences in sucrose content during the developmental stages.

Earlier Zhu *et al.* (1996) reported that, young internodes of all clones were low in sucrose while the older internodes accumulated sucrose to different levels depending on whether the clones is either high sucrose or low sucrose storing type. High sucrose clones usually started storage of sucrose three or four internodes earlier than the low sucrose clones.

Foliar application of enzyme effectors (Mg^{2+} and Mn^{2+} ions) was found effective in improving sucrose content in cane juice. CCS % juice and S/R ratio is also increased due to chemical treatment. Improvement in sucrose content might be due to change

in activity pattern of sucrose synthesizing enzymes which also helped increase CCS yield (Jain *et al.*, 2013)

Verma *et al.* (2011) reported that, sucrose accumulation was higher in mature internodes than immature internodes.

Prathima *et al.* (2012) reported that, sucrose concentration significantly increased from top internodes to middlet and bottom internodes in both high and low sugar genotyopes.

Batta *et al.* (2011) reported that, sucrose content increased from stem elongation stage to maturity stage in both parents and progenies.

4.3 Hexose content of varieties

The hexose content in the top and bottom portion of different sugarcane varieties were analyzed at different developmental stages from 240 to 390 DAP is presented in Table 11. It showed that the level of hexose decreased gradually both in top and bottom portion of the cane during the development. In a high sugar variety CoM 0254, the hexose content in the top portion decreased from 5.72 mg g^{-1} fr.wt. to at 240 DAP to 0.77 mg g^{-1} fr.wt. at maturity. In another high sugar variety Co 94012, the level of hexose decreased from 7.22 mg g^{-1} fr.wt. at 240 DAP to 2.27 mg g^{-1} fr.wt. at maturity. The hexose content of low sugar variety Co 62175 was comparatively much higher i.e. 21.59 mg g^{-1} fr.wt. at 240 DAP and which decreased to 6.00 mg g^{-1} fr.wt. at 390 DAP. The result clearly indicated that a higher free hexose pool in low sugarcane variety as compared to high sugar variety in the top portion of the cane. The hexose content at the bottom portion of the cane also decreased with advancement of maturity and the level of the hexose were much less in the bottom portion of the cane.

Table 11. Hexose content at different developmental stages in top and bottom portion of high and low sugar varieties

DAP/ Variety	Hexose (mg g^{-1} fresh wt.)							
	TOP				Bottom			
	CoM 0254	Co 94012	Co 62175	Mean	CoM 0254	Co 94012	Co 62175	Mean
240	5.72	7.22	21.59	11.51	2.92	4.42	17.49	8.28
270	4.82	6.32	21.00	10.71	2.32	3.82	16.29	7.48
300	3.72	5.22	15.79	8.24	1.32	2.82	10.39	4.84
330	1.02	2.52	10.59	4.71	1.23	2.72	6.69	3.55
360	0.92	2.40	8.29	3.87	0.8	2.30	5.26	2.79
390	0.77	2.27	6.00	3.01	0.35	1.85	3.50	1.90
Mean	2.828	4.325	13.877	7.01	1.489	2.988	9.937	4.805
		SE±	CD at 5%			SE±	CD at 5%	
	Variety	0.043	0.124		Variety	0.033	0.094	
	DAP	0.061	0.175		DAP	0.046	0.133	
	Variety x DAP	0.106	0.303		Variety x DAP	0.080	0.230	

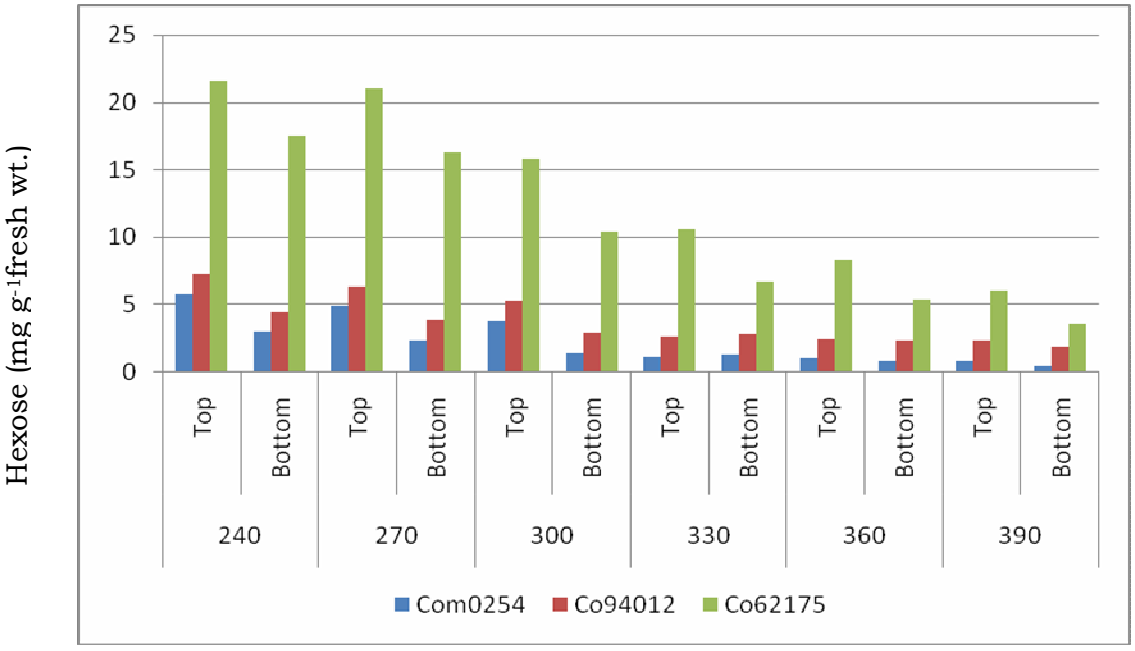

Fig. 5. Hexose content at different developmental stages in top and bottom portion of high and low sugar varieties

Analysis and comparison of the hexose level in the top and bottom portion of different varieties at different developmental stages clearly showed differences. The level of hexose in the bottom portion of the high sugar variety decreased almost 50 percent to the level in top portion, whereas, in a low sugar variety Co 62175, the hexose level at 240 DAP decreased from 21.59 mg g^{-1} fr.wt. in the top portion to 17.49 mg g^{-1} fr.wt. in the bottom portion. The higher level of free hexose pool during the grand growth phase in the low suagr variety is indicative of differences in sucrose synthesis rates. The data of hexose content showed significant varietal differences as well as differences during the developmental stages.

Earlier Pan *et al.*(2009) reported that, the hexose content in young internodes was found higher as compared to older internodes, where it decreased pronouncedly and become almost absent. The hexose content of low sugar variety was higher as compared to high sugar variety.

Hexoses in high and low sucrose accumulating cultivars decreased with age and concentration in mature internodes were lower than in immature internodes (McCormick *et al.,* 2006, 2009).

Prathima *et al.* (2012) reported that, low sugar genotypes had higher concentration of total hexoses as compared to high sugar genotypes. There was significantly decrease in total hexose concentration when the crop reached fully mature stage.

Verma *et al.* (2011) reported that, in both high and low sucrose accumulating cultivars, hexose sugar decreased with advancement of developmental stages.

Batta *et al.* (2011) reported that hexose sugar continuously decreased with advancement of the growth of the stem.

4.4 Invert ratio of juice of varieties

Invert ratio of juice at different developmental stages in top and bottom portion of high and low sugar varieties were analysed at different developmental stages from 240 to 390 DAP is presented in Table 12. It showed that, the level of invert ratio of juice decreased gradually both in top and bottom portion of the cane during the development. In a high sugar variety CoM 0254, the invert ratio of juice in the top portion decreased from 3.575 at 240 DAP to 0.376 at maturity.

In another high sugar variety Co 94012, the level of invert ratio of juice decreased from 4.979 at 240 DAP to 1.393 at maturity, whereas, the invert ratio of juice of low sugar variety Co 62175 was comparatively much higher i.e. 25.105 in top at 240 DAP and which was decreased to 4.412 at 390 DAP. In high sugar varieties, the invert ratio was less than 5 both in top and bottom portion and it declined gradually during maturity. However, the invert ratio in the low sugar variety Co 62175 was much higher during the early stage of the development from 240 DAP to 330 DAP and at maturity it declined much faster. The higher invert ratio in the low sugar sugarcane variety probably substantiate that the activities of sucrose phosphate synthase and a neutral invertase which are primarily involved in the sucrose synthesis are low which ultimately results in elevated hexose pool. The data of invert ratio of juice showed significant varietal differences as well as differences during the developmental stages.

Table 12. Invert ratio of juice at different developmental stages in top and bottom portion of high and low sugar varieties

DAP/ Variety	Invert ratio = (Reducing sugar x 100) / Sucrose							
	TOP				Bottom			
	CoM 0254	Co 94012	Co 62175	Mean	CoM 0254	Co 94012	Co 62175	Mean
240	3.575	4.979	25.105	11.220	1.678	2.947	15.900	6.842
270	2.754	4.213	22.34	9.769	1.289	2.344	13.352	5.662
300	2.033	3.346	15.48	6.953	0.670	1.640	7.529	3.280
330	0.537	1.585	8.68	3.601	0.567	1.545	4.460	2.191
360	0.46	1.491	6.377	2.776	0.359	1.299	3.206	1.621
390	0.376	1.393	4.412	2.060	0.152	1.045	2.096	1.098
Mean	1.623	2.835	13.732	6.063	0.786	1.803	7.757	3.449
		SE±	CD at 5%			SE±	CD at 5%	
	Variety	0.007	0.021		Variety	0.005	0.014	
	DAP	0.010	0.030		DAP	0.007	0.019	
	Variety x DAP	0.018	0.052		Variety x DAP	0.012	0.033	

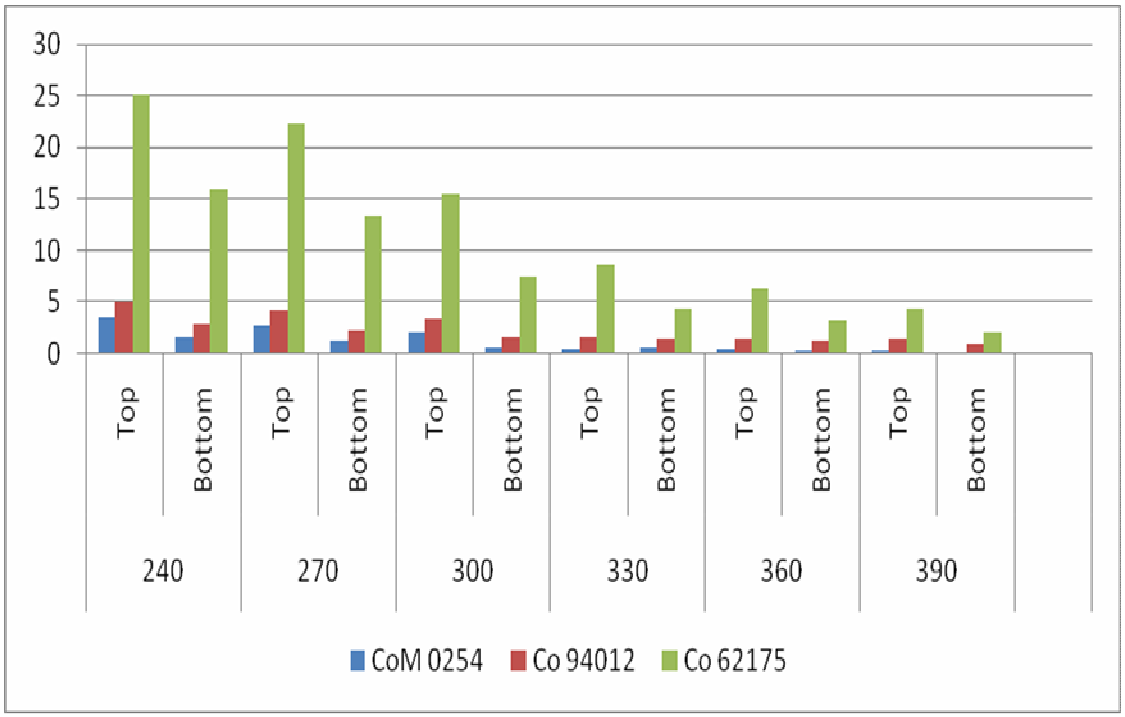

Fig. 6. Invert ratio in juice at different developmental stages in top and bottom portion of high and low sugar varieties

Higher concentrations of reducing sugars in immature stem tissue has physiological significance as these hexoses are utilized for supplying of energy for cell division and cell expansion during internodal elongation. During later stage of cane growth, as internodal expansion ceases, a fall in the level of reducing sugars has also been observed (Batta *et al.,* 1995; Lingle, 1999 and Batta *et al.,* 2008).

An increase accumulation of sucrose in stem tissue during growth has been reported. The high sugar early maturing variety Co 89003 has been reported to accumulate high sucrose content of 126.6 mg g^{-1} fr.wt during stem elongation and which reached of 160.6 mg g^{-1} fr.wt at maturity. The late maturing variety CoSe 94423 however recorded significantly low sucrose content of 44.0 mg g^{-1} fr.wt at stem elongation to 122.0 mg g^{-1} fr.wt at maturity (Batta *et al.,* 2011). The reducing sugar content also decreased from 15.3 mg g^{-1} fr.wt at stem elongation to 0.75 mg g^{-1} fr.wt at maturity in Co 89003 and from 31.5 mg g^{-1} fr.wt to 0.53 mg g^{-1} fr.wt in CoSe 94423. An increase in sucrose % from 10.21 % at 240 DAP to 16.70 % at 360 DAP with corresponding increase in CCS % from 6.35 to 11.59 has been documented in low sugar, mid late maturing genotype Bo91 (Jain *et al.,* 2013). An increase in sucrose content (10.21 to 16.76 %) with decrease of reducing sugar content (1.36 to 0.167 %) resulted in significant increase of S/R ratio from 7.51 to 100.4. On the contrary the mean invert ratio in juice at 4 stages has been reported to be 1.59 in CoC 671 an early maturing high sugar variety and 5.56 in Co 7717 in top portion (Thangavelu *et al.,* 2004). An invert ratio of 2 and less has been reported to be an index of cane maturity. An increase in sucrose

content from 172.5 mg g^{-1} fr.wt at 240 DAP to 204.6 mg g^{-1} fr.wt at 390 DAP in mature internodes of high sucrose cultivars and from 42.2 mg g^{-1} fr.wt at 240 DAP to 66.7 mg g^{-1} fr. wt at 390 DAP in low sucrose cultivars has been reported (Verma *et al.,* 2010). It has been reported that the difference between high sucrose and low sucrose clones was not only the final sucrose concentration reached in the old internodes but the internode development stage at which sucrose storage starts (Zhu *et al.,* 1996).

4.5 Sucrose phosphate synthase

4.5.1 Standardization of partial purification of sucrose phosphate synthase

The top and bottom portion of high yielding sugarcane variety CoM 0265 was used for the estimation of SPS activity at different developmental stages. Different ammonium sulphate fractions *viz.,* 0-30 %, 30-60 % and 60-80 % were used for assay of SPS activity from both top and bottom portion of cane and the data is presented in Table 13. The 30-60 % ammonium sulphate fraction recorded higher activity at all the developmental stages both in top and bottom portion of canes. Amongst the different ammonium sulphate fractions, 30- 60 % fraction recorded highest mean SPS activity (2.868 and 3.369 µmole sucrose formed mg^{-1} protein min^{-1}) both in top and bottom portion of the cane as compared to 0-30 % and 60-80 % fraction (2.027 to 2.621 µmole sucrose formed mg^{-1} protein min^{-1}) and (1.868 to 2.312 µmole sucrose formed mg^{-1} protein min^{-1}), respectively. Hence, this fraction (30-60 %) was used for assay of SPS activity in different high and low sugar sugarcane varieties and in the progenies of the crosses involving different parents.

Table 13. Standardization of sucrose phosphate synthase (SPS) activity in different ammonium sulphate fraction at different developmental stages in top and bottom portion of sugarcane variety CoM 0265

DAP/ Variety	SPS activity (µmole sucrose formed mg^{-1} protein min^{-1})							
	Top				Bottom			
	0-30 %	30-60 %	60-80 %	Mean	0-30 %	30-60 %	60-80 %	Mean
240	0.078	0.580	0.069	0.242	0.430	0.663	0.401	0.498
300	1.901	3.052	1.889	2.281	2.421	3.527	2.244	2.731
360	4.101	4.972	3.646	4.240	5.012	5.916	4.290	5.073
Mean	2.027	2.868	1.868	2.254	2.621	3.369	2.312	2.767

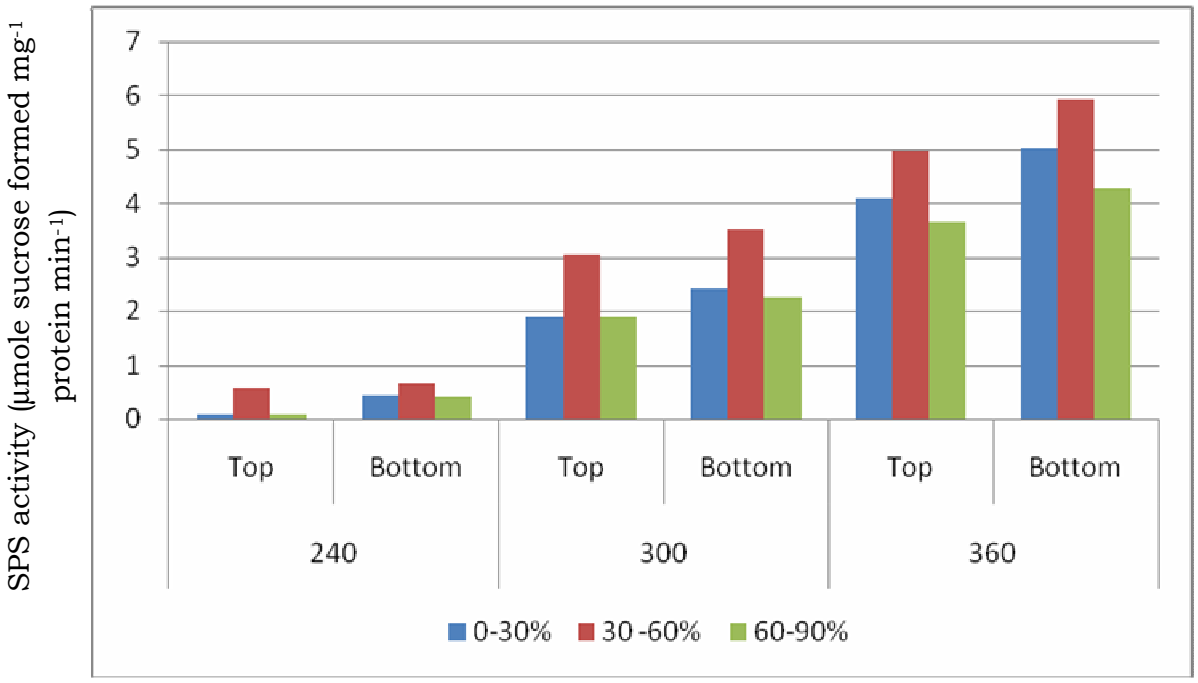

Fig. 7. Standardization of SPS activity in different ammonium sulphate fraction at different developmental stages in top and bottom portion of sugarcane variety CoM 0265

4.5.2 Sucrose phosphate synthase activity of varieties

Sucrose phosphate synthase activity at different developmental stages in top and bottom portion of high and low sugar varieties were evaluated from 240 to 390 DAP and the data is presented in Table 14. The SPS activity increased gradually both in top and bottom portion of the cane during development.

In high sugar variety CoM 0254, the SPS activity in the top portion increased from 0.591 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 0.969 µmole sucrose formed mg^{-1} protein min^{-1} at 390 DAP. In another early maturing sugarcane variety Co 94012 the activity of SPS increased from 0.320 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 0.525 µmole sucrose formed mg^{-1} protein min^{-1} at 390 DAP. The SPS activity was significantly much less in low sugar variety Co 62175 which increased from 0.227 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 0.372 µmole sucrose formed mg^{-1} protein min^{-1}at 390 DAP. The mean of SPS activity at different developmental stages in the top portion of the sugarcane variety CoM 0254 was 0.795 µmole sucrose formed mg^{-1} protein min^{-1} and in Co 94012 (0.431 µmole sucrose formed mg^{-1} protein min^{-1}) whereas, the mean SPS activity at different developmental stages in the top portion of the low sugar variety Co 62175 was 0.305 µmole sucrose formed mg^{-1} protein min^{-1}.

When the bottom portion of the cane was analyzed for SPS activity at different developmental stages, similar trend of increase in SPS activity was observed. The mean of SPS activity at different developmental stages in the bottom portion of the cane were 4.841 and 4.269 µmole sucrose formed mg^{-1} protein min^{-1} in

Table 14. Sucrose phosphate synthase (SPS) activity at different developmental stages in top and bottom portion of high and low sugar varieties

DAP/ Variety	SPS activity (µmole sucrose formed mg^{-1} protein min^{-1})							
	Top				Bottom			
	CoM 0254	Co 94012	Co 62175	Mean	CoM 0254	Co 94012	Co 62175	Mean
240	0.591	0.320	0.227	0.379	3.476	3.061	2.653	3.063
270	0.678	0.367	0.260	0.435	4.360	3.867	3.328	3.852
300	0.762	0.413	0.293	0.489	4.675	4.118	3.568	4.120
330	0.844	0.457	0.324	0.542	5.306	4.674	4.050	4.677
360	0.926	0.501	0.356	0.594	5.545	4.885	4.233	4.888
390	0.969	0.525	0.372	0.622	5.683	5.007	4.338	5.009
Mean	0.795	0.431	0.305	0.510	4.841	4.269	3.695	4.268
		SE±	CD at 5%			SE±	CD at 5%	
	Variety	0.005	0.015		Variety	0.048	0.139	
	DAP	0.007	0.021		DAP	0.068	0.197	
	Variety x DAP	0.013	0.037		Variety x DAP	0.118	0.342	

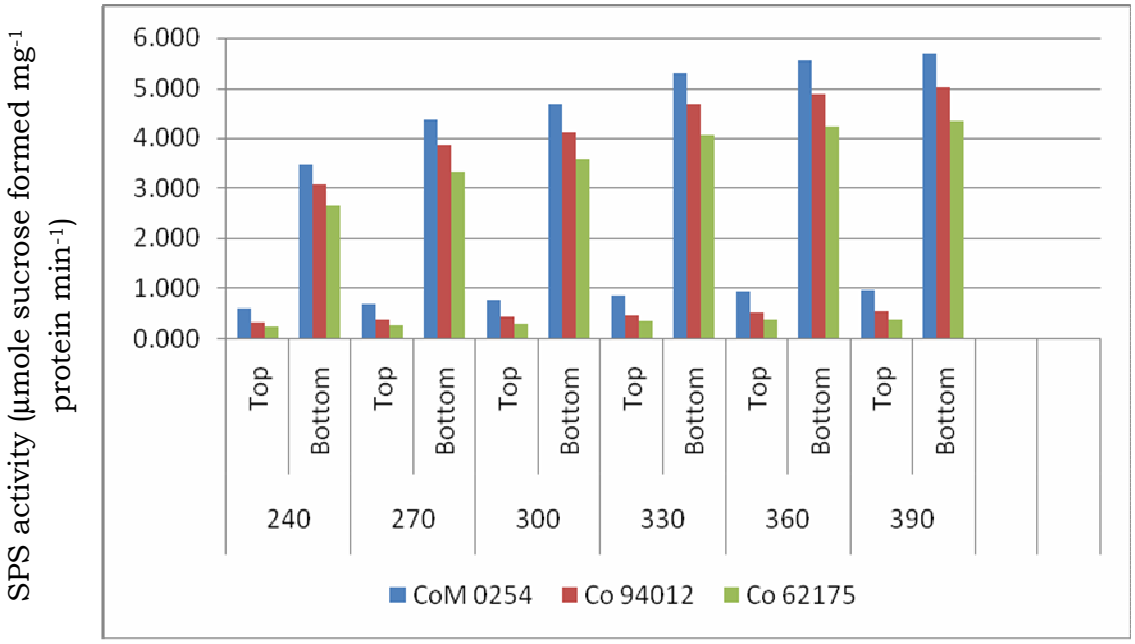

Fig. 8. SPS activity at different developmental stages in top and bottom portion of high and low sugar varieties

CoM 0254 and Co 94012, respectively. The mean of SPS activity at different developmental stages of the bottom portion of low sugar variety Co 62175 was 3.695 µmole sucrose formed mg^{-1} protein min^{-1}.

The data of SPS activity showed significant varietal differences as well as differences during the developmental stages.

The pattern of sucrose accumulation and degradation in relation to principle enzyme activities during cane maturation has been reported. The sucrose phosphate synthase activity in top internodes has been correlated to high sucrose accumulation (Joshi *et al.*, 2013). However, the sucrose synthase activity in cleavage direction increased in matured internodes of both the sugarcane varieties Co 86032 and CoG 93076.

Lingle (1998) reported that, the sucrose phosphate synthase activities increased during development.

Pan *et al.* (2009) earlier reported the lower SPS activity in the young internodes compared to maturing and matured internodes and it showed increasing trends with increasing in the age of the internodes.

Verma *et al.* (2010, 2011), Lingle (1998) reported that high sugar cultivars showed increased enzyme activity of SPS compared to low sugar cultivars at all developmental stages. SPS activity was positively correlated with sucrose and negatively correlated with hexose sugars.

Lau (2006) reported that the activity of SPS enzymes increased gradually with stalk development and decreased with maturity of sugarcane.

Grof *et al.* (2007) noticed that, SPS activity in immature internodes is higher than in mature internodes, while Botha and Black (2000) found that SPS activity was higher in mature internodes than in immature internodes. It has been reported that SPS becomes progressively more important in sucrose synthesis as the internodes mature based on the distribution of radiolabel in the hexose moieties of sucrose after labeling with glucose (Botha and Black, 2000). The highest sencitivity of SPS towards Pi inhibition during the stage where sucrose accumulation is higher has been reported.

Prathima *et al.* (2012) reported that SPS activity increased gradually from top to middle internodes and reach maximum at bottom internodes of both high and low sugar genotypes.

Botha and Black (2000) reported that high enzyme activity of SPS was associated with high level of sucrose in mature intermodal tissues of all sugarcane cultivars whereas, low level of SPS activity was associated with low level of sucrose in immature intermodal tissues.

The results obtained in the present investigation are in conformity with these earlier reports.

4.6 Sucrose synthase

4.6.1 Standardization of partial purification of sucrose synthase

The top and bottom portion of high yielding sugarcane variety CoM 0265 was used for the extraction and assay of SuSy activity at different developmental stages. For this, the SuSy activity of three different ammonium sulphate fractions viz. 0-30 %,

30-60 % and 60-80 % was estimated and the data is presented in Table 15. Among the different ammonium sulphate fractions, 30-60 % fraction recorded highest mean SuSy activity of 0.853 and 0.938 µmole sucrose formed mg^{-1} protein min^{-1} in top and bottom portion of the cane respectively as compared to 0.544 and 0.660 µmole sucrose formed mg^{-1} protein min^{-1} in both top and bottom portion and 0.618 and 0.364 µmole sucrose formed mg^{-1} protein min^{-1} in both top and bottom portion at 0-30 % and 60-80 % fractions. Hence, 30-60 % ammonium sulphate fraction was used for assay of SuSy activity in different high and low sugar sugarcane varieties and in the crosses involving different parents for comparison.

4.6.2 Sucrose synthase activity of varieties

Sucrose synthase activity from the top and bottom portion of high and low sugar varieties were evaluated at different development stages from 240 to 390 DAP is presented in Table 16. It is evident from the table that the SuSy activity gradually declined during the developmental stages from 240 DAP to 390 DAP. In high sugar variety CoM 0254, the SuSy activity in the top portion decreased from 2.396 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 1.388 µmole sucrose formed mg^{-1} protein min^{-1} at 390 DAP when extracted from top portion of the cane and from 3.668 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 2.125 µmole sucrose formed mg^{-1} protein min^{-1} at 390 DAP in Co 94012. The level of SuSy activity was comparatively much higher in low sugar variety Co 62175 which decreased from 3.993 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 2.313 µmole sucrose formed mg^{-1} protein min^{-1} at 390 DAP.

Table 15. Standardization of SuSy activity in different ammonium sulphate fraction at different developmental stages in top and bottom portion of sugarcane variety CoM 0265

DAP/ Variety	SuSy activity (µmole sucrose formed mg^{-1} protein min^{-1})							
	Top				Bottom			
	0-30 %	30-60 %	60-80 %	Mean	0-30 %	30-60 %	60-80 %	Mean
240	1.103	1.668	1.361	1.377	1.560	2.120	0.656	1.445
300	0.353	0.637	0.307	0.432	0.313	0.534	0.298	0.382
360	0.176	0.253	0.186	0.205	0.109	0.160	0.139	0.136
Mean	0.544	0.853	0.618	0.671	0.660	0.938	0.364	0.654

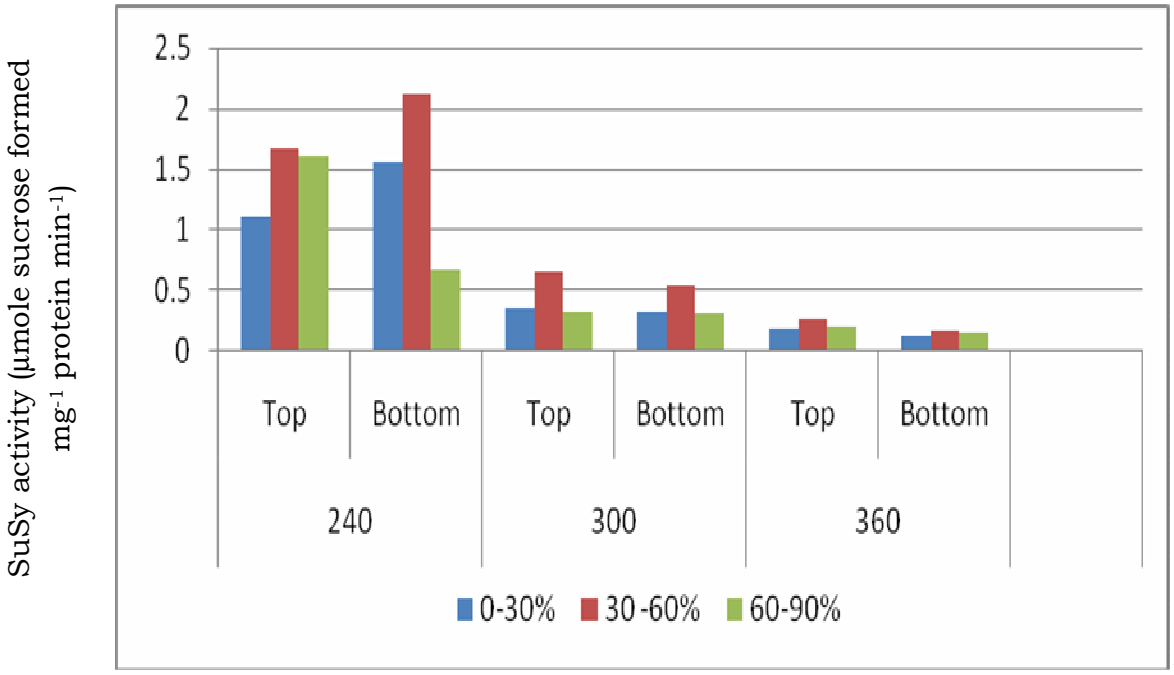

Fig. 9. Standardization of SuSy activity in different ammonium sulphate fractions at different developmental stages in top and bottom portion of sugarcane variety CoM 0265

Table 16. Sucrose synthase (SuSy) activity at different developmental stages in top and bottom portion of high and low sugar varieties

DAP/ Variety	SuSy activity (µmole sucrose formed mg^{-1} protein min^{-1})							
	Top				Bottom			
	CoM 0254	Co 94012	Co 62175	Mean	CoM 0254	Co 94012	Co 62175	Mean
240	2.396	3.668	3.993	3.352	1.310	1.350	1.770	1.477
270	2.216	3.393	3.693	3.101	1.209	1.246	1.633	1.363
300	2.032	3.143	3.420	2.865	1.009	1.040	1.364	1.138
330	1.839	2.817	3.066	2.574	0.895	0.924	1.212	1.010
360	1.626	2.491	2.711	2.276	0.469	0.706	0.998	0.724
390	1.388	2.125	2.313	1.942	0.374	0.555	0.784	0.571
Mean	1.916	2.940	3.199	2.685	0.878	0.970	1.294	1.047

		SE±	CD at 5%		SE±	CD at 5%
	Variety	0.010	0.028	Variety	0.014	0.040
	DAP	0.014	0.040	DAP	0.020	0.057
	Variety x DAP	0.024	0.070	Variety x DAP	0.034	0.098

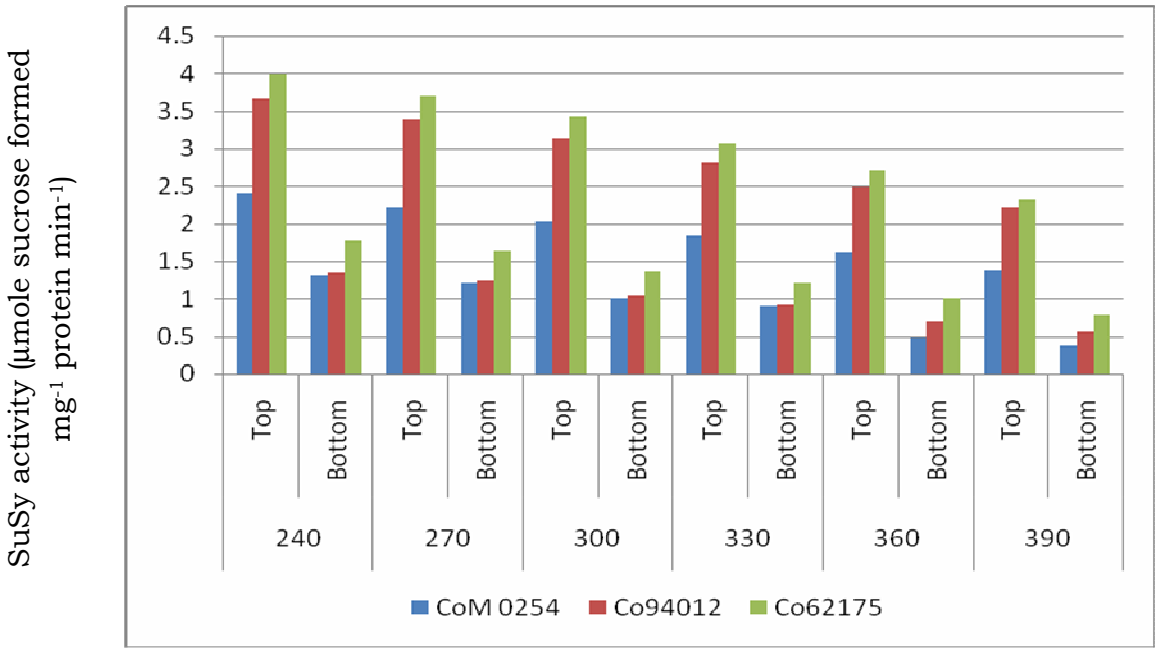

Fig. 10. SuSy activity at different developmental stages in top and bottom portion of high and low sugar varieties

The mean of SuSy activity at different developmental stages in the top portion of high sugar variety CoM 0254 was 1.916 µmole sucrose formed mg^{-1} protein min^{-1} and 2.940 µmole sucrose formed mg^{-1} protein min^{-1} in Co 94012 whereas the mean of SuSy activity at different developmental stages in the top portion of low sugar variety Co 62175 was 3.199 µmole sucrose formed mg^{-1} protein min^{-1}.

When the bottom portion of the cane were analysed for SuSy activity, similar trend of decrease in SuSy activity was noticed. The mean of SuSy activity at different developmental stages from the bottom portion of the high sugar variety CoM 0254 was 0.878 µmole sucrose formed mg^{-1} protein min^{-1} and 0.970 µmole sucrose formed mg^{-1} protein min^{-1} in Co 94012 an early maturing variety. The mean of SuSy activity at different developmental stages from the bottom portion of the low sugar variety Co 62175 although showed decrease in SuSy activity but was less as compared to high sugar variety with the mean of SuSy activity of 1.294 µmole sucrose formed mg^{-1} protein min^{-1}. The data of SuSy activity showed significant varietal differences as well as differences during the developmental stages.

High sucrose content in the older internodes has been reported to favour sucrose cleavage rather than synthesis reaction of SuSy. Higher SuSy activity was correlated with sucrose accumulation (Lingle, 1996). However, (Zhu *et al.,* 1997) did not found any correlation between SuSy and sucrose accumulation while analyzing the segregating F-1 population. The higher SuSy activity in the young internodes suggests that it may be an indicator of sink for sucrose import.

Sturm (1999) reported that sucrose synthase was expressed at high levels in low sucrose accumulating cultivars and was responsible for the high hexose concentration in the respective tissues. Sucrose synthase is also responsible for providing hexose sugar in actively growing cells for glycolysis and also acts as a carbon source and for energy depends on its cleavage into hexoses. This is in agreement with our results on sucrose and hexose accumulation at differenr developmental stages and in different internodal tissues of both high and low sucrose accumulating cultivars.

4.7 Invertase(s)

4.7.1 Soluble acid invertase activity of varieties

Soluble acid invertase activity at different developmental stages in top and bottom portion of high and low sugar varieties were evaluated from 240 to 390 DAP Table 17. The SAI activity gradually decreased during the developmental stages from 240 DAP to 390 DAP. In high sugar variety CoM 0254, SAI activity in the top portion of the cane decreased from 549 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 70 ηmole glucose formed mg^{-1} protein min^{-1} at 390 DAP and from 1099 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 145 ηmole glucose formed mg^{-1} protein min^{-1} at 390 DAP in Co 94012. The SAI activity in the top portion of low sugar variety Co 62175 was comparatively much higher (1498 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 200 ηmole glucose formed mg^{-1} protein min^{-1} at 390 DAP) than the high sugar variety. When the bottom portion of the canes was analyzed for SAI activity, similar trend of decrease in SAI activity was observed. In the bottom portion of the cane, the mean of SAI activity at different

Table 17. Soluble acid invertase (SAI) activity at different developmental stages in top and bottom portion of high and low sugar varieties

DAP/ Variety	SAI activity (ηmole glucose formed mg^{-1} protein min^{-1})							
	Top				Bottom			
	CoM 0254	Co 94012	Co 62175	Mean	CoM 0254	Co 94012	Co 62175	Mean
240	549	1099	1498	1049	490	858	1421	923
270	467	726	1295	829	379	654	924	652
300	311	540	922	591	243	494	738	492
330	226	483	728	479	146	428	510	361
360	140	395	485	340	59	185	249	164
390	70	145	200	138	29	55	219	101
Mean	294	565	855	571	224	446	677	449
		SE±	CD at 5%			SE±	CD at 5%	
	Variety	6.09	17.52		Variety	5.70	16.39	
	DAP	8.62	24.78		DAP	8.06	23.18	
	Variety x DAP	14.93	42.93		Variety x DAP	13.97	40.16	

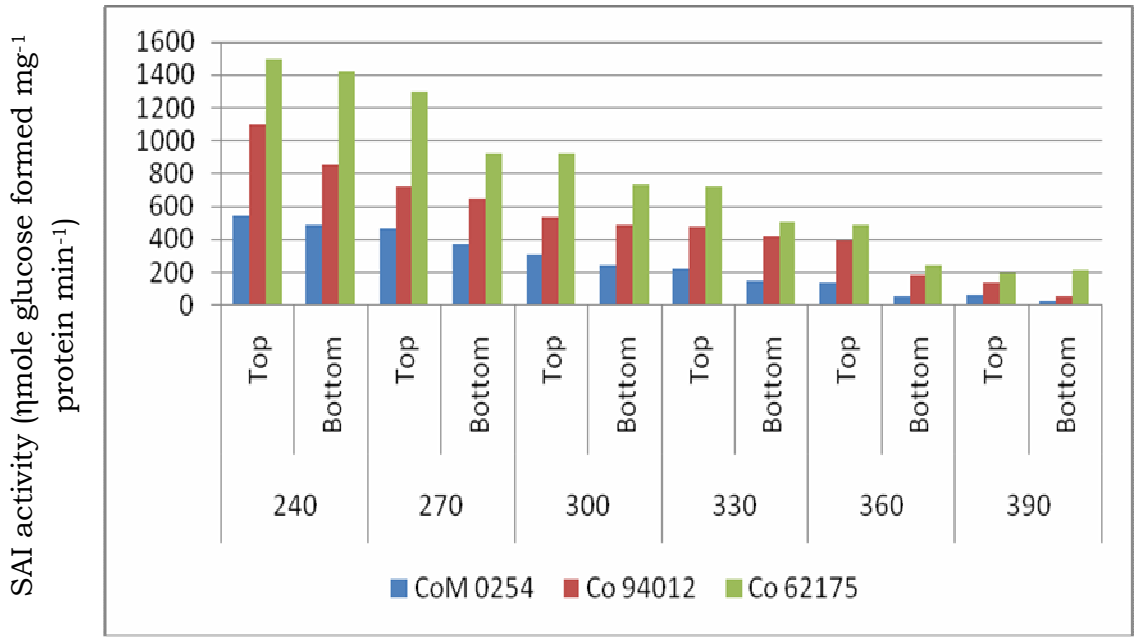

Fig. 11. SAI activity at different developmental stages in top and bottom portion of high and low sugar varieties

developmental stages of high sugar varieties was 224 and 446 ηmole glucose formed mg^{-1} protein min^{-1} in CoM 0254 and Co 94012 respectively. The SAI activity of the bottom portion of the low sugar variety Co 62175 (1421 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 219 ηmole glucose formed mg^{-1} protein min^{-1}) at 390 DAP was comparatively more than CoM 0254 and Co 94012 and decreased at maturity, with the mean of SAI activity was 677 ηmole glucose formed mg^{-1} protein min^{-1}. The data of SAI activity showed significant varietal differences as well as differences during the developmental stages.

Hawker and Hatch (1965) reported that, mature sucrose storing internodes of sugarcane contain negligible soluble acid invertase activity than immature internodes.

Sehtiya *et al.* (1991) emphasized that, the acid invertase activity was higher in immature internodes than mature internodes.

Zhu *et al.* (1997) reported that, SAI activity varied by internode age and young internodes of the low sugar genotype showed highest activities as compared to high sufar genotypes. When SAI activity was high, the sucrose content was always low, but when SAI was low, the sucrose content could be either low or high and during maturity stage there is marked decline in vacuolar acid invertase activity.

Thangvelu and Rao (2004) reported that, the activity of soluble acid invertase was much higher in the top potion than the mid and bottom portions at the stem elongation stage. With the advancement of cane growth, this activity fell but more drastically in the top part of the stem.

Batta *et al.* (2007) reported that, the rapid decline in the activity of soluble acid invertase with the maturity of the cane correlated with the high level of sucrose in early maturing varieties of sugarcane. The reduction of SAI activity was dramatic in early maturing and high sucrose storing cultivars, whereas the declimne in was lower in late maturing and low sucrose storing cultivars (Sachdeva *et al.* 2003b and Terauchi *et al.* 1999).

Lontom *et al.* (2008) reported that, the activity of SAI was highest in the young internodes and it decreased with internode age.

Veerma *et al.* (2011) reported that, the SAI activity was higher in immature internodes as compared to mature internodes.

4.7.2 Soluble neutral invertase activity of varieties

Soluble neutral invertase activity at different developmental stages in top and bottom portion of high and low sugar varieties were evaluated from 240 to 390 DAP Table 18. The SNI activity gradually increased during the developmental stages from 240 DAP to 390 DAP. In high sugar variety CoM 0254 in the top portion, the SNI activity increased from 175 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 1875 ηmole glucose formed mg^{-1} protein min^{-1} at 390 DAP and from 100 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 1785 ηmole glucose formed mg^{-1} protein min^{-1} at 390 DAP in Co 94012. The SNI activity in the top portion of low sugar variety Co 62175 (60 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 1618 ηmole glucose formed mg^{-1} protein min^{-1}) at 390 DAP was comparatively lower than the high sugar variety. When the bottom portion of the cane was analyzed for SNI activity, similar trend of increase in SNI

Table 18. Soluble neutral invertase (SNI) activity at different developmental stages in top and bottom portion of high and low sugar varieties

DAP/ Variety	SNI activity (ηmole glucose formed mg^{-1} protein min^{-1})							
	Top				Bottom			
	CoM 0254	Co 94012	Co 62175	Mean	CoM 0254	Co 94012	Co 62175	Mean
240	175	100	60	112	135	80	35	083
270	378	295	100	258	148	132	90	123
300	757	636	335	576	646	467	151	421
330	976	661	502	713	799	639	365	601
360	1171	735	717	874	1057	677	542	759
390	1875	1785	1618	1759	1388	1359	754	1167
Mean	889	702	555	715	696	559	323	526
		SE±	CD at 5%			SE±	CD at 5%	
	Variety	3.71	10.69		Variety	11.00	33.10	
	DAP	5.26	15.12		DAP	8.00	24.16	
	Variety x DAP	9.11	26.18		Variety x DAP	6.00	18.10	

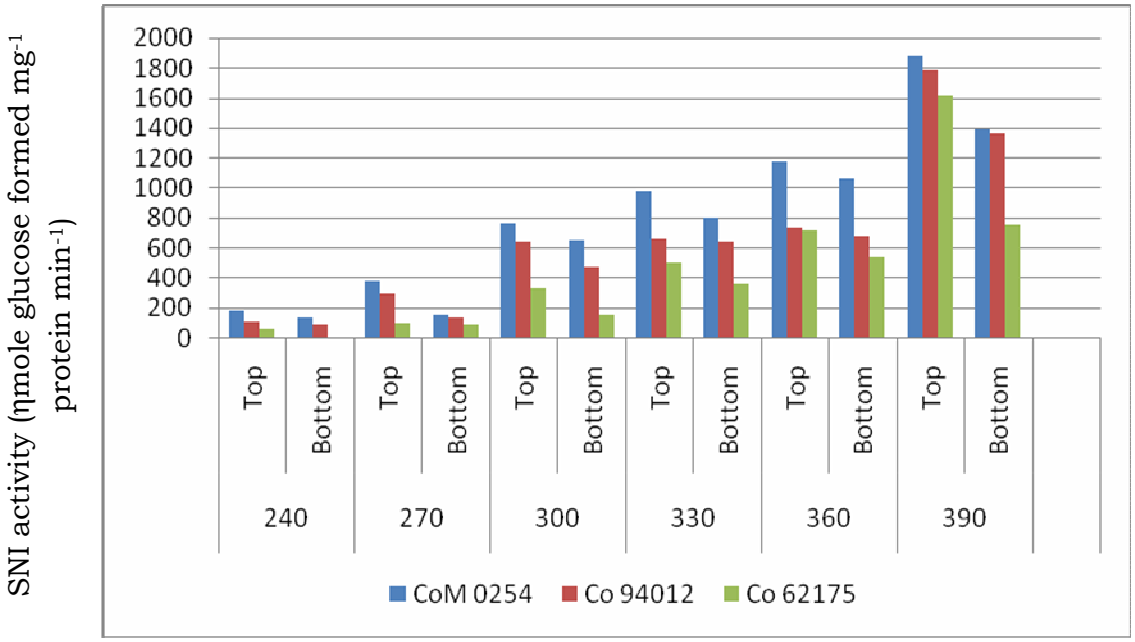

Fig. 12. SNI activity at different developmental stages in top and bottom portion of high and low sugar varieties

activity was observed. In the bottom portion of the canes, the mean of SNI activity of high sugar varieties CoM 0254 and Co 94012 was 696 and 559 ηmole glucose formed mg^{-1} protein min^{-1} whereas, the SNI activity of the bottom portion of the low sugar variety Co 62175 was 35 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 754 ηmole glucose formed mg^{-1} protein min^{-1} at 390 DAP which was comparatively less than high sugar varieties CoM 0254 and Co 94012 and showed increasing trends during the various developmental stages with the mean of SAI activity was 323 ηmole glucose formed mg^{-1} protein min^{-1}. The data of SNI activity showed significant varietal differences as well as differences during the developmental stages.

Early studies found that NI activity (expressed on FW basis) increase with internode maturity and therefore, correlates positively with sucrose concentrations in internodal tissues (Singh and Kanwar, 1991).

Dendsay *et al.* (1995) reported conflicting data where NI activity (expressed on FW basis) decrease with internode maturation. He also reported that NI activity was low or absent in mature storage tissue of many high sugar storing, early maturing varieties of sugarcane.

Neutral invertase was found at low concentrations in the cytoplasm of the young tissyes and at large concentrations in mature tissues (Glasziou, 1962).

Sehtiya *et al.* (1991) reported that the the neutral invertase activity was higher in mature internodes than in immature internodes, in agreement with the result of Verma *et al.* (2010).

Singh and Kanwar (1991) reported that neutral invetase (NI) activity (expressed on fresh weight basis) increases with internode maturity and correlate positively with sucrose concentration in intermodal tissues. Neutral invertase was present at low level in very young tissue and at greater levels in older tissues (Hatch and Glasziou, 1963 and Batta and Singh, 1986).

Terauchi *et al.* (1999) reported that, the soluble neutral invertase a positive factor for sucrose accumulation was higher in high sugar variety than low sugar variety.

Thangavelu and Rao (2004) reported that the activity of soluble neutral invertase was higher in the bottom than the mid and top portions and markedly increased in the bottom part of the mature cane.

Batta *et al.* (1986, 2008 and 2011) reported that, neutral invertase activity increased with the cane maturation.

The results obtained in the present investigation are in conformity with these earlier reports.

4.7.3 Cell wall invertase activity of varieties

Cell wall incertase activity at different developmental stages in top and bottom portion of high and low sugar varieties were evaluated from 240 to 390 DAP is presented in Table 19. It is evident from the table that the CWI activity gradually increased during the developmental stages from 240 DAP to 390 DAP. In high sugar variety CoM 0254 in the top portion, the CWI activity increased from 12 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 133 ηmole glucose formed mg^{-1} protein min^{-1} at 390 DAP and from 10 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 79 ηmole glucose formed mg^{-1} protein min^{-1} at 390 DAP in Co

Table 19. Cell wall invertase (CWI) activity at different developmental stages in top and bottom portion of high and low sugar varieties

DAP/ Variety	CWI activity (ηmole glucose formed mg^{-1} protein min^{-1})							
	Top				Bottom			
	CoM 0254	Co 94012	Co 62175	Mean	CoM 0254	Co 94012	Co 62175	Mean
240	12	10	8	10	38	27	19	28
270	33	17	13	21	69	42	36	49
300	36	36	25	32	109	73	73	85
330	52	48	40	47	127	81	77	95
360	83	50	43	59	146	131	110	129
390	133	80	69	94	196	145	122	154
Mean	58	40	33	44	114	83	73	90

		SE+	CD at 5%		SE+	CD at 5%
	Variety	1.42	4.09	Variety	1.96	5.64
	DAP	2.01	5.79	DAP	2.77	7.98
	Variety x DAP	3.49	10.03	Variety x DAP	4.81	13.82

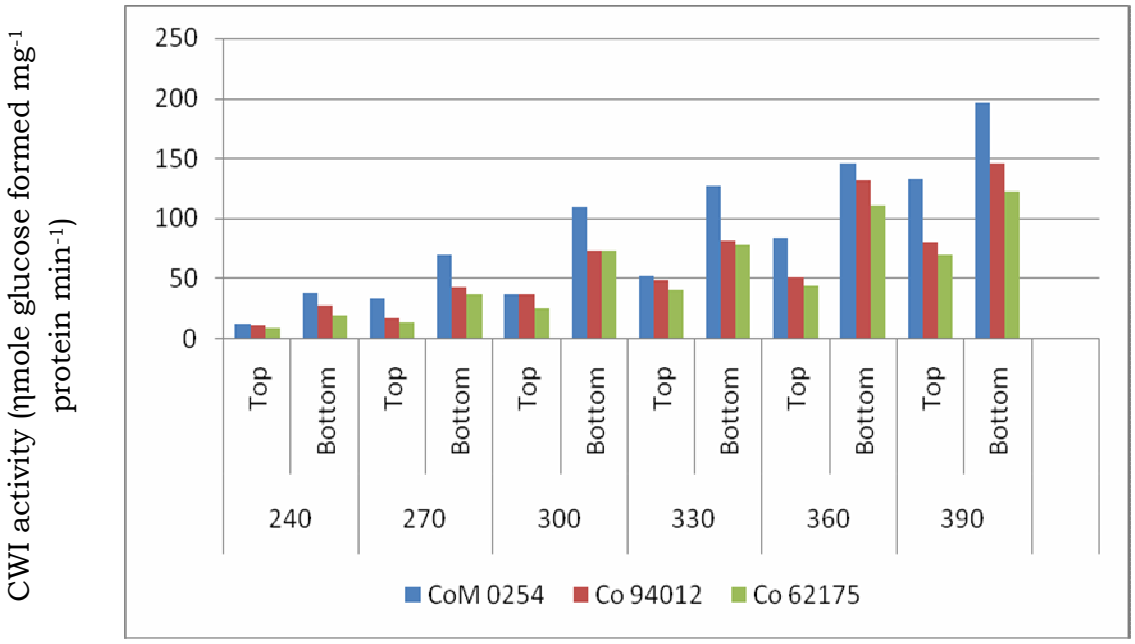

Fig. 13. CWI activity at different developmental stages in top and bottom portion of high and low sugar varieties

94012. The CWI activity in the top portion of low sugar variety Co 62175 was comparatively much lower i.e. 8 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 69 ηmole glucose formed mg^{-1} protein min^{-1} at 390 DAP than the high sugar variety and showed increasing trends during cane maturity. When the bottom portion of the cane were analyzed for CWI activity, similar trend of increase in CWI activity was observed. In the bottom portion of the canes, the mean of CWI activity of high sugar varieties CoM 0254 and Co 94012 was 114 and 96 ηmole glucose formed mg^{-1} protein min^{-1}. The CWI activity of the bottom portion of the low sugar variety Co 62175 was comparatively less i.e. 19 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 122 ηmole glucose formed mg^{-1} protein min^{-1} at 390 DAP than high sugar varieties CoM 0254 and Co 94012 and showed increase trends during the developmental stages with the mean of CWI activity was 73 ηmole glucose formed mg^{-1} protein min^{-1}. The data of CWI activity showed significant varietal differences as well as differences during the developmental stages.

Earlier Batta and Singh (1986) reported that, the activity of wall - bound invertase (pH 4.0) during stem elongation stage was low in each portion of the stem and increased with the maturity of the cane. In the top part of the cane, the activity of this enzyme was much higher throughout the period of cane development.

An increase in CWI activity with interode age has been reported by Vorster and Botha (1999) and Lingle (2004).

Batta and Singh (1986) reported that, the cell wall bound acid inveertase activity increased with the age of cane.

Lingle (2004) studied CWI in sugarcane in detail and reported that increased CWI activity, sucrose and sucrose to total sugar ratio with an increase in internode age. Higher activity of cell wall acid invertase in high sugar genotypes enhances sucrose unloading into the internode tissue. He was also reported that, the internodes of the high sucrose storing genotypes appears to be metabolically more active than those of the low sucrose genotypes, thus the cell wall invertase gene may be a good candidate for improving sucrose accumulation in sugarcane. Similar results have also been reported by Botha *et al*. (1996).

Thangavelu and Rao (2004) reported that the activity of the wall bound invertase (pH 4.0) during the stem elongation stage was low in each portion of the stem and increased with the maturity of the cane. In the top part of the cane the activity of this enzyme was much higher throughout the period of cane development. However, Joshi *et al.* (2012) reported tht CWI activity was high at immature top portion and gradually reduce towards the bottom portion of the cane.

4.8 Genetic diversity analysis

4.8.1 RAPD markers

In RAPD derived analysis similarity index ranged between 0.56 (in between CoM 0254 and Co 62175) and 0.76 (in between Co 94012 and CoM 0265). Clustering analysis Co 62175 was most divergent genotype with other three genotypes being some what more cluster together. It reflected moderate divergence among the genotypes studied (Table 20 and Plate 1 to 3).

Table 20. The Disc similarity coefficient value based on RAPD binary data of sugarcane varieties

	CoM 0254	Co 94012	CoM 0265	Co 62175
CoM 0254	1.00	-	-	-
Co 94012	0.75	1.00	-	-
CoM 0265	0.68	0.76	1.00	-
Co 62175	0.56	0.61	0.65	1.00

4.8.2 ISSR markers

In ISSR derived analysis similarity index ranged between 0.662 (in between CoM 0254 and Co 62175) to 0.874 (in between Co 94012 and CoM 0254). In clustering analysis CoM 0254 and Co 94012 were closely grouped together showing low divergences while another cluster comprised of distinctly grouped CoM 0265 and Co 62175 showing wide divergence. Similar to RAPD analysis ISSR analysis showed moderate divergence (Table 21 and Plates 4 to 6).

Table 21. The Disc similarity coefficient valve based on ISSR binary data of sugarcane varieties

	CoM 0254	Co 94012	CoM 0265	Co 62175
CoM 0254	1.000	-	-	-
Co 94012	0.874	1.000	-	-
CoM 0265	0.705	0.685	1.000	-
Co 62175	0.662	0.682	0.731	1.000

4.9 Brix©, sucrose % and CCS % content in progenies of crosses involving different parents

The crosses of sugarcane varieties were effected at C.S.R.S., Padegaon during the year 2012-13 to improve the sucrose content of the ruling variety CoM 0265. Two high sugar sugarcane varieties *viz.* CoC 671 and CoM 0254 were used as one of the parents during the crosses. The progenies of the crosses were then planted in the nursery and the crosses were evaluated for Brix©, sucrose and CCS % at different developmental stages. The crosses so evaluated were marked as high or low sugar clones and their Brix©, sucrose and CCS at different developmental stages is presented in Table 22. High sugar sugarcane cross Co 740 x CoC 671 recorded significantly higher mean brix© (20.4) followed by 19.3 in CoM 0265 x CoM 0254 and the least mean brix© i.e. 18.0 was recorded in another progeny of a cross CoM 0265 x CoC 671. The mean brix© of crosses involving different parents increased from 17.5 at 240 DAP to 20.9 at 390 DAP. The high sugar sugarcane cross Co 740 x CoC 671 recorded maximum brix© (18.5) at early stage of cane development i.e. 240 DAP as against 16.0 brix© in another high sugar cross CoM 0265 x CoC 671 at 240 DAP. The increase in brix© reading in high sugar sugarcane cross CoM 0265 x CoC 671 from 240 DAP to 390 DAP was 30 % whereas the increase in brix© in another high sugar sugarcane cross Co 740 x CoC 671 from 240 DAP to 390 DAP was 18.92 %.

The mean sucrose % of high sugar sugar sugarcane cross C0 740 x CoC 671 over the developmental stages was 18.5 % followed by 17.9 in CoM 0265 x CoM 0254 and least mean sucrose % i.e. 16.1 % was recorded in another progeny of a cross CoM 0265

Table 22. Brix©, sucrose, and CCS at different developmental stages in progenies of crosses involving different parents

DAP/ Variety	Brix ©				Sucrose %				CCS %			
	1	2	3	Mean	1	2	3	Mean	1	2	3	Mean
240	16.0	18.0	18.5	17.5	14.4	16.3	16.1	15.6	10.1	11.4	10.9	10.8
270	16.5	18.8	19.4	18.2	15.0	17.2	17.0	16.4	10.5	12.0	11.6	11.4
300	17.7	19.7	20.5	19.3	15.6	18.1	19.2	17.6	10.8	12.7	13.6	12.4
360	19.2	19.9	21.7	20.3	16.9	18.8	20.0	18.6	11.8	13.4	14.1	13.1
390	20.8	20.0	22.0	20.9	18.7	19.2	20.3	19.4	13.0	13.8	14.1	
Mean	18.0	19.3	20.4		16.1	17.9	18.5		11.2	12.7	12.9	

		SE±	CD at 5%		SE±	CD at 5%		SE±	CD at 5%
	Variety	0.09	0.27	Variety	0.11	0.32	Variety	0.08	0.24
	DAP	0.12	0.35	DAP	0.14	0.41	DAP	0.11	0.32
	Variety x DAP	0.21	0.61	Variety x DAP	0.24	0.71	Variety x DAP	0.19	0.55

Progenies: 1. CoM 0265 x CoC 671 2. CoM 0265 x CoM 0254 3. Co 740 x CoC 671

Table 22 contd.. Brix©, sucrose, and CCS at different developmental stages in progenies of crosses involving different parents

DAP/ Variety	Brix ©				Sucrose %				CCS %			
	4	5	6	Mean	4	5	6	Mean	4	5	6	Mean
240	15.0	16.0	16.0	15.7	12.6	14.4	14.2	13.7	8.5	10.0	9.7	9.4
270	18.8	17.4	17.0	16.7	13.5	15.6	14.9	14.7	9.0	10.8	10.1	9.9
300	17.1	18.1	18.1	17.8	15.0	16.7	16.1	15.9	10.3	11.7	11.0	11.0
360	18.5	19.5	18.8	18.9	16.8	18.4	17.0	17.4	11.7	13.1	11.8	12.2
390	19.2	19.9	19.1	19.4	17.5	18.9	17.3	17.9	12.2	13.5	12.1	12.6
Mean	17.1	18.2	17.8		15.0	16.8	15.9		10.3	11.8	10.9	
		SE±		CD at 5%		SE±		CD at 5%		SE±		CD at 5%
	Variety	0.09		0.28	Variety	0.09		0.29	Variety	0.08		0.26
	DAP	0.12		0.37	DAP	0.12		0.37	DAP	0.11		0.34
	Variety x DAP	0.22		0.66	Variety x DAP	0.22		0.64	Variety x DAP	0.20		0.59

Progenies: 4. CoM 0265 x CoC 671 5. CoM 0265 x CoM 0254 6. Co 740 x CoC 671

x CoC 671. The mean sucrose % of crosses involving different parents increased from 15.6 % at 240 DAP to 19.4 % at 390 DAP. The high sugar sugarcane cross Co 740 x CoC 671 recorded maximum sucrose % (16.1 %) at early stage of cane development i.e. 240 DAP as against 14.4 % in another progeny of a cross CoM 0265 x CoC 671. The progeny of a cross cross CoM 0265 x CoC 671 recorded maximum increase in sucrose % i.e. 29 .86 % followed by 26.08 % in Co 740 x CoC 671 and 17.79 % in CoM 0265 x CoM 0254 from 240 DAP to 390 DAP.

The CCS % which is calculated based on brix and sucrose also revealed similar trend with Co 740 x CoC 671 recording higher CCS %. The progeny of a cross Co 740 x CoC 671 recorded significantly higher increase in CCS % i.e. 29.36 % followed by 28.71 % in CoM 0265 x CoC 671 and minimum 21.05 % in CoM 0265 x CoM 0254 over the developmental stages.

The low sugar sugarcane cross CoM 0265 x CoM 0254 recorded maximum mean brix© i.e. 18.2 followed by 17.8 in Co 740 x CoC 671 and 17.1 in CoM 0265 x CoC 671. The brix© of crosses involving different parents increased from 15.7 at 240 DAP to 19.4 at 90 DAP. The low sugar sugarcane cross CoM 0265 x CoM 0254 recorded maximum increased in brix© reading i.e. 24.37 % from 240 DAP to 390 DAP whereas, another low sugar sugarcane cross Co 740 x CoC 671 recorded least increased in beix© i.e 19.37 % from 240 DAP to 390 DAP.

The low sugar sugarcane cross CoM 0265 x CoM 0254 recorded maximum mean sucrose % i.e 16.8 % over the developmental stages followed by 15.9 % in Co 740 CoC 671 and 15.0 % in CoM 0265 x CoC 671. The mean sucrose % of crosses

involving different parents increased from 13.7 % at 240 DAP to 17.9 % at 390 DAP. The low sugar sugarcane cross CoM 0265 x CoC 671 recorded maximum increased in sucrose % i.e. 38.88 % followed by 31.25 % in Com 0265 x CoM 0254 and least increased in sucrose i.e 21.85 % was recorded in Co 740x CoC 671 from 240 DAP to 390 DAP.

The low sugar sugarcane cross CoM 0265 x CoM 0254 recorded maximum mean CCS % i.e. 11.8 % over the developmental stages followed by 10.9 % in Co 740 x CoC 671 and 10.3 % in CoM 0265 x CoC 671. The mean CCS % of crosses involving different parents increased from 9.4 % at 240 DAP to 12.6 % at 390 DAP. The low sugar sugarcane cross CoM 0265 x Coc 671 recorded maximum increased in CCS % i.e. 43.52 % followed by 35 % in CoM 0265 xCoM 0254 whereas, the least increased in CCS % was recorded in cross Co 74 x CoC 671 i.e. 24.74 % from 240 DAP to 390 DAP.

The high and low sugar varieties and the crosses effected were then evaluated for the sucrose, hexose content, invert ratio of the juice and the activities of sucrose metabolizing enzymes *viz.* sucrose phosphate synthase, sucrose synthase and invertase(s) at different developmental stages in the top and bottom portion of the cane.

An interspecific cross between *S. officinarum* clone 'Gungera' with high sucrose content and *S. spontaneum* SEC603 with low sucrose content was effected and 29 progenies of the cross has been reported to vary considerably in sucrose content (Vandana *et al.,* 2010). The molecular markers differentiated the high and low sucrose progeny.

4.10 Sucrose phosphate synthase activity in progenies of crosses involving different parents

Sucrose phosphate synthase activity at different developmental stages of crosses involving different parents were evaluated from 240 to 390 DAP is presented in Tables 23 and 24. The data presented in the table showed that among the both high sugar and low sugar crosses the SPS activity increased gradually during different developmental stages from 240 to 390 DAP both in top and bottom portion of the cane.

In the high sugar cross, in the top portion, cross Co 740 X CoC 671 recorded highest SPS activity from 0.910 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 1.493 µmole sucrose formed mg^{-1} protein min^{-1} at 390 DAP and in another high sugar cross CoM 0265 X CoM 0254 from 0.736 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 1.208 µmole sucrose formed mg^{-1} protein min^{-1} at maturity. The level of SPS activity was much less in another high sugar cross CoM 0265 X CoC 671 i.e. 0.520 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 0.854 µmole sucrose formed mg^{-1} protein min^{-1} at 390 DAP but showed increased trends as the cane attend maturity. The mean of SPS activity in the top portion of the high sugar cross Co740 X CoC671 (1.224 µmole sucrose formed mg^{-1} protein min^{-1}) was significantly higher than CoM 0265 X CoM 0254 and CoM 265 X CoC 671 (0.991 and 0.700 µmole sucrose formed mg^{-1} protein min^{-1}).

When the bottom portion of the cane was analyzed for SPS activity, similar trend of increase in SPS activity was observed. The bottom portion of high sugar cross Co 740 X CoC 671 recorded highest SPS activity i.e. 5.261 µmole sucrose formed mg^{-1} protein

Table 23. Sucrose phosphate synthase (SPS) activity at different developmental stages in progenies of crosses having high sucrose content

DAP/ Variety	SPS activity (µmole sucrose formed mg^{-1} protein min^{-1})							
	Top				Bottom			
	CoM 0265 x CoC 671	CoM 0265 x CoM 0254	Co 740 X CoC 671	Mean	CoM 0265 x CoC 671	CoM 0265 x CoM 0254	Co 740 X CoC 671	Mean
240	0.520	0.736	0.910	0.722	1.547	1.656	5.261	2.821
270	0.597	0.485	1.044	0.828	1.775	1.899	6.004	3.226
300	0.674	0.950	1.174	0.932	1.996	2.136	6.665	3.599
330	0.743	1.052	1.299	1.032	2.210	2.366	7.380	3.985
360	0.812	1.154	1.426	1.132	2.424	2.595	8.242	4.420
390	0.854	1.208	1.493	1.184	2.538	2.717	8.629	4.628
Mean	0.700	0.991	1.224	0.972	2.081	2.228	7.030	3.780

		SE±	CD at 5%		SE±	CD at 5%
	Variety	0.013	0.038	Variety	0.021	0.061
	DAP	0.019	0.054	DAP	0.030	0.086
	Variety x DAP	0.032	0.093	Variety x DAP	0.051	0.149

Table 24. Sucrose phosphate synthase (SPS) activity at different developmental stages in progenies of crosses having high sucrose content

DAP/ Variety	SPS activity (µmole sucrose formed mg^{-1} protein min^{-1})							
	Top				Bottom			
	CoM 0265 x CoC 671	CoM 0265 x CoM 0254	Co 740 X CoC 671	Mean	CoM 0265 x CoC 671	CoM 0265 x CoM 0254	Co 740 X CoC 671	Mean
240	0.454	0.562	0.616	0.544	0.516	1.288	1.406	1.070
270	0.511	0.644	0.707	0.621	0.633	1.478	1.613	1.241
300	0.574	0.725	0.794	0.698	0.710	1.662	1.814	1.395
330	0.636	0.802	0.880	0.773	0.785	1.860	2.008	1.551
360	0.698	0.880	0.965	0.848	0.866	2.018	2.203	1.696
390	0.731	0.935	1.010	0.892	0.905	2.114	2.306	1.775
Mean	0.600	0.758	0.829	0.730	0.736	1.737	1.892	1.455

		SE±	CD at 5%		SE±	CD at 5%
	Variety	0.010	0.038	Variety	0.047	0.061
	DAP	0.014	0.054	DAP	0.067	0.086
	Variety x DAP	0.025	0.093	Variety x DAP	0.116	0.149

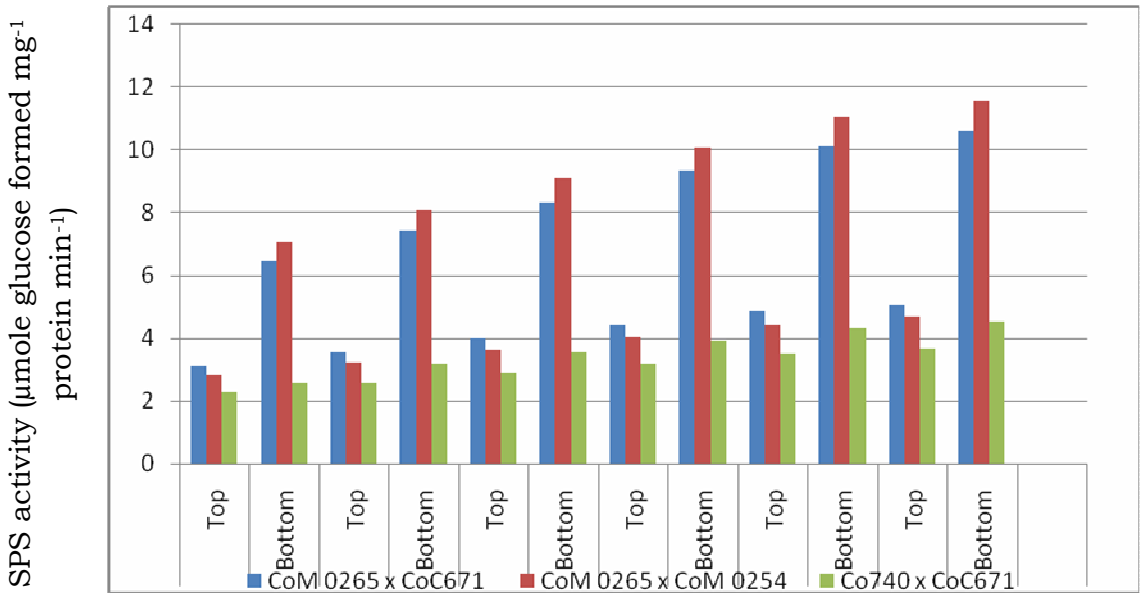

Fig. 14. SPS activity at different developmental stages in progenies of crosses having low sucrose content

min^{-1} at 240 DAP to 8.629 µmole sucrose formed mg^{-1} protein min^{-1} at 360 DAP than other high sugar crosses CoM 0265 X CoC 0254 (1.656 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 2.717 µmole sucrose formed mg^{-1} protein min^{-1} at 360 DAP) and CoM 0265 X CoC 671 (1.547 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 2.538 µmole sucrose formed mg^{-1} protein min^{-1} at 360 DAP), respectively. The highest mean of SPS activity was recorded in high sugar cross Co 740 X CoC 671 (7.030 µmole sucrose formed mg^{-1} protein min^{-1}) than CoM 0265 X CoM 0254 and CoM 0265 X CoC 671 (2.228 and 2.081 µmole sucrose formed mg^{-1} protein min^{-1}), respectively.

In the low sugar cross, in the top portion, cross Co 740 x Co 671 recorded highest SPS activity from 0.616 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 1.010 µmole sucrose formed mg^{-1} protein min^{-1} at 390 DAP and from 0.562 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 0.935 µmole sucrose formed mg^{-1} protein min^{-1} in another high sugar cross CoM 0265 X CoM 0254 at maturity. The level of SPS activity was less in another high sugar cross CoM 0265 X CoC 671 i.e. 0.454 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 0.731 µmole sucrose formed mg^{-1} protein min^{-1} at 390 DAP. The mean of SPS activity in the top portion of the high sugar cross Co 740 X CoC 671 (0.829 µmole sucrose formed mg^{-1} protein min^{-1}) was significantly higher than CoM 0265 X CoM 0254 and CoM 0265 X CoC 671 (0.758 and 0.600 µmole sucrose formed mg^{-1} protein min^{-1}), respectively.

When the bottom portion of the same crosses was analyzed for SPS activity, similar trend of increase in SPS activity

was observed. In the bottom portion, high sugar cross Co 740 x CoC 671 recorded highest SPS activity 1.406 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 2.306 µmole sucrose formed mg^{-1} protein min^{-1} at maturity than CoM 0265 X CoM 0254 i.e. 1.288 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 2.114 µmole sucrose formed mg^{-1} protein min^{-1} at 360 DAP, whereas CoM 0265 X CoC 671 recorded comparatively lower SPS activity (0.516 µmole sucrose formed mg^{-1} protein min^{-1} at 240 to 0.905 µmole sucrose formed mg^{-1} protein min^{-1} at 390 DAP and showed increasing trends as the cane attend the maturity. The highest mean of SPS activity was recorded in high sugar cross Co 740 X CoC 671 1.892 µmole sucrose formed mg^{-1} protein min^{-1}) than CoM 0265 X CoM 0254 and CoM 0265 X CoC 671 (1.737 and 0.736 µmole sucrose formed mg^{-1} protein min^{-1}), respectively.

The data of SPS activity showed significant differences in crosses involving different parents as well as differences during the developmental stages.

4.11 Sucrose synthase activity in progenies of crosses involving different parents

Sucrose synthase activity at different developmental stages of crosses involving different parents were evaluated from 240 to 390 DAP is presented in Tables 25 and 26. It is evident from the table that in both high and low sugar crosses, the SuSy activity decreased gradually during different developmental stages from 240 to 390 DAP both in top and bottom portion of the cane.

In the high sugar cross, in the top portion, cross Co 740 X CoC 671 recorded lowest SuSy activity from 8.543 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 5.101 µmole sucrose

Table 25. Sucrose synthase (SuSy) activity at different developmental stages in progenies of crosses having high sucrose content

DAP/ Variety	SuSy activity (µmole sucrose formed mg^{-1} protein min^{-1})							
	Top				Bottom			
	CoM 0265 x CoC 671	CoM 0265 x CoM 0254	Co 740 X CoC 671	Mean	CoM 0265 x CoC 671	CoM 0265 x CoM 0254	Co 740 X CoC 671	Mean
240	15.249	9.281	8.543	11.024	4.890	4.626	3.286	4.267
270	14.105	8.585	7.902	10.197	4.524	4.280	3.039	3.948
300	13.063	7.951	7.452	9.489	4.190	3.963	2.815	3.656
330	11.709	7.127	6.560	8.465	3.755	3.552	2.523	3.277
360	9.989	6.302	5.980	7.424	3.321	3.141	2.231	2.898
390	8.521	5.378	5.101	6.333	2.833	2.680	1.903	2.472
Mean	12.106	7.437	6.923	8.822	3.919	3.707	2.633	3.420

		SE±	CD at 5%		SE±	CD at 5%
	Variety	0.090	0.258	Variety	0.070	0.203
	DAP	0.127	0.365	DAP	0.100	0.287
	Variety x DAP	0.220	0.633	Variety x DAP	0.070	0.203

Table 26. Sucrose synthase (SuSy) activity at different developmental stages in progenies of crosses having low sucrose content

DAP/ Variety	SuSy activity (µmole sucrose formed mg^{-1} protein min^{-1})							
	Top				Bottom			
	CoM 0265 x CoC 671	CoM 0265 x CoM 0254	Co 740 X CoC 671	Mean	CoM 0265 x CoC 671	CoM 0265 x CoM 0254	Co 740 X CoC 671	Mean
240	8.203	6.258	3.823	6.095	4.504	3.114	1.842	3.153
270	7.587	5.789	3.536	5.637	4.166	2.881	1.704	2.917
300	7.027	5.361	3.275	5.221	3.859	2.668	1.578	2.702
330	6.318	4.805	2.935	4.686	3.458	2.391	1.414	2.421
360	5.570	4.250	2.596	4.139	3.050	2.115	1.251	2.139
390	4.752	3.625	2.214	3.530	2.609	1.804	1.067	1.827
Mean	6.576	5.015	3.063	4.885	3.608	2.496	1.476	2.526

		SE±	CD at 5%		SE±	CD at 5%
	Variety	0.096	0.277	Variety	0.085	0.246
	DAP	0.136	0.391	DAP	0.121	0.348
	Variety x DAP	0.236	0.677	Variety x DAP	0.209	0.603

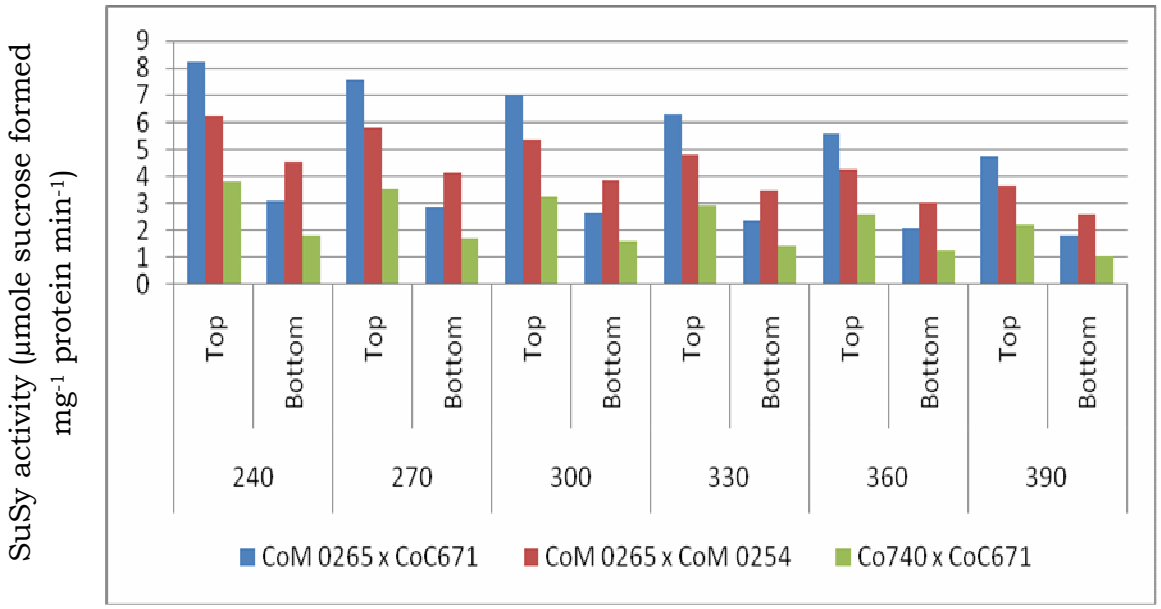

Fig. 15. SuSy activity at different developmental stages in progenies of crosses having low sucrose content

formed mg^{-1} protein min^{-1} at 390 DAP than CoM 0265 X CoM 0254 from 9.281 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 5.378 µmole sucrose formed mg^{-1} protein min^{-1} at maturity and in cross CoM 0265 X CoC 671 from 15.249 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 8.521 µmole sucrose formed mg^{-1} protein min^{-1} at 390 DAP. In the top portion of the high sugar crosses, cross Co 740 X CoC 671 (6.923 µmole sucrose formed mg^{-1} protein min^{-1}) recorded significantly lowest mean of SuSy activity than CoM 0265 X CoM 0254 and CoM 0265 X CoC 671 (7.437 and 12.106 µmole sucrose formed mg^{-1} protein min^{-1}).

When the bottom portion of the canes was analyzed for SuSy activity, similar trend of decrease in SuSy activity was observed. The lowest SuSy activity was recorded in the bottom portion of high sugar cross Co 740 X CoC 671 from 3.286 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 1.903 µmole sucrose formed mg^{-1} protein min^{-1} at 360 DAP than CoM 0265 X CoM 0254 (4.626 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 2.680 µmole sucrose formed mg^{-1} protein min^{-1} at 360 DAP) and CoM 0265 X CoC 671 (4.890 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 2.833 µmole sucrose formed mg^{-1} protein min^{-1} at 360 DAP) respectively. The lowest mean of SuSy activity was recorded in cross Co 740 X CoC 671 (2.633 µmole sucrose formed mg^{-1} protein min^{-1}) than CoM 0265 X CoM 0254 and CoM 0265 X CoC 671 (3.707 and 3.919 µmole sucrose formed mg^{-1} protein min^{-1}), respectively.

In the low sugar cross, in the top portion, cross Co 740 X CoC 671 recorded lowest SuSy activity from 3.823 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 2.214 µmole sucrose

formed mg^{-1} protein min^{-1} at 390 DAP than another high sugar cross CoM 0265 X CoM 0254 from 6.258 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 3.625 µmole sucrose formed mg^{-1} protein min^{-1} at maturity. Other high sugar cross CoM 0265 x CoC 671 showed comparatively more SuSy activity from 8.203 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 4.752 µmole sucrose formed mg^{-1} protein min^{-1} at maturity and showed decrease trends in SuSy activity towards the maturity of the cane. The significantly lowest mean of SuSy activity was recorded in the top portion of the low sugar cross, Co740 X CoC 671 (3.063 µmole sucrose formed mg^{-1} protein min^{-1}) than CoM 0265 X CoM 0254 and Co M 0265 X CoC 671 (5.015 and 6.576 µmole sucrose formed mg^{-1} protein min^{-1}), respectively.

When the bottom portion of the same crosses was analyzed for SuSy activity, similar trend of decrease in SuSy activity was observed. In the bottom portion, high sugar cross Co740 X CoC 671 recorded lowest SuSy activity from 1.842 µmole sucrose formed mg^{-1} protein min-1 at 240 DAP to 1.067 µmole sucrose formed mg^{-1} protein min^{-1} at maturity than CoM 0265 X CoM 0254 (3.114 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 1.804 µmole sucrose formed mg^{-1} protein min^{-1} at 360 DAP) and CoM 0265 X CoC 671 (4.504 µmole sucrose formed mg^{-1} protein min^{-1} at 240 DAP to 2.609 µmole sucrose formed mg^{-1} protein min^{-1} at 360 DAP) respectively. The lowest mean of SuSy activity was recorded in high sugar cross Co 740 X CoC 671 (1.476 µmole sucrose formed mg^{-1} protein min^{-1}) than CoM 0265 X CoM 0254 and CoM 0265 X CoC 671 (2.496 and 3.608 µmole sucrose formed mg^{-1} protein min^{-1}), respectively.

The data of SuSy activity showed significant differences in crosses involving different parents as well as differences during the developmental stages.

4.12 Soluble acid invertase activity in progenies of crosses involving different parents

Soluble acid invertase activity at different developmental stages of crosses involving different parents were evaluated from 240 to 390 DAP Tables 27 and 28. Among the both high sugar and low sugar crosses, the SAI activity decreased gradually during different developmental stages from 240 to 390 DAP both in top and bottom portion of the cane.

In the high sugar cross, in the top portion, cross Co 740 X CoC 671 recorded lowest SAI activity from 1469 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 191 ηmole glucose formed mg^{-1} protein min^{-1} at 390 DAP and from 1777 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 200 ηmole glucose formed mg^{-1} protein min^{-1} in another high sugar cross CoM 0265 X CoM 0254 at maturity. The level of SAI activity was much higher in another high sugar cross CoM 0265 X CoC 671 and showed decreasing trend from 2817 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 575 ηmole glucose formed mg^{-1} protein min^{-1} at 390 DAP. The high sugar cross Co 740 X CoC 671 (861 ηmole glucose formed mg^{-1} protein min^{-1}) recorded significantly lowest mean of SAI activity than other high sugar crosses CoM 0265 X CoM 0254 and CoM 0265 X CoC 671 (869 and 1523 ηmole glucose formed mg^{-1} protein min^{-1}) .

When the bottom portion of the cane was analyzed for SAI activity, similar trend of decrease in SAI activity was observed.

Table 27. Soluble acid invertase (SAI) activity at different developmental stages in progenies of crosses having high sucrose content

DAP/ Variety	SAI activity (ηmole glucose formed mg^{-1} protein min^{-1})							
	Top				Bottom			
	CoM 0265 x CoC 671	CoM 0265 x CoM 0254	Co 740 X CoC 671	Mean	CoM 0265 x CoC 671	CoM 0265 x CoM 0254	Co 740 X CoC 671	Mean
240	2817	1777	1469	2021	850	654	582	695
270	1955	1500	1260	1572	700	612	425	579
300	1614	1152	847	1204	580	583	352	505
330	1274	706	550	843	575	456	302	444
360	900	429	300	543	425	278	255	319
390	575	200	191	322	275	289	240	268
Mean	1523	961	770	1084	568	479	359	469

		SE+	CD at 5%		SE+	CD at 5%
	Variety	14.59	41.94	Variety	7.37	21.18
	DAP	20.63	59.32	DAP	10.42	29.96
	Variety x DAP	35.74	102.75	Variety x DAP	18.05	51.89

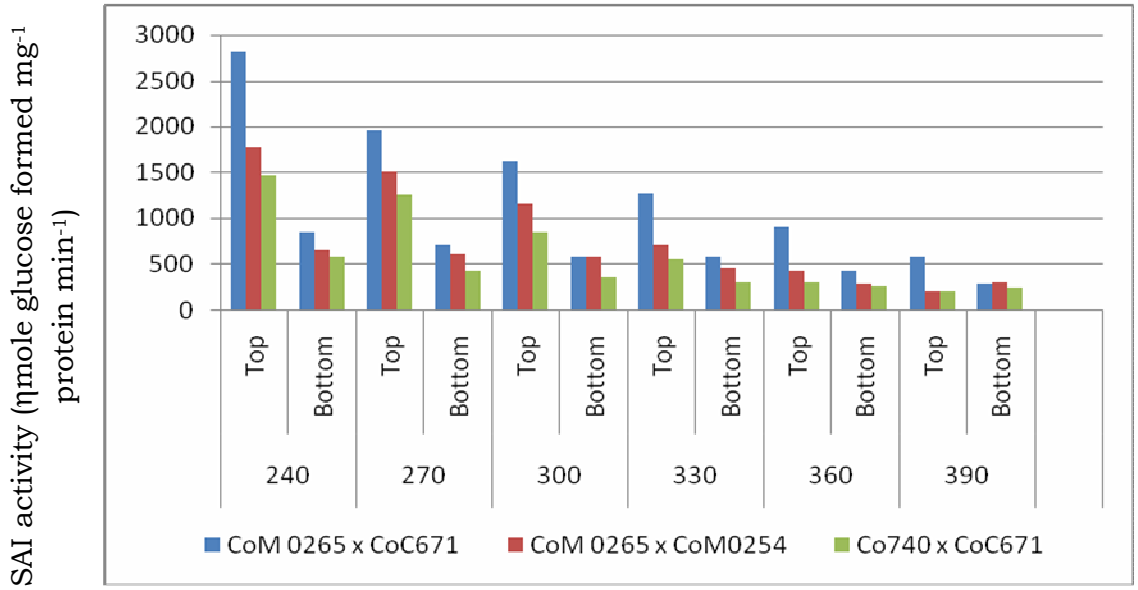

Fig. 16. SAI activity at different developmental stages in progenies of crosses having high sucrose content

Table 28. Soluble acid invertase (SAI) activity at different developmental stages in progenies of crosses having low sucrose content

DAP/ Variety	SAI activity (ηmole glucose formed mg^{-1} protein min^{-1})							
	Top				Bottom			
	CoM 0265 x CoC 671	CoM 0265 x CoM 0254	Co 740 X CoC 671	Mean	CoM 0265 x CoC 671	CoM 0265 x CoM 0254	Co 740 X CoC 671	Mean
240	3447	2151	1893	2497	1984	1423	750	1386
270	2347	1801	1524	1891	1834	1273	625	1244
300	2254	1233	1339	1609	1216	1109	542	955
330	2164	883	1034	1361	1066	973	459	833
360	2000	533	1000	1178	916	823	376	705
390	1018	283	824	708	766	673	294	578
Mean	2205	1147	1269	1540	1297	1046	508	951

		SE±	CD at 5%		SE±	CD at 5%
	Variety	12.09	34.77	Variety	9.35	26.89
	DAP	17.10	49.17	DAP	13.23	38.04
	Variety x DAP	29.63	85.17	Variety x DAP	22.92	65.88

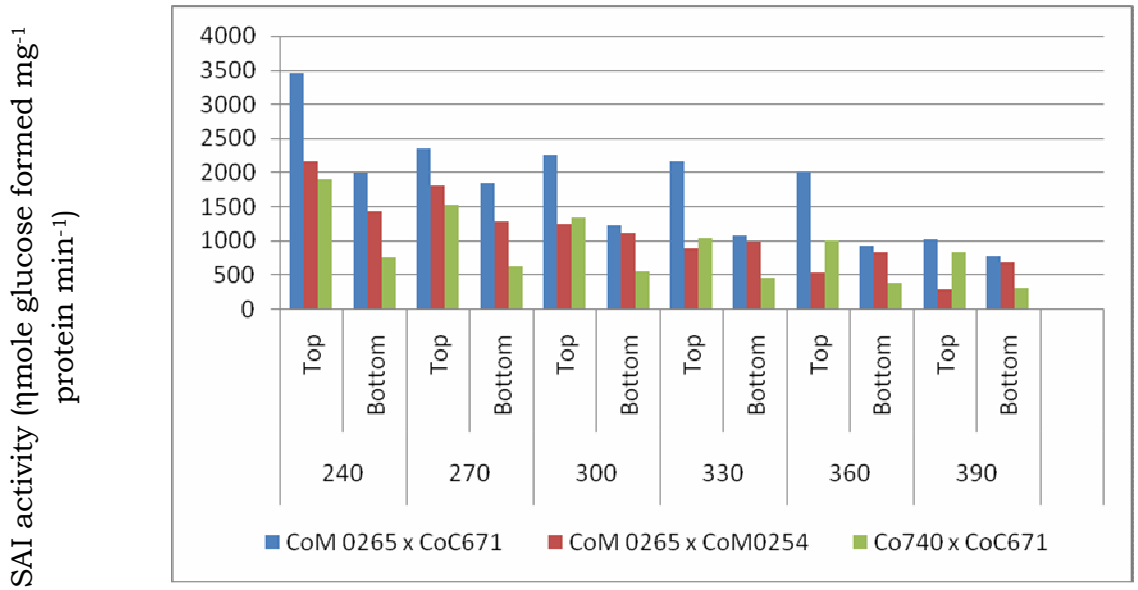

Fig. 17. SAI activity at different developmental stages in progenies of crosses having low sucrose content

The lowest SAI activity was recorded in the bottom portion of high sugar cross Co 740 X CoC 671 from 582 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 240 ηmole glucose formed mg^{-1} protein min^{-1} as the crop attend maturity than CoM 0265 X CoM 0254 (654 ηmole glucose formed mg^{-1} protein min^{-1}) at 240 DAP to 289 ηmole glucose formed mg^{-1} protein min^{-1} at maturity and CoM 0265 X CoC 671 (850 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 275 ηmole glucose formed mg^{-1} protein min^{-1} at maturity) respectively. The lowest mean of SAI activity was recorded in cross Co 740 X CoC 671 (359 ηmole glucose formed mg^{-1} protein min^{-1}) than CoM 0265 X CoM 0254 and CoM 0265 X CoC 671 (479 and 568 ηmole glucose formed mg^{-1} protein min^{-1}), respectively.

In the low sugar cross, in the top portion, cross Co 740 X CoC 671 recorded lowest SAI activity from 1893 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 824 ηmole glucose formed mg^{-1} protein min^{-1} at 390 DAP and from 2151 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 283 ηmole glucose formed mg^{-1} protein min^{-1} in another low sugar cross CoM 0265 X CoM 0254 at maturity. The level of SAI activity was more in another low sugar cross CoM265 X CoC 671 showed decreasing trend from 3447 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 1018 ηmole glucose formed mg^{-1} protein min^{-1} at 390 DAP. The low sugar cross Co740 X CoC 671 (1273 ηmole glucose formed mg^{-1} protein min^{-1}) recorded significantly lowest mean SAI activity than CoM 0265 X CoM 0254 and CoM 0265 X CoC 671 (1147 and 2205 ηmole glucose formed mg^{-1} protein min^{-1}), respectively .

When the bottom portions of the same crosses were analyzed for SAI activity, similar trend of decrease in SAI activity

was observed. In the bottom portion, low sugar cross Co740 X CoC 671 recorded lowest SAI activity from 750 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 294 ηmole glucose formed mg^{-1} protein min^{-1} at maturity than CoM 0265 X CoM 0254 (1423 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 673 ηmole glucose formed mg^{-1} protein min^{-1} at maturity) and CoM 0265 X CoC 671(1984 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 766 ηmole glucose formed mg^{-1} protein min^{-1} at maturity), respectively. The lowest mean of SAI activity was recorded in the bottom portion of low sugar cross Co740 X CoC 671 (508 ηmole glucose formed mg^{-1} protein min^{-1}) than CoM 0265 X CoM 0254 and CoM 0265 X CoC 671 (1048 and 1297 ηmole glucose formed mg^{-1} protein min^{-1}), respectively. The data of SAI activity showed significant differences in crosses involving different parents as well as differences during the developmental stages.

4.13 Soluble neutral invertase activity in progenies of crosses involving different parents

Soluble neutral invertase activity at different developmental stages of crosses involving different parents were evaluated from 240 to 390 DAP Tables 29 and 30. Among the both high sugar and low sugar crosses, the SNI activity increased gradually during different developmental stages from 240 to 390 DAP both in top and bottom portion of the cane.

In the high sugar cross, in the top portion, cross Co 740 X CoC 671 recorded highest SNI activity from 540 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 3729 ηmole glucose formed mg^{-1} protein min^{-1} at 390 DAP and from 300 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 2164 ηmole glucose

Table 29. Soluble neutral invertase (SNI) activity at different developmental stages in progenies of crosses having high sucrose content

DAP/ Variety	SNI activity (ηmole glucose formed mg^{-1} protein min^{-1})							
	Top				Bottom			
	CoM 0265 x CoC 671	CoM 0265 x CoM 0254	Co 740 X CoC 671	Mean	CoM 0265 x CoC 671	CoM 0265 x CoM 0254	Co 740 X CoC 671	Mean
240	225	300	540	313	182	173	322	268
270	563	612	980	729	325	645	630	522
300	850	1055	1150	956	446	869	968	823
330	983	1350	1540	1315	683	1423	1125	1053
360	1100	1700	2477	1859	900	2000	2250	1617
390	2130	2164	3729	2812	1459	2577	2708	2110
Mean	975	1197	1736	1331	666	1281	1334	1065

		SE+	CD at 5%		SE+	CD at 5%
	Variety	14.53	41.77	Variety	14.72	42.31
	DAP	20.55	59.07	DAP	20.81	59.83
	Variety x DAP	35.59	102.31	Variety x DAP	36.05	103.63

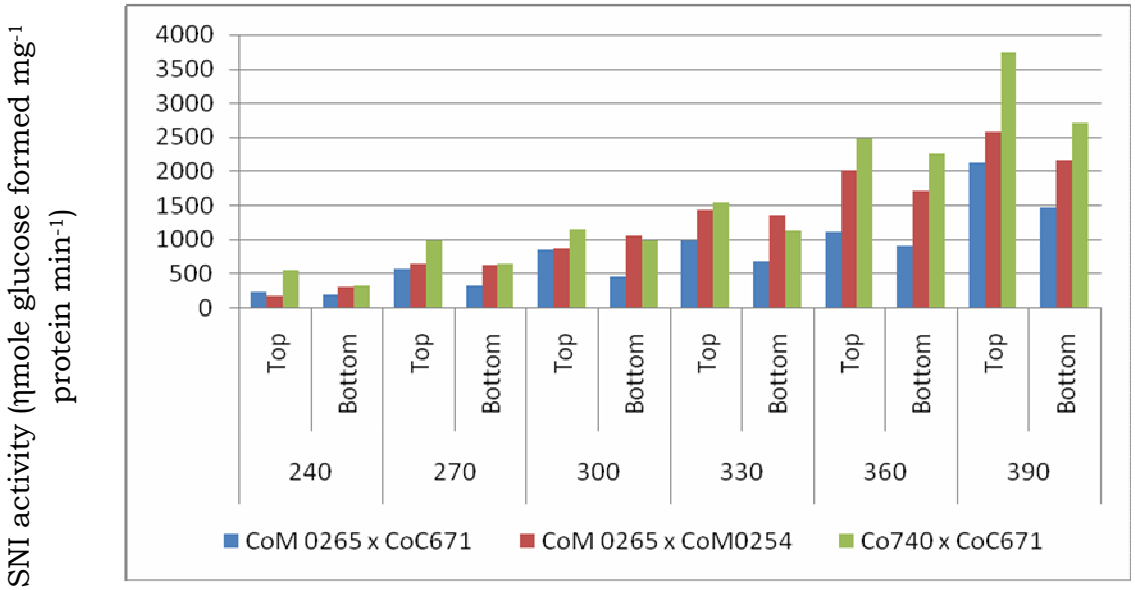

Fig. 18. SNI activity at different developmental stages in progenies of crosses having high sucrose content

Table 30. Soluble neutral invertase (SNI) activity at different developmental stages in progenies of crosses having low sucrose content

DAP/ Variety	SNI activity (ηmole glucose formed mg^{-1} protein min^{-1})							
	Top				Bottom			
	CoM 0265 x CoC 671	CoM 0265 x CoM 0254	Co 740 X CoC 671	Mean	CoM 0265 x CoC 671	CoM 0265 x CoM 0254	Co 740 X CoC 671	Mean
240	650	835	1069	851	272	469	572	438
270	933	1050	1175	1053	439	869	872	727
300	1100	1381	1485	1322	668	1148	1173	996
330	1233	1840	1875	1649	872	1543	1652	1355
360	1307	2120	2945	2124	1035	1914	2745	1898
390	2765	2835	3898	3166	1617	2184	3050	2284
Mean	1331	1677	2074	1694	817	1354	1677	1282

		SE±	CD at 5%		SE±	CD at 5%
	Variety	11.84	34.04	Variety	14.45	41.53
	DAP	16.75	48.15	DAP	20.43	58.73
	Variety x DAP	29.01	83.40	Variety x DAP	35.39	101.73

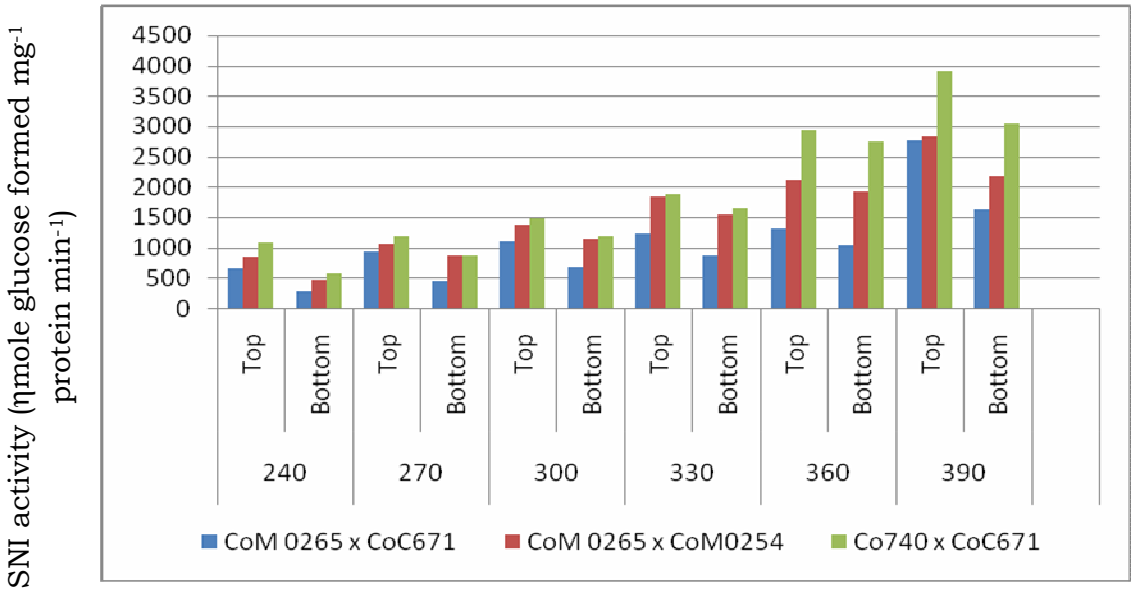

Fig. 19. SNI activity at different developmental stages in progenies of crosses having low sucrose content

formed mg^{-1} protein min^{-1} in another high sugar cross CoM 0265 X CoM 0254 to maturity. The another high sugar cross CoM 0265 X CoC 671 (225 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 2130 ηmole glucose formed mg^{-1} protein min^{-1} at maturity) showed lowest SNI activity as compared to other crosses but showed increasing trends during the development of canes. In the top portion of the high sugar crosses, cross Co 740 X CoC 671 (1736 ηmole glucose formed mg^{-1} protein min^{-1}) recorded significantly highest mean of SNI activity than CoM 0265 X CoM 0254 and CoM 0265 X CoC 671 (1281 and 975 ηmole glucose formed mg^{-1} protein min^{-1}), respectively.

When the bottom portions of the cane were analyzed for SNI activity, similar trend of increase in SNI activity was observed. The highest SNI activity was recorded in the bottom portion of high sugar cross Co 740 X CoC 671 from 322 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 2708 ηmole glucose formed mg^{-1} protein min^{-1} at 360 DAP than CoM 0265 X CoM 0254 (173 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 2577 ηmole glucose formed mg^{-1} protein min^{-1} at 360 DAP) and CoM 0265 X CoC 671(182 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 1459 ηmole glucose formed mg^{-1} protein min^{-1} at 360 DAP), respectively. The highest mean of SNI activity was recorded in the bottom portion of cross Co 740 X CoC 671 (1334 ηmole glucose formed mg^{-1} protein min^{-1}) than CoM 0265 X CoM 0254 and CoM 0265 X CoC 671 (1219 and 666 ηmole glucose formed mg^{-1} protein min^{-1}), respectively.

In the low sugar cross, in the top portion, cross Co 740 X CoC 671 recorded highest SNI activity from 1069 ηmole glucose

formed mg^{-1} protein min^{-1} at 240 DAP to 3898 ηmole glucose formed mg^{-1} protein min^{-1} at 390 DAP and from 835 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 2835 ηmole glucose formed mg^{-1} protein min^{-1} at maturity in another high sugar cross CoM 0265 X CoM 0254. The level of SNI activity was lower in another high sugar cross CoM 265 X CoC 671 from 650 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 2765 ηmole glucose formed mg^{-1} protein min^{-1} at 390 DAP as compared to other crosses and showed increase trend during the cane maturity. In the top portion of the low sugar crosses, cross Co 740 X CoC 671 (2080 ηmole glucose formed mg^{-1} protein min^{-1}) recorded significantly highest mean of SNI activity than CoM 0265 X CoM 0254 and CoM 0265 X CoC 671 (1677 and 1331 ηmole glucose formed mg^{-1} protein min^{-1}), respectively.

When the bottom portion of the same crosses was analyzed for SNI activity, similar trend of increase in SNI activity was observed. In the bottom portion, low sugar cross Co 740 X CoC 671 recorded highest SNI activity 572 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 3050 ηmole glucose formed mg^{-1} protein min^{-1} at maturity than CoM 0265 X CoM 0254 (469 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 2184 ηmole glucose formed mg^{-1} protein min^{-1} at maturity) and CoM 0265 X CoC 671(272 ηmole glucose formed mg^{-1} protein min^{-1} at 240 DAP to 1617 ηmole glucose formed mg^{-1} protein min^{-1} at maturity), respectively. The highest mean of SNI activity was recorded in the bottom portion of cross Co740 X CoC 671 (1656 ηmole glucose formed mg^{-1} protein min^{-1}) than CoM 0265 X CoM 0254 and CoM 0265 X CoC 671 (1376 and 815 ηmole glucose formed mg^{-1} protein min^{-1}), respectively. The data of SNI activity showed significant

differences in crosses involving different parents as well as differences during the developmental stages.

4.14 Validation of SSR markers for sugar content

Eleven SSR primers *viz.* NKS-31, NKS-9, STMS-34, NKS-5, NKS-45, NKS-46, USSM-359, USSM-458, UGSM-550, UGSM-632 and UGSM-644 previously reported to be linked with high sugar were used for validation in the high and low sugar sugarcane parents and their progenies of crosses developed (Plates 7 to 10).

The primer NKS-31 showed an amplified fragment of ~ 335 bp in high sugar sugarcane clone CoM 0254, Co 94012, CoM 0265 x CoM 0254 (HS) and CoM 0265 x CoC 671 (HS) and which was totally absent in low sugar sugarcane parents and clones.

The primer NKS-9 showed an amplified fragment of ~ 279 bp in high sugar sugarcane clone CoM 0254, Co 94012, CoM 0265 x CoM 0254 (HS) and which was totally absent in low sugar sugarcane parents and clones.

The primer STMS - 34 showed an amplified fragment of ~207 bp in high sugar sugarcane clones CoM 0254, Co 94012, CoM 0265 X CoM 0254 (HS) and CoM 0265 X CoC 671(HS) and which was totally absent in the low sugar sugarcane parent and clones.

Amongst the UGSM primers, primers UGSM 359, UGSM 458 generated an amplified fragments of ~ 617 and ~ 507 bp in high sugar sugarcane clones 138CoM 0254, Co 94012, CoM 0265 X CoM 0254 (HS) and CoM 0265 X CoC 671(HS) and which was absent in low sugar sugarcane clones Co 62175, CoM 0265 X CoM 0254 (LS) and CoM 0265 X CoC 671(LS). Among other UGSM primers, primers UGSM-550, UGSM 632 and UGSM 644 showed an amplified fragment of ~ 610, 631 and 824 bp in high sugar sugarcane parents and clones and which are not present in the low

sugar sugarcane parents and clones. The primer NKS- 5, NKS- 45 and NKS-46 previously reported to be linked with low sugar generated an amplified fragment of ~138, 329 and 221 bp only in low sugar sugarcane variety Co 62175 and progenies of crosses with low sugar CoM 0265 x CoM 0254 (LS) and CoM 0265 x CoC 671 (LS) which was absent in high sugar sugarcane parents clones.

From the analysis, it was observed that, the primers STMS-34, UGSM-359, UGSM-458, GUSM-550, UGSM-632 and UGSM-644 were specifically present in all high sugar sugarcane parents and clones alone and identified to be linked to high sucrose contain whereas, primers NKS-5, NKS-45 and NKS-46 were presented specifically in low sugar sugarcane parents and clones and identified to be linked to low sugar content.

4.15 Semi quantitative PCR expression of SPS, SS and SAI genes

The progenies of crosses involving high and low sucrose were utilized for gene expression study. Total RNA was extracted from leaves of progenies having high and low sucrose at 360 DAP. An equal amount of extracted total RNA was subjected to semi quantitative gene expression using gene specific primers by semi quantitative PCR analysis. The results of semi quantitative gene expression are depicted on Table 31 and Plates 11 to 14 showed that progeny of high sugar CoM 0265 x CoM 0254 shows higher expression of SPS and SS as compared to progeny of low sugar CoM 0265 x CoM 0254. However, progeny of low sugar CoM 0265 x CoM 0254 show higher expression of SAI gene as compared to progeny of high sugar CoM 0265 x CoM 0254 as evident from semi quantitative gene expression analysis.

Table 31. Amplification band intensity of SPS, SS and SAI genes

Name of Sample	SPS gene				SS gene				SAI gene			
	1	2	3	**Mean**	1	2	3	**Mean**	1	2	3	**Mean**
CoM0265 X CoM0254 (HS)	3478	3521	3354	3451	2728	2611	2732	2690	2078	2079	2071	2076
CoM0265 X CoM0254 (LS)	1778	1763	1760	1767	2383	2430	2473	2429	3041	3087	3180	3103

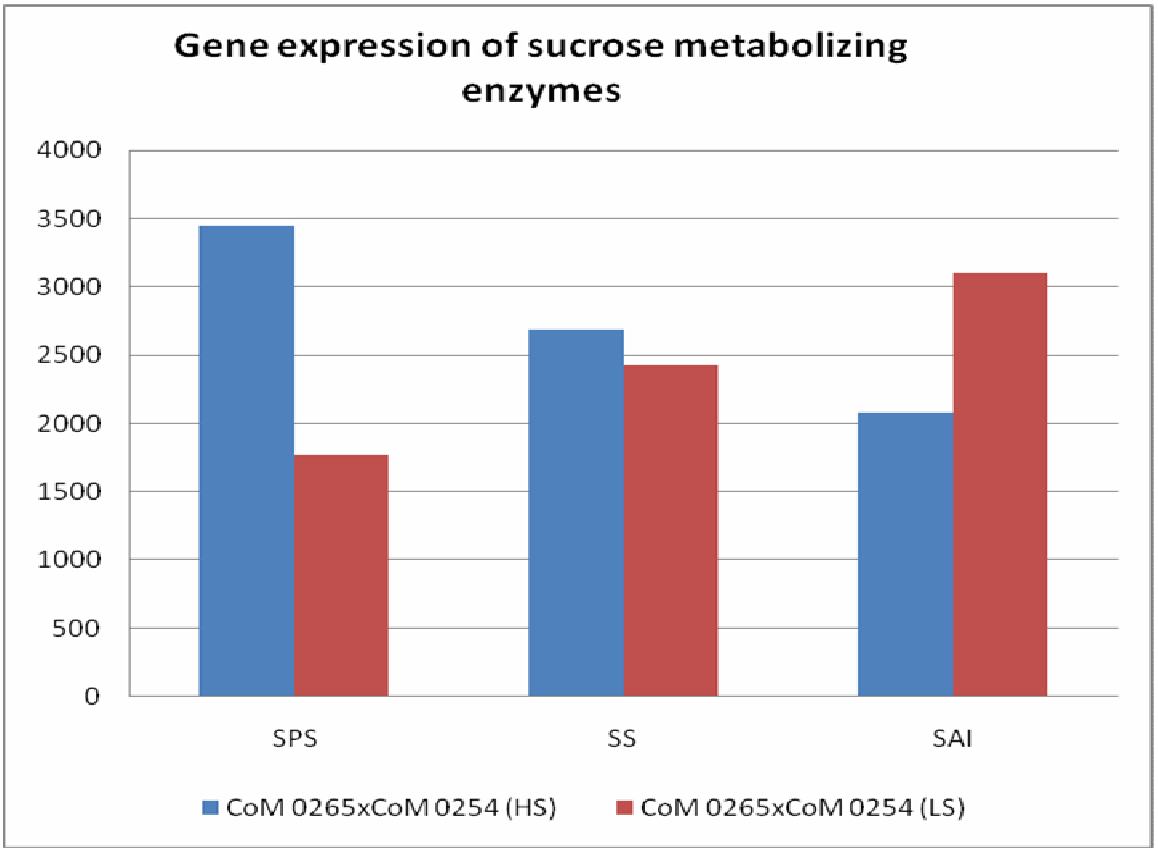

Fig. 20. Gene expression for sample CoM 0265 x CoM 0254 (HS) and CoM 0265 x CoM 0254 (LS) represented in the form of histogram

5. SUMMARY AND CONCLUSION

The results obtained during the present investigation entitled, “Investigations on the Interplay of SPS, SuSy and Invertase(s) in relation to Sucrose Accumulation in Sugarcane” is an attempt to study the developmental activity profiles of sucrose metabolizing enzyme in high and low sucrose sugarcane varieties and progenies of the crosses with high and low sucrose for validity, the correlation between activity profile, expression with sucrose accumulation. The validation of the earlier reported SSR markers associated with high sugar was also attempted. The results so obtained with these major objectives were discussed and summarized in this section highlighting the major outcome.

- The high sugar variety CoM 0254 recorded significantly higher sucrose content with minimum hexose pool and recorded the lowest invert ratio at maturity.
- The SPS activity was significantly higher even at the early stage of cane development in the high sugar varieties and also in progenies of the crosses having high sucrose. The activity profile of these enzymes was almost two fold higher in the early stage of the development in both high sugar varieties. The higher SPS activity both in top and bottom portion was correlated with higher sucrose and CCS %.
- The SuSy activity was significantly higher in the top i.e. maturing portion of the cane than in bottom portion and was higher in the low sugar varieties at all the developmental stages. The progeny of the cross Co 740 x CoC 671 with high sucrose content recorded lowest SuSy activity.

- The activity profile of soluble acid invertase revealed significantly higher SAI activity in low sugar varieties both in top and bottom portion with almost three fold difference in the mean SAI activity over developmental stages. The high sugar progeny of a cross Co 740 x CoC 671 also recoded lowest SAI activity both in the top and bottom portion.
- The neutral invertase activity was significantly low in low sugar variety both in top and bottom portion. However, significant differences in NI activity between high and low sugar varieties and the progenies were evident in the bottom portion of the cane at maturity. The CWI activity was higher in high sugar varieties and the progenies of the cross having high sucrose content.
- ISSR analysis was more informative and revealed higher similarity index between Co 94012 and CoM 0254 two high sugar varieties. The ISSR primer UBC–820 showed a promising amplicon of 740 bp only in moderate and low sugar varieties, on the contrary ISSR primer UBC-807 exhibited specific amplicon of ~ 990 and ~ 960 bp in moderate and low sugar varieties.
- The SSR primer NKS–31 showed an amplicon of ~335 bp when resolved on PAGE only in high sugar varieties and high sugar progenies. Similarly the primer NKS-9 was also discriminative with a prominent band of ~ 279 bp. STMS-34 which is already reported for tagging high sugar genes in sugarcane also revealed a specific amplicon of ~ 207 bp only in high sugar varieties and high sugar progenies.

- The expression profile of genes encoding SPS, SuSy and SAI at maturity stage revealed differences with comparatively higher expression of SPS in high sugar progeny as evident from the band intensity which was 3451 in high sugar progeny as against 1767 in low sugar progeny. The expression profile of SAI on the contrary showed higher expression in the low sugar progeny as evident from the band intensity which was 3103 in low sugar progeny as against 2076 in high sugar progeny.
- The expression profile of SuSy which operates both in sucrose synthesis and breakdown did not show differences in the expression in both high and low sugar progenies.

Conclusions

Based on the results obtained in the present investigation, it can be concluded that the activity profile and expression profile of SPS and SAI could be used as a tool in ascertaining the sucrose content and in the progenies for high sugar. These two enzymes can serve as enzyme indexing markers. The sucrose synthase activity and expression profile however did not show any remarkable differences in discriminating the varieties for sucrose content. The significant differences in the activity profile of SPS and SAI at early stage of cane development and the difference in SPS and SAI could be exploited. The identified ISSR and SSR markers which are validated can be used in molecular breeding efforts in sugarcane. The correlation between activity profile and expression probably suggest major contribution of *de-novo* synthesis rather than fine tuning of enzyme activity due to post-translational modification.

6. LITERATURE CITED

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