

**REMOBILIZATION OF SUCROSE FROM THE CULM DURING  
GERMINATION OF SUGARCANE SETTS**

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## Declaration

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

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## ABSTRACT

The main substrate use during shoot establishment from the lateral bud of sugarcane setts and enzymes involved in sucrose metabolism were investigated in the shoots and the internodes acting as source of carbohydrates. Radiolabelling studies were conducted to investigate the metabolism of sucrose and glucose during shoot establishment. The internode's total dry mass over the 21-day of shoot establishment period was reduced by 25% and 30% in plants incubated in dark/light and total darkness, respectively. Sucrose accounted for 90% of the remobilized internodes' dry mass. From the remobilized sucrose 35% and 50% were lost through respiration in plant incubated in dark/light and total darkness, respectively. All enzymes involved in sucrose cleavage/hydrolysis were induced in both the internodal tissues and the shoots. Northern analysis demonstrated that SAI transcripts were only detected in the internodal tissues after 14 days of shooting period when the sucrose content was reduced by one third compared to the day of planting. In contrast, CWI transcripts were observed throughout the shooting period in both tissue types. The highest CWI transcript levels were found after seven days in shoots and correlated with high hexose concentrations. The data suggests that both, SAI and CWI are expressed at transcript and protein level during shoot establishment. In both the internodes and the shoots, a cycle of breakdown and synthesis of sucrose was evident; despite the short labelling periods of one and three hours. From the data, it is evident that sucrose is the main form in which reduced carbon is translocated from the internodes (source) to the shoots (sink). Furthermore the data suggest that sucrose hydrolysis in the storage tissues is not required for export to the shoots.

## OPSOMMING

Die substraat wat as koolhidraatbron dien vir stingel-ontwikkeling vanuit die laterale knope van suikerriet litte en die ensieme betrokke in suikermetabolisme, is in beide die jong stingels en litte ondersoek. Radioisotoopstudies is gebruik om die metabolisme van sukrose en glukose tydens die ontwikkeling van die stingel te bestudeer. Die totale droëmassa van die litte het met 25 en 30% verminder, in plante wat onderskeidelik in 'n donker/lich siklus en totale donkerte tydens die een-en-twintig-dae ontwikkelingsperiode geïnkubeer is. Sukrose het 90% van die gemobiliseerde droëmassa van die internode verteenwoordig. Vyf en dertig en 50% van die gemobiliseerde sukrose het deur respirasie verlore gegaan in plante wat onderskeidelik in 'n donker/lich siklus en totale donkerte geïnkubeer is. Al die ensieme wat direk by sukrose splyting/hidrolise betrokke is, is in beide die litte en stingels geïnduseer. RNA-kladanalises het getoon dat oplosbare suurinvertase (OSI) transkripte slegs waarneembaar was in die litte na 14 dae se ontwikkeling. Tydens hierdie periode, het die sukrose inhoud met 33% verminder. In teenstelling was selwand invertase (SWI) transkripte deurgaans tydens die ontwikkelingsperiode in beide weefsels teenwoordig. Die hoogste vlakke van die SWI-transkrip is na sewe dae in spruite waargeneem en kon met hoë heksosevlakke gekorrigeer word. Die resultate dui daarop dat beide OSI en SWI geïnduseer word op transkript- en proteïenvlak tydens stingel ontwikkeling. 'n Siklus van sucrose-afbraak en -sintese is in beide die litte en stingels waargeneem, ten spyte van die kort merkingsperiodes van een en drie ure. Gebaseer op hierdie data, is dit duidelik dat gereduseerde koolstof hoofsaaklik in die vorm van sukrose vanaf die litte (bron) na die stingel (swelgpunt) vervoer word. Die resultate dui ook daarop dat sukrosehidrolise in die bergingsweefsel nie 'n voorvereiste is vir vervoer na die stingel is nie.

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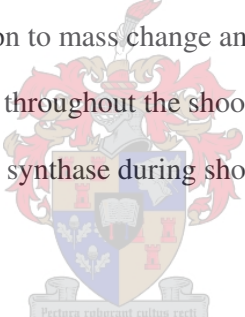
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## LIST OF ABBREVIATIONS

°C	degrees centigrade
AI	acid invertase
ATP	adenosine 5'-triphosphate
Bq	Bequerel
Bp	base pair
BSA	bovine serum albumin
cDNA	complementary deoxyribo nucleic acid
<sup>14</sup> C	radio-labelled carbon
CO <sub>2</sub>	carbon dioxide
CWI	cell wall invertase
dH <sub>2</sub> O	distilled water
DEPC	diethyl pyrocarbonate
DNA	deoxyribo nucleic acid
DTT	1,4-dithiothreitol
DW	dry weight
e.g.	for example
EDTA	ethylenediaminetetraacetic acid
Edn	edition
F6P	fructose-6-phosphate
FW	fresh weight
g	gram
xG	gravitational force
G6PDH	glucose-6-phosphate dehydrogenase (EC 1.1.1.49)
h	hour
H <sub>2</sub> O	water
HEPES	N-2-hydroxyethylperazine-N'-2-ethanesulfonic acid
HK	hexokinase (ATP: D-hexose-6-phosphotransferase, EC 2.7.1.1)
J	Joule
K <sub>m</sub>	substrate concentration producing half maximal velocity
L	litre
m	meter
M	molar



min.	minute
NAD <sup>+</sup>	oxidised nicotinamine adenine dinucleotide
NADP	reduced nicotinamide-adenine phosphate dinucleotide
NI	neutral invertase ( $\beta$ -fructofuranosidase, EC 3.2.1.26)
PCR	polymerase chain reaction
PGI	phosphoglucosomerase (D-glucose-6-phosphate ketol-isomerase, EC 5.3.1.9)
Pi	inorganic phosphate
PVPP	polyvinylpyrrolidone
RNA	ribonucleic acid
RNaseA	ribonuclease A
rpm	revolutions per minute
SAI	soluble acid invertase ( $\beta$ -fructofuranosidase, EC 3.2.1.26)
SDS	sodium dodecyl, sulphate
SPS	sucrose phosphate synthase (UDP-glucose: D-fructose-6-P 2- $\alpha$ -D-glucotransferase, EC 2.4.1.14)
SSC	saline sodium citrate
SuSy	sucrose synthase (UDP-glucose: D-fructose 2- $\alpha$ -D-glucosyl-transferase, EC 2.4.1.13)
TBE	tris-borate/EDTA electrophoresis buffer
TLC	thin layer chromatography
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
UDP	uridine 5'-diphosphate
UDPGlc	uridine 5'-diphosphate glucose
UV	ultra violet
v	volume
V	volt
w	weight

## CHAPTER 1

### General introduction

Sugarcane (*Saccharum* spp.) is a C<sub>4</sub> grass grown predominantly in tropical and subtropical regions for its ability to accumulate significant quantities of sucrose in the culm tissues (Moore and Maretzki 1997). Factors that contribute to the high yield of sugarcane are its perennial growth habit and the continuous accumulation of sucrose in the vegetative plant structure. As with other crops, the modification of carbon partitioning by conventional breeding has made a major contribution to increasing the yield over the last century. Moore *et al.* (1997) attribute these increases in sugarcane yield and sucrose content to the overcoming of productivity barriers in both the source and sink.

Improving commercial sugarcane varieties through genetic manipulation is dependent on an integrated understanding of plant metabolism at the physiological, biochemical and genetic level (Moore *et al.* 1997). Furthermore pathogen resistance, environmental tolerance and sucrose yield are the focus of many industrial-supported research groups worldwide. For many years, increases in the yield have been accomplished through traditional breeding and increased planting density. It would appear as if the genetic potential of sugarcane has been largely exploited in the conventional crossing programmes and therefore there is evidently a plateau in important traits such as sucrose yield and biotic and abiotic resistance. Therefore there is international interest in using modern technologies such as genetic manipulation to increase sucrose content in sugarcane.

One of the most popular targets in attempts to increase the sucrose content is to reduce the ability of sugarcane to hydrolyse sucrose through soluble and membrane bound acid invertases. Although a reduction in sucrose hydrolysis might eventually increase the sucrose load, it is not evident whether such an alteration could affect other important characteristics. For example one of the main reasons for the success of sugarcane as a sucrose producing crop is the fact that it is vegetatively propagated and that several ratoon crops can be obtained before replanting is needed. Currently there is no information available on how important sucrose mobilization from the storage parenchyma cells is in the germinating (sprouting) of the lateral bud and in shoot establishment.



The aim of this study was to investigate the mechanisms associated with shoots establishment from the lateral bud of sugarcane setts, also referred to as germination. This was to gain insight into the expression of enzymes involved in sucrose breakdown/hydrolysis since they are involved in sucrose utilization. In addition, invertase activities have been shown to correlate negatively with sucrose content (Zhu *et al.* 1997; Ebrahim *et al.* 1998) and can therefore be used in genetic manipulation.

The first specific goal of this study was to confirm to what extent sucrose is remobilized from the culm tissues by investigating changes in sugar contents and the activity of sucrose metabolizing enzymes during shoot establishment. Details of this investigation are given in chapter 3. The second goal of this study was to investigate the expression of soluble and cell wall bound acid invertase at transcriptional level, and correlate that expression with both extractable invertase activities and sugar contents. This investigation is detailed and discussed in chapter 4. The third goal was to investigate the sugar metabolism and elucidate the mechanism by which sugars are transported during shoot establishment. This last part was investigated and is discussed in chapter 5.



## CHAPTER 2

### Literature review

#### 2.1 Introduction

Sink organs of most plant species are supplied with reduced carbon in the form of sucrose (Avigad 1982; Hawker 1985). The mobilization of sucrose into sink metabolism requires its hydrolysis by several isoforms of invertase and cleavage by sucrose synthase, which are localized in different sub-cellular compartments. These two types of reaction will deliver hexoses that must be phosphorylated. They are the common entry point to metabolism, or UDP-glucose common entry to many reactions (Turner and Botha 2002; Stewart and Copeland 1998). The breakdown of sucrose has been implicated in the control of many processes such as sink strength, long distance transport, defence mechanisms etc. In addition, because sugars also regulate gene expression, the enzymes involved in sucrose breakdown could also play a fundamental role in controlling cell differentiation and development.

This chapter is primarily aimed at introducing the sucrose cleaving enzymes, invertase (EC 3.2.1.26) and sucrose synthase (UDP-glucose: D-fructose 2  $\alpha$ -D-glucosyltransferase, EC 2.4.1.13) that have been implicated in the control of sucrose accumulation and utilisation in sugarcane. The review presented therefore outlines aspects of sugarcane shoot establishment and importance, the source-sink transition and sugar transport processes. An integration of this knowledge with the gene expression patterns of invertase and sucrose synthase can be used to develop a model for their potential function during sugarcane shoot establishment.

#### 2.2 The importance of shoot establishment

The process during which roots are formed from stem pieces of sugarcane containing lateral bud of the seed-piece, the sprouting and growth of the young plantlets and forming of roots at the base of the young shoot is referred to as shooting or shoot establishment in this study. In many studies, that process is referred to as germination, although botanically

speaking this is not correct it has become the standard terminology for all involved in sugarcane agricultural practice and research.

Sugarcane is propagated commercially by the vegetative method, which involves the planting of sections of the stem of immature cane, this material being known as seed, seed cane, seed-pieces, and setts. The sequence of events in the development of a germinating sett will demonstrate how cane is propagated and the circumstances under which ratoon crops are produced. Considering a single-eye sett, the seed piece consists of a portion of young cane with internode on each side of the node or joint with its undamaged bud and embryo roots (Barness 1964).

When the cane is reaped there is still a portion of the stem or stalk left underground, and it is this which gives rise to the succeeding growth of cane known as ratoons. The underground portion consists of closely spaced joints, each with its complement of bud and root points, and the growth process of ratoons follows that of the original plants, except that its primary root system is already present, though this rapidly ceases to function as the new growth progresses. This enables the farmers to get three or four crops from each original plant before they have to replant (Barness 1964).

Shoot establishment is economically important in sugarcane since it is the crucial period that gives rise to the whole stalk. Apart from the shooting process, the ratooning system is also valuable since it enables the farmers to get three or four crops before they have to replant. The beneficial aspect of shoot establishment from planted setts and ratoons lies in the ability of the stored sucrose remobilization and utilization. It is now clear that genes from virtually any source can be modified for expression in plants. This makes genetic transformation a powerful accessory for sugarcane improvement, allowing specific alteration of existing genes to modify expression patterns or product, e.g. altered enzyme properties for enhanced sucrose synthesis or altered expression patterns for reduced remobilization out of the storage tissues (Birch 1997). In sugarcane, it is important to introduce genes into elite cultivars while avoiding any undesired changes to cultivar performance as a result of the gene transfer process, mainly for shoot establishment and ratoons.

## 2.3 The sink - source transition

Initially, all developing plant organs are sinks dependent on imported photosynthate to sustain their respiration and growth. In sugarcane, the major sites requiring import of photoassimilates to support respiration and growth are the roots, the shoot apical region, and the developing leaves and shoot internodes. The shoot apical region contains both the shoot apical meristems and the intercalary meristems of the stem internodes, which give rise to the major part of the plant body. In the absence of flowering, sugarcane growth is indeterminate so that the apical meristems of the shoot and root remain permanent growth sinks. However, the developing leaves and stem internodes are growth sinks for the finite period during which the organ reaches full size. As the leaves commence the photosynthesis, the export begins, and the import of photoassimilates diminishes (Moore and Maretzki 1997). As full elongation of the stem internode is reached, the imported photoassimilates remain in storage instead of being metabolized for growth.

### 2.3.1 *Developing leaves*

Both the cessation of import and the onset of export have been used to define the leaf sink-to-source transition. In gramineae, this transition progresses basipetally from the apex to the base of a developing leaf. Robinson-Beers *et al.* (1990) studied the import of photoassimilates into sugarcane leaves and concluded that each of the sink leaves imports reduced carbon from every one of the source leaves. The sink leaves included all leaves fully ensheathed by older leaves and the basal portion of the two or three leaves with their blades mostly emerged but their sheaths fully ensheathed (see Moore and Maretzki 1997). On the basis of morphometric studies of ensheathed leaves (Moore 1974), the number of leaves partially to fully functioning as sinks will be approximately 10. It is possible for a single leaf to be a source for so many sink leaves because grass node possesses an anastomosing network of vascular tissue, known as nodal plexus, derived from vascular strands from the leaves above and below a given node to connect all leaves in the vascular network efficiently (Hitch and Sharman 1971).

The strongest importing sink leaf was the most rapidly expanding, fully ensheathed leaf lacking chlorophyll (Robinson-beers *et al.* 1990). Older developing leaves continued to import until they reached about 90% of full blade length, a relatively late stage of

development to switch from importer to exporter. This late transition is consistent with the fact that much of the leaf growth occurs within older, ensheathing leaves, where lack of sunlight and CO<sub>2</sub> assures the dependency on imports. It has been suggested for other plants that the cessation of import may be related to increasing resistance to symplastic unloading (Schmalstig and Geiger 1987) and that this resistance results from a decrease in plasmodesmatal frequency and number (Ding *et al.* 1988). Data to test these hypotheses for sugarcane are lacking.

### 2.3.2 *Developing internodes*

The transition of internodes from importing photosynthate to support growth to importation for storage has been inferred indirectly by measuring internode relative length, enzyme activity and sucrose content. More exact determination based on rates of import, respiration, and storage has thus far not been reported. Therefore, the developmental or metabolic events which regulate this switch from growth to storage are not known. Several reasons for our lack of understanding include cell trauma and death in using tissue slices for in vitro assays, limited transport through tissue slices, and our inability to assimilate a morphological stage of internode development with an endogenous level of stored sucrose precisely and repeatedly (Moore and Maretzki 1997). To overcome these problems, workers have used cell suspension cultures as a model system to study sugar uptake into sugarcane cells (Maretzki and Thom 1972; Komor *et al.* 1981; Wendler *et al.* 1990; Goldner *et al.* 1991). While the results of these studies may not strictly represent what occurs *in planta*, they are compatible with results of studies on internode metabolism and give us clues about how partitioning may be regulated.

## 2.4 **Sugar transport in sugarcane**

Photoassimilate transport, partitioning and accumulation form the basis for the productivity and success of any plant, either as a competitor in natural communities or as a cultivated crop (Moore 1995). In plant cells sucrose is the major form in which assimilated carbon is transported to sink organs (Kuhn *et al.* 1999; Farrar *et al.* 2000).

Sucrose accumulation begins with the translocation of sucrose through the phloem sieve elements to the stem internodes (Moore 1995). The bulk of sucrose moves toward the plant

base and roots; while smaller amounts move upward toward the apical meristem and immature sink leaves (Hartt *et al.* 1963; Hatch and Glasziou 1964). In sugarcane, sucrose arrives intact at the sink and is then broken down and re-synthesized during movement into the storage cells (Hatch and Glasziou 1964; Moore 1995; Moore and Maretzki 1997). Sucrose is either metabolized in the cytosol to provide carbon skeletons and energy, or can be accumulated in the storage parenchyma cells and the apoplastic space surrounding them.

Anatomically it was shown, however, that the sclerenchyma bundle sheaths and most of the storage parenchyma cells of the stem become lignified and suberised prior to the bulk of internodal sucrose storage (Jacobsen *et al.* 1992). These modified cell walls prevent apoplastic solute flow and are therefore barriers to the radial transfer of sucrose (Moore 1995). Two well-separated apoplastic spaces, one in the bundle, the other in the stem parenchyma (Jacobsen *et al.* 1992; Welbaum *et al.* 1992), have led to the hypothesis that phloem unloading had to proceed symplastically and that unloading sugars first appear in the cytosol of the storage parenchyma.

Komor (2000) proposed a model for sugar transport in internode parenchyma that takes into account both apoplastic and symplastic phloem unloading. The model suggests that partial apoplastic transport may occur in immature, growing internodes since the cells only become lignified and suberised after cell expansion has ended. Symplastic transport would occur in the mature internodes. The driving force for symplastic transfer may be diffusion along the concentration gradient and/or bulk flow by hydrostatic pressure (Moore 1995; Komor 2000).

## **2.5 Sugar uptake in sugarcane**

The data from sugar uptake have been obtained from tissue slices of immature and mature internodes using radiolabelled sugars (Sacher *et al.* 1963; Hatch and Glasziou 1964; Lingle 1989; Thom and Maretzki 1992). Two membranes are involved in sugar uptake in sugarcane, namely the plasmalemma of storage parenchyma cells and the tonoplast of storage cells.

### 2.5.1 Sugar uptake through the plasmalemma

The storage parenchyma cells can take up hexoses (glucose and fructose) and sucrose at different rates and with different kinetic properties (Komor 2000). Work done with suspension cells (developmentally more comparable to fast growing meristematic cells) revealed a hexose uptake by an active, proton-symport system (Maretzki and Thom 1972; Komor *et al.* 1981). In this system sucrose is only taken up after hydrolysis by cell wall invertase. Reinvestigation of sugar uptake experiments with tissue slices (Lingle 1989) confirmed that cleavage of sucrose occurs in the apoplast. However, in contrast to the earlier experiments (Sacher *et al.* 1963; Hatch and Glasziou 1964); most of the sucrose accumulated without randomization of the supplied (<sup>14</sup>C-fructosyl)-labelled sucrose.

Apparently, conflicting results are presented with regard to sugar uptake in sugarcane. When the storage tissues were fed with asymmetrically labelled sucrose via phloem, label randomization was small. In contrast, feeding sucrose over the apoplast of the storage cells, a high or even complete randomization was observed (Lingle 1989; Thom and Maretzki 1992). The best understanding of sugar transport was observed when sucrose was fed under conditions, where extracellular hydrolysis was low, or using non hydrolysable fluoro-sucrose. This shown a sucrose uptake with low label randomization; or none in the case of fluoro-sucrose (Lingle, 1989; Thom and Maretzki, 1992). The uptake of hexose after sucrose hydrolysis is low when sucrose enters the storage tissues via the symplast, and high when sucrose enters from the apoplast (see Moore 1995).

### 2.5.2 Sugar uptake through the tonoplast

The movement of sucrose out and into the vacuole is controlled by the tonoplast. The integrity of sucrose in the vacuole is controlled by soluble acid invertase and in the cytosol by neutral invertase and SuSy. Even in the absence of any active transport and assuming diffusion across the tonoplast most of the sucrose in a storage parenchyma cell has to reside in the vacuole simply because the vacuole comprises 90% of the cell volume (Boller and Wiemken 1986).

The membrane transport studies on sugarcane vacuoles and vesicles have yielded conflicting conclusions due in part on the purity and integrity of the tonoplast preparations

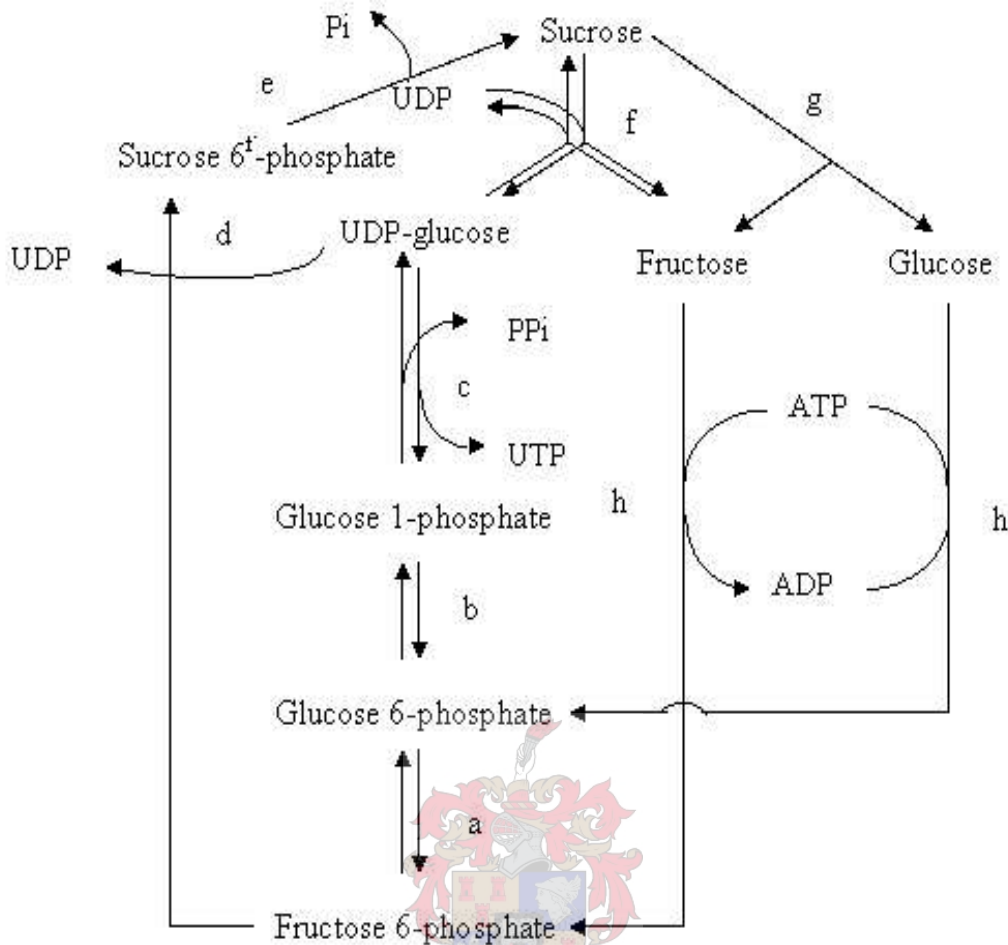
(Thom and Komor 1985; Thom *et al.* 1983; Preisser and Komor 1991). Evidence consistently supports the presence of a tonoplast-bound ATPase capable of maintaining the proton motive potential needed for sugar antiport systems (Thom and Komor 1985; Thom *et al.* 1982a). In most recent evidence using a specific carrier or channel protein, the results indicate that sucrose transport across the tonoplast is by facilitated diffusion (Preisser and Komor 1991).

Because during sucrose accumulation, intact cells develop a hydrostatic pressure against which the transport must work, as opposed to isolated vacuoles which change in volume and thus do not generate an energy gradient in opposition to sucrose transport (Moore 1995). It would appear that energy-based transport would be required for sucrose accumulation in the vacuole of intact cells. If facilitated diffusion is shown to be the general transport mechanism for sucrose accumulation in sugarcane, the fast, non-energy requiring equilibration of sucrose between the cytosol and the vacuole would mean that the vacuole storage of sucrose might not be regulated at the tonoplast but rather by the pool of sucrose in the cytosol (Preisser *et al.* 1992) which, in suspension culture cells at least, is controlled by a cycle of synthesis and degradation (Wendler *et al.* 1990; Komor 1994).

## 2.6 Sucrose metabolism

Sucrose has three fundamental and interrelated roles in plants. Firstly, it is a principal product of photosynthesis and can account for most of the CO<sub>2</sub> absorbed by a plant during photosynthesis (Kruger 1997). Secondly, sucrose is the major form in which carbon is translocated in plants (Hartt *et al.* 1963; Hawker 1985; Komor 2000). The importance of sucrose in translocation is not simply a reflection of its dominant role as a product of photosynthesis since it is also the principal form in which carbon reserves are exported from non photosynthetic tissues. Thirdly, many plants contain sucrose as a storage compound. However, starch is usually the main storage molecule and sucrose occurs at low concentrations relative to starch. In contrast, sugarcane and sugarbeet accumulate sucrose to high concentrations and contain virtually no starch (Komor 2000).





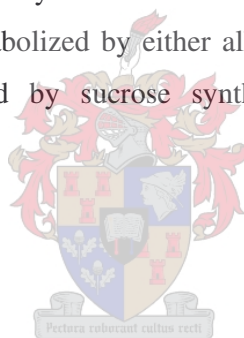
**Fig 2.1** Pathway of sucrose metabolism. The letters denote the following enzymes: a. glucose 6-phosphate isomerase, b. phosphoglucomutase, c. UDP-glucose pyrophosphorylase, d. sucrose phosphate synthase, e. sucrose phosphatase, f. sucrose synthase, g. invertase and h. hexose kinase.

Although most apparent in photosynthetic and gluconeogenesis tissues, the ability to synthesize sucrose is a widespread, possibly universal, characteristic of higher plants. Sucrose is derived from hexose phosphates through the combined activities of UDP-glucose pyrophosphorylase (EC 2.7.7.9;  $\Delta G' = -2.88 \text{ kJ mol}^{-1}$ ), sucrose phosphate synthase (EC 2.4.1.14;  $\Delta G' = -5.70 \text{ kJ mol}^{-1}$ ) and sucrose phosphatase (EC 3.1.3.24;  $\Delta G' = -16.5 \text{ kJ mol}^{-1}$ ) ( Kruger 1997; Fig. 2.1). The evidence that sucrose is synthesized via this pathway, and not through an alternative route involving sucrose synthase, comes from four sources. First the large overall free energy change of the reaction catalyzed by sucrose phosphatase ensures that production of sucrose is irreversible and allows synthesis to continue in tissues

that already contain large amounts of sucrose. Second, the kinetics of labelling pathway intermediates in tissue fed  $^{14}\text{CO}_2$  or  $^{14}\text{C}$ -glucose are consistent with the finding.

Plants contain two types of enzymes capable of cleaving sucrose. One is sucrose synthase (EC 2.4.1.13;  $\Delta G' = -3.99\text{kJ mol}^{-1}$ ) which catalyzed the readily reversible reaction (Fig.2.1). The other type of enzyme is invertase (EC 3.2.26;  $\Delta G' = -29.3\text{kJ mol}^{-1}$ ) which catalyzes the essentially irreversible hydrolysis of sucrose to glucose and fructose (Fig. 2.1). The contribution of these enzymes to the pathway of sucrose breakdown may, in part, be determined by the source of sucrose. Sucrose obtained through translocation can enter a cell via the symplast or the apoplast. Several studies using asymmetrically labelled sucrose have demonstrated that sugar obtained in this way moves primarily through the symplast and is not cleaved into glucose and fructose during transport. The second major source of sucrose for metabolism is that stored in the vacuole. At present there is not enough information to exclude the possibility that sucrose itself moves out of the vacuole. Sucrose entering the cytosol can be metabolized by either alkaline invertase or sucrose synthase, although the reaction catalyzed by sucrose synthase is readily reversible in vivo (Geigenberger and Stitt 1993).

## 2.7 Sucrose synthase (SuSy)



SuSy (UDP-glucose: D-fructose 2  $\alpha$ -D-glucosyltransferase, EC 2.4.1.13) catalyses a reversible reaction in vivo (Geigenberger and Stitt 1993); however the enzyme is thought to function primarily in the direction of sucrose cleavage in plant sink tissues supplied with ample sucrose substrate and with high demand for carbon biosynthetic and respiratory pathways. SuSy has been detected in all tissues of sugarcane (Buczynski *et al.* 1993; Schäfer *et al.* 2004), and performs very well at pH ranging from 7 to 7.5.

Two isoforms of sucrose synthase have been described in plants, they are encoded by two non-allelic genes (e.g. Chourey 1981; Wang *et al.* 1992). In maize, the form designated as sucrose synthase 1 (SuSy-1) has been localized primarily in endosperm, where it is believed to play a major role in providing sugar nucleotides for starch biosynthesis as well as in endosperm cell stabilization (Chourey *et al.* 1981; Chourey *et al.* 1986). The second isoform, SuSy-2, is also found in the endosperm and other regions of the embryo (Heinlein and Starlinger 1989), as well as in other tissues throughout the plant, including roots and

young leaves (Chourey *et al.* 1986). Susy-2 may provide substrate for respiration, as well as for cell-wall synthesis (Chourey *et al.* 1986). However, the two SuSy genes within the same tissue have differential responses to changing carbohydrate status, flow, and requirement (Koch *et al.* 1992). In sugarcane as in most plants, the two isoforms have been detected. SuSy-1 protein was present in all tissue and increases with internode maturity, however, SuSy-2 protein was more abundant in the immature tissue and it was not detectable in the fully mature internode and mature green leaves (Buczynski *et al.* 1993). Northern analysis of a full length cDNA corresponding to the Susy-1 isoform in sugarcane leaf and internodal tissues of varying maturity as well as roots and developing shoot detected transcripts in all tissues examined, although lower levels were evident in immature leaves (Lingle and Dyer 2001).

## 2.8 Invertase

Most plant species contain at least two isoforms of vacuolar invertase, which accumulate as soluble proteins (soluble acid invertases) in the lumen of this acidic compartment. Likewise, several isoforms of extracellular invertase (cell wall invertases) that are ionically bound to the cell wall have been detected. 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. Thus, these so-called acid invertases are  $\beta$ -fructofuranosidases and also hydrolyze other  $\beta$ -fructose-containing oligosaccharides such as raffinose and stachiose. Additionally, plants have at least two isoforms of cytoplasmic invertase with pH optima for sucrose cleavage in neutral or slightly alkaline range. Neutral and alkaline invertases are less well characterized but, in contrast to the acid invertases, these enzymes appear to be sucrose specific.

### 2.8.1 Acid Invertases

Soluble acid invertase (SAI,  $\beta$ -D-fructofuranosidase, E.C. 3.2.1.26) is present in most plant species in at least two isoforms, and localized in the vacuole (Avigad 1982). SAI activity is high in immature, actively growing stem tissue and declines with increasing maturity of the sugarcane stem (Hatch and Glasziou 1963; Moore 1995). This developmental variation in SAI activity presumably reflects both the demand for monosaccharides for carbohydrate deposition during cell elongation, and the greater requirement for metabolic substrates in

developing cells. Evidently, such SAI activity is important in the regulation of sucrose accumulation. SAI is also thought to mediate remobilization of sucrose from storage for growth or to maintain the cellular process during periods of stress.

It is reported that there is a negative relationship between sucrose concentration and SAI activity levels (Zhu *et al.* 1997; Ebrahim *et al.* 1998). High sucrose accumulation, is however not a direct result of low SAI levels. Additional factors play a role in the eventual accumulation of sucrose in the sugarcane culm (Zhu *et al.* 1997).

The cell wall isoform of acid invertase (CWI) is very poorly characterized in sugarcane and other species as it has been the subject of relatively little study as compared with the SAI isoform. There is considerable difficulty in distinguishing the CWI from the SAI due to the very similar properties of the two isoforms and further confusion arises as there may even be different isoforms of CWI present (Vattuone *et al.* 1981). The extracellular acid invertase was believed to be required for sucrose uptake into the storage parenchyma cells of the stem of sugarcane (Glasziou and Gayler 1972). More recent reports (Lingle 1989; Thom and Maretzki 1992) have cast doubt on the methodology used to draw this conclusion and thus relegated the extracellular invertase to play a lesser, undefined role in the sucrose accumulation processes. CWI in other plant species has been implicated in various processes including early development, environmental sensing, osmoregulation, phloem unloading and the reloading of sucrose leaked into the apoplast (Tymowska-Lalanne 1998).

Ionically bound invertase has been removed by high salt concentrations with varying degrees of success in some plant tissues (Tang *et al.* 1999). In sugarcane, attempts to isolate and measure ionically bound CWI have not been successful (Vattuone *et al.*, 1981). Residual soluble invertase within the cell wall pellets and acid hydrolysis of sucrose at low pH (Echeverria and Burns 1989) affect the measurement of CWI activity.

More recently, extraction and assay methods were developed for the determination of both soluble and cell wall invertase activity in sugarcane (Albertson *et al.* 2001). These methods using a pellet mix procedure overcome some difficulties and demonstrate a pH optima ranging between 3.2 and 3.6 for cell wall invertase. The pH optima for the soluble invertases were 4.5 and 7.3 for soluble acid and neutral invertase, respectively.

### 2.8.2 Neutral invertase (NI)

Neutral invertase ( $\beta$ -D-fructofuranosidase, E.C. 3.2.1.26) catalyses the hydrolysis of sucrose to glucose and fructose. NI displays a monophasic pH profile, indicating no significant competing soluble acid invertase activity; with a pH optimum of 7.2, showing half maximal activity at pH 6.4 and 8.2 (Vorster and Botha 1998). In many plants, an isoform of neutral invertase, namely alkaline invertase has been identified with pH optima for sucrose cleavage at slightly alkaline range. In carrot, the distinction between these two enzymes is based on the pH optima of 6.8 for neutral invertase and 8 for alkaline invertase (Lee and Sturn 1996); nevertheless, in many studies they are viewed as the same enzyme. In sugarcane, only neutral invertase has been found and it is localized in the cytosol (Vorster and Botha 1998).

The activity of neutral invertase in different tissues of sugarcane has been investigated to provide insight into the role played by that enzyme. Early work on fresh weight basis (Hatch *et al.* 1963; Batta and Singh 1986) reported an increase in the activity of neutral invertase with increased internode maturity. That finding suggests a regulatory role of NI on sucrose movement from vascular to storage tissue in mature internodes (Hatch *et al.* 1963) and its involvement in the turnover of hexoses in mature internodes (Gayler and Glasziou 1972). In contrast, subsequent studies have reported an almost opposite distribution of activity on both, fresh weight and protein basis, where the NI activity decreases with internode maturity and the highest activities were found in the youngest internodes (Lingle and Smith 1991; Zhu *et al.* 1997; Ebrahim *et al.* 1998; Vorster and Botha 1999; Rose and Botha 2000). It has also been found that NI activity was highly variable among internodes of the same age and between sampling dates (Lingle 1997). Recently, molecular studies demonstrate that NI was detected in all tissue types; and shown a higher transcript and protein expression in the younger culm tissue than the older internodal tissue (Bosch *et al.* 2004). This suggests that the enzyme could make a contribution to the supply of hexoses in young tissues.

## 2.9 Invertase inhibitors

Plant invertase gene expression and enzyme activity are both known to be influenced by a

variety of intracellular and extracellular factors. Among those factors are endogenous proteinaceous inhibitors and sugars (the end-products as well as the substrate itself).

### 2.9.1 Proteinaceous inhibitors

Low and variable invertase activity might be attributed at least in part to the presence of endogenous inhibitors. In potato tubers and other tissues, the enzyme seems to be controlled by interaction with endogenous proteinaceous inhibitors. The first evidence of an endogenous proteinaceous invertase inhibitor was obtained from a kinetic study on invertase in crude extracts of potato tubers (Schwimmer *et al.* 1961), from which the first protein inhibitor of invertase was isolated (Pressey 1966). Since then, invertase inhibitors have been isolated and purified from several plants including maize (*Z. mays*) (Jaynes and Nelson 1971), sweet potato (*Ipomoea batatas*) (Matsushita and Uritani 1976) and potato (*Solanum tuberosum*) (Bracho and Whitaker 1990). These invertase inhibitors from higher plants are all soluble proteins, and their inhibitor activity seems to be limited to acid invertase.



### 2.9.2 Sugars

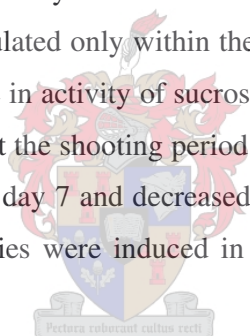
Recently, it has been suggested that changes in sugar fluxes may affect gene expression. The role of sugars in the regulation of gene expression is a phenomenon well established in yeast, where the repression of gene expression has been studied in some details. It has been shown, using genetic and molecular approaches, that glucose signalling is transduced via a cascade of events, some of which have been isolated and characterized (Carlson 1987; Trumbly 1992). Hence, in microorganisms, sugar-related expression of specific genes provides a way for optimizing metabolism relative to the supply of carbohydrates. In higher plants, sugar-responsive gene expression is less well understood. Multiple examples of sugar inhibition or activation of plant genes have been reported, but the mechanisms associated with sugar-signalling pathways are largely unknown.

## CHAPTER 3

### **Sugar concentrations and the activity of sucrose metabolizing enzymes during the development of side shoots from sugarcane setts**

#### **Abstract**

Sucrose, glucose and fructose concentrations, and sucrolytic enzyme activities were measured in the developing shoots and internodes of sugarcane (*Saccharum* spp.), hybrid variety N19. During shoot establishment the sucrose content decreased and the hexose content increased in the internodal tissues while in the shoots, both sucrose and hexoses continuously accumulate. The internode's dry mass was reduced by 25% and 30% over the 21-day shooting period in plants incubated in dark/light and total darkness, respectively. Sucrose accounted for 90% of the dry mass loss. Sucrose synthase activity, especially in breakdown direction was up-regulated only within the first seven days of shooting in both the tissue types. A slight increase in activity of sucrose synthase in synthesis direction was observed in the shoots throughout the shooting period. In the internodal tissues the sucrose synthase activity increased up to day 7 and decreased thereafter. Soluble acid, neutral and cell wall bound invertase activities were induced in both, the internodal tissues and the shoots.

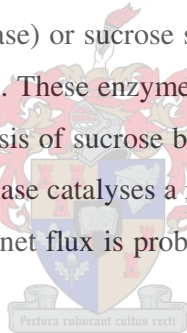




### 3.1 Introduction

The majority of reduced carbon fixed during photosynthesis is converted to sucrose before export from the source to sink organs. In higher plants the major form of reduced carbon in the phloem is therefore sucrose. The driving force of sucrose transport from source to sink is a turgor pressure gradient promoted by a sucrose concentration gradient (Ho and Baker 1982 and references therein). Among others factors, the maintenance of the assimilate concentration gradient is controlled by the rate of sucrose utilisation in sink tissues (Ho 1988) and by the energy dependent transport of sucrose through membranes (Patrick 1990).

In sugarcane (*Saccharum spp.*) the accumulation of sucrose in storage tissue involves cycles of continuous cleavage and synthesis (Batta and Singh 1986; Whittaker and Botha 1997). The first step of sucrose breakdown is the cleavage of the glycosidic bond by either invertase (EC 3.2.1.26,  $\beta$ -fructosidase) or sucrose synthase (UDP-glucose: D-fructose 2  $\alpha$ -D-glucosyltransferase, EC 2.4.1.13). These enzymes exist in several isoforms that differ in their sub-cellular location. Hydrolysis of sucrose by invertase is irreversible and generates glucose and fructose. Sucrose synthase catalyses a reaction, which is readily reversible, but under physiological conditions the net flux is probably in the glycolytic direction (Kruger 1990).



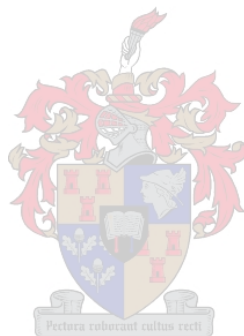
Invertases, a family of enzymes that hydrolyse sucrose into glucose and fructose, have been proposed to carry out critical roles during sucrose accumulation by sugarcane (Moore 1995). In sugarcane, sucrose unloaded from phloem passes through two distinct cellular compartments: the apoplastic space (including the cell wall) and the metabolic compartment (cytosol), to end up in the storage compartment (vacuole) (Bielecki 1960; Sacher *et al.* 1963). Each compartment contains a characteristic invertase isoform; one acid invertase is localised in the apoplastic space (cell wall invertase, CWI), another is located in the vacuole (vacuolar acid invertase, VAI, or soluble acid invertase, SAI), and a neutral invertase (NI) is present in the cytoplasm (Masuda *et al.* 1988; Tymowska-lalanne and Kreiss 1998).

Many efforts to increase sucrose content are aimed at reducing the activity of enzymes involved in sucrose cleavage/hydrolysis in the sink organs at sugarcane maturity.



Sugarcane agriculture depends on vegetative propagation and ratoon crops during which time we assume stored sucrose is remobilised to support growth and development. Based on work done in carrot (Tang *et al.* 1999) by exploiting genetic manipulation techniques, transgenic carrot lines with varying activities of CWI and SAI showed that invertases were important in the partitioning of carbon into sucrose in early plant development. It is imperative to assess the possible involvement of sucrose and the sucrolytic enzymes during sugarcane shoot establishment.

The purpose of this study was firstly to confirm to what extent sucrose is remobilized from the culm parenchyma cells during shoot establishment in both the internodal tissues and the shoots. Secondly, the activity of invertases and sucrose synthase during shoot establishment was assessed and their possible involvement in sucrose remobilization and utilization was evaluated.



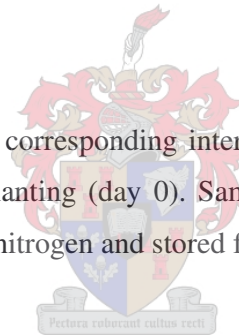
## 3.2 Materials and methods

### 3.2.1 Shoot establishment procedure

Mature non-flowering stalks from field grown (Kwazulu-Natal, South Africa) sugarcane of the commercial variety N19 were used in all shooting experiments. Setts were cut from nodes 14 to 22 with approximately two centimetres of internode on each side and sealed with candle wax on both ends. The setts were allowed to presume growth for 21 days in containers (750cm<sup>2</sup>, 10cm depth) filled with sand. After preliminary tests at 22°C, 27°C, 32°C and 37°C; the temperature of 32°C was identified and used as the optimum for shoot establishment. Two light regimes were used, i.e. a dark treatment (21days in complete darkness) and a dark/light treatment (12h/12h) starting on the 7<sup>th</sup> day after planting until the end.

### 3.2.2 Tissue sampling

Tissue types, the shoots and the corresponding internodal tissues were harvested every 7 days starting from the day of planting (day 0). Samples were collected in triplicate and ground into powder using liquid nitrogen and stored frozen at -80°C until use.



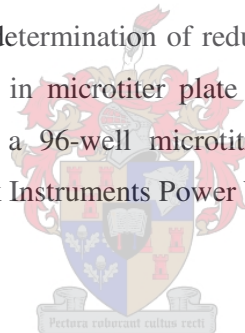
### 3.2.3 Enzyme extraction

Total soluble protein extractions were carried out in HEPES buffer (100mM, pH 7.5), containing 10mM MgCl<sub>2</sub>, 1mM ethylene ediamine tetra acetic (EDTA), Roche Complete<sup>®</sup> protease inhibitor at the recommended concentration, 14mM β-mercaptoethanol, 0.1% (v/v) Triton X-100 and 2% (m/v) polyvinylpolypyrrolidone (PVPP) (insoluble), modified from Albertson *et al.* (2001); with the following exceptions: soluble proteins were extracted in a 1:2 ratio of tissue to buffer and centrifuged at 10000 xG for 15 minutes at 4°C. The supernatant was immediately desalted on a Sephadex G-25 (Pharmacia PD-10) column equilibrated with desalting buffer (100mM HEPES buffer pH 7.0), 10mM MgCl<sub>2</sub>, and 2mM EDTA, and 10% glycerol. Aliquots of eluted protein were rapidly frozen in liquid nitrogen and stored at -80°C for soluble invertase assays and protein determinations.

For CWI, the pellet from the crude extract was re-suspended in 10ml of extraction buffer (without PVPP), kept on ice for 10min with occasional agitation, then centrifuged at 12000 xG for 15min at 4°C and the supernatant removed. This was followed by a second wash (Albertson *et al.* 2001). The pellet was kept on ice prior to activity assay.

### 3.2.4 Invertase assays

Soluble invertases were assayed in a final volume of 320µl consisting of 0.5M sucrose 50mM HEPES (pH 7.5) for neutral invertase and 50mM Citrate-phosphate (pH 4.5) for acid invertase, modified from Kingston-Smith *et al.* (1998). For cell wall bound invertase, the volume of the reaction medium was 2 fold that of the soluble acid (pH 3.6). Aliquots (80µl) were removed at 30, 60 and 90 min for all invertases and the reaction terminated by a stop solution (2mM Tris and 22mM ZnSO<sub>4</sub>) and a solution of Amidazole (4M, pH 7.6) was added for soluble and cell wall invertases, modified from Albertson *et al.* (2001). All aliquots were then used for the determination of reducing sugars as described above. The assay was scaled down for use in microtiter plate format in a final volume of 250µl. Reactions were carried out in a 96-well microtitre plate and NAD<sup>+</sup> formation was monitored at 340nm in a Bio-Tek Instruments Power Wave X spectrophotometer (Bio-Tek, Winooski, VT).



### 3.2.5 Sucrose synthase assays

Sucrose synthase was assayed in both synthesis and breakdown directions. In the synthesis direction, a 20µl aliquot of desalted extract was added to 50µl reaction cocktail [100mM Tris-HCl (pH 7.5), 15mM MgCl<sub>2</sub>, 10mM Fructose and 10mM UDP-Glucose] (Zeng *et al.* 1998). The reaction was incubated at 30°C for 1h and subsequently stopped by adding 70µl of 30% (m/v) KOH at 30 min and 1h. The tubes were then immersed in boiling water for 10 min to remove free hexoses (Zeng *et al.* 1998). Sucrose content was measured using 0.14% (m/v) Anthrone reagent in 80% H<sub>2</sub>SO<sub>4</sub> and incubated at 40°C for 20 minutes. The concentration of the reaction product (sucrose) was determined spectrophotometrically at 620nm.

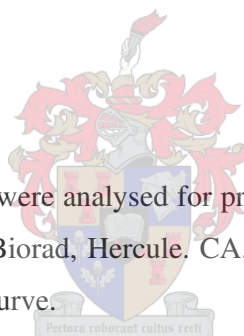
In the breakdown direction, a 10 $\mu$ l aliquot of the desalted extract was added to 220 $\mu$ l of reaction cocktail [100mM Tris-HCl (pH 7.5), 0.32M sucrose, 2mM NAD, 2mM MgCl<sub>2</sub>, 4U HK/G6PDH, 1mM ATP and 4U PGI]. To initiate the reaction, 20 $\mu$ l UDP was added, and the amount of free fructose was determined enzymatically (Bergmeyer and Bernt 1974).

### 3.2.6 *Sugar determination*

Sugars were extracted from aliquots of frozen tissues in a 1:10 ratio of mass to extraction buffer [50mM Tris-HCl (pH 7.0) and 70% (v/v) ethanol] and incubated overnight at 65°C. The samples were then centrifuged at 13000 xG for 10 min at 4°C. The supernatant was immediately used for sugar determination. Sucrose, glucose and fructose concentrations were determined using the standard enzymatic method as described by Bergmeyer and Bernt (1974).

### 3.2.7 *Protein determination*

Aliquots of the desalted extracts were analysed for protein content using a commercial Kit based on the Bradford method (Biorad, Hercules, CA, USA). Bovine albumin (fraction V) was used to generate a standard curve.



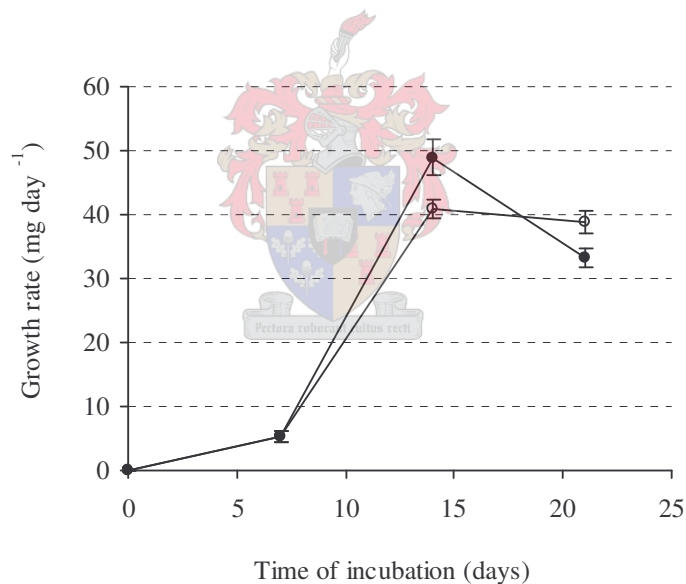
### 3.2.8 *Data analyses*

Data presented for sugars content, sucrose cleaving enzyme activities and growth rate are the mean values of three replicates with a replicate containing at least 3 independent samples. Correlation between internode dry mass and sucrose content was determined by linear regression analyses.

### 3.3 Results

#### 3.3.1 Absolute growth rate (AGR)

The increment in dry weight per unit of time was assessed and determined after 0, 7, 14 and 21 days of shoot establishment period. In both sets incubated in total darkness and dark/light, three main phases were distinguished. The first phase, an initial lag period, was observed during the first seven days of shooting; no significant changes in the growth rate were observed. This was followed by a period in which the growth rate increased exponentially between day 7 and 14. In this phase, the rate of biomass production in plant incubated in total darkness was 1.25 times higher than that of the shoots in the dark/light cycle. From day 14 to 21, a decrease in growth rate was observed in plants kept in total darkness, while the rate was constant in plants incubated in dark/light cycle (Fig.3.1).



**Figure 3.1** Growth rate (increase in dry weight per day) of the shoots incubated in total darkness (●) and dark/light (○). Each data point represents the mean values of three replicates  $\pm$  SE.

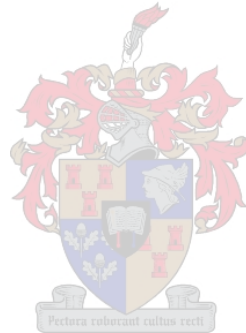
#### 3.3.2 Sugar content in the shoots

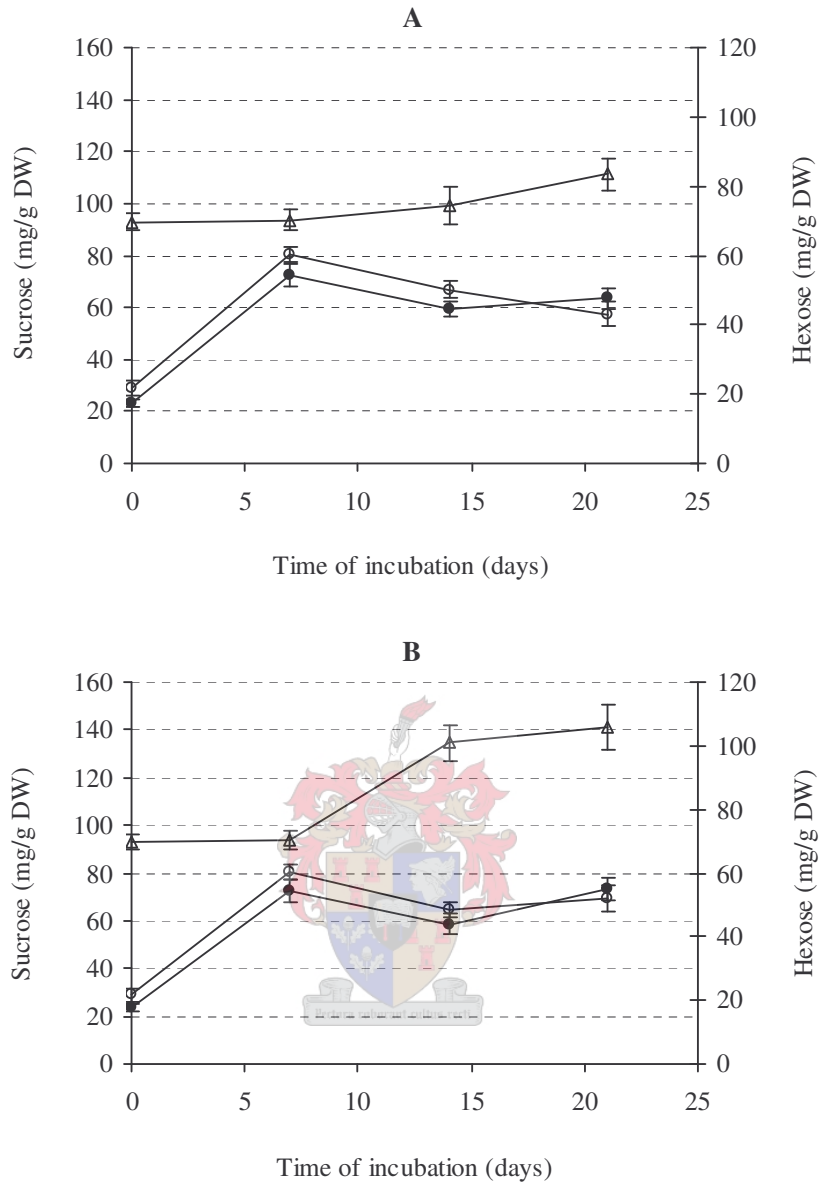
The soluble sugars changed throughout the shooting period. The hexose content was low (approximately 20mg g<sup>-1</sup> dry weight) at the day of planting (day 0). The hexose content

increased up to  $60 \text{ mg g}^{-1}$  dry weight after 7 days and stayed constant thereafter (Fig.3.2 A and B). The sucrose content of the plants incubated in dark/light was significantly higher than that of the plants kept in continuous darkness. In addition, the sucrose content increased by approximately 20% and 50% for plants kept in total darkness and in dark/light regime, respectively (Fig.3.2A and B).

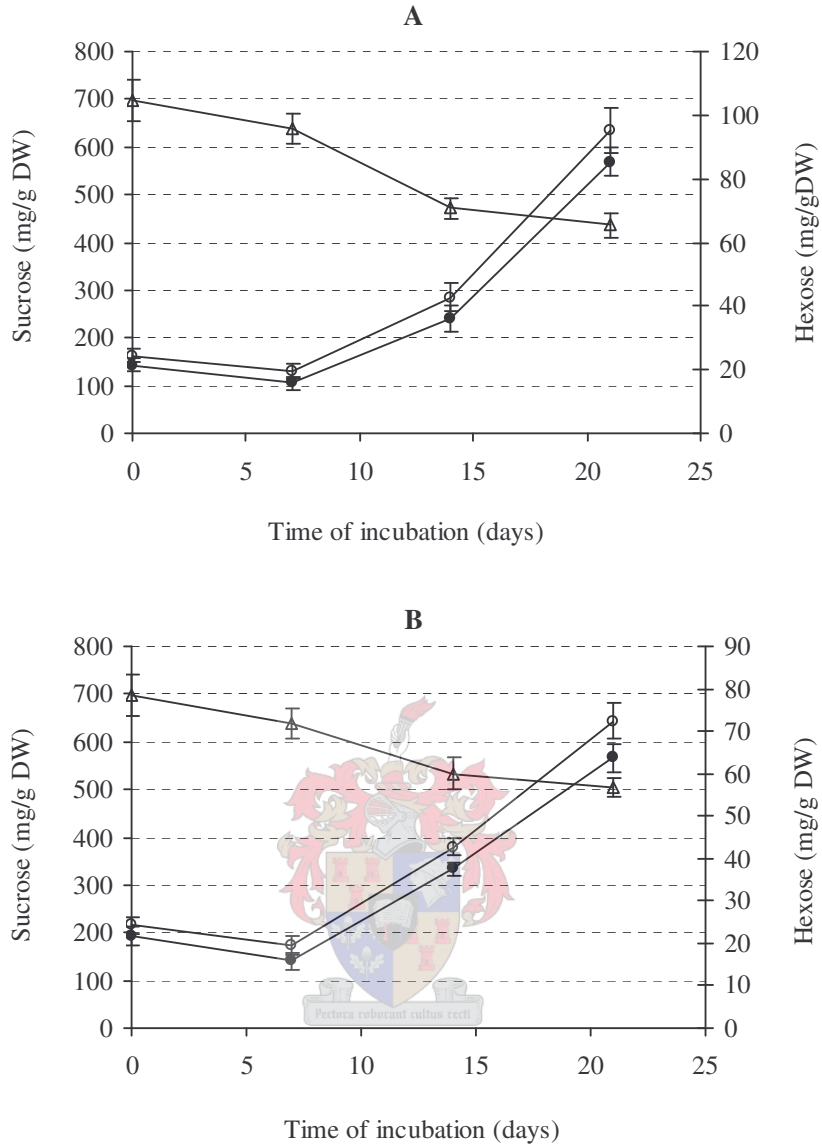
### 3.3.3 *Sugar contents in the internodes*

The hexose content in the internodes was similar to that of the shoots, this slightly decreased after 7 days and increased thereafter. Changes between day 14 and 21 showed 4 times increase in hexose in plants incubated in total darkness and 3 times in plants kept in the dark/light regime (Fig. 3.3 A and B). The sucrose content found on the day of planting was  $700 \text{ mg g}^{-1}$  dry weight, this decreased by approximately 40% in internodes incubated in total darkness and 30% in those incubated in dark/light after 21 days (Fig. 3.3 A and B).





**Figure 3.2** Sugar content in the shoots incubated in total darkness (A) and in dark/light (B). Each data point represents the mean values of three replicates  $\pm$  SE. ( $\Delta$ ) sucrose; ( $\bullet$ ) glucose; ( $\circ$ ) fructose.



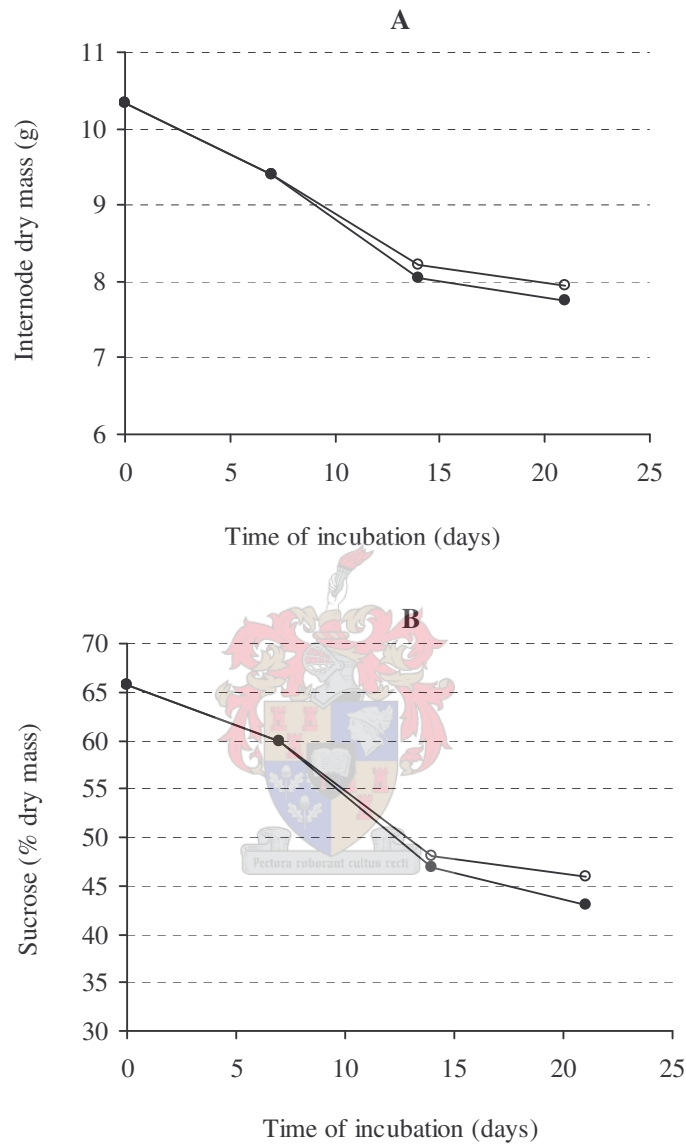
**Figure 3.3** Sugar content in the internodal tissues incubated in total darkness (A) and in dark/light (B). Each data point represents the mean values of three replicates  $\pm$  SE. ( $\Delta$ ) sucrose; ( $\bullet$ ) glucose; ( $\circ$ ) fructose.

### 3.3.4 Sucrose contribution to mass change and partitioning

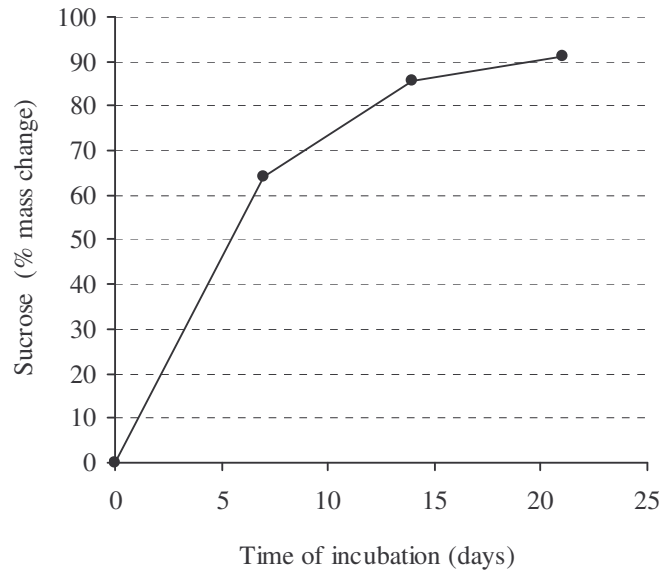
Prior to the shoot establishment procedure, the internode's sucrose contents represented approximately 66% of the total internode's dry mass and decreased thereafter (Fig. 3.4 B). In both internodes incubated in total darkness and dark/light cycle, the internode dry mass and the sucrose percentage dry mass decreased with the time of shooting (Fig. 3.4 A&B).



Sucrose contribution to internode mass change was 64% the first seven days of shoot establishment, 85% between day 7 and 14, and 91% thereafter (Fig.3.5).

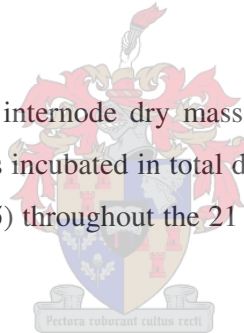


**Figure 3.4** Changes in internode total dry mass (A) and sucrose percentage dry mass (B) during shoot establishment. Each data point represents the mean values of three replicates. Internodes incubated in total darkness (●) and dark/light (○).

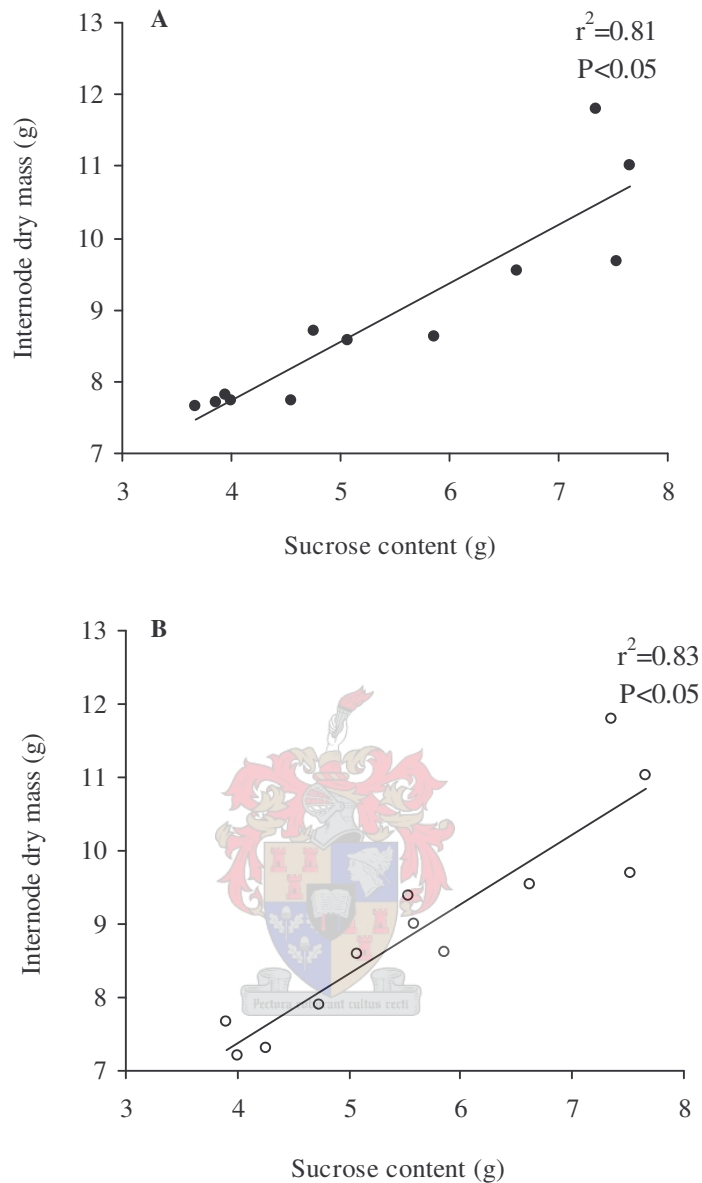


**Figure 3.5** Contribution of sucrose to internode mass change throughout the shooting period. Each data point represents mean values of three replicates.

In addition, the decrease in the internode dry mass strongly correlates with changes in sucrose content in both the plants incubated in total darkness ( $r^2= 0.81$ ,  $P<0.05$ ) and in the dark/light cycle ( $r^2= 0.83$ ,  $P<0.05$ ) throughout the 21 days of shoot establishment (Fig. 3.6 A and B).



During the shooting period, the distribution of the remobilized sucrose over the shoot and the roots (sett roots) was determined and the difference accounted for respiration. In both setts incubated in total darkness and the dark/light cycle, 32% of the remobilized sucrose was allocated to roots' biomass production during the first seven days; this decreased between day 7 and 14 and remained constant thereafter (Table 3.1). In setts incubated in total darkness, the percentage sucrose allocated to shoot biomass was approximately twofold that used for respiration during the first seven days. This remained fairly equal between days 7 and 14, and the percentage allocated to respiration exceeded that partitioned to shoot biomass thereafter (Table 3.1). Similar patterns were observed in setts incubated in the dark/light cycle during the first seven days and between day 7 and 14, but the percentage allocated to respiration decreased while the percentage partitioned to shoot biomass increased thereafter (Table 3.1).



**Figure 3.6** Correlation between internode dry mass and sucrose content throughout the shooting period in plants incubated in total darkness (A) and dark/light (B).

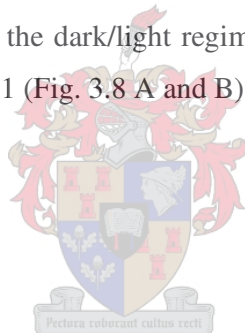
**Table 3.1** Distribution of the remobilized sucrose (%) over shoot, roots and respiration. Values are the mean of three replicates  $\pm$  SE.

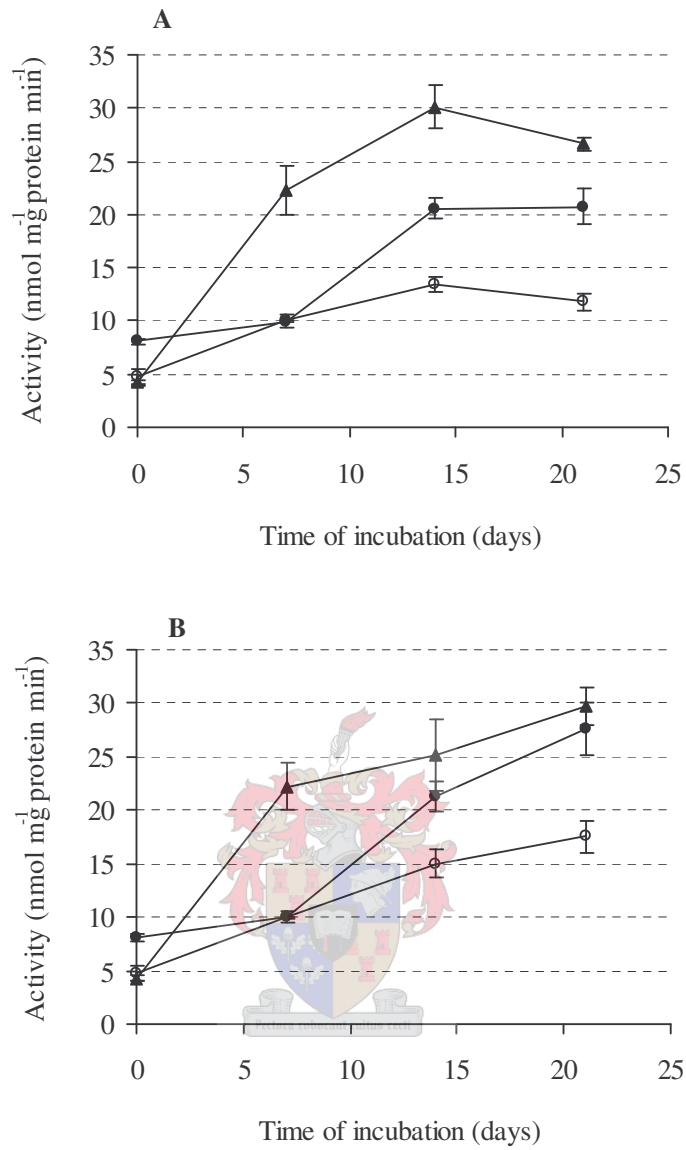
Time (days)	Dark			Dark/Light		
	Shoot	Roots	Respiration	Shoot	Roots	Respiration
0	0	0	0	0	0	0
7	43.2 $\pm$ 1.4	32.0 $\pm$ 3.3	24.8 $\pm$ 2.4	43.2 $\pm$ 1.4	32.0 $\pm$ 3.3	24.8 $\pm$ 2.4
14	45.3 $\pm$ 1.5	12.3 $\pm$ 2.5	44.4 $\pm$ 2.8	41.6 $\pm$ 1.1	14.8 $\pm$ 2.8	43.7 $\pm$ 5.8
21	38.3 $\pm$ 1.3	13.3 $\pm$ 2.1	48.4 $\pm$ 5.3	47.8 $\pm$ 1.8	17.7 $\pm$ 2.1	34.5 $\pm$ 3.3

### 3.3.5 *Invertase activities throughout the shooting period*

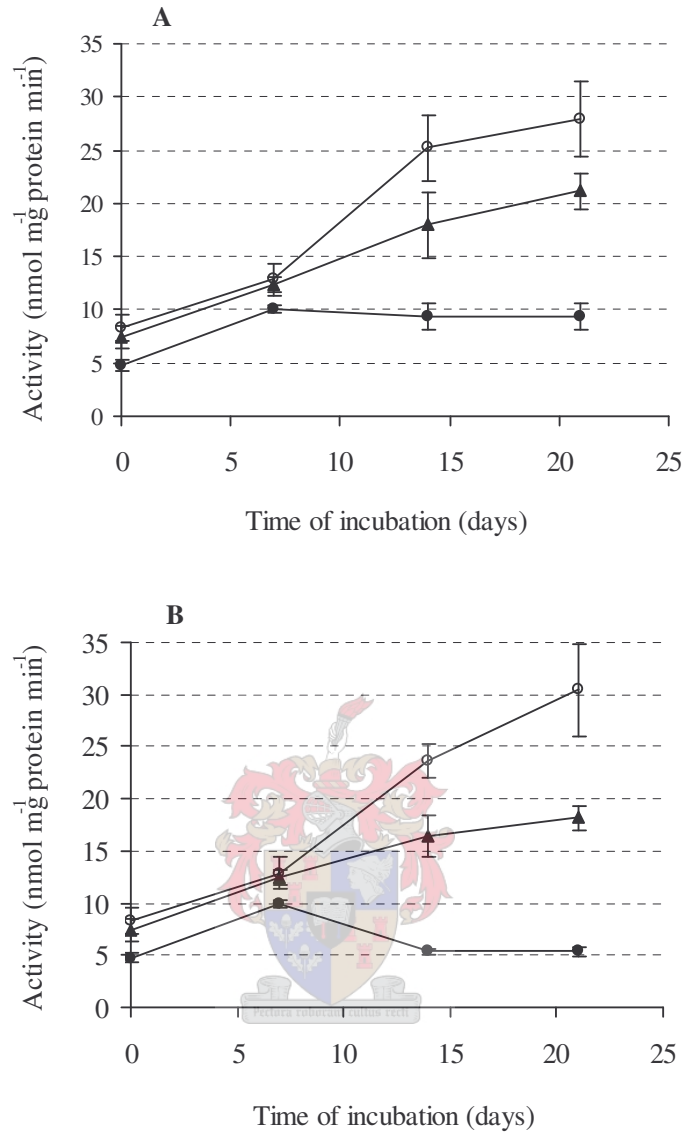
The activity of invertases during the shooting period was determined in both the shoots and the internodal tissues. In the shoots, a similar pattern of activity was observed in both plants incubated in total darkness and in the dark/light regime. The specific activity ( $\text{nmol mg}^{-1} \text{ protein min}^{-1}$ ) of all three invertases was low on the day of planting. A sharp rise in enzyme activity was evident during the first 14 days and it then remained constant thereafter in the shoot kept in total darkness (Fig. 3.7 A). In the shoot incubated in the dark/light regime, the enzyme activity increased throughout the 21 days of shooting period (Fig. 3.7 B).

In the internodes, the specific activity of SAI and CWI increased throughout the 21 days of shooting period regardless of the treatment (Fig. 3.8 A and B). NI specific activity increased up to day 7 and remained constant thereafter in plants incubated in total darkness, while in plants kept in the dark/light regime, the activity decreased back to the initial level between day 14 and 21 (Fig. 3.8 A and B).





**Figure 3.7** Invertase activities in the shoots incubated in darkness (A) and dark/light (B). Each data point represents the mean values of three replicates  $\pm$  SE. (▲) CWI; (○) SAI; (●) NI.

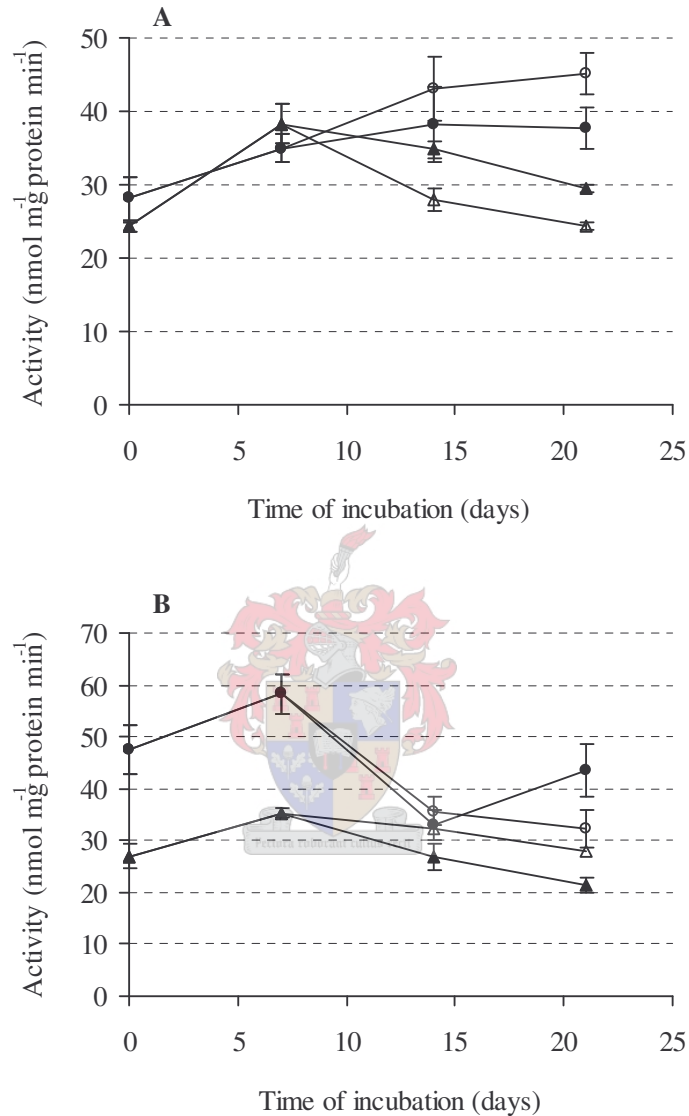


**Figure 3.8** Invertase activities in the internodes incubated in darkness (A) and dark/light (B). Each data point represents the mean values of three reps  $\pm$  SE. (▲) CWI; (○) SAI; (●) NI.

### 3.3.6 Activity of sucrose synthase during shoot establishment

SuSy activity was measured in breakdown and synthesis direction in both the shoots and internodes. In the shoots kept in total darkness, SuSy breakdown activity increased during the first seven days of incubation to reach a maximum level of  $37 \text{ nmol mg}^{-1} \text{ protein min}^{-1}$  and declines thereafter (Fig. 3.9 A). Similarly, SuSy activity in shoots incubated in

dark/light cycle increased up to day 7 and decreased back to the initial level between day 7 and 21 (Fig. 3.9 A).



**Figure 3.9** Activity of sucrose synthase determined from the shoots (A) and the internodal tissues (B). SuSy synthesis, (●) plants incubated in total darkness; (○) plants incubated in dark/light. SuSy breakdown, (▲) plants incubated in total darkness; (△) plants incubated in dark/light.

The level of SuSy activity in the synthesis direction in the shoots increased throughout the 21 days of shooting period and reached a high level of  $45 \text{ nmol mg}^{-1} \text{ protein min}^{-1}$  in plants incubated in dark/light regime (Fig. 3.9 A). Furthermore, SuSy synthesis activity in shoots

kept in dark/light exceeded the activity of those incubated in total darkness (Fig. 3.9 A). In the internodes, SuSy breakdown activity increased only during the first 7 days, and then decreased until it reached the initial level after 21 days of incubation (Fig. 3.9 B). In the synthesis direction, SuSy activity increased only during the first 7 days and decreased thereafter to a level below the initial activity in both plants incubated in total darkness and dark/light regime (Fig. 3.9 B).





### 3.4 Discussion

Since the setts were in darkness for most of the time, the increase in growth rate is probably directly related to the capacity of the shoots to import carbohydrate from the internodes. High growth rate therefore probably implies high import of carbohydrates. This would suggest that plants incubated in total darkness are importing more reduced carbon (carbohydrate) compared to those incubated in dark/light regime. Nevertheless, after day 14, the growth rate of the shoots incubated in total darkness decreased while in dark/light regime, the growth rate remained constant. This suggests that the capacity of the shoots incubated in total darkness to import carbohydrate decreased or it could also mean that the source (internode) can no longer deliver. The fact that the growth rate of the shoots incubated in the dark/light regime stayed constant after day 14 suggested that another source of carbohydrate most probably from the photosynthesis started to make a contribution. It has been suggested for other plants that the cessation of import may be related to increasing resistance to symplastic unloading (Schmalstig and Geiger 1987).

The decline/stabilization of the hexose contents in the shoots with shooting time and the increase in sucrose content between day 7 and 21 suggests sucrose import from the internodes and further conversion of hexoses into sucrose. Nevertheless, the difference in sucrose content between plants incubated in total darkness and in the dark/light regime also suggests the contribution of photosynthesis for shoot incubated in dark/light (Fig. 3.2). In addition, sucrose remains the principal free sugar during shoot establishment.

In the internodes, the highest decline in sucrose content from day 7 to 14 is in line with the increase in hexose contents and the highest increase in the shoots' growth rate regardless of the light regime (Fig.3.2 and Fig.3.1). This suggests that a breakdown of sucrose exists in the internodes at the same time sucrose is imported to the shoots. During the last stage of shooting, between day 14 and 21, the hexose produced from the hydrolysis of sucrose in the internodes increased while a slight decrease in sucrose content was observed. This led to the conclusion that the hexose concentration accounted for the sucrose breakdown at that period since the growth rate at that stage decreased in plants incubated in total darkness. The stabilization of the growth rate in plants kept in dark/light suggested another source of carbon rather than the one imported from the internode, probably from photosynthesis.

The changes in the internode dry mass and the sucrose contribution to shoot, roots and respiration were investigated during the 21 days of shoot establishment. At the end of the 21 days of the shooting period, 30% and 25% of total internode dry mass was remobilized from setts incubated in total darkness and dark/light regime, respectively. The difference found between the two light regimes is partly attributed to the difference in sink strength, the ability of a sink organ to attract sucrose. Nevertheless, independently of the light regimes, 90% of the remobilized internode dry mass was sucrose ( $P < 0.005$ ). In terms of the total remobilized sucrose utilization, a significant difference in setts incubated in total darkness and in the dark/light cycle was observed between day 14 and day 21. In setts incubated in total darkness, 38% and 48% were used for shoot biomass production and respiration, respectively. In setts incubated in the dark/light cycle, 48% and 34% were used for shoot biomass production and respiration, respectively. The 10% difference in respiration between treatments was a reflection of photosynthesis contribution for plants incubated in the dark/light cycle.

The enzymes of sucrose cleavage/hydrolysis were up-regulated in the shoots. Their activity generated 3 times the amount of hexoses after 7 days compared to the content found the day of planting in the shoots; while no changes in sucrose content was observed at this early stage. A significant difference in the activity of the three invertases were found (Fig.3.7 A and B), where the order in terms of specific activity was SAI, NI and CWI reaching the highest activity at day 14 in the shoots incubated in total darkness and at day 21 in those kept in the dark/light regime. The lower activity of SAI throughout the shooting period compare to NI and CWI counterparts can be related to the compartmentation. This suggests that up to 21 days the cells of the shoots are not well vacuolated, and sucrose hydrolysis/cleavage occurs mainly in the apoplast and the cytosol. CWI has been implicated in various processes including early development, environmental sensing, and osmoregulation, phloem unloading and reloading of sucrose leaked into the apoplast (Tymowska-Lalanne 1998).

The stabilization of the hexose contents from day 7 to 21 suggests the accumulation of sucrose where a difference was found between plants incubated in total darkness and dark/light. The variation in sucrose accumulation was partly attributed to the difference in SuSy synthesis activity between the two regimes and the alternate source of sucrose (photosynthesis) in the dark/light regime. This was supported by the finding that the rate of

sucrose hydrolysis by invertase between the shoots incubated in total darkness and those kept in the dark/light regime was similar. The activity of SuSy breakdown in the shoots incubated in total darkness was 1.2 times higher than in those kept in dark/light regime. This was supported by the finding that the shoots incubated in total darkness are the greater sucrose importers. It had been suggested that sucrose transport from source into sink organs was controlled by “sink strength”, the ability of a sink organ to attract sucrose (Ho 1988); and that sucrose synthase activity is an indicator of sink strength (Black *et al.* 1995; Sung *et al.* 1999).

In the internodes, the activity of the two acid invertases increased throughout the shooting period with the SAI activity exceeding the CWI activity (Fig.3.8 A and B). This clearly indicates the sucrose breakdown in both the apoplastic space and the vacuole. The NI counterpart was only expressed during the first seven days and decreased or remained constant thereafter. This suggests a low sucrose hydrolysis in the cytosol and that the remobilized sucrose is mainly transported to the shoots intact as it reaches the cytosolic compartment. The hexoses released in the internodes are not mainly for export into the shoots since they accumulate in the source tissues. This suggests that the activity of both SAI and CWI largely account for providing the exporting cells with reduced carbon for energy demands and internal maintenance (Echeverria 1998).

In conclusion, sucrose is the main substrate used during shoot establishment. The decrease in sucrose content was accompanied by a release of hexoses in the internodal tissues as a result of the activity of sucrose hydrolysis/cleavage enzymes. The shoot establishment period is characterised by the up-regulation of invertases and sucrose synthase, and the increase in sucrose and hexose contents in the shoots. Continuous sucrose accumulation in the shoots during the latest stage of shoot establishment in plants incubated in dark/light is a result of a source of carbon rather than the one from the internodes, probably photosynthesis. This indicates that, to undergo growth and development growing plants must switch from net importers to producers.

## CHAPTER 4

### **Expression of cell wall and soluble acid invertases during shoot development from planted sugarcane setts**

#### **Abstract**

Sugarcane acid invertase isoforms, soluble acid invertase (SAI) and cell wall bound invertase (CWI), are key enzymes that hydrolyse sucrose in the vacuole and apoplast, respectively. The correlation between sugar content and enzyme activity as well as RNA expression patterns in different tissues at different shooting stages was investigated. The specific aim of the work was to look at SAI and CWI expression throughout the shooting period in both the internodes and the shoots. SAI transcripts were only detected in the internodal tissues after 14 days of shooting. At this stage the sucrose content in the internodes was already reduced by one third compared to the day of planting. In contrast, CWI transcripts were observed throughout the shooting period in both tissue types.



## 4.1 Introduction

The physiological and biochemical studies on sugarcane reported a negative correlation between invertase activity and sucrose concentration in the culm and concluded that invertase activity is the key limiting step in the process of sucrose accumulation (Zhu *et al.* 1997; Ebrahim *et al.* 1998). However, since these studies frequently involve different stages of plant development, different environments, or different genotypes, it is difficult to determine a direct relationship between sucrose content in the culm and invertase activity.

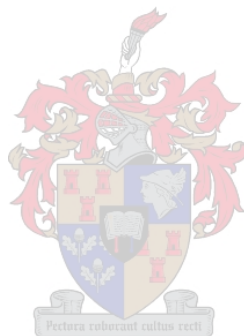
The potential role played by acid invertase isoforms in sucrose remobilization during the development of side shoots from sugarcane setts has never been investigated. This is in part because most of the earlier studies were focussed on invertase's role in sucrose accumulation. In an attempt to increase the productivity of sugarcane, acid invertase isoforms have been targeted for down regulation in mature tissues since there is a negative correlation between their activity and sucrose content (Gayler and Glasziou 1972a).

*Saccharum* spp. that retains high levels of soluble acid invertase (SAI) do not accumulate high levels of sucrose, but the reverse is not always true (Zhu *et al.* 2000), suggesting that SAI may contribute to sucrose accumulation but does not control it. SAI activity has been associated with remobilization of stored sucrose under conditions of increased vegetative growth or carbon stress and high SAI activity is found in the low sucrose water shoots that form in response to increased light at the extremity of field plots (Hatch and Glasziou 1963). In other plants SAI has been linked to general roles in early development relating to the creation of metabolic fluxes (Tang *et al.* 1999) and also to sucrose partitioning, sink strength and sugar composition of plant tissues (Sonnewald 1997). A role in sugar sensing has also been proposed to support the observation that expression of different SAI genes are differentially regulated by sugars (Koch 1996; Xu *et al.* 1996).

The CWI of sugarcane was thought to be required for sucrose uptake into the storage parenchyma cells of the stem (Glasziou and Gayler 1972a). However, more recent reports have cast doubt on this conclusion and thus have relegated the extracellular invertase to a lesser, undefined role in the sucrose accumulation process (Lingle 1989; Thom and

Maretzki 1992). Thus the CWI enzyme(s) from sugarcane have not been decisively characterised and their roles in sugarcane metabolism are not clearly defined.

The aim of this study was, therefore, to determine SAI and CWI expression at the transcript level, and to compare these patterns with enzyme activities as well as to the content of extractable sucrose and hexoses during shoot establishment.



## 4.2 Materials and Methods

### 4.2.1 Plant material and shoot establishment procedure

Mature non-flowering stalks from field grown (Kwazulu-Natal, South Africa) sugarcane of the commercial variety N19 were used in all shooting experiments. Setts were cut from nodes 14 to 22 with approximately two centimetres of internode on each side, sealed with candle wax on both ends and allowed to presume growth in containers (750 cm<sup>2</sup>, 10cm depth) filled with sand for 21 days at 32°C. Two treatments were used: a dark treatment (21days in complete darkness) and a dark/light cycle (12h/12h) starting from the 7<sup>th</sup> day after planting until the end of the shooting period. All tissue types were harvested every seven days starting from the day of planting, ground to powder in liquid nitrogen and stored at - 80°C until use. Sugars, proteins and RNA were all extracted from the shoots and the corresponding internodal tissues.

### 4.2.2 Sugar determination

Sugars were extracted from aliquots of frozen tissues at a 1:10 ratio with extraction buffer [50mM Tris-HCl (pH 7.0) and 70% (v/v) ethanol] and incubated overnight at 65°C. The samples were then centrifuged at 13000 xG for 10 min at 4°C. The supernatant was immediately used for sugar determination. Sucrose, glucose and fructose concentrations were determined using the standard enzymatic method as described by Bergmeyer and Bernt (1974).

### 4.2.3 Invertase extraction

Total soluble protein extractions were carried out in the HEPES buffer (100mM, pH 7.5), containing 10mM MgCl<sub>2</sub>, 1mM ethylene ediamine tetra acetic (EDTA), Roche Complete<sup>®</sup> protease inhibitor at the recommended concentration, 14mM β-mercaptoethanol, 0.1% (v/v) Triton X-100 and 2% (m/v) polyvinylpolypyrrolidone (PVPP) (insoluble), modified from Albertson *et al.* (2001); with the following exceptions: soluble proteins were extracted in a 1:2 ratio of tissue to buffer and centrifuged at 10000 xG for 15 min at 4°C. The supernatant was immediately desalted on a Sephadex G-25 (Pharmacia PD-10) column equilibrated with desalting buffer (100mM HEPES buffer pH 7.0), 10mM MgCl<sub>2</sub>,

and 2mM EDTA, and 10% glycerol. Aliquots of eluted protein were rapidly frozen in liquid nitrogen and stored at - 80°C for soluble invertase assays and protein determinations.

For CWI, the pellet from the crude extract was re-suspended in 10ml of extraction buffer (without PVPP), kept on ice for 10 min with occasional agitation, then centrifuged at 12000 xG for 15 min at 4°C and the supernatant removed. This was followed by a second wash (Albertson *et al.* 2001). The pellet was kept on ice prior to activity assay.

#### 4.2.4 Protein determination

Aliquots of desalted extracts were analysed for protein content using a commercial kit (Biorad, Hercules. CA, USA). Bovine serum albumin (fraction V) was used as the standard.

#### 4.2.5 Invertase assay

Soluble acid invertase was assayed in a final volume of 320µl consisting of 0.5M sucrose and 50mM Citrate-phosphate (pH 4.5), modified from Kingston-Smith *et al.* (1998). For cell wall bound invertase, the volume of the reaction medium was 2 fold that of the soluble acid (pH 3.6). Aliquots (80µl) were removed at 30, 60 and 90 min for both invertases and the reaction terminated by a stop solution (2mM Tris and 22mM ZnSO<sub>4</sub>) and a solution of Amidazole (4M, pH 7.6), modified from Albertson *et al.* (2001). All aliquots were then used for the determination of reducing sugars as described above. The assay was scaled down for use in microtitre plate format in a final volume of 250µl. Reactions were carried out in a 96-well microtitre plate and NAD<sup>+</sup> formation was monitored at 340nm in a Bio-Tek Instruments Power Wave X spectrophotometer (Bio-Tek, Winooski, VT).

#### 4.2.6 RNA extraction

RNA was extracted according to a modified method of Bugos *et al.* (1995). Tissue was ground to a fine powder in liquid nitrogen and added (1:2 (w/v)) to 25:24:1 phenol: chloroform: isoamyl alcohol and vortexed at high speed for 1 min. Thereafter an equal volume of homogenisation buffer [0.1M Tris-HCl (pH7.5), 1mM EDTA, 0.1M NaCl and 1% (w/v) SDS] was added. Sodium acetate (pH 5.2) was added to a final concentration of



0.1M; the emulsion was mixed and incubated on ice for 15 min. This was followed by a 15 min centrifugation step at 12000 xG at 4°C. The aqueous phase was transferred to a new tube and one volume isopropanol was added, followed by incubation at - 20°C overnight. The precipitated RNA was pelleted by centrifugation at 10000 xG for 10 min at 4°C. The pellet was washed with 70% (v/v) ethanol, centrifuged for 10 min at 10000 xG at 4°C and air-dried. RNA was re-suspended in 750µl DEPC (Diethyl pyrocarbonate) treated water and insoluble materials removed by centrifugation at 10000 xG for 10 min at 4°C. The supernatant was transferred to a microcentrifuge tube, and the RNA precipitated using LiCl (at a final concentration of 2M) and incubated overnight at 4°C. RNA was recovered by centrifugation at 12000 xG for 15 min at 4°C. The pellet was washed with 70% (v/v) ethanol, centrifuged at 10000 xG for 10 min and air-dried. The pellet was re-suspended in DEPC treated water and all remaining insoluble matter removed by centrifugation at 10000 xG for 10 min at 4°C. RNA was quantified spectrophotometrically.

#### 4.2.7 Northern blot analysis

Ten µg RNA per sample was loaded on a 1.2% (w/v) agarose gel (made up with 0.5x TBE buffer - Tris-Borate/EDTA electrophoresis buffer). RNA was separated at 100 V until the dye front had migrated 7cm. The gel was trimmed and stained in ethidium bromide solution (approximately 0.4µg ml<sup>-1</sup>) for 10 min and destained for 10 min in water. The RNA was viewed and photographed under UV light. The gel was then equilibrated in 10x SSC for at least 30 min, and the positively charged nylon membrane wet in water and equilibrated in 10x SSC for 30 min. RNA was transferred overnight onto the membrane by upward capillary blotting at room temperature using 10x SSC. After transfer the membrane was removed, rinsed in 5x SSC, excess liquid evaporated and UV cross-linked on both sides for 1.5 min at 1200 mJ cm<sup>-2</sup>.

#### 4.2.8 Probe preparation and hybridisation

The *Zea mays* invertase (Ivr1) gene (Genbank accession number U16123) described in Xu *et al.* (1995) and *Saccharum hybrid cultivar* cell wall invertase (Shcw1) mRNA (Genbank accession number AY302084) described by Peters *et al.* (2003) (unpublished) were used in northern hybridization.

The full length cDNA clone of cell wall invertase (1800 bp) was excised from the vector with Sac I and BamH I and purified after gel electrophoresis (1% (m/v) agarose) using a GFX™ PCR, DNA and Gel Band purification kit (Amersham). When soluble acid invertase was used as probe, the full length cDNA clone (1200 bp) was excised from the vector with EcoR I and purified as previously described. Probes were prepared by random primer incorporation of  $\alpha$  <sup>32</sup>P-dCTP (Prime it II Random Labelling, kit, Stratagene). The membrane was prehybridised in 10ml rapid-hybridisation buffer (Amersham) for at least 1h at 65°C. The probe was boiled for 3 min., added to the hybridisation bottle and incubated overnight at 65°C. Wash steps were as follows: rinse once in 2x SSC, 0.1% (w/v) SDS; two 10 min. wash in 2x SSC, 0.1% (w/v) SDS; one 10 min. wash in 1x SSC, 0.1% (w/v) SDS; one 15 min wash with 0.2x SSC, 0.1% (w/v) SDS. Hybridisation was visualised using a phosphor imager (Packard Cyclone).

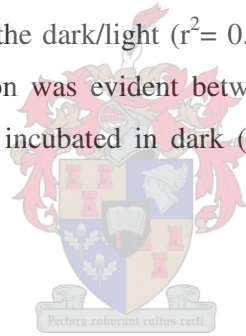


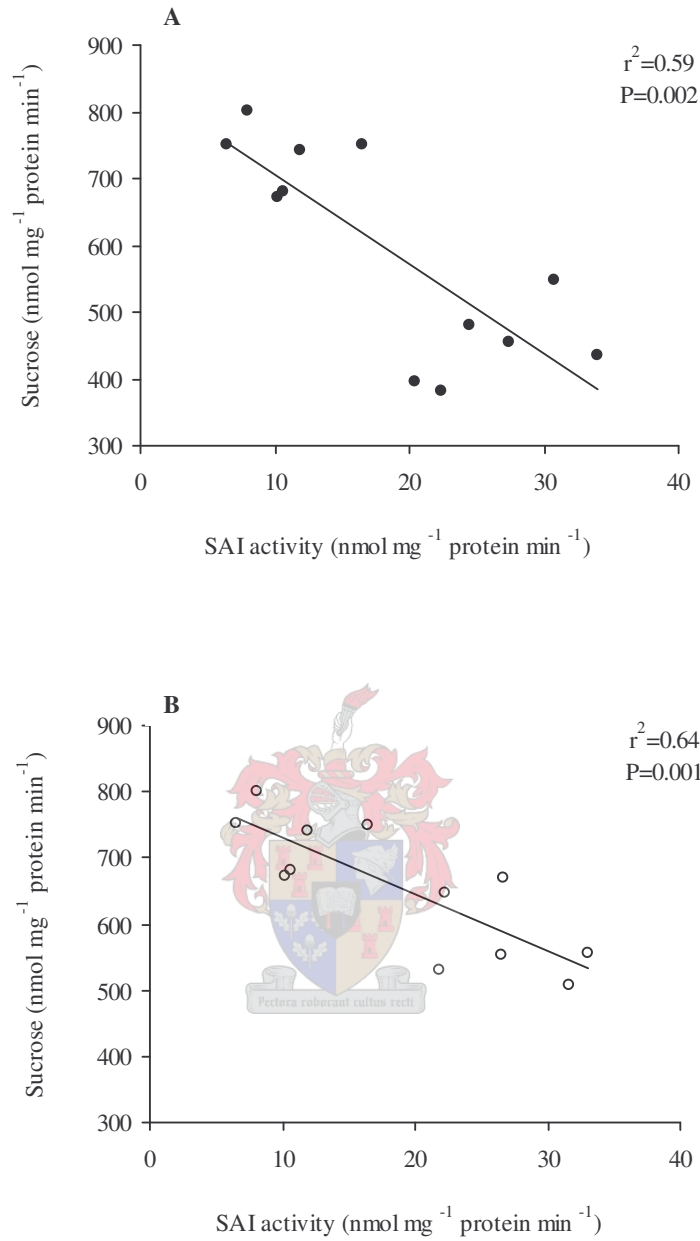
### 4.3 Results

Cellular constituents (sugars, proteins and RNA) were extracted and quantified from the internodal tissues and the shoots on the day of planting (d0) and thereafter every seven days until day 21 from both the setts incubated in total darkness and the setts incubated in the dark/light cycle.

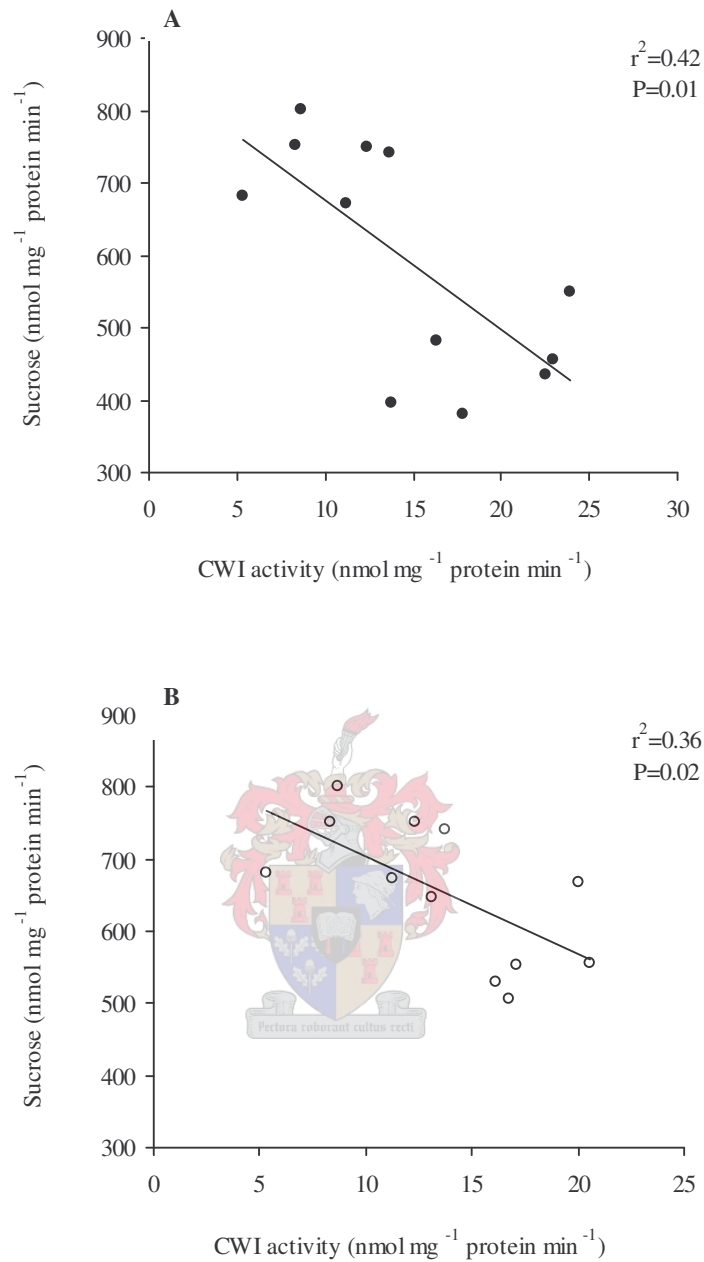
#### 4.3.1 Acid invertase activities and sucrose content in the internodes

Because a negative correlation exists between the activities of acid invertase isoforms and sucrose content (Gayler and Glasziou 1972a), the potential relationship between changes in sucrose concentration in the internodal tissues and acid invertase activities (SAI and CWI) was assessed during the 21-day shooting period. SAI activity showed a significant linear correlation with changes in sucrose concentration in both the setts incubated in dark ( $r^2=0.59$ ) and the setts incubated in the dark/light ( $r^2=0.64$ ) treatment (Fig. 4.1 A and B). In contrast no significant correlation was evident between changes in sucrose content and CWI activity in either the setts incubated in dark ( $r^2=0.42$ ) or in dark/light ( $r^2=0.36$ ) treatments (Fig. 4.2 A and B).





**Figure 4.1:** Relationship between changes in sucrose concentration and the activity of soluble acid invertase during shooting in the internodes incubated in total darkness (A) and in dark/light (B).

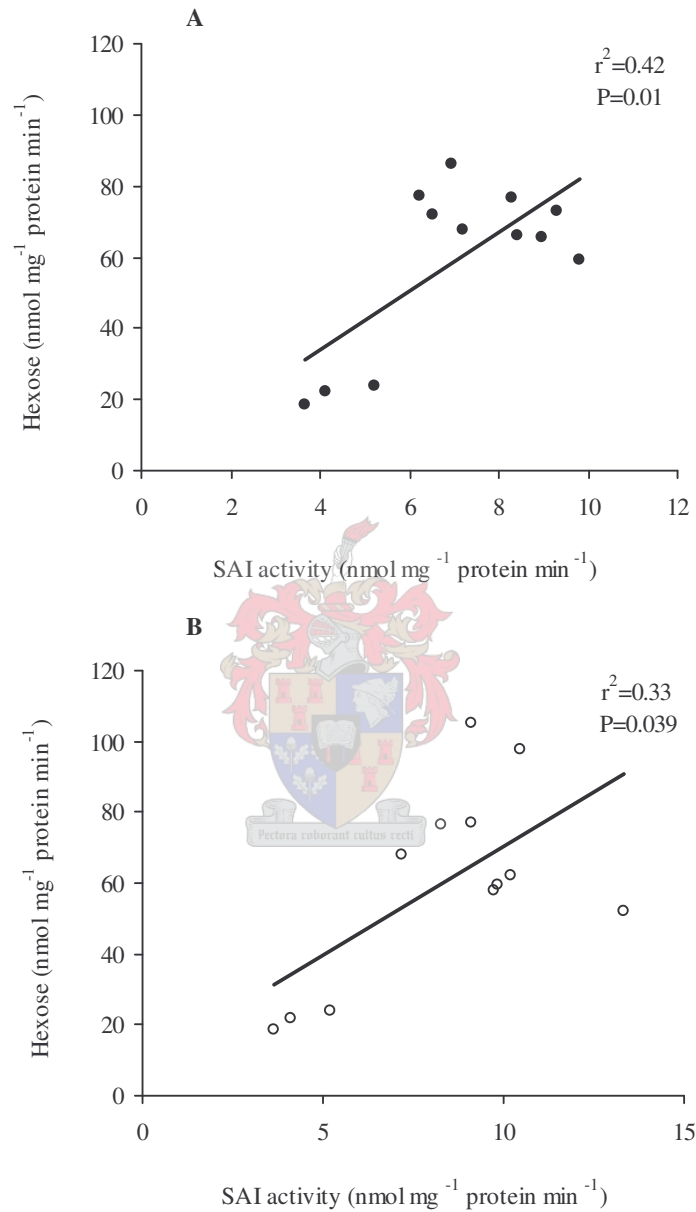


**Figure 4.2:** Relationship between changes in sucrose concentration and the activity of cell wall acid invertase during shooting in the internodes incubated in total darkness (A) and in dark/light (B).

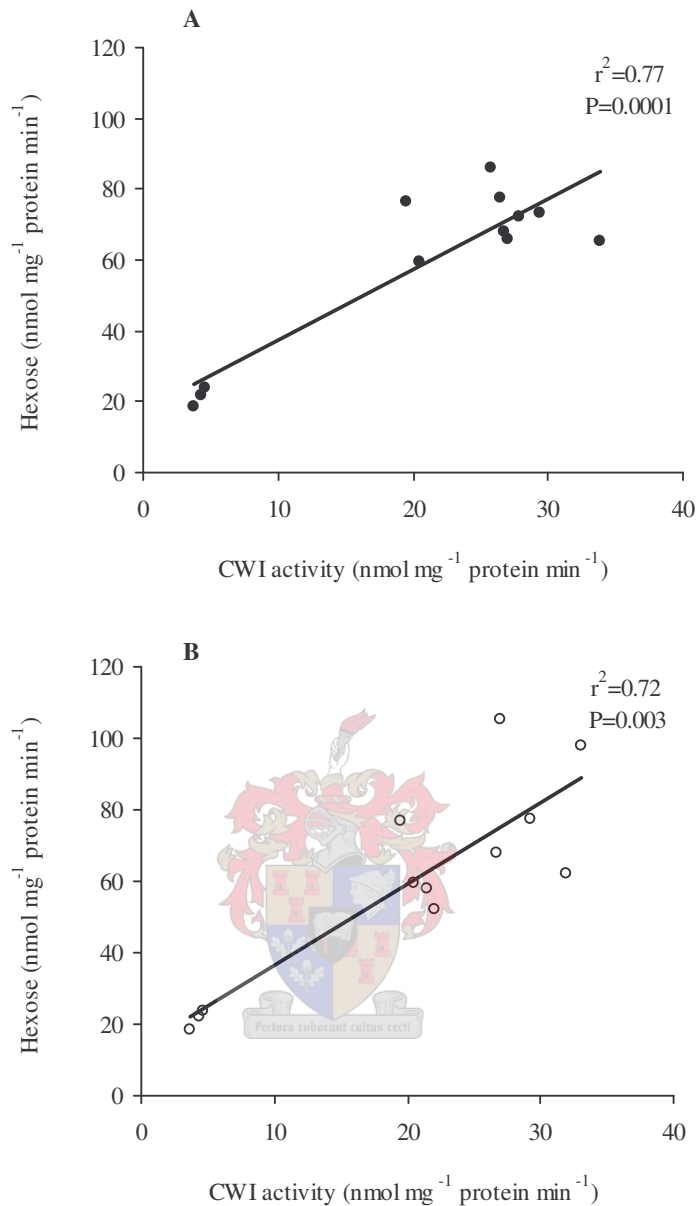
#### 4.3.2 Acid invertase activities and hexose content in the shoots

Since the utilization of sucrose as a source of carbon and energy requires its cleavage by either invertase or sucrose synthase (Kruger 1990) and the provision of growing tissues

with hexoses as a source of energy and carbon (Ap Rees 1974), the hexose concentration was plotted against acid invertase activities in the shoots (Fig.4.3 and Fig.4.4).



**Figure 4.3:** Relationship between changes in hexose concentration and the activity of soluble acid invertase during shooting in the internodes incubated in total darkness (A) and in dark/light (B).

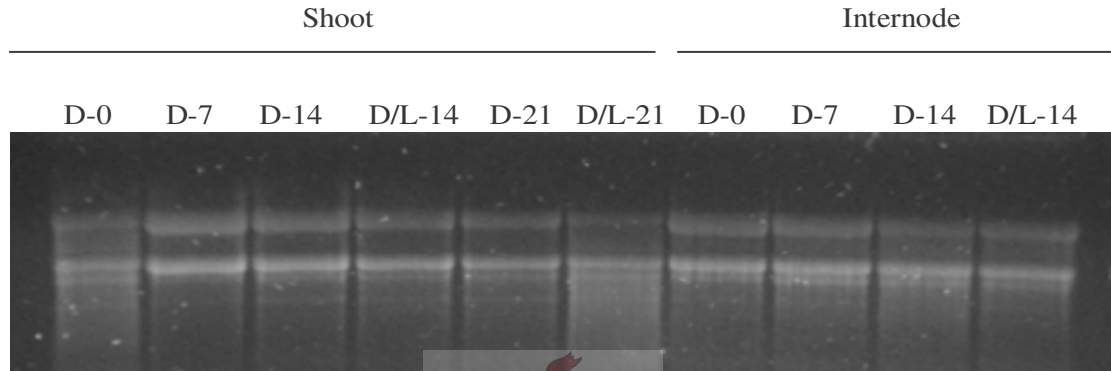


**Figure 4.4:** Relationship between changes in hexose concentration and the activity of cell wall acid invertase during shooting in the internodes incubated in total darkness (A) and in dark/light (B).

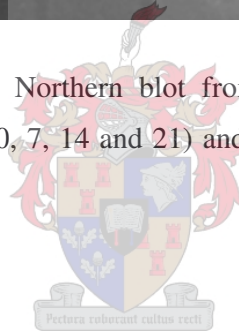
In the shoots, SAI activity showed a weak correlation with the hexose concentration in the plants incubated in total darkness ( $r^2=0.43$ ) and dark/light ( $r^2=0.33$ ) treatments (Fig. 4.3 A and B). Nevertheless, the increase in hexose content was observed throughout the shooting period. This correlated very well with the increase in CWI activity in both the plants kept in dark ( $r^2=0.77$ ) and dark/light ( $r^2=0.72$ ) treatments (Fig. 4.4 A and B).

### 4.3.3 Northern blot analysis

RNA extracted from 10 tissues at different times of shooting was visualised on 1.2% agarose gel and the loading was evidently uniform (Fig.4.5). The two rRNA bands were approximately 2.5kb and 1.5 kb in length.



**Figure 4.5:** RNA extracted for Northern blot from the shoots and the internodes at different times of shooting (day 0, 7, 14 and 21) and light regimes (D, total darkness and D/L, dark/light regime).



The maize SAI cDNA (*Ivr1*) was confirmed by overall sequence comparison to other clones on the GENBANK databases and shows 84% identity to *Saccharum robustum* and *Saccharum hybrid* cultivars soluble acid invertase mRNA.

When the *Ivr1* was used to probe the northern blot, the steady-state levels of SAI mRNA were undetectable in the shoots throughout the shooting period (Fig.4.6B). In contrast, SAI transcripts were detected in the internodal tissues at the latest stage of shooting in both the setts incubated in total darkness and the setts kept in the dark/light cycle (Fig. 4.6B). However, increasing SAI activity in the shoot throughout the shooting period did not demonstrate any transcripts as showed in the internodes (Fig. 4.6A and B).

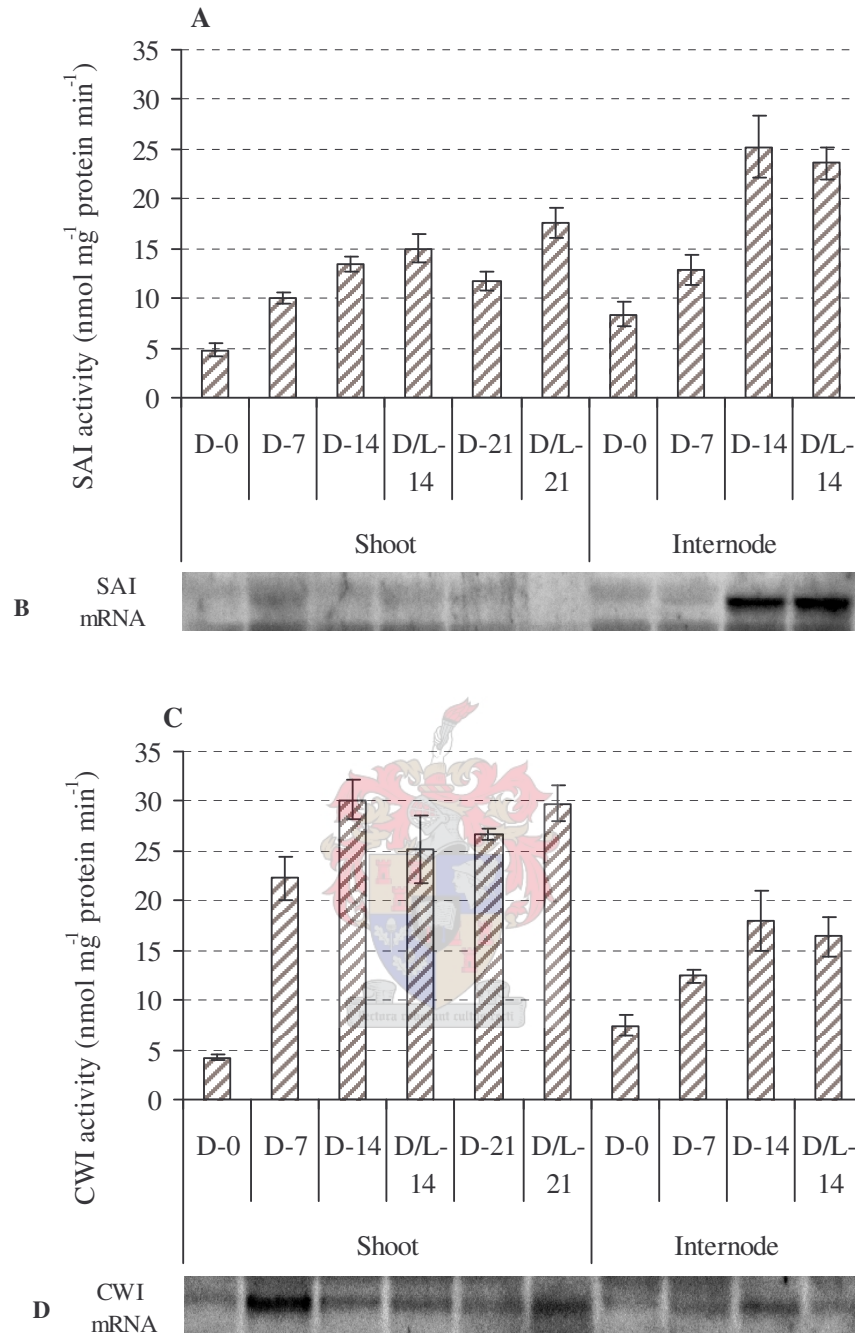
The *Saccharum hybrid* cultivar CWI cDNA (*shcw1*) mRNA was confirmed by sequence comparison to other clones on the GENBANK databases and shows 88%



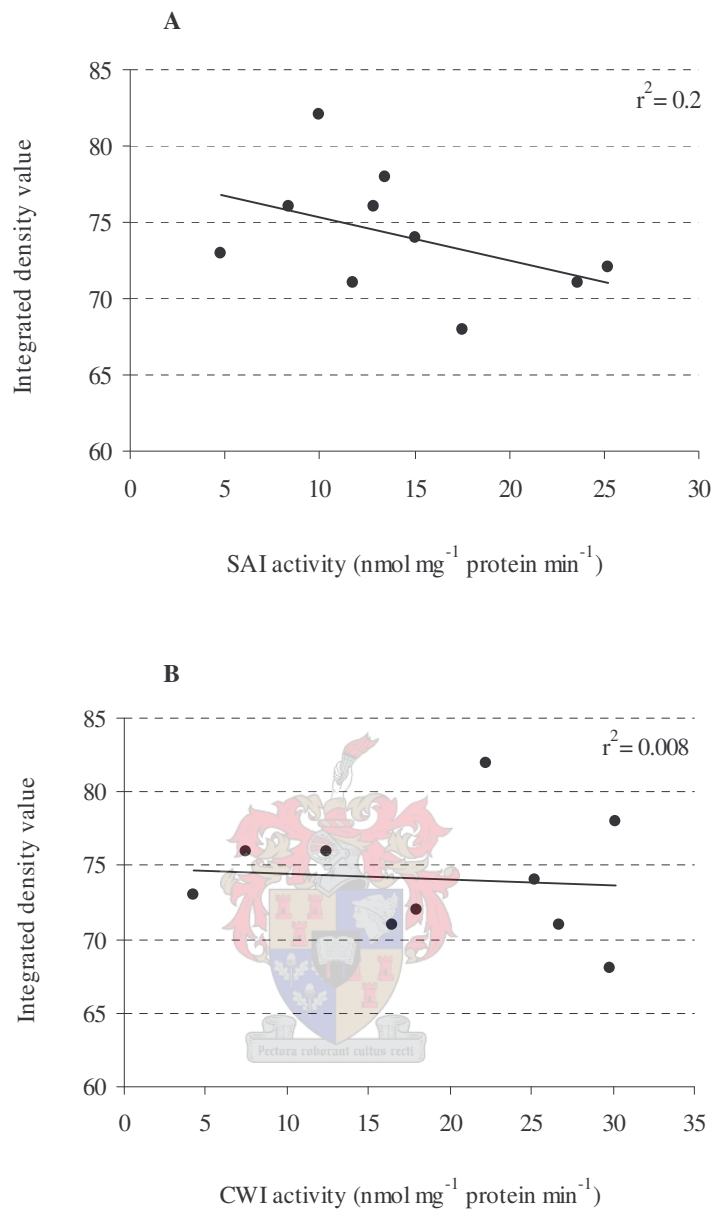
homology/identity to *Zea mays* CWI mRNA and 89% homology/identity to *Zea mays* CWI-inCW1 gene.

The steady-state levels of CWI mRNA in the shoots and internodes were determined by RNA gel blot using *shw1* as probe (Fig.4.6D). Both the shoots and the internodes showed transcript signals. In the shoots CWI mRNA pools started relatively high after seven days of shooting , slightly decreased and remained constant thereafter (Fig.4.6D). In the internodal tissues, the signals started very low after seven days of shooting and slightly increased thereafter (Fig.4.6D). Nevertheless, in the shoots and the internodes, the highest mRNA pools did not necessarily reflect the highest CWI activity (Fig. 4.6C and D). No correlation was observed between either CWI or SAI activity and total RNA pool (Fig. 4.7).





**Figure 4.6:** Enzyme activities and mRNA pool during shooting in shoots and in internodal tissues. SAI activity (A), RNA gel blot hybridized to SAI probe (B); cell wall invertase activity (C) and RNA gel blot hybridized to CWI probe (D).



**Figure 4.7:** Total RNA pool (integrated density value calculated from gel blot) plotted against SAI activity (A) and CWI activity (B).

#### 4.4 Discussion

In sugarcane, ripening involves a reduction in vegetative growth and an increase in sugar content as available water and temperature decline (Glasziou *et al.* 1965). If not harvested the plants return to vegetative growth and the stored sucrose is remobilized. The remobilization of the stored sucrose is facilitated through its breakdown into hexoses via either invertases or sucrose synthase (Leigh 1984, Hawker 1985, Martionoia 1992).

Both, CWI and SAI activities increase as sucrose content decreases in the internodal tissues, which act as the source. Despite the increase in CWI specific activity throughout the shooting period, no significant correlation with changes in sucrose concentration was observed either in setts incubated in total darkness ( $r^2= 0.42$ ) or those incubated in dark/light regime ( $r^2= 0.36$ ). However, the increase in SAI specific activity correlated very well with the decrease in sucrose concentration throughout the shooting period in both the setts incubated in total darkness ( $r^2= 0.59$ ) and those kept in the dark/light regime ( $r^2= 0.62$ ). This is consistent with the fact that the highest sucrose pool in sugarcane is found in the vacuole where soluble acid invertase is localized. Moreover, CWI and SAI activities in the internodes is not necessarily a reflection of hexose release for export to the shoots but probably supply the exporting cells with reduced carbon for energy demands and for internal maintenance (Echeverria 1998).

To investigate the role of acid invertases in the shoots throughout the shooting period, changes in hexose concentration were plotted against the acid invertase activities. The increase in hexose content positively correlates with the increase in CWI activity in both shoots incubated in total darkness ( $r^2= 0.77$ ) and those kept in the dark/light regime ( $r^2= 0.72$ ). This indicates that CWI plays a crucial role by cleaving sucrose and providing hexose to the developing shoots. One of the functions proposed for invertase is to provide growing tissues with hexoses as a source of energy and carbon (Ap Rees 1974). Furthermore, increasing CWI activity in sink tissue is expected to increase sink strength and result in a high rate of sucrose unloading, which may potentially increase the amount of sucrose delivered to the shoots. In contrast, SAI activity demonstrated a relatively weak correlation with changes in hexose concentration in the shoots incubated in total darkness ( $r^2= 0.43$ ) and in the dark/light regime ( $r^2= 0.33$ ). This weak correlation and the lowest

activity of SAI compared to CWI are probably a mere reflection of earlier stage of shoots development.

SAI transcripts were only observed in the internodal tissue after 14 days both in setts incubated in total darkness and in the dark/light cycle (Fig. 4.6 B) and correlated with increased SAI activity (Fig.4.6A). The absence of hybridization signal in the shoots can be attributed to the low abundance of mRNA below the detection limit, to tissue specific and carbohydrate status. In maize, *Ivr1* expression is induced by sucrose starvation (Xu *et al.* 1996).

The study has showed that the CWI is the predominant form of invertase during shoot establishment. This was confirmed by the presence of transcripts throughout the shooting period in both the shoots and the internodes. The highest transcripts were found after seven days in the shoots incubated in total darkness and after 21 days in those kept in the dark/light regime (Fig.4.6 D). The highest CWI activity did not suggest high transcripts; it is well known that high transcripts do not necessarily imply high enzyme activity since there is post-transcriptional control of gene expression. Potentially CWI may be fulfilling multiple roles during shoot establishment since its transcripts were detected in both the shoots and the internodes. Tang *et al.* (1999) has recently proposed a role for CWI in early plant development based on altered leaf/root partitioning in antisense CWI plants. Such a role may involve control of cell expansion via control of metabolic fluxes. In addition, CWI may be acting to prevent loss of sucrose from young leaves to facilitate continued growth and increased sink strength (Sturn *et al.* 1995). The expression of CWI in the internodes suggests enzyme activity for sucrose cleavage to provide the exporting cells with reduced carbon for energy demands and internal maintenance (Echeverria 1998). Hence, CWI expression in the shoots may represent enzyme activity involved in sucrose cleavage to provide growing tissues with hexoses as source of energy.

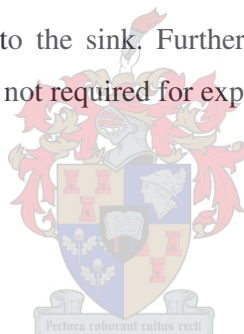
In conclusion, acid invertase isoforms are expressed in both source and sink during shoot establishment. CWI transcripts were observed throughout the shooting period in all tissue types and correlate with the enzyme activity. SAI transcripts observed at the latest stage of shooting in the internodes are merely a reflection of the tissue specific and developmental stage. CWI and SAI are important enzymes in sucrose remobilization and utilisation.

## CHAPTER 5

### Sugar metabolism in germinating sugarcane setts

#### Abstract

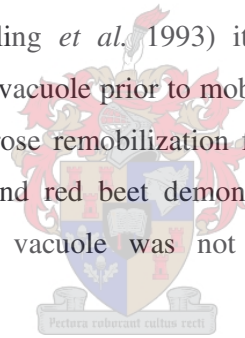
The metabolism of [U-<sup>14</sup>C]-sucrose and [U-<sup>14</sup>C]-glucose during shoot establishment in sugarcane was investigated using uniformly labelled sucrose and glucose. Sugarcane setts were planted and incubated at 32°C for 10 days prior to the injection of radiolabelled sugars in the internodes close to the developing shoot. Despite short labelling periods a cycle of breakdown and synthesis of sucrose was evident in both the internodes and the shoots. After three hours of labelling 17% of the total label was recovered in the developing shoots when applying labelled sucrose compare to 8.5% after applying labelled glucose. From the data it is evident that sucrose is the main form in which reduced carbon is translocated from the source to the sink. Furthermore the data suggests that sucrose hydrolysis in the source tissues is not required for export to the sink.



## 5.1 Introduction

For sustained growth and development, non-photosynthetic plant cells require an exogenous carbon source (Komor *et al.* 1981). In most instances this requirement is met by importation of hexoses or sucrose. Two types of plant cells are particularly well adapted for transport: phloem, which is responsible for mass transfer of assimilates to all parts of the plant, and stalk parenchyma, which serve to store excess sugar. Thus carbohydrates reside in phloem cells in transit, while parenchyma cells store carbohydrate.

Most of the studies done in sugarcane have been focussed on sucrose accumulation and enzymes involved in sucrose metabolism, very little work has been done on sucrose remobilization that supports vegetative growth. From analogy to other saccharide-mobilizing tissues such as tubers of Jerusalem artichokes (Darwen and John 1989) and Japanese artichokes (Keller and Matile 1985), and from the studies in aerated red beet slices (Leigh *et al.* 1979; Milling *et al.* 1993) it has been inferred that sucrose is enzymatically hydrolyzed in the vacuole prior to mobilization (Leigh 1984; Hawker 1985; Martinoia 1992). However, sucrose remobilization from the vacuole of storage cells of sugarcane stalks, carrot roots and red beet demonstrated that acid invertase and thus sucrose hydrolysis within the vacuole was not required for sucrose mobilization (Echeverria 1998).



Upon metabolic demand, stored sucrose is remobilized by storage cells to supply their own physiological needs or those of remote cells such as developing roots and shoots (Echeverria 1998). There are two important issues to consider in the remobilization of sucrose that is stored in the vacuole. Firstly, the sucrose, or its hydrolysis products glucose and fructose, must be transported across the tonoplast. From studies on Jerusalem and Japanese artichokes it was inferred that only the hexoses are exported out of the vacuole (Darwen and John 1989; Keller and Matile 1985). In contrast, in sugarbeet this happens through an  $H^+$ -sucrose antiport system (Briskin *et al.* 1985). Secondly, export of the sugars from the source cell can be through an apoplastic or symplastic route. Unloaded sucrose can be taken up by sink cells either directly via plasma membrane-localized, sink specific sucrose transporters (Gahrtz *et al.* 1996; Weber *et al.* 1997; Lemoine *et al.* 1999), or from monosaccharide transporters after extracellular sucrose hydrolysis by cell wall bound invertases (Sturn and Chrispeels 1990; Weber *et al.* 1995). In the tobacco seed plant, the

H<sup>+</sup>-sucrose cotransporter is of importance for sucrose loading into the phloem via an apoplastic route and possibly for intermesophyll transport as well (Burkle *et al.* 1998).

In an initial effort to understand the process of sucrose remobilization from the storage parenchyma cells (long-term storage), germinating sugarcane setts were investigated. This was done using uniformly labelled sucrose and glucose and thereafter analysing the labelled sugar distribution in both the internodes and the developing shoots. The results indicated that sucrose was the predominant sugar transported to the shoots. The labelled hexose after applying labelled sucrose was a reflection of the activities of the sucrose cleaving/hydrolysis enzymes.





## 5.2 Materials and methods

### 5.2.1 Shoot establishment procedure

Mature non-flowering stalks from field grown (Kwazulu-Natal, South Africa) sugarcane of the commercial variety N19 were used. Setts were cut from nodes 14 to 22 with approximately two centimetres of internode on each side, sealed with candle wax on both ends. The Setts were incubated in containers (750 cm<sup>2</sup>, 10cm depth) filled with sand and incubated in total darkness for 10 days at 32°C, and the planted Setts were watered every three days.

### 5.2.2 Radiolabelling experiments

To investigate sugar transport, 10-day-old plants were kept in containers and transferred to a fume hood at room temperature (20-25°C) for 2h prior to the labelling experiment. A small cylinder of tissue (diameter 1-2.5mm) was removed from both sides of the node with a fine cork borer, almost parallel to the developing shoot and 1cm from the node. 200µl (100µl for each side) of the carrier free buffer containing 250mM mannitol (pH 5.7), [U-<sup>14</sup>C]-sucrose or [U-<sup>14</sup>C]-glucose at final specific activity of 25Bq/nmol (Bindon and Botha 2000) was injected into the hole with a microsyringe, and the hole sealed with petroleum jelly.

### 5.2.3 Tissue sampling and sugars extraction

After 1 hour and then again after 3 hours incubation at room temperature, the young shoot was separated from the internode. The shoots were further divided into a top and bottom half. The internodal tissues were sampled from the site of isotope injection. Three replicates were sampled at each time point during the incubation period. The tissues were finely ground in liquid nitrogen and the resulting powder suspended in a 2:1 ratio (buffer to tissue). The buffer contained 50mM Tris (pH 7) and 70% (v/v) ethanol. The suspension was kept at 70°C for three hours and then centrifuged at 12 000 xG for 10 min. and the supernatant transferred to a new tube. The pellet was re-suspended in 500µl extraction buffer and incubated at 70°C for a further 30 min and then centrifuged at 12000 xG for 10

min and the supernatant added to the original one mL. The final supernatant was then reduced to approximately 25% through evaporation in a vacuum centrifuge.

For total sugars determination, sugars were extracted in buffer containing 50mM Tris (pH 7) and 70% (v/v) ethanol in a 1:10 ratio (m: v), at 65°C overnight and centrifuged at 12 000 xG for 10 min. Sucrose, glucose and fructose concentrations were determined from the supernatant using the standard enzymatic method as described by Bergmeyer and Bernt (1974).

#### 5.2.4 *TLC sugars separation*

Sugars were separated by loading 2µl of sample, onto a thin-layer chromatography (TLC) plate (Silica gel 60, Merck); each sample was loaded in triplicate. The TLC plates were developed twice for 3.5 h with a 30 min drying period between each development in a mobile phase containing ethyl acetate: 2-propanol: water (6:3:1), modified from Randerath (1964). After development, the TLC plate was dried at room temperature for 15 min and then sprayed with a fructose specific stain containing 0.5M urea, 10% (v/v) phosphoric acid, 70% (v/v) 1-butanol, and 5% (v/v) ethanol and incubated at 80°C for 15 min. The separated sucrose and fructose from each sample were scraped for radioactivity counting.



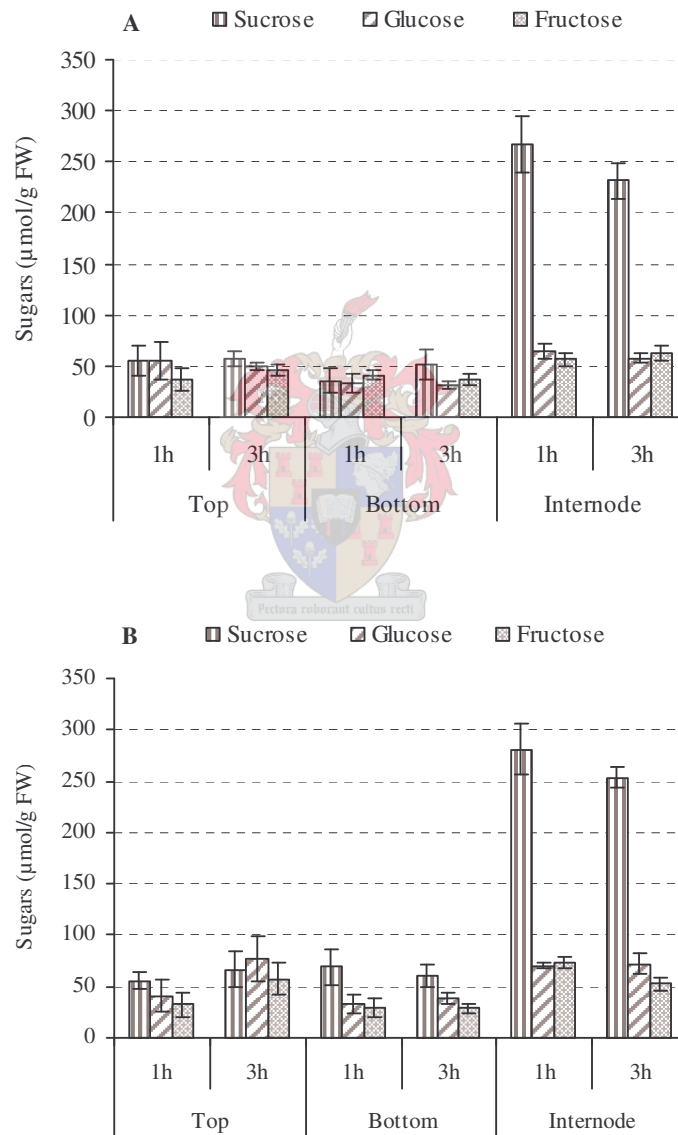
#### 5.2.5 *Scintillation counting procedure*

The radioactivity in the separated sucrose and fructose, as well the total soluble extract were counted in 1ml scintillation cocktail (Ultima Flo™ M) for 20 min in a Beckman LS 1801 scintillation counter. Counts were corrected for quenching using the internal standard of the instrument.

## 5.3 Results

### 5.3.1 Sugars

No significant differences in the total sugar concentrations in the various tissues were observed between the two time points when either labelled sucrose or glucose was applied (Fig 5.1 A and B).



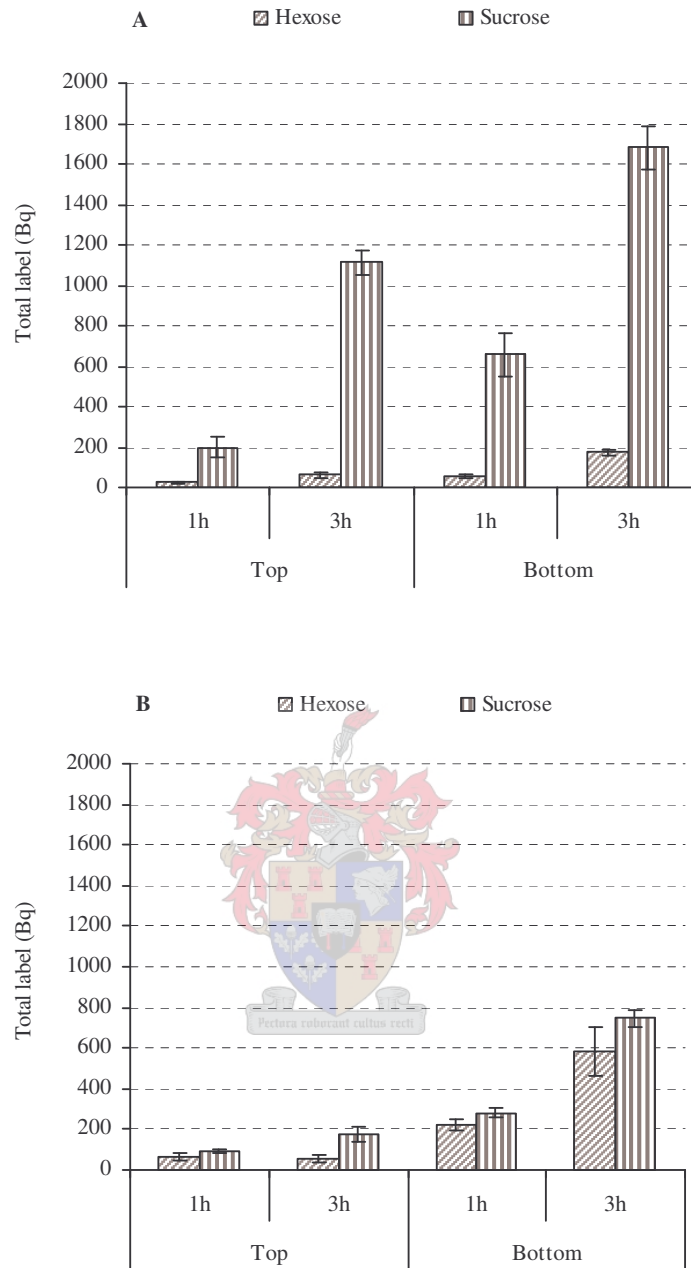
**Figure 5.1** Total sugar content in the young shoots (Top and Bottom) and internode tissues (Internode) after one and after three hours of injection of [U-<sup>14</sup>C]-sucrose (A) and [U-<sup>14</sup>C]-glucose (B). Values are the mean of three replicates  $\pm$  SE.

### 5.3.2 Radiolabelling in sugars

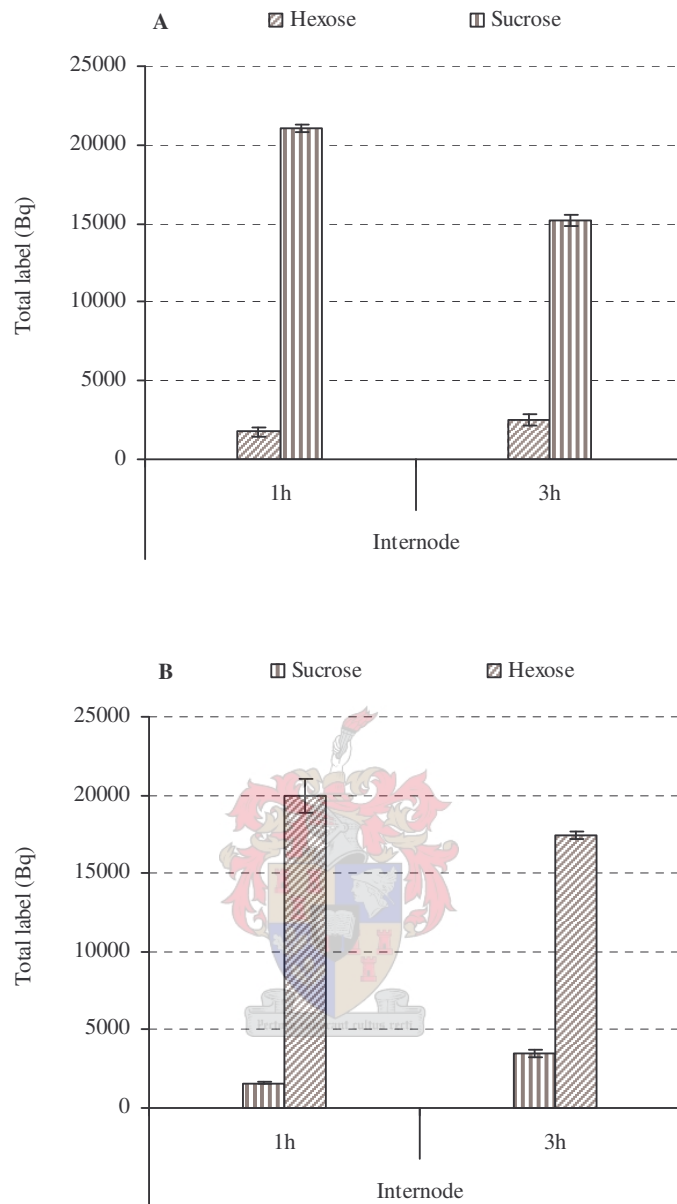
In the shoots, a significant difference between labelled sucrose and hexoses was evident when labelled sucrose was applied. In the top half, labelled sucrose was 7.7 times higher than labelled glucose after 1h and 18 times higher after 3h. In the bottom part, the ratio of labelled sucrose to glucose was 11.69 after 1h and decreased to 9.69 after 3h (Fig.5.2A). However, when labelled glucose was applied, no significant difference between labelled sucrose and hexoses was observed. An increase in sucrose to hexose ratio from 1.44 after 1h to 3.19 after 3h was observed in the top half, while no changes in the ratio was noticed in the bottom part (Fig. 5.2B).

In the internodal tissues, when labelled sucrose was applied, labelled sucrose decreased between 1h and 3h, and was accompanied by a release of labelled hexoses (Fig.5.3A). A similar pattern was observed when labelled glucose was applied, where a decrease in labelled hexose was accompanied by an accumulation of labelled sucrose (Fig.5.3B).





**Figure 5.2** Labelled sugars in the shoots (Top and Bottom) one hour and three hours after injection of  $[U-^{14}C]$ -sucrose (A) and  $[U-^{14}C]$ -Glucose (B). Values are the mean of three replicates  $\pm$  SE.



**Figure 5.3** Labeled sugars in the shoots the injection sites (Internode), one hour and three hours after injection of [U-<sup>14</sup>C]-sucrose (A) and [U-<sup>14</sup>C]-Glucose (B). Values are the mean of three replicates ± SE.

### 5.3.3 <sup>14</sup>C-label distribution

The percentage label distribution was calculated using the total count of the H<sub>2</sub>O-soluble components. Because fructose and glucose spots overlapped when separated using TLC, it was assumed that the free fructose staining represented the total labelled hexose pool. The

other H<sub>2</sub>O-soluble components represented the difference between the total H<sub>2</sub>O-soluble components and the label in sucrose and hexose.

**Table 5.1** Total percentage label allocated to the H<sub>2</sub>O-soluble component one hour and three hours after injection of [U-<sup>14</sup>C]-sucrose (A) and [U-<sup>14</sup>C]-Glucose (B). Tissue samples were from the internode (Internode) and from the shoots (Top and Bottom). Values are the mean of three replicates ± SE.

A					
Component	Time	Internode		Shoot	
			Top	Bottom	
Sucrose	1h	86.32 ± 0.77	1.20 ± 0.22	2.70 ± 0.43	
	3h	67.49 ± 1.45	4.95 ± 0.28	7.48 ± 0.47	
Hexose	1h	7.16 ± 1.12	0.10 ± 0.01	0.23 ± 0.03	
	3h	11.24 ± 1.51	0.27 ± 0.07	0.77 ± 0.05	
Other	1h	1.46 ± 0.34	0.06 ± 0.01	0.76 ± 0.03	
	3h	4.45 ± 0.72	0.14 ± 0.08	3.37 ± 0.76	

B					
Component	Time	Internode		Shoot	
			Top	Bottom	
Sucrose	1h	6.06 ± 0.46	0.40 ± 0.03	1.20 ± 0.09	
	3h	15.00 ± 1.00	0.75 ± 0.17	3.17 ± 0.18	
Hexose	1h	88.23 ± 1.53	0.27 ± 0.06	0.96 ± 0.09	
	3h	74.10 ± 1.00	0.23 ± 0.08	2.28 ± 0.50	
Other	1h	1.70 ± 0.22	0.12 ± 0.02	1.06 ± 0.40	
	3h	2.14 ± 0.33	1.20 ± 0.27	0.91 ± 0.09	

The percentage label present in the endogenous sucrose pool after feeding with labelled sucrose increased with time in the shoots as 4.95% was in the top half and 7.48% in the bottom half after three hours of incubation (Table 5.1 A). In addition, less than 1% of the recovered label was in hexose and 4% in other H<sub>2</sub>O-soluble components after 3h. In the internode, less than 68% of the label remained in sucrose, 11.24% was recovered in the hexose pool and 4.45% in other H<sub>2</sub>O-soluble components.

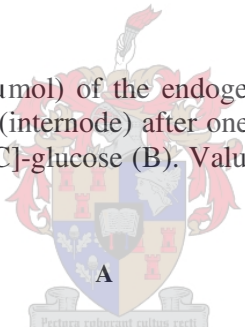
When labelled glucose was applied, less than 1% and 3.17% of the recovered label was found in sucrose respectively in the top half and the bottom half of the shoots after three

hours (Table 5.1 B). In addition, the percentage labelled hexose and the other H<sub>2</sub>O-soluble components increased in both the bottom half and top half (Table 5.1 B). In the internode, 74.1% of the recovered label was still in the hexose pool; 15% was incorporated into the sucrose pool and 2.14% in other H<sub>2</sub>O-soluble components after three hours.

### 5.3.4 Sugar specific activity

The specific activities of endogenous sucrose and hexose pools varied between the shoots and the internodes and were significantly lower than those of the original labelling solutions. Both the sucrose and hexose specific activities increased with the time in the shoots (Table 5.2 A and B). In the internodal tissues, when applying labelled sucrose, the hexose specific activity increased while the sucrose specific activity decreased (Table 5.2 A).

**Table 5.2** Specific activity (Bq/μmol) of the endogenous sugars in the shoots (Top and Bottom) and the site of injection (internode) after one hour and after three hours injection of [U-<sup>14</sup>C]-sucrose (A) and [U-<sup>14</sup>C]-glucose (B). Values are the mean of three replicates ± SE.



	Time	Hexose	Sucrose
Top	1h	0.17 ± 0.03	1.08 ± 0.03
	3h	0.35 ± 0.03	4.41 ± 0.54
Bottom	1h	0.42 ± 0.05	3.16 ± 0.24
	3h	0.68 ± 0.20	7.52 ± 0.98
Internode	1h	1.19 ± 0.34	3.12 ± 0.47
	3h	2.34 ± 0.49	2.13 ± 0.35

### B

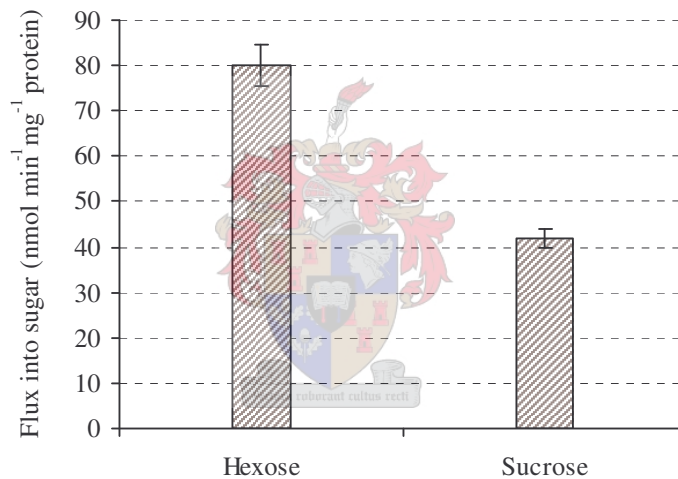
	Time	Hexose	Sucrose
Top	1h	0.60 ± 0.21	0.33 ± 0.02
	3h	0.35 ± 0.02	1.24 ± 0.26
Bottom	1h	1.75 ± 0.23	0.96 ± 0.11
	3h	3.14 ± 0.31	2.56 ± 0.22
Internode	1h	12.27 ± 1.45	0.34 ± 0.02
	3h	10.72 ± 0.24	0.43 ± 0.07



The same pattern was observed when applying labelled glucose, where the increase in sucrose specific activity was accompanied by the decrease in hexose specific activity during the time of incubation (Table 5.3 B).

### 5.3.5 Flux into sugars in the internode

The flux into sugars was determined using the calculated sucrose or hexose specific activity in the internode of the injected labelled sugar types. When injecting labelled sucrose, the flux into hexose was calculated in the internodes using internal sucrose specific activity. The sucrose hydrolysis rate was  $80 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$  (Fig. 5.4).



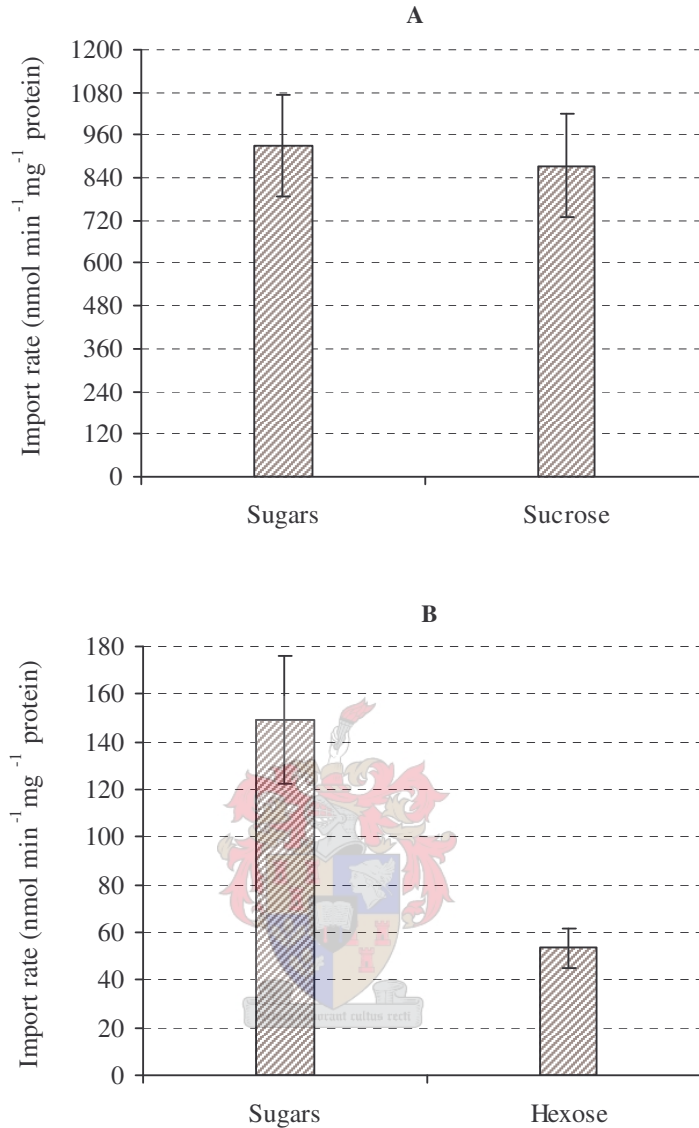
**Figure 5.4** Flux into sugars calculated from the change in amount of labelled sucrose or hexose pool over three hours as a function of the calculated internode specific activity. Flux into hexose was calculated using internode sucrose specific activity when  $[\text{U-}^{14}\text{C}]$ -sucrose was applied. Flux into sucrose was calculated using internode hexose specific activity when  $[\text{U-}^{14}\text{C}]$ -glucose was injected. Values are the mean of three replicates  $\pm$  SE.

When injecting labelled glucose, the flux into sucrose in the internode was calculated using the internal hexose specific activity. The rate of sucrose synthesis was  $41 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$  (Fig. 5.4). In addition, the rate of sucrose hydrolysis was twofold higher than that of sucrose synthesis.

### 5.3.6 *Sugar import to the shoots*

The import rates to the shoots were determined using the internal sucrose and hexose specific activities. Two assumptions were made when applying labelled sucrose. First, we assumed that the entire label in sugars found in the shoots were from both, sucrose and hexose import. Second, we assumed that the labelled sugars in the shoots were exclusively from sucrose transport. In experiments based on both assumptions, no significant difference was found between the rates of total sugars and sucrose import to the shoots (Fig. 5.5A).





**Figure 5.5** Sugar import rates in the shoots calculated from the change in amount of label in the total sugar pool (sugars) and sucrose or hexose pools over three hours as a function of calculated internode specific activity when [U-<sup>14</sup>C]-sucrose was applied (A) and when [U-<sup>14</sup>C]-glucose was applied (B). Values are the mean of three replicates  $\pm$  SE.

When labelled glucose was applied, again two assumptions were made. First, we assumed that the entire label in sugars found in the shoots were from both, sucrose and hexose import. Second, we assumed that the labelled sugars in the shoots were exclusively from hexose transport. The total sugar import rate was three times higher than the hexose import rate (Fig. 5.5B).

Sucrose and hexose import rates into the developing shoots were significantly different. The sucrose import rate was 16 times greater than the hexose import rate (Fig.5.5 A and B). In addition, the total sugar import rate when feeding labelled sucrose was six times greater than the total sugar import rate when the applied labelled sugar was glucose (Fig.5.5 A and B).



## 5.4 Discussion

Because of the high and low concentrations of sucrose and hexose respectively in the internodes (Fig.5.1A&B), the labelled sucrose was diluted more than the labelled glucose. This resulted in low specific activity of sucrose compare to hexose specific activity (Table 5.2). Eventually, after applying labelled sucrose, both sucrose and hexoses were labelled in the shoots and internodes (Fig.5.2 and B). Similarly, after applying labelled glucose, label in sucrose pool was observed in both the shoots and the internodes (Fig.5.3A and B). This is consistent with a hexose-sucrose cycle as described previously in sugarcane (Glasziou 1961; Sacher *et al.* 1963; Wendler *et al.* 1990).

The appearance of over 4 % and 11% of the applied labelled sucrose in the other H<sub>2</sub>O-soluble components and hexose pool respectively (Table 5.1A) gives an indication of sucrose metabolism in the internodal tissues. Sucrose is clearly hydrolysed by invertases (Batta and Singh 1986) or via cleavage by sucrose synthase (Kruger 1990). Injecting radiolabelled sugars in the internodes will result in most of the label ending up in the apoplast and the cytosol, and very little in the vacuole that makes up to 90% of the total cell volume (Komor 1994). This will indicate hydrolysis/cleavage from a pool with a much higher than the average specific activity, mainly in the apoplast and the cytosol. This could probably suggest that hydrolysis is mediated by sucrose synthase, neutral and cell wall invertase (Fig.5.6).

The fructose staining buffer used during the TLC analyses clearly identifies the free fructose as well as the fructosyl moiety of both the applied labelled sucrose and the newly synthesized sucrose. Label in the fructose pool after feeding labelled glucose indicates cleavage/hydrolysis of sucrose synthesized via either SuSy or SPS (Geigenberger and Stitt 1993). The involvement of SuSy in sucrose synthesis was confirmed by the activity observe at the same stage in the internodal tissues (chapter 3). Because the activity of SPS was not determined during this work, it is difficult to determine if it participates in sucrose synthesis during shoot establishment. Nevertheless, in Jerusalem artichoke SuSy is the enzyme responsible for sucrose synthesis during dormancy (Noël and Pontis 2000).

In the shoots, the specific activity of sucrose in top and bottom exceeds that of the sucrose pool in the internode (Table 5.2A). The specific activity in the shoots increases over time

despite the fact that the specific activity in the internodes decreases. This leads to the conclusion that sucrose is transported from a pool of sucrose, of which the specific activity exceeds the average. In other words, sucrose is probably exported primarily from the cytosol and apoplast in the internodes (Fig.5.6). This is partly because most of the applied label ends up in the apoplast and the cytosol. Moreover, the increase in specific activity indicates that sucrose import exceeds sucrose breakdown in the sink (Fig.5.5A). The low specific activity in the hexoses in the shoots and the lowest import rate when labelled glucose was applied further suggests that sucrose breakdown in the internodes is not a prerequisite for transport. Similarly, the study done in red beet hypocotyls, carrot roots and sugarcane stems (Echeverria 1998) even suggests no hydrolysis of sucrose by acid invertase from the vacuole prior to mobilization. This is possible because all three tissues contained a pool of hexoses to supply the exporting cells with reduced carbon for energy demands and for internal maintenance. It is proposed that sucrose transport to the shoots occurs simultaneously with an enzymatic cycle of sucrose synthesis and breakdown (Fig.5.6).

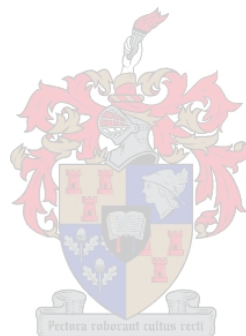
If hexoses were transported from the internodal tissues to the shoots, one could expect comparable labelled hexose in the latter when the labelled glucose was applied, which was not the case (Fig.5.2). In addition, the non-significant difference between the recovered sucrose and hexose pools (Table 5.1B) in the shoots is a reflection of an import rate that depends on the rate of sucrose synthesis in the internodes (Fig.5.4).

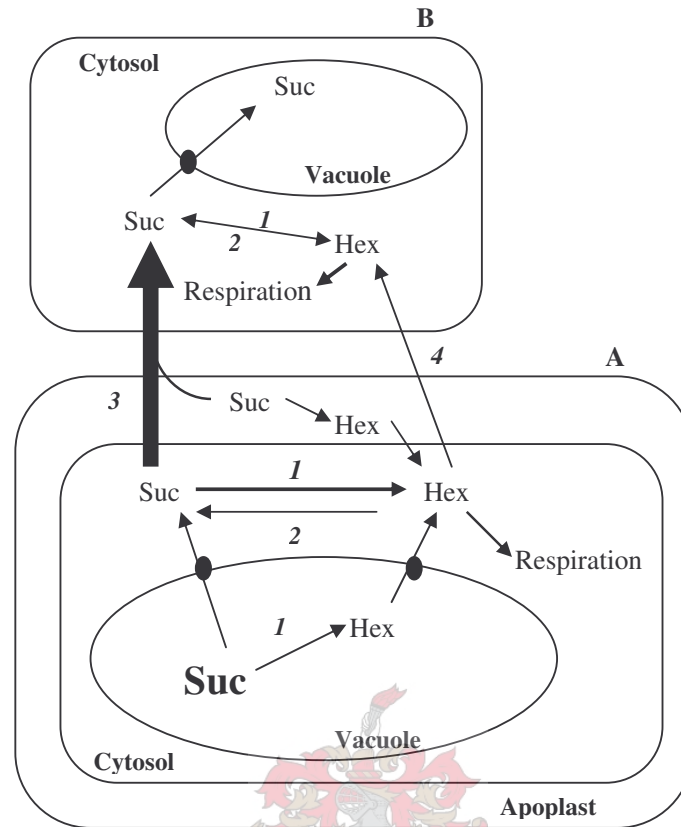
Sucrose compartmentation in sugarcane is not unequivocally established. Thom *et al.* (1982) reported sucrose to be entirely vacuolar. This would present a situation similar to that of red beet hypocotyls where all sucrose utilized had to be of vacuolar origin. If on the contrary, sucrose is equally distributed between the cytosol, vacuole and the apoplast (Preisser *et al.* 1992) and equilibrated by facilitated diffusion, removal of apoplastic and cytosolic sucrose during growth would create a down-gradient movement from the vacuole and sucrose will be mobilized from the vacuole without hydrolysis.

The two alternative pathways for sucrose uptake, either as intact sucrose or as its hexoses after cleavage, are not mutually exclusive. The support for sucrose cleavage was gained from later experiments on sugar uptake in tissue slices using (<sup>14</sup>C-fructosyl)-labelled sucrose that showed approximately 25% of label in the apoplast as [<sup>14</sup>C]-fructose (Lingle

1989). However, less than 2.5% of label was randomized in the sucrose accumulated in sucrose storing internodes and only 1.1% appeared in the glucose moiety of sucrose in meristematic internodes. The conclusion that intact sucrose could be taken up was supported by additional research on the uptake of (<sup>14</sup>C-fructosyl)-labelled sucrose by meristematic internode and by the fact that fluorosucrose, an analogue of sucrose that cannot be cleaved by invertase, was taken up by the tissue at 40% of the efficiency of sucrose (Thom and Marezki 1992). It is likely that sucrose hydrolysis in the internodal tissues (Table 5.1) is to supply the exporting cells with reduced carbon for energy demands and for internal maintenance (Echeverria 1998).

Finally, sucrose is the preferred sugar that is translocated from the source to the sink during sugarcane shoot development and its breakdown into hexose units is not a prerequisite for export to the shoots. In addition, glucose must first be incorporated into sucrose before transported to the developing shoots.





**Figure 5.6** Sugar metabolism during shoot establishment where (A) represent the internodal tissue and (B) the shoots. (1): sucrose cleavage via invertases or sucrose synthase, (2): sucrose synthesis via either sucrose synthase or sucrose phosphate synthase; (3): sucrose transport and (4): possible hexose diffusion. Suc = sucrose, Hex = hexose. Filled circles on the tonoplast could represent either transport proteins or protein channels.



## CHAPTER 6

### General Discussion

Apart from attempts to increase sucrose content of sugarcane through conventional plant breeding, several research programmes are investigating the opportunity to achieve this through genetic manipulation of key reaction steps. Grof and Campbell (2001) proposed that there are four principal rate limiting or co-limiting steps in the entire sucrose accumulation process. These are: (1) Leaf reactions-rate of photosynthesis, enzymes involved in the pathway of sucrose synthesis, and carbon partitioning within the leaf. (2) Rate of phloem loading and transport to the ripening stalk. (3) Rate of sucrose transport into the storage parenchyma and vacuole and (4) the rate of sucrose remobilization to support vegetative growth.

Understanding the steps involved in sucrose remobilization is crucial before attempts are made to alter sucrose accumulation. The reason for this is obviously linked to the possible requirement of sucrose mobilization for both shoot establishment and good ratoons. In an effort to gain such understanding, this work has focussed on three main aspects. The first aspect was to investigate changes associated with sugars and the activity of sucrose metabolizing enzymes during shoot establishment. The second aspect deals with the expression of soluble and cell wall invertase at transcriptional level throughout the shooting period. Thirdly to investigate sugars metabolism to elucidate the mechanism by which sugars are remobilized during shoot establishment.

Detailed analysis of sugars and the maximum extractable sucrose metabolizing enzyme activities showed that sucrose was the main carbon source to support root and shoot development. The sucrose cleaving enzymes (invertase and SuSy) were up regulated in the internodes and the shoots, respectively. During the 21-day shoot establishment period, sucrose accounted for 90% of the remobilized internode's dry mass. Sucrose, the main stored sugar in the storage parenchyma cells, therefore also represents the main source of carbon for internal maintenance and for the support of vegetative growth during shoot establishment. Shoots, roots and respiration are the main competitors for the remobilized sucrose. The carbohydrates allocated to shoots and roots exceeded that lost through respiration during the first seven days of shoot establishment. This repartitioning changed

between day seven and 14, when equal amount of carbohydrate were allocated to shoots and respiration and the rest, i.e. 12 to 15%, accounted for roots formation (chapter 3). During the latest stage of shooting between day 14 and 21, the carbohydrates distributed to respiration increase in plants incubated in total darkness, while in the dark/light cycle the carbohydrate allocation to respiration decreases. It is evident from the carbohydrate allocation to respiration that this phenomenon is for great importance during shoots establishment. Besides, respiration in plants, as in all living organism is essential to provide metabolic energy and carbon skeleton for growth and maintenance.

The sucrose metabolizing enzyme activities were determined throughout the shoot establishment period. The low activity of NI and SuSy (cleavage) in the internodes suggested that sucrose was mainly hydrolyzed in both the vacuole and the apoplast as CWI and SAI were highly induced. In the shoots, the high activity of acid invertases and SuSy (cleavage) during the initial stage of shoot establishment suggested the hydrolysis of imported sucrose by these enzymes. The increasing content of sucrose, glucose and fructose and the high activity of sucrose metabolizing enzymes in the developing shoots reflected a very active metabolic state of these tissues. The high activity of SAI and CWI in the internodes suggested the role played by those two enzymes during shoot establishment. Hexose accumulation in the internodes together with the rate of hexose transport to the developing shoots suggested that sucrose hydrolysis is mainly for internal maintenance and to help sucrose transport.

To determine whether sugarcane acid invertases (SAI and CWI) were subjected to fine or coarse control during shoot establishments, northern blot analyses were done. This was to confirm whether the invertase activities during shoot establishment were due to gene expression. SAI transcripts were only observed in the internodal tissues at the latest stage of shooting, and no signal was found in the shoots. The absence of a hybridization signal in the shoots can be attributed to the low abundance of mRNA below the detection limit. This was observed for SAI activity below  $20\text{nmol mg}^{-1}\text{ protein min}^{-1}$ , regardless of the tissue type. The cell wall acid invertase showed a transcript signal in both the shoots and internodal tissues throughout the shooting period. It is evident that CWI and SAI play crucial role during shoot establishment in both the internodes and the developing shoots. Consequently, the expression patterns of CWI and SAI have to be tightly controlled, both temporally and spatially. Induction of CWI or SAI is mediated via increased transcription

of the corresponding genes in response to a wide range of stress-related and developmental cues (Sturn 1999; Sturm and Chrispeels 1990; Godt and Roitsch 1997).

Since shoot establishment is crucial in sugarcane agriculture, the rate of sucrose remobilization and utilization could be important to achieve good shoot establishment successfully via either re-planting or ratooning systems. The understanding of sucrose metabolism during shoot establishment was undertaken using both labelled sucrose and glucose. The findings showed that a cycle of synthesis and breakdown of sucrose was evident. During the experimental period after applying [U-<sup>14</sup>C]-sucrose in the internodes below the developing shoot, labelled hexose in the sugar pool was observed in the sites of injection. This clearly indicates a sucrose hydrolysis/cleavage in the internodes. Because labelled sucrose represented 90% of the recovered label in the shoots, it was concluded that sucrose was transported uncleaved and the labelled hexose and other H<sub>2</sub>O-soluble components are merely a reflection of sucrose metabolism in the shoots. The import rate calculated, using internal sucrose specific activity, showed there was no significant difference between total sugar import and sucrose import. This confirms that sucrose is the main sugar transported to the shoots and that sucrose is transported uncleaved.

After [U-<sup>14</sup>C]-glucose was applied in the internodes below the developing shoot, label in the sucrose pool was observed. This indicated a sucrose synthesis via either sucrose synthase or sucrose phosphate synthase in the internodes since they are the only enzymes capable of synthesizing sucrose in sugarcane. Since no significant difference was found between labelled sucrose and hexose in the shoots, it was concluded that the rate of sucrose import was low and the hexoses were not the principal sugar transported to the shoots. The import rate calculated using internal hexose specific activity, showed that total sugar import exceeded hexose import and the import rate was low compared to that obtained when applying labelled sucrose. Since sucrose is the main imported sugar, the rate of sugar import after applying labelled hexose will depend on the rate of sucrose synthesis in the internodes. This was confirmed in chapter 5 where sugar transport after applying labelled sucrose was 6.25 times greater than that observed after applying labelled glucose.

In summary, it is evident that sucrose and the sucrose metabolizing enzymes play a crucial role during shoot establishment. Even shown that all the carbon moves primarily as sucrose; sucrose cleavage/hydrolysis is important in the culm storage tissues. Down

regulation of these enzymes in the storage cells could therefore be of great consequence on shoots establishment. In the South African sugarcane biotechnology programme, genetic manipulation of invertase levels is currently underway. The next step will be to analyze the rate of sucrose remobilization and the ratooning capacity in transgenic plants with altered invertase and SuSy (cleavage) activity. This will be important to assess the validity of the current speculation as to the effect of reduced sucrose cleaving enzyme activity in the shoot establishment performance.



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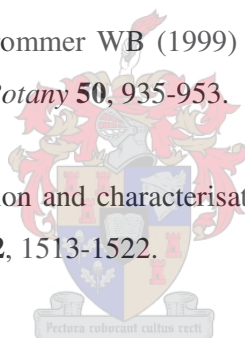
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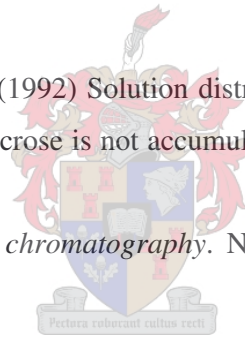
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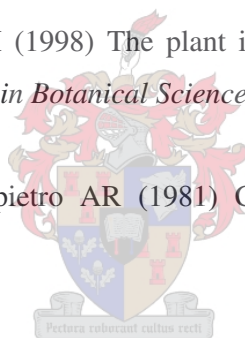
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