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Relationship Between Invertase Enzyme Activities and Sucrose Accumulation in Sugarcane (*Saccharum* spp.)

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ABSTRACT

The sucrose content, and the activities of sugar metabolism enzymes—namely, soluble acid invertase (SAI), neutral invertase (NI) and cell wall invertase (CWI), of various developmental stages of the stem and the relationships among them were investigated in cultivated sugarcane (*Saccharum officinarum* L.), line 20-2248 and wild sugarcane *S. spontaneum*, line S98-244. Stems of both sugarcane species 6–12 mth after planting (MAP) stems were collected and examined with samples consisting of three parts of the internode—the top, middle and bottom—representing relatively immature, maturing and mature internodes, respectively. The results showed that sucrose was the main sugar in the two species. Sugarcane stems of the two species revealed different patterns of sucrose accumulation and activities of invertase (SAI, CWI and NI). The sucrose content of line 20-2248 increased from 6 to 12 MAP and was highest in the bottom part and decreased in the middle and top parts, respectively, whereas the sucrose content of line S98-244 decreased from 6 to 12 MAP and was highest in the middle part than in the lower the bottom and top parts. The activities of SAI, CWI and NI in both sugarcane species were higher in the top part at each growth stage, lower in middle and lowest in the bottom part of the stem. These activities decreased from 6 to 12 MAP with line 20-2248, whereas the activities increased with increasing time after planting for line S98-244. There was a significantly negative correlation between the sucrose content and the activity of SAI, CWI and NI for line 20-2248. With line S98-244, the lowest concentration of sucrose was significantly negative only with SAI activity. The sucrose content correlation with CWI and with NI activity showed negative correlations but they were not significant.

Keywords: soluble acid invertase, neutral invertase, cell wall invertase, sucrose, sugarcane

INTRODUCTION

Sugarcane (*Saccharum officinarum* L.) is the most important economic crop for the sugar industry, with approximately 65–70% of the sugar in the world derived from sugarcane (Carson and Botha, 2002; Lakshmanan *et al*., 2005). In Thailand, according to Office of the Cane and Sugar Board (2013), the planted area of sugarcane in 2013/2014 was 1.52 million ha with an average production of 100 million t of crushed stem with an average commercial cane sugar (CCS) sugar

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percentage (sweetness) of 11.64%, which equates to an average sugar recovery of 100.25 $kg.t^{-1}$ or an annual amount of 10 million t of sugar. The CCS content is rather low when compared with its competitors such as Australia and Brazil where the CCS content is about 12–13% (Siamturakit, 2014). One of the important factors causing low sugar recovery is the limited number of varieties with the ability to accumulate high sucrose levels and this in turn is dependent on the ratio of sucrose synthesis to sucrose break down (Office of the Cane and Sugar Board, 2013).

Sucrose is a primary product which is synthesized by the photosynthesis in the photosynthetic tissue of the leaf mesophyll and is translocated to storage parenchyma cells in the stem (Halford *et al*., 2010). Sucrose accumulation in the sugarcane stem commences after vegetative growth has finished (Moore *et al*., 1997; Batta *et al*., 2011). Sucrose accumulation in the stem can reach up to 62% of the dry weight or 27% of the fresh weight (Bull and Glasziou, 1963; Sachdeva *et al.,* 2011). The sugar recovery percentage is mainly determined by the accumulation of sucrose in the stem (Batta *et al*., 2011). The ability of sucrose accumulation in the sugarcane stem depends on the ability of sucrose synthesis and sucrose break down by the plant itself and the sucrose cleavage in sugarcane is catalyzed by two enzymes (sucrose synthase and invertase) that cleave the sucrose to hexose (Strum and Tang, 1999).

Invertase (beta-fructofuranosidase, E.C 3.2.1.26) is the key enzyme involved in sucrose metabolism, by hydrolyzing sucrose to glucose and fructose as an energy source for cell growth and elongation and other metabolic processes (Strum, 1999; Roitsch and Gonzalez, 2004). Invertase is classified, by subcellular location, optimum pH, solubility and the isoelectric point, into three types of invertase—namely, vacuolar invertase or soluble acid invertase (SAI), cell wall invertase (CWI) and cytoplasmic invertase or neutral invertase (NI) (Quick and Schaffer, 1996; Tymowska and Kreis, 1998; Link *et al.*, 2004; Chandra *et al*., 2012). In sugarcane, sugar in the form of sucrose is translocated through the phloem to the sinks, where it is used for cell growth, metabolism, respiration or storage (Rae *et al*., 2005; Ayre, 2011; Wang *et al*., 2013). Several different physiological functions have been proposed for invertases, that is, to provide growing tissue with hexoses as a source of energy, to generate a sucrose concentration gradient and to partition sucrose between source and sink tissues, as well as to aid sucrose transport (Rees, 1974; Eschrich, 1980; Chandra *et al*., 2012.).

The aim of this research was to investigate the sucrose accumulation pattern, the activities of different invertases and the relationship of the sucrose accumulation and invertase activities at different stages of development and in different parts of the stem of a cultivated sugarcane line and the wild species.

MATERIALS AND METHODS

Plant materials

Sugarcane (*Saccharum officinarum* L.) line 20-2248 (high sucrose accumulation) and wild sugarcane (*S. spontaneum*) line S98-244 (low sucrose accumulation) were planted. All cultural practices undertaken followed the recommendations of Department of Agriculture (2009). Sugarcane stems were collected at 6, 8, 10 and 12 mth after planting (MAP) corresponding to the internodal late elongation, the ripening and the maturity stages of crop development. The stem samples were always collected early in the morning at 0900 hours to minimize the diurnal variations in the enzyme activity and sugar level. The stems were cut into three equal parts—namely, top, middle and bottom representing the relatively immature, maturing and mature storage tissues, respectively (Batta *et al*., 2002; Joshi *et al*., 2013). The samples were peeled and cut into small pieces and frozen in liquid nitrogen for later use. The first experiment involved sucrose content analysis using high performance liquid chromatography

(HPLC) and the second experiment consisted of enzyme activity assay for SAI, NI and CWI.

Sugar extraction and determination

Sugar was extracted using the protocol of Lu *et al*. (2011). The frozen tissue samples (1–2 g) were ground to powder in liquid nitrogen. Then, 10 mL of 80% ethanol was added and incubated at 80 °C in a water bath for 30 min. The samples were centrifuged at 12,000 revolutions per minute (rpm) at 4 °C for 20 min. The supernatant was removed and the volume was recorded; the residues were extracted with 10 mL of 80% ethanol by incubating at 80 °C in a water bath for 30 min and centrifuging at 12,000 rpm for 20 min. The supernatant was removed and combined with the first supernatant and recorded as the final volume. All the ethanol was removed from the supernatant by incubating at 95 °C in a water bath for 60 min. The sucrose content was determined using HPLC with a reflective index detector set at 80 °C and an Aminex HPX-87P column (300 × 7.8 mm), the mobile phase was deionized water at a flow rate of 0.6 $mL.min^{-1}$.

Enzyme extraction and assay

Enzymes were extracted using the protocol of Lontom *et al.* (2008). Frozen tissue samples, each 1–2 g, were ground to powder in liquid nitrogen. Soluble enzymes were extracted in 8 mL of extraction buffer I (50 mM (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid sodium salt, [HEPES-NaOH] at pH 7.5, 0.05% (weight per volume, w/v) Triton-X, 5 mM Na-ethylenediaminetetraacetic acid (EDTA), 5 mM dithiothreitol (DTT) and 10 mM $MgCl_2$). The cell wall residue was removed by centrifugation at 12,000 rpm at 4°C for 20 min. The supernatant volume was recorded. The soluble enzymes were concentrated using acetone precipitation. The pellet was re-solubilized in 300 µL of extraction buffer II (50 mM HEPES-NaOH at pH 7.5, 0.5 mM, Na-EDTA, 5 mM $MgCl_2$ and 2.5 mM DTT); these samples were prepared for SAI and NI assay, respectively. Insoluble enzyme was solubilized by incubating the cell wall residue in 2 mL of extraction buffer III (100 mM HEPES-NaOH at pH 7.5, 0.05% (w/v) Triton-X, 5 mM Na-EDTA, 10 mM $MgCl_2$, 5 mM DTT and 1 M NaCl) overnight at 4 °C; this sample was prepared for CWI assay.

SAI, NI and CWI were each assayed in a final volume of 100 µL of reaction mixture that consisted of 25 µL of 50 mM Na-citrate buffer at pH 3.5 for CWI activity assay, or 25 µL of 50 mM Na-citrate at pH 5.0 for SAI activity assay or 25 µL of 50 mM potassium phosphate buffer at pH 7.5 for NI activity assay, and also 50 µL of 125 mM sucrose and 25 µL of sample, with an incubation temperature of 37 ^{o}C. The reactions were stopped at 0 and 20 min by 100 µL of alkaline reagent followed by boiling for 15 min and then keeping on ice for 3 min. The reducing sugar products were determined using Nelson's arsenomolybdate reagent method (Nelson, 1944). The total soluble protein was determined using Lowry's method (Lowry *et al*., 1951). One unit of invertase enzyme was defined as the amount of enzyme used to hydrolyse sucrose to reducing sugar at 1 $nmol.min^{-1}$.

Statistical analysis

A factorial completely randomized design was used for each experiment. Correlations between the sucrose content and enzyme activity were determined using Pearson's correlation using the R-Program for Windows, version 2.10.1. (R Core Team, 2014).

RESULTS AND DISCUSSION

Sucrose content

The sucrose contents in different parts of the internode that is, the top (immature) relative to the middle (maturing) and bottom (mature) at different ages after planting in both species were different (Figure 1). In cultivated sugarcane line 20-2248, the average sucrose

content (123.87 mg·g^{-1} fresh weight, fr.wt.) was 2-fold higher than that of the wild sugarcane, line S98-244, (62.01 mg.g^{-1} fr.wt.). For line 20-2248, the sucrose content increased with increasing months after planting (MAP) from 6 MAP (76.81 mg.g^{-1} fr.wt.) to 12 MAP (153.16 mg.g^{-1} fr.wt). However, the sucrose content of every part did not significantly increase after 8 MAP. In each part of the internode, the sucrose content in the bottom part (145.42 mg·g^{-1} fr.wt.) was not significantly different from the middle part (140.42 mg·g^{-1} fr.wt.) but was significantly greater than that of the top part (88.50 mg·g^{-1} fr.wt.). In line S98-244, the sucrose content increased gradually from 6 MAP (74.33 mg·g^{-1} fr.wt.) to 8 MAP and after this period, it significantly decreased until 12 MAP (44.99 mg·g^{-1} fr.wt.). The sucrose content was highest in the middle internode part (74.75 mg·g^{-1} fr.wt.) and lowest in the top part (51.86 mg·g^{-1} fr.wt.), particularly at 10 and 12 MAP. The sucrose content was not significantly different between the middle and bottom parts (Figure 1). As expected, the sucrose content in line 20-2248 (high-sucrose accumulation) was higher than in line S98-244 (low-sucrose accumulation). The increase in the sucrose content with age in line 20-2248 and the decrease in S98-244 observed in the current experiment were similar to those reported by Verma *et al.* (2011) where the sucrose content in a high sucrose-accumulating cultivar increased during the period 240 to 390 DAP but decreased in low sucrose-accumulating cultivars. Furthermore, Batta *et al*. (2011) found that the sucrose content in the stem increased gradually with age from 44.6 to 160.5 to 188.2 mg·g^{-1} fr.wt at stem elongation, stem ripening and maturity, respectively. Gutierrez-Miceli *et al*. (2002) also found that the sucrose content in the stem changed over time depending on the variety.

Invertase activity

The activities of SAI in different parts of the internode and at different ages after planting in both species were different (Figure 2). In S98-244, the average enzyme activity was 2.6 fold (46.48 nmol·min^{-1}·mg^{-1} protein) higher than that of line 20-2248 (18.12 nmol·min^{-1}·mg^{-1} protein). With line 20-2248, the SAI activity gradually decreased with increasing time after planting from 6 MAP to 12 MAP. In each part of the internode, the mean SAI activity of the middle part (22.56 nmol·min^{-1} ·mg^{-1} protein) was not significantly different from

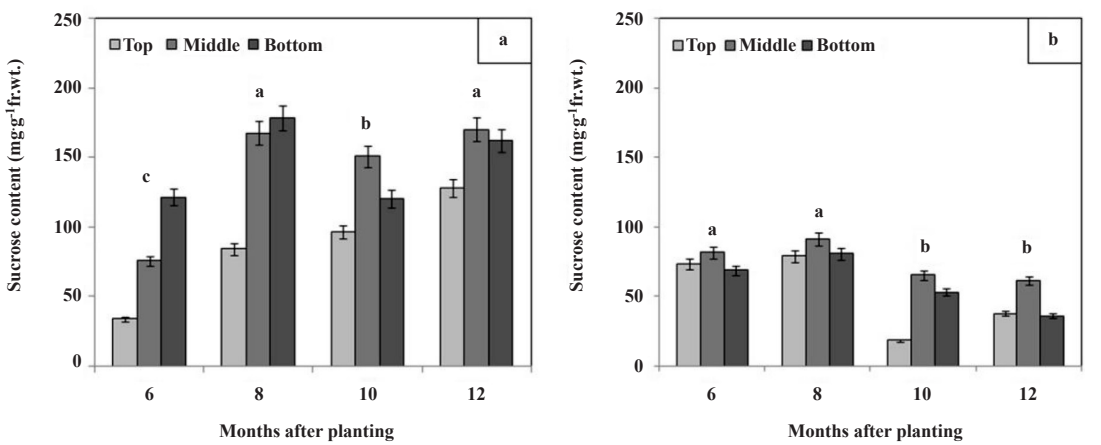

Figure 1 Sucrose content measured on a fresh weight (fr.wt.) basis in top, middle and bottom parts of sugarcane stems at different months after planting of: (a) Cultivated sugarcane line 20-2248; and (b) Wild sugarcane line S98-244. Error bars represent mean ± SE of three replicates; among sets of data at each time after planting, different lowercase letters indicate a significant difference at $P < 0.05$.

the top part (21.12 nmol·min^{-1}·mg^{-1} protein) but was significantly greater than the bottom part (10.68 nmol·min^{-1}·mg^{-1} protein) except at 10 MAP when the middle part had the lowest SAI activity. With line S98-244, the mean SAI activity increased with increasing months after planting from 6 MAP (8.39 nmol·min^{-1}·mg^{-1} protein) to 12 MAP (75.88 nmol·min^{-1}·mg^{-1} protein) and was greatest in the top part (88.51 nmol·min^{-1}·mg^{-1} protein) and lower in the middle and the bottom parts (42.21 and 8.72 nmol·min^{-1}·mg^{-1} protein, respectively). The greater SAI activity in the immature internode (top) compared with the mature internode (bottom) in both cultivated lines was in agreement with Verma *et al*. (2011) who reported that the SAI activity was higher in the immature internode compared to the mature internode. The SAI activity decreased with increased DAP indicating that decreased SAI activity was correlated with maturing sink tissue (Kubo *et al.,* 2001; Verma *et al.,* 2011). The SAI may play a role in the remobilization of stored sucrose from the vacuole (Sacher *et al*., 1963) and its activity was high in the elongation phase. Venkataramana *et al.* (1991) found that the SAI activity was high when growth of sugarcane was rapid and SAI activity was high, usually in tissue that was rapidly growing during cane growth and development, such as the immature internode (Hatch and Glasziou, 1963).

The activities of cell wall invertase (CWI) in different parts of the internode and at different ages after planting in both sugarcane lines were different (Figure 3). The average CWI activity of line 20-2248 (48.47 nmol·min^{-1}·mg^{-1} protein) was about 2-fold higher than that of line S98-244 (24.33 nmol·min^{-1}·mg^{-1} protein). With line 20-2248, the mean CWI activity decreased with increased period after planting from 6 MAP (131.90 nmol·min^{-1}·mg^{-1} protein) to 12 MAP (8.21 nmol·min^{-1}·mg^{-1} protein). The CWI activity of the top internode part (60.01 nmol·min^{-1} ·mg^{-1} protein) was not significantly different from the middle part (57.94 nmol·min^{-1}·mg^{-1} protein) but it was generally greater in the bottom part (33.09 nmol·min^{-1}·mg^{-1} protein). With line S98-244, the mean CWI activity increased from 6 MAP (12.53 nmol·min^{-1}·mg^{-1} protein) to 12 MAP (40.43 nmol·min^{-1}·mg^{-1} protein) and was greatest in the top part (39.95 nmol·min^{-1}·mg^{-1} protein) and decreased in the middle and bottom parts (27.64 and 5.40 nmol·min^{-1}·mg^{-1} protein, respectively),

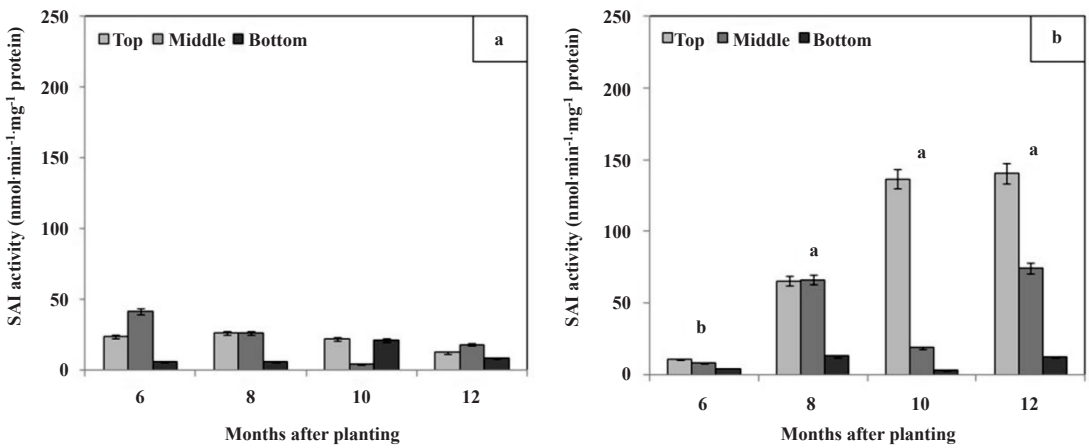

Figure 2 Soluble acid invertase (SAI) activity in top, middle and bottom parts of sugarcane stems at different months after planting of: (a) Cultivated sugarcane line 20-2248; and (b) Wild sugarcane line S98-244. Error bars represent mean ± SE of three replicates; among sets of data at each time after planting, different lowercase letters indicate a significant difference at $P < 0.05$.

particularly at 10 and 12 MAP (Figure 3). The results for line 20-2248 were in accordant with Batta *et al*.(2008) in that the maximum CWI occurred at the stem elongation stage (175 DAP) and declined thereafter in each part of stem. CWI is localized in the apoplast, is ionically linked to the cell wall and the activity remains measurable during different stages of plant development (Xu *et al.*, 1996). Vorster and Botha (1998) also referred to an apoplastic soluble form of acid invertase that was believed to be present in sugarcane. The cell-wall-associated isoform function is the hydrolysis of sucrose to glucose and fructose in the apoplast, yielding monosaccharides that are actively transported across the parenchyma plasmalemma (Roitsch and Gonzalez, 2004).

The neutral invertase (NI) activity of different parts of the internode at different months after planting between line 20-2248 and line S98-244 were not significantly different (Figure 4). With line 20-2248, the NI activity decreased from 6 MAP (74.61 nmol·min^{-1}·mg^{-1} protein) to 12 MAP (20.95 nmol·min^{-1}·mg^{-1} protein). In each part of the internode, the average NI activity was greater in the top part (59.31nmol·min^{-1}·mg^{-1} protein) and lower in the middle (34.47 nmol·min^{-1}·mg^{-1} protein) and lowest in the bottom part (18.55 nmol·min^{-1}·mg^{-1} protein). Similar results were reported by Dendsay *et al*. (1995) and Rossouw *et al*. (2010)—namely, that NI activity was low in the mature storage tissue of many high-sugar storing, early-maturing varieties of sugarcane. With line S98-244, the mean NI activity at different cane growth stages was relatively stable at 6 MAP (36.08 nmol·min^{-1}·mg^{-1}protein) to 8 MAP (33.77 nmol·min^{-1} ·mg^{-1} protein) and was highest at 10 MAP (47.35 nmol·min^{-1}·mg^{-1} protein) and less at 12 MAP (42.34 nmol·min^{-1} ·mg^{-1} protein). In each part of the internode, the mean NI activity was greater in the top part (88.81 nmol·min^{-1}·mg^{-1} protein), lower in the middle part (27.17 nmol·min^{-1} ·mg^{-1} protein) and lowest in the bottom part (5.64 nmol·min^{-1}·mg^{-1}protein) as shown in Figure 4.

Zhu *et al*. (1997) suggested that the highest SAI activity was associated with low levels of sucrose in immature stem internodes and, conversely, the lowest levels of SAI activity were associated with high levels of sucrose in mature internodes. Siswoyo *et al*. (2007) found a negative and significant correlation between NI and SAI with sucrose accumulation.

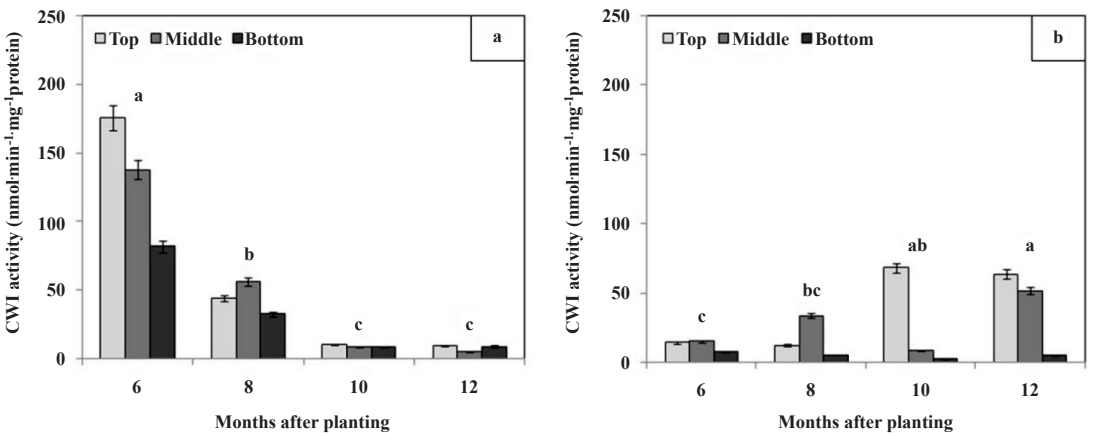

Figure 3 Activities of cell wall invertase (CWI) in top, middle and bottom parts of sugarcane stems at different months after planting of: (a) Cultivated sugarcane line 20-2248; and (b) Wild sugarcane line S98-244. Error bars represent mean ± SE of three replicates; among sets of data at each time after planting, the same lowercase letter indicate no significant difference at $P < 0.05$.

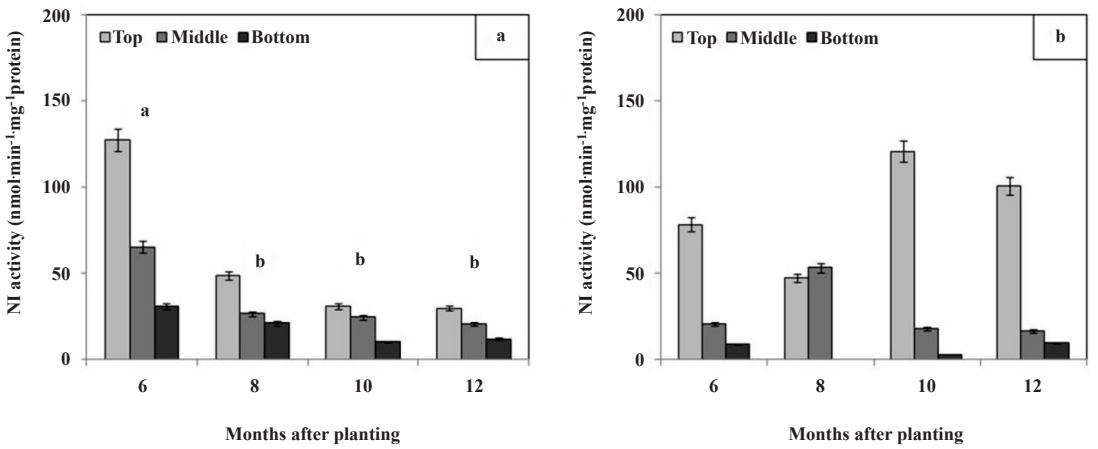

Figure 4 Activities of neutral invertase (NI) in top, middle and bottom stem parts of sugarcane stems at different months after planting of: (a) Cultivated sugarcane line 20-2248; and (b) Wild sugarcane line S98-244. Error bars represent mean ± SE of three replicates; among sets of data at each time after planting, different lowercase letters indicate a significant difference at $P < 0.05$.

Relationship of invertase activity to sucrose content

There was a significantly negative correlation in both sugarcane lines between the sucrose content and invertase enzyme activity (Figure 5). As the plant matured, the invertase enzyme activity increased but the sucrose content decreased. Plotting the activity of the different enzymes versus the concentration of sucrose gave a significantly negative correlation for SAI (correlation coefficient, r = -0.4743, $P < 0.05$), CWI (r = -0.6952, $P < 0.001$) and NI (r = -0.7352, $P < 0.001$) with line 20-2248. Similarly, with line S98-244, the concentration of sucrose gave a significantly negative correlation for SAI (r = -0.4498, $P < 0.05$); however, the concentration of sucrose was not significantly different compared to CWI (r = -0.3795, $P > 0.05$) and NI (r = -0.3857, $P > 0.05$). The results of the experiments showed that the accumulation of sucrose in the stem was low, where the activity of SAI was high. SAI activity is usually high in tissues that are rapidly growing in the immature stem internode (Zhu *et al*., 1997; Gutierrez-Miceli *et al*., 2002). Lontom *et al.* (2008) showed that the sucrose content of sugarcane during ripening was negatively correlated with the SAI activity in two studied cultivars (r = -0.449 and -0.503, respectively), and Pan *et al*. (2009) found a negative correlation for the sucrose content with both the SAI and NI activity. The results of the present study indicated that the SAI activity was lowest in the sugarcane cultivar line with high sugar accumulation and was highest in the sugarcane line with low sugar accumulation. This may indicate the important role of SAI with regard to the total sugar content of the sugarcane cultivar as Ma *et al*. (2000) stated that SAI had a negative correlation with and was important in the accumulation of sucrose.

CONCLUSION

In cultivated sugarcane line 20-2248, the average sucrose content was higher than that of wild sugarcane in all stem segment types regardless of the month after planting. There was a negative and generally significant correlation between the sucrose content and enzyme activity (SAI, CWI and NI) in both sugarcane species. As the plant was maturing, the invertase enzyme activity increased

but the sucrose content decreased. In the cultivated line 20-2248, the activities of SAI, CWI and NI decreased with increasing of months after planting and decreased with increasing maturation of the internode. With line S98-244, the enzyme activity increased with increasing time after planting and the activity levels of SAI, CWI and NI were significantly and negatively correlated with sucrose accumulation. Although, the activities of SAI, CWI and NI for line S98-244 were negatively correlated with sucrose accumulation, only SAI was significant. The data from these studies supported the hypothesis that invertase enzymes, especially SAI, play a key role in determining the sucrose concentration during ripening. The results also provide references for further research on regulating the sucrose accumulation in the sugarcane stalk at the molecular level.

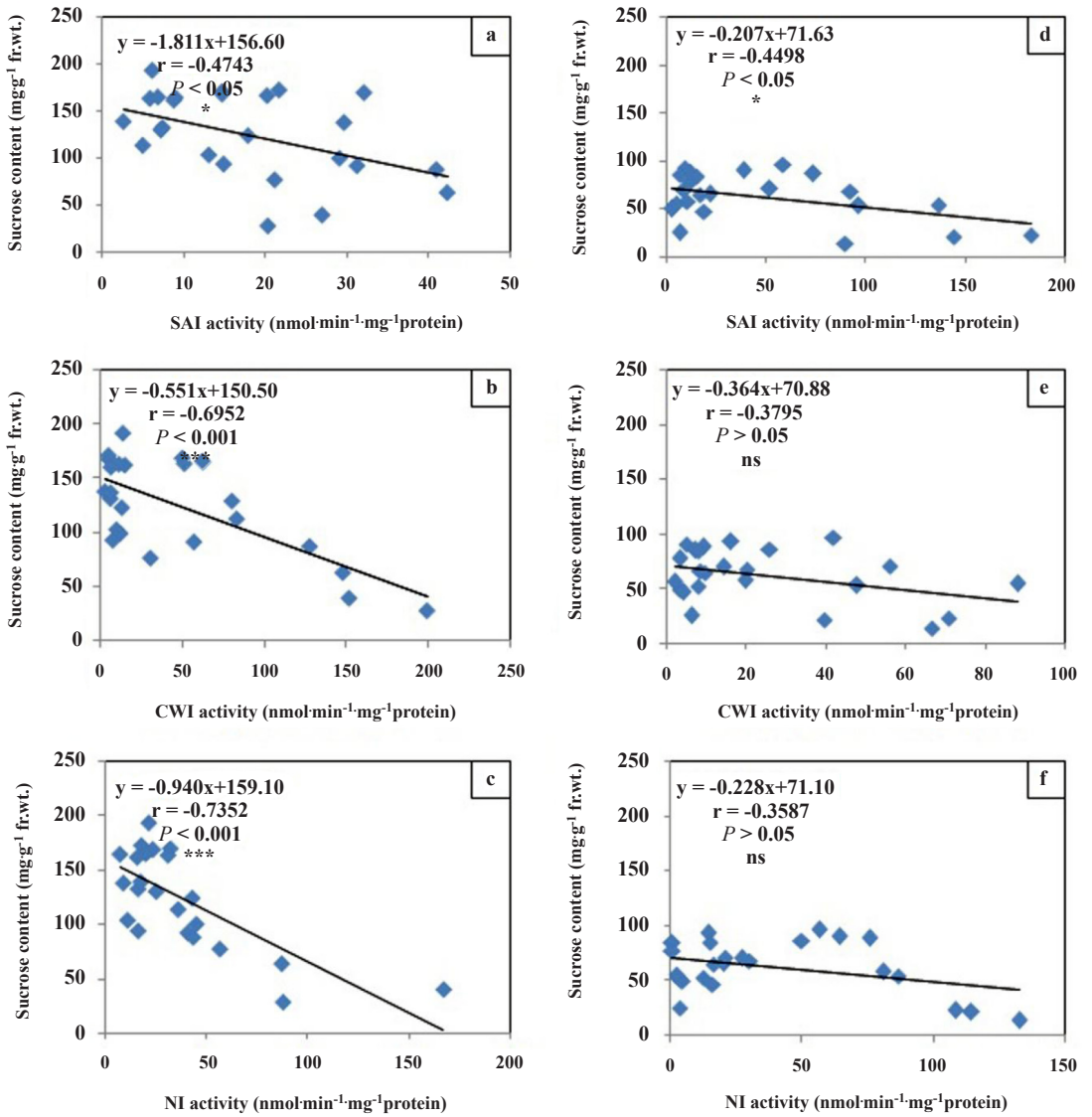

Figure 5 Relationship between sucrose content and specific enzyme activity of soluble acid invertase (SAI), cell wall invertase (CWI) and neutral invertase (NI) in sugarcane line 20-2248 (a, b and c, respectively) and line S98-244 (d, e and f, respectively). (r = Correlation coefficient; * and *** = Significant at the $P < 0.05$ and $P < 0.001$ levels of probability, respectively; ns = Not significant).

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