

CHANGES OF SUCROSE CONTENT AND INVERTASE ACTIVITY DURING SUGARCANE STEM STORAGE

Tri Agus Siswoyo^a, Ika Oktavianawati^a, Djenal^a, Untung Murdiyanto^b, and Bambang Sugiharto^a

^aResearch Center of Molecular Biology, University of Jember, Jalan Kalimantan III/23, Jember 68121, Indonesia

^bPT Perkebunan Nusantara XI (Persero), Jalan Merak 1 Surabaya 60175, Indonesia

ABSTRACT

Invertases (beta-D-fructofuranosidase, E.C. 3.2.1.26) are the key enzymes involved in sucrose metabolism in sugarcane plants. They are highly correlated with sucrose and reducing sugar contents during plant growth. The sugarcane plants have two kinds of invertases, namely neutral invertase (NI) and acid invertase (AI). They have different function in sucrose accumulation. The research aimed to study the role of AI and NI in accumulation of reducing sugar during storage of sugarcane stems. Plant materials of 18-month-old field grown sugarcane of the commercial variety R-579 (wet-land) and M 442-51 (dry-land) were used. Three internodes were sampled to represent immature (internode 1-8/F1), maturing (internode 9-16/F2), and mature (internode 17-24/F3) stem tissues. All tissues were stored for 0-9 days at room temperature (28-32°C) and each day, the sample was extracted to determine invertase activity, total soluble protein, and sugar contents. This observation was valid for invertase activity expressed on a protein basis. At the initiate harvested (0-3 days), NI had a higher specific activity than AI in the sucrose-accumulating region of the sugarcane stems. Negative significant correlation was found between NI specific activity and sucrose accumulation ($r^2 = 0.41$, $P < 0.05$). AI showed a higher specific activity after 4 days harvested and had negative correlation with sucrose accumulation ($r^2 = 0.40$, $P < 0.05$). These results showed that NI could be more responsible in sucrose hydrolisis than AI at early storage of sugarcane stems.

[Keywords: Sugarcane, neutral invertase, acid invertase, sucrose]

INTRODUCTION

Sugarcane (*Saccharum officinarum*) is a perennial agricultural crop belonging to the family of Gramineae. It is grown primarily for the sucrose-containing juice which is expressed from its stalks. Sugarcane biomass yields are the highest among any crops. However, recorded sucrose yields are approximately 60% of the theoretical maximum (Bull and Glasziou 1963). There is a considerable potential for increasing sucrose accumulation in sugarcane by modifying the physiological, biochemical and environmental limits (Moore *et al.* 1997; Zhu *et al.* 1997).

Sucrose yield of sugarcane stem depends on two interlinked processes: biomass production and su-

crose concentration (Ebrahim *et al.* 1998). The ability to accumulate such high sucrose concentration in harvested stem is the net result of sucrose synthesis and breakdown (Huber and Huber 1992a). Sugar metabolism is regulated by several enzymes such as invertases (E.C. 3.2.1.26), sucrose synthase (E.C. 2.4.1.13), and sucrose-phosphate synthase (E.C. 2.4.1.14) (Sugiharto *et al.* 1997; Rose and Botha 2000; Bosch *et al.* 2004; Mao *et al.* 2005). Invertases cleave sucrose to glucose and fructose. They are classified by stability, cellular location, and pH optimum. Based on pH optimum, they are acid invertase (AI) and neutral invertase (NI). Sucrose-phosphate synthase (SPS) synthesizes sucrose-6-phosphate, which is dephosphorylated by sucrose-phosphate phosphatase to form sucrose. Sucrose synthase can either cleave sucrose to UDP-glucose and fructose or catalyze the reverse synthetic reaction. It is widely believed to act in the cleavage direction *in vivo*.

Sucrose concentration in sugarcane internodes is correlated with AI activity and maturation (Zhu *et al.* 1997). Acid invertase activity was high in apoplast and vacuoles of young, actively growing internodes and almost absent from the mature internodes (Miron and Schaffer 1990). Neutral invertase presents at low level in very young tissue and at greater levels in older tissue (Hatch *et al.* 1963; Batta and Singh 1986). It has been proposed that NI regulates sucrose movement from vascular to storage tissue in mature internodes or that it is involved in the turnover of hexoses in mature tissue (Gayler and Glasziou 1972; Bosch *et al.* 2004).

The changes of AI and NI activities in sugarcane stems after harvest is still poorly understood and requires further investigation. Since high sugar contents are the main components in sugarcane stem, and are important for maintaining high quality of sugar product, we studied the changes of AI and NI activity to evaluate their involvement in metabolic changes of harvested sugarcane stems during storage.

MATERIALS AND METHODS

Plant Materials

Plant material consisted of 18-month-old field grown sugarcane. Two commercial varieties R-579 (wet-land) from plantation in PG Djatiroto (PTPN XI) and M 442-51 (dry-land) from plantation in PG Asembagus (PTPN XI) were investigated. Culms were harvested in August 2005 at 10:00 am. Three internodes were sampled to represent immature (internode 1-8/F1), maturing (internode 9-16/F2), and mature (internode 17-24/F3) stems. Internode one is defined as the internode from which the leaf with the first exposed dewlap originates. All tissues were stored for 0-9 days at room temperature (28-32°C). Tissue samples were ground in liquid nitrogen and stored at -80°C.

Sample Extraction

The frozen tissue powder was weighed off and ice-cold extraction buffer was added in a 1:3 (v/w) ratio. The extraction buffer contained 100 mM 4-morpholinepropanesulfonic acid (MOPS)-NaOH (pH 7.5), 12 mM MgCl₂, 1 mM EDTA, 2.5 mM β-mercaptoethanol (ME), 0.5 mM phenylmethanesulfonyl fluoride (PMSF), and 2.5% polyvinyl (poly) pyrrolidone (PVP) (Sugiharto *et al.* 1995). After vortexing, the slurry was centrifuged at 10,000 rpm for 10 minutes at 4°C. Supernatant was concentrated by 30-80% ammonium sulfate. After that sample solution was centrifuged at 10,000 rpm for 10 minutes at 4°C and the pellet was resuspended with 100 mM MOPS-NaOH (pH 7.5), 12 mM MgCl₂, 1 mM EDTA, and 2.5 mM β-ME. Finally, aliquots of the eluate containing proteins were rapidly frozen in liquid nitrogen and stored at -80°C for protein determination and enzyme activity assay.

Determination of Sugar Contents

Sugars were extracted from each fraction of sugarcane and extraction buffer was added in a 1:1 (v/w) ratio. The extraction buffer contained 100 mM MOPS-NaOH (pH 7.5), 12 mM MgCl₂, 1 mM EDTA, 2.5 mM β-ME, 0.5 mM PMSF, and 2.5% PVP. Sucrose content was determined using resorcinol as described by Sugiharto *et al.* (1995). Reducing sugars were measured using glucose-peroxidase method (Ikawa and Obara 1965).

Invertase Activity Assays

Activity of AI was assayed at 30°C in 50 mM buffer phosphate (Kpi) pH 4.5 and 120 mM sucrose. The assay system was the same for the NI except that a

50 mM Kpi pH 7.5 was used. The reaction of NI and AI was stopped by 10-minute incubation at 90°C and the addition of 30 μL 2.5 M Tris was used, respectively. Reducing sugars were measured using glucose-peroxidase method (Ikawa and Obara 1965). One unit of enzyme activity was defined as the enzyme quantity which liberated one μmol of β-D-glucose per minute.

Determination of Total Soluble Protein

Total soluble protein was measured out according to Bradford (1976). Protein concentrations were determined using bovine serum albumin as standard.

Statistical Analysis

The values were expressed as the means + standard deviation of three determinations. Correlation between enzyme activity and sucrose content was analyzed (Windows SPSS program version 10.05) by simple regression $Y = AX + B$, where Y is enzyme activity; X is sucrose contents; A is regression coefficient and B is interception factor.

RESULTS AND DISCUSSION

Sucrose and Reducing Sugar Contents

The rate of loss weight of sugarcane stem from wet-land (var. R-579) during storage at room temperature (28-32°C) was faster than that from dry-land (var. M 442-51) as shown in Figure 1. It increased over the entire storage period, with a faster rate after 15 days. The weight loss of wet-land sugarcane stem increased slightly about 7% than that of dry-land sugarcane stem after 8 days of storage. It may be because the morphology and transpiration rate of wet-land sugarcane stem differed from wet-land sugarcane stem. Transpiration occurs due to the differences in water content between stem and environment which causes weight loss during storage. According to Bull and Glasziou (1963), the differences in weight loss among varieties during storage periods correlated with morphological factors, such as biochemical function of plant age and the environment in which the plant is growing.

Sucrose content was lower in F1 internodes than the other internodes. It was observed in both of wet-land and dry-land sugarcane. Sucrose contents of F1, F2 and F3 were 47.42, 56.12 and 57.17 mg g⁻¹ fresh weight for wet-land sugarcane, and 65.30, 68.45 and 71.45 mg g⁻¹ fresh weight for dry-land sugarcane

respectively. The low sucrose content of F1 internode is consistent with the hypothesis which this internode region is more active in non-sucrose storing metabolism (e.g. processes involving respiration and growth) (Rose and Botha 2000). In fact, this evidence occurred even in the more mature internode F3 that already accumulates high sucrose levels. The difference in sucrose content between wet-land and dry-land sugarcane showed that the wet-land is more active in non-sucrose storing than the dry-land sugarcane variety. This evidence caused wet-land sugarcane was harvested older than that of dry-land.

The change in sucrose content between wet-land and dry-land sugarcane exhibited a dissimilar pattern

after the sugarcane stems were harvested. The sucrose content of wet-land sugarcane stems decreased slowly during storage and it was relatively constant after 2 days (Fig. 2A). In case of dry-land sugarcane stems, the sucrose content decreased rapidly during storage and it was relatively constant after 3 days (Fig. 2B).

Reducing sugar increased at initiate storage (0-3 days) due to sucrose hydrolysis which released glucose and fructose. After 2 days, reducing sugars have depleted 1-2 from initiate storage (Fig. 3), its resulting in the decline in content, which coincided with the occurrence of maximum respiration rate (Mao and Liu 2000). Storage of sugarcane internodes seems to coincide with a redirection of carbon from sucrose to reducing sugars, and respiration. This is significantly influenced by storage time. An interesting result was observed in the significant negative relationship between sucrose and reducing sugar for 0-3 days storage ($r^2 = 0.5$, $P < 0.05$), and 3-9 days storage ($r^2 = 0.4$, $P < 0.05$).

The ability to accumulate such high sucrose concentrations in stored stem is the net result of sucrose synthesis and breakdown (Huber and Huber 1992b; Rose and Botha 2000). The regulatory of sugar accumulation during storage showed that the present was regulated different functions of sucrose hydrolysis. The regulations of enzyme activity are elevated with the increase or decrease in sugar accumulation in stored tissue (Hatch and Glasziou 1963).

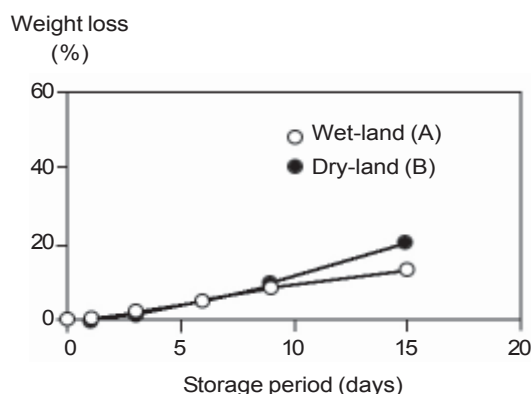


Fig. 1. Loss weight of wet-land and dry-land sugarcane stem during storage.

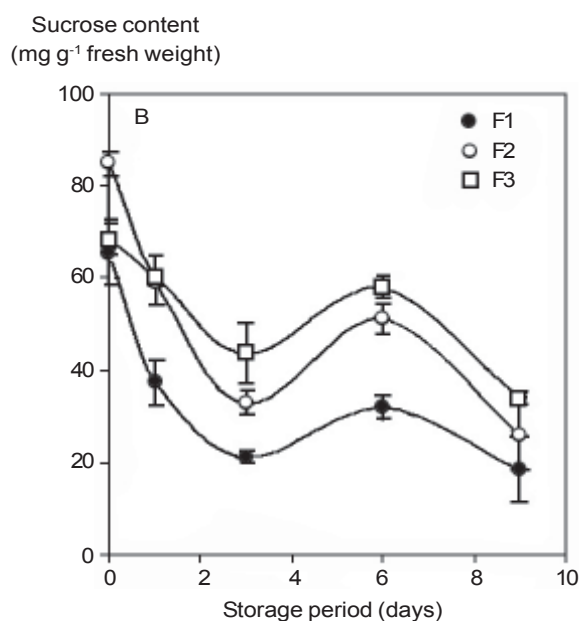
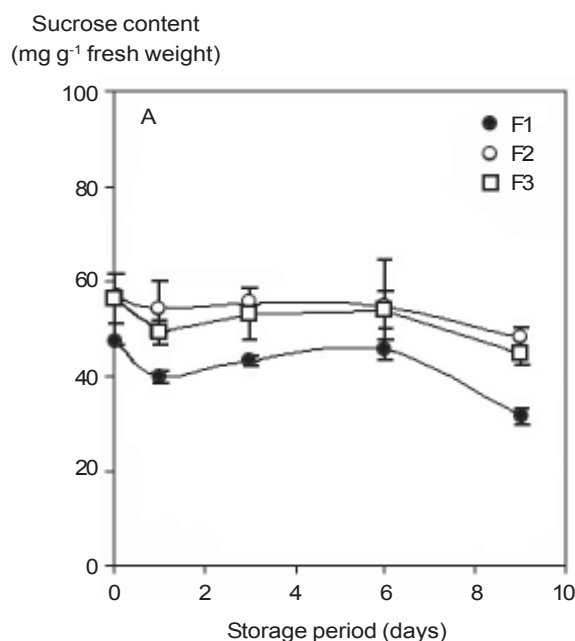


Fig. 2. Changes of sucrose content in harvested sugarcane stem after 0-9 days for wet-land (A) and dry-land varieties (B). Vertical bars represent \pm SE.

Acid and Neutral Invertase Activities

Changes in AI and NI activities exhibited a dissimilar pattern after sugarcane stems were harvested. NI activity increased rapidly, reaching a maximum level at one day storage period. After that, it declined rapidly (Fig. 4). However, AI activity showed a gradual increase after 3 days and increased continuously until 9 days of storage (Fig. 5). The opposite regulatory properties between NI and AI in sugarcane stem during storage were due to different characters of the enzymes in sucrose hydrolysis. NI activity is elevated with the increase in sugar accumulation in stored tissues (Hatch and Glasziou 1963), whereas AI activity is usually high in tissues that are rapidly growing, such as cell and tissue cultures, root apices, and immature stem internodes (Miron and Schaffer 1990).

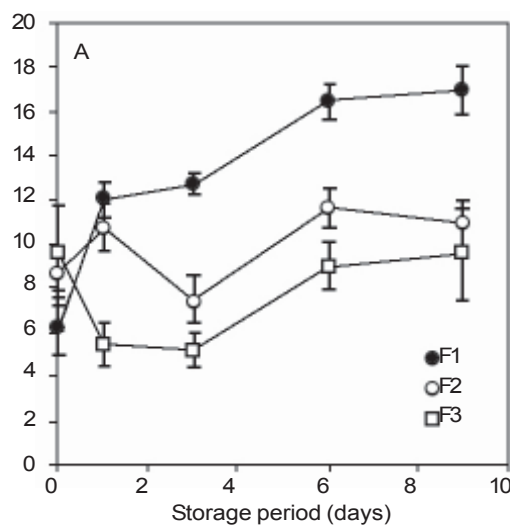
The final sucrose concentration should be correlated with the difference between the rate of sucrose synthesis and sucrose hydrolysis. However, little sucrose could be synthesized in stems during storage because of the limitation of substrates during photosynthesis process. In fact, sucrose hydrolysis could be more active for increased hexoses used in respiration and other ripening processes during storage. It might, therefore, be concluded that as stems ripen after harvest, respiration and other intermediate formation would be the primary cause of the sharp decline in sucrose content because both invertases (AI and NI) were responsible for sucrose hydrolysis.

Correlation Between Invertase Activities and Sugar Contents

The correlation between sucrose content and invertase (NI and AI) activities in sugarcane stems during storage are divided into two phase, i.e. the initiate (0-3 days) storage period and after 3-9 day-storage period (Fig. 6). When these data are plotted, a linear relationship is obtained. A negative correlation was observed between sucrose contents and NI activities ($r^2 = 0.41$, $P < 0.05$) and between sucrose contents and AI activities ($r^2 = 0.30$, $P < 0.05$) for both varieties at initiate storage (0-3 days). A weaker negative correlation existed between sucrose content and NI activity ($r^2 = 0.07$, $P < 0.05$) and between sucrose content and AI activity ($r^2 = 0.40$, $P < 0.05$) for both varieties at storage of 3-9 days.

These results showed that the NI could be more responsible for sucrose hydrolysis than AI at early storage period. NI activity reported to be high in storage tissue (Hatch and Glasziou 1963). This enzyme regulates movement of sucrose from vascular to storage tissue in mature internodes or that it is involved in the turnover of hexoses in mature tissue (Gayler and Glasziou 1972; Bosch *et al.* 2004). The regulatory function of NI was depending on some variables such tissue maturity and concentration of stored sugar (Venkataramana *et al.* 1991; Winter and Huber 2000). It is assumed that the change of invertase activities over a period would be depending upon the concentration of stored sugar. This enzyme

Reducing sugar content
(mg g⁻¹ fresh weight)



Reducing sugar content
(mg g⁻¹ fresh weight)

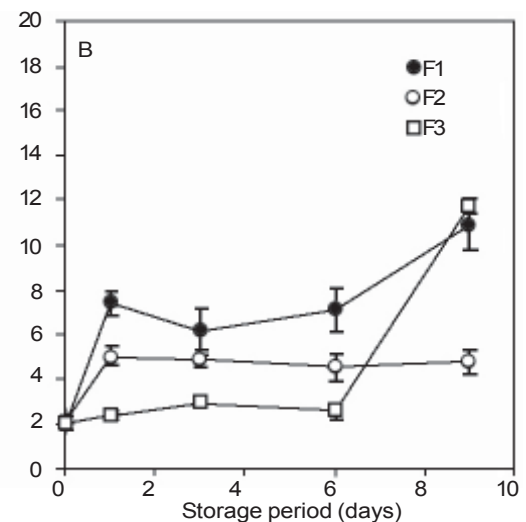


Fig. 3. Changes of reducing sugar contents in sugarcane stem during storage for wet-land (A) and dry-land varieties (B). Vertical bars represent \pm SE.

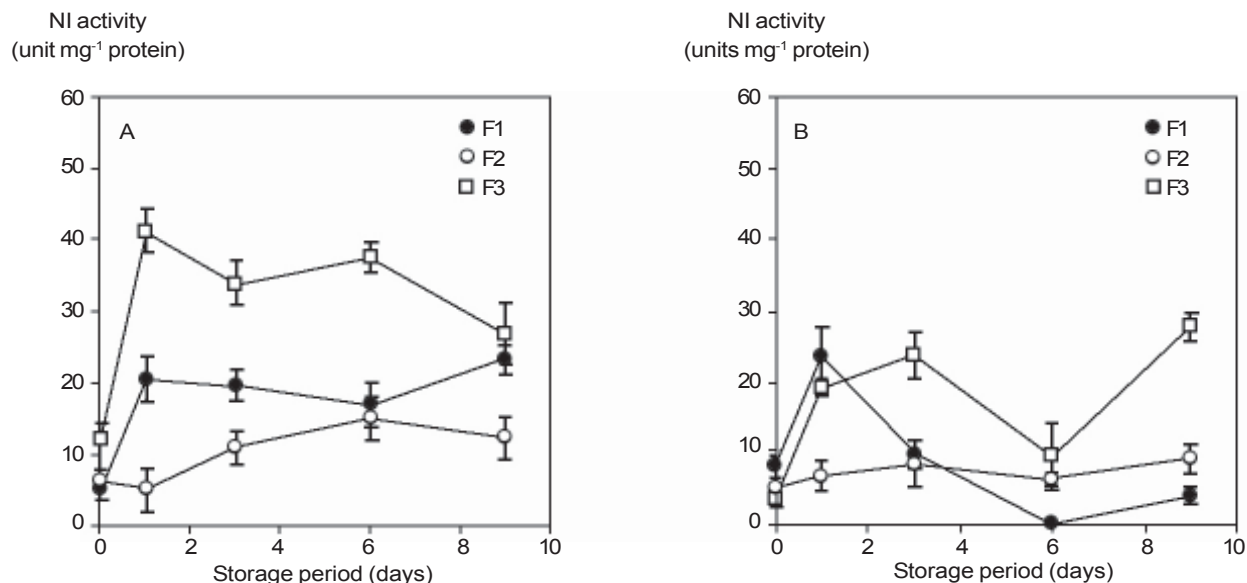


Fig. 4. Changes of neutral invertase (NI) activity in sugarcane stem during storage for wet-land (A) and dry-land varieties (B). Vertical bars represent ± SE.

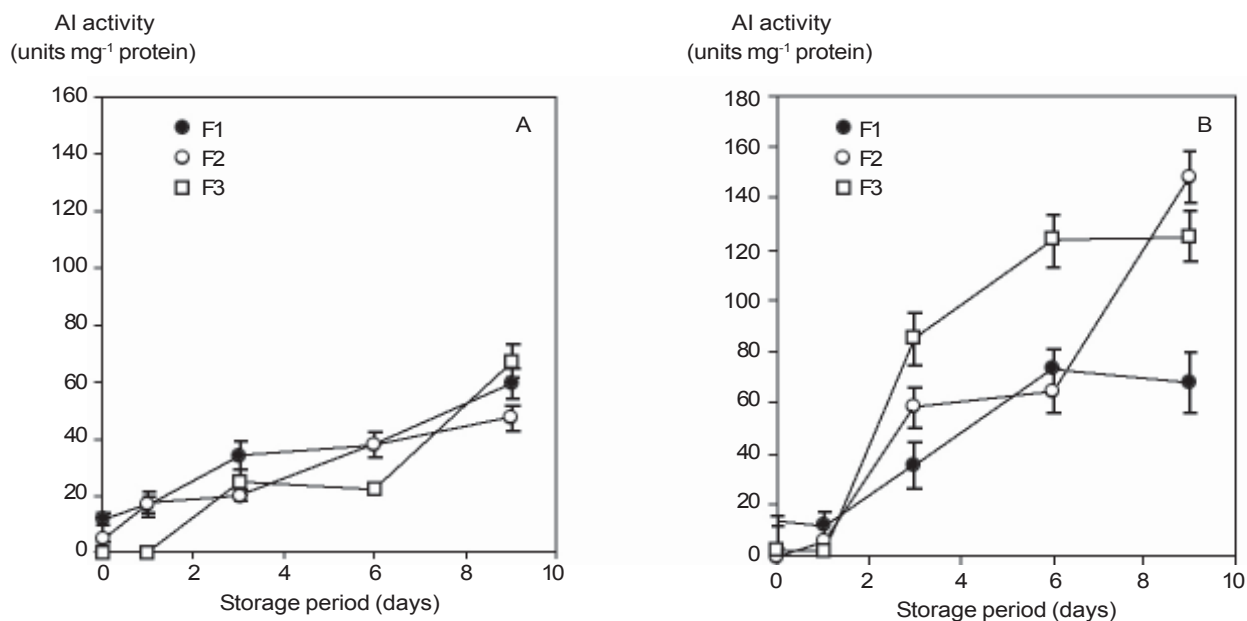


Fig. 5. Changes of acid invertase (AI) activity in sugarcane stem during storage for wet-land (A) and dry-land varieties (B). Vertical bars represent ± SE.

is considered as ‘maintenance’ enzyme involved in sucrose degradation when AI activity and sucrose synthase are low (Winter and Huber 2000).

The strong negative relationship between invertase activity and sucrose content in sugarcane stem during storage periods and the probably promotive effect of invertase promise a good potential for the manipulation of the invertase gene(s) expression to

inhibit the decrease in sucrose content by RNA-antisense technique in storage organs such as the stem internodes of sugarcane. These potentials have been demonstrated in tomato (Klann *et al.* 1996) and are under investigation for sugarcane (Hongmei *et al.* 2000). For tomato, transgenic tomato (*Lycopersicon esculentum* Mill.) plants expressing a constitutive antisense invertase transgene grew identically to

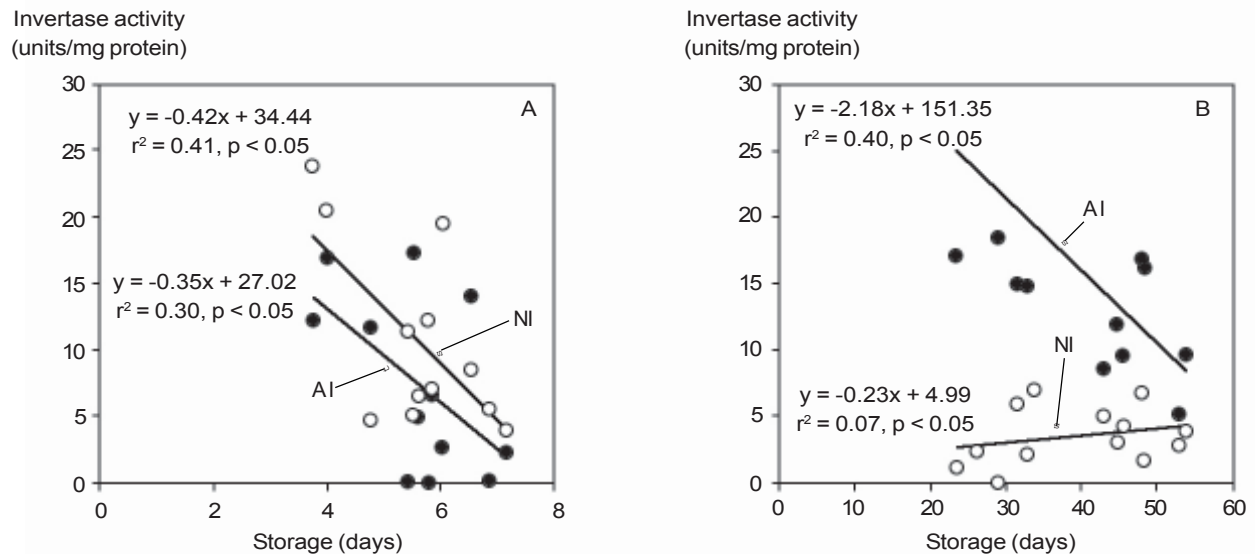


Fig. 6. Correlations between invertase activity and sucrose content in harvested sugarcane stem; A = 0-3 day initiate storage period and B = after 3-9 day storage period.

wild-type plants. Several lines of transgenic fruit expressing a constitutive antisense invertase gene had increased sucrose and decreased hexose sugar concentrations (Klann *et al.* 1996). For sugarcane, the transformation with the sugarcane antisense acid invertase gene produced a cell line with moderate inhibition of soluble acid invertase activity and a 2-fold increase in sucrose accumulation. Lowering acid invertase activity increased sucrose accumulation (Hongmei *et al.* 2000).

CONCLUSION

Neutral invertase had a higher specific activity at the initiate harvested (0-3 days) than acid invertase in the sucrose-accumulating region of the sugarcane stem. Negative significant correlation was found between NI specific activity and sucrose accumulation. AI showed a higher specific activity after 4 days harvested and had negative correlation with sucrose accumulation. The NI could be more responsible in hydrolisis of sucrose than AI at early storage. The storage period of sugarcane stems had significant influences on metabolic changes of sugar and enzyme activities. Direct losses due to loss weight and decay and indirect losses, such as sucrose hydrolysis, limit the shelf-life of sugarcane stems. The storage period after harvest is effective for maintaining the quality of sugarcane stems.

ACKNOWLEDGMENT

The work was supported by PT Perkebunan Nusantara XI (persero) no. contract XX-KONTR/03.013.

REFERENCES

- Batta, S.K. and R. Singh. 1986. Sucrose metabolism in sugarcane grown under varying climate conditions: Synthesis and storage of sucrose in relation to the activities of sucrose synthase, sucrose phosphate synthase and invertase. *Phytochemistry* 25: 2431-2437.
- Bosch, S., C.P.L. Grof, and F.C. Botha. 2004. Expression of neutral invertase in sugarcane. *Plant Sci.* 166: 1125-1133.
- Bradford, M.M. 1976. A rapid and sensitive method for the determination of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* 72: 248-254.
- Bull, T.A. and K.T. Glasziou. 1963. The evolutionary significance of sugar accumulation in *Saccharum*. *Aust. J. Bio. Sci.* 16: 737-742.
- Ebrahim, M.K., O. Zingsheim, M.N. El-Shourbagy, P.H. Moore, and E. Komor. 1998. Growth and sugar storage in sugarcane grown at temperatures below and above optimum. *J. Plant Physiol.* 153: 593-602.
- Gayler, K. and K.T. Glasziou. 1972. Physiological functions of acid and neutral invertase in growth and sugar storage in sugarcane. *Plant Physiol.* 27: 25-31.
- Hatch M.D. and K.T Glasziou. 1963. Sugar accumulation cycle in sugarcane. II. Relationship of invertase activity to sugar content and growth rate in storage tissue of plants grown in controlled environments. *Plant Physiol.* 38: 344-348.
- Hongmei, M., H.A. Henrik, P. Robert, and H.M. Paul. 2000. Metabolic engineering of invertase activities in different sub-

- cellular compartments affects sucrose accumulation in sugarcane cells. *Aust. J. Plant Physiol.* 27: 1021-1030.
- Huber, J.L. and S.E. Huber. 1992a. Role of sucrose phosphate synthase in sucrose metabolism in leaves. *Plant Physiol.* 99: 1275-1278.
- Huber, S.E. and J.L. Huber. 1992b. Role and regulation of sucrose phosphate synthase in higher plants. *Plant Physiol.* 47: 431-444.
- Ikawa, S. and T. Obara. 1965. Use of glucose oxidase-catalase system for determination of blood sugar (in Japanese). *Rinsho Byori.* 13: 197-200.
- Klann, E.M., B. Hall, and A.B. Bennett. 1996. Antisense acid invertase (TIV1) gene alters soluble sugar composition and size in transgenic tomato fruit. *Plant Physiol.* 112: 1321-1330.
- Mao, L. and W. Liu. 2000. Study on postharvest physiological changes and storage techniques of sugarcane. *Scientia Agriculture Sinica* 33: 41-45.
- Mao, L., F. Que, and G. Wang. 2005. Sugar metabolism and involvement of enzymes in sugarcane (*Saccharum officinarum* L.) stems during storage. *Food Chem.* 98: 338-342.
- Miron, D. and A.A. Schaffer. 1990. Sucrose phosphate synthase, sucrose synthase and invertase activities in developing fruit of *Lycopersicum esculentum* Mill and sucrose accumulating *Lycopersicum hirsutum*. *Plant Physiol.* 95: 623-627.
- Moore, P.H., F.C. Botha, R.T. Furbank, and C.R.L. Grof. 1997. Potential for overcoming physio-biochemical limits to sucrose accumulation. In B.A. Keating and J.R. Wilson (Eds.). *Intensive Sugarcane Production: Meeting the challenges beyond 2000*. CAB International, Wallingford, UK. p. 141-156.
- Rose, S. and F.C. Botha. 2000. Distribution patterns of neutral invertase and sugar content in sugarcane internodal tissues. *Plant Physiol. Biochem.* 38: 819-824.
- Sugiharto, B., Sumadi, G.F. Hutasoit, and M. Yusuf. 1995. Effect on activities of the phosphoenol pyruvate carboxylase and sucrose phosphate synthase and the levels of sucrose in sugarcane leaf. *Photosynthesis Research*, an International Journal in 10th International Photosynthesis Congress, 20-25 August 1995. Montpellier, France.
- Sugiharto, B., T. Handoyo, dan Sumadi. 1997. Variabilitas genetik dalam enzim fotosintetik dan enzim metabolisme sukrose pada beberapa varietas tebu. *Zuriat* 7: 76-78.
- Venkataramana, S., K.M. Naidu, and S. Singh. 1991. Invertases and growth factors dependent sucrose accumulation in sugarcane. *Plant Sci.* 74: 65-72.
- Winter, H. and S.C. Huber. 2000. Regulation of sucrose metabolism in higher plants: Localization and regulation of activity of key enzymes. *Critical Rev. Plant Sci.* 19: 31-67.
- Zhu, Y.J., E. Komor, and P.H. Moore. 1997. Sucrose accumulation in the sugarcane stem is regulated by the difference between the activities of soluble acid invertase and sucrose phosphate synthase. *Plant Physiol.* 115: 609-616.