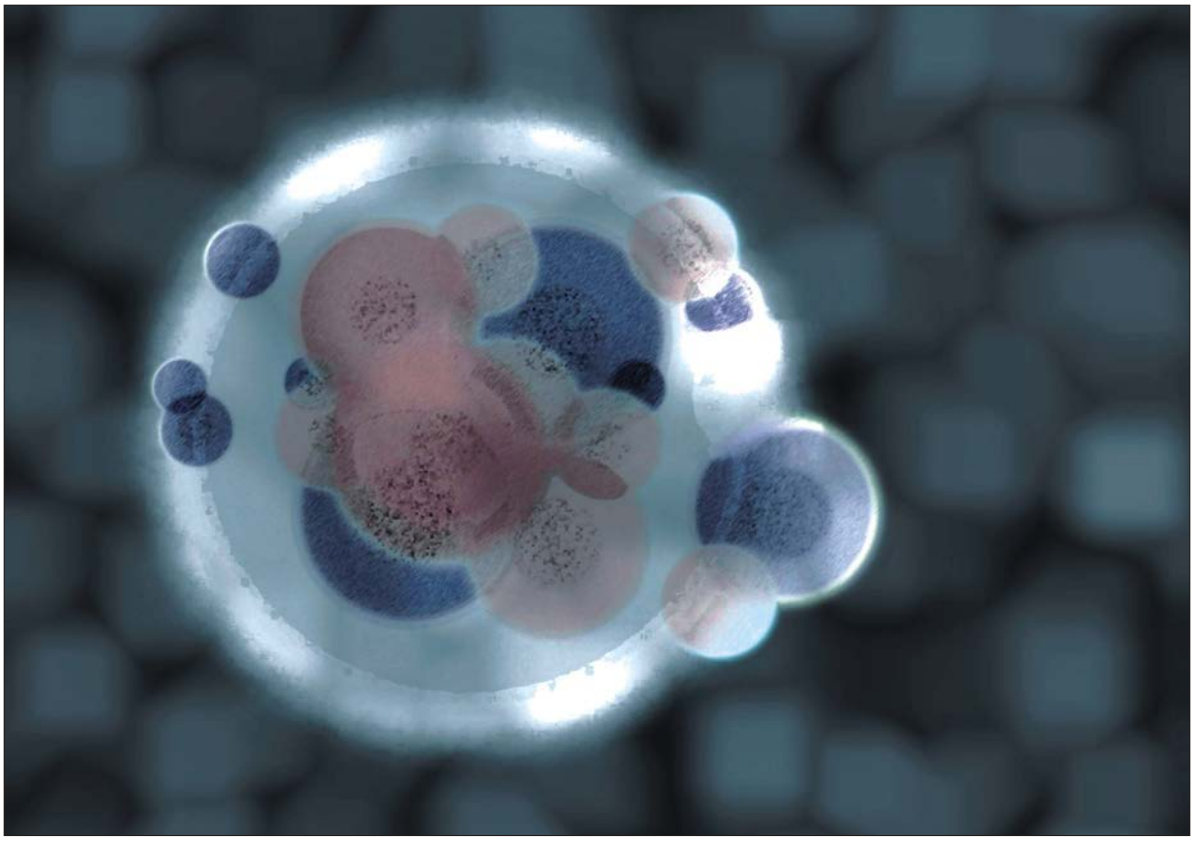

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Seasonal Fluctuations of Sucrose Metabolizing Enzymes Activities and Sugar Contents in Lettuce (*Lactuca sativa* L.)

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Abstract: The objective of this study was to understand the effect of environmental changes during growth and after harvest on the quality of lettuce. In both cultivars and portions, acid invertase activities (soluble and cell wall-bound fractions) increased throughout the harvest month, except in April in the leaf portion. SS and SPS activities declined with few fluctuations in any portions of both cultivars. The activities of three sucrose metabolizing enzymes were higher in the midrib than in the leaf portions. Sucrose concentration increased from December to a maximum in January and again decreased up to April. On the other hand, glucose and fructose concentrations declined up to March and increased in April. There was a highly significant negative correlation between the activity of acid invertase in cell wall-bound fraction and sucrose content. A higher concentration of reducing sugars was found in 'Bittsu' than 'Shisuko'. Fructose was higher than glucose and sucrose contents in the both portions of two cultivars.

Key words: Acid invertase, environmental change, lettuce, sucrose phosphate synthase, sucrose synthase, sugar content

INTRODUCTION

Preharvest factors, including environmental conditions and cultural practices, can influence the quality of horticultural crops (Hewett, 2006). Optimum growing temperatures, light conditions, amount of rainfall and irrigation, mineral nutrition and fertilization, pest management and maturity at the time of harvest can directly or indirectly affect postharvest quality, storage life and susceptibility of crops to disorders and diseases (Wang, 1997). Postharvest quality of fresh vegetables generally depends on the quality achieved at time of harvest (Weston and Barth, 1997). Therefore, field management practices play a very important role in determining quality attributes such as size, color, flavor, texture and nutritional values for vegetable crops (Wang, 1997; Mattheis and Fellman, 1999).

Lettuce (*Lactuca sativa* L.) is produced in cool days and nights areas to achieve firm but mild-flavored heads. While the heads grew in high temperatures or drought areas, bitter flavor can rapidly occur and the leaves become less tender (Weston and Barth, 1997). Moreover, the head weights of iceberg lettuce at maturity are associated with temperature around the time of heating (Wurr and Fellows, 1991). Koontz and Prince (1986) showed that increasing the photoperiod from 16 to 24 h increased 33 and 50% of fresh weight of butterhead and loose-leaf lettuce, respectively. However, the interaction between day temperature and radiation is very important factor which correlated with the growth of lettuce. The head responded more steeply to radiation in autumn and that grown in spring has a great response to temperature (Glenn, 1984).

Temperature fluctuations affect the chemical composition such as sugar contents of the products (Woolf and Ferguson, 2000). Sugar metabolism is characterized by a continuous process of degradation and biosynthesis of sucrose (Hill and ApRees, 1994). Sucrose phosphate synthase (SPS; EC 2.4.1.14) is the principal enzyme involved in the synthesis of sucrose in higher plants (Mao *et al*., 2006). Invertase (EC 3.2.1.26) and sucrose synthase (SS; EC 2.4.1.13), on the other hand, cleaved the glycosidic bond of sucrose (Jha and Dubey, 2004). Invertase simply splits sucrose into glucose and fructose to provide substrates for growth (Hirose *et al*., 2002). SS is a cytoplasmic enzyme that requires uridine 5'-diphosphate (UDP) as co-substrate and produces fructose and UDP-glucose (Sturm and Tang, 1999). SS may also act in the sucrose synthesis direction, but under normal conditions acts only in the breakdown of sucrose

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(Bhowmik *et al*., 2001). To our knowledge, limited information is available on the influence of environmental conditions during growth and after harvest of lettuce on its quality. Therefore, it is important to elucidate the metabolism of sucrose metabolizing enzymes and sugar accumulation of lettuce head in relation to temperature fluctuations.

MATERIALS AND METHODS

Plant materials: The head lettuce cultivars 'Bittsu' and 'Shisuko' were harvested from Kagawa Prefecture Agricultural Experiment Station, Busshouzan, Kagawa, Japan. Monthly harvest was done from December, 2005 to April, 2006 when the head reached the commercial maturity. Field temperature was recorded throughout the harvest months. After harvest, the heads were contained in a box and transported to the laboratory. The leaves were separated from the midribs and immediately stored at -30°C until analysis.

Enzyme extraction for acid invertase: Approximately 5 g of fresh sample from each portion were added with 1% of polyvinylpolypyrrolidone (PVPP) and 1 g of sea sand. The sample mixture was homogenized using a cooled mortar and pestle with 5 mL of 0.2 M citrate-phosphate buffer (C-P buffer, pH 5.0). The resulting homogenate was filtered through cotton cloth and was centrifuged at 12,000 x g, at 2°C for 10 min. The total supernatant was dialyzed with 40 times diluted 0.2 M C-P buffer (pH 5.0) for 12 h with frequent stirring and the inner solution was designated as soluble fraction. The residual tissues were reextracted after incubation for about 24 h at 4°C with 5 mL of 0.2 M NaCl C-P buffer (pH 5.0). The supernatant was dialyzed as described above. The dialyzed solution was designated as cell wall-bound fraction. All extraction procedures were carried out at 4°C.

Enzyme assay for acid invertase: The standard assay mixture for acid invertase consisted of 0.2 mL of 0.2 M C-P buffer (pH 5.0), 0.1 mL of 0.5 M sucrose, 0.1 mL of distilled water and 0.1 mL of crude enzyme solution. The blank experiment contained distilled water instead of sucrose. The assay mixture was incubated at 45°C for 15 min and neutralized with 0.1 N NaOH or 0.1 N HCl. A coloring Somogyi's copper reagent was added and the mixture was heated for 10 min in boiling water. After cooling, the mixture was added with 1 mL of Nelson's reagent. The amount of reducing sugars was estimated by the method of Somogyi (1952). Soluble protein content was determined according to the method of Lowry *et al*. (1951) using bovine serum albumin as the standard. The enzyme activity was revealed as the amount of glucose produced per minute per milligram of protein.

Enzyme extraction for SS and SPS: Approximately 5 g of fresh sample from each portion were added with 1% of polyvinylpolypyrrolidone (PVPP) and 1 g of sea sand. The sample mixture was homogenized using a cooled mortar and pestle with 10 mL of 0.3 M potassium-phosphate buffer (K-P buffer, pH 7.8) containing 1 M ascorbate, 1 M $MgCl_2$, 1 M DTT and 0.1 M Na-EDTA. The resulting homogenate was filtered through cotton cloth and was centrifuged at 12,000 x g, at 2°C for 20 min. The total supernatant was dialyzed with 40 times diluted 0.3 M K-P buffer (pH 7.8) for 12 h and the inner solution was used as the crude enzyme. All extraction procedures were carried out at 4°C.

Enzyme assay for SS and SPS: SS and SPS activities were assayed at 37°C following the method described by Hubbard *et al*. (1989) with slight modifications. For SPS determination, we used a mixture of 70.75 μL consisting 50 mM Heps-NaOH solution (pH 7.5), 15 mM $MgCl_2$, 25 mM fructose-6-phosphate, 25 mM glucose-6-phosphate, 25 mM UDP-glucose, distilled water and crude enzyme solution. For SS determination, 25 mM fructose was used instead of fructose-6-phosphate and glucose-6-phosphate. The blank experiment contained distilled water instead of UDP-glucose and crude enzyme. The reaction mixtures were incubated at 37°C for 30 min and were added with 70 μL of 30% KOH to terminate the reaction. After that, the assay mixtures were kept in boiling water (approximately 100°C) for 10 min to destroy any unreacted fructose or fructose-6-phosphate. After cooling, 2 mL of anthrone reagent (150 mg anthrone in 100 mL of 70% H_2SO_4) was added and incubated at 40°C for 10 min. The enzyme activities were determined by the absorbance of the sample at 620 nm using a spectrophotometer (UV-1200, Shimadzu Co., Ltd.). The soluble protein content was estimated following the method of Lowry *et al*. (1951) using bovine serum albumin as the standard. The SS and SPS activities were revealed as micromole of sucrose or sucrose-6-phosphate produced per minute per milligram of protein, respectively.

Determination of soluble sugar contents by high performance liquid chromatography (HPLC): The sucrose, glucose and fructose contents were determined by HPLC. Approximately 2 g of lettuce sample from each portion were mixed with 1 g of sea sand and homogenized in a cooled mortar and pestle. Ten milliliter of distilled water was added to the homogenate and centrifuged at 12,000 x g, at 2°C for 10 min. The mixture was filtered

through a cellulose nitrate membrane filter (0.45 μm pore size). Soluble sugar contents were analyzed by HPLC using a stainless steel column (10.7 mm ID ×30 cm) packed with silica gel (gel pack C610). The mobile phase (filtered water) was pumped through the column at a flow rate of 1.0 mL min^{-1}. The pressure was adjusted to 28-29 kg cm^{-2} and the temperature to 60°C. A refractive index monitor (Hitachi L-3300) was used to record the peak areas. Sucrose, glucose and fructose were identified by their retention times and were quantified according to standards.

Statistical analysis: A randomized complete block design (RCBD) was adopted in this experiment with three replications. The data were treated by analysis of variance with Duncan's Multiple Range Test (DMRT) between means, determined at the 5% level of significance. Linear correlation analysis was used to evaluate the relationships between sugar contents and enzyme activities.

RESULTS

Acid invertase activity: The acid invertase activities (soluble and cell wall-bound fractions) in both cultivars and portions continuously increased throughout the harvest months, except in the leaf portion which showed a decrease in April (Fig. 1-3). The activity in the cell wall-bound fraction was higher than in soluble fraction in two portions of each cultivar. In both cultivars, higher enzyme activity was observed in the midrib than in the leaf portion.

Sucrose synthase (SS) activity: In the leaf portion of both cultivars, the SS activity slightly declined throughout the harvest period (Fig. 4). Also, the SS activity showed the decrease in the midrib portion except in April where the activity was high. The SS activity was higher in the midrib than that in the leaf portion.

Sucrose phosphate synthase (SPS) activity: The SPS activity decreased with few fluctuations in both cultivars and portions (Fig. 5). However, a transient increase was observed in January in the midrib of 'Shisuko'. Midrib has higher SPS activity than in the leaf portion in both cultivars.

Soluble sugar contents: Sucrose content slightly declined in the leaf and midrib portions until the end of the harvest month except in January (Fig. 6 and 7). Glucose and fructose contents also decreased up to March and increased in April. A higher content of reducing sugars

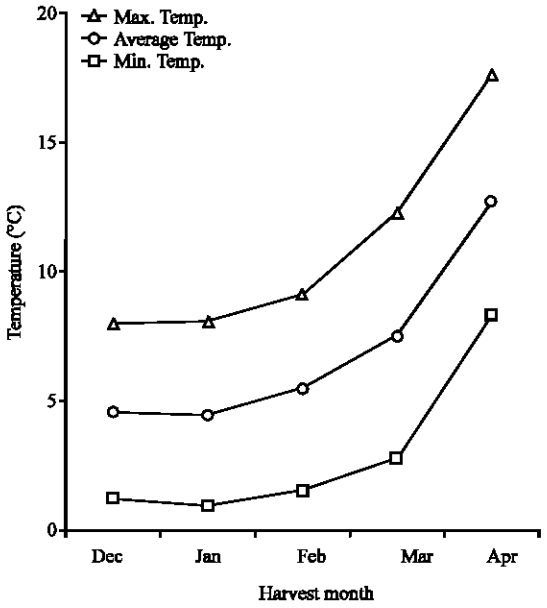

Fig. 1: Temperature of every harvest month from December, 2005 to April, 2006

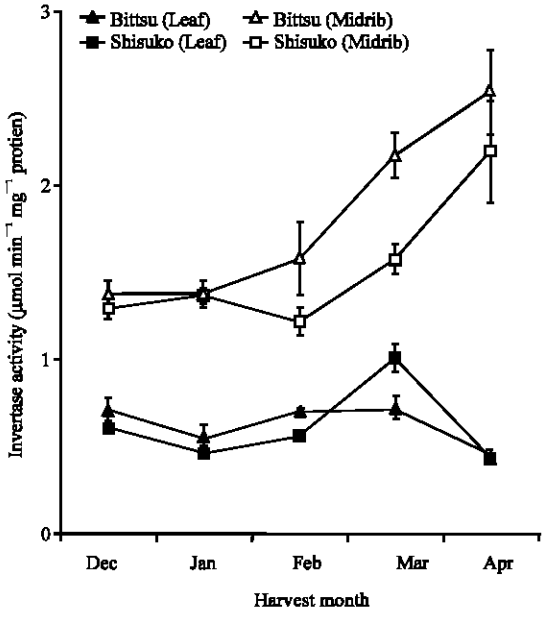

Fig. 2: Changes in acid invertase activity in the soluble fraction in the leaf and midrib portions of two lettuce cultivars ('Bittsu' and 'Shisuko') harvested at different months. Each point represents the mean of 3 replications. Vertical bars indicate standard errors (SE). Between leaf and midrib of two cultivars = significant difference at 99% level, all months

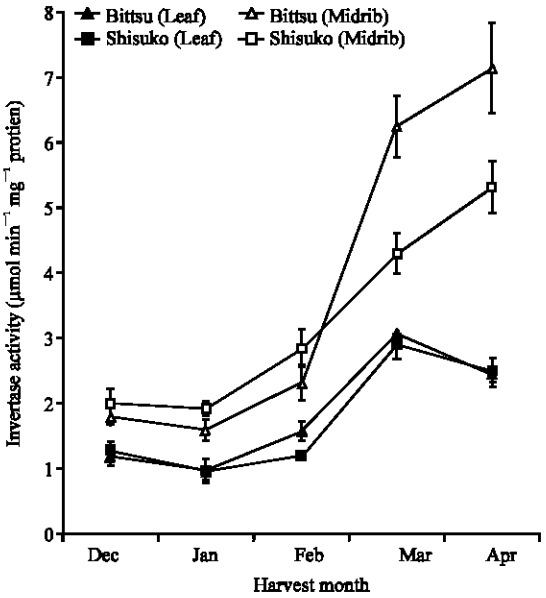

Fig. 3: Changes in acid invertase activity in the cell wall-bound fraction in the leaf and midrib portions of two lettuce cultivars ('Bittsu' and 'Shisuko') harvested at different months. Each point represents the mean of 3 replications. Vertical bars indicate standard errors (SE). Between leaf and midrib of 'Bittsu' = significant difference at 95% level, except February. Between leaf and midrib of 'Shisuko' = significant difference at 99% level, all months

Table 1: Correlation coefficients (r) between acid invertase (soluble and cell wall-bound fractions), SS, SPS activities and sugar concentrations in the leaf and midrib portions of two lettuce cultivars ('Bittsu' and 'Shisuko') harvested at different months

			Correlation coefficients (r) value			
			Acid invertase			
Sugar	Cultivar	Portion	SF	CWBF	SS	SPS
Sucrose	Bittsu	Leaf	0.067	-0.978**	0.539*	0.432
		Midrib	-0.863**	-0.866**	0.855**	0.769**
	Shisuko	Leaf	-0.348	-0.847**	0.919**	0.357
		Midrib	-0.752**	-0.937**	0.842**	0.977**
Glucose	Bittsu	Leaf	0.102	-0.522*	0.847**	0.824**
		Midrib	-0.664**	-0.634*	0.797**	0.978**
	Shisuko	Leaf	-0.125	-0.577*	0.582*	0.976**
		Midrib	-0.457	-0.719**	0.485	0.579*
Fmctose	Bittsu	Leaf	0.329	-0.259	0.879**	0.883**
		Midrib	-0.532*	-0.498	0.734**	0.942**
	Shisuko	Leaf	-0.158	-0.342	0.272	0.906**
		Midrib	-0.112	-0.418	0.293	0.268

SF = Soluble Fraction, CWBF = Cell Wall-bound Fraction, SS = Sucrose Synthase, SPS = Sucrose Phosphate Synthase, *, **denote significant correlation at $0.01<p\leq0.05$ and $p\leq0.01$, respectively, n = 15

(glucose and fructose) was found in 'Bittsu' than 'Shisuko'. Among the three sugars, the amount of fructose was higher than that of glucose and sucrose in the both portions of two cultivars.

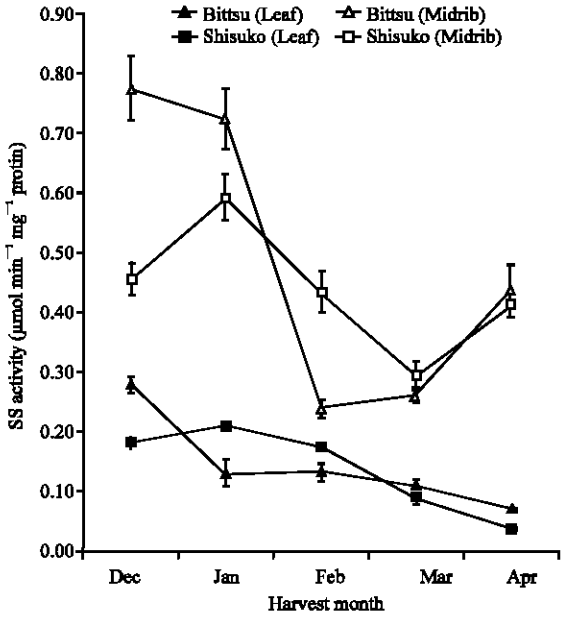

Fig. 4: Changes in sucrose synthase (SS) activity in the leaf and midrib portions of two lettuce cultivars ('Bittsu' and 'Shisuko') harvested at different months. Each point represents the mean of 3 replications. Vertical bars indicate standard errors (SE). Between leaf and midrib of two cultivars = significant difference at 99% level, all months

Correlation coefficients (R) between enzyme activities and sugar contents: There was a highly significant negative correlation between the acid invertase activity (cell wall-bound fraction) and sucrose content in the leaf and midrib portions of both cultivars (Table 1). Conversely, a highly significant positive correlation was observed between SS activity and sucrose content. Also, there was a highly significant positive relationship between SPS activity and sucrose content in midrib portion of both cultivars.

DISCUSSION

Environmental fluctuations have influence on the physiological and biochemical metabolisms in lettuce head. Changes of sugar metabolizing enzyme activities and soluble sugar contents in the lettuce head are related to seasonal temperature. In this study, acid invertase activity in all fractions of both cultivars and portions increased throughout the harvest months, except in April of the leaf portion, with a rise in temperature (Fig. 1-3). Correspondingly, Pramanik *et al.* (2004) found that the invertase activity in the florets and branchlets of broccoli increased in April with a rise in temperature. In addition,

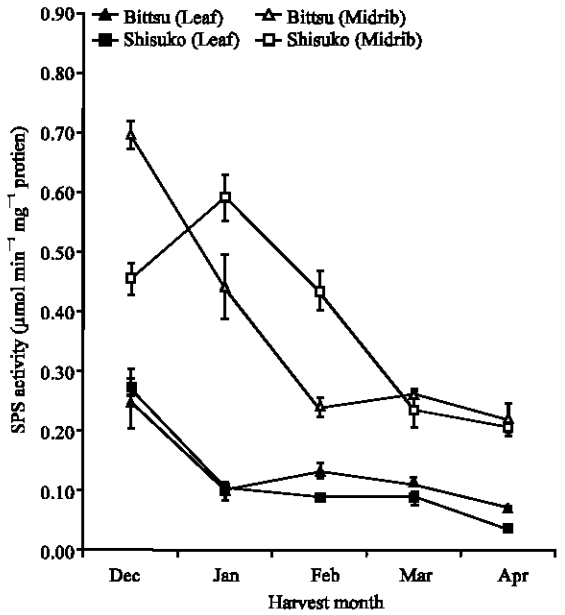

Fig. 5: Changes in sucrose phosphate synthase (SPS) activity in the leaf and midrib portions of two lettuce cultivars ('Bittsu' and 'Shisuko') harvested at different months. Each point represents the mean of 3 replications. Vertical bars indicate standard errors (SE). Between leaf and midrib of two cultivars = significant difference at 99% level, all months

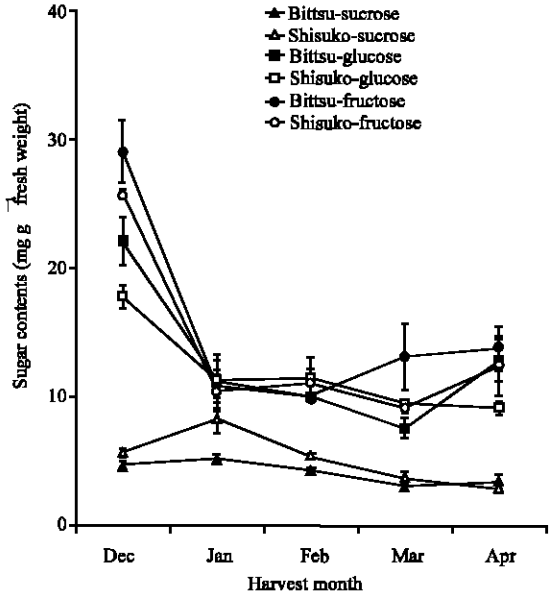

Fig. 6: Changes in soluble sugar contents in the leaf portion of two lettuce cultivars ('Bittsu' and 'Shisuko') harvested at different months. Each point represents the mean of 3 replications. Vertical bars indicate standard errors (SE). Among sugar contents of 'Bittsu' = sucrose, significant difference at 99% level, all months. Among sugar contents of 'Shisuko' = sucrose, significant difference at 99% level, except February

Ebrahim *et al.* (1998) showed that the activities of soluble acid invertase and neutral invertase were greater in sugarcane grown at 27°C than at 15°C. Generally, invertase activity increased with temperature up to a certain point and then decreased if temperature rose (Bhowmik *et al.*, 2001). The fluctuation of acid invertase activity observed in this experiment might be due to the fluctuation of temperature at that time. This pattern of activity was also revealed in other vegetables with the isolation of an invertase inhibitor, which is activated at high temperatures and inactivated at low temperatures (Phan, 1987). However, lower temperatures decrease the activity of invertase, it is deinhibited and will still cleaved sucrose into glucose and fructose.

The activity of sucrose synthase decreased throughout the experiment period, except in the midrib portion harvested in April (Fig. 4). The change of activity might be due to the seasonal temperature. Because temperature has a direct effect on enzyme activity (Lingle, 2004). In asparagus, SS activity gradually decreased in the top portion of spear harvested in autumn (Alam *et al.*, 1999). Both invertase and SS activities were higher in the midrib than in the leaf portions. The difference could be related in their physiological function; the leaf tissue serves as source while the midrib acts as sink organ. Castonguay and Nadeau (1998) observed that invertase, along with SS has an important impact on the level of sucrose in plant cells and their activity is particularly high in sink tissue than source tissue. Furthermore, SS is believed to be a marker of the sink strength of an organ (Wang *et al.*, 1993). On the other hand, the sucrose phosphate synthase activity declined with few fluctuations in all portions of both cultivars throughout the harvest season (Fig. 5). This result might be due to the increase in environmental temperature (Fig. 1). Low temperature induced high SPS activity in winter rye (*Secale cereale* L.) (Hurry *et al.*, 1994), winter wheat (*Triticum aestivum* L.) (Hurry *et al.*, 1995) and spinach leaves (*Spinacia oleracea* L.) (Guy *et al.*, 1992). Moreover, sucrose accumulation did not seem to reply on an increased SPS ability for synthesis.

Temperature is a major factor in determining growth rates, development and quality properties such as sugar contents of product (Woolf and Ferguson, 2000). From this result, the sugar contents decreased in both cultivars

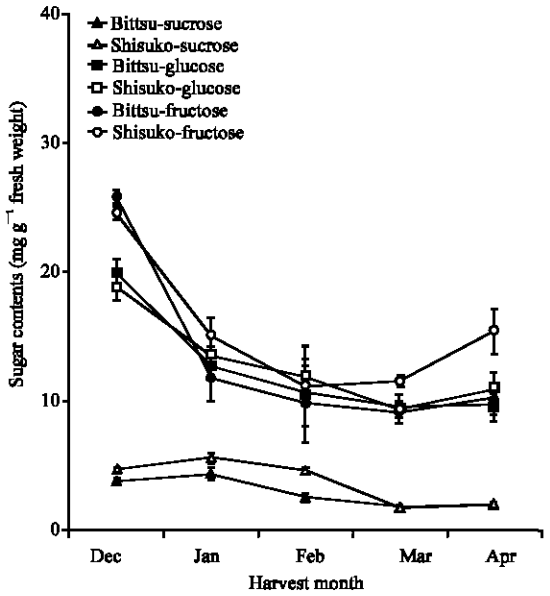

Fig. 7: Changes in soluble sugar contents in the midrib portion of two lettuce cultivars ('Bittsu' and 'Shisuko') harvested at different months. Each point represents the mean of 3 replications. Vertical bars indicate standard errors (SE). Among sugar contents of two cultivars = sucrose, significant difference at 99% level, all months

and portions (Fig. 6 and 7). In January, sucrose was the highest among all sugars during the coolest harvest period. At a low temperature, photosynthetic energy is reduced but to a lesser degree than the metabolic utilization process and a reserve accumulation of carbohydrate (Guy *et al*., 1992). In winter, carbohydrate concentrations were found to be the highest and this was attributed to cold hardiness or acclimatization to low temperature (Sivaci, 2006). The increase in carbohydrate contents in winter, especially sucrose, is important for cold hardiness (Kaurin *et al*., 1981). Thus, the plants become more resistant to very low temperature. There was a highly significant negative correlation between invertase activity and sucrose content in the leaf and midrib portions of both cultivars (Table 1). This correlation indicates that the acid invertase had a key role in sucrose breakdown metabolism.

In conclusion, the changes in activities of the three enzymes (acid invertase, sucrose synthase and sucrose phosphate synthase) and soluble sugar contents in the leaf and midrib portions of each cultivar could be influenced by seasonal fluctuations in temperature. Acid invertase activity increased throughout the harvest months, except in the leaf portion of the head harvested in April. On the other hand, SS and SPS activities decreased in all portions of both cultivars until the end of the harvest period. The maximum sucrose content was observed in January. 'Bittsu' showed higher reducing sugar contents than 'Shisuko'.

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