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Seasonal Fluctuations of Some Sucrose Metabolizing Enzymes and Sugar and Organic Acid Contents in Broccoli

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Abstract: This study investigated the seasonal fluctuations in invertase, sucrose synthase (SS) and sucrose phosphate synthase (SPS) activities and sugar and organic acid contents in the florets and branchlets of two broccoli cultivars 'Hartland' and 'Sairin' harvested from October to April. In both, cultivars and portions, invertase (soluble and cell wall-bound fraction) and SS activities were high in October thereafter decreasing until January and once again gradually increasing up to April. In both cultivars, there was no specific change in SPS activity throughout the harvest season but the activity was high in January. Organic acids (malic and citric) and sugar (specially, sucrose) concentrations increased from October to a maximum in January and again decreased up to April. However, no significant change was observed in glucose and fructose. There was a highly significant negative correlation observed between invertase and SS activities and sucrose content in the florets and branchlets of both cultivars. 'Sairin' showed higher invertase, SS and SPS activities in both florets and branchlets than 'Hartland'

Key words: Broccoli, cultivar, invertase, organic acid, sucrose, sucrose phosphate synthase, sucrose synthase

INTRODUCTION

Changes in climatic conditions during growth affect many metabolic processes in plants. The ability of plants to adapt to low temperatures has been attributed to changes in specific biochemical processes[1], including the synthesis of temperature shock proteins[2], alternations of structure and function of enzymes involved in key metabolic reactions[3] and development and repair of the photosynthetic apparatus[4]. Changes in cell membrane structure[5] or in the in soluble cell wall proteins are also involved in plant cell acclimation to temperature extremes[6]. Broccoli production is highly influenced by environmental temperature. The plants do not develop well when temperatures are below 0°C and growth slows down at temperatures above 20°C[7]. Floral development is also disrupted by temperature over 30°C[8]. Its yield and quality can also be determined by cultivar and growing season temperature[9]. Temperature fluctuations during growth of broccoli may result in various metabolic changes that may affect the quality of its head. Color, soluble sugars and organic acid contents are the important quality attributes in broccoli. Dufault[10] demonstrated that broccoli head color at harvest changed with mean temperature during production. Sucrose, glucose and fructose are the main soluble sugars present in broccoli which play an important role in the quality as well as in the shelf life of the commodity. Sucrose accumulation in some fruits and vegetables is responsible for their sweetening prior to harvest. In fact, sucrose content is often one of the major determinants of fruit and vegetable quality. Therefore, it is important to elucidate the metabolic fate of sugar metabolizing enzymes and sugar accumulation in relation to seasonal fluctuations in temperature. Sugar metabolism is also characterized by a continuous process of degradation and biosynthesis of sucrose observed in very different plant cells[11].

In plants sucrose is cleavaged either by sucrose synthase or invertase (β-fructofuranoside fructohydrolase). Sucrose synthase (SS) is a cytoplasmic enzyme converting sucrose in the presence of UDP (uridine 5′-diphosphate) into UDP-glucose and fructose whereas invertase hydrolyzes, cleaving sucrose into glucose and fructose. SS may also act in the direction of sucrose synthesis in different fruit and vegetables. An understanding of preharvest changes of the commodity is essential for accurate interpreting of the postharvest life of perishable produce. There is much information concerning the effects of postharvest treatments such as cooling method, chemical application, controlled

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atmosphere storage and modified atmosphere packaging on the shelf life of broccoli[12-14]. However, limited information is available on the influence of seasonal fluctuations temperature during growth and harvest of broccoli on its quality, hence, this study was conducted.

MATERIALS AND METHODS

Plant materials: Two broccoli (*Brassica oleracea* L.) cultivars 'Sairin' and 'Hartland' grown at Agricultural Experiment Station, Miki Branch, Kagawa, Japan, were harvested monthly from October to April. Right after harvest, the heads were brought to the laboratory. Temperature in the field was recorded at every harvest month (Fig. 1). After collection florets were shaved off using a razor blade and placed in an individual bag and immediately stored at -30°C until analysis.

Enzyme extraction for acid invertase: Approximately 5 g of sample from each portion was mixed with 1% of polyvinylpolypyrrolidone (PVPP) and 1g sea sand. The sample was then homogenized using a cooled mortar and pestle with 5 mL of 0.2 M citrate-phosphate buffer (C-P buffer) at pH 5.0. The resulting homogenate was then filtered through four layers of cotton cloth and the filtrate was centrifuged at 11000xg for 10 min. The total supernatant was dialyzed with 0.2 M C-P buffer (pH 5.0), diluted 40 times for 12 h and the inner solution was designated as soluble fraction. The residual tissues were re-extracted in 5 mL of 0.2 M NaCl C-P buffer for about 24 h with occasional stirring. The supernatant was dialyzed as described above and dialyzed solution was designated as cell wall-bound fraction. All extraction procedures were carried out at 0-4°C followed immediately by the enzyme assays.

Enzyme assays for acid invertase: The standard assay medium for acid invertase consisted of 0.2 M C-P buffer (pH 5.0), 0.1 mL of 0.5 M sucrose, 0.1 mL of 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. After the reaction mixture was neutralized with 0.1 N NaOH or 0.1 N HCl, a coloring Somogy's copper reagent was added and the mixture was heated for 10 min in boiling water. The amount of reducing sugars was estimated by the method of Somogy[15]. Soluble protein content was determined by the method of Lowry[16]. Bovine serum albumin was used as the standard. The enzyme activity was expressed as the amount of glucose produced per min per mg of protein.

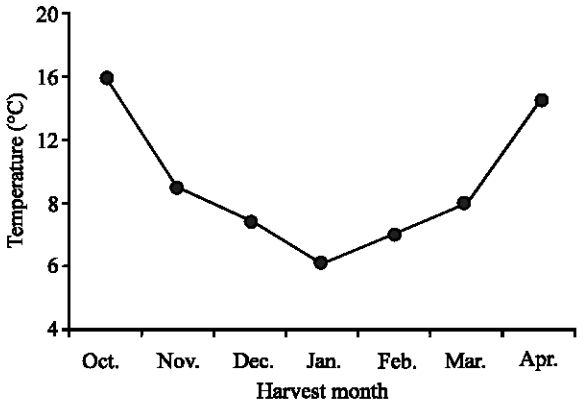

Fig. 1: Average temperature of every harvest month from October to April

Enzyme extraction for SS and SPS: Approximately 5 g sample from each portion was mixed with 1% of PVPP and 1 g sea sand. The sample was then homogenized with 10 mL of 0.2 M K-P buffer (pH 7.8) containing 10 mM ascorbate, 15 mM $MgCl_2$, 1 mM EDTA and 1 M DTT using a cooled mortar and pestle. The resulting homogenate was then filtered through four layers of cotton cloth and the filtrate was centrifuged at 11000xg for 20 min. The total supernatant was dialyzed with 40 times diluted 0.2 M K-P buffer (pH 7.8) for 16 h and the inner solution was used as the crude enzyme. All extraction procedures were carried out at 0-4°C.

Enzyme assays for SS and SPS: SS and SPS activities were assayed at 37°C by the method described by Hubbard[17] with slight modifications. Reaction mixtures (70 μL) contained 50 mM Hepes-NaOH buffer (pH 7.5), 15 mM $MgCl_2$, 25 mm fructose-6-phosphate, 25 mM glucose-6-phosphate and 25 mM UDP-glucose. The mixture was incubated for 30 min at 37°C and the reaction was terminated with the addition of 70 μL of 30% KOH. Enzyme blanks were terminated with KOH at 0 min. Tubes were kept at 100°C for 10 min to destroy any unreacted fructose or fructose-6-phosphate. After cooling, 2 mL of anthrone reagent (150 mg anthrone with 100 mL of 15% H_2SO_4) was added and incubated in a 40°C water bath for 15 min. After cooling, color development was measured at 620 nm. SS was assayed as above but with 25 mM fructose instead of fructose-6-phosphate and in the absence of glucose-6-phosphate. The soluble protein content was determined by the method of Lowry[18] using bovine serum albumin as the standard. The enzyme activity was measured as micromole of sucrose or sucrose-phosphate produced per minute per milligram of protein.

Determination of soluble sugars by HPLC: About 4 g of broccoli sample (for each portion) was mixed with 1 g sea sand and homogenized in a cooled mortar and pestle. Ten milliliter of distilled water was added to the homogenate and was centrifuged at 11000xg for 10 min. The mixture was filtered through a cellulose nitrate membrane filter (0.5 μm pore size). Soluble sugars were analyzed by HPLC using a stainless steel column (10.7 mm IDx30 cm) packed with silica gel (gel pack C 610). The filtered water was pumped through the column at a flow rate of 1.0 mL min^{-1}. The pressure was adjusted to 14-15 kg cm^{-2} and the temperature to 60°C. A RI monitor (Hitachi L-3300) was used. Sucrose, glucose and fructose were identified by their retention times and were quantified according to standards.

Determination of organic acid by HPLC: About 4 g of broccoli sample (for each portion) was mixed with 1 g sea sand and homogenized in a cooled mortar and pestle. Ten milliliter of distilled water was added to the homogenate and was centrifuged at 11000xg for 10 min. The mixture was filtered through a cellulose nitrate membrane filter (0.5 μm pore size). Organic acids were analyzed by HPLC using a stainless steel column (10.7 mm IDx30 cm) packed with silica gel (gel pack C 610). The mobile phase was 0.1% phosphoric acid adjusted to a flow rate of 0.5 mL min^{-1}. The pressure was adjusted to 15-19 kg cm^{-2} and the column temperature was ambient. The ultraviolet detector was set at 210 mn. Oxalic, citric and malic acids were identified by their retention times and were quantified according to standards.

Statistics: A randomized complete block design was adopted with three replications. The level of significance was calculated from the F-value of ANOVA. The relationship between sugar content and enzymes activities was described by a linear correlation analysis

RESULTS

Acid invertase activities: The acid invertase activities (soluble and cell wall bound fractions) were high in October, decreased until January then increased up to April in both floret and branchlet portions of each cultivar (Fig. 2 and 3). The invertase activity in the cell wall-bound fraction was higher than in soluble fraction in all portions of each cultivar. In both cultivars, a higher activity was found in branchlets than in the floret portion. 'Sairin' showed a significantly higher activity than 'Hartland'.

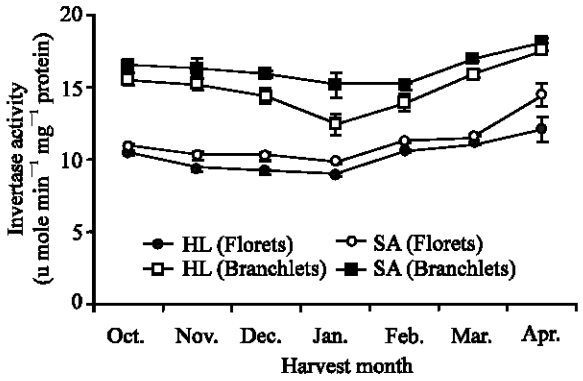

Fig. 2: Seasonal changes in acid invertase activity in the soluble fraction in the florets and branchlets of broccoli. Each point represents the mean of three replications. Vertical bars indicate S.E. which, when absent, is concealed by the graph symbol. HL= Hartland; SA= Sairin

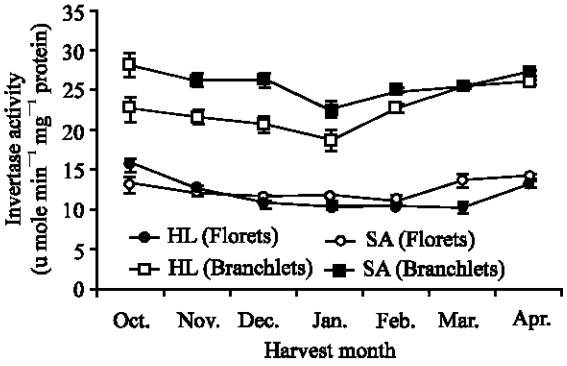

Fig. 3: Seasonal changes in acid invertase activity in the cell wall-bound fraction in the florets an branchlets of broccoli. Each point represents the mean of three replications. Vertical bars indicate S.E. which, when absent, is concealed by the graph symbols

Sucrose synthase activity: The SS activity decreased continuously from October until January and increased thereafter in both portions of each cultivar (Fig. 4). In both cultivars, branchlets showed higher enzyme activity than in the floret portion.

Sucrose phosphate synthase activity: SPS activity did not change drastically in any portion of the two cultivars until the end of the storage period (Fig. 5). However, higher SPS activity was found in the branchlet than in the floret portion.

Table 1: Correlation coefficients (r) between the enzyme activities and sugar content in the florets and branchlets of two broccoli cultivars harvested at different seasons

			Correlation value (r)			
			Acid invertase			
Sugar	Cultivars	Portions	SF	CWBF	SS	SPS
Sucrose	Hartland	Florets	-0.590**	-0.810**	-0.553**	0.105
		Branchlets	-0.606**	-0.843**	-0.710**	0.429
	Sairin	Florets	-0.462*	-0.487*	-0.875**	0.176
		Branchlets	-0.614**	-0.756**	-0.849**	0.061
Glucose	Hartland	Florets	-0.146	0.331	-0.249	-0.142
		Branchlets	0.920**	0.814**	0.738**	-0.406
Fructose	Sairin	Florets	0.652*	0.681**	0.671**	-0.115
		Branchlets	0.375	0.773	0.804**	0.215
		Florets	0.481	0.898**	0.382	0.107
		Branchlets	0.780**	0.815**	0.624**	-0.279
Hartland	Sairin	Florets	0.561*	0.615**	0.494*	0.254
		Branchlets	0.353	0.761**	0.709**	-0.342

SF= soluble fraction, CWBF= cell wall-bound fraction, SPS=sucrose phosphate synthase, SS=sucrose synthase * ** denote significant at $p<0.05$ and $p<0.01$, respectively, n=21

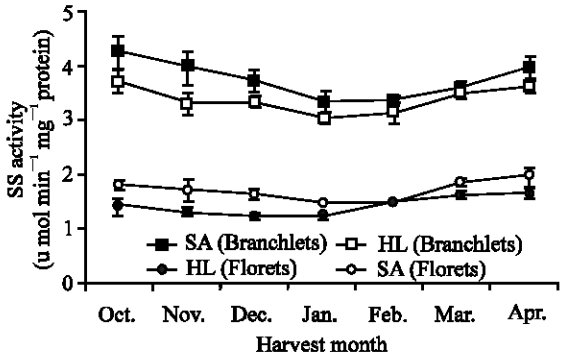

Fig. 4: Seasonal changes in sucrose synthase activity in the florets and branchlets of broccoli. Each point represents the mean of three replications. Vertical bars indicate S.E. which, when absent, is concealed by the graph symbols

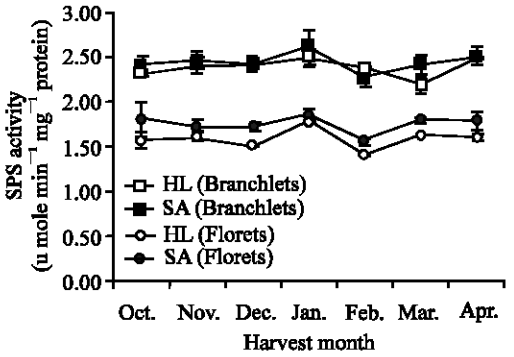

Fig. 5: Seasonal changes in sucrose phosphate synthase activity in the florets and branchlets of broccoli. Each point represents the mean of three replications. Vertical bars indicate S.E. which, when absent, is concealed by the graph symbols

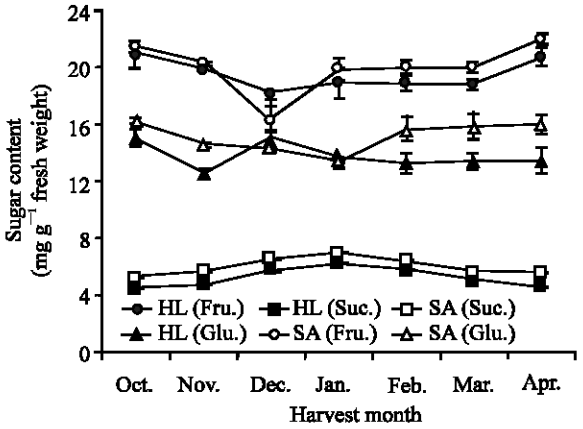

Fig. 6: Seasonal changes in soluble sugar contents in the florets of broccoli. Each point represents the mean of three replications. Each point represents the mean of three replications. Vertical bars indicate S.E. which, when absent, is concealed by the graph symbols. Fru= Fructose; Glu= Glucose; Suc= Sucrose

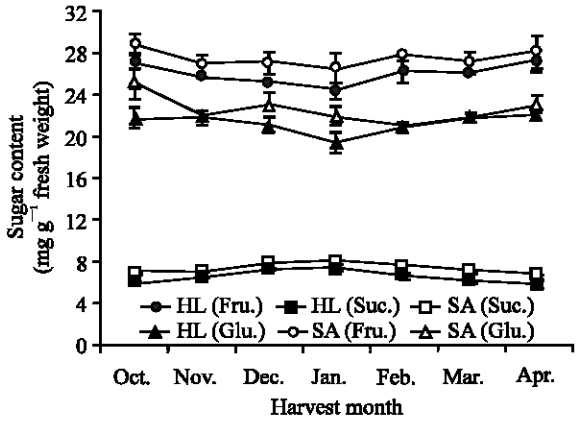

Fig. 7: Seasonal changes in soluble sugar contents in the branchlets of broccoli. Each point represents the mean of three replications. Vertical bars indicate S.E. which, when absent, is concealed by the graph symbols

Changes in soluble sugars content: Sucrose concentration gradually increased until January and after that it again decreased up to April while there was no significant change was observed in glucose and fructose (Fig. 6 and 7). A higher amount of soluble sugars (sucrose, glucose and fructose) were observed in the branchlets than florets of both cultivars. Among the three sugars, the level of fructose and glucose remained higher than that of sucrose in all portions of the two cultivars.

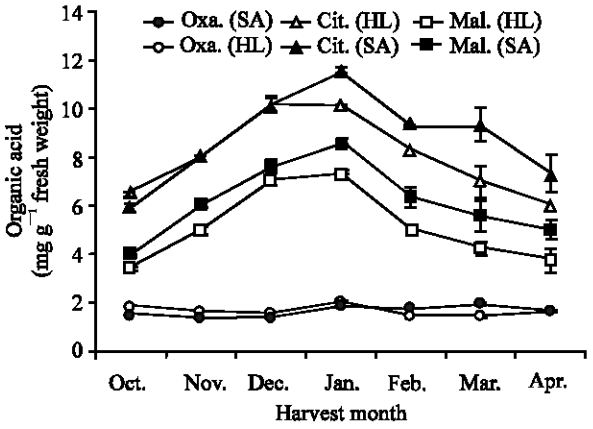

Fig. 8: Seasonal changes in organic acid contents in the florets of broccoli. Each point represents the mean of three replications. Vertical bars indicate S.E. which, when absent, is concealed by the graph symbols. Cit= Citric; Mal= Malic; Oxa= Oxalic

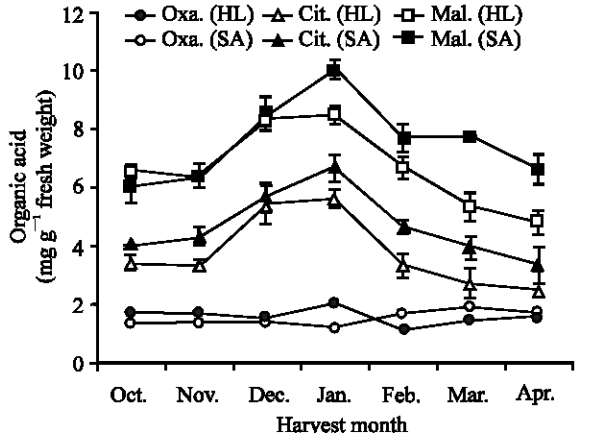

Fig. 9: Seasonal changes in organic acid contents in the branchlets of broccoli. Each point represents the mean of three replications. Vertical bars indicate S.E. which, when absent, is concealed by the graph symbols

Changes in organic acid contents: Malic and citric acid are the major organic acids present in all portions of broccoli. In both portions of each cultivar, malic and citric acid concentrations increased from October until January and after that decreased up to April (Fig. 8 and 9). In the floret portion, malic acid content was lower than in the branchlets of both cultivars. In case of oxalic acid, no significant change was found throughout the harvest season. 'Sairin' showed the higher organic acid contents than 'Hartland'.

Correlation coefficients (r) among enzyme activities and sugar content: There was a highly significant negative correlation observed between the invertase activities and sucrose content in the florets and branchlets of both cultivars (Table 1). A highly significant negative correlation was also found between SS activity and sucrose content of both portions of each cultivar. There was no significant correlation between sugar contents and SPS activity in any portions of both cultivars.

DISCUSSION

Broccoli plants are highly sensitive to environmental temperature. The invertase activities (SF and CWBF) of florets and branchlets of each cultivar gradually decreased up to January and increased until April with a rise in temperature. Generally, invertase activity increased with temperature up to a certain point. After that it decreased if temperature rose. The fluctuation of invertase activity observed in this experiment might be due to the fluctuation of temperature at that time. This type of result was also observed in other vegetables[19,20]. Similarly, SS activity decreased to a minimum in January and gradually increased thereafter. The almost similar pattern of acid invertase and SS activities observed might be due to breakdown of sucrose by both enzymes in the same direction. Both invertase and SS activities were higher in branchlets than florets. The branchlets portion is mainly composed of sink tissues. Gastonguay and Nadeau[21] reported that SS, along with invertase has an important impact on the steady state level of sucrose in plant cells and their activity is particularly high in sink tissue than source tissue. There was no specific inclining and declining pattern of SPS activity in any portions of both cultivar throughout the harvest season except in January in which activity was in maximum. The high activity in this harvest month might be due to a low temperature because low temperature induced SPS activity occurs in a number of crop species including winter rye (*Secale cereale* L.)[22,23], winter wheat (*Triticum aestivum* L.) and winter rape (*Brassica napus* L.)[24] and spinach leaves (*Spinacia oleracea* L.)[25]. In both portions of each cultivar, malic and citric acid concentrations started to increase from October until January and after that decreased up to April (Fig. 8 and 9). Higher organic acid content could be a consequence of higher sugar level during this harvest period because in most cases the immediate precursors of an organic acid is sugar or an other organic acid from which organic acid is formed or synthesized[26]. In the florets, malic acid content was

lower than branchlets in both cultivars. A similar result was found in asparagus spears where the top portion contained a lower amount of malic acid than in the bottom portion[27]. Sucrose concentration gradually increased and maximum in January and after that it again decreased up to April. At a low temperature photosynthetic energy capture is reduced but to a lesser degree than the metabolic utilization process and production of photosynthate in excess of needs would lead to a reserve accumulation of carbohydrate[25]. The most abundant and as well as most commonly accumulated free sugar in response to low temperature is sucrose[28]. A higher amount of soluble sugars (sucrose, glucose and fructose) were observed in the branchlets than florets in both cultivars. There was a highly significant negative correlation between enzyme (invertase and SS) activity and sucrose content in the floret and branchlet portions of both cultivars (Table 1). The correlation results indicate that invertase along with SS hydrolyzes sucrose.

Based on the above discussion, it was found that the enzyme activities, sugar and organic acid contents in the floret and branchlet portions of each cultivar vary at different harvest month which could be influenced by seasonal temperature fluctuations. Further research is necessary in a wider range of cultivars to determine the storage behaviour of the commodity as influenced by changing climatic conditions.

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