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Fluctuations in the Activities of Some Ammonia-assimilating Enzymes and Ammonia Content in Broccoli Harvested at Different Seasons

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Abstract: Temperature changes during growth and development influence various metabolic processes in plants. To determine its influence in broccoli (*Brassica oleracea* L.) at harvest, we examined the activities of some ammonia-assimilating enzymes and ammonia content in the floret and branchlet portions of two cultivars (Hartland and Sairin) harvested from October to April. Glutamine synthetase (GS; EC 6.3.1.2) in the two portions showed decreasing and increasing activity in the florets and branchlets, respectively with decrease in temperature. Asparagine synthetase (AS; EC 6.3.5.4) activity did not show significant variation in both cultivars throughout the experimental period except the branchlet portion wherein activity reached a peak in January and then fluctuated until April. AS activity was significantly higher in the floret than in the branchlet portion in both cultivars. Glutamate dehydrogenase (GDH; EC 1.4.1.2) aminating activity was higher during warmer harvest months while no distinct declining or inclining trend was found in the deaminating activity throughout the experimental period. GDH activities were significantly higher in the branchlet than in the floret portion except for GDH-amination in Hartland harvested in October and December. In the floret portion GDH activity was not detected in Sairin and Hartland after December and February harvests, respectively. The results of this study may provide insights in obtaining optimum yield and product quality of broccoli.

Key words: Ammonia, asparagine synthetase, commercial/harvestable maturity, cultivar, glutamate dehydrogenase, glutamine synthetase, temperature

INTRODUCTION

In temperate and subtropical regions, plants are exposed to varying degrees of temperature during growth and development particularly during the transition of one season to another. The changes in temperature influence various metabolic processes in the plant system which eventually affects yield and product quality. Total available heat and the extent of low and high temperatures are the most important factors in determining growth rate and chemical composition of horticultural crops[1]. In field production, environmental conditions such as temperature are often unmanageable but have strong implications for crop quality[2]. For instance, broccoli planted in autumn would generally produce a better quality head at harvest. However, exposure to very low temperature may result to premature formation of small flowering head. Higher temperatures during growth, on the other hand, can cause rapid swelling of buds, elongation of branchlets, loose head and bracts protruding from the head[3]. Tan *et al.*[4] reported that the response of the crop to temperature would depend on cultivar and consequently determines yield and product quality. Furthermore, chemical composition of harvested commodity is highly related to preharvest climatic conditions specifically fluctuations in temperature. In asparagus, sucrose content in roots[5] and accumulations of amino acids in spears[6] have also been demonstrated to be affected by temperature.

Although there are a lot of metabolic processes affected by temperature, nitrogen metabolism would be an interesting aspect to elucidate since N is an indispensable element incorporated in most important structural and functional macromolecules, such as proteins and nucleic acids[7]. One of the most common sources of N in plants is ammonia which is eventually synthesized into organic

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nitrogen compounds such as amino acids. In vegetable crops, excessive levels of N delay maturity and induce several disorders that diminish postharvest quality[8].

Hence, studying the enzymes responsible for its assimilation would help understand product quality after harvest. Some of the key enzymes responsible for ammonia assimilation are glutamine synthetase (GS; EC 6.3.1.2) asparagine synthetase (AS; EC 6.3.5.4) and glutamate dehydrogenase (GDH; EC 1.4.1.2). GS catalyzes the conversion of inorganic nitrogen (ammonium) into organic form (Gln) and for this reason, is a good candidate to be a critical and possibly rate-limiting enzyme in ammonium assimilation[9]. Moreover, N can also be channeled to the biosynthesis of asparagine which is catalyzed by AS[10]. Another enzyme potentially involved in ammonia metabolism is GDH. Aside from its possible role in ammonia detoxification process[11], GDH is one of the few enzymes capable of releasing amino nitrogen from amino acids to give a keto-acid and NH_3 that can be separately recycled to be used in respiration and amide formation, respectively[12].

Postharvest management is a continuum process which should give due considerations of preharvest factors affecting product quality. Hence, this study was conducted to investigate the fluctuations in the activities of GS, AS and GDH and ammonia content in the floret and branchlet portions of two broccoli cultivars (Hartland and Sairin) harvested at different months from autumn to spring. The results of this study provide insights on the cultural management of the crop such as fertilizer use efficiency and help growers optimize yield and quality by matching cultivars to time of planting.

MATERIALS AND METHODS

Plant material: Samples of two field-grown broccoli cultivars (Hartland and Sairin) were obtained from Kagawa Agricultural Experiment Station, Miki, Kagawa, Japan. Field temperature throughout the harvest months was recorded. Monthly harvest was done from October to April when the head reached the commercial/harvestable maturity. Duration from planting to harvest varied depending on season. Commercial/harvestable maturity is referred to, in this study as the stage of development when the head is compact or before the opening of the florets. At this stage, the product is considered at its best for commercial purposes. Right after harvest, the heads were packed in a box with crushed ice and transported to the laboratory. The florets were separated from the branchlets and immediately kept at -30°C until analysis.

Ammonia assay: Two grams of fresh-weight sample from each portion of the broccoli head was extracted with 10% trichloroacetic acid (TCAA) at 0°C (ice bath) and centrifuged at 12000 × g at 2°C for 10 min. Ammonia content was assayed using the procedure of Kun and Kearney[13] which required a 1 mL assay mixture containing 200 μL 0.5 M tris-buffer (pH 8), 100 μL 0.1 M 2-oxoglutarate solution (pH 7.4), 30 μL 8 mM β-NADH solution, 20 μL (10 mg mL^{-1} *GlDH*), 150 μL distilled water and 500 μL of neutral extract sample. The decrease in NADH, as determined by the change of extinction at 365 nm, was used as a measure of the reaction.

Enzyme extraction and assay: Five grams fresh-weight sample from each portion of the broccoli head was added with 1% polyvinyl polypyrrolidone (PVPP) proportional to the sample weight, 1 g sea sand and 5 mL buffer A solution. The mixture was homogenized using mortar and pestle. Extraction was performed using a procedure by Hurst and Clark[14] in which the buffer A contained 50 mM tris-HCl (pH 7.6), 10 mM $MgSO_4$, 1 mM EDTA, 1 mM dithiothreitol (DTT), 12 mM 2-mercaptoethanol, 5 mM Na L-glutamate and 100 mL glycerol per liter and buffer B contained 50 mM KH_2PO_4 (pH 8), 50 mM KCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 12 mM 2-mercaptoethanol and 100 mL glycerol per liter. Buffer A was used for the extraction of GS and GDH while buffer B was used for extraction of AS. The homogenate was squeezed through four layers of cotton cloth. The residual tissues were re-extracted with an additional 5 mL of the same buffer and the filtrate was centrifuged at 12000 × g at 2°C for 10 min.

Enzyme activities were assayed in a total volume of 1 mL assay mixture. For determination of GS activity, 80 mM Na L-glutamate, 100 mM tricine-KOH buffer (pH 7.0), 6 mM NH_2OH, 20 mM $MgSO_4.7H_2O$, 1 mM diethylenetriamine pentaacetic acid (DTPA), 8 mM ATP, and 8 mM mercaptoethanol were used. The AS activity was assayed with a mixture containing 20 mM Na L-aspartate, 100 mM tris-HCl (pH 7.0), 12 mM $MgCl_2.6H_2O$, 10 mM ATP-2 Na and 800 mM NH_2OH-HCl. After incubating the samples at 35°C for 8 min and at 30°C for 10 min for GS and AS, respectively, the reaction was stopped by adding 1 mL ferric reagent containing 0.37 M $FeCl_3$, 0.67 N HCl and 0.2 M TCAA. Absorbance of the sample was recorded at 540 nm using a double beam spectrophotometer (Shimadzu model UV-150-02). Protein content was determined using bovine serum albumin as the standard following the method of Lowry *et al.*[15]. GDH activity was determined in both aminating and deaminating directions in a total volume of 1 mL assay

mixture. For GDH-amination activity determination, a mixture of 20 mM α-ketoglutaric acid, 100 mM tris-HCl (pH 8), 200 mM NH_4Cl, 1 mM $CaCl_2$, 0.20 mM NAD(P)H, 20 μL distilled water and 200 μL enzyme solution was used. The GDH-deamination activity was assayed with a mixture containing 100 mM L-glutamate, 100 mM tris-HCl (pH 9.3), 1 mM $NAD(P)^+$, 0.5 mM $CaCl_2$, 240 μL distilled water and 200 μL enzyme solution. Both amination and deamination activities were monitored using a double beam spectrophotometer (Shimadzu model UV-150-02) at 350 nm to NADH oxidation or NAD^+ reduction. One unit of GDH activity is defined as the oxidation or reduction of 1 μmol of coenzyme (NADPH/NADP, respectively) per min at 30°C.

Analysis of data: A randomized complete block design with three replications was adopted. The significant differences between sample means were determined based on the F-value calculated using ANOVA.

RESULTS

Ammonia content: Ammonia content in all portions of both cultivars significantly changed throughout the harvest months. The ammonia content in the floret portion of Sairin increased with the drop in temperature until December while the rest of the samples reached a maximum level in January (Figs. 1 and 2). The branchlet portion contained higher amount of ammonia than the floret portion.

Glutamine synthetase: GS activity in the floret portion of both cultivars gradually declined until January and increased thereafter (Fig. 3). In the branchlet portion, maximum activity was observed in December and January in Sairin and Hartland, respectively. However, the activity dropped when the temperature of the harvest month became warmer (Figs. 2 and 3). Enzyme activity was remarkably higher in the floret portion of both cultivars except during the coldest harvest period wherein GS activity in Sairin dropped to a level below that found in the branchlet portion.

Asparagine synthetase: There was no remarkable variation in the activity of AS in the floret portion of both cultivars with the change in temperature of the harvest months (Figs. 2 and 4). However, in the branchlet portion, an increasing activity was observed reaching its peak in January and then fluctuated until April. Hartland has a relatively higher enzyme activity in both portions compared with Sairin. Consistent in the two cultivars, AS activity was significantly higher in the floret than in the branchlet portion.

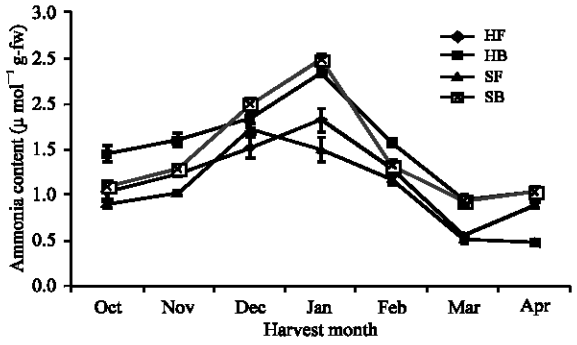

Fig. 1: Ammonia content in the floret and branchlet portions of two broccoli cultivars (Hartland and Sairin) harvested at different seasons. Each point represents the mean of 3 replications. Vertical bars indicate SE. SE bars were not shown when masked by the graph symbols. Legend: HF = Hartland florets; HB = Hartland branchlets; SF= Sairin florets; and SB = Sairin branchlets

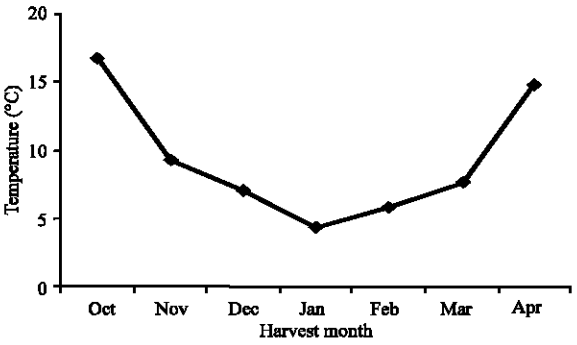

Fig. 2: Average temperature of every harvest month

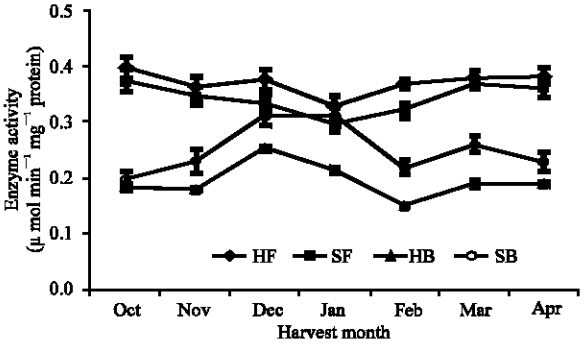

Fig. 3: Seasonal fluctuations in the activity of glutamine synthetase in the floret and branchlet portions of two broccoli cultivars (Hartland and Sairin). Each point represents the mean of 3 replications. Vertical bars indicate SE. SE bars were not shown when masked by the graph symbols. Legend: As shown in Fig. 1

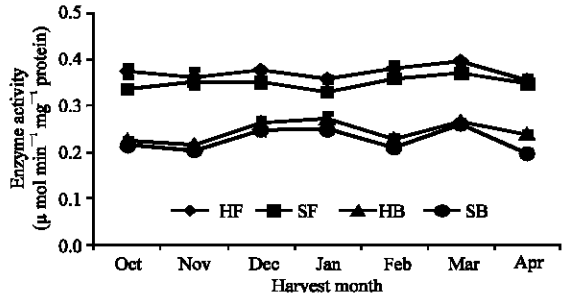

Fig. 4: Seasonal fluctuations in the activity of asparagine synthetase in the floret and branchlet portions of two broccoli cultivars (Hartland and Sairin). Each point represents the mean of 3 replications. Vertical bars indicate SE. SE bars were not shown when masked by the graph symbols. Legend: As shown in Fig. 1

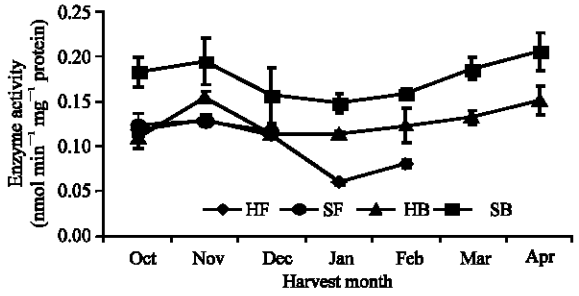

Fig. 5: Seasonal fluctuations in the activity of glutamate dehydrogenase-amination in the floret and branchlet portions of two broccoli cultivars (Hartland and Sairin). Each point represents the mean of 3 replications. Vertical bars indicate SE. SE bars were not shown when masked by the graph symbols. Legend: As shown in Fig. 1

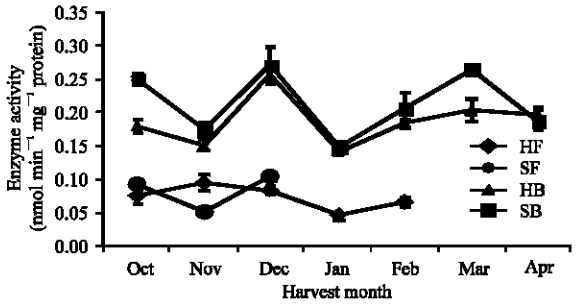

Fig. 6: Seasonal fluctuations in the activity of glutamate dehydrogenase-deamination in the floret and branchlet portions of two broccoli cultivars (Hartland and Sairin). Each point represents the mean of 3 replications. Vertical bars indicate SE. SE bars were not shown when masked by the graph symbols. Legend: As shown in Fig. 1

Glutamate dehydrogenase: GDH-aminating activity was higher during warmer harvest months. It dropped to the lowest level in January and increased thereafter (Fig. 5). On the other hand, GDH-deaminating activity did not show a distinct declining or inclining trend in all harvest months (Fig. 6). GDH activities were significantly higher in the branchlet than in the floret portion except for GDH amination in Hartland harvested in October and December. In the floret portion, GDH activity was not detected in Sairin and Hartland after December and February harvests, respectively.

DISCUSSION

Variations of temperature during growth and development affect ammonia content and activities of its assimilating enzymes in plant tissues. In this study, the response of broccoli to temperature changes during growth until harvest was remarkable as exhibited by higher ammonia content at colder harvest months (Figs. 1 and 2). This could be due to the increased NH_4^+ uptake of the crop at low temperature as observed in barley[16]. Except for the florets of Sairin which had the highest ammonia content in December, the other portions of the two cultivars increased consistently and reached the maximum during the coldest harvest period in January. However, the changes in the activities of the enzymes examined in relation to change in temperature vary with tissues. In the floret portion, GS activity of both cultivars decreased with temperature until January and gradually increased towards spring (Fig. 3). Though the activity in the floret portion was remarkably higher than in the branchlet portion, it was not maintained during the coldest harvest month wherein the activity dropped to a level as that in the branchlets of Sairin. The increased level of ammonia in the florets could be due to the decreased GS activity or due to the lesser demand of N by the tissues at lower temperature. On the other hand, an opposite trend was found in the branchlet portion wherein enzyme activity reached the maximum at the coldest harvest month. These differences of response between the two portions can be attributed to the type of tissues in the branchlets and florets; the former is composed of more mature tissues than the latter. Branchlets have also been found to contain higher amount of sugars than the florets[17]. It is likely that the high sugar content increases the energy level and consequently GS activity in the tissues[18]. Another probable cause of the different patterns of GS activity between the two portions could be genetically related. In soybean, cytosolic GS is encoded by a multigene family and the members are regulated in an organ-specific and developmental manner[19]. Expression of a chimeric gene in *Lotus corniculatus* is increased in

response to treatment with ammonia, however, no induction was observed in tobacco roots[20]. A detailed study at the molecular level would give a conclusive evidence of the relation between GS activity and ammonia accumulation in different tissues as affected by changes of temperature during growth.

There was no significant variation in the activity of AS in the floret portion of both cultivars with the change in temperature throughout the harvest months (Fig. 4). However, in the branchlet portion the activity increased slightly reaching a maximum in January and then fluctuated until April. It is likely that, in addition to temperature, AS activity is affected by light and carbohydrate level. In conditions of ample energy, GS may have an inhibitory effect on AS activity. Downs and Somerfield[21] reported that AS gene expression in broccoli increases as sucrose declines after harvest. The differential regulation of the activities of GS and AS could be a way of maintaining a balance in carbon and nitrogen metabolism in plants[22].

As an alternate pathway for ammonia assimilation, GDH was examined in both aminating and deaminating directions. There was higher GDH-aminating activity during warmer harvest months. It dropped to the lowest level in January and increased thereafter (Fig. 5). Lower temperature has been observed to adversely affect GDH activity in the roots of *Glycine* and the decrease in enzyme activity was possibly caused by relative decrease in enzyme protein content as well as changes in the ratio between NADH/NADPH forms[23]. In addition, the high sugar content of the branchlets during colder harvest months[17] favors GS to assimilate ammonia rather than GDH. Sugars appear to play a more central role in the regulation of GDH than ammonia and other nitrogenous sources[24]. It was proposed that there is reciprocal regulation of GS and GDH[18]. Furthermore, considering the low affinity[25] or high K_m value[26] of GDH for ammonium, it could also be hypothesized that although ammonia concentration in both portions was increasing, the concentration may not be enough to trigger the enzyme activity. GDH-deaminating activity did not show a distinct declining or inclining trend in all harvest months (Fig. 6). The fluctuation in GDH-deaminating activity may imply that other growth factors such as light intensity and duration, relative humidity and rainfall could influence enzyme activity in addition to temperature change. In the floret portion, GDH activities were not detected in Sairin and Hartland after the December and February harvests, respectively. In a separate study, GDH activity was also found deficient in the floret portion of Sairin harvested in midwinter and was not induced despite the significant increase in ammonia content during postharvest senescence[27]. Instead of relying mainly on physical harvest indicators such as compactness of the head, and/or number of days from planting, GDH activities could be a useful index in approximating the optimum physiological maturity that coincides with harvestable/commercial maturity of the crop. A detailed study must be performed to validate this suggestion. Harvesting the crop at its optimum physiological maturity has some important postharvest implications. Sairin which was deficient in GDH activity had a shorter shelf life than Hartland during storage at 20°C[27]. In the present study, the samples were harvested based on the harvestable/commercial maturity indices of the crop such as compactness of the head or before the opening of the florets. In February, when the temperature started to increase, the heads became commercially mature earlier than the previous months harvests, thus, early harvest was done to avoid premature opening of the florets. During this time the heads were not as compact as those harvested at colder months.

The result of this study pointed out that although the response varies with the type of tissues, changes in temperature during growth influence the activities of some ammonia-assimilating enzymes. Ammonia content was higher in the branchlet and floret portions of both cultivars during the coldest harvest month. For efficient fertilizer use, N application could be adjusted depending on growing season. Sairin could be suitably planted starting late summer such that harvest can be done until midwinter and a good quality head of Hartland could be available until late winter. Further studies on the interaction between temperature and other growth factors such as light intensity, photoperiods, relative humidity and plant nutrition are needed to give a holistic picture of the changes in the activities of ammonia-assimilating enzymes, ammonia content and other qualities in broccoli harvested at different seasons. The influence of different growth factors on the shelf life and storage qualities of the head harvested at different seasons need to be studied. The specific thermal requirements for each stage of development and duration of exposure to abuse temperatures for a wider range of cultivars also need to be elucidated as these factors have implications on the harvest quality and shelf life.

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