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Gene Profiling for Invertase Activity: Assessment of Potato Varieties for Resistance towards Cold Induced Sweetening

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Abstract

Background: Potato is the most important staple food in the world. Cold-induced sweetening occurs when potatoes are stored at low temperature for longer period of time. Due to non-enzymatic Millard reaction it causes unwanted changes in colour, taste and in flavor when fried and roasted at high temperature. However, long-term cold storage is mandatory to keep an adequate supply of potatoes throughout the year. The cause of cold-induced sweetening is invertase enzyme.

Methods: Five potato varieties (Hermes (A) Lady Rosetta (B) Oscar (C) Kuroda (D) and Multa (E)) were investigated for invertase activity during two month cold storage at 4°C. Crude protein was extracted by PD Midi Trap G25 column technique. Quantification of mRNA expression was employed through QPCR. Determination of sucrose, reducing sugars and organic acids was simply done by 80% ethanol method and concentration were find out by using HPLC with already set standards. The correlation between invertase enzyme, sugar content and mRNA expression was calculated through Statistical methods.

Results: Significant activity of invertase was observed at 4°C with up to 6.3 nmol/min/mg of protein in the type-1 & 4 (cv. Hermes and Kuroda); 2.5 times less in type 2 (Rosetta) and 3.5 times less in type 3 (Multa) when compared with same at 4°C. In addition, malic acid concentration was found positively correlated with invertase activity at 4°C as compared with its concentration at harvesting time. However, citric acid and oxalic acid concentrations were independent of invertase enzyme activity. The transcript level of invertase enzyme was found significantly high in potato tubers stored at 4°C in result 1 & 4 type, less in result type 2(C) and negligible in result 3(E) potato variety when revealed through reverse transcription PCR.

Conclusions: In conclusion, Oscar (C) and Multa (E) were found more resistant to CIS at 4°C storage and may be used for future variety improvement programs for CIS resistant through breeding and molecular approaches.



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Introduction

Potato has turned into a very important crop for both consumers and farmers in Pakistan. All over the world Pakistan stands fourth in production of potato. It is extremely nutritious and high yielding crop. Potatoes are usually harvested at rest period or even after the end of that period. Poor post-harvest handling including improper storage practices and transport that cause abounding damage and abridgement of burning quality. The potentially damaging factor among above listed is the improper storage. Potato needs metabolic stability with respect to its carbohydrate contents for uninterrupted supply to the industry all the year around. Low temperature storage is mandatory as it diminishes bacterial soft rots, decrease water, dry matter loss and prevents sprouting [1]. Sowokinos reported that storage at low temperature triggers increase in reducing sugar content of the potato termed as "low temperature sweetening" [2]. It is reported that reducing sugars react with free amino acids by the Millard reaction and a neurotoxin acrylamide accumulates during this process [3]. This undesired increase in reducing sugars of potato severely affects its market demand by end user.

Accumulation of hexose sugars in potato during cold storage at 4°C for two months is done by the enzyme invertase. Correlation exists between hexose accumulation, acid invertase activity and sucrose ratio in cultivars exhibiting varying resistance to cold-induced sweetening [4-7]. At low temperature storage, the increase in activity of invertase is cultivar dependent [8]. Presumably differences in mRNA expression patterns and post-transcriptional events determine the degree of particular cultivar's resistance to cold-induced sweetening [7,8]. Certain reports exist where hexose accumulation was prevented during cold storage in transgenic plants with suppressed acid invertase activity [9]. In this process, the activity of vacuolar invertase (StvacINV1) and the role of invertase inhibitors are considered equally important [10,11].

The current study focused on identification of elite potato varieties from Pakistan with resistance towards low temperature induced sweetening based on vacuolar acid invertase enzyme activity. Here we used five potato varieties; Hermes (A) Lady Rosetta (B) Oscar (C) Kuroda (D) and Multa (E). Vacuolar invertase activity and sugar contents of each variety's tubers were recorded during storage period at 4°C, 10°C and at 20°C.

Similar parameters were also tabulated during growing stage for each variety under study. The correlation between invertase enzyme activity, sucrose and reducing sugar content is highlighted. This study is one of its kinds regarding determination of relevance of Invertase mRNA expression level and reducing sugars.

Methods

Plant Material

Five local potato varieties; Hermes (A) Lady Rosetta (B) Oscar (C) Kuroda (D) and Multa (E) were kindly provided by Potato Research Institute Sahiwal, Pakistan. Potato varieties with significant agronomical potential were selected. All-inclusive five potato varieties were harvested through three successive years (2010-12) in the Centre of Excellence in Molecular Biology, University of the Punjab, Lahore fields. All the required agronomic practices including fungicide, herbicide, insecticides and fertilizers were applied to obtain better yield output. The harvested tubers were cured at 20°C for 15 days in the dark. Fresh, good skinned and healthy looking tubers weighing between 100-120 g were selected and were stored at 20°C, 10°C and at 4°C in the dark with 90% relative humidity. For analysis of sugar content at growing stage, three immature tubers weighing between 75-100 g were selected while post harvest analyses were performed on five randomly selected potatoes.

Sample Preparation & Extraction of Crude Protein

Longitudinal sections of subjected potato tubers were made to measure amount of sucrose, reducing sugar, organic acid and invertase enzyme activity assay. The samples were snap frozen with liquid nitrogen. For invertase enzyme activity assay, total tuber protein was isolated. Approximately 100 g of the grinded tuber sample was used for crude protein extraction. PD Midi Trap G25 column cat#45-001-530 was used for this task. Extraction buffer was prepared by using 50mM HEPES-KOH, pH7.5, 5mM MgCl₂, H₂O, 1mM EDTA, 1mM EGTA, 0.1% W/V Triton X-100 and 10% W/V glycerol. PMSF was added just prior to use at the final concentration of 1mM. Further extraction of crude protein was done as per manufacturer's instructions. Total crude protein was estimated by Bradford assay [12].

Vacuolar acid invertase enzyme activity

Invertase enzyme activity was measured with and without invertase inhibitor as originally described by Liu [13]. The assay was based on the principle that the crude protein extract of potato has an endogenous proteinaceous inhibitor which has an inhibitory effect on soluble invertase activity [14]. Each sample comprised of 20 µg of total protein. Negative control samples were also processed in parallel. Final absorbance was taken at 560 nm by spectrophotometer after putting the reaction mix plate in dark for 30 minutes. Activity was calculated as nmol glucose formed per minute per mg of protein.

Determination of sucrose, reducing sugars and organic acids

Potato tuber samples were subjected to lyophilisation for two days prior to snap freezing in liquid nitrogen. After centrifugation, samples were resuspended in 4 mL of 80% ethanol followed by vortexing for 1 min. Further, samples were incubated at 60°C with continuous shaking overnight. Subsequently, supernatant was transferred into new tube and centrifuged at 4000 rpm and 20°C for 10 min. The previous step was repeated once. Further, 80% ethanol was added in the supernatant to make volume up-to 10ml. Subsequently, the samples were subjected to vacuum-fuge for drying without heat for overnight. For HPLC analysis, 1ml of mQ H₂O was added and incubated at room temperature for 30 min. The pellet was dissolved through vortex and centrifuged at 13000 rpm for 10 minutes. Filtration of samples was done with the help of tuberculin syringe, by using 0.2 µm regenerated cellulose syringe filter. Glucose, fructose, and sucrose were calculated in mg/ml along with Malic acid, Citric acid and Oxalic acid through High Performance Liquid Chromatography (HPLC).

Real Time PCR analysis

Total cellular RNA was extracted from snap frozen tuber slices by using Agilent Plant RNA isolation Mini Kit (Cat#5155-2780). RNA was treated with DNase (Ambion DNase treatment kit Cat#AM1906). The cDNA was prepared by using Superscript III (Invitrogen, cat18080-051) as per instructions. This cDNA was used as template for real-time PCR assay. Relative quantification of mRNA (invertase gene) was done with Bio-Rad PCR system. SYBR

Green/fluorescein qPCR Master mix (Cat#k0241) was used. Data were normalized by actin as internal control. Cycling profile was 95°C for 10 min, followed by 95°C for 15s, 55°C for 30s and 72°C for 30s.

Statistical analysis

The deduced data was statistically analysed by using analysis of Variance technique [15] whereas heritability [16] and genetic advance was analysed according to Falconer [17].

Results

Estimation of sugar content in potato tuber at growing period

Maximum 5 nmol/mL of glucose content was obtained in 35 days old immature potato tubers. Fructose was present less than 1 nmol/mL and it was found to be almost constant in all the cultivars. Sucrose content ranged from 4-11 nmol/mg of fresh biomass of tuber which was higher than glucose at early growing stages. However, amount of glucose and sucrose was almost same at harvesting stage. Before the end of the growing season the minimum contents of reducing sugars in tubers were reached and deliberate addition occurred hereafter [18]. Similar changing trend in sugars were obtained in this research (Fig. 1 extreme left).

Estimation of sugar content at different storage time and temperatures

Less than 1.5 nmol/mg of sugar contents of fresh weight were obtained in almost all tubers among all varieties when analysed during dormancy stage, depicting low metabolic activities during this period. Beside this a little increase in the sucrose contents was observed in all varieties during sprouting. Sugar contents of both varieties A and B were same with low glucose and sucrose contents initially. But after 35 days of first analysis, glucose content were increased to 3 nmol/mg as compared to sucrose and fructose which were 2.7 nmol/mg and 2.4 nmol/mg, respectively at 10°C. While variety C showed high sucrose contents (3.5 mg/g) throughout sampling period. Results of variety D were interesting in a sense that instead of sucrose (2 nmol/mg) and glucose (2.7 nmol/mg), fructose (4 nmol/mg) was high throughout sampling dates from initial to ending period. Variety E showed no prominent fluctuation from normal as its sucrose was high (2.6

nmol/mg) but it was low as compared to other varieties (4 nmol/mg). Four different patterns of glucose, fructose and sucrose contents were observed in all five potato varieties when subjected to storage at 4°C i.e. (1) increased reducing sugars level during storage; (2) increase in sucrose level but not reducing sugars; (3) increase fructose level as compared to glucose and sucrose; (4) low level of sucrose as well as reducing sugars. In result-1 category (variety A and D) sucrose and reducing sugars were 2 mg/g Fresh weight (FW) initially during storage period of 30-80 days. There was observed an increase in reducing sugar level up to 6.5 mg/g FW in the same varieties during storage period of 180 days. The levels of reducing sugars increased gradually up to 7 mg/g FW in variety A while 8 mg/g FW in variety D after storage period of 230 days. However in result-2 category, in variety (Oscar C) sucrose contents were high in initial sampling that is almost 5 mg/g of FW. It reached at maximum level of 5.8 mg/g FW after 180 days of storages and then remained stable. Interestingly, levels of reducing sugar in this cultivar (C) remained stable. In variety D the pattern was quite similar to type 1, but the only difference which was noticed is that instead of glucose, fructose was high (8 mg/g FW) until last days of storage and remained stable. In result-3 category, variety E showed unique pattern. Sucrose contents increased rapidly and reached to its maximum level within 5 weeks (about 3 mg/g FW), but then decreased. The reducing sugar were also increased during that period, but later on decrease in reducing sugars was also observed until 2 mg/g fresh weight. A slight increase in sucrose was also observed at this stage and after that it became stable. The cultivar E is the progeny of ND 860-2, which is a so-called, "low sugar-accumulating, cold-tolerant" breeding line. These results are well correspond to Espen [19]. Corresponding with the results described by (Burton. 1989) type1 & 4, results are resembling with Hill [20]. The results of cultivar E are similar to those observed in studies of cold-tolerant lines, ND 860-2 (Fig. 1) [6].

Variation in enzyme activity

Three types of vacuolar acid invertase activities were observed which represents four types of sugar contents of five varieties. During the growing season the activity of acid invertase was observed to be 2.5 to 3 nmol glucose/min/mg. Decrease in acid invertase activity was observed which attained its lowest level till the

harvesting time. Similar behaviour of all the varieties was seen during growing season. In the Fig. 2 activity of acid invertase enzyme represents the sugar contents behaviours of all the varieties at curing period which demonstrate that the activity of acid invertase enzyme was low as in the growing season but fluctuation in acid invertase enzyme activity was observed at 10°C in the similar pattern as was the case with sugar contents at this stage for all these varieties. Vacuolar acid invertase activity was 3.5 nmol glucose/min/mg at 10°C storage period, but at 4°C their gradual increase in the activity of vacuolar acid invertase (VINV) was observed which reached to its maximum level of 6.3 nmol glucose /min/mg. Then it attained a constant level and slight decrease was observed after 180 days of storage after that stability was attained.

Relative transcript levels of vacuolar acid invertase in potato tubers

In cultivar (A, B and D), very low signals were observed during real time quantification of vacuolar acid invertase gene in potato tubers stored at 10°C, but increase in transcriptional level was observed at 4°C. A very medium to low transcriptional level for vacuolar acid invertase gene was observed for cultivar C at 10°C as well as at 4°C storage of potato tubers. Vacuolar acid invertase gene activity was negligible for variety (E) when analysed through real time quantification for its transcriptional level at 10°C as well at 4°C (Fig. 3). Random samples from varieties (A & D) were picked for organic acid analysis because these varieties were mostly affected by invertase activity and protein was isolated from stored potato tubers (4°C). Protein samples were subjected to HPLC and concentrations of malic acid (20 mg/ml of protein) was found high as compared to citric acid (4 mg/mL) and oxalic acid (3 mg/mL).

Statistical analysis

Significant genetic differences were reported among potato genotypes at growth stage and under different temperature treatments (Table 1 in supplementary data). It was found that maximum amount of sucrose (4.38 ± 0.8482 mg) was recorded at growth stage of potato as compared with glucose and fructose. Highest heritability (81.47%) and genetic advance (136.446%) was observed for sucrose while lower heritability and higher genetic advance was found for glucose and fructose. Heritability (58.665%, 62.679%) and genetic

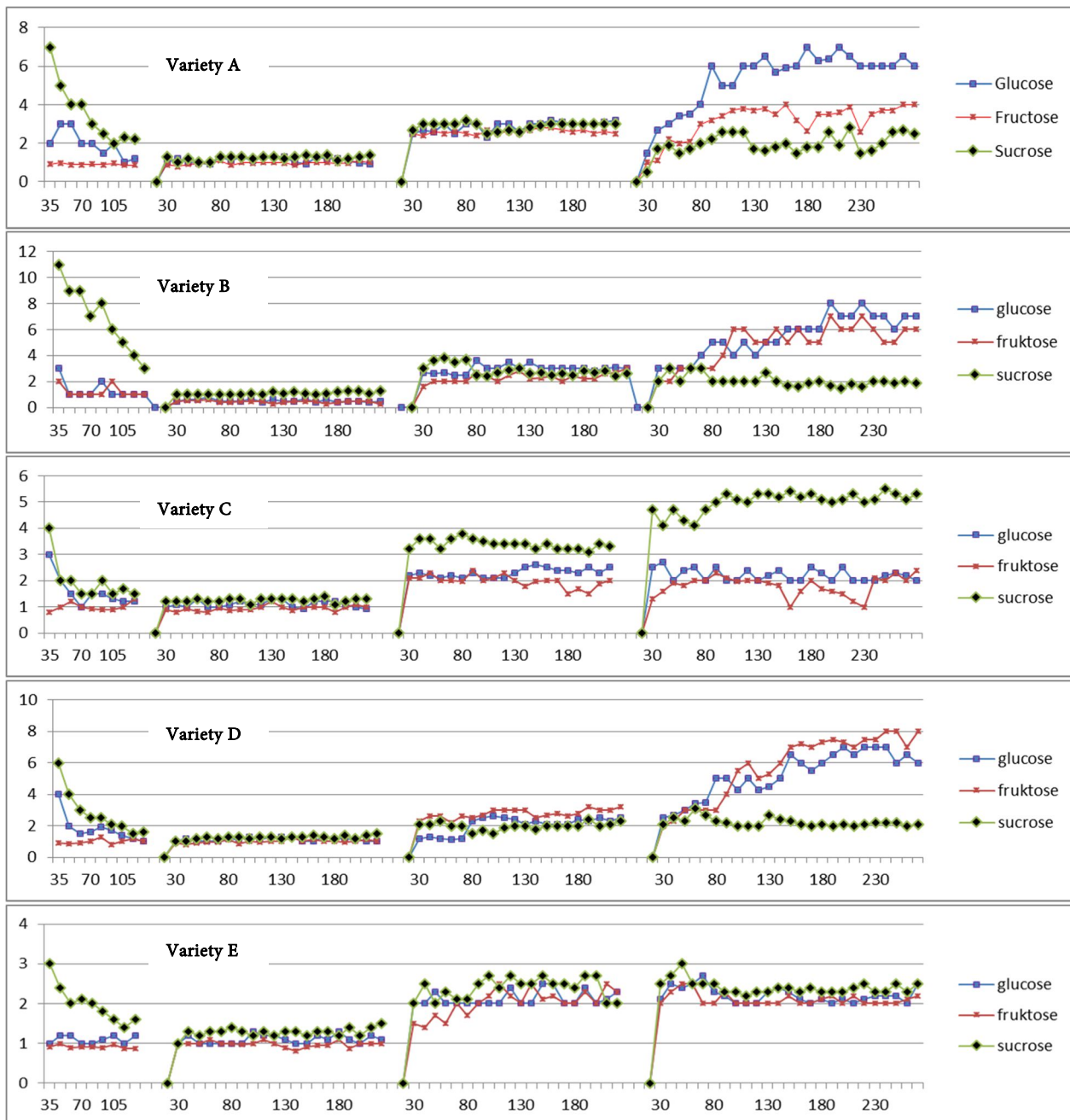


Figure 1: Graphic representation of change in sugar contents of potato tubers of five varieties (A: HERMES, B: LADY ROSETTA, C: (OSCAR), D: Kurodaand E: Laal-e-faisal) during storage at different low incubation temperatures. Potato tubers were stored at 20oC, 10°C and 4°C for 20 180 and 230 days followed by HPLC for sugar contents. Each value represents the mean value of three replicates.

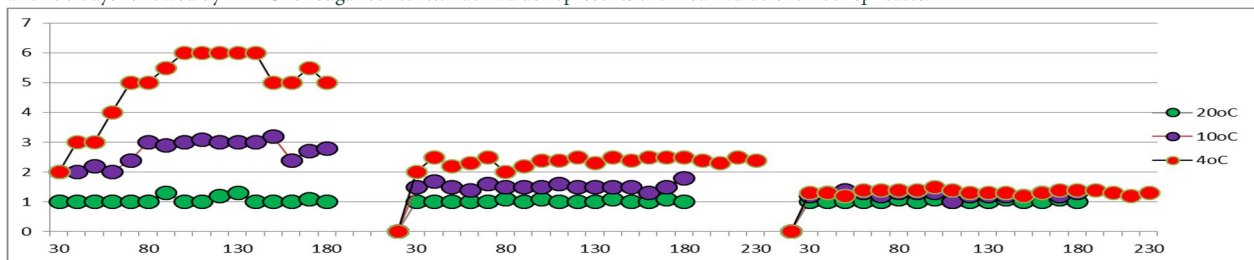


Figure 2: Fluctuation in the activity of vacuolar acid invertase in potato tubers of five varieties at growing stage and during storage period.

advance (54.137%, 16.984%) was higher for fructose in the potato tubers at 10°C and 4°C temperature storage, respectively. At 20°C higher amount of sucrose (1.1667±0.0586 mg) was found as compared with glucose and fructose amounts while higher heritability (82.852%, 85.259%) and genetic advance (84.050%, 89.679%) were found for fructose and glucose, respectively. Table 2 (in supplementary data) shows that higher amount of fructose (1.2222 mg) and sucrose (6.8889 mg) was recorded for Multa while lowest fructose (0.9087 mg) and sucrose (1.9889 mg) was recorded from Multa (E) at growth stage of potato. Higher glucose (1.9667 mg) was found for Hermes (A) while lowest was found for Multa (E) at growth stage of potato. It was persuaded from results that higher amount of fructose was found for Oscar (B) while lowest for Multa and Kuroda (D) at 20, 10 and 4°C temperature storage. Higher amount of glucose was found in Oscar (C) and Kuroda (D) at 4°C, while lowest glucose amount was reported for Multa (E) at 4°C temperature storage. Higher amount of sucrose was found for Multa at 4°C, 10°C while lowest for Oscar (C) and Kuroda (D). Principle component Bi-plot (Fig. 1) suggested that over all better performance for sucrose was observed by Multa at 4°C storage. Higher amount of fructose, glucose and low sucrose was reported for Lady Rosetta (B) and Hermes (A) (Fig. 2). It can be inferred from figure 3 that higher amount of sucrose was found for Multa, Glucose for kuroda (A & D) while the other genotype showed higher fructose contents.

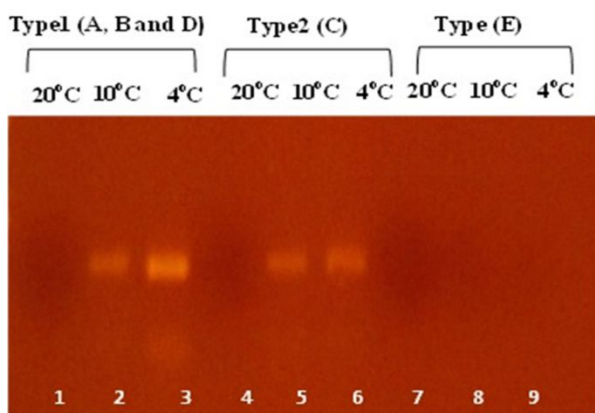


Figure 3: Reverse transcription PCR depicting mRNA expression pattern of vacuolar acid invertase gene among five potato varieties at various storage temperatures. Amplified fragment size was 1kb. mRNA expression was obtained in A, B and D potato varieties at 4°C and 10°C; potato variety C at 4°C and 10°C with no amplification at 20°C in all mentioned varieties. However potato variety E exhibited no mRNA expression at any of the storage temperatures.

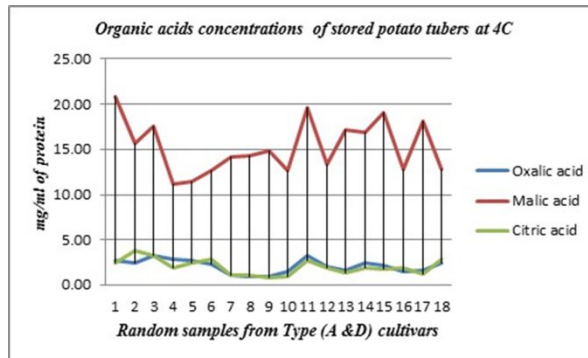


Figure 4: The concentration of Oxalic acid, Citric acid and Malic acid in potato variety A and D at 4°C. Values of each organic acid were revealed after HPLC analysis.

Discussion

An effort was made to monitor the sugar contents and vacuolar acid invertase enzyme activity in young tubers in local potato cultivars as done by Endo [4] in Japanese potato cultivars. The contents of sucrose and glucose were estimated to be high in all the varieties in initial stages but a gradual decrease in sucrose and glucose contents was also observed with tuber growth. This may be due to the fact that youngest tubers were having relatively large connection with acid invertase activity. Vacuolar acid invertase activity is being dominated by sucrose synthase activity in vegetative growing stages [4,21,22]. However fructose contents were estimated to be lower than glucose and sucrose in early stages. High ratio of glucose to fructose is due to increase in the activity of fructose specific hexokinase, which is higher than glucose specific hexokinase as reported by Davis and Opark [23]. Davis and Viola reported that the decline in activities of sucrose synthase, fructose kinase and glucose-phosphorylation might induce the reduction ratio of glucose to fructose from ca.80:1 to 2:1. Similar results were achieved in our study where values of glucose, sucrose and fructose contents were decreased toward tuber maturation but slight variations in the ratio might be due to genetic variability of the varieties and fluctuation in the enzyme activity (Fig. 2). In the current study, various values of sugars were obtained at varied storage temperatures. These results followed the same pattern of sugar accumulation as reported by Hammond and Zrener [8,9]. In varieties (A, B & D) similar changes of sugar contents and invertase activity was observed. In variety D instead of glucose, fructose values were high which indicate positive correlation of vacuolar acid invertase enzyme activity with glucose,

fructose contents. A comparison of type 2 acid invertase activity i.e. 2.5 nm glucose/min/mg with cultivar (C) during storage period was same but a slight increase occur during 10°C while at 4 °C activity was low as compared to type 1. Cultivar (C) showed high sucrose contents and low reducing sugar throughout storage period. Cultivar E was most attention-able. Sucrose and reducing sugars were low along with low invertase activity pattern, similar during curing period and throughout the storage period.

Increase in sucrose contents of Multa might be due to the genetic background of *Solanum tuberosum* sub sp. *Andigena* [4]. Its yellowish orange colour was because of high carotenoid contents [24] so it can be referred to as highly resistant potato cultivar tolerant to cold. Variety E was called low sugar accumulating cold tolerant resistant cultivar due to its genetic background, ND 860-2. Thus the data obtained in cultivar E was similar to that observed in studies of cold-tolerant lines, ND 860-2 [10]. Sugar contents for result A, B & D were highly corresponding with the vacuolar acid activity observed in type 1 vacuolar acid activity. With increasing storage incubation period vacuolar acid activity increased. These findings are highly corresponding with the reported study of Endo [4]. Real Time PCR results expression corresponds to similar findings. The transcription level at 20°C was negligible, while at 10°C slight increase was observed, which continued at 4°C during different storage period. Statistical analysis suggested that the conversion of glucose and fructose into sucrose was higher at vegetative growth stage of potato genotypes. Higher genetic advance also revealed that the genotypes may be used to fix increase in sucrose at growing stage of potato [25]. Further findings suggested that potato storage at various temperature conditions resulted in the higher glucose contents at 4°C and 10°C as compared to sucrose and fructose contents [26]. Storage of potato tubers at 20°C found helpful to improve sucrose amount in potato and to develop good quality potato producing genotypes [27]. It was further concluded from results that there is significant change in the contents of fructose, glucose and sucrose at growth stage and under different temperature treatments [28]. Organic acids especially malic acid concentration was high during storage at 4°C as compared to harvesting stage [29]. Citric and oxalic acid

concentrations were not affected during storage [30]. A positive correlation of malic acid was found with increased activity of vacuolar acid invertase. Initially at harvesting, malic acid concentration was low as compared to citric and oxalic as shown in Figure 4. The genotypes that showed higher sucrose at different temperatures (Multa) may be used for the development of higher yielding and good quality potato chipping varieties [29-34].

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