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Effects of harvesting stage and storage duration on postharvest quality and shelf life of sweet bell pepper (*Capsicum annuum* L.) varieties under passive refrigeration system

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A laboratory experiment was carried out to determine the effects of harvesting stages (0, 25, 50, 75 and 100% fruit colourations) and storage durations (0, 1, 2, 3 and 4 weeks) on physicochemical quality and shelf life of sweet pepper varieties (Telmo-Red and Velez-Yellow) under passive refrigeration system (PRS). The aim of the study was to identify the optimum stage of maturity at harvest and storage period under PRS that can ensure better quality and longer shelf life of two greenhouse sweet pepper varieties. The experiment was arranged in 2 x 5 x 5 factorial combinations in complete randomized design (CRD) with three replications. Thirty (30) fruits of sweet pepper were packed in card-board boxes for each treatment and stored under PRS optimum storage conditions. Fruits were assessed for weight loss percentage, fruit firmness, total soluble solids, titratable acidity, postharvest decay percentage and shelf life. Total soluble solids were increased; whereas fruit firmness decreased with increasing harvesting stages. Weight loss percentage, postharvest decay percentage and shelf life increased; while fruit firmness decreased with increasing storage periods. Telmo variety showed significantly better postharvest quality and storability potential than Velez variety.

Key words: Harvesting stage, postharvest, passive refrigeration system, sweet bell pepper.

INTRODUCTION

Sweet bell pepper (*Capsicum annuum* L.) is one of the most commercially important horticultural crops grown in tropical and sub-tropical regions of the world. From the nutritional point of view, peppers are generally considered as a balanced source of most of essential nutrients, high content of vitamins, important antioxidants, rich in flavonoids and phytochemicals (Maria et al., 2010). Sweet peppers are currently the object of much attention due to possible links to prevention of certain types of cardiovascular diseases, atherosclerosis, cancer, haemorrhage, delaying of ageing process, avoiding cholesterol, improving physical resistance and increasing appetite (Marin et al., 2004).

Growing and marketing of fresh produce is complicated by high postharvest losses which are estimated to reach as high as 25-35% of the produced volume for vegetables (Agonafir, 1991). Sweet peppers like other vegetables are quite perishable, about 28.6 and 38.7% post-

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harvest losses were reported during the dry and wet seasons, respectively (Tunde-Akintunde et al., 2005). Optimum temperature and relative humidity can be achieved using passive refrigeration system (PRS) cooling machine, which is a very efficient technique to store and transport products. The system works without ventilation thus assuring shelf life which is better than the active refrigeration system equipment. The thermal autonomy allows the storage and transport without use of power during operations (Nomos, 2008).

However, there is no available scientific literature regarding the effect of harvesting stages and storage durations on retaining the postharvest physicochemical quality properties of sweet bell pepper varieties under passive refrigeration system storage condition. The main objective of the present study was to evaluate the effect of harvesting at different maturity stages and storing in PRS, on shelf life and quality of sweet bell pepper varieties.

MATERIALS AND METHODS

Experimental design and treatments

The treatments were comprised of two varieties of sweet pepper (Telmo and Velez) picked at five harvesting stages (0, 25, 50, 75, 100% colourations) and stored for five storage durations (0, 1, 2, 3, and four weeks) under PRS. The treatments were combined in CRD factorial experiment, resulting in a total of 50 treatment combinations (2x5x5) with three replications and 150 total observations (2x5x5x3). Each treatment consisted of 30 fruits packed in standard card board boxes for storage under PRS.

Experimental procedures

Fruits of two sweet pepper varieties with similar size (160 g) and shape (bell shaped) were harvested from Hawassa Jittu Horticulture PLC greenhouse. Maturity stages of fruits were determined by fruit colouration guide and days from anthesis. Fruits were harvested manually with care to minimize mechanical injuries. After harvest, fruits were immediately transported using standard plastic crates to packing house within 10 min and held at 10°C pre-cooling room overnight. Fruits with bruises, sign of infection or those different from the group were discarded from the samples. Fruits were washed with tap water, surface dried with soft cloth and subdivided, sorted, and weighed in the packinghouse; thereafter stored under PRS (model *DS-TP-001-03*) on three shelves as replication. Samples were taken to food technology laboratory for quality analysis. The treatments were tested at test room environmental conditions (20°C temperature and 70% relative humidity) combined with 24 h lighting to assess the shelf life of fruits after removing from the PRS.

Data collection

Weight loss percentage (WLP)

Five sweet pepper fruits were weighed at day zero and in each storage duration using sensitive balance. The difference between initial and final weight of fruits was considered as total weight loss during storage interval and expressed as percentage (AOAC, 2007):

$$\text{WLP} = \frac{\text{Initial} - \text{Final Weight}}{\text{Initial Weght}} \times 100\%$$

Fruit firmness

Firmness of three fruits was measured using a computer-controlled automatic fruit texture analyzer (model: *TA-LEVEL-05*) according to Manolopoulou et al. (2010). The firmness measurement was carried out using a cylindrical stainless steel probe of 2 mm in diameter. Puncture tests were taken from the two opposite equatorial sides of the same fruit.

Total soluble solids (TSS)

Juice of sweet pepper fruits was extracted from three fruits in a blender as described by Antoniali et al. (2007). The homogenized sample was filtered using funnel with filter paper in a beaker. The filtrate was taken for TSS determination using digital refractometer (model: RFM-860, Japan) in °Brix by placing a few drops of clear juice on the prism surface.

Titratable acidity (TA)

10 ml of juice was extracted from three fruits and then homogenized and filtered using funnel with filter paper in a beaker. The TA was measured using NaOH (0.1 N) as a standardized titration solution. When the end point of titration was reached at pH 8.2, the amount of NaOH used on the burette was read off and recorded to calculate TA:

$$TA = \frac{\text{Titre} \times 0.1\text{N NaOH} \times 0.67}{1000} \times 100\%$$

Postharvest decay percentage (PDP)

Fruits were visually evaluated for symptoms of decay at the end of each storage interval based on the method prescribed by El-Mougy et al. (2012). Samples having symptoms of chilling injury and of diseases were counted. Pathogens causing decay were not identified.

$$\text{PDP} = \frac{\text{Number of Decayed Fruits}}{\text{Number ofTotal Fruits}} \times 100\%$$

Shelf life

Shelf life of fruits was evaluated by counting the number of days required to attain fruits remaining still acceptable for marketing as described by Rao et al. (2011). It was decided based on the appearance and spoilage of fruits. When 50% of fruits showed symptoms of shrinkage or spoilage due to pathogens and chilling injury, lot of fruits was considered to have reached end of shelf life.

Statistical analysis

Data were subjected to ANOVA using SAS software version 9.

Table 1. Interaction effect of harvesting stage and storage duration on mean weight loss percentage of sweet pepper fruits under passive refrigeration system.

Harvesting stage (%)	Weight loss percentage Storage duration (weeks)					Mean
	0	1	2	3	4	
0	0.00^{n}	2.67^{k}	3.61^{hi}	4.60^{ef}	6.01^{b}	3.38
25	0.00^{n}	1.39^{m}	2.25^{l}	3.30^{ij}	4.54^{fg}	2.30
50	0.00^{n}	2.03^{l}	3.00^{j}	3.89^{h}	4.88^{de}	2.70
75	0.00^{n}	2.28^{l}	3.30^{ij}	4.27^{g}	5.47^{c}	3.06
100	0.00^{n}	3.28^{j}	4.23^{g}	5.16^{cd}	6.50^{a}	3.83
Mean	0.00	2.33	3.35	4.24	5.48	
$LSD_{(0.05)}$		0.33				
CV (%)		9.29				

Means within a column followed by same letter(s) are not significantly different at 5% LSD test.

Verification of significant differences was done using LSD test at 5% probability level.

RESULTS AND DISCUSSION

Weight loss percentage

The interaction effect of harvesting stage and storage duration on mean weight loss percentage (WLP) of sweet pepper fruits was highly significant (P<0.001); while all other interaction effects were non-significant (P>0.05). At one week of storage, mean WLP of fruits harvested at 0, 25, 50, 75 and 100% colouration stages were 2.67, 1.39, 2.03, 2.28 and 3.28%, respectively; similar trends were observed at other storage times (Table 1). Mean WLP of fruits harvested at full green stage were 0.00, 2.67, 3.61, 4.60 and 6.01 at 0, 1, 2, 3 and 4 weeks of storage, respectively; the same results were apparent at other harvesting stages (Table 1).

The highest and lowest WLP were recorded for combinations of harvested at completely ripened stage and four weeks storage as well as harvested at 25% colouration stage and one week storage under PRS, respectively (Table 1).

Across all storage periods, the WLP of sweet pepper fruits harvested at completely ripened and full green stages were significantly higher than fruits harvested at intermediate stages (Table 1). This is in agreement with the findings of Moneruzzaman et al. (2009) who observed a higher WLP in fruits harvested at early matured stage than intermediate stages. This might be due to poorly developed waxy layer and cuticle on the surface of green pepper fruits as supported by Melaku et al. (2006). The high WLP in completely ripened fruits could be due to changes in permeability of cell membranes, making them more sensitive to the loss of water as confirmed by Antoniali et al. (2007).

Fruit firmness

The main effects of variety, harvesting stage and storage duration on mean firmness of fruits were highly significant (P<0.001); while all interaction effects were non-significant (P>0.05). The highest fruit firmness of 36.06 N was recorded for variety Telmo-Red whereas the lowest value (30.97N) was recorded for Velez-Yellow variety (Table 2). The mean firmness of fruits harvested at 0, 25, 50, 75 and 100% colouration stages were 38.41, 36.33, 33.60, 31.06 and 28.17 N, respectively. The maximum and minimum fruit firmnesses were recorded at full green and completely ripened harvesting stages, respectively (Table 2). The mean fruit firmness of sweet peppers stored for 0, 1, 2, 3 and 4 weeks under PRS were 35.75, 34.73, 33.35, 32.58 and 31.16 N, respectively. The highest and lowest values were recorded at four weeks and zero week storage periods, respectively (Table 2).

Telmo-Red variety was 14.12% firmer than Velez-Yellow variety (Table 2). This finding is in agreement with results of Lahay et al. (2013) who reported that the value of fruit firmness varied in magnitude between varieties of tomato fruits. The observed variation might be due to genetic or environmental factors as confirmed by Beckles (2012). Ilic et al. (2012) disclosed that the higher pericarp thickness of a variety, the better is the firmness of fruit.

Fruit firmness decreased with increase in harvesting stages (Table 2). The present result is in coherence with the findings of Zhou et al. (2011) who found a decrease in fruit firmness with increasing harvesting stages. The apparent decline in fruit firmness with age might be due to cell wall softening directly influencing the levels of fruit firmness. This is in line with the work of Rao et al. (2011) who found that cell wall softening is due to the activity

Table 2. Effect of variety, harvesting stage and storage duration on mean fruit firmness and total soluble solids under passive refrigeration system.

Variety	Fruit firmness (N)	Total soluble solids (°Brix)
	Mean	Mean
Telmo-Red	36.06[a]	7.22[a]
Velez-Yellow	30.97[b]	6.56[b]
$LSD_{(0.05)}$	0.52	0.10
Harvesting stage (%)	Mean	Mean
0	38.41[a]	5.36[e]
25	36.33[b]	6.40[d]
50	33.60[c]	7.02[c]
75	31.06[d]	7.63[b]
100	28.17[e]	8.03[a]
$LSD_{(0.05)}$	0.82	0.16
Storage duration (Weeks)	Mean	Mean
0	35.75[a]	6.48[e]
1	34.73[b]	6.88[c]
2	33.35[c]	7.35[a]
3	32.58[d]	7.07[b]
4	31.16[e]	6.66[d]
$LSD_{(0.05)}$	0.82	0.16
CV (%)	4.76	4.60

Means within a column followed by the same letter(s) are not significantly different at 5% LSD test.

of softening enzymes such as pectin methylesterase.

The mean fruit firmness progressively decreased with increase in storage time (Table 2). This result is consistence with reports of Lahay et al. (2013) who found a reduction in firmness of fruits during prolonged storage periods. This could be due to high respiration rate and weight loss as supported by Cantwell et al. (2009).

Total soluble solids

The main effects of variety, harvesting stage and storage duration on mean total soluble solids (TSS) were highly significant ($P<0.001$); while all interaction effects were non-significant ($P>0.05$). The maximum TSS of 7.22 °Brix was recorded for Telmo-Red variety whereas the lowest (6.56 °Brix) was recorded for Velez-Yellow variety (Table 2). The mean TSS content of fruits harvested at 0, 25, 50, 75 and 100% colouration stages were 5.36, 6.40, 7.02, 7.63 and 8.03 °Brix, respectively. The maximum and minimum TSS contents were recorded at completely ripened and full green harvesting stages, respectively (Table 2). The mean TSS of fruits stored for 0, 1, 2, 3 and 4 weeks under PRS were 6.48, 6.88, 7.35, 7.07 and 6.66 °Brix, respectively. The highest and lowest TSS values were recorded at two weeks and zero week storage periods, respectively (Table 2).

The maximum TSS content was recorded in Telmo-Red variety which showed 0.66 °Brix higher than Velez-Yellow variety (Table 2). This is in agreement with the results of Bernardo et al. (2008) who reported that the value of TSS varied in magnitude between varieties of sweet pepper fruits. The observed TSS variation between varieties might be due to genetic or environmental factors as confirmed by Beckles (2012).

The level of TSS content progressively increased with increase in harvesting stage (Table 2). The Mean TSS in completely ripened fruits was 2.67 °Brix higher than those harvested at full green stage (Table 2). The TSS content in this study is in line with reports of Antoniali ° (2007) who found minimum and maximum TSS values in yellow sweet pepper fruits assessed at full green and completely ripened maturity stages, respectively. The increment in TSS might be due to disassociation of some molecules and structural enzymes in soluble compounds, which directly influence the levels of TSS.

TSS content was increased during the first two weeks storage under PRS followed by a decreasing trend with increase in storage duration (Table 2). This result is in agreement with reports of Rao et al. (2011) who found an increase in TSS as fruits were stored for short period followed by a decreasing trend during prolonged storage periods. The increment in TSS for stored fruits was probably due to increase of respiration and metabolic activity. In this regard, Ali et al. (2011) found that the higher respiration rate increases the synthesis and use of

Table 3. Interaction effect of variety and harvesting stage on mean titratable acidity of sweet pepper fruits stored under passive refrigeration system.

Variety	Titratable acidity (%) Harvesting stage (%)					
	0	25	50	75	100	Mean
Telmo-Red	0.56[c]	0.62[b]	0.69[a]	0.51[d]	0.39[g]	0.55
Velez-Yellow	0.43[f]	0.45[e]	0.51[d]	0.36[g]	0.29[h]	0.41
Mean	0.49	0.54	0.60	0.43	0.34	
$LSD_{(0.05)}$			0.03			
CV (%)			7.58			

Means within a column followed by same letter(s) are not significantly different at 5% LSD test.

metabolites result in higher TSS due to the higher change from carbohydrates to sugars.

Titratable acidity

The interaction effect of variety and harvesting stage on mean titratable acidity (TA) was highly significant (P<0.001); while all other interaction effects were non-significant (P>0.05). For Telmo-Red variety, mean TA of fruits harvested at 0, 25, 50, 75 and 100% colouration stages were 0.56, 0.62, 0.69, 0.51 and 0.39%, respectively; while for Velez-Yellow variety, TA of fruits harvested at 0, 25, 50, 75 and 100% colouration stages were 0.43, 0.45, 0.51, 0.36 and 0.29%, respectively (Table 3).

TA values of fruits harvested at full green stage were 0.56 and 0.43% for Telmo-Red and Velez-Yellow varieties, respectively; the same results were apparent at other harvesting stages (Table 3). The highest and lowest TA values were recorded at combinations of Telmo-Red variety and harvested at 50% colouration as well as Velez-Yellow variety and harvested at completely ripened stage, respectively (Table 3).

For both varieties, the TA values of fruits harvested at 50 and 25% colouration stages were significantly higher than fruits harvested at other stages. There was an increasing trend in TA value until fruits attained their half ripening stage and thereafter decreased with increasing harvesting stages for both varieties (Table 3).

The results are in coherence with reports of Anthon et al. (2011) who found that TA of tomato fruits was increased with maturity stages and reached the peak at half ripening stage and thereafter started to decrease. The increment in TA value might be due to the presence of pectin methylesterase enzyme activity; while the reduction in TA of fruits harvested after half ripening stage could be due to high respiration rate and reduction in organic acids as supported by Anthon and Barrette (2012).

Postharvest decay percentage

The three-way interaction effect of variety, harvesting stage and storage duration on mean postharvest decay percentage of fruits under PRS was highly significant (P<0.001). At zero and one week storage periods, all fruits of both varieties were free from any postharvest decay across all harvesting stages. At two weeks of storage, mean PDP of Telmo-Red variety harvested at 0, 25, 50, 75 and 100% colouration stages were 1.63, 0.00, 0.20, 0.90 and 2.33%, respectively; similar trends were observed at three and four weeks under passive refrigeration system (Table 4). Postharvest decay percentages of Telmo-Red fruits harvested at full green stage were 1.63, 4.45 and 5.45 at 2, 3, and 4 weeks of storage, respectively; the same results were apparent at other harvesting stages (Table 4). Similarly, at two weeks of storage, mean postharvest decay percentage of Velez-Yellow variety harvested at 0, 25, 50, 75 and 100% colouration stages were 2.78, 1.16, 1.96, 2.44 and 3.35%, respectively; similar trends were observed at three and four weeks under passive refrigeration system (Table 4).

Postharvest decay percentage of Velez-Yellow sweet pepper fruits harvested at full green stage were 2.78, 5.89 and 7.20% at 2, 3, and 4 weeks, respectively; the same results were apparent at other harvesting stages (Table 4). Starting from two weeks storage period, the highest and lowest postharvest decay percentage were recorded at combinations of Velez-Yellow variety harvested at completely ripened stage and four weeks storage as well as Telmo-Red variety harvested at 25% colouration and two weeks storage under Passive Refrigeration System, respectively (Table 4).

Starting from two weeks of storage, PDP of both varieties harvested at all maturity stages was increased with increasing storage periods (Table 4). Starting from two weeks of storage, fruits of both varieties harvested at completely ripened and full green stages had significantly higher PDP than the other harvesting stages; however it was significantly lower for Telmo-Red variety (Table 4). The present findings are in conformity with reports of Ciccarese et al. (2013) who found that PDP in fruits harvested at completely ripened stage and stored for longer period of time was always higher than fruits harvesting at intermediate stages and stored for less time. Bayoumi (2008) concluded that the higher PDP in late harvesting stage of fruits was due to higher rate of respiration, more skin permeability for water loss and high susceptibility to decay. Moneruzzaman et al. (2009) also determined that fruit PDP increases when fruits are harvested at early matured stage due to poorly developed fruit cuticular wax layer. The increment in PDP during prolonged period of time could be due to the influence of high respiration rate, fruit senescence and enzymatic degradation of fruits' cell wall (Ciccarese et al., 2013).

Table 4. Interaction effect of variety, harvesting stage and storage duration on postharvest decay percentage of sweet pepper fruits stored under passive refrigeration system.

Variety	Harvesting stage (%)	Postharvest decay (%)					
		Storage duration (weeks)					
		0	1	2	3	4	Mean
Telmo-Red	0	0.00^{v}	0.00^{v}	1.63^{rs}	4.45^{g}	5.45^{e}	2.31
	25	0.00^{v}	0.00^{v}	0.00^{v}	1.39st	2.21^{op}	0.72
	50	0.00^{v}	0.00^{v}	0.20^{uv}	1.85^{qr}	3.23^{ij}	1.06
	75	0.00^{v}	0.00^{v}	0.90^{u}	2.07^{opq}	3.45^{i}	1.28
	100	0.00^{v}	0.00^{v}	2.33^{mo}	4.77^{f}	7.30^{b}	2.88
	Mean	0	0	1.01	2.91	4.33	
Velez-Yellow	0	0.00^{v}	0.00^{v}	2.78^{kl}	5.89^{d}	7.20^{b}	3.17
	25	0.00^{v}	0.00^{v}	1.16^{tu}	2.57^{lm}	2.94^{jk}	1.33
	50	0.00^{v}	0.00^{v}	1.96^{pq}	2.87^{k}	3.89^{h}	1.74
	75	0.00^{v}	0.00^{v}	2.44^{mn}	3.27^{i}	4.45^{g}	2.03
	100	0.00^{v}	0.00^{v}	3.35^{i}	6.49^{c}	8.38^{a}	3.64
	Mean	0.00	0.00	2.34	4.22	5.37	
$LSD_{(0.05)}$				0.29			
CV (%)				8.95			

Means within a column followed by same letter(s) are not significantly different at 5% LSD test.

Table 5. Interaction effect of harvesting stage and storage duration on mean shelf life of sweet pepper fruits stored under passive refrigeration system.

Harvesting stage (%)	Shelf life (days)					
	Storage duration (weeks)					
	0	1	2	3	4	Mean
0	11.17^{r}	14.00^{no}	19.85^{l}	26.32^{h}	30.17^{de}	20.30
25	14.00^{no}	16.17^{m}	24.84^{i}	31.00^{d}	36.00^{a}	24.40
50	13.34^{p}	15.83^{m}	23.31^{j}	29.50^{ef}	34.00^{b}	23.20
75	12.33^{q}	14.52^{n}	21.50^{k}	28.00^{g}	33.00^{c}	21.87
100	9.67^{s}	13.00^{pq}	19.00^{l}	24.82^{i}	29.00^{f}	19.10
Mean	12.10	14.70	21.70	27.93	32.43	
$LSD_{(0.05)}$			0.99			
CV (%)			3.97			

Means within a column followed by same letter(s) are not significantly different at 5% LSD test.

Shelf life

The interaction effect of harvesting stage and storage duration on mean overall shelf life (shelf life under PRS plus after being transferred to room temperature) of sweet pepper fruits was highly significant ($P<0.001$); while all other interaction effects were non-significant ($P>0.05$). At zero week of storage, mean shelf life of fruits harvested at 0, 25, 50, 75 and 100% colouration were 11.17, 14.00, 13.34, 12.33 and 9.67 days, respectively; similar trends were observed at other storage periods (Table 5). Mean shelf life of fruits harvested at full green stage were 11.17, 14.00, 19.85, 26.32 and 30.17 days stored for 0, 1, 2, 3 and 4 weeks under PRS, respectively; the same results were apparent at other harvesting stages (Table 5). The maximum and minimum overall shelf lives were recorded at combinations of harvested at 25% colouration stage and four weeks storage under PRS as well as harvested at completely ripened stage and zero week storage under PRS, respectively (Table 5).

Across all storage periods, the shelf life of fruits harvested at 25 and 50% colourations were significantly higher than fruits harvested at full green and late harvesting stages

stages (Table 5). The present results are in line with the findings of Dilmacunal et al. (2011) who observed that tomato fruits harvested at breaker stage had a better storability potential under cold storage than the unripe and full red fruits. This could be due to the high weight loss percentage and respiration rate of completely ripened fruits and lack of a well developed fruit cuticular wax layer at full green stage which in turn might have resulted in lower shelf life. Moreover, the increasing trend in overall shelf life of fruits during prolonged storage period might be due to the presence of the new, modern and innovative passive refrigeration system storage equipment. This reality is supported by Shen et al. (2013) who found that refrigeration is used to reduce spoilage and extend the shelf life of fresh fruit by slowing down the metabolism and reducing fruit deterioration.

Conclusion

The postharvest quality and shelf life of sweet pepper fruits was affected by varieties, harvesting stage and storage duration. TSS content was increased while fruit firmness decreased with increasing harvesting stages. Weight loss percentage, postharvest decay and overall shelf life were found to increase; whereas fruit firmness declined correspondingly with increasing storage periods. The present results showed that Telmo-Red variety harvested at 25 and 50% harvesting stages and stored under Passive Refrigeration System storage condition could maintain better postharvest quality and extend their shelf life for more than one month.

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Diversity analysis of sugarcane genotypes by microsatellite (SSR) markers

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Thirty (30) simple sequence repeat (SSR) primer pairs chosen randomly from the SSR primer collection were used to detect polymorphism in 17 sugarcane accessions. A total of 62 DNA fragments were generated by the 30 primers with an average of about 2.14 bands per primer. Bands that a primer yielded in the study ranged from 1 to 4. The genetic distances for SSR data using 17 sugarcane accessions, was constructed based on Nei (1978) and relationships between accessions were portrayed graphically in the form of a dendrogram. The value of genetic similarity ranging from 62.90 to 90.30% was observed among the 17 sugarcane accessions. The highest genetic similarity of 90.03% was seen among genotypes S-2003-US-118 and S-2003-US-312. From the present study, it may be concluded that SSRs markers are best tool for investigation of genetic diversity in sugarcane.

Key words: Simple sequence repeat (SSR), polymorphism, genetic diversity.

INTRODUCTION

Sugarcane (*Saccharum* spp. hybrids) is a genetically complex crop of major economic importance in tropical and sub-tropical countries (Khan et al., 2004). It is mainly used for sugar production but recently gained increased attention because of its employment generation potential and recent emphasis on production of bio-fuels. The importance of sugarcane has increased in recent years because cane is an important industrial raw material for sugar and allied industries producing alcohol, acetic acid, butanol, paper, plywood, industrial enzymes and animal feed (Arencibia, 1998). Considering the current needs of cane industry it is imperative to breed high sugar producing varieties that also have other desired agro-nomic traits.

Saccharum is a complex genus characterized by high ploidy levels and composed of at least six distinct species - *Saccharum officinarum, Saccharum barberi, Saccharum sinensi, Saccharum spontaneum, Saccharum robustum* and *Saccharum edule* (Daniels and Roach, 1987). Sugar recovery can be increased from current average of 8.32 to 10-11% with the development of improved cane varieties. For development of improved varieties, genotypic studies of sugarcane are required. Described as an allopolyploid, modern cultivated sugar-cane have approximately 80-140 chromosomes with 8-18 copies of a basic set (x = 8 or x = 10 haploid chromo-some number) (Ming et al., 2001). Continuous selection for the same traits may narrow genetic diversity to the extent that it may be difficult to predict diversity based on pedigree history alone. With the advent of molecular markers, it is now possible to make direct comparison of genetic diversity at the DNA level without some of the over simplifying assumptions associated with calculating genetic diversity based on pedigree history (McIntyre et al., 2001). Rapid advances in the field of molecular bio-logy and its allied sciences made the use of molecular markers a routine practice providing plant breeders a precise tool in analyzing genetic diversity for plant improvement (Andersen and Lubberstedt, 2003).

The molecular markers are of many types e.g. RFLPs,

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Table 1. Description of seventeen genotypes used in genetic diversity study.

Genotype	Source of collection
CPF-247	AARI , Faisalabad
SPF-245	AARI , Faisalabad
S-2003-US-618	AARI , Faisalabad
S-2003-US-628	AARI , Faisalabad
S-2002-US-247	AARI , Faisalabad
HSF-240	AARI , Faisalabad
CPF-237	AARI , Faisalabad
CPF-234	AARI , Faisalabad
S-2003-US-718	AARI , Faisalabad
S-2003-US-778	AARI , Faisalabad
S-2003-US-165	AARI , Faisalabad
S-2003US-312	AARI , Faisalabad
HSF-242	AARI , Faisalabad
CP-77-400	AARI , Faisalabad
CP-72-2086	AARI , Faisalabad
SPF-246	AARI , Faisalabad
SPF-213	AARI , Faisalabad

TRAPs, RAPDs, SNPs, simple sequence repeats (SSRs) and AFLPs. In the present study, microsatellite or SSR marker was used to analyze genetic diversity of different sugarcane genotypes. Microsatellites or simple sequence repeats (SSRs), are stretches of DNA, consisting of tandemly repeated short units of 1-6 base pairs in length. They are ubiquitous in eukaryotic genomes and can be analyzed through PCR technology. The sequences flanking specific microsatellite loci in a genome are believed to be conserved within a particular species, across species within a genus and rarely even across related genera. Simple sequence repeats (SSR) markers reveal polymorphisms due to variation in the lengths of microsatellites at specific individual loci. Microsatellites are born from regions in which variants of simple repetitive DNA sequence motifs are already over represented (Tautz et al., 1989). It is now well established that the predominant mutation mechanism in microsatellite tracts is ‘slipped-strand mispairing’. This process has been well described by Eisen (1999). When slipped-strand mispairing occurs within a microsatellite array during DNA synthesis, it can result in the gain or loss of one, or more, repeat units depending on whether the newly synthesized DNA chain loops out or the template chain loops out, respectively. The relative propensity for either chain to loop out seems to depend in part on the sequences making up the array, and in part on whether the event occurs on the leading (continuous DNA synthesis) or lagging (discontinuous DNA synthesis) strand. SSR allelic differences are, therefore, the results of variable numbers of repeat units within the microsatellite structure; they are therefore, multiallelic and co-dominant in nature, thus proving to be very informative. Among the range of DNA-based molecular marker techniques, a promising polymerase chain reaction (PCR)-based technique used extensively for genetic mapping (McIntyre et al., 2001), as well as fingerprinting of sugarcane clones (Piperidis et al., 2000; Pan *et al*., 2002), is microsatellites or SSRs. SSR genetic markers are the best tool to demonstrate the genetic diversity in sugarcane (Smiullah et al., 2012).

The present study was undertaken to investigate the genetic diversity and establish the relationship between different sugarcane genotypes in Pakistan, using SSR markers. Obtaining accurate estimates of the genetic diversity among germplasm sources may increase the efficiency of plant breeding. Knowledge of genetic diversity and relationships among breeding genome, their polymorphic nature, codominance and materials has a significant impact on crop improvement.

MATERIALS AND METHODS

The genetic diversity studies were done as a collaborative research, in Department of Plant Breeding and Genetics, University of Agriculture, Faisalabad and Agriculture Biotechnology Research Institute (ABRI), Ayub Agricultural Research Institute (AARI), Faisalabad during 2010-2012. The plant material used for the study of genetic diversity was comprised of seventeen sugarcane accessions (Table 1). These accessions were collected from the germplasm source in the Sugarcane Section of Ayub Agricultural Research Institute, Faisalabad. The genetic material includes commercial cultivars and elite lines.

PCR amplification

Fresh young leaves were collected from the field experiment for isolation of the DNA. Total genomic DNA of the plants was extracted by using modified (CTAB) method (Hoisington et al., 1994; Doyle and Doyle, 1990). DNA concentration was determined, using a Nano Drop spectrophotometer (ND1000). Primer selection was based on previous investigation on SSR analysis, carried out with sugarcane genotypes and somaclones in this laboratory. Primer pairs obtained from Gene link company (USA) were used in PCR reaction for each genotype. For SSR analysis, concentration of genomic DNA, l0 × PCR buffer with $(NH_4)_2SO_4$, $MgCl_2$, dNTPs primers and taq DNA polymerase were optimized.

A reaction mixture of 20 µl was used to amplify genomic DNA in a thermal cycler (Eppendorf DNA Thermal Cycler 9600). To confirm that the observed bands were amplified genomic DNA and not the primer artifacts, genomic DNA was omitted from control reaction. A negative control was also run to confirm if the master/reaction mixture is correctly prepared or not. The PCR products were electrophoresed at 90 V, in 2% agarose gel for approximately 2 h, using 0.5 × tris-boric acids EDTA (TBE) buffer, along with a DNA molecular size marker.

The gel contained 0.5 µg/ml ethidium bromide to stain the DNA and photographed under UV light using gel documentation system. Reactions were duplicated to check the consistency of the amplified products. Only easily resolved bright DNA bands were scored as presence (1) and absence of bands (0). Coefficient of similarity among somaclones was calculated according to Nei and Li (1978). Similarity coefficient was utilized to generate a dendrogram by means of unweighted pair.

Data analysis

The data on bands generated by the 30 primers were selected for analysis of genetic diversity (Table 2). The bands were counted by starting from the top and ending with the bottom of the lanes. All segregating bands that were well resolved and unambiguous were scored for the presence (1) or absence (0) in the 17 genotypes. The data of the primers were used to estimate the dissimilarity on the basis of number of unshared amplified products and a dissimilarity matrix was generated using Nei's similarity indices (Nei, 1978). In addition, population relationships were inferred using the un-weighted pair group of arithmetic means (UPGMA) clustering method using the Popgen software (version 3.5).

RESULTS AND DISCUSSION

In recent years, the popularity of SSR-based markers has increased considerably. The main reasons which make microsatellites an especially attractive tool for a number of applications are: their high levels of allelic variation and their co-dominant character, which means that they deliver more information per unit assay than any other marker systems, thus reducing costs; microsatellites are assayed using PCR, so only small amounts of tissue are required.

Thirty (30) SSR primer pairs chosen randomly from the SSR primer collection were used to detect polymorphism in 17 sugarcane accessions. The PCR product was observed by running on agarose gel to study polymorphism, most of the primers were polymorphic except five primers which were monomorphic and produced only one fragment per primer (Figure 1). All the primers were found to give reproducible bands. A total of 62 DNA fragments were generated by the 30 primers with an average of about 2.14 bands per primer. Bands that a primer yielded in the study ranged from 1 to 4. Generally, the size and the number of bands produced were dependent upon the nucleotide sequence of the primer pair, size of the primer used and the source of the template DNA. In this study the primer used were of the size ranging from 300-420 bp. Reactions were duplicated form to check the consistency of the amplified products. Only easily resolved bright DNA bands were scored.

Cluster analysis

Pattern of polymorphism by SSRs

About 85.25% polymorphism was estimated as 55 out of 62 fragments were polymorphic with 30 primers used among the 17 sugarcane accessions. The rest of the 7 bands were monomorphic in the 17 accessions. In the present study, the 17 sugarcane accessions appeared to show variability with the 30 primers used. Although none of the primers individually was as informative as to differentiate all the accessions; highly polymorphic profiles were obtained with of the primers SMs35. (Sugarcane Microsatellite primer no.35) while five primer pairs such as SMs46, SMs47, SMs48 SMs49 and SMs50 were found to be monomorphic. Therefore, it may be concluded from the present results that SSRs can be used for identification of genetic diversity and the relationship between the members of the complex species. Jannoo et al. (2001) studied diversity in 96 sugarcane genotypes with just two primer pairs and reported a high level of heterozygosity. Cordeiro et al. (2001) applied 21 primer sets to five sugarcane genotypes, and among them, 17 pairs were polymorphic, but the level of polymorphism (PIC value) in the cultivars detected by these SSRs was low (0.23). The level of polymorphism indicates that distinction between any two varieties is possible with appropriate SSR primer pair. This supports the use of SSR markers, as an excellent tool, for diversity analysis and loci mapping.

Genetic distances/similarities between the accessions

The genetic distance for SSR data using 17 sugarcane accessions, was constructed based on Nei (1978) and relationships between accessions were portrayed graphically in the form of a dendrogram in Figure 2, the value of genetic similarity ranging from 62.90 to 90.30% was observed among the 17 sugarcane accessions. The lowest genetic distance of 62.90% was seen among genotypes S-2003-US-118 and S-2003-US-312. These two genotypes differed from each other only in 5 bands with 14 different primers. The most dissimilar of all the accessions was S-2003-US-118 and SPF-213 with genetic distance of 90.30%. Genomic SSRs have been shown to produce a greater number of alleles and higher PIC values than those from EST derived SSRs in sugarcane (Pinto et al., 2006).

In several other studies, elite sugarcane (*Saccharum* hybrids) germplasm showed genetic diversity as well (Selvi et al., 2003; Cordeiro et al., 2003). Selvi et al. (2003) revealed a broad range (0.324-0.8335) of pair-wise similarity values when tested on 30 or 40 commercial sugarcane cultivars.

Clustering pattern

The cluster analysis based on similarity values has classified all the sugarcane accession in two of the four major groups (I, II, III and IV). The first major group consisted of two accessions CPF-247 and S-2003-US-165 forming the most distinct cluster I. Second major group was further grouped into IIA, IIB and IIC. Group IIA consisted of three accessions namely SPF-245, S-2003-US-618 and HSF-242. Group IIB consists of four genotypes viz. HSF-240, CP-72-2086, S-2003-US-778 and SPF-213. Group IIC contained two accessions CPF-

Table 2. Name of the primers used for detection of polymorphism in sugarcane genotypes.

Primer no.	Band size	Primer sequence (F/R)	Annealing temperature
SMs1	600-2000	GGTTTGTTACTCTACTCCCGT GGTTTGTTACTCTACTCCCGT	55
SMs2	550-900	CATCTGCTCCCTCTTCCT TGAGCAAAGAAAGAGAAGTAGTC	55
SMs3	400-550	CATCTGCTCCCTCTTCCT CTCTGGCGGCTTGGTCCTG	52
SMs5	400-800	CTCTGCGGCTTGGTCCTG CATCCTCCAAGCATCTGT	54
SMs6	500-600	GACTCCTGTCACCGTCTTC ATACTTCAACCGTCTCCTCC	55
SMs7	400-500	CTAAGCAAGAACACAGGAAAG AGCAACAGCAGAGAGCAG	54
SMs8	400-550	CTGACTAAGGAGGAAGTGGAG GACGACGATAGATGAAACA	55
SMs9	400-500	GAGCCGCAAGGAAGCGAC CATACAAGCAGCAAGGATAG	50
SMs10	500-700	CTCTCTTCTCGTCTCCTCATT GTCCTTCTTCTTCTCGTGGT	55
SMs11	400-500	ACACGCATCGCAAGAAGG AAGAACACTCAACAGAAGCAC	55
SMs12	400 - 600	AAATGTCTTCGCACTAACC AAGGAGATGCTGATGGAGA	55
SMs16	400 - 500	CCCAGAGGACAAGGAACT GTAATGGAAGGAAGCAACTGA	50
SMs17	400-450	GGCTCCTCCTACTCGTTC GAGCCTTTGGATGTGGTC	55
SMs18	400-600	CTACACATCTCCATTCCACAG TTTAGGGTTCGTTAGGGTAAG	55
SMs19	300-500	GGCTCCTCCTACTCGTTC GAGCCTTTGGATGTGGTC	53
SMs20	350-500	CTACACATCTCCATTCCACAG TTTAGGGTTCGTTAGGGTAAG	50
SMs21	400-600	GGCTCCTCCTACTCGTTC GAGCCTTTGGATGTGGTC	50
SMs22	300-400	CTACACATCTCCATTCCACAG TTTAGGGTTCGTTAGGGTAAG	55
SMs23	350-600	GGCTCCTCCTACTCGTTC GAGCCTTTGGATGTGGTC	50
SMs24	400-450	CTACACATCTCCATTCCACAG TTTAGGGTTCGTTAGGGTAAG	53
SMs31	550-650	TTCTCGCCCTCCCGCTAC TTCTCTCCTCCTCCTCTTTC	55
SMs35	400-850	TTCTCGCCCTCCCGCTAC TTCTCTCCTCCTCCTCTTTC	53
SMs42	400-500	GTTTCTCCACCTCCAACTC ACAGACACAGGCGGGCGA	55
SMs43	400-500	CCCAGTGCTTCCTCTCTC TAGCACTCCATTCAGCAAA	55
SMs45	400	CTTCCCTCCCTCTCCTCT AGCCTTCTACTAAACTATCTGCT	55
SMs46	400	GTGAGTGAGACCAGACCAG CCGTGCTGTAGTTGTTGTAG	50
SMs47	400	ATACGCTACTCTGAATCCCAC CAATCACTATGTAAGGCAACA	50
SMs48	400	ACTCCTCTTCCTCTTCCTCTT GTTGTTCCCGTTCCCGCC	53
SMs49	250 - 400	ACTCGGTCATCTCATCACTC GTTCTTCGGGTCATCTGG	55
SMs50	400-500	ACGGTGAGCGAGGACTAC CTTGGGTGGCATCAGGAA	55

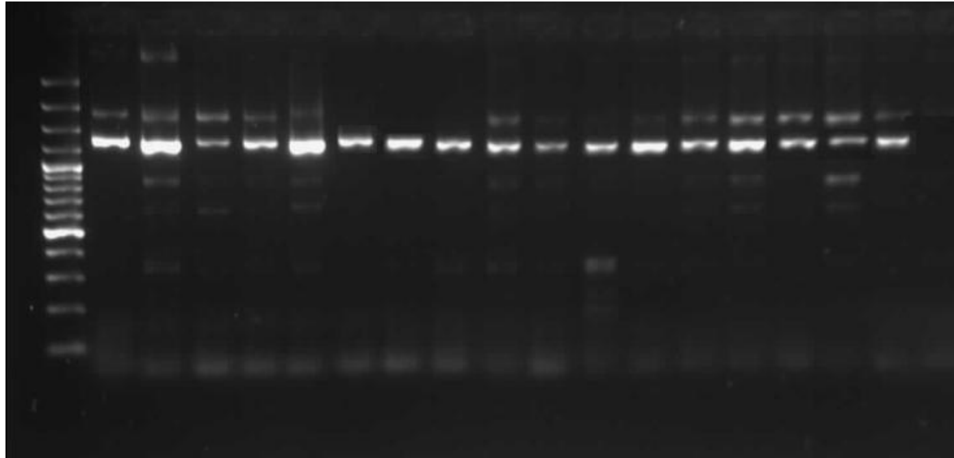

Figure 1. Result of electrophoresis of SSR product of 17 genotypes using sugarcane microsatellite primer no.18.

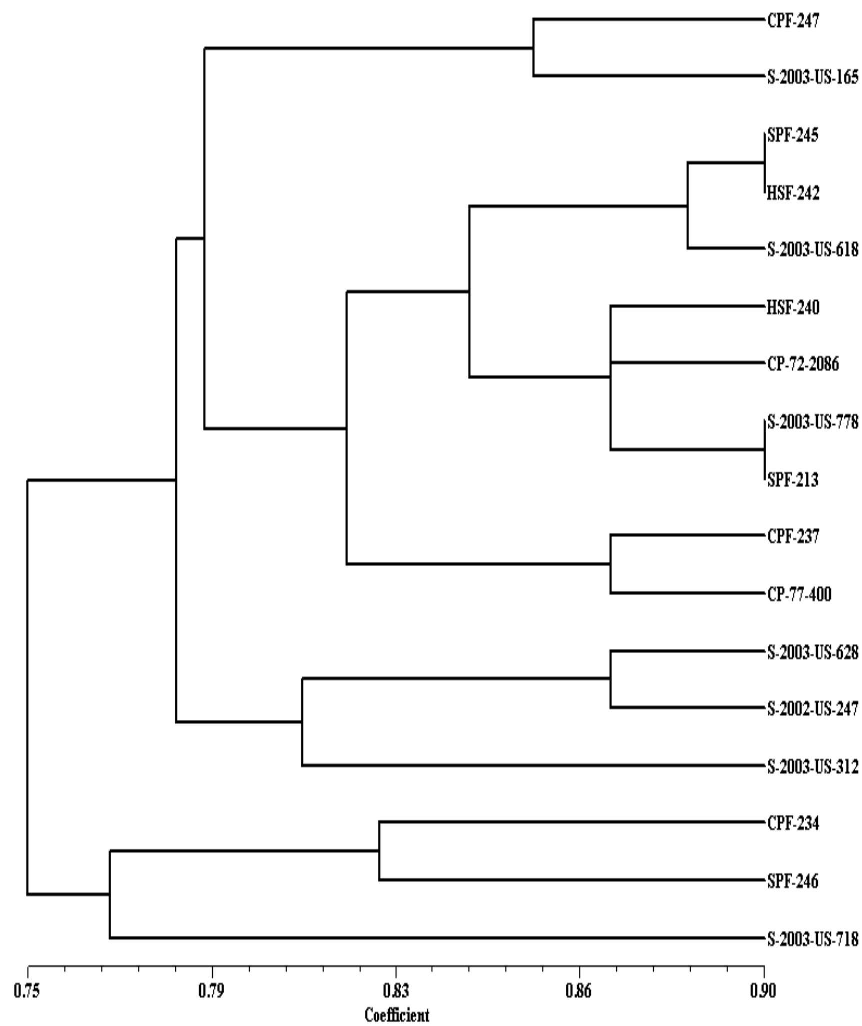

Figure 2. Dendrogram of 17 sugarcane accessions developed from SSRs data using unweighted pair group of arithmetic means (UPGMA) based on Nei's (1978) genetic distance.

237 and CP-77-400. Group III comprised of three genotypes viz. S-2003-US-628, S-2003-US-312 and S-2002-US-247. Group IV consisted of three accessions viz. CPF-234, SPF-246 and S-2003-US-718. Genotypes included in same cluster are more similar to each other but these are less similar to the genotypes in other clusters.

Conclusions

The analysis of variations in SSR fragments provides an effective tool for examining diversity to improve plant breeding strategies. Identifying useful SSRs is critical but in sugarcane this can be a lengthy and difficult process due to their abundance and the complexity of the sugarcane genome. Less information is available on the genetic diversity within and between *Saccharrum* cultivars which has been based mainly on morphological characteristic. Thus, it can be concluded that estimates of genetic similarity based on molecular markers may provide more accurate information to plant breeder. This data will support the exploitation of sugarcane germplasm on molecular basis. SSR markers used in the study may also be used by researcher for genetic mapping and gene tagging in sugarcane. Locus mapping ability of these SSR markers will provide more information than those available through diversity. These markers may be used for construction of genetic map in sugarcane. Future breeding efforts involving crosses between and within the groups identified in this study may provide useful strategies for combining beneficial genes and alleles in new sugarcane varieties while maintaining genetic diversity.

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