Shelf life determinants and enzyme activities of pearl millet: a comparison of changes in stored flour of hybrids, CMS lines, inbreds and composites

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Abstract Shelf life of pearl millet flour is very short because of rapid development of rancidity. This investigation was carried out in view of generating breeding material for development of low rancid pearl millet hybrids/varieties. Flour of twenty-one genotypes; seven hybrids, seven CMS lines, five inbreds and two composites stored in covered aluminium boxes at 37 °C for 30 days along with respective fresh flour was analysed for shelf life indicators/determinants. Crude fat content and fat acidity (FA) of fresh flour of the genotypes varied from 3.8 to 7.2% and 11 to 75 mg KOH/100 g d.m., respectively. FA in stored flour ranged between 180 and 330 mg KOH/100 g d.m. After storage, magnitude of decrease in pH of water extract of flour of the genotypes varied from 0.15 to 0.44. Activity of peroxidase (POX) varied from 378 to 588 units in control flour and irrespective of the genotypes decreased upon storage. Increase in FA (difference between FA of fresh and stored flour) rather total build up of FA was positively associated with crude fat content ($r = 0.440^*$) indicated comparatively more prominent role of lipolytic enzymes. Chemical changes taking place in water soluble fraction of flour were independent of fat content as no correlation was discerned between fat content and decrease in pH. Among the hybrids, HHB 197 had

lowest crude fat content (4.7%), lowest total build up FA (212 mg KOH/100 g d.m.), slowest increase in FA (191 mg KOH/100 g d.m.), least decrease in pH (0.31) of water soluble fraction flour during storage and lowest activity of POX in fresh flour (377 units/g d.m.). Among all the tested CMS lines, inbreds and composites, HBL 11 showed pattern of quantitative changes in FA, pH and POX activity similar to the hybrid HHB 197 and was identified a promising inbred for developing low-rancid pearl millet variety or hybrid.

Keywords Pearl millet · Storage · Fat acidity · pH · Oxidative enzymes

Introduction

In India, pearl millet is the third most important food crop in terms of area and production after paddy and wheat. It is a dual purpose crop of arid and semiarid areas as it provides cheap food for humans, feed for poultry birds and also dry as well as green fodder for cattle. Rapid development of off flavour in pearl millet flour, an old and unresolved problem, is the major hindrance for wider consumer acceptability. Thus use of pearl millet in food industry and consequently its consumption by urban population is very low. In India, pearl millet was grown over an area of 7.9 and 7.1 million hectares with total production of 9.2 and 9.1 million tons per year and productivity of 1161 and 1272 kg/ha during 2014 and 2015, respectively. Hydrolytic cleavage of lipids (Carnovale and Quaglia 1973), presence of volatile compounds (Thiam et al. 1976), changes in composition of lipids and oxidative changes in unsaturated fatty acids (Lai and Varriano-Marston 1980), high activity of POX and enzyme catalysed

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changes in phenolics (Reddy et al. 1986; Bangar et al. 1999) and presence of high concentration of 2-acetyl pyrroline (Seitz et al. 1993) have been documented as possible causes for generation of off-odour. Of these hydrolytic cleavage through the action of lipase is of utmost importance. Jain (2013) suggested that initial activity levels of lipase, and POX and/or phenolics content in grains might be having more important role in generating off flavour in pearl millet flour during storage. Generally hydrothermal treatment, irradiation, refrigeration or combinations of more than one technique have been used to extend the shelf life of milling fractions of millet flour as well as products made of flour. This can be attributed to inactivation effect of these treatments on endogenous enzymes. However, use of these techniques may result in unlikable changes in quality attributes of millet grains and their food products. One better approach to improve shelf life is to increase the inherent capacity of the crop to produced low rancid flour. But exact causes of poor shelf life of pearl millet flour are not known yet because paucity of genetic variability in magnitude of off flavor and its indicators particularly fat acidity, pH and possible determinants viz. lipase, POX, LOX and PPO. However, for establishing quantitative relationship between magnitude of off flavour and its determinants and for producing hybrids with longer shelf life knowledge of genetic variation among the genotypes is essential. The present investigation was carried out to investigate 21 pearl millet genotypes (hybrids, CMS lines, inbreds and composites) for identifying promising genotypes in respect of shelf life indicators/determinants. Once qualitative and quantitative relationship between particular constituent(s) of grain, rancidity indicators/determinants and organoleptic evaluation is established it would become possible to develop low rancid pearl millet hybrid/variety through conventional plant breeding. The present investigation is the beginning to achieve this objective.

Materials and methods

Mature grains of pearl millet genotypes procured from Bajra Section, Department of Genetics and Plant Breeding, CCS HAU, Hisar were used. Flour made of mature pearl millet grains in a cyclotec grinding mill (Foss Analytical AB, Sweden) using 0.8 mm screen of seven hybrids (F_2 generation of HHB 67 imp, HHB 94, HHB 146, HHB 197, HHB 223, HHB 226 and HHB 234), seven CMS lines (ICMA 89111, ICMA 95222, ICMA 97111, ICMA 94555, ICMA 843-22, HMS 7A and ICMA 94222), five inbreds (G-73-107, HTP 94/54, HBL 11, H77/833-2-202 and 78/711) and two white composites (WHC 901-445, HMP 802) was stored in aluminium boxes at 37 °C. The stored

flour (SF) of these genotypes was analyzed simultaneously along with the fresh flour (FF) prepared on the respective day of storage for the characters mentioned below. All the chemicals used were of high purity analytical grade.

Chemical analyses

Crude fat content (%) and fat acidity (FA) (mg KOH/100 g d.m.) were determined using the standard methods of AOAC (1990) and AAAC (1999), respectively. Total phenolics (mg CE/100 g d.m.) were estimated by the method of Malik and Singh (1980), using catechol as an authentic standard phenol. The pH of water extract of flour was determined by the method of AOAC (1990).

Enzymatic activities

Preparation of extract

Preliminary experiments were conducted to optimize the extraction conditions with respect to pH, molarity and type of buffer. The primary objective was to affect adequate cell breakage in a minimum amount of time with no warming of the extract. One gram of flour was homogenized in a prechilled pestle and mortar in 10 mL of 0.2 M potassium phosphate buffer (pH 7.5). The homogenate was centrifuged at 10000 rpm for 20 min in a refrigerated centrifuge. The supernatant was carefully filtered through four layers of cheese cloth and used as crude enzyme preparation for determining enzyme activities. The extract was diluted to a suitable extent to obtain linear response of each enzyme with respect to time and concentration of respective substrates.

Assay methods

POX was extracted and assayed by the method of Malik and Singh (1980). The assay mixture in a total volume of 2.8 mL contained: 2.5 mL of 0.05 M potassium phosphate buffer (pH 6.8), 0.1 mL of 0.1% (w/v) *o*-dianisidine, 0.1 mL of 0.1 M H_2O_2 and 0.1 mL enzyme preparation. The reaction was started by adding H_2O_2 . Thus omission of H_2O_2 from the reaction mixture served as blank. Increase in absorbance was monitored at 430 nm at an interval of 15 s for 2 min. LOX activity in crude extract was measured spectrophotometrically at 234 nm. Substrate (linoleic acid) was prepared by the method of Surrey (1964). The reaction mixture in a final volume of 2.66 mL contained 2.5 mL of 0.05 M acetate buffer (pH 4.2), 60 μ L of 7.5 mM linoleic acid and 0.1 mL of enzyme extract. The reaction was started by addition of the enzyme and progress of the reaction was monitored by recording increase in absorbance at 234 nm for 2 min against blank. For POX and

LOX only the linear portion of change in absorbance was considered to calculate enzyme activity. Activity of PPO was determined by the method of Kruger (1994) with minor modifications. A solution of 0.01 M catechol was prepared in 0.05 M phosphate buffer (pH 6.2). This solution was prepared fresh each time just before starting the assay. The reaction was initiated by adding 0.2 mL of enzyme extract to 2.8 mL of substrate solution. This mixture was incubated at 37 °C for 30 min. The absorbance was measured at 410 nm on a UV–Vis spectrophotometer. A blank was run simultaneously with boiled enzyme extract. One unit of enzyme activity of POX, LOX and PPO was defined as the amount of enzyme required to increase 0.1 unit absorbance min^{-1} g d.m.⁻¹ (dry matter) under the test conditions.

Statistical analysis

Estimation of all the chemical/biochemical parameters was done in triplicates. The data recorded during the present investigation was statistically analyzed using Statistical Package for Agriculture Scientists', OPSTAT (Sheoran et al. 1998).

Result and discussion

The mean sum of squares (ANOVA) due to genotypes was significant for all the parameters studied. This indicates the prevalence of enough genetic variability in the genotypes under study for further analysis.

The crude fat content of hybrids was higher than that of CMS lines, inbreds and white composites (Fig. 1a). In hybrids crude fat content varied from 4.7 to 7.2%, whereas that of CMS lines, inbreds and white composites ranged from 4.0 to 6.2, 3.8 to 5.4 and from 4.8 to 4.9%, respectively. The hybrid HHB 94 possessed 7.2% crude fat; highest among the tested genotypes. The fat percentage is in close proximity to that reported by Jain (2013) and Čepková et al. (2014). Crude fat content of 5.88 and 5.77% in two pearl millet cultivars was observed by Lestienne et al. (2007). In contrast the inbred HBL 11 had only 3.8% crude fat content; lowest among the tested genotypes. Low fat content of few pearl millet genotypes ranging from 4.2 to 4.8% has been documented in literature (Yadav et al. 2012; Amadou et al. 2013). The per cent fat content of HBL 11 is perhaps the least reported in literature.

Total phenolic content ranged between 165–276, 154–266 and 200–261 mg/100 g d.m. in hybrids, CMS lines and inbreds, respectively (Fig. 1b). Both white genotypes had 199 mg/100 g d.m. total phenolic content. Lowest content of total phenolics was present in ICMA 97111 (154 mg CE/100 g d.m.) while highest amount was

detected in HHB 67 imp (276 mg CE/100 g d.m.). Bangar et al. (1999) studied comparative distribution of phenolics in pearl millet grain fractions and reported that concentration of water soluble phenolics in the defatted meal (136 mg/100 g) was lower than in the germ (1216 mg/100 g). The most common phenolic compound in pearl millet is ferulic acid (Gorinstein et al. 2008). Ferulic acid in bound form underwent destructive oxidation reaction during storage (Ragaei et al. 2014). The identified low and high phenolic genotypes i.e. ICMA 97111 and HHB 67 imp, respectively might be useful in studying relationship with the off flavour generation.

FA of fresh flour of pearl millet genotypes varied from 11 to 75 mg KOH/100 g d.m (Fig. 2a). Storage had profound effect on buildup of FA in flour of all the genotypes analyzed. Significant increase in FA of flour of each genotype was observed after 30 days of storage. The milling of grains leads to tissue damage and contact between lipolytic enzymes and its substrate i.e. fats (Dvoracek et al. 2010) causing release of fatty acids resulting in increase in FA. Comparison of mean value of FA of fresh flour revealed that it increased from 24.6 to 276.5 mg KOH/100 g d.m. in hybrids, 52 to 298.2 mg KOH/100 g d.m. in CMS lines, 52.7 to 272 mg KOH/100 g d.m. in inbreds and 36.7 to 328 mg KOH/100 g d.m. in white composites. Increase in FA of pearl millet flour stored for different time periods has been reported by many investigators (Nantanga et al. 2008; Yadav et al. 2012; Čepková et al. 2014; Tiwari et al. 2014). Kaced et al. (1984) reported that fat acidity increased during first 90 h of storage and then levelled off at 450 mg KOH/100 g of meal. The reason for leveling-off of FA was not given. Perusal of data presented in Fig. 2a clearly revealed differential behavior, in respect of development of FA in stored flour, of all the genotypes irrespective of the group (hybrid, CMS line, inbred or white composite) they belong to. For example lowest increase in FA was observed in the inbred HBL 11 (180 mg KOH/100 g d.m.) followed by the hybrid HHB 197 (212 mg KOH/100 g d.m.) while higher values of FA ranging between 328 and 330 mg KOH/100 g d.m. were detected in flour of the composite HMP 802, the inbred G 73-107 and the hybrid HHB 234 after 30 days of storage. The observed variation in total FA in stored flour of the genotypes might be due to differences in the activities of lipolytic enzymes present in the dormant pearl millet which needs further investigation.

pH is an important parameter in determining quality of flour. Low pH value imparts sour taste to the flour which makes it less preferred for consumption (Apea-Bah et al. 2011). pH of water extract of flour of the 21 genotypes varied from 6.47 to 6.67 (Fig. 2b). Substantial decline in pH (0.15–0.44) of water extract of flour of each genotype was observed when stored for 30 days. These results are in

Fig. 1 Crude fat (a) and total phenolic (b) content of fresh flour of pearl millet genotypes. CD at 5% 0.304 (a) and 0.647 (b)

accordance with those of Goyal et al. (2017) who reported higher decrease in pH of flour of pearl millet compared to wheat and maize. Decrease in pH of all instant dambu samples prepared from pearl millet over the storage period (70 days) has also been observed earlier by Agu et al. (2014). Most likely there is increase in the content of organic acids other than fatty acids since these are not extracted in water. Formation of acidic chemical constituents as a result of enzymatic action might be one of the possible reasons for decreased pH after 30 days of storage of flour.

Like differential build up of FA in stored flour of the individual genotype of each group analyzed, genotypic variability in acidity of water soluble fraction of these genotypes was also discerned (Fig. 2b). Among all the genotypes tested hybrid HHB 234 and inbred line G73-107 showed high decrease in pH (0.44 and 0.42, respectively) while CMS line ICMA 95222 and inbred line HBL 11 and 78/711 showed low decrease (0.15, 0.23 and 0.24, respectively).

Enzymatic activities

The changes which occur in stored flour are probably due to enzymes liberated in the flour. POX has attracted interest

because of its capacity to modified food in both desirable and undesirable ways. Chavan and Hash (1998) and Bangar et al. (1999) reported that activity of peroxidase was responsible for off odour generation in pearl millet flour. POX is highly active in fresh pearl millet flour (Goyal and Chugh 2014). POX can directly interact with unsaturated fatty acids causing production of hydroperoxides. High variability was found in the genotypes in respect of POX activity (Fig. 3a). In control flour activity of POX varied between 378 and 580 units/g d.m. in hybrids, between 243 and 580 units/g d.m. in CMS lines, between 260 and 400 units/g d.m. in inbreds and between 488 and 588 units/g d.m. in white composites. Activity of POX was higher in hybrids in comparison to their parents and highest activity was observed in HHB 223. Except HHB 197, POX activity in fresh flour of all the hybrids was either equal to or more than 400 units/g d.m. Similar level of activity of POX was present in fresh flour of the white composites. On the other hand except ICMA 95222 and ICMA 94555 activities of POX in fresh flour of all the CMS lines as well as inbreds were well below 400 units/g d.m. Activity of POX decreased upon storage of flour. Significant and marked decrease in POX activity in flour of all the genotypes was observed after 30 days of storage. The activity of the genotypes after storage varied from 115.0 to 355.0 units/g

Fig. 2 Fat acidity (FA) (a) and pH of water extract (b) of fresh and stored flour of pearl millet genotypes. CD at 5% 2.362 and 4.456 (a) and 0.017 and 0.2 (b), respectively

d.m. (Fig. 3a). These results are in agreement with those of Jain (2013). It was found that both in vitro and in situ activity of POX decreased after storage of flour. Percent decrease in POX activity was minimum in flour of HBL 11 (15.2%) which may be due to lesser substrate availability for peroxidation suggesting its higher shelf life. According to Richardson and Hyslop (1985), decrease in enzyme activity during storage might be due to a change in the stability of enzyme conformers, increased intra-enzymic hydrogen bonding or decreased accessibility of enzyme to substrate. It could also be due to increased hydrogen bonding between water and either substrate or enzyme active site, formation of enzyme polymers, changes in mechanism, shift in pH or increased in viscosity.

LOX is one of the major enzymes responsible for off flavour development by catalyzing oxidation reaction which produces medium chain aldehydes, ketones and their alcoholic counterparts (pentanal, hexanal, heptanal and even nonanal along with their unsaturated forms) (Zilic et al. 2010; Yadav et al. 2012; Tiwari et al. 2014). These compounds are released soon after grinding of grains. In Wang et al. (2014) studied aroma stability of foxtail millet

powder during storage and assumed that LOX in millet kernel catalyzes fatty acid oxidation, resulting in deterioration in flavour and quality. Two forms of lipoxygenase are present in pearl millet grains (Sharma and Chugh 2017). LOX activity varied from 405 to 650 units and from 280 to 580 units in fresh and stored flour respectively (Fig. 3b). Maximum activity was recorded in ICMA 95222 while minimum was found in ICMA 97111. These results are in agreement with those of Schwarz and Pyler (1984) who reported that activity of LOX was cultivar related in case of barley. Significant decrease in activity of LOX was observed in flour of all the genotypes when stored for 30 days (Fig. 3b). The maximum loss (36%) in activity of the enzyme was found in CMS line HMS 7A. On the other hand the inbred HBL 11 lost only 11% of the activity. Thus the pearl millet genotypes behaved differentially in retaining the activity of LOX. Data on LOX activity in pearl millet flour of diverse genotypes are not available in scientific literature. Even after significant loss during 30 days substantial activity of LOX was present in most of the genotypes. Thus high activity of LOX might have caused deterioration of quality of flour much earlier.

Fig. 3 Peroxidase (POX) (a), lipxygenase (LOX) (b) and polyphenol oxidase (PPO) (c) activity of fresh and stored flour of pearl millet genotypes. CD at 5% (0 and 30 days) 9.512 and 9.868 (a), 17.953 and 15.397 (b) and 0.082 and 0.12 (c), respectively

PPO catalyzes the hydroxylation of monophenols to *o*-diphenols (“monophenolase” activity) and the oxidation of *o*-diphenols to *o*-quinones (“diphenolase” activity). The quinone products of PPO react with a number of functional groups, such as amines, thiols, and phenolics, and form complex colored products (melanins) (Whitaker

and Lee 1995). This is the basis for PPO-mediated deterioration of many food products. Data on activity of PPO in fresh and stored flour of the pearl millet genotypes presented in Fig. 3c show that the activity in fresh flour varied from 4.4 to 6.5 units/g d.m. Lowest activity of PPO was present in the CMS line ICMA 97111. Most of

Table 1 Correlation matrix of biochemical analyses

	Crude fat	FA (FF)	FA (SF)	Increase in FA (SF–FF)	PH (FF)	PH (SF)	Decrease in pH (FF–SF)	Total phenolic content	POX (FF)	POX (SF)	LOX (FF)	LOX (SF)	PPO (FF)	PPO (SF)
Crude fat														
FA (FF)	–0.030 ^{NS}													
FA (SF)	0.410 ^{NS}	0.299 ^{NS}												
Increase in FA (SF–FF)	0.440*	–0.089 ^{NS}	0.924**											
pH (FF)	0.240 ^{NS}	–0.472*	–0.044 ^{NS}	0.144 ^{NS}										
pH (SF)	0.120 ^{NS}	0.004 ^{NS}	–0.572**	–0.599**	0.177 ^{NS}									
Decrease in pH (FF–SF)	0.065 ^{NS}	–0.333 ^{NS}	0.457*	0.610**	0.547*	–0.727**								
Total phenolic content	0.489*	0.076 ^{NS}	0.043 ^{NS}	0.014 ^{NS}	–0.063 ^{NS}	0.260 ^{NS}	–0.265 ^{NS}							
POX (FF)	0.173 ^{NS}	–0.360 ^{NS}	–0.044 ^{NS}	0.099 ^{NS}	–0.030 ^{NS}	–0.124 ^{NS}	0.085 ^{NS}	0.101 ^{NS}						
POX (SF)	0.055 ^{NS}	–0.315 ^{NS}	–0.391 ^{NS}	–0.282 ^{NS}	–0.172 ^{NS}	0.036 ^{NS}	–0.150 ^{NS}	0.338 ^{NS}	0.666**					
LOX (FF)	0.338 ^{NS}	–0.404 ^{NS}	0.048 ^{NS}	0.213 ^{NS}	0.123 ^{NS}	0.032 ^{NS}	0.059 ^{NS}	0.223 ^{NS}	0.651**	0.509*				
LOX (SF)	0.173 ^{NS}	–0.391 ^{NS}	–0.420 ^{NS}	–0.281 ^{NS}	–0.058 ^{NS}	0.450*	–0.423 ^{NS}	0.284 ^{NS}	0.270 ^{NS}	0.431 ^{NS}	0.581**			
PPO (FF)	0.450*	–0.419 ^{NS}	–0.032 ^{NS}	0.134 ^{NS}	–0.031 ^{NS}	0.005 ^{NS}	–0.025 ^{NS}	0.309 ^{NS}	0.499*	0.596**	0.644**	0.689**		
PPO (SF)	0.351 ^{NS}	–0.343 ^{NS}	–0.278 ^{NS}	–0.153 ^{NS}	0.012 ^{NS}	0.096 ^{NS}	–0.073 ^{NS}	0.425 ^{NS}	0.515*	0.773**	0.512*	0.617**	0.817**	

* $P < 0.05$; ** $P < 0.01$

the hybrids possessed high activity of this enzyme. Among the hybrids activity of PPO in fresh flour ranged from 5.5 to 6.5 units/g d.m. The individual genotypes under inbreds and CMS lines differed from each other by narrow and wide margin respectively. Like POX, activity of PPO also decreased in flour upon storage, however, to different extent. Among the genotypes tested decrease in activity of PPO was maximum in HHB 234 (25.5%) and minimum in HTP 94/54 (2%). Similarly substantial variation in PPO activity among wheat cultivars and grain lots was observed by Fuerst et al. (2006). Goyal et al. (2017) compare PPO activity in Pearl millet, wheat and maize and found it similar in fresh flour of pearl millet (5.7 units/g d.m.) and wheat (5.2 units/g d.m.) while fresh flour of maize possesses lowest PPO activity (4.0 units/g d.m.). Fold decrease in PPO activity was almost similar in pearl millet (1.1-fold), wheat (1.1-fold) and maize (1.3-fold) after 30 days of storage.

In the present study, genotypic variations in chemical and biochemical parameters were detected. Correlation matrix of these parameters is presented in Table 1. The crude fat content was found to be positively correlated with total phenolic content and PPO activity (fresh flour) with correlation coefficient of $r = 0.489^*$ and $r = 0.450^*$ respectively. Positive correlation between crude fat and total phenolic content ($r = 0.401^*$) was also reported by Sharma (2015). In oats it has been observed by Decker et al. (2002) that phenols play a role in protection of fats. Increase in FA was positively associated with crude fat content ($r = 0.440^*$) whereas decrease in pH of water extract of flour was not. This indicates that chemical changes taking place in water soluble fraction of flour are independent of fat content. Activities of POX, LOX and PPO were strongly correlated with each other.

Conclusion

The present investigation showed that genotypic variation exist among pearl millet hybrids, CMS lines, inbred lines and white composites for rancidity indicators/determinants viz., fat content, total phenol content, development of FA and activities of POX, LOX and PPO. Variability of CMS and inbred lines may prove to be useful in correlating quantitatively these parameters with volatile and semi-volatile compounds produced during storage of flour. This would lead to identification of chemical/biochemical markers for selecting the desired material from a large pool of germplasm and other breeding material. Chemical changes taking place in water soluble fraction of flour were independent of fat content as no correlation was discerned between fat content and decrease in pH. As per the criteria suggested by Goyal et al. (2017) the promising genotypes

were identified. Among the hybrids, HHB 197 had lowest crude fat content, lowest total build up FA, least decrease in pH of water soluble fraction flour during storage and lowest activity of POX in fresh flour. Among all the tested CMS lines, inbreds and composites, HBL 11 showed pattern of quantitative changes in FA, pH and POX activity similar to the hybrid HHB 197 and was identified a promising inbred for developing low-rancid pearl millet variety or hybrid. Though increase in FA and decrease in pH of flour of one CMS line ICMA 95222 was low but was not identified as it had high enzyme activities and did not fit into the adopted criteria.

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