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Effect of gamma irradiation and variability in Polyphenol oxidase and peroxidase in determining flour quality of pearl millet during storage

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Abstract
Pearl millet is a nutritionally dense crop which ensures high production potential under the changing climatic conditions. Inspite of their rich nutritional composition in terms of fats, carbohydrates, proteins, fibre etc., they are not well accepted by the consumers. Higher content of fatty acids and phenolics in pearl millet gets oxidized in the presence of certain enzymes leading to rancidity, browning and off-flavor generation. Mainly, polyphenol oxidase (PPO) and peroxidase (POX) enzyme activities are associated with the browning effect and thus, limiting the widespread popularity and consumption of pearl millet flour. In this study, we have characterized PPO and POX enzymes activities in 22 pearl millet genotypes during 0, 10 and 20 days of their storage period. Also, we have analyzed the effect of gamma irradiation in inhibiting these enzymes for increasing the shelf life of pearl millet associated with its appearance, taste and flavor. During the course of our study, we have analyzed a large variation in the activities of polyphenol oxidase (0.08-0.23 U/mg protein) and peroxidase (47-121 nM DMAP/min/mg protein) enzymes in fresh flour of genotypes under study. Additionally, we found that gamma irradiated grains caused reduction in PPO activity by 13-15% and POX activity by 53-55%. This study is useful for the selection of pearl millet genotypes with less oxidative enzyme activities which may be ideal for the breeding purpose in future. This may also help in selection and optimization of gamma treatment for improving the shelf life and nutritional properties of pearl millet flour.

Keywords: Polyphenol oxidase, peroxidase, gamma irradiation, pearl millet

1. Introduction
Pearl millet (*Pennisetum glaucum* L.) being a Nutri-cereal is a well-known source of macro and micronutrients. In India, it's the 3rd most important crop after wheat and rice and, it can be grown under adverse condition where major crops like maize and sorghum fail to persist. Nutritional and phytochemical composition of pearl millet has gained significant attention towards its utilization but development of rancidity due to lipid oxidation and associated off-flavor development and browning of flour due to phenolics oxidation during storage are the major limitation for its wide popularity (Goswami *et al*. 2020) [6]. In addition to rancidity, presence of higher concentration of specific anti-nutritional factors like, tannins, phytate and polyphenols in the flour also leads to the chelation of essential minerals in the gastrointestinal tract, thereby decreasing its bioavailability (Tomar *et al*. 2021) [21]. Higher activity of Peroxidase (POX) was found to be the major cause for development of rancid odor in pearl millet and Polyphenol oxidase (PPO) associated with browning of flour (Goyal and Chugh 2017) [7, 9]. During milling of flour, phenolics in pericarp comes in contact with these oxidizing enzymes leading to off flavor and browning generation during storage and hence influencing the quality of flour.

PPO and POX are the critical enzymes involved in these oxidations of phenolic compounds especially, flavonoids and such oxidative reactions can later form quinones. These are highly reactive and can further condense with other phenolics, amino acids or protein finally yielding highly complex brown products (Taranto *et al*. 2017) [20]. Even though their role in browning and odor generation are well established in other major crops like wheat and maize, the role in pearl millet flour is still limited (Mallick *et al*. 2013) [13]. PPO and POX of pearl millet grain are reported to be higher in activity than those in wheat and maize (Goyal *et al*. 2017) [9]. In initial studies, Bangar *et al*. (1999) [2] reported the polyphenol oxidizing enzymes that are responsible for unpleasant smell in pearl millet flour.

In a recent study, a large variation in the PPO and POX activity was reported in different Indian varieties, hybrids and composites (Goyal and Chugh 2017) [9]. In addition to phenolic oxidation, peroxidases have also been reported to oxidize fatty acids and in the formation of hydroperoxides which promotes the rancidity (Rodriguez-Saona *et al*. 1995) [19]. Inhibition of these enzyme activities are essential for enhancing the shelf life of pearl millet flour. Earlier in studies, several attempts including decortication (Jain and Bal 1997) [10] blanching, dry heating (Rai *et al*., 2008) [18] low temperature (Mohamed *et al*. 2011) [15], microwave (Yadav *et al*. 2012) [22] were done to address the rancidity issue of pearl millet flour. Even though, inhibiting the enzymatic action of pearl millet flour by using different physical treatments are still limited.

Browning, odor and flavor generation due to polyphenol oxidation during processing and storage are great concern for the food industries as it negatively influences the nutritional quality as well as appearance of food products (Fuerst *et al*. 2018) [5]. Gamma irradiation is found to be an effective way of inhibiting PPO and POX in fruits and vegetable thereby, increasing their shelf life (Moon *et al*. 2020). In a recent study Suneha *et al*. (2020) reported that, initial 2 weeks after flour preparation is critical for assessing lipid oxidation and thereby over all rancid nature of the flour. So, this study is primarily focused on the enzymatic changes in the activity of PPO and POX of diverse pearl millet genotypes during different storage period of 20 days, and also the effect of gamma irradiation on the enzymatic inhibition of PPO and POX.

2. Material and Methods

Twenty-two Pearl millet genotypes grown in the year 2019 were obtained from All India Coordinated Research Project (AICRP), Jodhpur. Flour with moisture content 12±0.5% was prepared and sieved through 0.3 mm mesh sieve and stored under room temperature (25±3 °C), with a relative humidity of 45-85% in airtight plastic zip bags kept under dark till further analysis. Pure enzyme of Polyphenol oxidase (Cas-9002-10-2, Merck) was obtained from Sigma Aldrich. All other reagents used were of analytical grade.

2.1 Gamma irradiation treatment

For studying the effect of gamma irradiation on enzymatic activity, 86M38 hybrid genotype of pearl millet was used. Grains were divided into 10 groups, i.e., control (non-irradiated, 0 kGy), 0.0025 kGy, 0.005 kGy, 0.01 kGy, 0.05 kGy, 0.1 kGy, 0.2 kGy, 0.4 kGy, 0.6 kGy and 1.0 kGy in duplicates. Ten-gram seeds of uniform size were selected and packed in polyethylene high density bags (4 mm thickness) and were irradiated at different dose level of gamma at room temperature (24 ± 2 ^{O}C). Gamma irradiation was performed using 60Co gamma radiation chamber (Model GC-5000, BRIT Mumbai) facility at Nuclear Research Laboratory, IARI, New Delhi India. Treated grains were then milled, sieved and stored at room temperature till further analysis.

2.2 Enzymes extract preparation

Crude enzyme preparation was done by homogenizing 100 mg of flour in 1 mL of 0.2M potassium phosphate buffer (pH 7.0) in a pre-chilled mortar and pestle. Homogenate was centrifuged at 13,000 rpm for 20 minutes at 4 °C. The supernatant was carefully filtered through four layers of cheesecloth and used as an enzyme extract for activity assay. Total soluble protein of crude enzyme extract was estimated by Bradford method (Bradford 1976) [3].

2.3 Peroxidase activity assay

Peroxidase (POX) activity was assayed as per the method of Malick and Singh (1980) [12]. In brief, 2.8 mL assay mixture was made by adding 2.5 mL of 0.05M potassium phosphate buffer (pH 6.8), 0.1 mL of 0.1 M H2O2, 0.1 mL of 0.1% (w/v) o-dianisidine and 0.1 mL of enzyme extract. Reaction was started by addition of H2O2. A reaction mixture without H2O2 served as a blank. Increase in absorbance at 430 nm was recorded at an interval of 30 seconds for 2 minutes. Molar extinction coefficient of bis-(3,3'-dimethoxy-4-amino) azodiphenyl (DMAP) is 30 mM-1 cm-1, which is the oxidized form of o-dianisidine, used for calculation. The POX activity was expressed as nanomole DMAP/min/mg soluble protein.

2.4 Polyphenol oxidase activity assay

Polyphenol oxidase (PPO) activity assay was followed by the method of Kruger *et al*. (1994) [11] with slight modifications. In brief, freshly prepared 0.01M catechol solution in 0.05M potassium phosphate buffer (pH 6.2) was used as substrate solution. Reaction was started by addition of 0.2mL of crude enzyme extract to 2.8mL of freshly prepared catechol solution. The reaction mixture was then incubated at 37°C for 30 minutes. Absorbance was recorded at 410 nm on a UV-vis spectrophotometer. Pure PPO enzyme of known units was used in calculation.

2.5 Statistical analysis

Three replicates were used to carry out biochemical assays. The analysis of variance was computed by using one-way ANOVA for statistically significant differences. Analysis was done by SPSS v.16.

3. Results and Discussion

3.1 Changes in the enzymatic activities of POX and PPO during storage

POX activity of genotypes under study is given in Fig.1. Large variation in the POX activity was observed in which highest activity in freshly milled flour was found in GHB538 (121 nM DMAP/min/mg protein) and lowest in GHB 732 (47 nM DMAP/min/mg protein) which is about 2.4-fold reduction in activity as compared to the highest activity showing genotype. A reduction in the activity of POX was observed during storage period of flour and the extent of reduction vary from genotypes to genotypes. Maximum reduction in the POX activity was observed in HHB 226 (81%) followed by 431B (76.9%), ICMR0733 (76.6%) GHB 719 (65.5%) and minimum reduction was observed in NANDI 90 (9%). Reduction in POX activity during storage was reported by Goyal and Chugh (2017) [9] in pearl millet flour and in rice grains by Chen and Chen (2003). During storage, significant decline in the pearl millet flour pH was observed as compared to wheat and rice, and this difference is due to the enzymatic and non-enzymatic formation of fatty acids and phenolic acids (Goyal *et al*. 2017) [9]. Being pH sensitive in nature peroxidase activity is reduced in response to changes in pH (Goyal and Chugh 2014) [8].

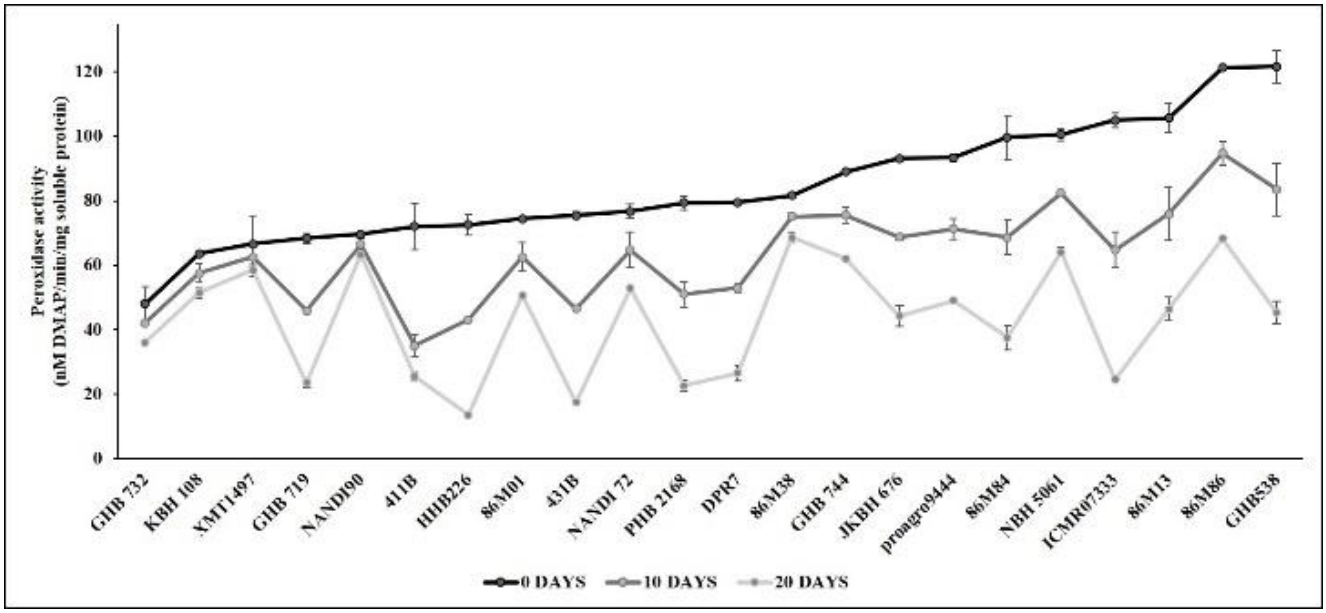

Fig 1: POX activity of genotypes under study

PPO is considered to be the major browning enzymes in tissues of most of the plants (Pourcel *et al*., 2007) [17]. PPO activity of genotypes under study is given in Fig.2. Like in POX, study also observed reduction in activity of PPO during storage period. Highest activity of PPO was observed in ICMR 0733 (0.23U/mg protein) followed by 86M84, NANDI 90 and lowest PPO activity was observed in 86M38 (0.08U/mg protein). A storage period of 20 days further reduced PPO activity by 50-82% in genotypes under study. This is an agreement with findings of (Goyal and Chugh 2017) [9] where 25% reduction was reported. Decrease in enzyme activities during storage might be due to reduced accessibility of enzyme to substrate, increased hydrogen bonding between water and either enzyme active site or substrate, shift in pH, increased intra-enzymic hydrogen bonding or formation of enzyme polymers etc.

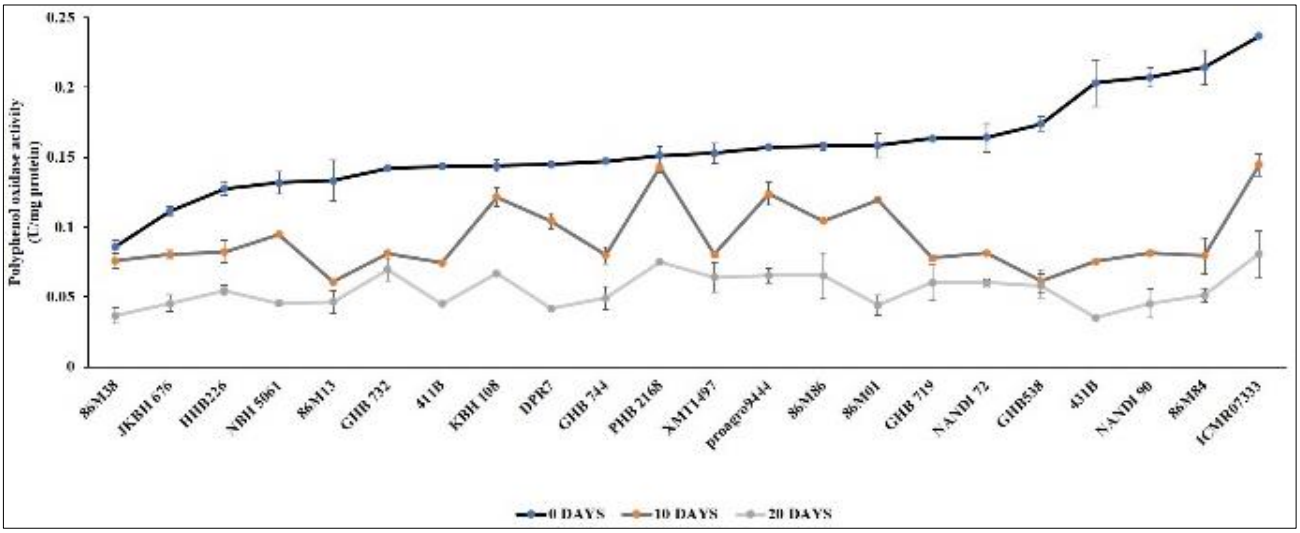

Fig 2: PPO activity of genotypes under study

3.2 Effect of Gamma irradiation on activity of PPO and POX: Inhibitory effect of gamma irradiation on PPO and POX is given in Fig.3. Study observed that, gamma irradiation is found to have less inhibitory effect on PPO as compared to POX. In PPO, 1 kGy gamma irradiation treatment caused a reduction of 13-15% in enzymatic activity in comparison to the control (0.07U/ mg protein). This result is in agreement with finding of (Mishra *et al*. 2012) [14] in which 0.5kGy gamma irradiation caused light reduction in litchi PPO activity. However, in other studies PPO activity remain unchanged after treatment with 2 kGy of gamma irradiation (Banerjee *et al*. 2015) [1]. Data on inhibitory role of gamma irradiation on cereals and millet PPO are still limited. In POX, gamma irradiation effectively reduced enzymatic activity from 71nM DMAP/min/mg protein in control sample to 34nM DMAP/min/mg protein in 1 kGy gamma irradiation treated sample. So here 53-55% reduction in POX activity was observed. Studies showed 15-20% reduction in POX activity in response to 0.5 kGy gamma irradiation in litchi pericarp POX (Banerjee *et al*. 2015) [1].

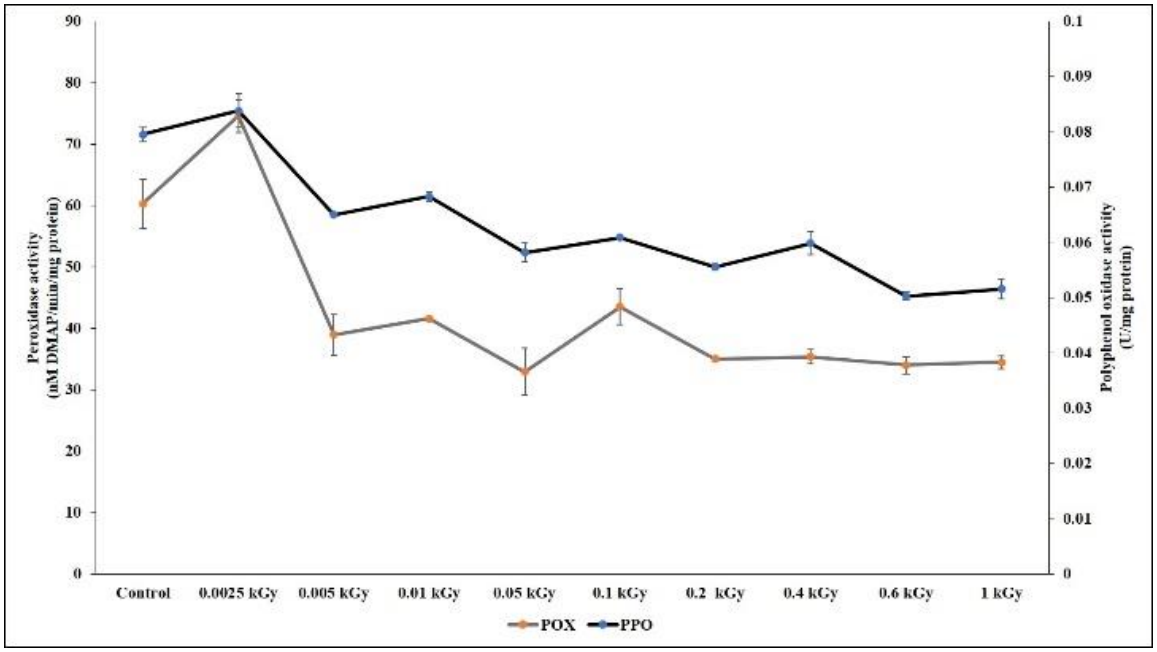

Fig 3: Inhibitory effect of gamma irradiation on PPO and POX

4. Conclusions

Development of off-flavor, off-odor and browning of pearl millet flour during storage is the major drawback that limits the consumer acceptability. Enzymatic and non-enzymatic changes in the flour during storage period accelerate this quality deterioration processes. This study is primarily focused on the determination of wide variability in phenolics degradative enzymes i.e., POX and PPO present in pearl millet flour and the inhibitory role of gamma irradiation on such enzymes. This study found the existence of wide variation in the POX and PPO activity of pearl millet genotypes under study. This result could help in the selection of pearl millet lines with low degradative enzymatic activities or phenolic content so that selective breeding could be performed in a way that lesser advancements in improving the shelf life and appearance of pearl millet grain would be needed at the later stages and thereby, increasing their popularity among consumers. This study also focused on the inhibitory role of gamma irradiation on POX and PPO enzymatic activities. Here, 1 kGy gamma irradiation caused only 13-15% reduction in PPO activity and 53-55% reduction in POX activity. There is a further need to explore more such physical and chemical treatments of pearl millet so that its shelf life could be extended without compromising its nutritional properties.

5. Conflicts of interest

The authors declare that they have no conflict of interest.

6. Acknowledgment

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