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# Changes in Enzyme Activities during Seed Senescence in Pearl Millet (*Pennisetum glaucum* L.) Hybrids and their Parental Lines

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**Study was undertaken to elucidate the effect of accelerated ageing on seed deterioration and enzymatic changes associated with seed ageing in hybrids and their parental lines of pearl millet. The results revealed that the activities of dehydrogenase, peroxidase, catalase, polyphenol oxidase and α amylase enzymes differed significantly among the genotypes of *Pennisetum glaucum* seed. The enzyme activities except for polyphenol oxidase, declined significantly during accelerated ageing. Dehydrogenase activity decreased at faster rate to about 50 per cent of its initial level. Catalase activity declined gradually throughout the ageing period, while peroxidase activity slightly increased up to eight days of ageing and then decreased sharply. Aged seeds recorded delayed onset and reduced activity of α amylase enzyme. The polyphenol oxidase activity showed an inconsistent trend during seed senescence.**

**Key words**: Pearl millet, seed senescence, dehydrogenase, peroxidase, catalase, polyphenol oxidase, α amylase

Seed deterioration during ageing processes is a natural phenomenon that involves complex changes in many cellular constituents. These physiological and biochemical changes in overall metabolic efficiency of seeds lead to decreased seed vigour and eventually loss of viability. Many seed biologists have made detailed investigations on the biochemical changes during seed deterioration which includes changes in enzymatic activity (Grabe, 1964; Meulen *et al*., 2000); chromosomal damage and loss of membrane integrity (Woodstock and Grabe, 1967); decline in nucleic acid and protein synthesis (Sen and Osborne 1977); accumulation of free radicals (Harman *et al.*, 1982; Pal and Basu, 1989) etc. Measurement of the activity of specific enzyme is one of those biochemical techniques used to trace seed deterioration and predict the viability. However, these changes associated with seed deterioration have not been investigated in pearl millet. The knowledge on the pattern of seed deterioration of pearl millet hybrids and their parental lines is important to adduce an ideal physiological seed treatment. With this background, studies were undertaken by employing the accelerated ageing technique to trace the pattern of seed deterioration and to elucidate the enzymatic changes associated with seed ageing in parental lines and hybrids of pearl millet.

## Materials and Methods

Hybrids (X 7 and Pusa 23) and their parental line seeds of pearl millet ($V_1$ - ms L 111 A, $V_2$ - ms 841 A, $V_3$ - L 111 B, $V_4$ - 841 B, $V_5$ - PT 1890, $V_6$ - D 23, $V_7$ - X 7 hybrid, $V_8$ - Pusa 23 hybrid) were subjected to different periods of accelerated ageing ($A_0$-non aged, $A_1$- aged for 4 days, $A_2$ -aged for 8 days, $A_3$ - aged for 12 days, $A_4$ - aged for 16 days, $A_5$ - aged for 20 days) to study the enzyme activities during the process of seed ageing.

### *Accelerated ageing of seeds*

Seed samples in required quantities from each genotype were taken in perforated butter paper bag placed in a make shift humidity chamber (2.5 l capacity desiccator filled with 250 ml water) and kept in a BOD incubator maintained at 40 + 1ºC for 20 days. Two replications were maintained under identical conditions. The seed samples were drawn at different intervals (4days) to obtain seeds with differential ageing. Then the seeds with differential ageing in each genotype were dried to uniform moisture content and subjected to the enzyme estimations (Delouche and Baskin, 1973).

### *Lipid peroxidation value*

Lipid peroxidation value was estimated by thiobarbituric acid (TBA) colour reaction with suitable modifications (Bernheim *et al.,* 1948). Seven ml of 0.5 per cent TBA-TCA reagent (5 g of thiobarbituric acid in 100 ml of 20% trichloro acetic acid) was added to 200 mg of dry seed powder in a hard glass test tube. The mixture was thoroughly shaken and placed in a water bath at 100ºC for 30 minutes. After

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cooling and centrifugation for 10 minutes, the absorbance of the clear supernatant was read in an ELICO SL 150 spectrophotometer with blank using 530 nm filter.The lipid peroxidation value was expressed as OD value.

***Enzyme estimation***

Dehydrogenase activity was measured as per the method described by Kittock and Law (1968). Seeds were preconditioned in between filter paper for 18 h (ISTA, 1999). Twenty seeds from each treatment were cut longitudinally using a sharp knife and one half from each seed was immersed in 5 ml of 0.5 per cent aqueous solution of 2,3,5-triphenyl tetrazolium chloride and kept in darkness for six hours at ambient temperature. After staining, the solution was decanted and the seeds were washed thoroughly with distilled water. The colour was eluted from the stained embryos by steeping in 3 ml of 2-methoxy ethanol (methyl cellosolve) for 1 hour and decanted. The colour intensity was then measured in ELICO SL 150 spectrophotometer with suitable blank, using blue filter (470 nm) and expressed as OD value

Estimation of peroxidase activity was assessed by grinding 100 mg of seed samples with 10 ml of 0.1 M phosphate buffer (pH 7.0) in a pre-chilled mortar and pestle and centrifuged at 15,000 rpm for 30 min at 4ºC to extract the enzyme. The assay mixture contained 2 ml 0.1 M phosphate buffer (pH 7.0) 1 ml 0.01 M pyrogallol, 1 ml 0.005 M $H_2O_2$ and 0.2 ml enzyme extract. The reaction was stopped by adding 1 ml 2.5 N $H_2SO_4$ after 10 min. incubated at 25ºC and the absorbance was measured at 420 nm. The enzyme activity was expressed in OD g-110 $min^{-1}$ (Kumar and Khan, 1982).

For estimation of catalase activity, the enzyme extract was prepared as in peroxidase assay. From this enzyme extract, 0.2 ml was added to 2 ml 0.005 M $H_2SO_4$ and 3 ml phosphate buffer (pH 7.0). The reaction was stopped by adding 10 ml 0.7 N $H_2SO_4$ after 1 min. incubation at 20ºC. The residual $H_2O_2$ was titrated with 0.01 N $KMnO_4$. A blank was prepared by adding the extract to an acidified solution of the reaction mixture at zero time. The difference between the titre value of enzyme reaction mixture and blank was the permanganate equivalent to enzyme activity. The catalase activity was expressed in $\mu$ mole $H_2O_2$ $min^{-1}$ $g^{-1}$ (Kumar and Khan, 1982).

Estimation of polyphenol oxidase activity was done with seed samples of 100 mg each that were homogenized with 10 ml of 0.1 N phosphate buffer (pH 7.0) in a pre-chilled mortar and pestle and centrifuged at 15,000 rpm at 4ºC for 30 min to extract the enzyme. The assay mixture contained 2 ml of 0.1 M phosphate buffer (pH 7.0), 1 ml of 0.01 M pyrogallol and 1 ml enzyme extract. The reaction was stopped by adding 1 ml of 2.5 N $H_2SO_4$ to the reaction mixture after 60 min. incubation at 25ºC. The amount of purpurogallins formed was estimated by measuring the absorbance at 420 nm and the enzyme activity was expressed in OD $g^{-1}$ h-1 (Kumar and Khan, 1982).

The method outlined by Paul *et al.* (1970) was adopted with suitable modification for estimation of $\alpha$ amylase enzyme activity. Seeds were pre hydrated by placing in between moist filter paper for 24 h to trigger $\alpha$ amylase synthesis. Later the seeds were dried slowly to the uniform moisture content and used for $\alpha$ amylase assay. Seed samples of 250 mg each were homogenied in 10 ml of pre-cooled 0.02 M sodium phosphate buffer (pH 6.0) and centrifuged at 2000 rpm for 10 minutes. The reaction mixture consisted of 1 ml of 0.067 per cent starch solution and 0.1 ml of enzyme extract. The reaction was stopped after 15 min, incubated at 25ºC by adding 1 ml iodine HCl solution (60 mg KI and 6 mg $I_2$ in 100 ml of 0.05 N HCl). The change in colour was measured along with the maltose standards at 620 nm. The activity was calculated from maltose standard graph and expressed as mg maltose $g^{-1}$ 15 $min^{-1}$.

**Results and Discussion**

The individual enzyme assay carried out in this investigation revealed that dehydrogenase, peroxidase, catalase, polyphenol oxidase and $\alpha$ amylase activities significantly differed among the genotypes. In general, all these enzymes except for polyphenol oxidase declined during the entire 20 days of accelerated ageing at different rates and magnitudes.

Dehydrogenase activity decreased at a faster rate to about 50 per cent of its initial level in the seeds of ms 841 A, L 111 A and L 111 B. The decline was relatively slow in other genotypes (Table 1). This difference might be due to the differential death of the sensitive embryo cells during ageing. Similar loss in dehydrogenase activity in differentially aged pearl millet seeds was reported by Malarkodi and Dharmalingam (1998).

The critical analysis of data showed a gradual decline in catalase activity throughout the ageing period; whereas, the peroxidase activity slightly increased upto 8 days of ageing and then decreased sharply. In general, these two enzyme activities were less in isogenic lines (A and B lines) and higher in hybrids and male lines (Table 2). Since the peroxidase enzyme is also involved in dehydrogenation of large number of organic compounds such as aromatic amines and free radicals, the decrease in the level of this enzyme could lead to the accumulation of toxic substances in the seeds. Such accumulation of toxic free radicals accompanied by the decreased activity of antioxidant enzyme was reported by Hendry (1993). Kole and Gupta (1982) and Kalpana and Madhavarao (1994) which implicated that the decreased catalase and peroxidase activity might lead to accumulation of hydrogen peroxide in the seeds.

**Table 1. Lipid peroxidation value and dehydrogenase enzyme activity in the seeds of pearl millet genotypes during accelerated ageing**

| Ageing Period/ | Lipid Peroxidation (OD) | | | | | | | Dehydrogenase (OD) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotypes | $A_0$ | $A_1$ | $A_2$ | $A_3$ | $A_4$ | $A_5$ | Mean | $A_0$ | $A_1$ | $A_2$ | $A_3$ | $A_4$ | $A_5$ | Mean |
| $V_1$ | 0.127 | 0.128 | 0.136 | 0.152 | 0.163 | 0.184 | 0.148 | 0.845 | 0.810 | 0.705 | 0.580 | 0.470 | 0.405 | 0.636 |
| $V_2$ | 0.127 | 0.134 | 0.139 | 0.150 | 0.163 | 0.181 | 0.149 | 0.800 | 0.780 | 0.705 | 0.625 | 0.560 | 0.400 | 0.644 |
| $V_3$ | 0.129 | 0.132 | 0.134 | 0.158 | 0.169 | 0.177 | 0.150 | 0.825 | 0.805 | 0.700 | 0.590 | 0.540 | 0.390 | 0.642 |
| $V_4$ | 0.123 | 0.126 | 0.132 | 0.146 | 0.160 | 0.169 | 0.143 | 0.845 | 0.805 | 0.760 | 0.715 | 0.650 | 0.555 | 0.722 |
| $V_5$ | 0.110 | 0.111 | 0.119 | 0.125 | 0.141 | 0.149 | 0.126 | 0.850 | 0.820 | 0.710 | 0.670 | 0.620 | 0.560 | 0.705 |
| $V_6$ | 0.111 | 0.118 | 0.124 | 0.136 | 0.143 | 0.148 | 0.130 | 0.885 | 0.865 | 0.780 | 0.720 | 0.640 | 0.505 | 0.733 |
| $V_7$ | 0.114 | 0.115 | 0.122 | 0.129 | 0.144 | 0.158 | 0.130 | 0.890 | 0.860 | 0.760 | 0.700 | 0.660 | 0.585 | 0.742 |
| $V_8$ | 0.112 | 0.117 | 0.127 | 0.135 | 0.144 | 0.152 | 0.131 | 0.895 | 0.865 | 0.785 | 0.725 | 0.630 | 0.510 | 0.735 |
| Mean | 0.119 | 0.122 | 0.129 | 0.141 | 0.153 | 0.165 | | 0.854 | 0.826 | 0.738 | 0.665 | 0.596 | 0.489 | |
| | V | | A | | VA | | | | | V | | A | | VA |
| SEd | 0.003 | | 0.003 | | NS | | | | | 0.027 | | 0.024 | | NS |
| CD (0.05) | 0.001 | | 0.006 | | | | | | | 0.055 | | 0.048 | | |

Conversely, the increase in peroxidase activity during the initial period of seed ageing might be due to the conversion of tetrameric molecules of catalase activity to monomeric molecules of peroxidase activity. The subsequent decline in peroxidase activity after eight days of ageing might be attributed to the severe loss of catalase activity. These results were in corroboration with the findings of Kumar and Khan (1982) in finger millet seedlings and Kalpana and Madhavarao (1994) in pigeonpea seeds.

In the present study, even though the peroxidase activity was found to be higher in the better storers (R lines and hybrids), the enzymatic lipid peroxidation value remained low (Table 1). This result further strengthened the logic that the lipid peroxidation in the pearl millet seeds was mainly due to the non-enzymatic process such as Millard reaction.

**Table 2. Peroxidase and catalase enzyme activity in the seeds of pearl millet genotypes during accelerated ageing**

| Ageing Period/ | Peroxidase (units $g^{-1}$ 10 $min^{-1}$) | | | | | | | Catalase ( $\mu$ mole $H_2O_2$ $g^{-1}$ $min^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotypes | $A_0$ | $A_1$ | $A_2$ | $A_3$ | $A_4$ | $A_5$ | Mean | $A_0$ | $A_1$ | $A_2$ | $A_3$ | $A_4$ | $A_5$ | Mean |
| $V_1$ | 82 | 88 | 100 | 87 | 74 | 61 | 82 | 409 | 392 | 379 | 368 | 357 | 349 | 375 |
| $V_2$ | 90 | 91 | 105 | 91 | 85 | 72 | 89 | 447 | 431 | 423 | 413 | 397 | 378 | 415 |
| $V_3$ | 89 | 85 | 98 | 83 | 77 | 63 | 83 | 402 | 395 | 378 | 368 | 357 | 344 | 374 |
| $V_4$ | 92 | 94 | 103 | 94 | 88 | 80 | 92 | 467 | 455 | 446 | 441 | 426 | 414 | 441 |
| $V_5$ | 101 | 100 | 106 | 93 | 84 | 80 | 94 | 497 | 493 | 479 | 466 | 456 | 452 | 474 |
| $V_6$ | 104 | 107 | 110 | 100 | 88 | 81 | 98 | 512 | 496 | 480 | 471 | 460 | 449 | 478 |
| $V_7$ | 100 | 95 | 107 | 91 | 83 | 75 | 92 | 494 | 484 | 475 | 464 | 458 | 453 | 471 |
| $V_8$ | 108 | 110 | 116 | 100 | 90 | 73 | 99 | 517 | 501 | 485 | 473 | 466 | 459 | 483 |
| Mean | 96 | 96 | 105 | 92 | 83 | 73 | | 468 | 456 | 443 | 433 | 422 | 412 | |
| | V | | A | | VA | | | V | | A | | VA | | |
| SEd | 2.31 | | 2.00 | | NS | | | 4.60 | | 3.98 | | NS | | |
| CD (0.05) | 4.64 | | 4.02 | | | | | 9.25 | | 8.01 | | | | |

Polyphenol oxidase is another enzyme that causes similar type of reaction as peroxidase. In the present study, the poly phenol oxidase activity showed inconsistent trend during ageing. However, higher activity was noticed in hybrids and restorer line seeds than the male sterile line seeds. Irrespective of the genotypes, a slight decline (55 to 48 units $g^{-1}$ 10 $min^{-1}$) was noticed up to 8 days of ageing and then slightly increased up to 20 days of ageing (60 units $g^{-1}$ 10 $min^{-1}$) (Table 3). Since this enzyme was relatively stable during ageing, it might not be appropriate to relate this with seed senescence and this change in activity might be attributed to other biochemical factors.

Shepherd *et al.* (1995) reported that radical extension was closely correlated with $\alpha$ amylase activity and suggested that this might be a rate limiting step in germination. In the present findings as well, the $\alpha$ amylase activity declined steeply in all the genotypes (Table 3). The delayed onset and

**Table 3. Polyphenol oxidase and α amylase activity in the seeds of pearl millet genotypes during accelerated ageing**

| Ageing Period/ | Peroxidase (units $g^{-1}$ 10 $min^{-1}$) | | | | | | | Catalase ( μ mole $H_2O_2$ $g^{-1}$ $min^{-1}$) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Genotypes | $A_0$ | $A_1$ | $A_2$ | $A_3$ | $A_4$ | $A_5$ | Mean | $A_0$ | $A_1$ | $A_2$ | $A_3$ | $A_4$ | $A_5$ | Mean |
| $V_1$ | 47 | 43 | 41 | 48 | 50 | 50 | 46 | 61 | 52 | 43 | 38 | 27 | 21 | 40 |
| $V_2$ | 52 | 48 | 44 | 53 | 58 | 59 | 52 | 57 | 49 | 44 | 36 | 28 | 22 | 39 |
| $V_3$ | 49 | 46 | 44 | 51 | 53 | 53 | 49 | 62 | 54 | 43 | 34 | 28 | 23 | 41 |
| $V_4$ | 55 | 53 | 48 | 57 | 60 | 62 | 56 | 51 | 45 | 38 | 32 | 26 | 22 | 35 |
| $V_5$ | 60 | 55 | 52 | 62 | 64 | 64 | 59 | 46 | 41 | 37 | 30 | 26 | 22 | 33 |
| $V_6$ | 65 | 61 | 58 | 67 | 68 | 67 | 64 | 43 | 38 | 34 | 28 | 23 | 20 | 31 |
| $V_7$ | 53 | 50 | 48 | 55 | 59 | 61 | 54 | 51 | 47 | 43 | 38 | 29 | 25 | 39 |
| $V_8$ | 60 | 58 | 55 | 62 | 66 | 65 | 61 | 50 | 45 | 41 | 33 | 29 | 24 | 37 |
| Mean | 55 | 52 | 48 | 57 | 60 | 60 | | 52 | 46 | 40 | 33 | 27 | 22 | |

| | V | A | VA | V | A | VA |
|---|---|---|---|---|---|---|
| SEd | 1.82 | 3.66 | NS | 1.70 | 1.47 | NS |
| CD (0.05) | 1.58 | 3.17 | | 3.42 | 2.96 | |

reduced activity of α amylase in the aged seeds might have reduced the amount of soluble sugars. Thus, the availability of energy substrates needed for germination was limited in aged seeds.

Karrer *et al*. (1993) showed that seedling vigour, -amylase activity and accumulation of α amylase mRNA were positively correlated in the rice cultivars. Similar relationship between α amylase activity and seed vigour was reported by Krishnasamy and Seshu (1990) in rice, Meulen *et al*. (2000) in barley and Ritchie *et al*. (2000) in other cereals.

The slight decrease in the α amylase activity in the early hours of germination in the high vigour pearl millet seeds (R lines) might be due to high polyphenol content. However, during subsequent germination process, polyphenol oxidase could activated to hydrolyse the polyphenols which have been increased the α amylase activity at the later stages of germination. Such inhibition of α amylase by polyphenol was also reported by Thompson and Yoon (1984) and Sutardi and Buckle (1985); while the increase in α amylase activity at later stages of pearl millet seed germination was reported by Sharma and Kapoor (1995).

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