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# Comparison of Peroxidase Enzyme Activity and Spot Test in Soybean [*Glycine max* (L.) Merill] Genotypes

**JAGADISH HOSAMANI[1]*, M DADLANI[1], MB ARUN KUMAR[1], IM SANTHA[2], A TALUKDAR[3] AND SK LAL[3]**

[1]Division of Seed Science and Technology, ICAR-Indian Agricultural Research Institute, New Delhi-110012, India
[2]Division of Agricultural Biochemistry, ICAR-Indian Agricultural Research Institute, New Delhi-110012, India
[3]Division of Genetics, ICAR-Indian Agricultural Research Institute, New Delhi-110012, India
*jagiari@gmail.com

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**ABSTRACT:** Thirty three soybean [*Glycine max* (L.) Merill] genotypes, comprising 14 black seeded and 19 yellow seeded ones, were selected on the basis of their reported storability for a comparative study on peroxidase activity and validation of spot test. It was observed that though the mean peroxidase activity was higher in poor storer genotypes, it cannot clearly differentiate a good storer from the poor, and irrespective of the initial status, the peroxidase activity declines drastically during storage. The peroxidase spot test, which is used for grouping soybean varieties into positive and negative reaction groups, which is not indicative of their storability behaviour. It was also evidenced that the spot test reaction changes drastically with storage, revealing the importance of sampling only fresh/high vigour soybean seeds for peroxidase test.

**Keywords:** Soybean, peroxidase activity, spot test, seed coat colour, germination

Soybean [*Glycine max* (L.) Merill] has assumed a key position in the oilseed cultivation in India, due to its high productivity among the oilseed crops and contribution in sustaining the soil fertility. At physiological maturity the soybean seed reaches its maximum potential for germination and vigour [1], but has a poor storability [2]. Germination is often reduced below the Indian minimum prescribed standards (70 per cent) even within one planting season under warm and humid sub-tropical climate [3, 4].

Peroxidases are haemoproteins, widely distributed in the plant species that catalyse the oxidation of a wide variety of substrates, using hydrogen peroxide ($H_2O_2$) as the oxidant. The typical peroxidase reaction involves a well established cycle in which many electron donors, mainly phenols, can be oxidized [5]. The enzyme has been reported to be involved in a range of metabolic activities, such as in regulation of the level of indole acetic acid [6], in cell wall formation [7], in regulation of membrane permeability [8], in biotic and abiotic stress reactions [9] and in seed dormancy and germination [10].

The seed coat of soybean is rich in peroxidase activity. The peroxidase activity in the seed coat is a characteristic used to classify soybean genotypes into two broad categories viz., low activity (-) and high activity (+) [11]. Inheritance of high peroxidase activity in soybean seed coat is attributed to the dominant gene *Ep* [12]. Low activity results from the homozygous recessive condition *epep*. The *Ep* locus, controlling seed coat peroxidase, is frequently used as an enzyme marker in genetic studies and as a diagnostic tool in the identification of plant cultivars.

Several researchers have tried using seed coat peroxidase test (spot test) to differentiate different crop genotypes to varying levels of success [13-15]. Several rapid laboratory tests were examined to determine their usefulness in characterising 36 soybean cultivars [16]. The tests which successfully differentiated the cultivars included hilum colour, hypocotyls colour, seed coat peroxidase and electrophoresis of â-amylase and urease in the unimbibed seeds [16]. Ortega *et al*. [17] reported that 24 soybean cultivars were characterized by the peroxidase test, with 79 per cent exhibiting high and 21 per cent low peroxidase activity. Based on these findings, it was suggested that peroxidase test could be incorporated in routine seed testing work for monitoring varietal purity of soybean seeds.

Notwithstanding the above information, peroxidase test of the seed coat is referred both by ISTA [18] and AOSA [19] for grouping/characterization of soybean varieties

leading to their identification. Information on peroxidases in soybean seeds, therefore, could be of value in relation to varietal purity and identification as well as seed viability and dormancy [20].

## MATERIALS AND METHODS

### Plant material

Thirty three soybean genotypes were collected from the senior Soybean Breeder, Division of Genetics, IARI, New Delhi. The genotypes were selected on the basis of their reported storability as per previous studies [21, Lal, unpublished]. Among these 14 were black seeded and 19 were yellow seeded genotypes (Table 1). To obtain the seeds of uniform physiological status, the stock seeds were multiplied under similar agronomic conditions in wet season (*kharif)* in the Research Farms of IARI, New Delhi. Seeds were hand threshed and manually cleaned.

### Germination testing

Germination was tested in three replications of 100 seeds each, following between-paper method at 25 °C [22]. The

**Table 1.** Germination (%) of soybean genotypes before and after ambient storage

| S. No. | Genotypes | Testa colour | Fresh seeds | | Stored seeds | |
|---|---|---|---|---|---|---|
| | | | First count | Final count | First count | Final count |
| **Good storers** | | | | | | |
| 1 | AMSS – 34 | Black | 98 (83.44) $^{ba}$ | 98 (83.44) $^{bac}$ | 80 (63.43) $^{dec}$ | 88 (69.73) $^{dc}$ |
| 2 | DS – 74 | Black | 99 (85.37) $^{a}$ | 99 (85.37) $^{ba}$ | 80 (63.43) $^{dec}$ | 90 (71.62) $^{bdc}$ |
| 3 | DS – MM – 64 | Black | 98 (81.87) $^{bac}$ | 99 (85.37) $^{ba}$ | 90 (71.62) $^{a}$ | 94 (75.95) $^{ba}$ |
| 4 | G – 2253 | Black | 96 (78.71) $^{bdac}$ | 97 (81.95) $^{bac}$ | 86 (68.06) $^{bac}$ | 90 (71.62) $^{bdc}$ |
| 5 | G – 2265 | Black | 96 (78.46) $^{bdac}$ | 98 (81.87) $^{bac}$ | 84 (66.52) $^{bdac}$ | 88 (69.90) $^{dc}$ |
| 6 | G – 2601 | Black | 99 (85.37) $^{a}$ | 99 (85.37) $^{ba}$ | 86 (68.06) $^{bac}$ | 90 (71.62) $^{bdc}$ |
| 7 | G – 2603 | Black | 96 (78.46) $^{bdac}$ | 98 (81.87) $^{bac}$ | 88 (69.73) $^{ba}$ | 96 (78.46) $^{a}$ |
| 8 | G – 2614 | Black | 98 (81.87) $^{bac}$ | 98 (81.87) $^{bac}$ | 82 (64.91) $^{bdc}$ | 90 (71.62) $^{bdc}$ |
| 9 | G – 2651 | Black | 99 (85.37) $^{a}$ | 99 (85.37) $^{ba}$ | 88 (69.73) $^{ba}$ | 96 (78.46) $^{a}$ |
| 10 | M – 253 | Black | 95 (77.12) $^{bdc}$ | 100 (90.00) $^{a}$ | 78 (62.04) $^{fde}$ | 88 (69.73) $^{dc}$ |
| 11 | M – 1090 | Black | 98 (81.87) $^{bac}$ | 99 (85.37) $^{ba}$ | 84 (66.42) $^{bdac}$ | 86 (68.06) $^{d}$ |
| 12 | M – 11913 | Black | 95 (77.12) $^{bdc}$ | 99 (85.37) $^{ba}$ | 82 (64.91) $^{bdc}$ | 86 (68.06) $^{d}$ |
| 13 | TG X 444 – 422 | Black | 98 (81.87) $^{bac}$ | 99 (85.37) $^{ba}$ | 88 (69.73) $^{ba}$ | 92 (73.57) $^{bac}$ |
| 14 | P – 761 | Black | 97 (80.12) $^{bdac}$ | 97 (80.12) $^{bc}$ | 80 (63.43) $^{dec}$ | 84 (66.42) $^{ed}$ |
| | Mean of good storers | | 97.29 (81.21) | 98.5 (84.19) | 84.00 (66.57) | 89.85 (71.77) |
| **Poor storers** | | | | | | |
| 15 | P – 218 | Yellow | 96 (78.46) $^{bdac}$ | 96 (78.46) $^{bc}$ | 70 (56.79) $^{fhg}$ | 74 (59.35) $^{gf}$ |
| 16 | P – 222 | Yellow | 97 (80.12) $^{bdac}$ | 97 (80.12) $^{bc}$ | 68 (55.55) $^{ihg}$ | 74 (59.35) $^{gf}$ |
| 17 | P – 241 | Yellow | 96 (78.46) $^{bdac}$ | 99 (85.37) $^{ba}$ | 74 (59.35) $^{feg}$ | 78 (62.04) $^{ef}$ |
| 18 | P – 250 | Yellow | 98 (81.87) $^{bac}$ | 98 (81.87) $^{bac}$ | 64 (53.15) $^{ijhk}$ | 70 (56.79) $^{gifh}$ |
| 19 | P – 732 | Yellow | 97 (80.12) $^{bdac}$ | 99 (85.37) $^{ba}$ | 62 (51.94) $^{lijhk}$ | 66 (54.33) $^{gijh}$ |
| 20 | P – 871 | Yellow | 97 (80.12) $^{bdac}$ | 97 (80.12) $^{bc}$ | 60 (50.78) $^{lijk}$ | 64 (53.15) $^{ijh}$ |
| 21 | P – 876 | Yellow | 98 (81.87) $^{bac}$ | 98 (81.87) $^{bac}$ | 66 (54.33) $^{ijhg}$ | 72 (58.09) $^{gfh}$ |
| 22 | P – 884 | Yellow | 96 (78.46) $^{bdac}$ | 97 (80.12) $^{bc}$ | 60 (50.78) $^{lijk}$ | 74 (59.35) $^{gf}$ |
| 23 | P – 898 | Yellow | 97 (80.12) $^{bdac}$ | 97 (80.12) $^{bc}$ | 70 (56.79) $^{fhg}$ | 74 (59.35) $^{gf}$ |
| 24 | PK – 262 | Yellow | 93 (74.68) $^{d}$ | 94 (75.95) $^{bc}$ | 48 (43.85) $^{m}$ | 62 (51.94) $^{ij}$ |
| 25 | PK – 416 | Yellow | 94 (75.95) $^{dc}$ | 95 (77.12) $^{bc}$ | 58 (49.42) $^{lik}$ | 64 (53.15) $^{ijh}$ |
| 26 | PK – 472 | Yellow | 94 (75.82) $^{dc}$ | 94 (75.82) $^{bc}$ | 54 (47.30) $^{lm}$ | 60 (50.78) $^{j}$ |
| 27 | TAMS – 38 | Yellow | 93 (74.68) $^{d}$ | 94 (75.82) $^{bc}$ | 64 (53.15) $^{ijhk}$ | 72 (58.09) $^{gfh}$ |
| 28 | EC – 13969 | Yellow | 93 (74.89) $^{dc}$ | 93 (74.89) $^{c}$ | 74 (59.35) $^{feg}$ | 78 (62.04) $^{ef}$ |
| 29 | EC – 1023 | Yellow | 98 (81.87) $^{bac}$ | 98 (81.87) $^{bac}$ | 74 (59.35) $^{feg}$ | 78 (62.04) $^{ef}$ |
| 30 | EC – 34141 | Yellow | 93 (74.89) $^{dc}$ | 93 (74.89) $^{c}$ | 56 (48.45) $^{lmk}$ | 66 (54.33) $^{gijh}$ |
| 31 | EC – 93751 | Yellow | 96 (78.46) $^{bdac}$ | 96 (78.46) $^{bc}$ | 74 (59.35) $^{feg}$ | 78 (62.04) $^{ef}$ |
| 32 | EC – 18761 | Yellow | 96 (78.71) $^{bdac}$ | 96 (78.71) $^{bc}$ | 60 (50.78) $^{lijk}$ | 70 (56.79) $^{gifh}$ |
| 33 | MACS – 681 | Yellow | 95 (77.12) $^{bdc}$ | 97 (80.12) $^{bc}$ | 78 (62.04) $^{fde}$ | 78 (62.04) $^{ef}$ |
| | Mean of poor storers | | 95.63 (78.24) | 96.21 (79.32) | 64.95 (53.82) | 71.15 (57.63) |
| | Total mean | | 96.33 (79.50) | 97.18 (81.38) | 73.03 (59.23) | 79.09 (63.63) |
| | MSD at 5% | | 7.096 | 9.833 | 5.420 | 5.439 |
| | SE (d) | | 1.207 | 1.427 | 1.055 | 1.057 |

Note: Values in the parenthesis are arc sin transformed. Similar alphabets indicate non-significant differences between the values, following Tukey's Studentized Range (HSD) Test.

germinated seeds were evaluated into normal seedlings, abnormal seedlings, hard seeds and dead seeds, and were counted on 8th day. Germination percentage was recorded on the basis of normal seedlings only.

### Seed storability

About 150 grams of untreated fresh soybean seeds were packed in cloth bags and stored under ambient conditions of laboratory with an average relative humidity of 65±5 per cent and temperature of 25±2 °C. Samples were drawn after 12 months for the conduct of germination and other tests.

### Peroxidase test for grouping of varieties

Two replications of 10 seeds from each genotype were decoated and the seed coats were incubated in 10 drops of 0.5 per cent guaiacol at 25 °C. After 10 min one drop of 0.1 per cent hydrogen peroxide was mixed. The colour development (reddish brown) was recorded after 30 seconds, one minute and two minutes, as peroxidase positive (high peroxidase activity) as against peroxidase negative (low peroxidase activity) indicated by a colourless solution in the test tube [18, 19 and 23].

### Peroxidase (POX) activity

Peroxidase (POX, EC 1.11.1.7) activity was assayed in the decoated cotyledons, as increase in optical density due to oxidation of guaiacol to tetra-guaiacol at 470 nm absorbance using a reaction mixture containing 12 mM hydrogen peroxide and 96 mM guaiacol in phosphate buffer pH 7.0 [24].

Absorbance due to the formation of tetra-guaiacol was recorded at 470 nm and enzyme activity was calculated as per the extinction coefficient of its oxidation product, tetra –guaiacol E = 26.6 nM/cm. Enzyme activity was expressed as µmoles/cm/min/gram fresh weight.

### Statistical analysis

The data was analyzed by using SAS software package version 9.2 for calculation of minimum significant difference (MSD). All the replicated data was subjected to Tukey's Studentized Range (HSD) Test.

## RESULTS AND DISCUSSION

### Germination

Germination of freshly harvested seeds was comparable among the genotypes including good and poor storers. The initial germination percentage and the first count of all the varieties were above 90 per cent (Table 1). However after 12 months of ambient storage (25±2 °C and 65±5% RH), significant reduction in germination was observed among the genotypes, which was more pronounced in the poor storers (Table 1). In case of good storer genotypes, the mean germination and the first count were 89.85 and 84 per cent and in poor storers these were 71.15 and 64.95 per cent, respectively. It was evident that, there is genotypic variability with respect to storability among different soybean varieties and black seeded varieties in general showed better storability. This is in general confirmation of observations made by previous researchers' viz., [25-27]

### Peroxidase spot test

Though the spot peroxidase test for soybean genotypes is recommended to be conducted on the seed coats, recording the colour reaction after one minute, the test was modified further by recording the colour reaction after 30 seconds, one and two minutes after adding the $H_2O_2$. This was done primarily to ascertain the enzyme activity in fresh and old seeds (by extending the reaction time) and also to differentiate the peroxidase activity in the genetically different tissues, *i.e*., seed coat (maternal) and the cotyledon (zygotic). The 'spot test' was further extended with respect to (a) tissue specificity, (b) reaction time and (c) age of the seed.

In case of fresh seeds, out of 33 genotypes, peroxidase test was positive in 21 and negative in 12 genotypes up to 30 seconds. However, at one minute interval, as per the recommended procedure, peroxidase test was positive in 24 and negative only in 9 genotypes, which remained unchanged up to two minutes of incubation (Table 2). Six out of 14 (~43 per cent) good storer genotypes recorded negative peroxidase reaction, whereas among the poor storer genotypes only three out of 19 (~16 per cent) showed negative reaction.

In case of stored seeds, out of 33 genotypes, peroxidase test was positive in 17 and negative in 16 genotypes at 30 seconds of incubation. At one minute interval, 22 genotypes were positive and 11 genotypes were negative to the test and at two minutes interval, the test was positive in 26 and negative only in seven genotypes (Table 2). Thus, in genotypes DS-MM-64, M-11913 and P-898, the colour reaction changed from negative to positive only after two minutes of incubation in the old seed lots, as against one minute in the fresh ones. On the other hand, in P-218 while the fresh seeds exhibited negative

**Table 2.** Peroxidase spot test in fresh and stored seed lots of 33 soybean genotypes

| S. No. | Genotypes | Fresh seeds | | | Stored seeds | | |
|---|---|---|---|---|---|---|---|
| | | 30 sec | 1 min | 2 min | 30 sec | 1 min | 2 min |
| 1 | AMSS – 34 | - | - | - | + | + | + |
| 2 | DS – 74 | - | - | - | + | + | + |
| 3 | DS – MM – 64 | - | + | + | - | - | + |
| 4 | G – 2253 | - | - | - | + | + | + |
| 5 | G – 2265 | - | - | - | - | + | + |
| 6 | G – 2601 | + | + | + | + | + | + |
| 7 | G – 2603 | + | + | + | + | + | + |
| 8 | G – 2614 | - | - | - | - | + | + |
| 9 | G – 2651 | + | + | + | - | - | - |
| 10 | M – 253 | + | + | + | - | - | - |
| 11 | M – 1090 | + | + | + | - | - | - |
| 12 | M – 11913 | + | + | + | - | - | + |
| 13 | TGX 444 – 422 | - | - | - | - | - | - |
| 14 | P – 761 | + | + | + | + | + | + |
| 15 | P – 218 | - | - | - | - | - | + |
| 16 | P – 222 | + | + | + | + | + | + |
| 17 | P – 241 | + | + | + | + | + | + |
| 18 | P – 250 | - | - | - | - | - | - |
| 19 | P – 732 | - | - | - | - | - | - |
| 20 | P – 871 | + | + | + | + | + | + |
| 21 | P – 876 | + | + | + | + | + | + |
| 22 | P – 884 | + | + | + | - | - | - |
| 23 | P – 898 | - | + | + | - | - | + |
| 24 | PK – 262 | + | + | + | + | + | + |
| 25 | PK – 416 | + | + | + | + | + | + |
| 26 | PK – 472 | + | + | + | + | + | + |
| 27 | TAMS – 38 | + | + | + | + | + | + |
| 28 | EC – 13969 | + | + | + | + | + | + |
| 29 | EC – 1023 | + | + | + | - | + | + |
| 30 | EC – 34141 | - | + | + | + | + | + |
| 31 | EC – 93751 | + | + | + | - | + | + |
| 32 | EC – 18761 | + | + | + | + | + | + |
| 33 | MACS – 681 | + | + | + | - | + | + |

Note: **-** denotes negative peroxidase colour reaction, + denotes positive peroxidase colour reaction

colour response even up to two minutes, the old seeds showed positive reaction after two minutes. Also after one minute of incubation, while fresh seeds of G-2614 showed a negative reaction, the stored seeds recorded a positive response. In case of fresh seeds, there were three mismatches after one and two minutes of incubation, while in stored seeds there were five mismatches after one minute of incubation, whereas after two minutes of incubation, the mismatch increased to nine out of 33 genotypes.

The peroxidase colour reaction is a simple test which could be used to supplement seed characteristics in the grouping and identification of varieties [18, 19 and 23]. From this study, it was evident that some genotypes produce virtually no colour reaction even with prolonged reaction times, while on the other hand, a few of those classified in the low activity group will produce some colour if the spot test is allowed to stand. It is possible that some of this colour development may be due to peroxidase in scrapings of the cotyledons which may occasionally be removed with the seed coat. This is in confirmation of observations made by previous researchers' *viz*., [11].

**Peroxidase activity**

The activity of peroxidase enzyme was determined in fresh and stored (12 months) seed lots of 33 soybean genotypes. The results revealed that (a) there is

significant genotypic variation with respect to peroxidase activity, irrespective of their storability behaviour, ranging from 0.7713 to 11.87 µmoles/cm/min/g seed, (b) the mean activity level was higher in poor storer genotypes (5.426 µmoles/cm/min/g seed) than in good storer genotypes (3.436 µmoles/cm/min/g seed) and (c) there was drastic reduction in the enzyme activity during storage, irrespective of the initial level and storability behaviour of a genotype (Table 3).

**Table 3.** Peroxidase activity (µmoles/cm/min/g seed) among 33 soybean genotypes

| S. No. | Genotypes | Fresh seeds | Stored seeds |
|---|---|---|---|
| **Good storers** | | | |
| 1 | AMSS – 34 | 2.154 $^{sr}$ | 0.0406 $^{c}$ |
| 2 | DS – 74 | 3.054 $^{nmo}$ | 0.0315 $^{c}$ |
| 3 | DS – MM – 64 | 2.719 $^{qpo}$ | 0.0202 $^{c}$ |
| 4 | G – 2253 | 3.339 $^{nml}$ | 0.0180 $^{c}$ |
| 5 | G – 2265 | 2.192 $^{qsr}$ | 0.0620 $^{c}$ |
| 6 | G – 2601 | 2.388 $^{qpr}$ | 0.0406 $^{c}$ |
| 7 | G – 2603 | 0.9652 $^{t}$ | 0.0383 $^{c}$ |
| 8 | G – 2614 | 1.811 $^{s}$ | 0.0315 $^{c}$ |
| 9 | G – 2651 | 5.224 $^{gf}$ | 0.0451 $^{c}$ |
| 10 | M – 253 | 0.8457 $^{t}$ | 0.0552 $^{c}$ |
| 11 | M – 1090 | 7.574 $^{d}$ | 0.0406 $^{c}$ |
| 12 | M – 11913 | 6.583 $^{e}$ | 0.0512 $^{c}$ |
| 13 | TG X 444 – 422 | 3.739 $^{kjl}$ | 0.0383 $^{c}$ |
| 14 | P – 761 | 5.525 $^{f}$ | 0.0305 $^{c}$ |
| | Mean of good storers | 3.436 | 0.0388 |
| **Poor storers** | | | |
| 15 | P – 218 | 11.87 $^{a}$ | 0.0958 $^{c}$ |
| 16 | P – 222 | 6.563 $^{e}$ | 2.249 $^{a}$ |
| 17 | P – 241 | 4.818 $^{gh}$ | 0.1330 $^{c}$ |
| 18 | P – 250 | 4.655 $^{ih}$ | 0.0473 $^{c}$ |
| 19 | P – 732 | 3.357 $^{ml}$ | 0.0654 $^{c}$ |
| 20 | P – 871 | 9.716 $^{b}$ | 0.1691 $^{cb}$ |
| 21 | P – 876 | 5.477 $^{f}$ | 0.3585 $^{b}$ |
| 22 | P – 884 | 7.222 $^{d}$ | 0.0180 $^{c}$ |
| 23 | P – 898 | 4.235 $^{ij}$ | 0.0372 $^{c}$ |
| 24 | PK – 262 | 8.887 $^{c}$ | 0.0270 $^{c}$ |
| 25 | PK – 416 | 10.09 $^{b}$ | 0.0146 $^{c}$ |
| 26 | PK – 472 | 3.958 $^{kj}$ | 0.0315 $^{c}$ |
| 27 | TAMS – 38 | 7.327 $^{d}$ | 0.0484 $^{c}$ |
| 28 | EC – 13969 | 3.705 $^{kjl}$ | 0.0473 $^{c}$ |
| 29 | EC – 1023 | 1.052 $^{t}$ | 0.0552 $^{c}$ |
| 30 | EC – 34141 | 3.644 $^{kl}$ | 0.0744 $^{c}$ |
| 31 | EC – 93751 | 0.7713 $^{t}$ | 0.0789 $^{c}$ |
| 32 | EC – 18761 | 2.804 $^{npo}$ | 0.0338 $^{c}$ |
| 33 | MACS – 681 | 2.944 $^{nmo}$ | 0.0349 $^{c}$ |
| | Mean of poor storers | 5.426 | 0.1904 |
| | Total Mean | 4.582 | 0.1262 |
| | MSD at 5% | 0.5359 | 0.2022 |
| | SE (d) | 0.3301 | 0.2037 |

Note: Similar alphabets indicate non-significant differences between the values, following Tukey's Studentized Range (HSD) Test.

Peroxidases are haemoproteins that catalyze the reduction of $H_2O_2$ to water and molecular oxygen. Unlike other antioxidants, distribution of this enzyme in the seed coat and cotyledons was variable among cultivars. There was no clear cut difference with respect to peroxidase reaction among the good and poorer storer genotypes. The inheritance of peroxidase activity in the seed coat is controlled by a single dominant gene *Ep* [12], but its presence and activity in other seed parts are not well understood. In a previous study, no clear cut relationship could be established between peroxidase activity and seed longevity pattern in soybean varieties [27]. However, in the present study a moderate level of association was seen between the peroxidase reaction, level of peroxidase activity and longevity behaviour of soybean genotypes.

Although a number of different functions have been attributed to peroxidases, its major role is far from clear. It was evident from this study that while its activity, in general, is higher in poor storer varieties, it cannot clearly differentiate a good storer from the poor and irrespective of the initial peroxidase activity, it declines drastically during storage. The peroxidase spot test can be used for grouping soybean genotypes into positive and negative reaction groups, but it does not indicate their storability behaviour. However, a genotype with better storability was more likely to indicate a negative colour reaction. A change in colour reaction was also seen with the age of the seed, highlighting the need to use only the fresh seeds for this purpose.

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