DEDICATION

Affectionately dedicated to
my beloved parents,
brother and sisters who
have strived to make
me What
I am.

.... Nitin

STUDIES ON PEROXIDASES IN PEARL MILLET SEEDS

by

CHAVAN NITIN HARIDAS
(Reg. No. 98112)

A Thesis submitted to the

**MAHATMA PHULE KRISHI VIDYAPEETH,
RAHURI-413722, DIST. AHMEDNAGAR,
MAHARASHTRA, INDIA.**

in Partial Fulfilment of the Requirements for the Degree

of

MASTER OF SCIENCE (AGRICULTURE)

in

BIOCHEMISTRY

**DEPARTMENT OF BIOCHEMISTRY
POST GRADUATE INSTITUTE
MAHATMA PHULE KRISHI VIDYAPEETH
RAHURI - 413 722, DIST. AHMEDNAGAR (M.S.) INDIA**

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2000

CANDIDATE'S DECLARATION

I hereby declare that this thesis or part thereof

has not been submitted by me or any other

person to any other University or

Institute for a Degree

or Diploma

Place : MPKV, Rahuri **(Chavan N.H)**

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This is to certify that the thesis entitled **" Studies on peroxidases in pearl millet seeds",** submitted to the Faculty of Agriculture, Mahatma Phule Krishi Vidyapeeth, Rahuri, Dist. Ahmednagar, Maharashtra, India, in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE (AGRICULTURE)** in **BIOCHEMISTRY**, embodies the results of a piece of *bona fide* research work carried out by **Shri. CHAVAN NITIN HARIDAS**, under my guidance and supervision and that no part of the thesis has been submitted for any other Degree or Diploma.

The assistance and the help rendered during the course of this investigation have been duly acknowledged.

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Dated : / /2000

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Place : M.P.K.V. Rahuri

Date : 19 /08 /2000

(Chavan N.H.)

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ABSTRACT

STUDIES ON PROXIDASES IN PEARL MILLET SEEDS

By

Mr. CHAVAN NITIN HARIDAS

A candidate for the degree
of
MASTER OF SCIENCE (AGRICULTURE)
in
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MAHATMA PHULE KRISHI VIDYAEETH, RAHURI

2000

Research Guide	**:**	***Dr. J. K. Chavan***
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The present investigations were undertaken to study the total peroxidase activity in dry seeds of promising pearl millet cultivars, two CMS lines and some market samples differing in the seed physical properties; to standardize the conditions for partial purification of pearl millet seed peroxidases by gel filtration technique and to study isoenzyme patterns of peroxidases in seeds of these pearl millet cultivars using polyacrylamide gel electrophoresis (PAGE).

Studies on different genetically pure cultivars showed a wide variation in total seed peroxidase activity (72.8 to 137.0 units/g/min). The cultivars, RHRBH-9907 and RHRBH-9911 with lowest peroxidase activity were identified as desirable types in relation to shelf-life of the meal. In

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CMS systems, the peroxidase activity in dry seeds of hybrid was found to be similar or close to the female parent indicating its dominance. In market samples, the seed peroxidase activity was found to be lower for globular seed types as compared to obvate or lanceolate types. The activity however did not show any definite trend for seed colour. The seed peroxidase was purified by about 10-fold using a Sephacryl S-100 gel filtration column. The isoenzyme patterns for various cultivars on PAGE showed that the isoforms with Rm values 0.59 and 0.65 are common in all types with one to three additional isoforms of slow moving type. These differences in peroxidase isoforms can be used as a marker to determine the genetic purity of pearl millet cultivars. However, this needs to be confirmed by analyzing a larger number of pearl millet cultivars.

INTRODUCTION

1. INTRODUCTION

Pearl millet [(*Pennisetum glaucum* (L) R.Br.] locally called bajra is one of the important cereal crops grown for human consumption in Asia and Africa, while it is grown mainly as fodder crop in USA and Europe. It exhibits a superior performance to other cereals, particularly under moisture stress and low soil fertility conditions. It has phenomenal capacity to respond to improved agricultural inputs. Hence, pearl millet is an important cereal in the areas of rainfed agriculture.

The world production of pearl millet was 15 MMT in the year 1995-96 (FAO, 1996). Most of the world's pearl millet production is contributed by Asia and Africa. India contributes about 35 per cent of the world's pearl millet production. In India, it is mainly grown in the states of Maharashtra, Gujrat, Rajasthan, Haryana, Uttar Pradesh, Andhra Pradesh, Karnataka and Tamil Nadu on an area of 10 million hectares with a production of 6.57 million tonnes. (Singhal, 1999). In Maharashtra, a production of about 7.6 lakh tonnes in 1980 was reported which increased to 11.19 lakh tonnes in 1997-98 (Anonymous, 1998). It indicates that the pearl millet is becoming popular among the cultivators and rural consumers.

Pearl millet is an economical and staple source of dietary nutrients such as proteins, calorie, minerals and certain B group vitamins to a large segment of population, particularly from lower socio-economic groups and farming community. (Salunkhe *et al.* 1985). The grain is mainly used for making *roties*, beverages and porridge. Recently, Malleshi and his associates from CFTRI, Mysore have developed extruded ready-to-eat weaning food and popped millet-based supplementary food using pearl millet (Malleshi,

1993; Hadimani, 1994). Thus, pearl millet also holds a promise for utilization on industrial scale by processing into value-added convenient foods.

The processing and utilization of pearl millet is however limited by its coarse nature, gray, brown to yellow pigments in the grains and a very short shelf-life of its meal. The intact grains can be stored for a longer time, but the meal becomes rancid and exhibits a strong mousy, acidic odour upon a short storage at ambient conditions. The generation of mousy, acidic odour decreases the acceptability of the meal and meal products. The odour generation was earlier attributed to the lipolytic or oxidative break down of germ lipids upon milling and suitable seed treatments to inactivate lipase were standardized. (Kadlag *et al.* 1995; Palande *et al.* 1996; Dalvi *et al.* 1997).

Reddy *et al.* (1986) have fractionated and isolated the odour forming compounds in ground, stored whole as well as defatted pearl millet meals. The compounds were found to be extractable in methanol and water-soluble. Since the defatted meal also exhibited a mousy odour in water-soluble fraction, the odour generating constituents were regarded as phenolics. This was further confirmed by scanning the water extract for various UV absorption spectra and by its analysis on HPLC with phenolic standards. The compounds separated and showing characteristic odour were identical with apigenin, a aglycone of C-glycosylflavones. The odour generation in the meal was found to be accelerated with addition of water. Hence, it was speculated to be an enzymatic degradation of phenolics.

The pearl millet phenolics have been isolated and characterized as C-glycosylflavones and alkali-labile ferulic acid (ALFA). Out of these, the

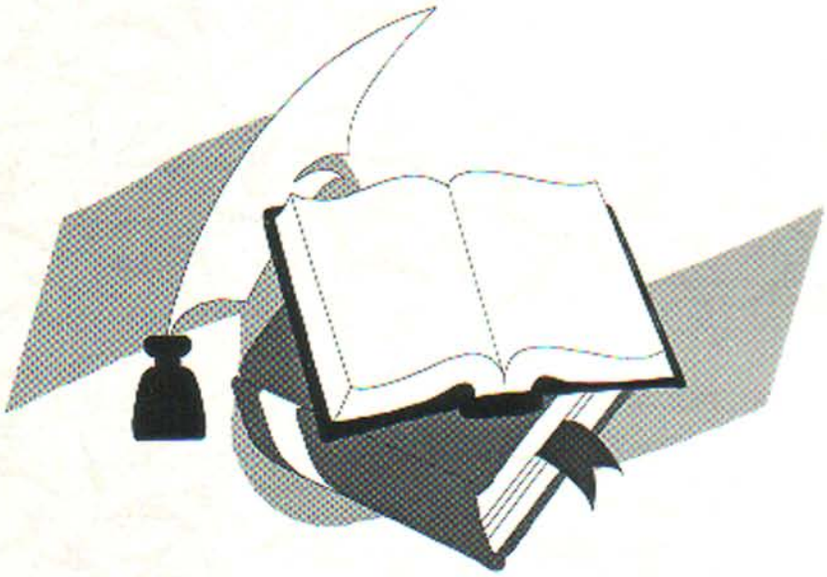
C-glycosylflavones are the major contributing to over 60 and 70 per cent of the total phenolics in the seed (Reichert *et al.* 1980). The C-glycosylflavones are made up of an aglycone apigenin attached with one or two glucose residues through a carbon-carbon covalent bond, resistant to hydrolytic clevage (Salunkhe *et al.* 1989). Hence it is speculated that during storage, the enzyme oxidize the hydroxyl groups on the aromatic ring and the oxidized product exhibits mousy odour.

Usually, two enzymes, namely polyphenol oxidase and peroxidase are known to oxidize plant phenolics. The polyphenol oxidase in wheat has been reported to oxidize meal phenolics and cause dough darkening (Abrol, 1970). A group of enzymes that oxidize a range of compounds including phenolics in the presence of hydrogen peroxide are collectively called peroxidases, are also present in dry seeds. Recently, Bangar *et al.* (1999) have shown that peroxidase activity mainly from the germ fraction of the seed is responsible for odour generation in pearl millet meal.

The peroxidases are ubiquitous in occurrence. The peroxidases from the seeds of soybean (Gillikin, 1991) maize (Koshiba, 1993), durum wheat (Iori *et al.* 1995), rice (Padiglia *et al.* 1995), barley and wheat germ (Billaud *et al.* 1999) have been isolated, purified and characterized. The results have shown a wide range of peroxidase isoforms present in crop plants differing in activity, heat stability, pH optima, hydrogen donor specificity and substrate specificity. However, the information on isolation, purification and characterization of pearl millet seed peroxidases is lacking. Hence, there is a need to purify the pearl millet seed peroxidases and study their isoenzyme patterns. Such information will be useful to identify or develop a

low peroxidase plant types with improved shelf life of its meal. The present investigations were therefore undertaken with following objectives :

1. To study the total peroxidase activity in dry seeds of promising pearl millet hybrids, CMS lines and market samples differing in the seed physical properties.
2. To standardize the conditions for purification of pearl millet seed peroxidases.
3. To study isoenzyme pattern of peroxidases in the seeds of pearl millet cultivars including genetically improved cultivars and some CMS lines and few market samples.



REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

Peroxidase (EC 1.11.1.7), member of a large group of enzymes called oxidoreductases, is considered to have an empirical relationship to off-flavours and off-colours in raw and unblanched vegetables (Weaver and Hautala, 1970). Peroxidase is widely distributed and has been isolated from many higher plants. The wide distribution of the enzyme suggests that it could be of great biological importance. However role that it plays in metabolism is not clear due to a large number of reactions it catalyzes and the considerable number of isozymic species. Much of the difficulty in understanding peroxidase is due to the presence of multiple isoenzymes in various products.

The peroxidase is found primarily in the roots and sprouts of higher plants (Tauber, 1949). Reed (1975) stated that unlike many of the digestive enzymes, peroxidase is usually intracellular, as are the other oxidoreductases like polyphenol oxidase, lipoxygenase, ascorbic acid oxidase, glucose oxidase and many others. The work of Scandalios (1969) showed that the enzyme is tissue or organ specific. The most documented sources of peroxidase in plants are horseradish roots and the sap of the fig tree (Sumner and Somers, 1947; Tauber, 1949).

2.1 Classes of Peroxidase

Reed (1975) reported that there are basically three classes of peroxidases : ferriprotoporphyrin peroxidases; verdoperoxidases, and flavoprotein peroxidases.

2.1.1 Ferriprotoporphyrin peroxidases

The ferriprotoporphyrin (hematin) peroxidase is brown in nature and includes the peroxidases from higher plants (horseradish root, turnip root, radish root), animals (tryptophan pyrrolase, thyroid iodine peroxidase), and microorganisms (yeast cyctochrome C peroxidase). The prosthetic group for the ferriprotoporphyrin peroxidases is ferriprotoporphyrin III.

2.1.2 Verdoperoxidases

The verdoperoxidase is green in nature and is considered important in foods. It is primarily found in milk as lactoperoxidase and in animals as reported by Karlson (1965). Reed (1975) reported that the prosthetic group contains an iron porphyrin group other than ferriprotoporphyrin III, which in the past was known as green hematin (Karlson, 1965).

2.1.3 Flavoprotein peroxidases

Flavoprotein peroxidase as described by Reed (1975) has been purified from several Streptococci, such as *Streptococcus faecalis*, and from several animal tissues. The prosthetic group is flavin adenine dinucleotide (FAD).

2.2 Mechanism of Peroxidase Action

The peroxidase reaction consists of two successive steps each involving 1 electron. A general equation for peroxidase catalysed reaction can not be formulated because the course of the reaction depends on the type of substrate. In the simplest case, the same molecule is hydrogen donor for both steps. The equation of overall reaction is usually :

$$H_2O_2 + DH_2 \xrightarrow{\text{Peroxidase}} 2\,H_2O + D$$

(Bergmayer, 1974).

2.3 Peroxidases in Crop Plants

Peroxidases occure in all higher plants including cereals, pulses, oilseed crops, fruits and vegetables.

2.3.1 Cereals

Kondo *et al.* (1982) studied buckwheat flour peroxidase. The enzyme lost 83 per cent of its activity after heating at 70^{O}C for 30 min but had 10 per cent greater activity after heating at 60 ^{O}C than at 40 ^{O}C. It's activity was not greately affected by NaCl upto 1 M. Extraction of the enzyme was doubled by adding tween-80 at 1 per cent to the water but no activity was found when 1 per cent dodecyl sulphate was used instead of shaking with 1 per cent tween-80.

Shighal *et al.* (1986) studied eight bread wheat varieties differing in grain weight and plant height for grain peroxidase activity at 5 stages from 10 days after anthesis to mature grain. The correlation between peroxidase activity and 100-grain dry weight was significant and negative at 24 and 31 days after anthesis and at maturity (r = -0.929 to -0.964). Olesen's Dwarf (the shortest variety) showed the highest peroxidase activity at all stages of development, and the lowest 100-grain weight at maturity.

Lal and Gupta (1993) observed differential behaviour of pearl millet genotype for peroxidase activity under different fertility levels and temperatures at different developmental stages. The peroxidase activity was low at initial stages and gradually rose during subsequent stages. The

highest peroxidase activity was observed at 30 ^{O}C at all the fertility levels. The peroxidase activity differences were more between fertility levels.

Poongodi (1996) assayed flag leaves of 11 male sterile lines of *Pennisetum glaucum*, collected during early flowering for peroxidase activity. From assay, it was noted that 861A was having the highest peroxidase activity (14.57 ΔA/mg protein/min). Male steriles viz., TRI 117A, TRI 125A, 732A, ICMA 87001, PNMS-2A and ICMA-3 had more or less similar peroxidase activity (6.64, 6.36, 6.20, 5.62, 6.04 and 5.61 ΔA/mg protein/min respectively). The activity was low in the lines ICMA-1, ICMA-2, ICMA-87002 and ICMA-4 (2.32, 3.69, 2.45 and 2.94 ΔA/mg protein/min respectively). It was suggested that the lines which has better expression of peroxidase may be used for hybridization to evolve disease resistant varieties of pearl millet.

Chavan and Hash (1998) analyzed several ICRISAT pearl millet cultivars of mapping populations for polyphenol oxidase and peroxidase activities in dry grains. A considerable genetic variations was observed among the cultivars studied. The cultivars with low peroxidase activity in relation to odour generation were identified. Bangar *et al.* (1999) evaluated 20 promising pearl millet hybrids for seed peroxidase activity and reported a significant genetic variations (90 to 158 units/g/min) among the cultivars studied. The low peroxidase activity in dry seeds was reported to be a desirable in relation to off odour development in the meal during storage.

Subhadra and Reddy (1998) estimated quantitatively the varying levels of peroxidase activity in anther derived calli of four *indica* rice cultivars viz., Tellahamsa, Rasi, Basmati-370, Pattanbi-33 and their 12 F_1 hybrids. It was observed that both high green plant regeneration and amount

of peroxidase present in these calli were directly proportional. Higher amounts of peroxidase activity were observed in high regenerating cultivars and hybrids. The lower amount of peroxidase activity, on other hand, was found to be the characteristic feature of low regenerating calli.

Several sorghum cultivars were screened for seed peroxidase activity (Anonymous, 2000), in relation to resistance to blackening due to mould infestation. A significant and negative correlation was observed for total seed peroxidase activity and degree of moulding and blackening. The cultivars with resistance to moulding were found to contain higher seed peroxidase activity.

2.3.2 Legumes

Buttery and Buzzel (1968) studied peroxidase activity in seeds of soybean (*Glycine max* (L.) Merril.). They seperated varieties of soyabean into two main groups on the basis of high or low peroxidase activity in the seed coat. Ahuja *et al.* (1980) demonstrated the presence of superoxide dismutase and peroxidase activities in the cotyledons, hypocotyl and epicotyl of germinating mung bean (*Phaseolus aureus*) seedlings. In whole seeds and in its various parts, both of these activities exhibited a rhythmic behaviour during early period of germination. The distribution of dismutase activity was highest in epicotyl and lowest in cotyledons. The results suggested the existence of a correlation between the two enzymes though their distribution in different tissues was not similar.

Kermasha and Metche (1988) extracted and partially purified seed peroxidases from *Phaseolus vulgaris* cv., haricot. The precipitation of an active peroxidase fraction with solid amonium sulfate (at 35-90% saturation)

increased its activity by a factor of 3. The pH for optimum activity was 5.4. The addition of 4 μM hydrogen peroxide increased the activity by 92 fold; however, enzyme activity was decreased by higher concentrations of this reagent. The carbonyl compounds resulting from peroxidase activity were isolated as their dinitro phenylhydrazones and then purified by preparative thin layer chromatography. Subsequent GC-MS analysis revealed that acetone was the principal carbonyl compound resulting from enzyme activity. Chakrabarty and Agrawal (1989) had developed seed keys to identify 16 black gram varieties on the basis of morphological and chemical characters namely seed size, colour, seed coat peroxidase activity, phenol colour reaction, sodium hydroxide and potassium hydroxide colour reaction.

2.3.3 Oilseed crops

Sarvesh and Reddy (1988) studied the activities of polyphenol oxidase [catechol oxidase] and acid phosphatase during leaf disc senescence. A considerable variation among the 4 varieties of castor was reproted. Alkaline inorganic pyrophosphatase was thought to be involved in triggering leaf senescence, and early maturing varieties showed low peroxidase and higher alkaline inorganic phosphatase activities compared to late-maturing varieties. Chlorophyll and protein contents decreased with leaf age. It is suggested that these characters could be used in the identification of genotypes.

Satakopan *et al.* (1990) studied the peroxidase activity in germinating groundnut seeds in 30 mM NaCl and/or $CaCl_2$ solution. The peroxidase activity in cotyledons was found to decrease with germination in water. The $CaCl_2$ showed the greatest inhibitory effect followed by NaCl + $CaCl_2$ and NaCl.

Singh and Abidi (1996) conducted a field experiment on selected cultivars of Indian mustard (*Brassica juncea* L. czern and coss) to study the activities of enzyme peroxidase and lipase and their influence on biochemical parameters. It was found that the activity of enzyme peroxidase was enhanced and lipase was declined. Total sugar content was increased in all the cultivars. A decreasing trend in protein content was found in all the varieties. Maximum oil content was shown by cultivar NDR-8501 followed by Varuna and Kranti.

Mehta *et al.* (1997) performed study on the peroxidase activity during embryogenesis in sesame (*Sesamum indicum* L.). The peroxidase activity was found to be about 2.5 fold higher in somatic embryo 35 days after floweirng (DAF) than zygotic embryo at 5 DAF. The expression of peroxidase activity during embryonal development occurred in three growth phases : Linear phase (5 to 15 DAF), decreasing reverse phase (15 to 25 DAF) and sprouting dessication phase (25 to 35 DAF).

2.3.4 Fruits and vegetables

Marangoni *et al.* (1989) developed a rapid method for the isolation of tomato anionic peroxidase (TAPR) using Fast Protein Liquid Chromatography. TAPR was purified 295 times to an RZ value of 0.7. The rate constant for the formatin of compound I was 1.53×10^{7} M^{-1} Sec^{-1} and for compound II was 6.93×10^{5} M^{-1} Sec^{-1}. TAPR had maximum activity at pH 5.0-5.2 and an isoelectric point at pH 3.5. A SDS gel electrophoresis showed three major bands at 16.6, 40.0, and 42.0 KD. TAPR demonstrated a calcium dependancy for activity, exhibiting a maximum at a concentration of 1.0×10^{-5} M.

Narayanankutty and Gopalakrishnan (1990) have reported a relationship between the activities of catalase and peroxidase and yield of the coconut crop. Low, medium and high-yielding coconut palms cv. West Coast Tall were selected and the activities of the 2 enzymes were estimated in fresh leaf samples. No overall relationship between yield and activities of these enzymes was detected.

Pilar *et al.* (1995) studied changes in peroxidase and polyphenol oxidase activities of papaya (*Carica papaya*) cv. sunrise, during ripening, freezing and short frozen storage. The fruits were stored at 14^{O}C and 85-90% RH until maturity was reached (about 21 days). The fruits were frozen cryogenically and frozen slices were stored at -18^{O}C. The peroxidase activity increased in pulp tissue upto the ripe stage, showing a maximum value after 7 days of cold storage. Similarly, polyphenol oxidase activity showed an important increase (4 times of initial value) on the same date. Quantity of extractable proteins was at a maximum after 15 days of storage at 14^{O}C. Freezing and frozen storage (-18^{O}C) produced an increase in peroxidase activity while polyphenol oxidase activity was only slightly affected.

Dighe *et al.* (1997) analysed mature fruits of 57 promising brinjal cultivars differening in their morphological characteristics and revealed a wide variation in the contents of various nutritional constituents, total phenolics and activities of polyphenol oxidase and peroxidase. The fruit shape, colour and spinyness were found to be related with the nutritional composition. The ablong shape, green colour and spinelessness in fruits seem to be desirable morphological traits that would produce nutritious

brinjal. The correlation coefficients between different morhological and nutritional constituents were worked out.

2.3.5 Other crops

Kochhar *et al.* (1982) studied the activity and isoenzyme pattern of peroxidase, the enzyme involved in the maintanance of dynamic level of auxin, in the male fertile (mf) and male sterile (ms) lines of African marigold. The peroxidase activity in the stem of both (mf and ms) was three times as high as that of flowers and lower both in the stem and flower of ms than that of mf. This variation in activity was found associated with its molecular forms, thereby suggesting their role in sex determination.

Parthasarathi *et al.* (1986) demonstrated the activity of a specific peroxidase isoenzyme in the living bark tissue, to bear a strong negative correlation with the oil content in the heartwood in mature sandal (*Santalum album* L.) plants. The utility of this relation in forecasting, even at an young age, the oil bearing capacity of a sandal plant has been indicated.

2.4 Purification of Peroxidases

2.4.1 Cereals

Ascorbate peroxidase (APX) was purified to homogeneity from maize (*Zea mays* L. cv.) coleoptiles by Koshiba (1993). APX was a monomer with a molecular mass of 28 KD , as determined by gel filtration and SDS-PAGE. It contained one protoheme moiety per molecule, with the oxidized form giving a soret peak at 403 nm with small peaks at 502 and 638 nm, and the reduced from giving peaks at 435 and 556 nm. The enzyme was not inactivated by depletion of ascorbate. A cell fractionation and

immunohistochemical studies using polyclonal antibodies raised against maize APX revealed that the enzyme was not located in the chloroplasts of green leaves. It was abundant in cytoplasm but not in the vacuoles of cells in the coleoptile, mesocotyl and young leaves of seedlings. In mature green leaves, small amounts of the enzyme were distributed in vascular systems, particular in the companion cells. The N- terminal amino acid sequence of maize APX exhibited high homology to pea cytosolic APX, spinach APX and Arabidopsis APX, but not to APX from tea chloroplasts.

Iori *et al.* (1995) purified two cathodic peroxidases (C1, C2) from durum wheat (*Triticum durum*) flour by ion exchange chromatography. Both the peroxidases appear to be homogeneous when analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and isoelectric focusing. The isolated isoenzymes, both with a purity index (A 402 nm / A 280 nm) of 3.7, were polypetides of 39.7 KD (C1) and 38.6 KD (C2), with $P^I > 9.3$. The calcium ion strongly activated the C1 and C2 isoenzymes, which increased their specific activity by 92 and 690 times, respectively. The enzyme activity was considered of great importance both in the production technology of high quality pasta and more generally in other bakery products.

In *Oryza sativa* plant two isoperoxidases called perox I and perox II have been reported. Padiglia *et al.* (1995) purified only perox I to homogeneity and its enzymatic, physical and chemical properites have been studied.

Billaud *et al.* (1999) separated, partially purified peroxidases of crude extracts from barley and wheat germ using salt fractionation, ion-exchange and hydrophobic interaction chromatographies and their properties

were examined. Barley and wheat germ peroxidase contained basic, neutral and anionic isoforms as confirmed by isoelectric focussing. Toyopearl Butyl 650 M chromatography resolved peroxidase into four cationic fractions. The chromatography of wheat germ extract on CM-Sepharose isolated an anionic and a neutral fraction.Following chromatography on Concanavalin-A Sepharose, enzymes in both cereals showed differences in their elution properties. The optimum P^H ranges were 4.0 to 5.5 (barley) and 5.3 to > 6.3 (wheat germ) and peroxidase reacted differently under acidic or basic conditions. Their catalytic behavior in the presence of calcium also differed. Kinetics of peroxidase were of Michaelian type with a ping-pong mechanism and Michaelis constants of guaicol oxidation in the presence of hydrogen peroxide varied from one enzymatic group to another.

2.4.2 Legumes and oilseed crops

Halpin *et al.* (1989) purified peroxidase isoenzymes from green peas with ion-exchange chromatography on DEAE-Sepharase. Three isoenzymes were identified, one neutral (N) and two cationic (C1, C2). The N was extremely heat labile, with 50% original activity lost after heating for 1.5 min at 25^0 C. The N had Km values (pH 5.0) of 10.2 mM and 2.6 mM for guaicol and H_2O_2 respectively. The C1 and C2 retained activity on heating at 30-70^0 C. The C1 was able to remain reactivate after thermal inactivation. The Km values for guaicol and H_2O_2 were 10.8 mM, 7.2 mM (pH 5.0) and 10.8 mM, 4.3 mM (pH 6.0) for C1 and C2, respectively. The three isoenzymes exhibited different peroxidase activity with different H-donors, different sensitivities to cynide and different abilities to catalyze oxidation of indole acetic acid.

Gillikin and Graham (1991) showed that the majority of peroxidase activity in soybean (*Glycine max* var. William 82) seeds was localized in the seed coat. A single isoenzyme is responsible for this activity and has been purified to electrophoretic homogeneity by successive chromatography on DEAE-Sepharose Fast Flow, Concanavalin- A Sepharose, and Sephadex G-75. The peroxidase exhibited a pI of 4.1, an appearent molecular mass of 37 KD and had properties characteristic of a glycoprotein. The enzyme was found to began to accumulate approximately 21 days after anthesis and continued to do so through the maturation of the seed coat where it represented at least 5 per cent of soluble protein in dry seed coats. Due to its localization in the seed coat, it was implicated in the hardening of the seed coat.

2.4.3 Vegetables and fruits

Ramanuja *et al.* (1988) assayed peroxidase activity in 15 vegetables. The concentration of enzyme in vegetables was compared on wet and dry weight basis and found to vary widely among them. Studies on the partial purification of the enzyme from some vegetables showed that acetone precipitation gave better recovery and higher fold purification than ammonium sulfate. Rate of loss of activity of the enzyme with heating was studied using the acetone precipitate and the activity time curves obtained at different temperatures in the range of 60-100^{O}C. Among the vegetables studied, the enzyme from lobia and cluster beans was found to have higher heat resistance than others.

Sung *et al.* (1993) purified peroxidase isoenzyme from the extract of pineapple stem through successive steps of ammonium sulfate fractionation,

CM-Sephrose CL-6B chromatography and DEAE-Sepharose CL-6B chromatography. By these steps, twelve isoenzymes of peroxidase were obtained.

2.5 Electrophoretic Characterization - Isoenzyme Patterns

Kobrehel and Gautier (1974) studied variability in peroxidase isoenzymes in wheat and related species by polyacrylamide-slab gel electrophoresis and showed that the nature of peroxidases in wheat is a genetic character, different genomes control the synthesis of specific peroxidases. The growing conditions have no influence on isozyme patterns. Within a single species, the electrophoretic patterns of peroxidases may differ from one variety to another. Thus, two classes of *Triticum aestiuum* can be distinguished with *Triticum durum*, the dissimilarity of enzymograms is particularly important.

Nadualvari *et al.* (1984) studied significance of wheat peroxidase activity for dough quality, behaviour of wheat grain peroxidase isoenzymes (from whole grain of cv. Martonvasari) during grain ripening and storage, by polyacrylamide gel electrophoresis at pH 7.9. During grain ripening, only isoenzymes of peroxidase that had zero mobility or that migrated to the anode were detected, mobility of anodic isoenzymes decreased with ripening. At harvest and during storage, only cathodic and zero-mobility isoenzymes were detected.

Phul *et al.* (1987) characterized electrophoretically peroxidase and acid phosphatase isoenzymes from anthers of 5 cytoplasmically male-sterile (CMS) lines and their maintainers of *Sorghum bicolor* (L.) Moench.

Differences in presence/absence and intensity of bands were observed between all the CMS lines and their maintainers. In general, band intensity was greater in the CMS lines, suggesting that in these lines, the enzymes are active in breaking down metabolites otherwise important for the formation of fertile anthers.

Rao *et al.* (1989) studied isoenzyme pattern in embryogenic glume calli of maize. Glumes inoculated on MS medium supplemented with 2 mg 2,4-D/L formed callus within 2 weeks. Embryogenic calluses showed high activities of peroxidases and polyphenol oxidase. Two slow migrating bands of isoperoxidases and specific esterases were present in embryogenic calluses and absent in non-embryogenic cultures indicating a possible association of these enzymes with somatic embryogenesis in vitro.

Rao *et al.* (1992) analyzed nine isoenzyme systems (esterase, peroxidase, amylase, acid phosphatase, glutamicoxalotransaminase [aspartate aminotransferases] , leucine [cytosol] aminopeptidase, superoxide dismutase, malate dehydrogenase and alcohol dehydrogenase) using PAGE in 10 inbred lines of pearl millet (*Pennisetum glaucum*) at 10 developmental stages/tissues (from dry seeds to pollination). No differences in the isozyme pattern were observed amongst the genotypes, with the exception of esterase which gave 4 dry seed phenotypes. However, the isozyme patterns showed developmental variation and tissue specificity, and stage-specific zymograms were observed.

Bhandal (1995) studied isoenzymes of peroxidase in roots of 72 h-old seedlings of rye and several species of wheat (diploid *Triticum aegilopoides* and *T. orientale;* tetraploid *T.durum, T.dicocum, T.polonicum, T.turgidum* and *T.timopheevii* and hexaploid *T.sphaerococcum, T. spelta* and

T.vulgare [*T.aestivum*]) employing horizontal starch gel electrophoresis. There were interspecific differences and some of these could be correlated with specific genome and/or ploidy levels.

Shaista and Halim (1997) evaluated seven isoenzymes from 14 genotypes of pearl millet (*Pennisetum slaucm*), including hybrids, male sterile lines and restorers, by SDS-PAGE for quantitative and qualitative polymorphisms. The isoenzymes of esterase [carboxylesterase], glutamate oxaloacetate transaminase [aspartate aminotransferases] and peroxidase were genotype specific.

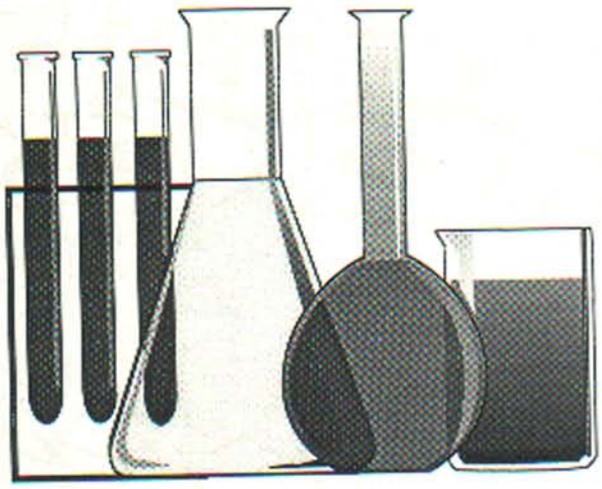
2.6 Peroxidases in Odour Development

Peroxidase appears to be one of the most heat-stable enzyme present in fruits and vegetable; therefore, it serves as a prime index of blanching products prior to canning or freezing. However, it can contribute to deteriorative changes in flavour, colour and nutrition both in raw foods such as fruits and vegetables and in processed products (Burnette, 1977). It is also well known that, under certain conditions of limited heat treatment, peroxidases can regain activity during storage, resulting in loss of flavour or development of off-flavour (Lu and Whitaker, 1974).

Aparicio-Cuesta *et al.* (1992) studied total, soluble and bound peroxidase activities in frozen green beans stored under proper conditions (-18^{O}C, -22^{O}C and in display freezer). The blanching of beans inactivated the enzyme, there was no regeneration for 12 months at -22^{O}C, but slight regeneration at -18^{O}C. The sensory quality of the properly stored product was acceptable upto 1 year or longer. The storage in display freezer over 60 days did not affect peroxidase activity. Temperature fluctuations were

deleterious for sensory quality and total peroxidase activity was only slightly regenerated.

Bangar *et al.* (1999) concluded that water-soluble phenolics and peroxidase activity concentrated mainly in germ fraction of the grain appeared to be responsible for odour generation in stored pearl millet meal. The polyphenol oxidase distributed uniformly in the endosperm and germ was found to be relatively heat stable and not involved in odour generation. The heat-sensitive peroxidase activity responsible for odour formation was effectively inactivated by hot water blanching of seed at 98^{O}C for 40 sec or by steam heating of seeds at 15 psi for 5 min. The information on the purification of seed peroxidase and isoenzyme patterns in pearl millet seeds is however lacking.



MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1 Material

3.1.1 Pearl millet seeds

Following pearl millet seeds of 12 different genetically pure cultivars and that of 2 CMS lines grown in kharif season of 1998 were obtained from the Senior Millet Breeder, Mahatma Phule Krishi Vidyapeeth, Rahuri.

List of cultivars

A) Gentically pure cultivars

Sr. No.	Cultivar	Sr. No.	Cultivar
1.	RHRBH-9901	7.	RHRBH-9907
2.	RHRBH-9902	8.	RHRBH-9908
3.	RHRBH-9903	9.	RHRBH-9909
4.	RHRBH-9904	10.	RHRBH-9910
5.	RHRBH-9905	11.	RHRBH-9911
6.	RHRBH-9906	12.	RHRBH-9912

B) CMS lines

I. Shradha

Sr. No.	CMS line
1.	RHRBH - 8609 (Shradha Hybrid)
2.	RHRBI - 138 (Male)
3.	RHRB - 1A (Female)

II. Saburi

Sr. No.	CMS line
1.	RHRBH - 8924 (Saburi Hybrid)
2.	RHRBI - 458 (Male)
3.	RHRB - 5A (Female)

In addition to this, 12 market samples differing in seed colour, size and shape were collected from local market of Rahuri. The seeds were cleaned and stored at 4^0C until use.

3.1.2 Chemicals

Most of the chemicals used in this study were of analytical grade. They were obtained from Sarabhai Chemicals, Baroda; M/S Glaxo Laboratories, Mumbai; Sisco Research Laboratories, Mumbai and from Pharmacia Fine Chemicals, Swedan.

3.2 Methods

3.2.1 Peroxidase assay in dry seeds

Method for preparation of enzyme extract given by Kumar and Khan (1982) was used. One gram of dry seeds were homogenized with 8 ml of 0.1 M chilled phosphate buffer, pH 7.0 in a prechilled mortar and pestle and the homogenate was centrifuged at 15,000 x g at 4^0C for 30 min. The supernatant was used as a source of enzyme.

Enzyme assay mixture contained 2.7 ml of 0.1 M phosphate buffer (pH 7.0), 1 ml of 0.01 M pyrogallol, 0.1 ml of 0.05 M H_2O_2 and 0.2 ml of enzyme extract. The assay was initiated by adding the crude enzyme extract

into other components already mixed and the change in extinction at 440 nm was recorded at 27^{O}C upto one min. One unit of enzyme activity was defined as change in extinction by 0.1 per min. The results were expressed as units/min/g of meal.

3.2.2 Purification of seed peroxidase

3.2.2.1 Extraction and preparation of enzyme

The pearl millet seeds (50g), of cultivar Shardha, were soaked in 0.1 M phosphate buffer over night at 4^{0}C and homogenized in chilled mortar and pestle with the chilled buffer (1 : 20 w/v). The slurry was centrifuged at 15000 x g for 30 min and the supernatant was collected. The supernatant was then dialized against water at 4^{0}C for 24 hrs with 4 changes of water and finally freeze dried.

The freeze dried enzyme (50 mg) was dissolved in 5 ml of same phosphate buffer, centrifuged and the supernatant was applied to a gel filtration column.

3.2.2.2 Gel filtration chromatography

A pharmacia column of 64 x 1.5 cm id (volume 115 ml) size was used. It was packed with pre-swollen Sephacryl S-100 gel as per the directions of manufacturer and washed by 4 column volumes with phosphate buffer to ensure uniform packing. The packed column was then connected to buffer tank through a flow adaptor at the top, while to a fraction collector through UV absorption and chart recorder unit, from the bottom.

A 5 ml of enzyme preparation was applied on the top of the column with an adaptor and the column eluted at a flow rate of 50 ml/hr. A fraction

of 2.5 ml were collected with fraction collector. The fractions were analyzed for soluble proteins (Lowry *et al.* 1953) and peroxidase activity (Kumar and Khan, 1982) to locate the fraction of peroxidase. The specific activity of the enzyme was defined as number of units/mg soluble protein. The appropriate fractions were pooled, dialyzed against water and freeze dried.

3.2.3. Isoenzyme pattern of peroxidases

Polyacrylamide gel electrophoresis (PAGE) system was used to study the isoenzyme pattern in pearl millet as per the procedure prescribed by Dadlani and Varier (1993) with some modifications.

3.2.3.1 Preparation of crude extract

The crude enzyme extract was prepared for all the samples as described in section 3.2.1 and directly used for detection of isoforms on a PAGE.

3.2.3.2 PAGE System

I. Apparatus

The vertical rod gel regular dual model electrophoresis apparatus (Bangalore Genei Pvt. Ltd.) with 70 x 5 mm id glass tubes was used for performing PAGE.

II. Stock solutions for separation gel

A) 1. Tris	:	18.3 g
2. 1 M HCl	:	24.0 ml
3. TEMED	:	0.15 ml

4. Distilled water to make volume 100 ml. The pH was adjusted to 8.9 with 1 N HCl. The solution was filtered and stored in brown bottle at 4^0C.

B) 1. Acrylamide : 28.0 g

2. Bisacrylamide : 0.74 g

3. Distilled water to make volume 100ml. The solution was filtered in brown bottle and stored at 4^0C.

C) Sucrose, 40 g dissolved in 100 ml distilled water. The solution was filtered and stored at 4^0C.

D) Ammonium persulfate, 0.14 g was dissolved is distilled water to make volume 100 ml. This solution was prepared fresh every time.

III. Stock solutions for spacer gel

E) 1.Tris : 1.5 g

2. 1 M HCl : 12.0 ml

3. TEMED : 0.12 ml

4. Distilled water to make volume 25 ml. The pH was adjusted to 6.9 with 1 N HCl. The solution was filtered and stored in brown bottle at 4^0C.

F) 1. Acrylamide : 2.5 g

2. Bisacrylamide : 0.63 g

3. Distilled water to make volume 25 ml. The solution was

filtered and stored in brown bottle at 4^0C.

G) Riboflavin, 1 mg, was dissolved in distilled water to make volume 25 ml. The solution was filtered and stored at 4^0C.

IV. Working solution for separation gel (7% , pH 8.9)

A Solution	:	2.5 ml
B Solution	:	5.0 ml
C Solution	:	2.5 ml
D Solution	:	10.0 ml

V. Working solution for spacer gel (2.5% , pH 6.9)

E Solution	:	0.5 ml
F Solution	:	1.0 ml
G Solution	:	0.5 ml
C Solution	:	2.0 ml

VI. Tray/Electrode buffer (pH 8.3)

Stock buffer

1. Tris	:	6.0 g
2. Glycine	:	28.8 g

3. Distilled water to make volume 1000 ml. The pH was adjusted to 8.3, filtered and stored at 4^0C. The stock buffer was diluted 10 times with distilled water before use.

VII. Staining solution

The peroxidase isoenzymes present in the polyacrylamide gels were detected by using benzidine solution in the presence of hydrogen peroxide. The preparation of incubation mixture for the activity staining of peroxidase was as follows.

A) Saturated benzidine

Four grams of benzidine was dissolved in 200 ml boiling water for 4-5 minutes. The solution was cooled, filtered and stored in an amber coloured bottle in a refrigerator until use.

B) Ammonium chloride (30%)

Thirty grams of ammonium chloride was dissolved in 80 ml of distilled water and final volume made 100 ml with distilled water.

C) Hydrogen peroxide (0.2%)

The reagent grade 30% hydrogen peroxide (0.7 ml) was added to distilled water to make up final volume 100 ml. This solution was prepared fresh just before use.

For preparing staining solution or incubation media, the solutions A, B and C were mixed in the proportion of 100:15:20. After incubation, the bands were fixed by placing gels in 7% acetic acid to stop the activity of staining and preserved in 2% acetic acid.

3.2.3.3 Electrophoretic procedure

The separation gel solutions were mixed in appropriate proportion and degassed. The degassed solution was immediately poured in

the electrophoresis tubes mounted on a platform. Then, 0.5 ml distilled water was carefully layered over the top of the gels to get a horizontal gel surface and to avoid contact of air. The tubes were kept for gel polymerization under fluorescent light for 1 hr. After polymerization of gel, the water layer from the top of the polymerized gel was removed. The stock solutions of spacer gel were mixed in appropriate proportion to get a working solution and the mixture was degassed immediately. The degassed solution, about 0.5 ml immediately poured over the separation gel in each tube. About 0.2 ml water was layered over the top of the gel and the tubes were kept for polymerization under fluorescent light for 2 hrs. After the polymerization of the gels the water layer from the top of the polymerized gels was removed.

The tubes were connected immediately to upper buffer tray with the help of rubber stopper. Both the trays were filled with electrode buffer (pH 8.3) upto prescribed level. Sample containing crude enzyme extract was loaded along with sucrose solution on the top of the gel under buffer. A few drops of 1.0 per cent bromophenol blue dye was added in the upper tray buffer to function as tracking dye.

Initially the current of 2 mA/tube was applied for 10 min and then increased to 5 mA/tube. The electrophoresis was carried out until the bromophenol blue (tracking dye) migrated almost to the end of the tube and the current was switched off.

The gels were removed from tubes by inserting a syringe needle between the gel and the walls of the tubes with careful discharge of water from the syringe while rotating the tubes. The gels were collected in petridishes and stained using benzidine solution in the presence of hydrogen

peroxide for 10-15 minutes. Sites (bands) indicating the presence of the peroxidase appeared reddish brown. Peroxidase isozymes present in the acrylamide gel reacts with hydrogen peroxide present in the incubation medium and produce necent oxygen at the site of peroxidase. The necent oxygen reacts with benzidine in presence of ammonium chloride and oxidize the benzidine into brown coloured end product which appears on the acrylamide gels as bands. The activity of staining was stopped and bands were fixed by using 7% acetic acid and preserved in 2% acetic acid.

The gels were photographed immediately to make permanent record of the bands. The relative mobility of a given isozyme band was calculated as follows.

$$Rm = \frac{\text{Distance migrated by isoenzyme (cm)}}{\text{Distance migrated by tracking dye from the top of the separation gel (cm)}}$$

The Rm values for each genotype were recorded separately.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

The experiments were conducted to study the peroxidase activity in dry seeds of promising pearl millet hybrids including two CMS lines and several market samples differing in seed characteristics. An attempt has been made to standardize chromatography conditions for partial purification of peroxidase using gel filtration column. The isoenzyme patterns for peroxidases in the above mentioned cultivars have also been studied using polyacrylamide gel electrophoresis. The results obtained are presented and briefly discussed in this section.

4.1 Peroxidase Activity in Dry Seeds

4.1.1 Promising hybrids

The results on peroxidase activity in dry seeds of promising pearl millet hybrids developed at this University are presented in Table 1. The peroxidase activity was found to range from 72.8 to 137.0 units/g/min indicating a wide genetic variability. The hybrids RHRBH-9907 and RHRBH-9911 showed the lowest activity while the hybrids, RHRBH-9903 and RHRBH-9901 exhibited the maximum level of peroxidase activity in dry seeds.

Several investigators have studied the peroxidase activity in pearl millet seeds (Lal and Gupta, 1993; Poongodi, 1996, Chavan and Hash, 1998 Bangar *et al.*, 1999). The values obtained on the peroxidase activity units in the present study are fairly in agreement with those reported by Chavan and Hash (1998) and Bangar *et al.* (1999). The seed peroxidase activity mainly located in the germ fraction of the seed has been implicated in the oxidation

Table 1 : Peroxidase activity (POD) in dry seeds of promising pearl millet cultivars.

Sr. No.	Cultivar	POD activity units/g/min.	Sr. No.	Cultivar	POD activity units/g/min.
1	RHRBH-9901	137.0	7	RHRBH-9907	72.8
2	RHRBH-9902	120.0	8	RHRBH-9908	108.2
3	RHRBH-9903	125.0	9	RHRBH-9909	110.6
4	RHRBH-9904	110.4	10	RHRBH-9910	119.2
5	RHRBH-9905	108.6	11	RHRBH-9911	77.2
6	RHRBH-9906	92.0	12	RHRBH-9912	93.2

Range 72.8 to 137.0 units/g/min.

Mean 106.2 units/g/min.

of meal phenolics to cause a development of acidic, mousy odour in the meal during a short storage (Bangar *et al.*, 1999). Therefore, the seeds with lower seed peroxidase activity are desirable. In this context, the hybrids RHRBH-9907 and RHRBH-9911 are identified as desirable types among the 1999 series of promising hybrids.

4.1.2 CMS lines

With a view to study the nature of genetic inheritance of peroxidase activity, the dry seeds of two CMS systems including hybrid viz., Shradha and Saburi and their parents developed at this University were analyzed for peroxidase activity and results are summarized in Table 2. In CMS lines of Shradha, the hybrid seeds were found to contain 108.6 units , while the male parent 88.4 and the female parent 110.6 units/g/min. The peroxidase activity in the hybrid was found to be similar or close to the female parent indicating its dominance for this character. In other CMS system (Saburi), the hybrid showed 120.0 units while the male parent 72.4 units and that of female parent 133.8 units/g/min. In this CMS line also, the peroxidase activity in dry seeds of hybrid was found to be similar or close to the female parent indicating its dominance. These results therefore indicated that a female parents with lower seed peroxidase activity may be useful in developing a hybrid with improved shelf life of the meal. However, work on additional CMS lines is essential to confirm these findings. The results obtained on the units of peroxidase activity in both the CMS systems are in the line with earlier reports (Bangar *et al.*, 1999).

Table 2 : Peroxidase activity (POD) in dry seeds of two CMS lines of pearl millet

I. Shradha

Sr. No.	CMS line	POD activity units/g/min.
1.	RHRBH - 8609 (Shradha Hybrid)	108.6
2.	RHRBI - 138 (Male)	88.4
3.	RHRB - 1A (Female)	110.6

II. Saburi

Sr. No.	CMS line	POD activity units/g/min.
1.	RHRBH - 8924 (Saburi Hybrid)	120.0
2.	RHRBI - 458 (Male)	72.4
3.	RHRB - 5A (Female)	133.8

4.1.3 Market samples

The survey of local weekly food grain market indicated the availability of cultivars significantly differing in their seed colour, size and shape showing wide genetic diversity in the pearl millet types that are cultivated by the farmers. It was thought interesting to analyze such samples for the peroxidase activity. The observations on seed size, colour and shape and their peroxidase activity are presented in Table 3. The size of the seeds was found to range between 7.79 to 14.38 g/1000 seeds. The colour variation was found white to yellowish, brown or gray with varying intensities and mixed shades. The shapes of seed were grouped as globular round to quite slender (Lanceolate) with some intermediate types (obvate). The peroxidase activity in 12 such samples was found to range between 78.2 to 137.0 units/g/min. In general, the samples with globular seed types were found to contain lower seed peroxidase activity than the obvate or lanceolate types. The lanceolate types (slender) were found to have higher seed peroxidase activity. The colour or size of seeds however did not exhibit any definite relationship with the seed peroxidase activity.

The data obtained on the seed peroxidase activity of different samples are similar to the values obtained for promising hybrids or CMS lines. Chavan and Hash (1998) analyzed several ICRISAT pearl millet lines from mapping populations differing in seed colour and found that the peroxidase activity in seeds was not related to the seed colour. The results of the present study are in agreement with this report. Most of the seed peroxidase activity is located in the germ and surface layers of the seeds (Bangar *et al.*, 1999). The higher level of peroxidase activity in lanceolate type of seeds observed in the present study can be attributed to the more

Table 3 : Peroxidase activity (POD) in dry seeds of market samples of pearl millet.

Market sample No.	Seed colour	Seed shape	Seed size (1000 grain wt. in g.)	POD activity units/g/min.
1.	White	Lanceolate	7.79	125.0
2.	Gray	Globular	9.33	98.0
3.	Light brown gray	Lanceolate	9.44	127.2
4.	Light brown gray	Obvate	9.55	119.4
5.	Light brown gray	Lanceolate	10.27	108.6
6.	Yellowish white	Obvate	11.52	97.0
7.	Brown	Globular	11.56	99.2
8.	Light brown gray	Obvate	12.05	102.0
9.	Gray	Obvate	12.20	110.6
10.	Light brown gray	Globular	12.25	78.2
11.	Brownish gray	Obvate	13.49	137.0
12.	Yellowish white	Globular	14.38	88.4
		Range	7.79-14.38g	78.2-137.0 units/g/min.
		Mean	11.15g	107.6 units/g/min.

surface area and possibly greater proportion of the germ in such seeds. Hence, the globular seed-type cultivars with lesser surface area and proportion of germ may be advantageous to have lower peroxidase activity and better shelflife of meal.

4.2 Purification of Seed Peroxidase

A preliminary experiment was conducted to standardize a procedure for partial purification of pearl millet seed peroxidase. The seeds of cv. Shradha were homogenized with phosphate buffer (0.1 M, pH 7.0) in prechilled mortar and pestle, the contents centrifuged at 15,000 x g for 30 min and the supernatant was freeze dried after dialysis against water at $10^{O}C$ for several hours.

The freeze dried preparation was dissolved in the same buffer and applied to the Sephacryl S-100 gel filtration column. The column was eluted and suitable fractions (2.5 ml) were collected as described in section 3.2.2.2 the original supernatant and the appropriate fractions were analyzed for the content of soluble proteins and peroxidase activity (Table 4). The fraction number 20 to 40 showed the distribution of most of the peroxidase activity eluted from the column. A maximum peroxidase activity was found to be eluted in fractions 32 to 34 with a elution volume of 82.5 ml (Fig.1). The purification data related to specific activity in initial extract and in chromatography fraction with the level of purification obtain are summarized in Table 5. The original crude supernatant contained 3.25 units/mg soluble proteins while the fraction number 33 contained 34.1 units/mg soluble protein of peroxidase activity. Thus a purification of about 10-fold was obtained.

Table 4 : Soluble proteins and peroxidase activity (POD) distribution in different fractions obtained from gel filtration column.

Sr. No.	Fraction No.	Soluble protein $A_{660\ nm}$	POD activity A_{440nm}
1.	20	0.249	0.025
2.	21	0.215	0.029
3.	22	0.223	0.037
4.	23	0.228	0.034
5.	24	0.223	0.042
6.	25	0.197	0.054
7.	26	0.230	0.074
8.	27	0.174	0.098
9.	28	0.223	0.114
10.	29	0.205	0.105
11.	30	0.187	0.101
12.	31	0.186	0.146
13.	32	0.159	0.221
14.	33	0.116	0.228
15.	34	0.137	0.204
16.	35	0.120	0.101
17.	36	0.125	0.065
18.	37	0.143	0.038
19.	38	0.148	0.025
20.	39	0.191	0.018
21.	40	0.167	0.016

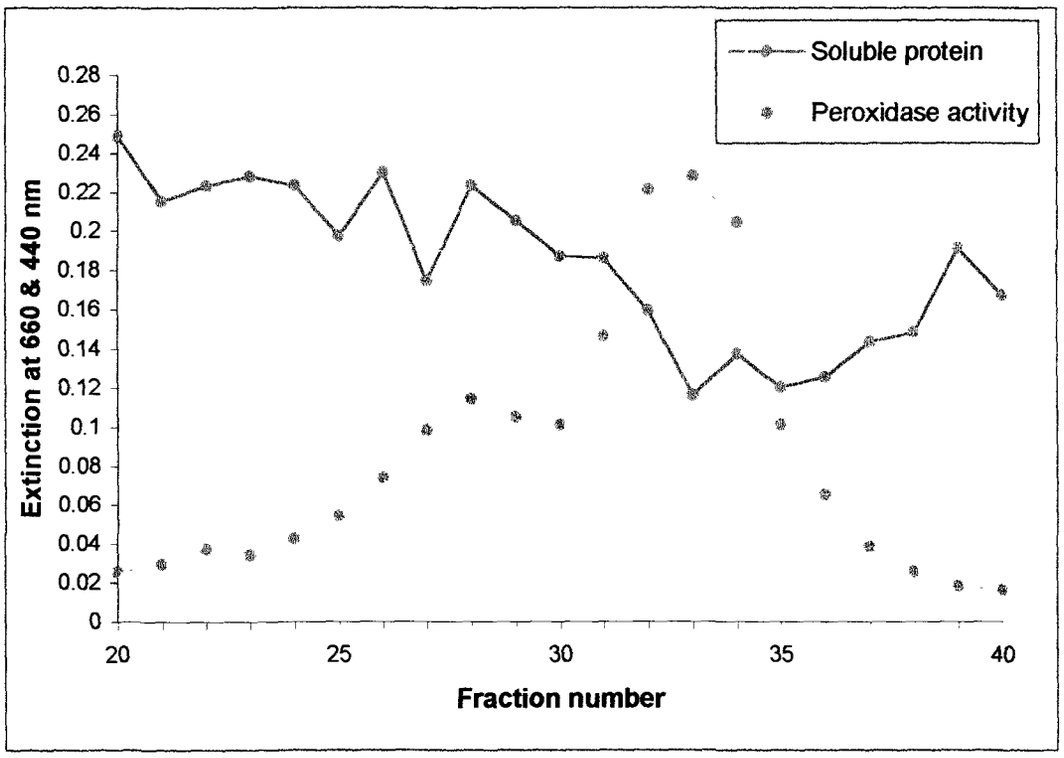


Fig. 1 : Gel chromatographic separation of peroxidase of pearl millet seeds

Table 5 : Purification data for pearl millet peroxidase on Sephacryl S - 100 gel filtration column.

1. Sample	50 g extracted in 400 ml.
2. Initial supernatant	
a. Soluble proteins	2 mg/ml
b. Peroxidase activity	6.5 units/ml/min
c. Specific activity	3.25 units/mg soluble proteins
3. Chromatography fraction No. 33	
a. Soluble proteins	0.33 mg/ml
b. Peroxidase activity	11.25 units/ml/min
c. Specific activity	34.1 units/mg soluble proteins
4. Purification folds	About 10

Several investigators have purified peroxidases from various sources such as cereals, legumes, oilseeds, fruits and vegetables using either gel filtration, ion exchange, affinity column using Con-A Sepharose. Koshiba (1993) purified peroxidases from maize coleoptiles by gel filtration and SDS-PAGE while Iori *et al.* (1995) used ion exchange chromatography for purification of peroxidases of durum wheat. Billaud *et al* (1999) used Con-A Sepharose affinity column for peroxidases of barley and wheat germ. The DEAE-Sepharose ion exchange column was used by Halpin *et al.*(1989) for purification of peroxidases from green peas and by Gillikin and Graham (1991) for peroxidases in soybean. Such information for purification of peroxidase from pearl millet seeds is however lacking. In this investigation, a preliminary experiment has indicated that about 10-fold purification of pearl millet peroxidase is possible with Sephacryl-S-100 gel filtration column. However, use of Sephacryl S-300 column followed by ion exchange on DEAE-Sephadex or Sepharose may yield higher level of purification. The use of Con-A-Sepharose affinity column for purification of barley or wheat germ peroxidases (Billaud *et al.*, 1999) indicated their glycoprotein nature. Such information is however not available for pearl millet seed peroxidases.

4.3 Peroxidase Isoenzyme Patterns

The peroxidase isoenzyme patterns in crude extracts obtained from the seeds of several promising hybrids, two CMS systems and different market samples were studied using polyacrylamide gel electrophoresis.

4.3.1 Promising hybrids

Twelve promising pearl millet hybrids of 1999 series were analyzed for peroxidase isoenzyme patterns. The banding pattern observed for peroxidase isoforms is shown in Plate 1, while the number of isoenzymes with their relative mobility values are presented in Table 6. Of the 12 cultivars, 9 exhibited 3 isoforms while 3 hybrids showed 4 isoforms for peroxidase. All the cultivars studied exhibited 2 common isoforms with Rm values 0.59 and 0.65 with almost similar band intensities. Most cultivars contained one additional isoform of slow moving type and of lesser and diffused band intensity. The RHBH-9905, RHRBH-9906 and RHRBH-9909 exhibited fourth isoform with still lesser mobility (0.41 to 0.47). Based on Rm values the number of peroxidase isoforms observed for 12 different hybrids are summarized in Table 7. The isoform number 8 and 9 were found to be common in all the cultivars while a significant genetic differences were seen for 7 other isoforms. These differences in peroxidase isoforms can be used as markers or test to determine the genetic purity of the seeds. Further studies are however essential to confirm the possibility of using peroxidase isoenzyme pattern as a marker for testing purity of the seeds.

4.3.2 CMS lines

The peroxidase isoenzyme pattern for two CMS systems namely Shradha and Saburi as obtained in PAGE are shown in Plate 2 and Plate 3, respectively. The number of isoforms and their relative mobilities are summarized in Table 8. The hybrid Shradha exhibited 3 distinct isoforms with Rm values 0.44, 0.59 and 0.65, out of which the band intensity corresponding to 0.44 and 0.65 were greater. The male parent showed two

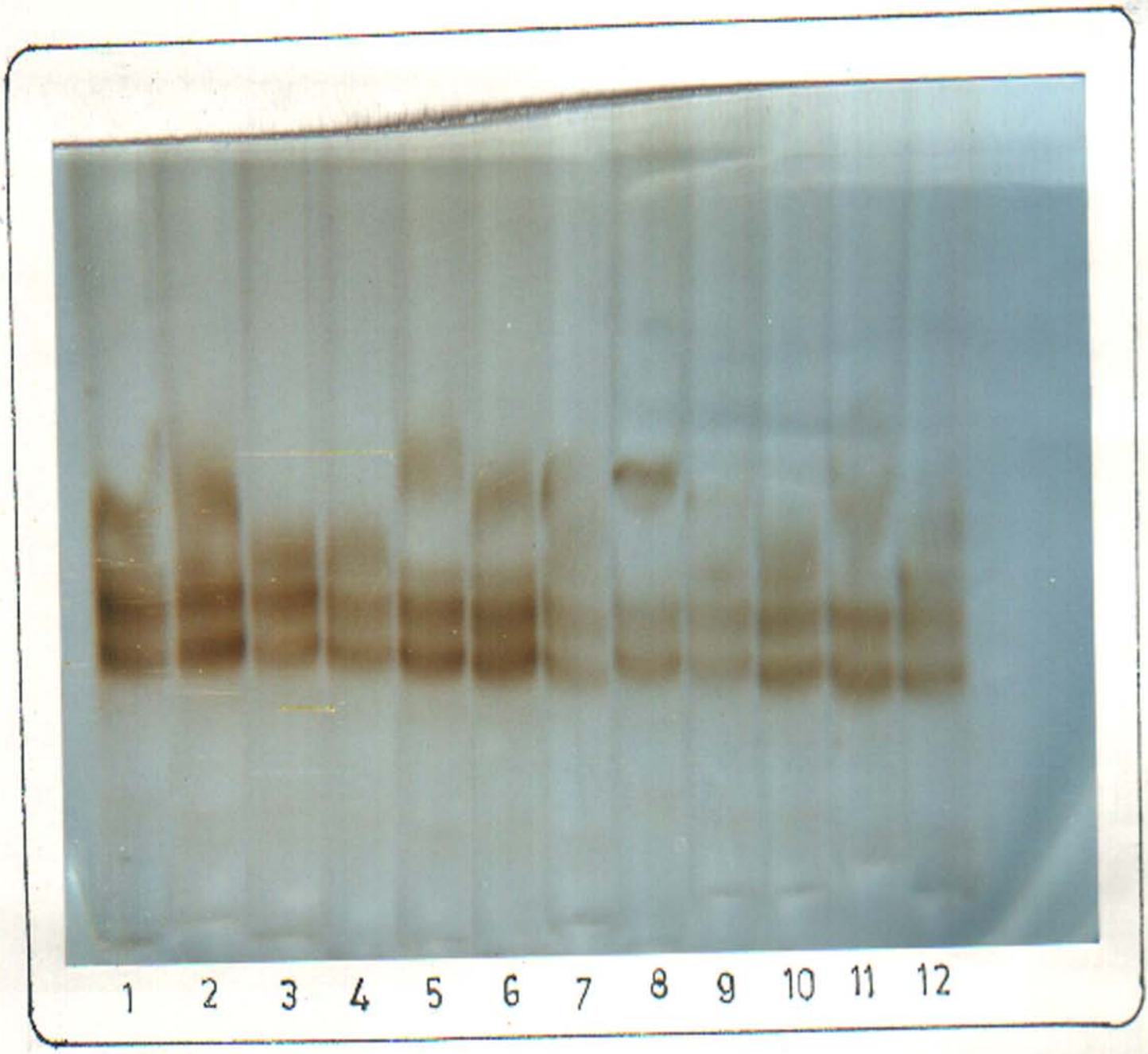


Plate 1 : Peroxidase isoenzyme pattern in dry seeds of promising pearl millet cultivars

1. RHRBH-9901
2. RHRBH-9902
3. RHRBH-9903
4. RHRBH-9904
5. RHRBH-9905
6. RHRBH-9906
7. RHRBH-9907
8. RHRBH-9908
9. RHRBH-9909
10. RHRBH-9910
11. RHRBH-9911
12. RHRBH-9912

Table 6 : Number of peroxidase isoenzymes and their relative mobility (Rm) values in promising pearl millet cultivars.

Sr. No.	Cultivar	Total No. of isoenzyme bands	Rm values of band No.			
			1	2	3	4
1.	RHRBH - 9901	3	0.48	-	0.59	0.65
2.	RHRBH - 9902	3	0.46	-	0.59	0.65
3.	RHRBH - 9903	3	-	0.53	0.59	0.65
4.	RHRBH - 9904	3	-	0.53	0.59	0.65
5.	RHRBH - 9905	4	0.41	0.55	0.59	0.65
6.	RHRBH - 9906	4	0.44	0.55	0.59	0.65
7.	RHRBH - 9907	3	0.44	-	0.59	0.65
8.	RHRBH - 9908	3	0.44	-	0.59	0.65
9.	RHRBH - 9909	4	0.47	0.55	0.59	0.65
10.	RHRBH - 9910	3	-	0.55	0.59	0.65
11.	RHRBH - 9911	3	0.47	-	0.59	0.65
12.	RHRBH - 9912	3	-	0.55	0.59	0.65

Table 7 : Comparative relative mobility (Rm) values of isoforms of peroxidase in seeds of different cultivars of pearl millet.

Isoform No.	Cultivar											
	RHRBH 9901	RHRBH 9902	RHRBH 9903	RHRBH 9904	RHRBH 9905	RHRBH 9906	RHRBH 9907	RHRBH 9908	RHRBH 9909	RHRBH 9910	RHRBH 9911	RHRBH 9912
1.	-	-	-	-	0.41	-	-	-	-	-	-	-
2.	-	-	-	-	-	0.44	0.44	0.44	-	-	-	-
3.	-	0,46	-	-	-	-	-	-	-	-	-	-
4.	-	-	-	-	-	-	-	-	0.47	-	0.47	-
5.	0.48	-	-	-	-	-	-	-	-	-	-	-
6.	-	-	0.53	0.53	-	-	-	-	-	-	-	-
7.	-	-	-	-	0.55	0.55	-	-	0.55	0.55	-	0.55
8.	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59	0.59
9.	0.65	0.65	0.65	0.65	0.65	0.65	0.65	0.65	0.65	0.65	0.65	0.65

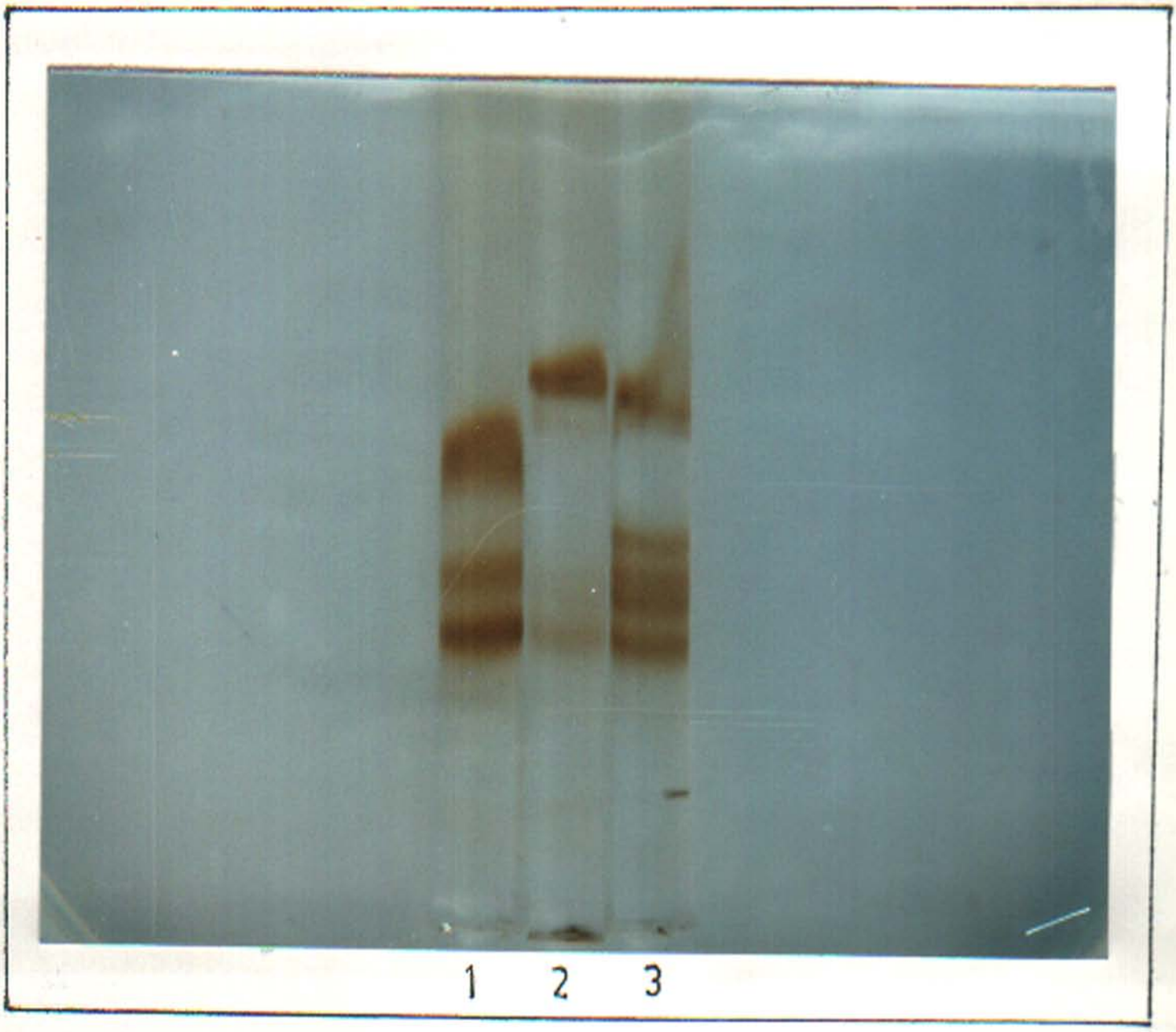


Plate 2 : **Peroxidase isoenzyme pattern in dry seeds of CMS line, Shradha of pearl millet**

1. RHRBH-8609 (Shradha Hybrid)
2. RHRBI-138 (Male)
3. RHRB-1A (Female)

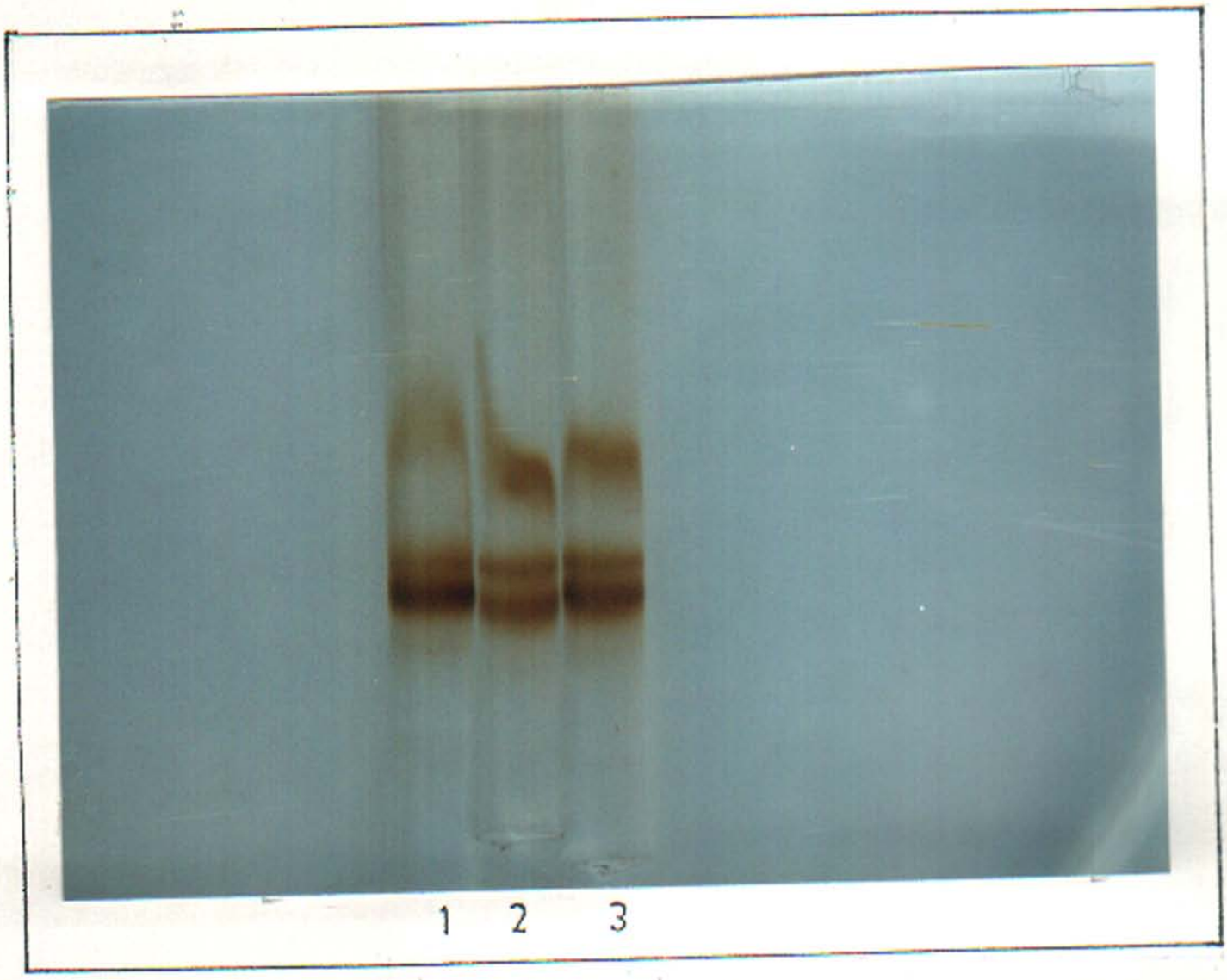


Plate 3 : Peroxidase isoenzyme pattern in dry seeds of CMS line, Saburi of pearl millet

1. RHRBH-8924 (Saburi Hybrid)
2. RHRBI-458 (Male)
3. RHRB-5A (Female)

Table 8 : Number of peroxidase isoenzymes and their relative mobility (Rm) values in CMS lines of pearl millet

I. Shradha

Sr. No.	CMS lines	Total No. of isoenzyme bands	Rm values of band No.				
			1	2	3	4	5
1.	RHRBH-8609 (Shradha Hybrid)	3	0.44	-	-	0.59	0.65
2.	RHRBI-138 (Male)	3	0.35	-	-	0.59	0.65
3.	RHRB-1A (Female)	5	0.38	0.53	0.59	0.61	0.65

II. Saburi

Sr. No.	CMS line	Total No.of isoenzyme bands	Rm values of band No.		
			1	2	3
1.	RHRBH-8924 (Saburi Hybrid)	3	0.47	0.59	0.65
2.	RHRBI-458 (Male)	3	0.46	0.59	0.65
3.	RHRB-5A (Female)	3	0.48	0.59	0.65

faint bands corresponding to Rm values 0.59 and 0.65 while one with intense band at Rm value 0.35. The female parent however exhibited 5 isoforms with almost similar band intensities. In all the three lines, the isoform with Rm value 0.59 and 0.65 were found to be common. Although a definite relationship is not seen, the banding in female parent showed similarity with the hybrid with respect to band intensities. This indicated that the character is dominated by the female parent. This observation is in the line with total peroxidase activity observed for these lines.

In another CMS group, Saburi all the three lines exhibited 3 isoforms with almost similar Rm values. However, the band intensity for isoform with Rm value 0.65 in female parent and the hybrid were found to be similar dense while it was faint in male parent. This observation also indicated a dominance of female parent which is in the line with total peroxidase activity observed in seed.

4.3.3 Market samples

The isoenzyme patterns for peroxidase in 12 different market samples differing in shape, size and colour of seeds are shown in Plate 4 while the number of isoforms with their Rm values are summarized in Table 9. Although a complex pattern of peroxidase isoforms was seen among the market samples, the two isoforms with Rm values 0.59 and 0.65 were found to be predominantly present in all the samples which is similar to observations recorded for genetically pure hybrids or CMS groups. These results indicated the common occurrence of two major isoenzymes in pearl millet with additional one or two minor forms in certain cultivars. The isoenzyme pattern observed in market samples seem to have no relationship

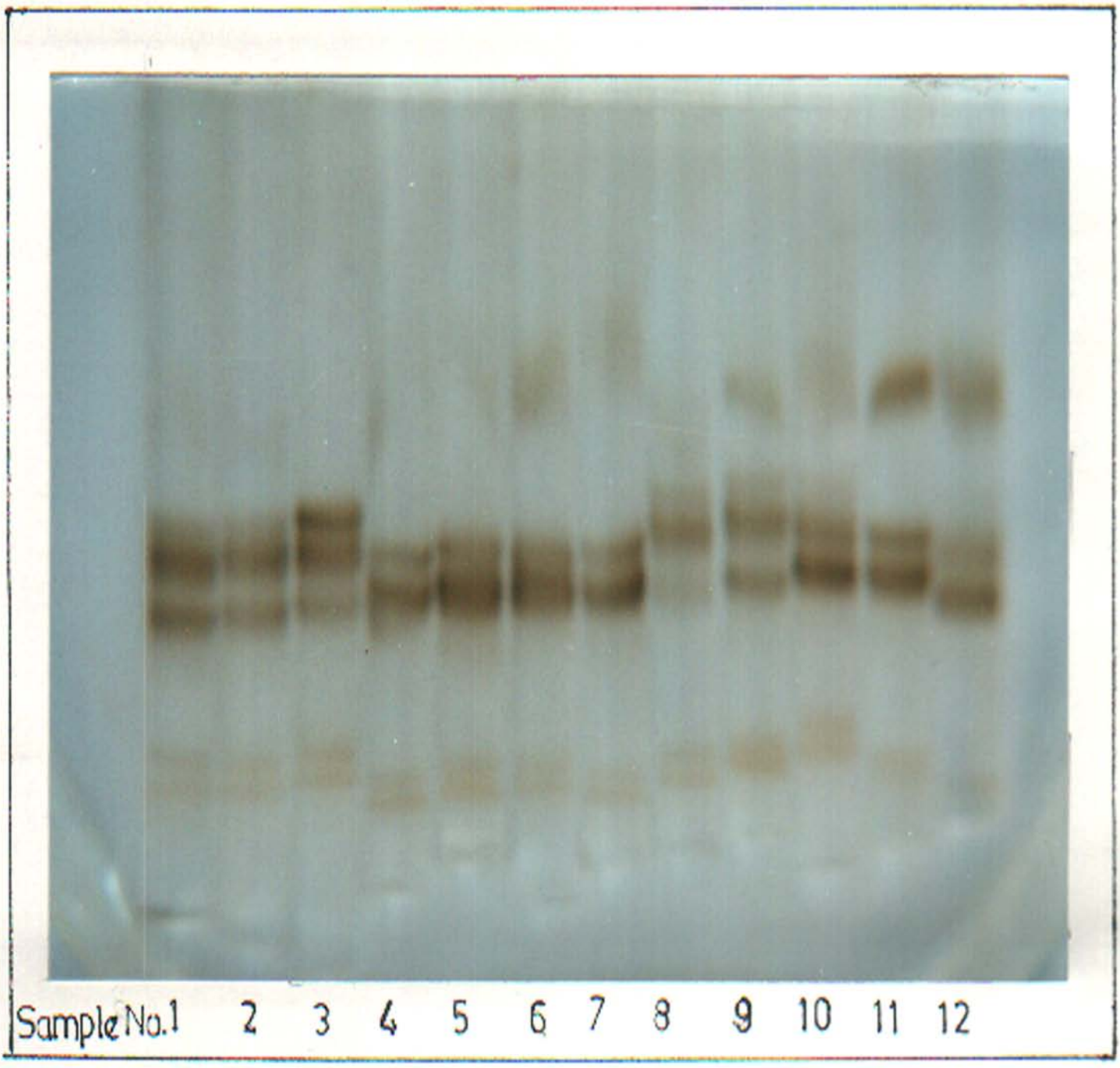


Plate 4 : Peroxidase isoenzyme pattern in dry seeds of market samples of pearl millet

Table 9 : Number of peroxidase isoenzymes and their relative mability (Rm) values in market samples of pearl millet

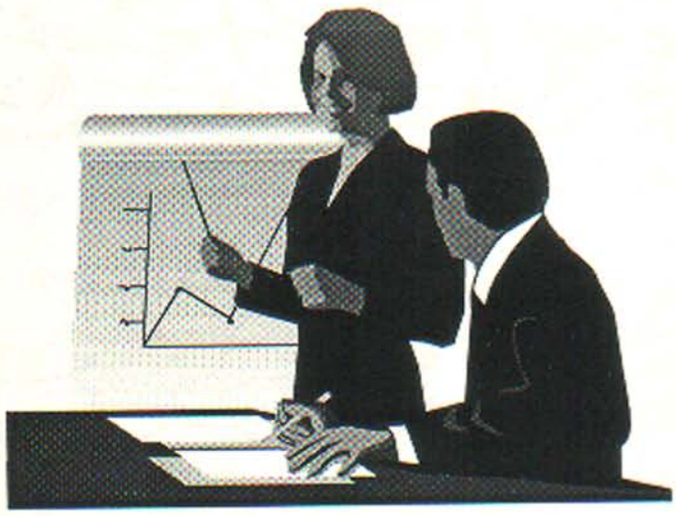
Sr. No.	Market sample No.	Total No. of isoenzyme bands	Rm values of band No.			
			1	2	3	4
1.	1	3	-	0.56	0.59	0.65
2.	2	3	-	0.56	0.59	0.65
3.	3	3	-	0.56	0.59	0.65
4.	4	2	-	-	0.59	0.65
5.	5	2	-	-	0.59	0.65
6.	6	3	0.40	-	0.59	0.65
7.	7	2	-	-	0.59	0.65
8.	8	3	-	0.55	0.59	0.65
9.	9	4	0.45	0.55	0.59	0.65
10.	10	3	0.45	-	0.59	0.65
11.	11	3	0.45	-	0.59	0.65
12.	12	3	0.40		0.59	0.65

with size, shape or colour of the seeds. It may be further mentioned that number of isoforms did not show a definite relationship with total peroxidase activity observed in the crude extract for the respective cultivars. This may be due to the activity variations in the isoforms. Further investigations are therefore essential to study such relationship between total enzyme activity and number of isoforms in several pearl millet cultivars.

Several investigators have studied the peroxidase isoenzymes using electrophoretic techniques. Kobrehel and Gautier (1974) reported that electrophoretic patterns of peroxidases differ from variety to variety in wheat and suggested that it could be used to distinguish classes of wheat. The differences in isoenzyme patterns and intensity of bands were observed in the CMS lines and their maintainers in sorghum (Phul *et al.* 1987) and suggested that the peroxidases are involved in generation of male sterility. Rao *et al.* (1992) studied peroxidase isoenzyme patterns in 10 inbred lines of pearl millet and observed no significant differences in the isoforms among the various genotypes. These reports indicate that variety of peroxidases differing markedly with respect to ionic charges (cathodic or anodic), number of isoforms, molecular size and their quantitative proportion occur in the seeds of cereal grains including pearl millet. The results of the present study have also indicated considerable genetic differences with respect to total peroxidase activity and the isoforms in a groups of pearl millet cultivars studied.

In pearl millet seeds, the peroxidases are implicated in the development of acidic mousy odour in the meal upon a short storage. Hence the cultivars with low total peroxidase activity are desirable. The cultivars RHRBH-9907 and RHRBH-9911 are identified as low peroxidase types.

However the activity measurements in seeds did not show the definite relationship with number of isoforms. At least two isoforms were found to be commonly present in all the cultivars. Thus, it may be interesting to investigate the contribution of additional isoforms in total peroxidase activity of seed and activity variations in two common isoforms found to occur in all the cultivars.



SUMMARY AND CONCLUSIONS

5. SUMMARY AND CONCLUSIONS

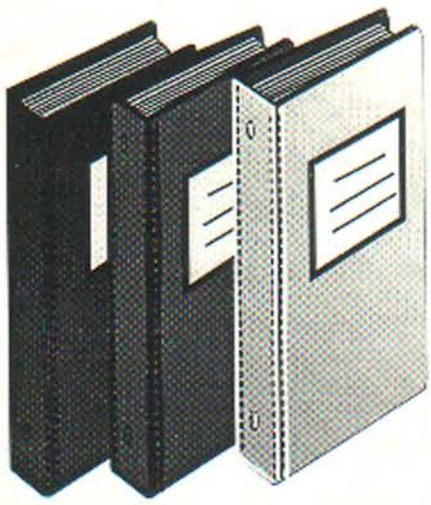
Pearl millet is an economical and staple source of dietary nutrients to a large segment of population particularly from lower socio-economic groups and farming community of India. During the recent years, pearl millet production has been increased markedly which indicated that it would be an important food source for the people in the future.

Processing and utilization of pearl millet is however constrained due to a rapid development of off odour in the meal during a short storage due to the degradation of phenolic compounds by the peroxidase activity. Hence, cultivars with a low seed peroxidase activity are desirable. Therefore, an attempt has been made to study the variations in seed peroxidase activity in several new pearl millet hybrids, some CMS lines and several market samples showing variations in size, shape or colour of the seeds. Further, attempt has been made to standardize conditions for the partial purification of seed peroxidase from pearl millet and study the isoenzyme patterns in several pure hybrids, CMS systems and market samples using PAGE. The results obtained on such studies are briefly summarized in this section.

1. A wide genetic variability was observed for a total seed peroxidase activity in 12 newly developed promising pearl millet hybrids. The activity was found to range from 72.8 to 137.0 units/g/min. The hybrids RHRBH-9907 and RHBH-9911 are identified as low seed peroxidase types which is desirable from the point of view of shelf-life of the meal.
2. The seed peroxidase activity in CMS system of Shradha ranged between 88.4 to 110.6 while that for Saburi it varied from 72.4 to 133.8 units/g/min. In both the groups, the seed peroxidase activity in the hybrid

was found to be similar to the female parent. These results indicated that the character is dominated by the female parent.

3. Studies on market samples revealed a lower seed peroxidase activity in globular seeds than that of obvate or lanceolate types. The total activity however did not show any definite relation with colour or size of the seeds.
4. The extraction of the peroxidase from the seeds in 0.1 M phosphate buffer pH 7.0 (1:20 w/v) followed by a gel filtration of dialyzed and freeze dried material on Sephacryl S-100 column (64 x 1.5 cm id) at a flow rate of 50 ml/hr with 2.5 ml fractions, yielded a fraction with 10-fold purification of the enzyme.
5. The isoenzyme patterns for peroxidases in different pearl millet cultivars on PAGE clearly indicated the presence of two common isoforms with Rm values 0.59 and 0.65 in all the cultivars. One to three additional isoforms with varying Rm values were also found in these cultivars. The information may be useful to standardize a chemical test for identification of varieties and hybrids or genetic purity of the material. A definite relationship between total seed peroxidase activity and number of isoforms was not seen. Further investigations on a large number of cultivars to confirm such relationship, if any, are suggested.



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*** Originals not seen.**

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