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**Research Article**

# VARIETAL CHARACTERIZATION IN MUSTARD (*BRASSICA JUNCEA* L.) GENOTYPES

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**ABSTRACT**

Varietal identification or discrimination of cultivars is essential for quality seed production. Seed protein and isozyme electrophoresis have been utilized as a tool for varietal identification. The study was undertaken at the Department of Seed Science and Technology, Tamil Nadu Agricultural University, Coimbatore in nine mustard genotypes (Rohini, Varuna, Bio 902, PCR 7, Kranti, Pusa Bold, Maya, RN 393 and GM-2).Electrophoresis of seed proteins showed a total of 7 bands in Maya, GM-2, Varuna, PCR 7 and Pusa Bold whereas for rest of the genotypes some bands were absent. The critical bands for identification were the bands at Rf values 0.237, 0.327 and 0.667 which formed the 1st, 3rd and 7th band, respectively. The intensity of the band also varied among all the genotypes. Hence the present study obviously indicated the use of SDS page profile through electrophoresis for discrimination of mustard genotypes and Electrophoresis of seedling peroxidase expressed 7 bands in all the genotypes except in GM-2, PCR 7 and Pusa Bold. The critical band for identification was at Rf value of 0.944 at 7th band It is confirmed that seedling peroxidase could be used effectively to analyze variation among the mustard genotypes.



## INTRODUCTION

Varietal identification or discrimination of cultivars is essential for quality seed production through the successful operation of seed certification programme and granting the plant breeders' right under the PVP and FR Act. The varieties can be distinguished based on morphological, physiological, biochemical and molecular characters. However, it is always very difficult to identify a particular genotype based on any one of the characters. Usually the varieties are identified by a single or with the combination of morphological, biochemical or molecular characters. In this case the distinguishing characters should be a heritable, stable and reproducible one. Seed protein and isozyme electrophoresis have been utilized as a tool for varietal identification. The analysis of protein composition as a mean of plant variety identification is now established and has been thoroughly reviewed (Cooke, 1984, 1988 and Wrigley *et al*., 19823). The success of electrophoretic procedures depends on the wide ranging polymorphism of seed protein and isozymes and the fact that these proteins represent primary gene products. Analysis of protein composition has proved to be a good indicator unless altered by growth condition only to a relative minor extent. Rapidity and repeatability of results are the foremost among its advantages. Hence, there is a need to develop protein profiles for the cultivars for varietal characterization.

## MATERIALS AND METHODS

***Biochemical characterization of seed and seedlings***

***Analysis of total soluble proteins (Tris – HCl Soluble) in seeds through Polyacrylamide gel electrophoresis (PAGE)***

**Tris – HCl soluble proteins of seed**

Tris – HCl soluble proteins in the seed were separated using SDS-PAGE (Varier *et al.* (1992).

***Preparation of solution***

***Extraction Buffer (Tris – HCl pH 7.5)***

Tris base (1.21 g) was dissolved in 50 ml distilled water and pH adjusted with concentrated HCl to 7.5 and then the volume made upto 100 ml.

***Protein sample buffer (5x concentration)***

***Tris- HCl buffer (pH 6.6) 0.6M***

Tris- base (7.2g) was dissolved in 50 ml of distilled water and pH adjusted with concentrated HCl to 6.6 and volume made upto 100 ml with distilled water.

***Stock-sample buffer***

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| | | |
|---|---|---|
| Tris-HCl buffer (pH 6.6) 0.6M | : | 10.4 ml |
| Distilled water | : | 7.9 ml |
| Sodium dodecyl sulphate | : | 4 g |
| Glycerol | : | 10.0 ml |

The above mentioned components were mixed thoroughly.

***Working sample buffer***

| | | |
|---|---|---|
| Stock-sample buffer | : | 4.25 ml |
| β- mercapto ethanol | : | 0.75 ml |

These components were mixed thoroughly and the volume was made upto 10 ml. to this a pinch of Bromophenol blue was added, to act as a tracking dye.

***Stock gel solution (30 per cent acrylamide)***

| | |
|---|---|
| Acrylamide | : 30.08 g |
| Bis – Acrylamide | : 0.80 g |

Dissolved in distilled water and volume made up to 100 ml.

***Defatting solution***

| | |
|---|---|
| Chloroform | 2 parts |
| Methanol | 1 part |
| Acetone | 1 part |

Mixed thoroughly and used for defatting.

***Separating gel buffer (pH 8.8) 0.5 M***

Tris – base (22.69g) was dissolved in 70 ml distilled water. The pH was adjusted to 8.8 with concentrate HCl or with KOH and volume made up to 100 ml with distilled water.

***Stacking gel buffer (pH 6.8) 0.5 M***

Tris-base (6.05 g) was dissolved in 70 ml of distilled water, pH adjusted with concentrated HCl to 6.8 and volume made up to 100 ml distilled water.

***SDS 10 per cent***

SDS (Sodium Dodecyl Sulphate) 1.0 g was dissolved in 6 ml of distilled water and volume made up to 10 ml.

***Preparation of separating or running gel mixture***

| Solutions | Gel concentration (10 per cent ) for 15 ml |
|---|---|
| Acrylamide solution (C) | 5.0 ml |
| Separating gel buffer | 3.75 ml |
| Distilled water | 6.25 ml |
| 10 per cent SDS (G) | 100 µl |
| 10 per cent APS (H) | 100 µl |
| Tetramethyl Ethylene Diamide (TEMED) | 20 µl |

The separating gel mixture was poured to the glass sandwich assembly to a level of about 4 cm from the notch. One ml of butanol saturated with distilled water was gently applied over the separating gel. After polymerization, (10 minutes) the outer layer of butanol was washed off by inverting the casting gel and washed 3 to 4 times with distilled water to remove traces of butanol. The water droplets were removed using a filter paper without touching the separating gel.

***Preparation of stacking gel mixture (10 ml)***

| Stock acrylamide solution (C) | 1.3 ml |
|---|---|
| Stacking gel buffer (F) | 2.5 ml |
| 10 per cent SDS (G) | 100 ml |
| 10 per cent APS (H) | 50 ml |
| Distilled water | 6.1 ml |
| Tetramethyl Ethylene Diamide (TEMED) | 10 µl |

The above solution were mixed thoroughly and poured on the top of the separating gel. An acrylic well forming comb was inserted, ensuring no air bubble trapped beneath. The top portion of the gel solution was overlayed with 1 ml butanol saturated with distilled water. The gel was allowed to polymerize for 45 minutes. Then the acrylic comb was removed carefully not to distort the wells and resultant wells were cleaned with distilled water. The excess water was sucked out using a micro syringe and each well was cleaned carefully using bits of filter paper. The gel was then installed by removing the gasket in the electrophoresis apparatus to which electrode buffer was poured and pre-run for 10 min.

***Preparation of seed sample***

0.2 gram of seed coat removed samples was ground using a pestle and mortar. The seed powder of each variety was taken in an eppendorf tube and l ml of defatting solution (solution D) was poured in each tube. After thorough shaking, the eppendorf tubes were left for 3 h. The supernatant was decanted and this procedure was repeated three times. The samples were then kept overnight at a room temperature for drying. Next day, 1 ml of extraction buffer (solution A) was added and eppendorf tubes were kept overnight at 10°C (inside the refrigerator). The next day, the samples were centrifuged in a refrigerated centrifuge (4°C) at 12,000 rpm for 30 min.

Supernatant solution from each sample was taken into a separate eppendorf tube, to this equal volume of sample buffer (solution B) was added and mixed well. This was boiled in water for 2-3 minutes, then cooled and used for loading.

***Electrophoresis***

Electrophoresis was conducted using electrophoretic unit. The gel castle was then immersed in the electrode buffer solution (solution 1) and then upper reservoir was connected to the power pack fitting the electrodes in sockets identical colour. The voltage was fixed at 100 volt till the tracking dye reached the bottom of the gel and then the electrophoresis was stopped.

***Fixing and staining***

The gel was removed after the run and fixed overnight in 15 per cent Tri-Chloro Acetic acid (TCA). On the following day, the TCA was drained off and the gel was rinsed with distilled water and immersed in the stacking solution.

***Staining solution***

| *Comassie Brilliant Blue R. 250* | *0.1g* |
|---|---|
| Methanol | 40 ml |
| Glacial Acetic Acid | 10 ml |
| Distilled water | 50 ml |

***Destaining solution***

| Methanol | 40 ml |
|---|---|
| Glacial acetic acid | 10 ml |
| Distilled water | 50 ml |

In the staining solution the gel was kept minimum of 6 h. Then using destaining solution the gel was destained up to background become clear. The gels were placed in a trans-illuminator and photographed.

***Evaluation and documentation***

The gel was documented using Biovis gel documentation software from which the Rf values of the bands were known. Based on Rf values dendrograms were prepared wherever necessary using Biovis gel match software. Apart from these, intensity, position, presence or absence of bands were critically observed and recorded to discriminate the cultivars.

***Varietal characterization through peroxidase isozymes profiling***

The peroxidase isozyme was analyzed by Alkaline PAGE procedure described by (5) and the staining procedure of (Dadlani and Varier (1993) and the staining procedure of Reddy and Gasber (1971).

***Preparation of solution***

***Extraction buffer (Tris – HCl pH 7.5) 0.1 M***

1.21g of Tris was dissolved in 75 ml of distilled water. Concentrated HCl was used to adjust the pH to 7.5. Then the volume was made up to 100 ml with distilled water.

**Stock gel solution (30 per cent acrylamide)**

| **Acrylamide** | **30.08g** |
|---|---|
| Bis acrylamide | 0.80g |

Dissolved in distilled water and volume was made up to 100 ml

***Separating gel buffer (pH 8.8) 1.875 M***

Tris 22.69 g was dissolved in 75 ml distilled water. pH was adjusted to 8.8 with concentrated HCl and volume was made up to 100 ml with distilled water.

***Stacking gel buffer (pH 6.8) 1.875 M***

Tris 6.0 g was dissolved in 50 ml of distilled water, pH adjusted to 6.7 with concentrated HCl or with KOH and the volume was made to 100 ml with distilled water.

***APS 10 per cent***

APS 100 mg was dissolved in 1 ml of distilled water. This solution was prepared freshly each time.

***Electrode buffer***

| **Tris-base** | **3 g** |
|---|---|
| Glycine | 14.4 g |

Both components were dissolved in distilled water and volume made up to 1000 ml with distilled water.

***Preparation of gel***

***Resolving/separating gel (8 per cent) for 15 ml***

| **Stock gel solution (B)** | **3.75 ml** |
|---|---|
| Tris-HCl buffer (pH 8.8) (C) | 3.75 ml |
| Distilled water | 7.5 ml |
| 10 per cent APS (E) | 100 µl |
| Tetramethyl Ethylene Diamide (TEMED) | 20 µl |

After arranging the glass plates in gel casting stand with spacers the above components were mixed thoroughly and poured into a vertical cassette leaving a margin of 4 cm on upper side. The gel surface was left for polymerization for 1 h at room temperature.

***Stacking gel (for 10 ml)***

| **Stock Gel solution (B)** | **1.3 ml** |
|---|---|
| Tris-HCl buffer (pH 6.8) 0.6 M (D) | 2.5 ml |
| Distilled water | 6.1 ml |
| APS 10 per cent (E) | 100 ml |
| Tetra Methyl Ethylene Diamide (TEMED) | 20 µl |

The stacking gel solution was mixed thoroughly and poured on the top of separating gel. An acrylic well forming comb was inserted ensuring that no air was trapped beneath. The gel allowed to polymerise for about 1 h.

***Sample preparation***

Four replication of twenty five seeds of each variety were subjected to germination and grown in roll towel method and kept in germination room. Seven days old seedling of each genotype were taken and kept on blotting paper to remove excess of water on the surface. They were ground using a pestle and mortar on ice. Extraction buffer (solution A) 1 ml was added and the sample was finely ground. It was centrifuged at 12,000 rpm for 30 min in refrigerated centrifuge at a temperature 4°C. The supernatant was used for loading.

***Electrophoresis***

Electrophoresis was conducted by using ATTO electrophoresis unit. Sample of 70 µl and two drops of bromophenol blue (tracking dye) was loaded in each well. Electrode buffer (solution F) was filled in buffer tank and the space between the two plates. The electrophoresis apparatus was connected to the power pack fitting the electrodes in sockets of identical colour. The electrophoresis unit was kept in a refrigerator to dissipate the heat produced during electrophoresis. The voltage was set at 60 Volt till the tracking dye reached the end of the gel. Then the gel was taken out and kept in staining solution.

***Preparation of staining solution and staining***

***Acetate buffer (0.1 M)***

a. 0.2 M solution acetic acid (1.2 ml in 100 ml)
b. 0.2 M solution sodium acetate (2.12 g in 100 ml)

82 ml of solution 'a' was mixed with 18 ml of solution 'b' and final volume made upto 200 ml.

***Benzidine 200 mg dissolved in 1 ml of acetic acid***

Both solutions (i) and (ii) were mixed to which the gel was placed. Then the gel was incubated for 30 minutes at 35$^{o}$C in dark with shaking.

After 30 minutes, 1 to 2 ml of hydrogen peroxide was poured drop by drop till the blue colour bands appeared. After appearance of the blue colour bands for stopping the reaction the gel was transferred to seven per cent acetic acid solution.

***Evaluation and documentation***

As detailed under 1.7.

## RESULT AND DISCUSSION

***Seed protein profile through SDS PAGE***

The total seed protein was extracted and separated by SDS-PAGE method. The detailed electrophoretic profile of nine mustard genotypes studied has been presented in Plate 1.

The detailed electrophoregram of total soluble seed protein has been presented in Figure.6. The entire protein was divided into five regions starting from A to E. This was in the order of increasing Rf values and decreasing molecular weight of proteins. Among the five regions B and E were most useful to distinguish cultivars due to clear banding appearance. The Rf value for all the bands found in the entire profile have been presented in Table.1. The presence or absence of a band and the intensity of band were used for varietal characterization.

**Table 1** Rf values, Intensity and position of protein bands for mustard genotypes using SDS PAGE

| Band | Rf value | Rohini | Bio 902 | Kranti | Maya | GM-2 | Varuna | PCR 7 | Pusa Bold | RN 393 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.205 | - | - | - | + | + | + | + | + | - |
| 2 | 0.229 | ++ | ++ | ++ | +++ | +++ | +++ | +++ | +++ | - |
| 3 | 0.270 | + | - | - | + | + | + | + | + | - |
| 4 | 0.297 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ |
| 5 | 0.377 | ++++ | +++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ |
| 6 | 0.416 | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | ++++ | +++ |
| 7 | 0.486 | ++ | ++ | ++ | ++++ | ++++ | ++ | ++ | + | - |

\+ Low intensity ++ Medium intensity +++ High intensity ++++ Very high intensity

The band with low intensity of Rf value 0.237 was categorized as Region A (97.4 kDa). Region B (43.00 kDa) was characterized with two bands of low, medium and high intensity whose Rf values lies between 0.314 and 0.382. Only one band of high and very high intensity was present in Region C (29.0 kDa). In Region D (20.1 kDa) single band with high and very high intensity was appeared with the Rf value of 0.477 and Region E was characterized with one band of Rf value 0.662.

SDS-PAGE of proteins is the most commonly used method to discriminate the varieties. The protein banding pattern is unique for the particular genotype and is independent of seed vigour and physiological seed activity (Ferguson and Grabe, 1986). The criteria for distinctness among the cultivars were the presence or absence of a particular band or set of bands occurring at a position on the electrophoretic gel and the band intensity.

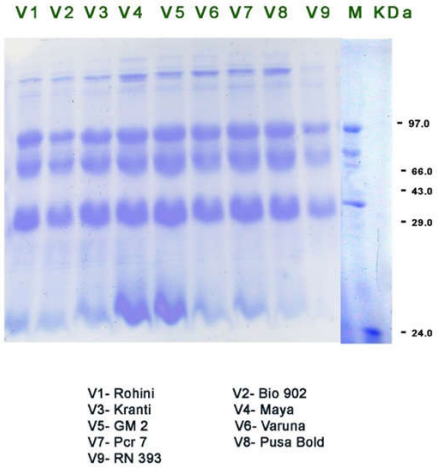
SDS-PAGE was used for characterization of mustard genotypes, as this produced denatured protein. The advantage of examining denatured proteins is that, it is independent from seed vigour and physiological activity. Denaturing system provides a simple reproducible technique for cultivar identification as reported by Gayathri Devi (2000) in sunflower.

The present study revealed that there was no difference in the banding pattern in the region C and D. the difference in banding pattern was mainly confined only to A, B and E region (Fig. 1), these regions of the total seed protein gel profile obviously indicated the usefulness of the high and low molecular proteins than the medium range molecular weight proteins for varietal discrimination of the mustard genotypes.
In the present investigation for total soluble seed proteins, three common bands (Rf value 0.384, 0.454 and 577) was detected for all the nine genotypes. The band at Rf value 0.237 was present in Maya, GM-2, Varuna, PCR 7 and Pusa Bold, while it was absent in Rohini, Bio 902 and RN 393. Similarly the band at Rf value 0.384 was present in all the genotypes except in Bio 902, Kranti, and RN 393. The band at Rf value 0.662 was present in all the genotypes except RN 393 (Table.1). Thus, the absence of these bands was effectively used for discriminating the mustard genotypes.

The variation in number and intensity of the bands might be due to differential extraction or difference in solubility of protein or lack of separation of several proteins having similar migration rates (Ladizinsky and Hymowitz, 1979). Similar observations based on band intensity were reported by Wang Chun *et al*. (1994), Asghar *et al*. (2003) and Varma *et al*. (2005) in maize genotypes, Vijayan (2005) in rice, Paul and Datta (2006) in celery and ajowan, Nisha (2006) in wheat and Sumathi (2007) in oats.

It could be suggested from the present investigation that the electrophoresis of total seed proteins could less discriminate the genotypes of mustard except that discrimination was possible to some extent based on number and intensity of the bands.

**PLATE 1. TOTAL SEED PROTEIN PROFILE OF MUSTARD GENOTYPE THROUGH SDS PAGE**




***Electrophoresis of seedling peroxidase through NATIVE PAGE***

Peroxidase isozyme patterns were defined by taking into account the number, position and intensity of bands. The banding pattern and corresponding results of mustard genotypes has been presented in Plate 2, Figure 2 and Table 2.

**Table 2** Rf values, Intensity and position of protein bands for mustard genotypes using peroxidase

| Band | Rf value | Rohini | Bio 902 | Kranti | Maya | GM-2 | Varuna | PCR 7 | Pusa Bold | RN 393 |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0.102 | ++++ | ++++ | ++++ | ++++ | ++++ | +++ | ++++ | ++++ | +++ |
| 2 | 0.304 | ++ | ++ | ++ | + | ++ | ++ | +++ | + | ++ |
| 3 | 0.353 | ++ | ++ | ++ | ++ | ++ | ++ | +++ | ++ | ++ |
| 4 | 0.766 | ++ | ++ | ++ | + | ++ | ++ | +++ | ++ | ++ |
| 5 | 0.851 | ++ | ++ | ++ | + | ++ | ++ | +++ | ++ | ++ |
| 6 | 0.875 | +++ | +++ | ++ | +++ | ++++ | +++ | ++++ | ++++ | +++ |
| 7 | 0.944 | ++ | +++ | ++ | ++ | - | +++ | - | - | +++ |

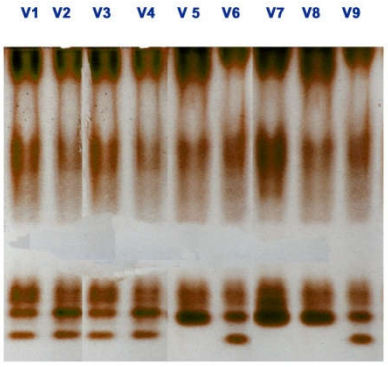
**\+ Low intensity ++ Medium intensity +++ High intensity ++++ Very high intensity**

A total of seven bands were obtained with Rf value of 0.102, 0.304, 0.353, 0.766, 0.851, 0.857 and 0.944. The bands with Rf value 0.102 and 0.875 was of very high intensity. All The

seven bands show high intensity bands with Rf value range from 0.102 to 0.944.

The expression of isozymes is exclusively based on the genetic makeup of the plant and less affected by environmental conditions (Loeschcke and Stegemann, 1966). The presence or absence and intensity of the major bands are taken as the criteria for varietal discrimination.

PLATE 2. SEEDLING PEROXIDASE PROFILE FOR MUSTARD GENOTYPES THROUGH NATIVE PAGE




Peroxidase isozymes are related to the defence mechanisms in plants (Bestwick *et al*., 1998; Schopfer *et al*., 2001). The present study indicated that common bands were detected at Rf value 0.102, 0.304, 0.358, 0.766, 0.851 and 0.875 (Table. 2) (Fig. 2). Only one band at Rf value 0.944 showed the polymorphism. The peroxidase banding pattern revealed that absence of band at Rf value 0.944 in cv. GM-2, PCR 7 and Pusa Blod was useful in discriminating the mustard genotypes. Similar results were reported by Roy *et al*. (2001) in peas, Thangavel (2003) in sorghum, Senthil Kumar (2003) in lucerne, Mukhlesur *et al*. (2004) in *Brassica rapa* cultivars, Manonmani *et al.* (2004) in Indica rice, Praveena (2005) in muskmelon Nisha (2006) in wheat and Sumathi (2007) in oats.

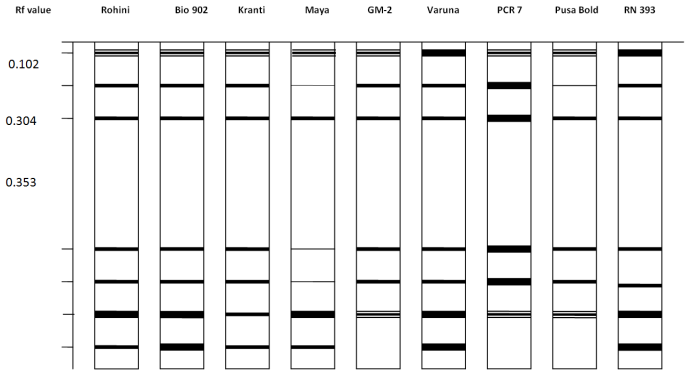



**Fig 2** Zymogram of isozyme peroxidase of mustard genotypes through NATIVE PAGE

## CONCLUSION

Electrophoresis of seed proteins showed a total of 7 bands in Maya, GM-2, Varuna, PCR 7 and Pusa Bold whereas for rest of the genotypes some bands were absent. The critical bands for identification were the bands at Rf values 0.237, 0.327 and 0.667 which formed the 1st, 3rd and 7th band, respectively. The intensity of the band also varied among all the genotypes. Hence the present study obviously indicated the use of SDS page profile through electrophoresis for discrimination of mustard genotypes.

Electrophoresis of seedling peroxidase expressed 7 bands in all the genotypes except in GM-2, PCR 7 and Pusa Bold. The critical band for identification was at Rf value of 0.944 at 7th band It is confirmed that seedling peroxidase could be used effectively to analyze variation among the mustard genotypes.

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