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S. R. Upadhyay · A. Shukla · A. K. Gaur

Assessment of peroxidase isozyme marker-based model for cross identifications in hybrids (F_1) of urdbean [*Vigna mungo* (L.) Hepper]

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Abstract Four hybrids (4 F_1s) were chosen out of crosses in the urdbean [*Vigna mungo* (L.) Hepper, 2n = 22] having contrasting morphological characters. Zymograms for isozyme peroxidase were drawn from the patterns obtained from parents and their respective F_1 hybrids on the basis of relative similarities to parental bands. The selfed or crossed nature of hybrid pods was determined from the zymograms and their analysis. The number of bands and their intensities gave an idea about the extent of crossing in F_1 populations. Genetic identity (I) values were indicative of their selfed nature. Dendrograms were constructed on the basis of genetic identity values to display the relative similarities between the populations. Analysis was based on individual pods to confirm their hybrid or selfed nature. Possible use of this technique for identification of F_1 pods and elimination of selfed pods might be implemented to shorten the breeding operations during crossing.

Keywords Hybrid identification · Isozyme · Peroxidase · Urdbean

Introduction

Urdbean [*Vigna mungo* (L.) Hepper, 2n = 22] belongs to the family *Leguminosae* that is being cultivated for human consumption. Dry mature seeds contains a up to three-times higher protein content in comparison to cereal crop grains, and thus constitutes an important source of protein for a vegetarian diet of common people. Further, it also plays a crucial role in sustaining the productivity of cropping systems by adding atmospheric nitrogen to the soil. The crop involved has spread to various geographical regions in the world, except for temperate regions, because of the versatility of its adaptation to various environments. The main productivity losses (15–20% of the total yield) in leguminous crops are due to insect-pest damage. Thus the principle characters required for improvement is insect resistance involving conventional and modern genetic tools during breeding programmes. The crossing programmes for hybridization to develop a high yielding and insect-resistant variety with desirable traits, is an ongoing activity in any breeding operation including a developing transgenic biotype(s). Morphological markers are used to detect the hybrids on the basis of visual scores; however, these characters are usually dominant or recessive (Chawla 2000). Sometimes it is difficult to select an appropriate morphological marker(s) resulting in the propagation of the selfed generation, which ultimately becomes futile due to ungainful selection. Isozymic markers have been commonly practiced for the characterization of particular plant genotypes. These variations have been applied successfully in plant breeding as biochemical markers for hybrid confirmation and for convenient screening of seedlings with required characters (Arus et al. 1982, 1985, *Brassica oleraceae*; Colby and Pierce 1988, asparagus; Weeden and Provvidenti 1988, pea). Gel electrophoretic separation methods have also been widely used in taxonomic and genetic identification of various crops (Pierce and Brewbaker 1973). The crossing in the crop urdbean is manually carried out and, being a self-pollinated crop, the amount of heterosis in the hybrid (F_1) population is not clearly distinguishable between parents. The peroxidase isozyme marker-based model for hybrid (F_1) identification can provide a convenient and cost-effective protocol over other PCR-based methods to eliminate self-progeny in order to manage the size of the breeding program.

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S.R. Upadhyay (✉) · A. Shukla
Department of Genetics and Plant Breeding,
G.B. Pant University of Agriculture and Technology,
Pantnagar-263145, India
e-mail: deanag@gbpuat.eruet.in

A.K. Gaur
Department of Biochemistry,
G.B. Pant University of Agriculture and Technology,
Pantnagar-263145, India

The hybrid (F_1) populations obtained from tagging the individual pods from various crosses were electrophoresed for isozyme peroxidase banding patterns to test the feasibility of this method in recognition of the selfed or crossed nature of F_1 populations, which are to be further carried out for selection in advanced generations.

Materials and methods

The experimental material for the present investigation comprised five genotypes of four crosses, namely cross 1 (Shu-9511 × IC-201893), Cross 2 (Shu-9511 × Shu-9701), Cross 3 (Shu-9619 × Shu-9641) and Cross 4 (Shu-9619 × Shu-9701) derived from crossing with the respective parents. These parents had morphological distinctions: parents Shu-9511 and Shu-9641 had a black pod, a black seed colour with no seed lustre and a later one had bold seeds; Shu-9619 had a straw coloured pod and a green seed colour with no seed lustre; IC-201893 had black pods, black and miniature seed with no seed lustre; and Shu-9701 had a black pod, and black and shining seed-lustre traits. The F_1 hybrid seeds were obtained from tagged crossed pods harvested individually from every cross. These crosses were made at the rainy season in 2000 at the Crop Research Station, Pantnagar, India. These individual harvested pods were sown in separate pots and their F_1 plants were subjected to peroxidase marker analysis.

Tender upper-leaf samples (1 g) were ground with 0.4 ml of extraction buffer (0.9% NaCl + 0.1% KCl in equal volume) with a pinch of fine sand for about 15 min, and the slurry was centrifuged at 10,000 rpm for 20 min at a 2 to 4 °C temp. The enzyme was extracted in the supernatants and pellets were discarded. The electrophoresis (Native-PAGE) was performed on male–female parents and their hybrids, according to Laemli (1970). Samples were prepared by mixing 2 parts of extract + 1 part of 50% glycerol + 1 drop of Bromophenol blue (0.05 mg/ml of dH_2O) and stored in the deep freezer at –20 °C. Electrophoresis was carried out on a 7% separating gel and a 4% stacking gel. A pre-run at 100 V for 1/2 h was given before loading the samples. After loading the samples (5 µl), Native-PAGE was run at 150 V. It took 4–5 h for the dye to enter the lower buffer before electrophoresis was completed. After that the gel was stained using 5 ml of H_2O_2 in 95 ml of d H_2O and 0.5% w/v of benzidine in 10% v/v acetic acid.

The gel was then transferred to 7% acetic acid for 3 min which ensures fixation of bands in the gel. Photographs of the gels were taken on a gel documentation system.

The gels were analysed and zymograms were prepared on the basis of the relative mobility of each form of peroxidase visualized on the gel.

Genetic identity (I) was determined as described by Nei (1972, 1975) according to the formula:

$$I_{xy} = \frac{\sum xi \cdot yi}{\sqrt{\sum x^2 i \cdot \sum y^2 i}}$$

where, xi·yi are the frequencies of the i^{th} allele in populations X and Y respectively. If the frequency of alleles at a locus is I = 1 in two taxa, equal allelic frequencies are given. On the other hand, if I = 0 (zero) then allelic frequencies are completely different. This was calculated on the basis of the relative mobility of isozyme bands together.

Dendrograms were constructed by measuring genetic similarity or the genetic links between operational taxonomic units (OTUs) of populations of parents and their F_1 hybrids. The values of genetic similarity for all possible OTUs were presented in the form of a matrix to display the results (Nei 1973).

Results

Four different crosses have been assessed. In cross Shu-9511 and IC-201893, female parent Shu-9511 and F_1 pod 3 were similar, and was also found to be the same in F_1 pods 2 and 4. In case of F_1 pod 3 (Fig. 1a) the banding pattern is completely similar to female parent Shu-9511 indicating that it is selfed pod of parent Shu-9511. The banding patterns of F1 pods 2 and 4 have one extra band than both of the parents. The minimum similarity was observed in between F_1 pods 2 and 5 and also in F_1 pods 4 and 5.

The results for cross1 Shu-9511 and IC-201893 has been depicted in the form of dendrogram (Fig. 2a), which is based on genetic identity values. F1 pods 2 and 4 have more genetic identity and bifurcate from the rest of the parents and the F_1 pods. F1 pod 5 was more related with parent Shu-9511, which indicates very little contribution from the pollinator parent; thus it is near to a self-category.

In cross 2 (Shu-9511 and Shu-9701) the similarity coefficient value of parents and hybrid F_1 populations ranged around 0.273 between pollinator parent Shu-9701 and F_1 pod 7, and it was a maximum (0.800) between crossed pod 5 and F1 pod 6. While observing the zymogram banding patterns of the peroxidase isozyme, it was also found that the female parent had 10, and the male parent had 11, numbers of bands. F1 pods 1 and 6 had 13 bands; F1 pods 3 and 5 had 12 bands; F1 pods 4, 7 and 8 had 11 bands. F_1 pod 2 has same number of bands as the female parent but one band is found to be missing though with an additional band of different relative mobility, and thus it was not found to match the female parental bands (Fig. 1b). The highest genetic identity value obtained was 0.896 between F_1 pods 5 and 6 while it was a minimum of 0.658 between pollinator parent Shu-9701 and F_1 pod 8. The maximum genetic identity value from the dendrograms of this cross was observed in pollinator parent Shu-9701 from which populations 7, 8 and 4 bifurcated on one side and populations 5, 10, 6, 9, 1 and 3 bifurcated on the other side (Fig. 2b).

In cross 3 between Shu-9619 and Shu-9641, the similarity coefficient value between parent Shu-9619 and F_1 pod 4 was 1.0. This was also evident from the zymogram (Fig. 1c) that is completely similar to the female parent Shu-9619. The hybrids of F_1 pods 1, 2, 3, 5 and 6 have more extra bands than both of the parents. In F_1 pod 7 the relative mobility and intensity of bands was different from the female parents. The genetic identity (I) value was 1.0 between the female and F_1 pod 4; again confirming the selfed nature of pod 4. The dendrogram also depicted that F_1 pod 6 had a maximum genetic dissimilarity from which all the other populations bifurcated (Fig. 2c).

In cross 4, between Shu-9619 and Shu-9701 (Shining grain), the similarity coefficient value between parent Shu-9619 and hybrid plants of F_1 pod 4 was 1.0; similarly it was also 1.0 between F_1 pods 2 and 3. It is also evident from the zymogram (Fig. 1d) that the banding patterns of F_1 pod 4 were completely similar to female par-

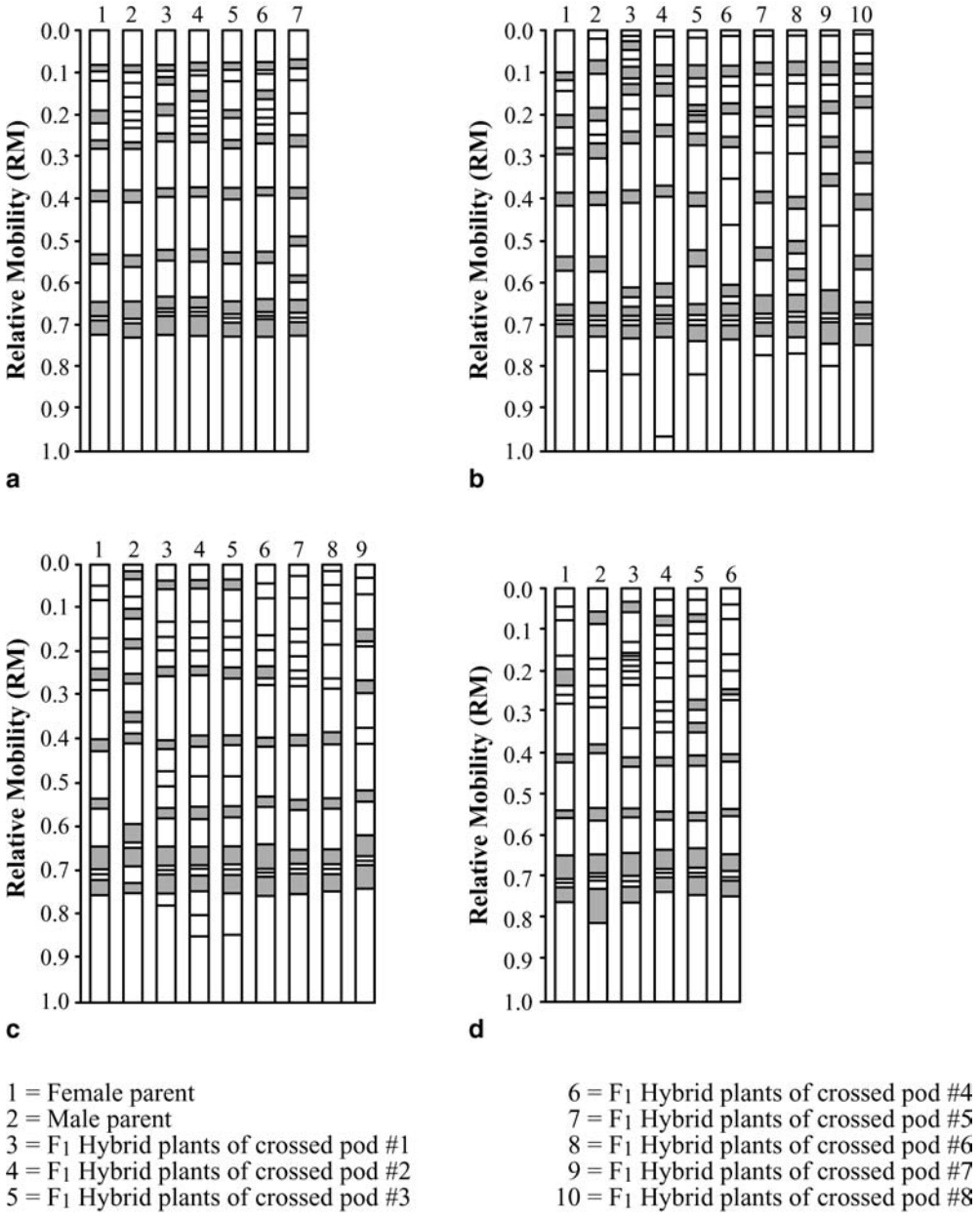

Fig. 1a–d Zymogram of electrophoretic band patterns for peroxidase isozyme of parents and various F_1 hybrids of urdbean. **a** Cross #1 – Shu-9511 (1) × IC-201893 (2). **b** Cross #2 – Shu-9511 (1) × Shu 9701 (2). **c** Cross #3 – Shu-9619 (1) × Shu-9641 (2). **d** Cross #4 – Shu-9619 (1) × Shu-9701 (2)

ent Shu-9701, indicating the selfed pod of parent Shu-9619. The hybrids of F_1 pods 2 and 3 were also similar in their banding patterns but they consisted of 2 and 3 extra bands than did the female and male parents respectively. However, F_1 pod 1 has a maximum number (15) of bands. Similar results for the populations of parent Shu-9619 and F_1 pod 4, and F_1 pods 2 and 3, have also been obtained from the genetic identity values. The results for the cross between Shu-9619 and Shu-9701 are depicted in the form of dendrogram (Fig. 2d). It is noted that populations 1 and 6 were genetically more identical (I value = 1), again confirming that at population 6 (F_1 pod 4) is a selfed one. Populations 4 and 5, as well as 2 and 3 were also found similar, because they have fallen in the same cluster group.

Discussion

The use of biochemical markers, particularly isozymes, have been extensively used in many crop plants for genetic studies and the screening of hybrids with target characters. In the crop urdbean their use has not been utilized and, hence, biochemical marker analysis has been attempted in the present investigation which was aimed at the identification of a isozyme marker(s) to develop an understanding about the selfed or crossed nature among F_1 populations, as these biochemical markers are relatively stable. The results that were obtained after such an analysis in four crosses of urdbean have clearly demonstrated that problems of the crossing programme are due to selfing, which occurs in the female parents, as well as

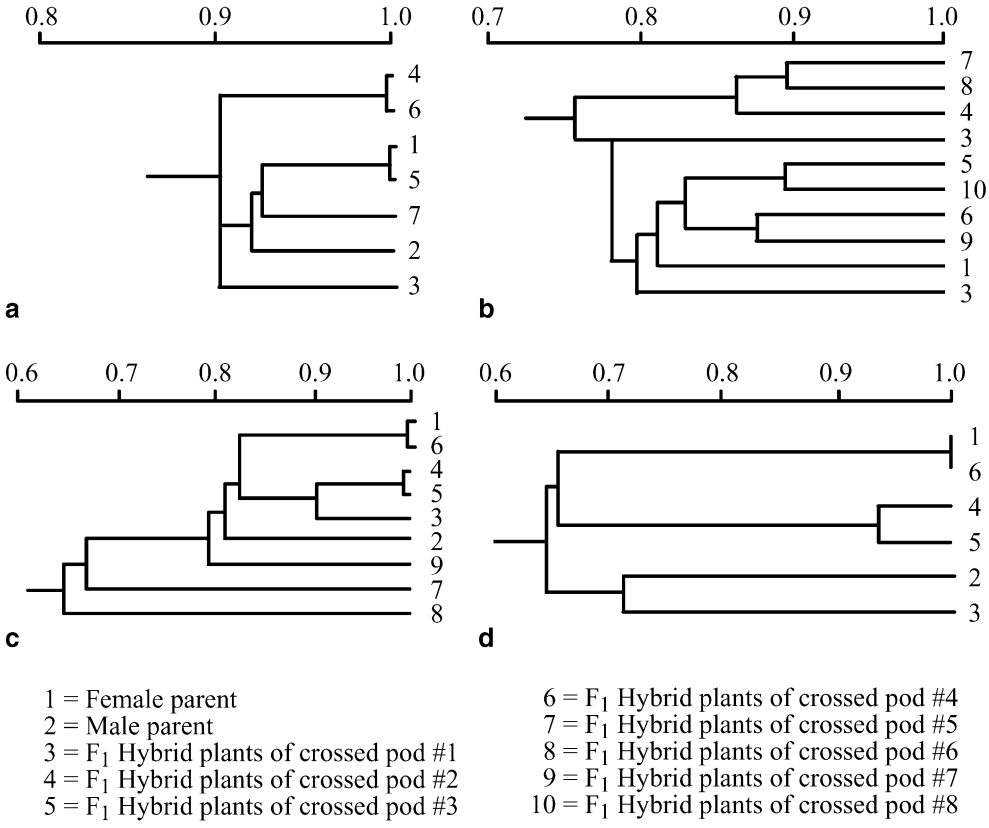

Fig. 2a–d Dendrograms showing the genetic similarity among parents and F_1 populations of urdbean. **a** Cross #1 – Shu-9511 (1) × IC-201893 (2). **b** Cross #2 – Shu-9511 (1) × Shu-9701 (2). **c** Cross #3 – Shu-9619 (1) × Shu-9641 (2). **d** Cross #4 – Shu-9619 (1) × Shu-9701 (2)

recombination that is usually achieved when the pollen fertilizes the female parent, which might be tackled with these isozymic markers. Such gene recombinations can also be studied through isozymic variation obtained in their banding patterns. The similar bands with those of the female parent were confirmatory evidence of selfing in the F_1 population, although slight morphological variations seen in the F_1 may be due to abiotic stresses. In the present study the identification of selfed pods was carried out in various crosses. The detection of the crossed nature of pods can be done on the basis of differences in the banding pattern of F_1 hybrids with their parents. It was observed in most of the cases other than selfed ones that the F_1s had a few extra bands compared to both the parents, as well as the fact that the relative mobility and thickness of bands was different the case of cross 1 between Shu-9511 and IC-201893, and F_1 pods 1, 2, 4 and 5 (Fig. 1a). In yet another case some new bands were also found to appear, which might be due to reshuffling of parental genes for a particular locus. The studies of Khanna et al. (1994) on peroxidase and esterase in wheat–barley crosses have also indicated that the hybrids showed a combination of both parental bands, and some new bands also appeared at different positions with different intensities, with some missing parental bands in a few hybrids.

The recombined nature of populations has also been observed in terms of coefficients, where similarity and genetic identity values were significantly less than 1.00. In cross 2 between Shu-9511 and Shu-9701, no cross combination with an exact similarity was obtained; thus confirming that the hybrid nature of this cross was different from that of the parents, with different degrees of recombination for this gene locus. The data on similarity coefficient genetic-identity values can be a good indicator for the degree of diversity in crosses, which is a direct function of gene recombination or a good indicator of the extent of gene reshuffling. The depiction of genetic identity (I) in the form of a dendrogram gives a convenient method for the measurement of relative diversity obtained after crossing.

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