Journal of Experimental Biology and Agricultural Sciences

http://www.jebas.org

ISSN No. 2320 – 8694

ASSESSMENT OF GENETIC PURITY OF INTER-SPECIFIC F_1 HYBRIDS INVOLVING *VIGNA RADIATA* AND *VIGNA UMBELLATA*

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Received – July 15, 2017; Revision – August 20, 2017; Accepted – October 03, 2017
Available Online – October 31, 2017

http://dx.doi.org/10.18006/2017.5(5).636.643

KEYWORDS

Crossability

Hybrid

Inter-specific

Ricebean

Mungbean

Yellow mosaic disease

ABSTRACT

Genetic purity test of true hybrids from controlled crosses before further generations of selfing or crossing and selection is essential for Mungbean improvement. The present study was conducted to transfer mungbean yellow mosaic (MYM) disease resistance in mungbean from ricebean and to assess the genetic purity of developed inter-specific F_1 hybrids using morphological features and microsatellite markers. Inter-specific crosses were made involving two genotypes of mungbean viz., K 851 and TM 96-2 and one genotype of ricebean (RBL 1) where the crossability results revealed significant differences. Crossability was recorded 8.2% (TM 96-2 × RBL 1) and 4.6% (K 851 × RBL 1). Pollen fertility was recorded 1.6% and 3.4% in TM 96-2 × RBL 1 and K 851 × RBL 1, respectively. Morphological features such as epicotyl colour, hypocotyl length, petiole length, germination habit, etc., were used as indicators of true hybridity. Further, the microsatellite markers were used to confirm the genetic purity of the developed inter-specific hybrid. These hybrids exhibited resistance against mungbean yellow mosaic disease under natural epiphytotic field conditions. The present study will be useful in developing high yielding varieties or lines of mungbean coupled with stable MYMV resistance.

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Peer review under responsibility of Journal of Experimental Biology and Agricultural Sciences.

Production and Hosting by Horizon Publisher India [HPI] (http://www.horizonpublisherindia.in/).

1 Introduction

Mungbean (*Vigna radiata* (L.) Wilczek) (2n=22), third in the series of important pulse crop, is an excellent source of easily digestible proteins. It has low flatulence which complements the staple rice and wheat diet in Asia (Selvi et al., 2006). Mungbean which is also known as green gram is one of the most important pulse crops of India grown in area of 3.83 m ha with 1.60 MT production and productivity 418 kg ha^{-1} (Directorate of Economics and Statistics, 2016). Mungbean is a self-pollinated grain legume and occupies an important position as it possess high seed protein content (22-24%) as well as has the ability to restore the soil fertility by fixing atmospheric nitrogen through symbiotic relationship with *Rhizobium* in the root nodule of the crop (Malik, 1994; Bhanu et al., 2016). The reasons for low productivity of mungbean are biotic (Mungbean yellow mosaic virus, *Cercospora* leaf spot, powdery mildew) and abiotic (heat, drought and pre-harvest sprouting) stresses (Sahoo et al., 2002). Among the biotic stresses, the most prevalent problem contributing much to this yield reduction up to 85% is yellow mosaic disease (YMD) caused by Mungbean yellow mosaic virus (MYMV), a member of family Geminiviridae, has emerged into a great threat because of its severity and ability to cause high yield loss (Haq et al., 2010).

Limiting variability prevailing among the existing germplasm coupled with low harvest index also restricts in improving the productivity of mungbean. Extensive screening of the germplasm collections of mungbean has not yielded stable source of resistance to YMD. Use of in-vivo and in-vitro techniques to induce mutagenesis for the induction of resistance has also been not so effective (Javed et al., 2016). The resistance source for mungbean yellow mosaic India virus (MYMIV) has been reported in urdbean but the significance of resistance is indistinct (Anjum et al., 2010). However, in the recent times, new sources of resistance and molecular markers associated with MYMIV have been identified (Chen et al., 2012). Karthikeyan et al. (2011) proclaimed that even though urdbean can be used effectively as resistant source for introgressing MYMV resistance into mungbean, the resistance breaks down very often due to rapid evolution of new pathotype (Kumar, 2010). Thus, to diversify and broaden the genetic base of cultivated mungbean genotypes, there is a need to look for alien gene transfer from other *Vigna* species. Introgression of alien genes from cultivated /wild species would not only minimize the risks of biotic and abiotic stresses but will also make discernible yield advances and quality in the crop (Stalker, 1980; Kumar et al., 2011). Therefore, expeditious consideration on identification of sources resistance to biotic and abiotic stresses coupled with favourable agronomic traits is indispensable (Sehrawat et al., 2014).

Ricebean [*Vigna umbellata* (Thunb.) Ohwi & Ohashi], an underutilized crop possess many useful characteristics such as disease resistance, particularly to MYMV, *Cercospora* Leaf Spot, bacterial leaf spot and bruchids along with the highest potential grain yield among the *Ceratotropis* spp. (Somta et al., 2006; Sehrawat & Yadav, 2014). However, ricebean holding immense potential is still considered as a scientifically underutilized crop which has not been subjected to systematic breeding. The plant breeding techniques such as inter-specific hybridization can be effectively used to transfer the useful characters of ricebean in other susceptible crops, consequently facilitating in developing improved varieties of food legumes for biotic stress-prone areas (Singh et al., 2013; Sehrawat & Yadav, 2014).

Genetic purity of parental variety and hybrids is of crucial importance, as one percent reduction in purity of hybrid seed, results in a reduction of about 100 kg ha^{-1} in yield of commercial crop. In the last few years, several molecular markers have been recognized that can scrutinize complex traits into individual components. Marker assisted breeding is one of the significant molecular approaches which accelerates and aids conventional breeding (Ashraf & Foolad, 2013). Application of SSRs in *V. radiata* and *V. umbellata* includes determining the identification of hybrids, phylogeny and gene mapping establishing marker-trait association using segregating populations (Michelmore et al., 1991). Keeping above mentioned points into consideration, the present investigation was undertaken to introgress YMD resistance into mungbean from ricebean using inter-specific hybridization and to evaluate the genetic purity of developed inter-specific F_1 hybrids employing morphological features and microsatellite markers.

2 Materials and Methods

2.1 Plant material and hybridization of genotypes

Seeds of ricebean genotype namely, RBL 1 and two varieties of mungbean viz., K 851 and TM 96-2 were procured from Department of Genetics & Plant Breeding, Institute of Agricultural Sciences, Banaras Hindu University, Varanasi. Seeds of parental lines were planted in crossing block in cemented pots during the Kharif, 2015. Hybridization was done using the method given by Boling et al. (1961). The stigma of the mungbean remains highly receptive during the early hours of the day. Consequently, emasculation was done between 04:00-06:00 pm and pollination in the subsequent morning between 06:00 and 08:00 am. Large number of flowers were pollinated for each cross, in order to obtain higher number of hybrid (crossed) pods. The pure seeds of two varieties of mungbean namely K 851 and TM 96-2 and one of ricebean i.e. RBL 1 and crossed seed (F_0) of two inter specific hybrids viz., K851 × RBL 1 and TM 96-2 × RBL 1 were raised, during Kharif, 2016-2017.

2.2 Morphological characterization

Observations were recorded on crossability, growth habit, plant type, leaf shape, leaf colour, length/ breadth ratio of primary leaf, petiole length (cm), flower colour, pod character, upper epicotyl colour, hypocotyl length, raceme, number of tubercles per inflorescence, keel tip colour, germination, 100-seed weight besides pollen fertility (%). Viability of the fresh pollen samples was determined on the basis of observations on stainability of fresh pollen grains by acetocarmine technique as described by Roberts (1977). Two hundred pollen grains were counted per slide with five slides each. Percent pollen fertility was calculated as (total number of stained pollen/total number of pollen) × 100. Normal deeply stained pollen grains were counted as viable, while weakly stained were recorded as non-viable (Pearson & Harney 1984).

2.3 Field evaluation for YMD resistance

Field trials for screening of YMD were done under natural conditions. One row of infector line CO-5 variety of urdbean was raised after every two test entries to evaluate MYMV symptoms. Plants were randomly selected and their leaves showing clear symptoms (veinal yellowing and scattered bright yellow spots) and total leaves were counted and percent disease incidence was calculated (Wheeler, 1969). Genotypes were scored on 0-5 arbitrary scale as Highly Resistant (HR), Resistant (R), Moderately Resistant (MR), Susceptible (S) and Highly Susceptible (HS) based on disease severity (Bashir et al., 2005; Akhtar et al., 2009).

2.4 Microsattelite Markers (SSRs) used in the study

For the molecular confirmation of hybrid purity of the hybrids obtained in the present study, the adzukibean-specific simple sequence repeat markers (SSRs) CEDG035 and G 1034 showing polymorphism between the parental lines were utilized.

2.5 Genomic DNA extraction and PCR Amplification

Young leaves from 15-20 days old seedlings of all the F_1 plants and their respective parents were collected. Total genomic DNA was extracted using the CTAB DNA extraction method (Doyle & Doyle, 1987). The quantification of the DNA was assayed on 0.8% agarose gel electrophoresis in 1X TAE. PCR reactions with SSR markers were performed in a 15 µl volume of PCR mix which consisted of 50 ng of template DNA and 10X Taq Buffer (with $MgCl_2$) in a final concentration of 1X 1.0 µM of each forward and reverse primer (Sigma), 10 mM of dNTPs, and 0.03 units of Taq DNA polymerase (5U/µL). The PCR reaction was run on a thermocycler (Mastercycler eppendorf, USA). PCR amplification was done following the protocol consisting of initial denaturation at 94 °C for 30 s followed by 35 cycles 94 °C for 30s (denaturation), annealing at 55-60 °C for 1 min, and 72 °C for 30 s (elongation), followed by the final extension at 72 °C for 5 min and cooling at 4 °C for 10 min. The amplified products from SSR markers were resolved with 2.5% agarose gel electrophoresis. Ethidium bromide was used for staining the gels, which were subsequently visualized and photographed on a Gel Documentation System (GEL DOC TM XR^+, BIORAD USA). Consequently, the DNA banding patterns of the PCR-amplified products of the derived inter-specific hybrid plants were compared with their respective male and female parents to confirm their true hybrid nature.

3 Results and Discussion

3.1 Crossability and morphological verification of hybrid purity

In present investigation, interspecific hybridization involving two genotypes of mungbean and one of ricebean have been effected to study the germination, as well as fertility behavior and attempt has been also made to assess the usefulness of testing the purity of the newly developed interspecific hybrids using morphological features and microsatellite markers.

The 30 crossed seed of each of the F_1 and 10 seeds of each of the parents were sown in the pots (05 seed per pots) during Kharif, 2016. Out of 30 seeds only 15 F_1 plants could survive in each of the two cross.

Considerable variation was found in interspecific crosses. The number of seeds per pod in F_1 hybrid varied from 1 to 3. The crossability was recorded 8.2% and 4.6% in the interspecific cross, TM 96-2 × RBL 1 and K 851 × RBL 1 respectively. Varying degree of success in interspecific hybridization was reported in previous studies also (Chen et al., 1977; Ahn & Hartmann, 1977; Ahn & Hartmann, 1978) owing to reproductive obstructions between the species (Adinarayanamurty et al., 1993). Nevertheless, the present study revealed that all the selected male and female parents were cross-compatible with each other. Inter-specific hybrids showed poor germination as only 50% crossed seeds germinated. These findings showed that *V. radiata* is crossable with *V. umbellata* and corroborate with earlier findings on hybridity among different *Vigna* species (Pandiyan et al., 2010; Singh et al., 2013; Sehrawat & Yadav, 2014).

The hybrid seedlings initially showed poor growth. However, after seedling stage vigorous growth of the hybrid plants was observed. The inviability or weakness of the F_1 seedlings could be due to disharmonies between genomes of the parental species; between genomes of one species and cytoplasm of the other or between genotypes of F_1 zygote and genotype of endosperm or maternal tissue (Cooper & Brink, 1940; Stebbins 1958; Gill & Waines, 1978, Monika et al., 2001).

The morphological characterization of F_1 hybrids and the parental genotype are depicted in **Table 1.** The germination habit of F_1 seedlings was intermediate between the parental species. The position of cotyledon of the hybrid seedlings were at the soil surface, and the average length of hypocotyl was 2.5 cm. The indeterminate growth character of *V. umbellata* was dominant in the F_1 plants. Change in growth habit was exhibited in the hybrids. The hybrids showed indeterminate vegetative growth which was in contrast to the determinate growth of the female parent. The growth habit of hybrid plant was intermediate, semi erect and compact type and characterized by a thicker stem with good branching (Figure 1).

Pollen fertility was observed 1.6% and 3.4% in TM 96-2 × RBL 1 and K 851 × RBL 1, respectively. The unstained pollen grains were variable in size, while the stained pollen grains were usually larger than the parental pollen. The pollen fertility was lower in F_1 as compared to parents (Figure 2). The reduced fertility has been ascribed due to pairing abnormality and the formation of the anaphase bridges and laggards leading to the unequal distribution of the chromosome by various workers (De & Krishnan, 1967; Biswas & Dana, 1975; Anandabaskaran & Rangasamy, 1996; Kaur & Satija, 1998; Gupta et al., 2002**).**

Pseudo-pod formation took place which later degenerated. This may be due to embryo disharmony. However, wherever fertile pods formed, a small increase in seed size was observed over the female parent. Large seed size may provide yield advantage and other yield-related attributes.

Evaluation of the morphological features of hybrid plants from germination to maturity assists in testing the genetic purity of hybrids (Dongre et al., 2010). Consequently, inheritance of bi-parental morphological characteristics by the inter-specific hybrids confirmed their genetic purity.

3.2 Molecular confirmation of hybrid purity

The SSR marker G 1034 primer pair produced a specific but reproducible band of 100 bp in the male parent and 200 bp in both parents. Likewise, the adzukibean- specific SSR CEDG 035 amplified a reproducible band of one male (165 bp) and female (146 bp) specific repeats. Consequently, all the F_1 inter-specific hybrids produced both bands specific to their respective parents (Figure 3 and 4). The complementary banding pattern of the male and female parents makes a way to identify the hybrid (Sudharani et al., 2014).

Table 1 Comparison among *V. radiata*, *V. umbellata* and their hybrid

Sl. No.	Character	*V. radiata*	*V. umbellata*	Hybrid
1	Growth habit	Determinate	Indeterminate	Indeterminate
2	Plant type	Erect and compact	Semi-erect and semi-compact	Semi-erect and semi-compact
3	Leaf shape	Ovate	Lanceolate	Lanceolate
4	Leaf colour	Dark green	Light green	Light green
5	Length / breadth ratio of primary leaf (cm)	6.15 /5.8 = 1.06	5.4/1.4 = 3.85	3.16/1.11= 2.84
6	Length of petiole (cm)	12.2	6.5	9.6
7	Flower colour	Light yellow	Dark yellow	Dark yellow
8	Pod	Hairy	Non-hairy	Hairy
9	Pollen fertility	>95%	>95%	1.6-3.4%
10	Upper epicotyl colour	Green	Purple	Light Purple
11	Hypocotyl length (cm)	6.5	0.0	2.5
12	Raceme	Often compound	Simple	Simple
13	Number of tubercles per inflorescence	5-7	12-16	8-10
14	Keel tip colour	Grayish	Bright yellow	Light yellow
15	Germination	Epigeal	Hypogeal	Intermediate
16	100- seed weight	3.5 g	5.6 g	4.3 g

Figure 1 Comparison of F_1 hybrid with parents

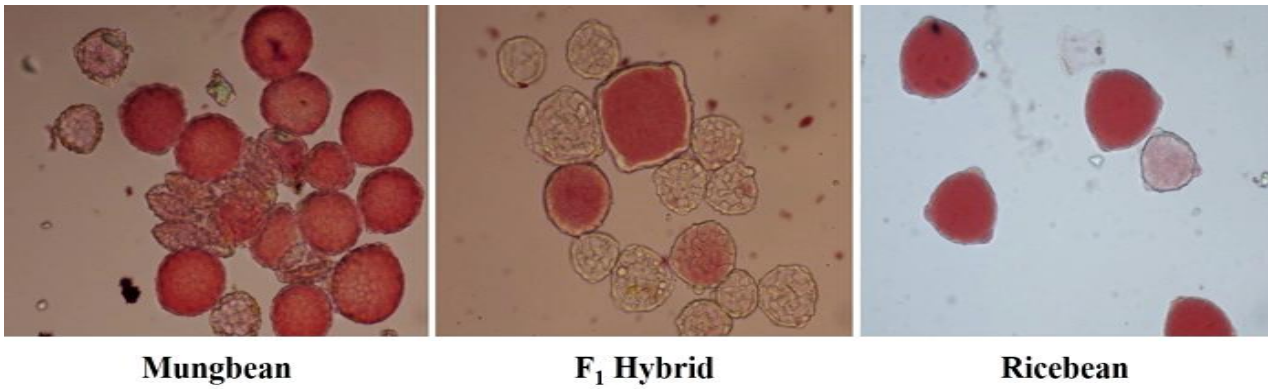

Figure 2 Comparison of pollen of F_1 hybrid with parents

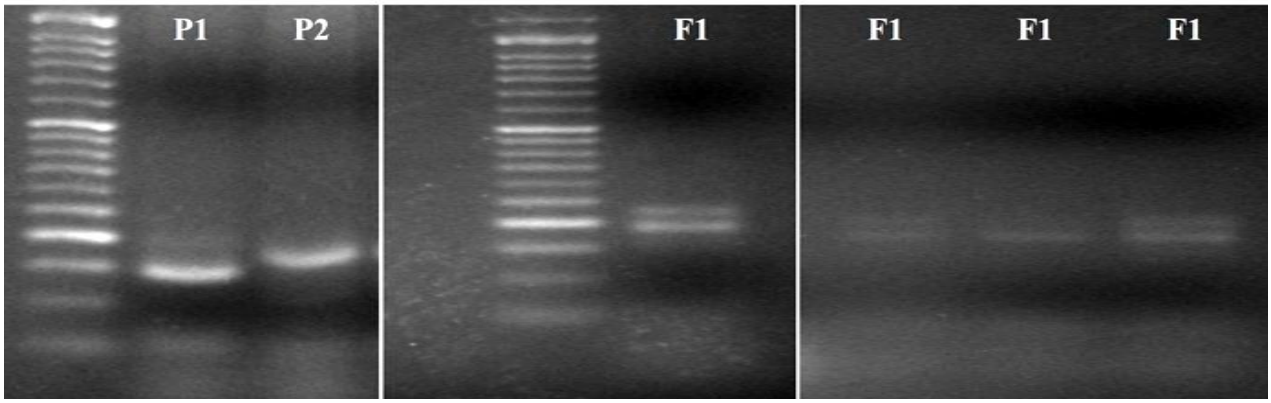

Figure 4 Confirmation of the purity of the hybrids using adzukibean-specific microsatellite G 1034 marker

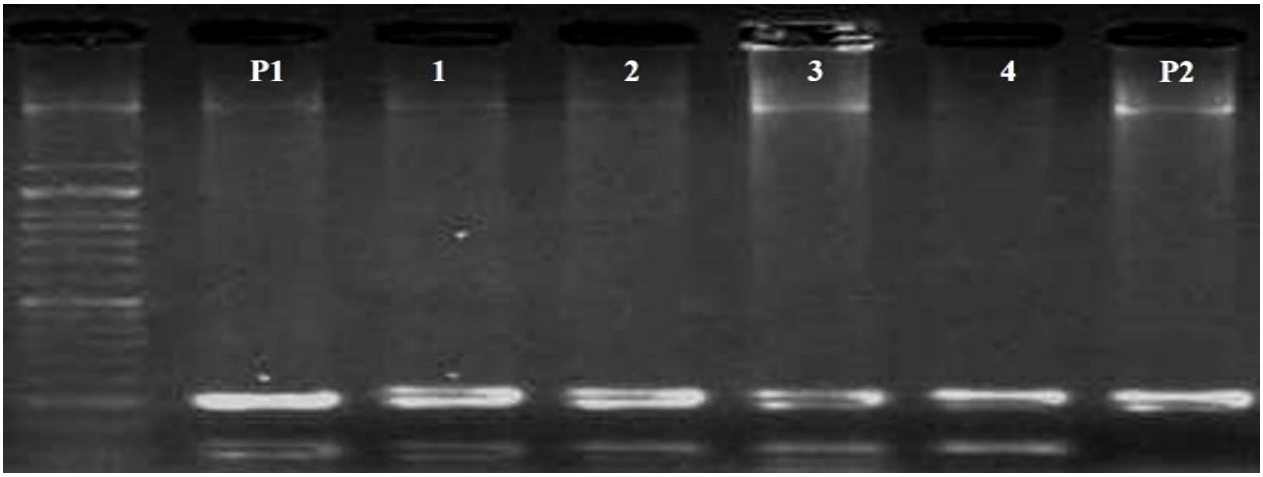

Figure 4 Confirmation of the purity of the hybrids using adzukibean-specific microsatellite G 1034 marker

The higher degree of resemblance between the male parent and offspring as well as their male specific band marker imparts a clear indication that the offspring is successful crop and true hybrid of *V. radiata* × *V. umbellata*. Consequently, these SSR markers were highly expedient for the identification of the true hybrid during the study and can be used unambiguously as referral markers for the identification of hybrids. The amplification of microsatellite marker across related legumes will upsurge their efficacy in breeding program (Dikshit et al., 2012).

3.3 Field evaluation of hybrids for YMD resistance

In the present investigation, screening of genotypes of mungbean and ricebean showed that ricebean genotype, RBL 1 was highly resistant. However, the mungbean genotype viz., TM 96-2 and K 851 were moderately resistant and highly susceptible respectively. During Kharif 2016, three interspecific crosses along with the parents were screened for MYM disease symptoms under natural epiphytotic field conditions. The results showed that all the hybrids and the ricebean genotype (RBL 1) were highly resistant to MYM disease, while the mungbean genotype viz., K851 was highly susceptible. Present study corroborates earlier findings of Pandiyan et al. (2010) and Sehrawat & Yadav (2014). Further evaluation of segregating generations of these hybrids will help in determining the inheritance of resistance and lead to development of stable and improved variety of mungbean.

Conclusions

Exploitation of novel genes and alleles from exotic germplasm is needed which can be a major source of robust donors for both biotic and abiotic resistance. The present study concludes that ricebean genotypes exhibits a high level of resistance against YMD which can be utilized as donor parent in inter-specific hybridization programme to develop resistant varieties in other vulnerable *Vigna* species coupled with high yield potential. The morphological characterization and the SSR–PCR based molecular verification efficiently proved the genetic purity of the inter-specific hybrids. Morphological features such as epicotyls, colour, petiole length and germination habit are a good indicator of true hybridity. Adzukibean derived microsatellite markers can aid in genetic improvement of mungbean or other *Vigna* species by means of genomic studies for tagging and mapping agronomically important traits using marker-assisted breeding for desired traits. To overcome the limitations of the narrow genetic base of mungbean crop, conventional breeding approaches accompanied with biotechnological techniques will prove to be valuable. Last but not the least, F_2 populations developed from the inter-specific hybridization can be very much useful in mapping population or developing recombinant inbred lines for the identification of gene/ quantitative trait loci for MYM disease resistance.

Acknowledgement

We are grateful to the Department of Science and Technology, Government of India, for funding and supporting the research programme by providing the DST INSPIRE Fellowship to the first author (A. N. B).

Conflict of Interest

Authors would hereby like to declare that there is no conflict of interests that could possibly arise.

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