Legume Research, 41(4) 2018: 526-531
Print ISSN:0250-5371 / Online ISSN:0976-0571

AGRICULTURAL RESEARCH COMMUNICATION CENTRE
www.arccjournals.com/www.legumeresearch.in

Generation of interspecific hybrids for introgression of mungbean yellow mosaic virus resistance in [*Vigna radiata* (L.) Wilczek]

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Received: 03-12-2016 Accepted: 15-06-2017 DOI: 10.18805/LR-3808

ABSTRACT

Vigna radiata genotypes *viz.*, SML 668 and SML 832 and *V. mungo* genotypes *viz.*, Mash 114 and Mash 218 were crossed in all possible combinations during summer 2015 to generate F_1 hybrids. Interspecific hybridization was attempted by using *V. radiata* genotypes as female parent. Pod set percentage varied from 5.5 percent (SML 832 x Mash 218) to 24.1 percent (SML 832 x Mash 114). The germination percentage ranged from 14.29 to 30.56. Maximum pollen fertility was observed in cross SML 668 x Mash 114 (28.36 percent) followed by SML 668 x Mash 218 (27.03 percent), SML 832 x Mash 218 (24.32 percent) and minimum in SML 832 x Mash 114 (22.59 percent).The purity of hybrids were tested through microsatellite markers. For parental polymorphism, microsatellite markers were selected from related *Vigna* species such as *Vigna unguiculata, Vigna radiata and Vigna mungo*. Out of 84 markers used, 46 were polymorphic i.e 54.76 per cent polymorphism between parents. These polymorphic markers were used for confirmation of hybrids produced from different crosses. All the F_1 plants gave resistant reaction to Mungbean yellow mosaic virus (MYMV) indicating the introgression of resistance gene(s) from *V. mungo* to *V. radiata.*

Key words: Interspecific hybrids, MYMV, SSR markers, *V. mungo, V. radiata*.

INTRODUCTION

Mungbean [*Vigna radiata* (L.) Wilczek] also known as green gram is an important food legume crop with wide adaptability, well suited to a large number of cropping systems and low input requirement. It is an excellent source of easily digestible good quality protein. Mungbean is consumed as bean, sprouts, noodles, green beans and boiled dry beans. It improves soil health and also offers the potential for increased income for small-scale farmers. Recently, development of short duration varieties of mungbean with synchronous maturity, reduced photosensitivity, high yield and disease resistance offered a great scope for its introduction as a catch crop in rice-wheat cropping system.

In India, mungbean is grown on an area of 3.0 million hectare with average production of 1.50 million tonnes (Anonymous 2016). The average yield of mungbean is very low, not only in India but in entire tropical and sub-tropical Asia. This is due to inherent low yield potential of existing cultivars and their susceptibility to MYMV (Mungbean Yellow Mosaic Virus) and other foliar diseases.

Mungbean yellow mosaic virus (MYMV) is the most dreadful disease of mungbean, which is transmitted by whitefly, *Bemisia tabaci* (Nene 1972). Whitefly can be controlled effectively by insecticides. But the cost and unavailability of pesticides, hinders the effective control of the disease. Moreover, use of pesticides is ineffective under severe whitefly infestations. Thus, there is urgent need to introduce long term resistance for MYMV into mungbean in order to stabilize its cultivation and productivity in northern India. Urdbean, *V. mungo* (L.) Hepper possesses some desirable traits including synchronous maturity, non-shattering pods and more durable resistance which can be transferred to mungbean through hybridization (Singh 1990). So, interspecific hybridization is one of the methods of creation of genetic variability and widening of genetic base of a crop species.

MATERIALS AND METHODS

Plant material: Two genotypes of mungbean (SML 668 and SML 832) and two genotypes of urdbean (Mash 114 and Mash218) were crossed in all the possible combinations at the field area of Department of Plant Breeding & Genetics, Punjab Agricultural University. Mungbean genotypes SML 668 and SML 832 are high yielding varities but they are susceptible to MYMV, if grown during *kharif* season. Mash 218 and Mash 114 both are urdbean genotypes cultivated in Punjab state during summer and *Kharif* seasons, and they are highly resistant to MYMV and other foliar diseases. The emasculations and pollinations were carried out as per the procedure given by Boiling *et al.* (1961) using *Vigna radiata* as the female parent. To enhance the crossability and pod setting percentage 4 ppm NAA (α- Naphthyl acetic acid)

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was applied to pedicel of emasculated buds as well as to pollinated buds twice a day at least for ten days consecutively.

SSR marker analysis: Young leaves of parents and F_1 hybrids were collected from each cross for SSR marker analysis. DNA of individual F_1 plant and their parental lines was extracted using CTAB method (Doyle and Doyle 1987) with slight modifications. The quality of DNA was checked on 0.8% agarose gel and the quantity was determined using UV spectrophotometer. For parental polymorphism, microsatellite markers were used which were selected from related *Vigna* species such as *Vigna unguiculata, Vigna radiata* and *Vigna mungo*. PCR reactions were performed in 10 µl volume containing 2.0 µl genomic DNA, 1.5 µl dNTPs, 2.0 µl PCR buffer (5X), 0.8 µl $MgCl_2$, 0.8 µl of both forward and reverse primers, 1.0 µl Taq polymerase and 1.1 µl autoclaved distilled water. PCR amplifications were performed in an Eppendorf Mastercycler (Eppendorf, Hamburg, Germany) using the following thermal profile: 1 cycle of 94 ° C for 4 min, followed by 35 cycles of 94 °C for 1 min, Annealing for 1 min at respective temperature of primer, 72 °C for 1 min and a final extension of 72 °C for 10 min. PCR products obtained were resolved by electrophoresis on 2.5 percent high resolution metaphore agarose stained with ethidium bromide and photographed by using Gel Documentation system. 100 bp DNA ladder was used to for approximate sizing of bands.

RESULTS AND DISCUSSION

Generation of interspecific hybrids and crossability studies: Interspecific hybridization has played an essential role in combining different desirable traits of related species in genus *Vigna* and thus broaden its genetic base. *Vigna radiata* (mungbean) and *V. mungo* (urdbean) are primarily self-pollinated crops. Crossability measured as pod set percentage seems to be genotype dependent. In total 296 crosses were attempted between genotypes of *V. radiata* and *V. mungo* during summer 2015 (Table 1). The F_1 seeds obtained from these crosses were planted during *kharif* 2015 to study morphological traits of putative hybrid plants.

In the first cross, i.e. SML 668 x Mash 114, 167 flowers buds were emasculated, out of which only 107 flower buds were left, which were pollinated with pollen from Mash 114. Thus pod set percentage was 10.3 percent. In yet another cross SML 832 x Mash 218, 133 flower buds were emasculated and 90 buds were pollinated which produced 5 pods with pod set percentage of 5.5. While pod set percentage was 20.0 in cross SML 668 x Mash 218 and 24.1 in cross SML 832 x Mash 114. Thus pod set percentage varied from 5.5 percent (SML 832 x Mash 218) to 24.1 percent (SML 832 x Mash 114). Similarly, Pandiyan *et al* (2012) reported that by using wide hybridization in mungbean, intermediates can be obtained and different morphological plant types can be developed to create genetic variation. They reported crossability percentage of 10.25 in the cross of *Vigna radiata* x *V. trilobata*. In yet another study, Sehrawat *et al* (2016) reported the crossability percentage of 0.6 to 2.89 with an average value of 1.87 percent in interspecific crosses between genotypes of urdbean and ricebean.

Varying degree of success has been reported earlier in different interspecific crosses of *Vigna* species. Bharathi *et al* (2006) attempted crosses between different *Vigna* species and found that mungbean (*Vigna radiata)* x ricebean (*V. umbellata)* was the best cross combination that showed highest pod set percentage of 29.6. They also reported that flower drop after pollination and low seed set suggests the presence of post-fertilization barriers. Pandiyan *et al* (2010) crossed *Vigna radiata* as female with thirteen wild *Vigna* species with an objective to transfer genes for MYMV and bruchid resistance. They observed highest pod set of 25 and crossability of 21.9 percent in the cross *V. radiata* x *V. radiata* var. sublobata. However, lowest pod set of 2.0 percent was recorded in the cross *V. radiata* x *V. dalzellina*. So, crossability between two species is a pre-requisite for gene transfer through wide/interspecific hybridization. Understanding the crossability relationship among different species would be helpful in choosing methods to produce F_1 hybrids.

Number of seeds in the crossed pods: In total 36 seeds were obtained from 11 pods of cross SML 668 x Mash 114, 12 seeds from 5 pods in case of SML 832 x Mash 218, 28 seeds from 14 pods in case of SML 668 x Mash 218 and 15 seeds from 7 pods in cross SML 832 x Mash 114. The length of crossed pod was very small as compared to the pods of parental genotypes (Fig.1A). On an average each pod contained 1-4 seeds. Similar observations on number of seeds per pod in the cross between *Vigna radiata* x *V. mungo* were reported by Gosal and Bajaj (1983). Sehrawat *et al* (2016) reported that number of F_1 seeds per pod in interspecific crosses between genotypes of urdbean and ricebean varied from 1 to 4.

Table 1: Crossability between *V. radiata* and *V. mungo*.

Crosses	Total no. of flowers buds emasculated	Total no. of flowers buds pollinated	No. of mature pods	Pod set percentage	No. of seeds sown	No. of seeds germinated	Germination percentage
SML 668 x Mash 114	167	107	11	10.3	36	11	30.56
SML 832 x Mash 218	133	90	5	5.5	12	3	25.00
SML 668 x Mash 218	91	70	14	20.0	28	4	14.29
SML 832 x Mash 114	47	29	7	24.1	15	3	20.00

Fig 1: A. Pods and **B.** Seeds of parental genotypes SML 668, Mash 114 and their F_1

Table 2: Different *V. radiata* and *V. mungo* cultivars used in crosses to produce F_1 hybrids, total number of F_1s produced and number of true F_1s as identified from SSR markers analysis.

V. radiata	*V. mungo*	Total no. of F_1s produced	No. of true F_1 (SSR marker analysis)
SML 668	Mash 218	4	4
SML 668	Mash 114	11	5
SML 832	Mash218	3	3
SML 832	Mash 114	3	3

The F_1 seeds obtained from all four cross combinations were small, wrinkled and shrunken (Fig.1B). The F_1 seeds were small in size and shriveled because of the poor development of the endosperm and embryo which is due to incompatibility between the two parental genomes (Rashid *et al* 1987). Generally the hybrid seeds from interspecific hybridization were shriveled or partially filled and empty as was reported by earlier workers (Biswas and Dana 1976; Satijia and Vikal 1993).

Germination of hybrid seed: In different cross combinations, the germination percentage ranged from 14.29 to 30.56. In the cross SML 668 x Mash 114, number of seeds sown were 36 and germination percentage was found to be 30.56. While it was 25.0 in case of SML 832 x Mash 218, 20.0 in SML 832 x Mash 114 and 14.29 in case of SML 668 x Mash 218. Pandiyan *et al* (2010) recorded germination percentage in range of 16.6 to 60 percent in interspecific crosses between *Vigna radiata* and 13 wild *Vigna species*. Similar studies were conducted by Subramanian (1980) where he studied seed set and germination in interspecific crosses between *Vigna radiata* and *V. mungo*. The hybrid seeds showed germination of 22 percent. In a crossability study between green gram and black gram by Shanmugan *et al* (1983), the success rate varied from 0.7 to 11.5 percent. Pandiyan *et al* (2012) reported the crossability percentage of 10.25, hybrid seed germination 34.21 and hybrid lethality 53.84 percent in cross between *Vigna radiata* and *V. trilobata*.

Pollen fertility: In mungbean and urdbean parents, the pollen fertility varied from 94.12 to 100 percent (Table 3). However it was very low in the interspecific hybrids, ranging from 22.59 to 28.36 percent. Maximum pollen fertility was observed in SML 668 x Mash 114 (28.36 percent) followed by SML 668 x Mash 218 (27.03 percent), SML 832 x Mash 218 (24.32 percent) and minimum in SML 832 x Mash 114 (22.59 percent) (Table 4).

Completely sterile F_1 hybrids have been reported by Biswas and Dana (1976). The reduced fertility of F_1 hybrids results from meiotic abnormalities such as more number of univalents, formation of anaphase bridges, presence of laggards, thus leading to unequal distribution of the chromosomes in about 50 percent of the cells. However, Gosal and Bajaj (1983) observed that F_1 hybrids from the cross *Vigna radiata* x *V. mungo* were partially fertile and produced viable seeds for the next generation.

The low crossability between mungbean and urdbean is also due to diversity between parental genomes. It may also be due to flower shedding before or after pollination. The flower sheds as a result of particular physiological injuries to style and stigma, when interspecific crosses are made. Such forms of physiological shocks could be reduced through the application of growth regulators like gibberallic acid, E-amino caproic acid and naphthalene acetic acid to the pedicle of emasculated buds (Gosal and Bajaj, 1983). In the present study, use of 4 ppm NAA helped in retention of emasculated flowers to a great extent.

Few of these hybrid plants were easily identified as true hybrids as their plant type such as, leaf shape, pod size and branching pattern did not resemble either of parents but identification of other F_1s was not possible visually. So, confirmation of these F_1 hybrids was done using SSR markers.

Table 3: Pollen fertility (percent) of *Vigna radiata* and *V. mungo* genotypes.

Genotype	Pollen fertility
SML 668	100.0
SML 832	96.25
Mash 114	99.14
Mash 218	94.12

Table 4: Pollen fertility (percent) of the hybrids from crosses between *Vigna radiata* and *V. mungo*.

Total No. of pollen grains	No. of fertile pollens	No. of stertile pollens	Pollen fertility
		SML 668 X Mash 114	
50	14	36	**28.36**
92	26	66	
98	30	68	
82	23	59	
87	23	64	
		SML 668 X Mash 218	
22	5	17	**27.03**
26	7	19	
25	9	16	
18	5	13	
20	4	16	
		SML 832 X Mash114	
55	8	47	**22.59**
100	19	81	
37	8	29	
65	17	48	
75	23	52	
		SML 832 X Mash218	
67	19	48	**24.32**
31	10	21	
72	11	61	
48	15	33	
78	17	61	

Confirmation of interspecific hybrids through SSR markers: Out of 84 SSR markers used for parental polymorphism, 46 were polymorphic i.e. 54.76 percent polymorphism between parents: SML 668, SML 832 and Mash 114, Mash 218. These polymorphic markers were used for hybrid confirmation (Table 5). F_1 hybrids from cross SML 668 x Mash 114 were confirmed using two primer pairs namely VR 0200 and VR 0223. The primer pair VR 0200 produced a PCR product of 110 bp in parent SML 668 but PCR product of 120 bp in Mash 114, while PCR products of both size were present in true hybrids (Fig 2). Similarly, primer pair VR 0223 was used for confirmation of hybrids which produced a PCR product of 150 bp in parent SML 668 and 135 bp in parent Mash 114 and PCR products of both size were present in true hybrids. Out of eleven putative hybrids (Table 2), only five hybrid plants were found to be true on the basis of molecular analysis. F_1 hybrids from cross, SML 832 x Mash114 were confirmed using primer pair VR 0293, VR 0223 and CEDG 008. The primer pair VR 0223 produced a PCR product of 150 bp in parent SML 832 and 135 bp in parent Mash 114 and PCR products of both size were present in true hybrids. Similar, results were shown by primer pairs VR 0293 and CEDG 008. Three true hybrid plants from cross SML 832 x Mash114 were confirmed using SSR markers. Primer pair VR 0293 was used for confirmation of four hybrid plants produced from cross SML 668 x Mash 218. PCR product of size 135 bp was produced in parent SML 668 and that of 145 bp in parent Mash 218. Presence of PCR products of both sizes confirmed the hybrid to be true. Primer pair VR 0200 was used for confirmation of three hybrid plants produced from cross SML 832 x Mash 218. PCR product of size 110 bp was produced in parent parent SML 832 and that of 120 bp in parent Mash 218. Presence of PCR products of both sizes confirmed the hybrid to be true.

The similar DNA banding pattern between hybrids and their parents confirmed the purity of the tested hybrids. The amplification of SSR markers across related *Vigna*

Table 5: Details of polymorphic SSR markers used for hybrid confirmation.

Code	Primer sequence	Annealing Temp	Reference
VR 0200R	TGGGAAATAAAGAAAGCGTAGG	55^0C	Tangphatsornruang *et al* (2009)
VR 0200F	CTCTTCTCCTTTGCCTCTACAAA		BMC Plant Biology2009, 9:137
VR 0223R	GCGTGATCGAGGCAGACTAT	55^0C	Tangphatsornruang *et al* (2009)
VR 0223F	GTGGGTAGCTCGGTAATAGCAC		BMC Plant Biology2009, 9:137
VR 0293R	GTGGCTCACAAGGTAGTGCTAA	52^0C	Tangphatsornruang *et al* (2009)
VR 0293F	GAGAGAAACAACCAACCAAAGG		BMC Plant Biology2009, 9:137
Ced G 008R	AGGCGAGGTTTCGTTTCAAG	52^0C	Somta *et al* Plant Breeding 125, 77-84, 2006
Ced G 008F	GCCCATATTTTTACGCCCAC		

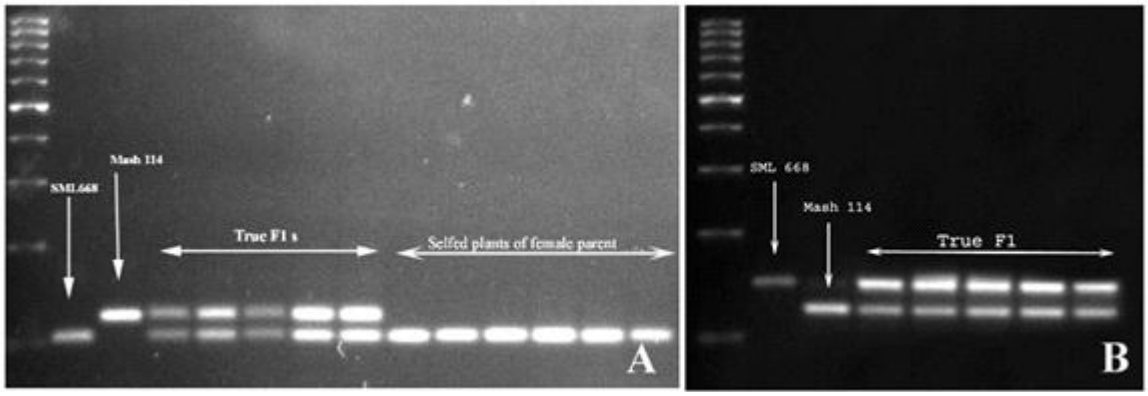

Fig 2: PCR amplification of SML 668, Mash 114 and their hybrids with VR 0200(A) and VR 0223(B) marker on 2.5 percent agarose gel

Fig 3: MYMV reaction of SML 668, F_1 (SML 668 x Mash 114) and Mash 114.

species increase their utility in verification of interspecific hybrids (Dikshit *et al*. 2012). In previous reports microsatellite markers have been successfully used for cultivar characterization, purity testing in other crop plants and identification of hybrids, (Tabbasam *et al*. 2006; Sehrawat *et al*. 2014).

Field Evaluation of hybrids for MYMV resistance: The interspecific hybrids and their parents were screened for MYMV reaction under natural epiphytotic field conditions during *kharif* 2015. The rating scale 1-9 (Shukla *et al*. 1978) was used to score MYMV reaction. The hybrid plants from all the cross combinations and their pollen parents (Mash 114 and Mash 218) gave highly resistant reaction to MYMV. Whereas, the female parents (SML 668 and SML 832) gave highly susceptible reaction to MYMV (Fig.3). Similar results were reported by Sehrawat *et al*. (2016) in interspecific hybrids developed from crosses between urdbean and rice bean genotypes. Pal *et al* (2000) also transferred MYMV resistance from related *Vigna* species; *V. sublobata* and *V. mungo* to *V. radiata* through interspecific hybridization. The hybrids obtained were resistant to MYMV. The F_2 populations of interspecific hybrids generated in the present study will be further screened to determine the inheritance of resistance gene(s) introgressed from urdbean into mungbean genotypes. Identification of molecular marker(s) associated with resistance gene, will increase the efficiency and accuracy in MYMV-resistance breeding program and could be used in future for the development of high yielding mungbean yellow mosaic virus resistant cultivars of mungbean and urdbean.

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