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Introgression of mungbean yellow mosaic virus resistance in *Vigna mungo* (L.) Hepper and purity testing of F1 hybrids using SSRs

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Abstract: The present study was designed to transfer mungbean yellow mosaic virus (MYMV) resistance in urdbean from ricebean. Four MYMV-resistant ricebean genotypes (RBL1, RBL6, RBL35, and RBL50) were hybridized as males with the MYMV-susceptible urdbean line PS 1 using hand emasculation and pollination techniques under field conditions. The results revealed significant differences in the crossability of ricebean genotypes with an urdbean genotype. The highest number of crossed pods was obtained from the interspecific cross PS1 × RBL35. Crossability ranged from 0.6% to 2.89% with an average value of 1.87%. The number of F1 seeds in the crossed pod varied from 1 to 4. Molecular and morphological characterization verified the genetic purity of the developed hybrids. These hybrids exhibited resistance against MYMV infection under natural epiphytotic field conditions. The present study will help to develop improved varieties or lines of urdbean with stable MYMV resistance.

Key words: Interspecific hybridization, hybrid purity, ricebean, urdbean, MYMV resistance

1. Introduction

Urdbean (*Vigna mungo* L. Hepper) is a staple grain legume crop in Central and Southeast Asia (Delic et al., 2009). It is native to India and commonly known as urd (Vavilov, 1926). It is an important protein source for vegetarians and the poor in a cereal-based society due to easy digestibility of protein without flatulence (Karamany, 2006). Symbiosis with soil *Rhizobium* spp. results in atmospheric nitrogen fixation and, therefore, a restoration of soil fertility. Its rapid growth, early maturity, conservation of soil nutrients, and utilization of the leftover soil moisture after rice cultivation also makes it valuable in various cropping systems (Ahmad et al., 2001). Among the pulses, blackgram occupies the fourth position in production and acreage (Deepalakshmi and Anandakumar, 2004). However, the productivity of blackgram is still low and cannot fulfill the domestic consumption demand of India (Muruganatham et al., 2005). Considering its socio-economic importance, this crop is neglected in breeding research, both at the national and international levels.

The major yield-limiting factors are various biotic (viruses, fungi, bacterial pathogens, and insects) and abiotic (salinity, drought, temperature, waterlogging, etc.) stresses. Among the biotic constraints, yellow mosaic disease (YMD) caused by the mungbean yellow mosaic virus (MYMV) is the major threat for huge economic losses in the Indian subcontinent (Nene, 1973). An infection of MYMV may cause up to 85%–100% yield loss in urdbean (Singh et al., 2011). The disease is caused by begomoviruses with bipartite genomes. Begomoviruses are a large group of whitefly-transmitted plant viruses containing single-stranded circular DNA encapsidated in geminate particles (Khattak et al., 2000; Karthikeyan et al., 2004). Initial symptoms of the disease appear as small yellow specks along the veins, which then spread over the leaf. In severe infections, the entire leaf may become chlorotic, which later turns into necrotic regions (Qazi et al., 2007). Recently, Borah and Dasgupta (2012) summarized the major developments in begomoviral research in India in during the last 15 years. They also suggested that future research particularly focus on begomovirus resistance, virus–vector or plant–virus interactions, and identification of natural wild varieties of crop plants or genes resistant to begomovirus.

The yield of urdbean ought to be enhanced and stabilized to meet the demand of the ever-growing population in the

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traditional growing areas. Any improvement in urdbean against YMD via genetic transformation approaches is limited due to its recalcitrant nature and lack of an effective regeneration system (Eapen, 2008). Breeding has emerged as a viable option for overcoming limitations in urdbean improvement. A lack of genetic variability, a low harvest index, and the absence of suitable ideotypes for different cropping systems also restrict urdbean improvement. Few efforts have been made, with limited success, to enhance the tolerance of urdbean against diseases and insect pests through this approach (Pandiyan et al., 2010). Resistance to mungbean yellow mosaic India virus (MYMIV) has been detected in urdbean in Kanpur, Uttar Pradesh, but the value of this resistance is unclear (Anjum et al., 2010). New sources of resistance to MYMIV have been identified and the molecular markers linked to resistance genes are becoming available (Chen et al., 2012). Early resistance to MYMV within *Vigna mungo* has been reported, but the resistance breaks down easily and frequently due to the rapid formation of new pathotypes (Karthikeyan et al., 2011). Recently, Singh et al. (2013) reported introgression of MYMV resistance in urdbean. Future progress in breeding efforts will require immediate attention on identification of accessions with favorable agronomic traits (Sehrawat et al., 2014c).

Wild relatives of a crop species can be effectively used as a natural source of resistance. The major genes or traits governing biotic stress resistance can be introgressed into the susceptible genotypes by breeding (Sehrawat et al., 2013a, 2014b). Wide hybridization is the only promising option to transfer desirable genes from related species for the quantitative and qualitative improvement of urdbean. Ricebean is an important crop of Southeast Asia. It has many useful characteristics such as bruchid resistance and disease resistance, particularly to YMD and bacterial leaf spot, along with the highest potential grain yield among the *Ceratotropis* spp. (Somta et al., 2006; Sehrawat and Yadav, 2014). Ricebean is described as a scientifically neglected crop of great potential. However, it has not been subjected to systematic breeding. The useful traits of ricebean can be introduced in susceptible crops through breeding to develop improved varieties of food grain legumes in biotic stress-prone areas (Singh et al., 2013; Sehrawat and Yadav, 2014). The recent era of advanced biotechnology provides various molecular markers such as RAPD, RFLP, AFLP, STMS, SSRs, SCAR, and SNPs that can dissect complex traits into individual components. Molecular approaches facilitate conventional breeding as marker assisted breeding (Ashraf and Foolad, 2013).

The present study was undertaken to introgress YMD resistance into urdbean through crossing with ricebean and testing the purity of the newly developed interspecific hybrids using morphological features and microsatellite markers.

2. Materials and methods

2.1. Plant material

The seeds of four ricebean genotypes (*V. umbellata* (Thunb.) Ohwi & Ohasi), namely RBL35, RBL50, RBL1, and RBL6 (having a high level of MYMV resistance), were procured from the National Bureau of Plant Genetic Resources in New Delhi. The urdbean genotype (*V. mungo* (L.) Hepper) PS1 (MYMV susceptible) was procured from the Division of Genetics of the Indian Agricultural Research Institute in New Delhi.

2.2. Hybridization of MYMV-resistant and susceptible genotypes

Seeds of the parental lines were sown in rows with proper spacing (45 cm) in the experimental field plot (7.6 m × 10.7 m) at the Herbal Garden of the Centre for Biotechnology (CBT) at Maharshi Dayanand University (MDU) in Rohtak, Haryana, India, during July–October, 2012. Four ricebean genotypes (RBL35, RBL50, RBL1, and RBL6) were used as male parents, while the urdbean genotype PS1 was used as the female parent to develop interspecific hybrids. The resistant and susceptible genotypes were hybridized using hand emasculation and pollination (Boling et al., 1961). The stigma of the urdbean is highly receptive in the early hours of the day. Therefore, the emasculation and pollination steps were performed in the early morning hours between 0500 and 0900 before the pollen shedding. Attempts to cross the MYMV-resistant and susceptible genotypes were done with a line × tester design. A significant number of flowers (25–50) were pollinated for each cross in order to obtain a higher number of hybrid pods.

2.3. Growth of hybrids and microsatellite markers (SSRs) used

Seeds of the F_1 hybrids of the four interspecific crosses [PS1 × RBL1, PS1 × RBL6, PS1 × RBL35, and PS1 × RBL50], along with their respective parents, were sown in the experimental field at the Herbal Garden, CBT, MDU, Rohtak, during July–September 2013 (Table). The azukibean-specific simple sequence repeat markers (SSRs) CEDG030 and CEDG248 that showed polymorphism between the parental lines were used to test the purity of their respective hybrids.

2.4. DNA extraction and quantification

Total genomic DNA was extracted from the young leaves of all of the F_1 plants and their respective parents using a GenElute Plant Genomic DNA Extraction Kit (Sigma) following the manufacturer's instructions. The quantification of the DNA was assayed on a 0.8% agarose gel electrophoresis in 1X TAE.

2.5. PCR using azukibean-specific SSRs

The DNA samples were diluted to 20 ng/µL prior to polymerase chain reaction (PCR) amplification. The PCR

was performed by preparing a reaction setup of 10 µL volume containing 20 ng of template DNA and 10X Taq Buffer B (Banglore Genei) in a final concentration of 1X 2.5 µM of each forward and reverse primer (Sigma), 2.5 mM of dNTPs (Fermentas), and 0.03 units of Taq DNA polymerase (5 U/µL, Banglore Genei). The PCR reaction was run on a thermocycler (Applied Biosystem Gene AMP PCR System 9700). The program used consisted of initial denaturation at 94 °C for 30 s followed by 38 cycles each consisting of denaturation at 94 °C for 30 s, annealing at 55–60 °C for 1 min, and elongation at 72 °C for 30 s. The final extension setup was carried out at 72 °C for 7 min and cooling was done at 4 °C indefinitely. The PCR-amplified products were resolved with 3.0% agarose gel electrophoresis. Ethidium bromide was used for staining the gels, which were then visualized and photographed on a Gel Documentation System (CFW- 1312M, BioRad). The DNA banding patterns of the PCR-amplified products of the hybrid plants were compared with their respective male and female parents for similarities and differences to verify their true hybrid nature.

2.6. Morphological characterization and field evaluation of hybrids for MYMV resistance

The F_1 hybrids were observed for their leaf morphology and color, leaf texture on the back surface, growth habit, flowering, and seed size, and were compared with their respective progenitors. The hybrid seeds and their progenitors were also screened for MYMV symptoms under field conditions during the rainy season.

3. Results and discussion

3.1. Crossability

All four types of crosses were done successfully between the selected genotypes. Significant variation was observed with each type of cross done in this study. The highest numbers of crossed pods were obtained from the interspecific cross PS1 × RBL35 followed by PS1 × RBL50. The number of F_1 seeds in the crossed pod varied from 1 to 4. The crossability ranged from 0.6% to 2.89% with an average value of 1.87% (Table). A few hybrid seeds were found to be immature. The results of the crossing demonstrated that doing the breeding during early hours minimized accidental self-pollination in urdbean. Moreover, the hand emasculation and pollination procedure of Boling et al. (1961) produced hybrid pods with healthy seeds set. Crossing species of different origin produces reproductive obstructions (Adinarayanamurty et al., 1993); therefore, hybridization between different species produces either weak or inviable hybrids. Hybrid lethality and sterility are natural mechanisms to maintain the integrity of crop species (Adinarayanamurty et al., 1993).

The results showed that all of the selected male and female parents were cross-compatible with each other. Out of the four male parental lines used for crossing, RBL35 was highly crossable with urdbean line PS1 and produced the highest number of crossed pods, followed by RBL50. In addition, the F_1 seeds were healthy. On the other hand, RBL6 and RBL1 showed poor crossability as they produced fewer crossed pods. The cross-compatibility of the pollen grains from RBL35 was higher than that of the other accessions as shown by the number of hybrid pods obtained (Table). The order of cross-compatibility was RBL35 > RBL50 > RBL6 > RBL1. These findings showed that *Vigna mungo* is easily crossable with *Vigna umbellata.* These results corroborate earlier findings on crossability among different *Vigna* species (Pandiyan et al., 2010; Singh et al., 2013; Sehrawat and Yadav, 2014).

3.2. Molecular verification of hybrid purity

The CEDG030 primer pair produced a reproducible band of 108 bp in both parents but a specific band of 130 bp was also obtained only in the male parent. The azukibean- specific SSR CEDG248 produced a reproducible band of 260 bp in the female parent and a band of 302 bp in male parent. All of the F_1 hybrids produced either the male-specific banding pattern or both bands specific to their respective parents (Figure 1). The similarity of the DNA banding pattern between hybrids and their parents verified the purity of the tested hybrids. The azukibean-specific SSR markers were highly useful for the identification of true hybrids during this study. The use of the same microsatellite markers in

Table. Details of the successful interspecific crosses of urdbean × ricebean.

Sr. no.	Cross involved	No. of crossed pods obtained	Total no. of F_1 seeds obtained	Frequency of crossover obtained
1	PS1 × RBL35	10	26	2.89
2	PS1 × RBL50	7	18	2.2
3	PS1 × RBL6	5	12	1.8
4	PS1 × RBL1	3	7	0.6
		Average frequency		1.87

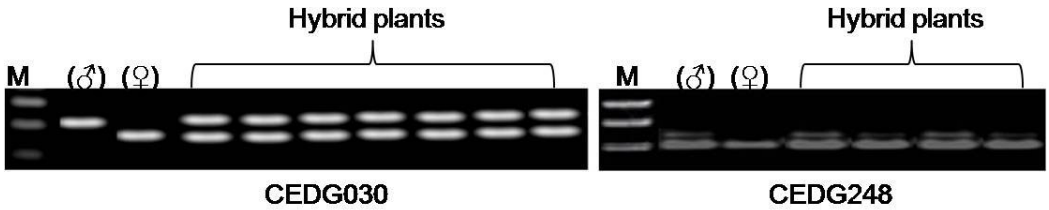

Figure 1. Confirmation of the purity of the hybrids using azukibean-specific microsatellite markers.

different plant species depends on the extent of the sequence conservation in the primer binding sites and its stability during evolution (Gupta et al., 2008). The amplification of microsatellite markers across related legumes increases their utility (Dikshit et al., 2012). Microsatellite analysis has been successfully employed for verification of parents, cultivar characterization, identification of hybrids, and purity testing in other crop plants (Tabbasam et al., 2006; Sehrawat et al., 2013b, 2014a).

3.3. Morphological characterization of hybrid purity

All of the hybrid plants inherited the trifoliate pattern similar to the male and female parents (Figure 2). The leaves of the hybrids showed little variation in color along with smoothness on the back surface similar to the donor parents. However, the hybrids also exhibited more vegetative growth and delayed flowering compared to the recurrent parent, which showed changes in growth habit and flowering period. A small increase in seed size of the hybrid plants showed hybrid vigor over the parental lines. Large seed size can improve the grain yield per plant and other yield-related attributes. Inheritance of bi-parental morphological features by the hybrids further confirmed their genetic purity. Assessment of the morphological characteristics of hybrid plants grown to maturity helps in testing the genetic purity of hybrids (Dongre et al., 2010).

3.4. Field evaluation of hybrids for MYMV resistance

The hybrids and their parents were screened for MYMV symptoms under natural epiphytotic field conditions during the rainy season. All of the hybrids and their male parents were found to be resistant to MYMV, while the female parent PS1 was found to be highly susceptible to MYMV (Figure 2). These results corroborate earlier findings (Pandiyan et al., 2010; Sehrawat and Yadav, 2014). The segregating generations of these hybrids will be tested further to determine inheritance of resistance against MYMV and MYMIV at hotspots. This may lead to the development of a stable and improved variety of urdbean for MYMV resistance.

3.5. Conclusions

The present study concludes that the developed hybrids exhibited a high level of resistance against YMD similar to the donor parents. The morphological characterization and the SSR–PCR analysis efficiently proved the genetic purity of the hybrids. The results further validate that microsatellite markers developed from azukibean can be used in genomic studies for mapping agronomically

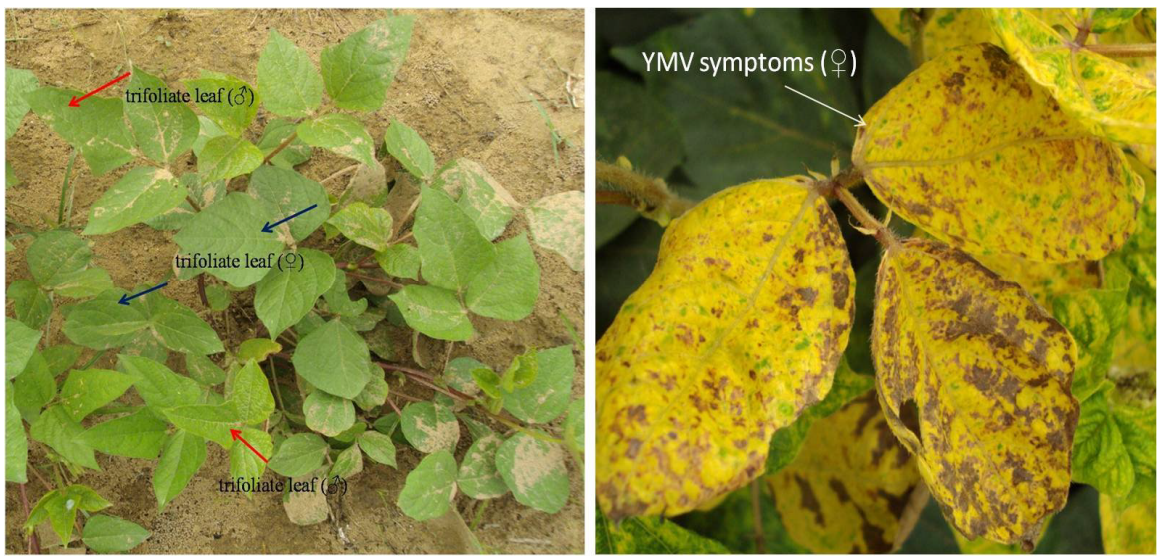

Figure 2. Expression of the trifoliate leaf pattern and resistance against MYMV in the urdbean × ricebean hybrids.

important traits via marker-assisted breeding for a desired trait in urdbean, and thus aid its genetic improvement. Conventional and traditional breeding methods should be supplemented with biotechnological techniques to overcome the limitations of the narrow genetic base of urdbean. An increase in seed size will help to improve grain yield and may increase its market price. The F_2 populations can be further used as a mapping population or to develop recombinant inbred lines for the identification of gene/quantitative trait loci for YMD resistance. Breeding-mediated MYMV resistance can be introduced in other vulnerable *Vigna* species to improve YMD resistance.

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References

Adinarayanamurty VV, Rao MVB, Satyanarayana A, Subramanyam D (1993). The crossability of *V. mungo* and *V. radiata* with *V. trilobata*. Int J Trop Agri 11: 209–213.

Ahmad T, Hefeez FY, Mehmood T, Malik KA (2001). Residual effect of nitrogen fixed by mungbean (*Vigna radiata*) and blackgram (*Vigna mungo*) on subsequent rice and wheat crops. Australian J Exp Agri 41: 245–248.

Anjum T, Gupta KS, Datta S (2010). Mapping of mungbean yellow mosaic India virus (MYMIV) and powdery mildew resistant gene in black gram [*Vigna mungo* (L.) Hepper]. Electron J Plant Breed 1: 1148–1152.

Ashraf M, Foolad M (2013). Crop breeding for salt tolerance in the era of molecular markers and marker-assisted selection. Plant Breed 132: 10–20.

Boling M, Sander DA, Matlock RS (1961). Mungbean hybridization technique. Agron J 53: 54–55.

Borah BK, Dasgupta I (2012). Begomovirus research in India: a critical appraisal and the way ahead. J Biosci 37: 791–806.

Chen HM, Ku HM, Schafleitner R, Bains TS, Kuo GS, Liu CA, Nair RM (2012). The major quantitative trait locus for mungbean yellow mosaic Indian virus resistance is tightly linked in repulsion phase to the major bruchid resistance locus in a cross between mungbean [*Vigna radiata* (L.) Wilczek] and its wild relative *Vigna radiata* ssp. *Sublobata*. Euphytica 192: 205–216.

Deepalakshmi AJ, Anandakumar CR (2004). Creation of genetic variability for different polygenic traits in blackgram (*Vigna mungo* L. Hepper) through induced mutagenesis. Legume Res 27: 188–192.

Delic D, Stajkovic O, Kyzmanovic D, Rasulic N, Knezevic S, Milicic B (2009). The effects of rhizobial inoculation on growth and yield of *Vigna mungo* L. in Serbian soils. Biotechnol Anim Husb 25: 1197–1202.

Dikshit HK, Singh D, Singh A, Jain N, Kumari J, Sharma TR (2012). Utility of adzuki bean [*Vigna angularis* (Willd.) Ohwi and Ohashi] simple sequence repeat (SSR) markers in genetic analysis of mungbean and related *Vigna* spp. Afri J Biotechnol 11: 13261–13268.

Dongre AB, Raut MP, Bhandaarkar R, Meshram KJ (2010). Identification and genetic purity testing of cotton F_1 hybrid using molecular markers. Indian J Biotechnol 10: 301–306.

Eapen S (2008). Advances in development of transgenic pulse crops. Biotechnol Adv 26: 162–168.

Gupta SK, Souframanien J, Gopalakrishna T (2008). Construction of a genetic linkage map of blackgram, *Vigna mungo* (L.) Hepper based on molecular markers and comparative studies. Genome 51: 628–637.

Karamany MFEL (2006). Double purpose (forage and seed) of mungbean production 1-effect of plant density and forage cutting date on forage and seed yields of mungbean (*Vigna radiata* (L.) Wilczeck). Res J Agri Biol Sci 2: 162–165.

Karthikeyan A, Sudha M, Pandiyan M, Senthil N, Shobana VG, Nagarajan P (2011). Screening of MYMV resistant mungbean (*Vigna radiata* L. Wilczek) progenies through agro-inoculation. Int J Plant Pathol 2: 115–125.

Karthikeyan AS, Vanitharani R, Balaji V, Anuradha S, Thillaichidambaram P, Shivaprasad PV, Parameswari C, Balamani V, Saminathan M, Veluthambi K (2004). Analysis of an isolate of mungbean yellow mosaic virus (MYMV) with a highly variable DNA B component. Arch Virol 149: 1643–1652.

Khattak GSS, Haq MA, Ashraf M, Elahi T (2000). Genetics of mungbean yellow mosaic virus (MYMV) in mungbean (*Vigna radiata* (L.) Wilczek). J Genet Breed 54: 237–243.

Muruganantham M, Ganapathi A, Amutha S, Vengadesan G, Selvaraj N (2005). Shoot regeneration from immature cotyledonary nodes in black gram (*Vigna mungo* (L.) Hepper). Indian J Biotechnol 4: 551–555.

Nene YL (1973). Viral diseases of some warm weather pulse crops in India. Plant Dis Rep 57: 463–467.

Pandiyan M, Senthil N, Ramamoorthi N, Muthiah AR, Tomooka N, Duncan V, Jayaraj T (2010). Interspecific hybridization of *Vigna radiata* × 13 wild Vigna species for developing MYMV donar. Electron J Plant Breed 1: 600–610.

Qazi J, Ilyas M, Mansoor S, Briddon B (2007). Legume yellow mosaic viruses: genetically isolated begomoviruses. Mol Plant Pathol 8: 343–348.

Sehrawat N, Bhat KV, Kaga A, Tomooka N, Yadav M, Jaiwal PK (2014a). Development of new gene-specific markers associated with salt tolerance for mungbean (*Vigna radiata* L. Wilczek). Span J Agric Res 12: 732–741.

Sehrawat N, Bhat KV, Sairam RK, Jaiwal PK (2013a). Identification of salt resistant wild relatives of mungbean (*Vigna radiata* L. Wilczek). Asian J Plant Sci Res 3: 41–49.

Sehrawat N, Bhat KV, Sairam RK, Tomooka N, Kaga A, Shu Y, Jaiwal PK (2013b). Diversity analysis and confirmation of intra-specific hybrids for salt tolerance in mungbean (*Vigna radiata* L. Wilczek). Int J Integ Biol 14: 65–73.

Sehrawat N, Jaiwal PK, Bhat KV, Tomooka N, Kaga A, Yadav M (2014b). Breeding mediated improvement of mungbean [*Vigna radiata* (L) Wilczek] for salt tolerance. Thai J Agric Sci 47: 109–114.

Sehrawat N, Yadav M (2014). Screening and cross-compatibility of various *Vigna* species for yellow mosaic virus resistance. J Innov Biol 1: 31–34.

Sehrawat N, Yadav M, Bhat KV, Sairam RK, Jaiwal PK (2014c). Evaluation of mungbean genotypes for salt tolerance at early seedling growth stage. Biocatal Agric Biotechnol 3: 108–113.

Singh I, Sandhu JS, Gupta SK, Singh S (2013). Introgression of productivity and other desirable traits from ricebean (*Vigna umbellata*) into black gram (*Vigna mungo*). Plant Breed 132: 401–406.

Singh MK, Singh K, Haq QMR, Mandal B, Varma A (2011). Molecular characterization of tobacco leaf curl Pusa virus, a new monopartite begomovirus associated with tobacco leaf curl disease in India. Virus Genes 43: 296–306.

Somta P, Talekar NS, Srinives P (2006). Characterization of *Callosobruchus chinensis* (L.) resistance in *Vigna umbellata* (Thunb.) Ohwi & Ohashi. J Stored Prod Res 42: 313–327.

Tabbasam N, Rahman M and Zafar Y (2006). DNA-based genotyping of sorghum hybrids. Pak J Bot 38: 1599–1604.

Vavilov NI (1926). Studies on the origin of cultivated plants. Bull Appl Botany 16: 139–248.