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Identification of Host Plant Resistant to Dolichos Yellow Mosaic Virus (DYMV) in Dolichos Bean (*Lablab Purpureus*)

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Abstract

Three hundred Dolichos bean (*Lablab purpureus*) genotypes were screened against Dolichos Yellow Mosaic Virus (DYMV) disease. Initial screening was done under field conditions, where disease incidence was calculated for each genotype. Subsequently, selfed progenies of 34 symptomless lines were challenged by sap inoculation under field conditions, out of which only three genotypes, viz. VRSEM-894, VRSEM-887 and VRSEM-860 did not show any symptoms. Using root stalk of susceptible genotype (Ankur Goldy), these three putative symptomless genotypes were further challenged by grafting. The resistant reactions of VRSEM-894, VRSEM-887 and VRSEM-860 were confirmed, as even after 60 days of successful grafting, no viral symptom appeared on all the grafted plants of these genotypes. When subjected to PCR amplification with DYMV coat protein gene specific primer, these three symptomless genotypes did not show any amplification, suggesting that there was no infection of Dolichos Yellow Mosaic Virus in those genotypes.

Keywords: Dolichos bean; *Lablab purpureus*; Geminivirus; Dolichos yellow mosaic virus

Introduction

Dolichos bean (*Lablab purpureus*) is used as a green vegetable, as well as pulse crop, respectively. In many developing countries including India, leguminous crops e.g. pulse and beans play an important role by providing protein nutrition source [1]. Pulse and beans contain 20-30% protein, on dry matter basis [2]. Legumes play great role in controlling soil erosion, managing soil fertility and solving nutrient imbalances in live stock [3]. Unfortunately, numerous pathogens infect and influence the cultivation of Dolichos bean resulting in more than a ten different diseases. These diseases are caused by bacteria, fungi, viruses and nematodes. Fortunately, most of the diseases have only local importance, but a few diseases make a global impact; one of them is Dolichos yellow mosaic virus (DYMV) disease, which is the most dominant disease of Dolichos bean and caused by the geminivirus, which is transmitted by vector whitefly [4]. The disease is characterized by yellow to bright yellow patches, and vein clearing on leaves [5]. Initially, few yellow patches are seen on the leaf lamina but later on, the entire leaf becomes yellow [6]. Plants which are infected in early stages remain dwarf with small leaves, and less number of branches and fruits [5]. In the recent years after realization of severity of disease (80 % crop loss), farmers have become reluctant in cultivation of Dolichos bean in India [7]. However, the satisfactory control of aphids, mites and partial control of viral disease have been achieved with the application of certain pesticides, which is mostly ineffective, uneconomical, causes environmental hazard and pose a health risk to the farmers and consumers, while an initial pesticide application may be necessary to control heavy infestations; repeated applications may lead to strains of whiteflies that are resistant to pesticides [8].

So, cropping disease resistant plants remains the best approach. In this order, Dolichos yellow mosaic resistant genotypes Pusa Sem-2 and Pusa Sem-3 have been reported previously, under open field conditions [9]. Hence, in order to combat DYMV disease as an initiative towards yellow mosaic resistant breeding, germplasm lines were systematically screened, and the results are reported herein.

Material and Methods

Plant materials and crop rising

A total of 300 Dolichos bean genotypes belonging to two cultivated species; *Lablab purpureus* var. *typicus* and *lignosus* were raised during 2007 and 2008. Twenty healthy seeds were sowed in second week of july in both years, at a spacing of 2 m x 1m in randomized block design, consisting of 3 replications. All the recommended agronomic practices were followed for raising a healthy crop; in addition, insecticide was not applied throughout the crop stand to ensure white fly population build-up.

Natural screening of genotypes against DYMV

Plants were grown from fungicide treated seeds in the research form of Indian Institute of Vegetable Research, ICAR Varanasi, Uttar Pradesh, India; for subsequent growth and phenotypic evaluation during August 2007 and 2008, when the virus inoculums pressure was at height. The initial screening was done under field conditions based on visual observations; the data on incidence of DYMV was recorded on individual plant basis, starting from first week of August from 30 days after seed sowing and continued up to April at 15 days interval, during 2007 and 2008. The incidence of disease was scored mainly through visual observation of typical symptoms of DYMV disease like yellowing, smaller leaflets with or without marginal chlorosis of the plant. An arbitrary scale was employed to score the disease, as

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described by [4] where, symptoms absent (symptom severity grade 0) was classified as symptomless, small yellow spots scattered on leaf lamina (up to 5%) (symptom severity grade 1) was classified as highly resistant, yellow spots scattered on leaf lamina (6-25%), (symptom severity grade 2) was classified as resistant, yellow mosaic spots on 26-50% leaves (symptom severity grade 3) was classified as moderately resistant, yellow mosaic patches on 51-75% leaves (symptom severity grade 4) was classified as moderately susceptible, severe yellow mosaic patches on >75% leaves (symptom severity grade 5) was classified as susceptible, and genotypes having stunting of plants, deformed small laves, premature death of plant (symptom severity grade 6) was classified as highly susceptible plant.

Artificial screening of genotypes against DYMV

Through sap inoculation: For artificial screening under glasshouse conditions in first phase, 34 symptomless selfed progenies (Table 1) were raised, and challenged by sap inoculation [10]. In this method, DYMV susceptible culture was inoculated on 34 symptomless lines. For preparing inoculum suspension, DYMV infected leaves were taken and crushed by mortar and pastel. After crushing, the suspension was sieved through a muslin cloth. Cotton swabs were soaked in suspension, and put on five twigs/leaves of each genotype after scratching with blade/carborundum powder, at 60 days twice a month, after seed sowing in September and October, 2008.

Through grafting: For further confirmation, the symptomless line (VRSEM-894, VRSEM-887 and VRSEM-860) was grafted, according to method [10]. For this, a bush type highly susceptible line (Ankur Goldy) was used as root stock during November to December, 2008 and successful grafted plants were kept upto 60 days, for observation of the incidence of disease.

Genomic DNA extraction: Young leaves from three symptomless, four highly resistant and highly susceptible lines were collected before and after infection of DYMV disease from IIVR, Varanasi research farm, and genomic DNA was extracted following the CTAB protocol [11], with required modification. For genomic DNA isolation, 250 mg DYMV infected from susceptible genotypes, fresh leaf tissues from resistant genotypes, were ground with liquid nitrogen in preheated CTAB extraction buffer (2.2% CTAB, 1.5 M NaCl, 20 mM EDTA, 100 mM Tris-HCl, pH 8.0 and 0.6% β-mercaptoethanol added, prior to use) and transferred to a 2 ml Eppendorf tube. After thorough mixing, RNAase was added, and then this mixture was incubated at 65°C for 1 hour. It was centrifuged at 12,000 rpm for 16 minutes at 15°C. 1 ml supernatant was taken out and equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) was added to it, and after mixing thoroughly, it was centrifuged at 12,000 rpm for 15 minutes at 15°C, this step was again repeated for, as condition first, for equal volume of chloroform:isoamyl alcohol. The DNA present in upper aqueous phase was precipitated in 2/3 vol. of chilled isopropanol. The DNA was washed twice with 70% alcohol, dried in incubator at 37°C for 30 minutes, and finally, palette was dissolved in autoclaved double distilled water.

Polymerase chain reaction: PCR reactions were carried out using conditions, as described earlier [12]. It was performed in a 25 µl reaction mixture containing 50 ng genomic DNA, 50 mM $MgCl_2$, 75 µm dNTPs, 10 x assay buffer (10 mM Tris-HCl pH 8.0, 50 mM KCl, 2.5 mM $MgCl_2$ and 0.01% gelatin), 0.1 µM of each primer (forward and reverse) sequence coat protein gene marker, F 5' ATG GCG AAG CGA CCA AGC 3' R 5' TTA ATT TGT ACG CAA TCA TAA 3' and 0.6 units of Taq polymerase was used (Bangalore Genei Pvt. Ltd, Banglore, India). Amplification was performed in Thermo Thermal Cycler - programmed as, one cycle of pre-denaturation at 94°C for 5 minutes, 38 cycles each of denaturation at 94°C for 1minute, annealing at 54°C for 35 seconds, elongation for 1minute at 72°C, and final elongation at 72°C for 7 minutes. The reactions were held at 4°C, following completion of PCR. Amplified products were resolved through gel electrophoresis at 60 volts for 120 minutes, using 1 X TAE buffer in 1.4% agarose gel containing 5 µl (1mg/ml) ethidium bromide.

Results and Discussion

PCR amplification analysis

When PCR amplification was carried out with degenerate primers for confirmation of viral genome, three symptomless genotypes did not show any amplification, while four moderately resistant and susceptible lines showed amplification of 750 bp DNA fragment, specific to viral genome (Figure 1). Similar results have been observed in Chilli, when the pepper genotypes viz. BS-35, GKC-29, and EC497636 were screened against pepper leaf curl virus [13]. In the present investigation, it has been observed that three genotypes were symptomless carrier and possess mechanism to avoid transmission of viral genome in their sap, and exhibit true resistance. The results also validated the usefulness of degenerate primers, for early and reliable demarcation of Gemini-virus resistant and susceptible Dolichos bean plants. For the first time, to the best of our knowledge, symptomless resistant sources to DYMV have been identified through artificial screening and grafting, and resistance mechanism has been confirmed due to the absence of DYMV coat protein gene, in the leaf tissues of all three symptomless genotypes on molecular basis. Two of these genotypes, viz. VREM-894 and VREM-860 are *Dolichos lablab* var. *lignosus*, in which seeds are at right angles to the suture; known as field bean and mainly cultivated for dry seed as pulse. The VRSEM-887 is *Dolichos lablab* var. *typicus*, in which the long axis of the seed is parallel to the suture, a garden type and cultivated for its soft and edible pods. These two Dolichos varieties are cross-compatible. The remaining symptomless, highly resistant and resistant genotypes with desirable market types, identified during this study under field conditions, are intended to be selected for confirmation through artificial screening, and confirmed resistant genotypes may directly be promoted after seed multiplication.

Natural and artificial screening

On the basis of CI values obtained under field conditions, the maximum numbers of genotypes (83) were found to be moderately resistant, followed by resistant (63), moderately susceptible (58), symptomless (34), highly resistant (30), susceptible (18) and highly

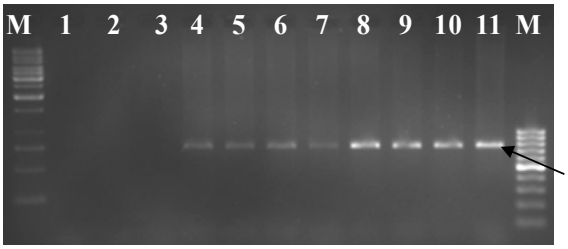

Figure 1: Confirmation of the absence of DYMV coat protein gene in the identified DYMV symptomless genotypes of Dolichos bean (1-3). M-100 bp marker; 1: VRSEM-894 field symptomless; 2: VRSEM-887 field symptomless; 3: VRSEM-860 field symptomless; 4: VRSEM-893 field moderately resistant; 5: VRSEM-6 field moderately resistant; 6: VRSEM-764 field moderately resistant; 7: VRSEM-772 field moderately resistant; 8: HADB-4 field highly susceptible; 9: VRSEM-933 field highly susceptible 10: VRSEM-11 field highly susceptible; 11: VRSEM-8 field highly susceptible.

S. No.	Genotypes	Field response	Response after sap inoculation	Response after grafting
1	'VRSEM-894'	SL	SL	SL
2	'VRSEM-887'	SL	SL	SL
3	'VRSEM-860'	SL	SL	SL
4	'VRSEM-37'	SL	MR	TNR
5	'VRSEM-906'	SL	MR	TNR
6	'VRSEM-18'	SL	R	TNR
7	'VRSEM-5'	SL	HR	TNR
8	'VRSEM-17'	SL	R	TNR
9	'VRSEM-744	SL	R	TNR
10	'VRSEM-745'	SL	R	TNR
11	'VRSEM-748'	SL	MR	TNR
12	'VRSEM-757'	SL	S	TNR
13	'VRSEM-810'	SL	R	TNR
14	'VRSEM-808'	SL	MR	TNR
15	'VRSEM-802'	SL	MR	TNR
16	'VRSEM-953'	SL	MS	TNR
17	'VRSEM-766'	SL	HR	TNR
18	'VRSEM-783'	SL	HR	TNR
19	'VRSEM-786'	SL	MS	TNR
20	'VRSEM-801'	SL	R	TNR
21	'VRSEM-799'	SL	MR	TNR
22	'VRSEM-789'	SL	HR	TNR
23	'VRSEM-796'	SL	HR	TNR
24	'VRSEM-792'	SL	HR	TNR
25	'VRSEM-904'	SL	R	TNR
26	'VRSEM-778'	SL	HR	TNR
27	'VRSEM-785'	SL	HR	TNR
28	'VRSEM-709'	SL	HR	TNR
29	'VRSEM-898'	SL	HR	TNR
30	'VRSEM-902'	SL	MR	TNR
31	'VRSEM-869'	SL	HR	TNR
32	'VRSEM-826'	SL	HR	TNR
33	'VRSEM-889'	SL	R	TNR
34	'VRSEM-864'	SL	HR	TNR

SL= Symptomless, HR = highly resistant, R = Resistant, MR= moderately resistant, MS= moderately susceptible, S = Susceptible, TNR = Testing not required

Table 1: Response of putative symptomless 34 genotypes (field conditions), against DYMV under sap inoculation and grafting experiments.

susceptible (14). Finding of this investigation was similar to those reported for Dolichos bean [14] and Mung bean [15]. Selfed progenies of 34 genotypes showing no symptoms under field conditions were raised under glasshouse, and challenged with sap inoculation. Under this condition, out of 34 genotypes only 3, viz. VRSEM-894, VRSEM-887 and VRSEM-860 were symptomless against DYMV (Table 1). These results are in closed conformity with the finding in Indian bean [13]. The resistant reactions of symptomless lines, VRSEM-894, VRSEM-887 and VRSEM-860 were further confirmed, as even after 60 days of successful grafting on susceptible root stock of Ankur Goldy, no viral symptom appeared on all the grafted plants (Figure 2).

Conclusion

The identified symptomless sources can be used as basic material to study the inheritance of host plant resistant against DYMV, and to execute resistance breeding against this devastating virus.

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Figure 2: (A) Typical yellow mosaic symptom caused by DYMV in Indian bean, (B) Symptomless genotype VRSEM-894, (C) Symptomless genotype VRSEM-894 after successful grafting on susceptible Ankur Goldy, (D) Symptomless genotype VRSEM-887, (E) Symptomless genotype VRSEM-860.

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