Evidence of seed transmission of dolichos yellow mosaic virus, a begomovirus infecting lablab-bean in India

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Abstract Yellow mosaic disease in field bean caused by begomoviruses belonging to the family Geminiviridae is a major threat to the cultivation of crop in South India. Appearance of bright yellow mosaic symptom in emerging seedlings in farmers field was suggestive of seed transmission of the begomovirus associated with the disease which was investigated in the present study. The begomovirus causing yellow mosaic disease was identified as dolichos yellow mosaic virus (DoYMV) and the presence of DoYMV in matured seeds was confirmed by DAS-ELISA with OD value up to 3.268. In PCR with DoYMV specific primer (DoYMV-CP) the virus was detected in different parts of the seeds viz., seed coat, endosperm and embryo. In embryo the virus was detectable up to 100 per cent followed by endosperm (69.23%). When the non symptomatic leaves of 30 days old grow-out test plants were subjected to DAS-ELISA, the virus was detected up to 46.6%. This is the first evidence of seed transmission of DoYMV.

Keywords Seed borne nature · Begomovirus · Dolichos yellow mosaic virus (DoYMV) · Field bean · ELISA

Introduction

Lablab-bean (*Lablab purpureus* L.) also called as Dolichos bean or Field bean is one of the multipurpose leguminous crops cultivated throughout India. Worldwide, it is widely cultivated in dry tropical parts of South Central America, West Indies, Asia, Africa and China [8]. In India, the crop is cultivated throughout Karnataka, Tamil Nadu, Andhra Pradesh, Maharashtra, and Uttar Pradesh. The crop is mainly grown for its green pods and dry seeds. The seeds are one of the major sources of protein for vegetarian diet in South India. Lablab-bean is infected by many plant viruses such as Dolichos enation mosaic virus (Tobamovirus) [7], Peanut stunt virus (Cucumovirus) [2], Clover yellow vein virus (Potyvirus) [16], Bean common mosaic necrosis virus (Potyvirus) [22] and Dolichos mosaic virus (Potyvirus) [19].

Among the viral diseases, the yellow mosaic disease caused by dolichos yellow mosaic virus (DoYMV) is the major constraint under field conditions in India. Though the association of another begomovirus, mungbean yellow mosaic India virus has been reported by Singh et al. (2006) [23], DoYMV is more predominant. The yellow mosaic disease is caused by the species DoYMV belonging to the genus *Begomovirus* of the family *Geminiviridae*. The family *Geminiviridae* comprises of nine genera viz., *Becurtovirus*, *Begomovirus*, *Capulovirus*, *Curtovirus*, *Eragrovirus*, *Grablovirus*, *Mastrevirus*, *Topocuvirus* and *Turncurtovirus*. Among them, the highest number of plant-infecting ssDNA viruses belong to the genus *Begomovirus* [1], which are transmitted by the vector *Bemisia tabaci*. Begomoviruses consist of circular single-stranded DNA genomic components encapsidated within geminate particles of 18 × 30 nm size. Its genome is divided into two (Bipartite genome, DNA A and DNA B). The DNA-A of

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begomovirus encodes for genes governing replication, encapsidation and DNA B encodes for proteins facilitating intra and intercellular movement.

During a survey in farmers field in villages of Coimbatore district, young seedlings (15 days old) of field bean plants were observed to show yellow mosaic symptoms. From that observation, it was speculated that the causative agent may be transmitted by seeds. The geminiviruses are confined to phloem tissue and were therefore considered not to be able to penetrate seed tissue as the vascular connectivity in seed tissue is only up to hilum. However, recently evidences contrary to our earlier understanding on seed borne nature of begomoviruses are obtained. Kim et al. (2015) demonstrated for the first time 15% of transmission level of sweet potato leaf curl virus (SpLCV) in true seedlings, while more than 70% of the seeds harvested from infected plant were positive in PCR [14]. Kothandaraman et al. (2016) revealed the presence of mungbean yellow mosaic virus (MYMV) in whole seeds, seed coats, cotyledon and embryonic axis. However in the grow-out tests, there was no symptom development in the seedlings, though the virus was detected in 32% of tested seedlings [15]. Kil et al (2016) reported a very high rate of transmission of tomato yellow leaf curl virus (TYLCV-IL) to the seedlings (80–84%) when tomato plants were inoculated either by whitefly or by agroinoculation. The seedlings from seeds of infected plants served as inoculum when they were caged with whiteflies, the vector acquired the virus and transmitted it [11]. Kil et al. (2017) reported TYLCV seed transmission in asymptomatic hosts sweet pepper and soybean plants. The results for the first time indicated that, asymptomatic hosts could act as a reservoir of virus which could infect tomato [13]. In the present communication, results of investigations on seed borne nature of DoYMV and its importance as the inoculum source are discussed.

Materials and methods

Confirmation of the begomovirus association by DAS-ELISA and PCR

A total of 30 field bean leaf samples exhibiting yellow mosaic symptoms were collected from the fields (Fig. 1a) of TNAU (CBE1), Thondamuthur (TM1) and Dhaliyur (DH1) villages of Coimbatore district. Initially the samples were tested for the association of begomovirus by performing DAS-ELISA with tomato leaf curl New Delhi virus (ToLCNDV) polyclonal antibody (DSMZ, DAS-ELISA kit, Cat No # AS-1109) using 96 well micro titre plates (Micro Test Plate Flat bottom, Tarson Pvt. Ltd, Kolkata). ToLCNDV antiserum (AS-1109, IgG) was used

Fig. 1 a Field bean plant showing yellow mosaic symptom. b Detection of begomovirus in naturally infected leaf sample collected from different locations through PCR with Rojas primer (PALic1960 and PARIv722); Lane 1—Ladder (1 kb), Amplicon with DNA extracted from; Lane 2, 3, 4—TNAU isolate (CBE1), Lane 5, 6, 7—Thondamuthur isolate (TM1), Lane 8, 9, 10—Dhaliyur isolate (DH1), Lane 11—Positive control (Infected leaf from tomato plant) and Lane 12—Negative control (Healthy uninoculated leaf from field bean plant). c Detection of DoYMV in naturally infected leaf sample collected from different locations through PCR with DoYMV-CP primer (DoYMV-CP 377F and DoYMV-CP 580R); Lane 1—Ladder (1 kb), Amplicon with DNA extracted from; Lane 2, 3, 4—TNAU isolate (CBE1), Lane 5, 6, 7—Thondamuthur isolate (TM1), Lane 8, 9, 10—Dhaliyur isolate (DH1), Lane 11—Positive control (Infected leaf from field bean plant) and Lane 12—Negative control (Healthy uninoculated leaf from field bean plant)

at 1:1000 dilution in carbonate buffer (pH 9.6). 100 µl of the coating antibody was added into the wells of microtiter plates. The coated plate was covered with aluminium foil and incubated at 37 °C for 4 h and was washed thrice with 1X phosphate buffered saline with Tween 20 (PBST). Then 100 µl of seed extract, positive control (tomato leaf samples with leaf curl symptom), negative control (leaf samples from healthy, uninoculated field bean plants) and buffer control (extraction buffer) were added to the respective wells and the plate was incubated overnight at 4 °C. After washing three times with 1X PBS-T buffer, each well was added with 200 µl of enzyme conjugate (AS-1109, IgG-AP) at the dilution of 1:500, in enzyme conjugate buffer. The absorbance was read at 405 nm in ELISA reader (Sunrise™, Tecan groups Ltd, Switzerland) after addition of p-Nitro phenyl Phosphate (p-NPP) (MDL No. MFCD00284586, Sigma-Aldrich, USA). DAS-ELISA was also performed to detect the virus presence in seeds. The extracts were prepared in 1:1 dilution. Seeds and leaf tissues of field bean from uninoculated plants which were negative in PCR and DAS-ELISA was used as negative control, in subsequent experiments.

Total nucleic acid of 35 infected leaf samples from all the three locations were extracted using GEM-CTAB method [21] and subjected to PCR with Rojas primer [20], PALic1960 5' ACNGGNAARACNATGTGGGC 3' and PARIv722 5' GGNAARATHTGGATGGA 3'. The polymerase chain reaction was performed in 25 µl reaction mixture containing 12.5 µl of master mix, (ready mix of taq polymerase, dNTPs and PCR Buffer, Genei

Laboratories Pvt. Ltd., cat no # 0667700041730), 5 µl of template DNA, 3.5 µl of sterile distilled water and 2 µl of each forward and reverse primer. Following were the PCR conditions; initial denaturation at 94 °C for 2 min and 30 cycles of denaturation at 94 °C for 50 s, annealing at 52 °C for 45 s and extension at 72 °C for 90 s followed by a final elongation for 15 min at 72 °C.

Cloning and sequencing of genomic components of the begomovirus

For the enrichment of DNA, the Rolling Circle Amplification (RCA) was performed for Thondamuthur isolate (TM1) using Ø29 DNA polymerase [9]. Both the viral insert and pUC18 vector were restricted with *Pst*I and *Hind*III enzymes for cloning full length genomic components. Transformation was performed using competent cells of *Escherichia coli* (DH5α strain). The recombinants were confirmed by restriction digestion with the respective enzymes. The selected clones were sequenced by primer walking method availing commercial facility (Bioserve Biotechnologies, Hyderabad). Complete nucleotide sequences of the full length DNA A component of the begomovirus under study was compared with DoYMV isolates, and then other begomoviruses with which BLAST hit was obtained. The sequences retrieved from database (www.ncbi.nlm.nih.gov) were aligned using Multiple Sequence Comparison by Log-Expectation (MUSCLE) Edgar software, 2004 and the percentage pairwise nucleotide identity was computed.

Detection of DoYMV in seeds from infected plants through PCR using DoYMV-CP primer

From the sequences of DNA A clones of DoYMV of the present study, the specific primers (DoYMV-CP) were designed for the region of Coat Protein (CP) with DoYMV-CP 377F- 5' CAGTTCGTCGAACTCCGAG 3' and DoYMV-CP 580R- 5' CGATGAGTAAGGTTGTTTC 3'; this specific primer was used for further seed detection studies. Seeds collected from infected plants of all the three locations were dissected into seed coat, endosperm and embryo. Twenty number of mature whole seed, 24 samples of seed coat, 26 samples of endosperm and 24 samples of embryo were subjected to DNA extraction by GEM-CTAB method [21]. The extracted DNA from infected leaves, mature whole seed, seed coat, endosperm and embryo were subjected to PCR with DoYMV-CP primer in thermocycler (Eppendorf). The PCR conditions were initial denaturation at 94 °C for 5 min and 30 cycles of 30 s denaturation at 94 °C, 1 min of annealing at 56 °C and 90 s of extension at 72 °C followed by 15 min of final elongation at 72 °C.

Grow-out test

Around 100 of field bean seeds were collected from Thondamuthur location from symptomatic plants and 20 seeds were collected from symptom free healthy plants which were negative for DoYMV in DAS-ELISA. The seeds were sown in earthen tube pots separately and maintained in insect proof cages in glass house, Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore. The plants were numbered by tagging. The periodical observations were recorded for symptom appearance.

The leaves from the tagged plants were collected on 30 DAS and were analyzed for the presence of virus in the samples by DAS-ELISA and PCR using DoYMV specific primer. In DAS-ELISA, 108 plant samples comprising 88 from infected plants and 20 from healthy plants were checked for the presence of virus with two replications.

Results

Confirmation of the presence of begomovirus in field bean leaf samples by DAS-ELISA and PCR

Among the 30 samples of infected leaves collected from different locations *viz.*, TNAU (CBE1), Thondamuthur (TM1), and Dhaliyur (DH1) 14 samples were positive with the OD value which ranged from 0.434 to 0.869. Further, among the three locations tested, DH1 sample recorded a maximum of 0.869 OD value which is higher than positive control value (0.791). In the healthy negative control sample, the OD value was only 0.212 (Fig. 2). In PCR with Rojas primer, out of 35 samples of 3 locations tested, 25 samples were positive with the expected amplicon size of 1.2 kb (Fig. 1b). In the case of DoYMV specific primer, out of 35 samples tested, thirty were positive (Fig. 1c).

Identity of the virus as DoYMV

Sequences were generated for one *Pst*I and one *Hind*III clones by primer walking method. The sequences were edited and joined end to end and analysed initially in BLAST programme (www.ncbi.nlm.nih.gov). From BLAST results, *Pst*I clone was found to represent DNA A as they exhibited nearly 97% identity with DoYMV isolates from Coimbatore and Mysore (Accession No: KP78466, AJ968370). Next to legumoviruses hit was observed with tomato viruses from Indonesia, Seychelles, Toga, Ghana and with African cassava mosaic virus from Burkino Faso. Surprisingly, no hit with any other begomovirus from Indian subcontinent was observed. The *Hind*III clone was identified to represent DNA B and had

Fig. 2 Detection of begomovirus in yellow mosaic infected field bean leaf samples collected from different location by DAS—ELISA using ToLCNDV antiserum; Absorbance at 405 nm was recorded 30 min after adding substrate. Values presented is average of three

replication. Leaf samples from tomato showing leaf curl symptoms were used as positive control. Leaf samples from uninoculated healthy field bean plants served as negative control

only one hit with DNA B of DoYMV from Kanpur (Accession No: KJ481205).

In order to ascertain the identity of the virus under study, the complete nucleotide sequence of DNA A of the study isolate was compared with nine other begomoviruses that includes legumoviruses and begomoviruses with which BLAST hit was obtained. In pairwise identity matrix generated in MUSCLE option, 97% identity was observed between the study isolate and DoYMV, Coimbatore isolate (KP78466) and Mysore isolate (Accession No: AJ968370); However identity with New Delhi isolate (Accession No: AY309241), Kanpur isolate (Accession No: KJ481204) and Bangladesh isolate (Accession No: AY271891) were only 93, 94 and 95% respectively. Next to DoYMV about 61% identity was observed with legumoviruses like MYMV (Accession No: FM242701), MYMIV (Accession no: KCO19304), VbSMV (Accession No: FN543425) and HgYMV (Accession No: 7520088). With the tomato and cassava viruses taken for comparison, 57 to 58% identity was observed.

On the basis of threshold value of 91% set for the begomovirus species demarkation by the geminivirus study group [5] the virus isolate described in the present study is designated as an isolate of dolichos yellow mosaic virus for which the following isolate descriptor is proposed: *Dolichos yellow mosaic virus* (India: Tamil Nadu: TM1 isolate: 2018); DoYMV-(IN-TN:18). DNA B component is yet to be sequenced completely.

Complete nucleotide sequence of DNA A of the study isolate (Genbank Accession No: MH795972) revealed the presence of six ORFs typical of Old World begomoviruses. They are AV2 and AV1 in the virion sense and four (AC1, AC2, AC3 and AC4) in complementary sense. The intergenic region is 251 nucleotide long and comprises nonanucleotide and conserved stem-loop structure, constituting the origin of replication.

Seed borne nature of dolichos yellow mosaic virus

Presence of virus in whole seed was confirmed by serological detection. About 50 seeds collected from infected plants from the Thondamuthur location were subjected to DAS-ELISA. All the 50 whole seeds were positive in DAS-ELISA, in seeds collected from healthy plants, the OD value was 0.206. DoYMV infected samples exhibited a maximum increase in absorbance value up to 3.268 (Fig. 3).

On PCR analysis with DoYMV-CP primer, the presence of DoYMV was confirmed in different parts of the seed. The percentage of samples which are positive for the presence of virus in suspected seed coat dissected from the seed of infected plant is 37.5 (9 out of 24) and in endosperm, it was 69.23% (18 out of 26). For detection of virus in the embryo, DNA was extracted from single embryo and in PCR, all the 24 samples tested were positive which indicates 100% seed borne nature of the virus (Fig. 4).

Seed transmission of dolichos yellow mosaic virus through grow-out test

In grow-out test, out of 100 seeds from infected plants sown, only 88 germinated. A total of 108 plants including 20 plants from healthy seeds were maintained in insect proof cages in glass house, there was no symptom expression even after 30 DAS. After 30 DAS leaves from non symptomatic plants were subjected to DAS-ELISA. Results revealed that 41 seedlings were positive out of 88 seedlings from infected seeds tested and there was more than twofold increase in the OD value observed in the samples collected from plants of DoYMV infected seed. The virus titre as indicated by the absorption value in grow-out test seedlings ranged from 0.212 to 2.924. The leaves of the plant grown from seeds of healthy plants were negative

from field bean plant showing mosaic symptom was used as positive control. Leaf samples from uninoculated healthy field bean plants served as negative control

TM1 isolate, Lane 13 to 17—DH1 isolate, Lane 18—Positive control (Infected leaf from field bean plant), Lane 19—Negative control (Healthy uninoculated leaf from field bean plant). **a** Seed coat. **b** Endosperm. **c** Embryo

from 15% during 1990 s to 40% in 2000 [17]. In the present communication, a Tamil Nadu isolate of DoYMV and its seed transmission are described. Though yellow mosaic disease was recorded in field bean as early as 1950 [6], the etiological role of geminivirus was first indicated by serological and electron microscopy only in 1990 s [10, 18, 24]. Maruthi et al. (2006) characterized the DoYMV isolates from Mysore and Bangalore and reported that their isolates shared 92.5 to 95.3% identity with previously described isolates from North India and Bangladesh [17]. Akram et al. (2015) characterized full genome of DoYMV isolate from Kanpur (Accession No:KJ481204) which revealed 93 to 96% identity with DoYMV isolate sequences available in database [3]. From the perusal of literature and our own study it can be inferred that DoYMV isolates are highly homogenous across different parts of

Field bean is an important vegetable crop grown extensively in the Indian subcontinent for its nutrient rich pod and grain. It is severely affected by the yellow mosaic disease and in South India and incidence has increased

Fig. 5 Detection of begomovirus in leaves of grow-out test plants by DAS-ELISA using ToLCNDV antiserum; Absorbance at 405 nm was recorded 30 min after adding substrate. Leaf samples from field bean

plant showing mosaic symptom was used as positive control. Leaf samples from uninoculated healthy field bean plants served as negative control

Fig. 6 Detection of DoYMV in grow-out test plants by PCR using DoYMV-CP primer (DoYMV-CP 377F and DoYMV-CP 580R); Lane 1—Marker (100 bp), Lane 2 to 11—amplicon with DNA

extracted from leaf samples of grow-out test, Lane 12—Positive control (Infected leaf from field bean plant), Lane 13—Negative control (Healthy uninoculated leaf from field bean plant)

Indian subcontinent. From the comparison, it is evident that isolates from South India share more identity between them and may be considered as belonging to one strain as they share more than 97% identity in DNA A component. Characterization of DNA B component (Data not shown) is in progress and the segment for which sequences are completed, exhibited nearly 94% identity with DNA B of Kanpur isolate (Accession no: KJ481205), the only DNA B sequence available for DoYMV.

The most significant result of the study is the detection of DoYMV in seed parts. In the seeds collected from infected plants, the virus was detected upto 100% in embryonic axis; whereas percentage detection in seed coat and endosperm were 37.5 and 69.23% respectively. In the grow-out tests, though the plants were symptom free, virus detection was upto 46 and 55% by DAS-ELISA and PCR, confirming the seed transmission of the virus. This study constitute the sixth report on the seed transmission of geminiviruses. Kim et al. (2015) showed that more than 70% of the seeds from infected sweet potato plants tested positive for SpLCV. The transmission from seeds to seedlings was upto 15%. They also clearly demonstrated that virus replicated in test seedlings [14]. Kil et al. reported seed infectivity of TYLCV-IL upto 20-100% in tomato and average transmission rate to seedlings was upto 84 and 80% with whitefly mediated transmission and agroinoculation respectively [11]. Kothandaraman et al.

(2016) demonstrated presence of MYMV in blackgram by nucleic acid hybridization and PCR. In the grow out tests virus was detected in 32% of tested seedlings, though all of them remained symptom free [15]. Kil et al. revealed the presence of TYLCV in agroinoculated soybean and capsicum plants which were considered to be non-hosts for TYLCV-IL [12, 13]. From the results of Anabestani et al. (2017) on the presence of beet curly top virus and beet curly top Iran virus upto 38.2 to 78% and 8.8 to 18.5% respectively in Petunia, it is speculated that seed transmission of many geminiviruses may be soon a reality [4].

Of all the methods of virus transmission, seed transmission is the major constraint. Even if the seed transmission is low, the infected seedlings may serve as potential inoculum source as demonstrated by Kil et al. (2016) for TYLCV [11]. True seed transmission occurs when embryo is infected either through pollen or entry of virus into egg mother cell. In the absence of vascular tissue and plasmodesmata connection within seed tissue, the mechanism of entry of geminiviruses into embryo needs to be understood. In all the instances above cited for seed transmission, absence of symptoms in grow-out seedlings is a common phenomenon. Though replication of viral forms have been demonstrated, often symptom expression is not met with. It is hypothesized that the RNAi defence of the plant may be highly active in emerging seedlings, thereby interfering in symptom expression. The

symptomless host plants may serve as inoculum source for active vector transmission.

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