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SHORT COMMUNICATION

Detection of *Cardamom mosaic virus* and *Banana bract mosaic virus* in cardamom using SYBR Green based reverse transcription-quantitative PCR

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Abstract Cardamom being perennial, propagated vegetatively, detecting viruses in planting material is important to check the spread of viruses through infected material. Thus development of effective and sensitive assay for detection of viruses is need of the time. In this view, assay for the detection of *Cardamom mosaic virus* (CdMV) and *Banana bract mosaic virus* (BBrMV), infecting cardamom was developed using SYBR Green one step reverse transcription-quantitative PCR (RT-qPCR). The RT-qPCR assay amplified all isolates of CdMV and BBrMV tested but no amplification was obtained with RNA of healthy plants. Recombinant plasmids carrying target virus regions corresponding to both viruses were quantified, serially diluted and used as standards in qPCR to develop standard curve to enable quantification. When tenfold serial dilutions of the total RNAs from infected plants were tested through RT-qPCR, the detection limit of the assay was estimated to be 16 copies for CdMV and 10 copies for BBrMV, which was approximately 1,000-fold higher than the conventional RT-PCR. The RT-qPCR assay was validated by testing field samples collected from different cardamom growing regions of India. This is the first report of RT-qPCR assay for the detection of CdMV and BBrMV in cardamom.

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Cardamom (*Elettaria cardamomum* Maton) acclaimed as the 'Queen of Spices' is the true cardamom belonging to the family Zingiberaceae. It is native of evergreen forests of Western Ghats of South India [6]. Mosaic/katte disease caused by *Cardamom mosaic virus* (CdMV) (genus: *Macluravirus*) and chlorotic streak disease caused by *Banana bract mosaic virus* (BBrMV) (genus: *Potyvirus*) are the major viral diseases affecting the crop [3, 10]. The mosaic disease is characterized by interveinal prominent discontinuous yellowish stripes running out from midrib to the margin of leaves [12]. Besides India, mosaic disease is predominant in Guatemala and Sri Lanka. Crop losses of 10–60, 26–91 and 82–92 % were reported in first, second and third years of production, respectively [14]. The chlorotic streak disease is characterized by continuous or discontinuous intraveinal chlorotic streak with an incidence ranging from 0 to 15 % in the plantations where either banana was grown nearby or banana was the previous crop [10]. Coat protein gene sequence studies of different isolates revealed that CdMV as variable (identity ranging from 74.8 to 99.3 %) [4, 11] while BBrMV was highly conserved (97–99 %) [10].

Detection of virus is an important component in effective management. As cardamom is mainly propagated vegetatively through tillers, identification of virus-free plants is crucial in selecting mother plants to be used for further propagation. Symptoms alone cannot be a reliable criteria for identification of virus-free plants due to the variable incubation period of the virus depending on the cultivar and season. Hence sensitive assays are needed for

selecting virus-free mother plants. So far, ELISA and RT-PCR based assays were reported for the detection of CdMV and BBrMV infecting cardamom [1, 8, 10]. The sensitivity of real-time quantitative PCR (qPCR) and real-time reverse transcription (RT) qPCR have been explored by many workers in developing detection assays for several viruses [5]. It combines the simplicity of PCR amplification with the sensitivity of fluorescence detection during amplification. It provides the added advantage that of precise quantification and does not require post-PCR processing. These features make RT-qPCR assay very suitable and widely accepted for pathogen detection [13]. With this in view we developed sensitive detection assays for CdMV and BBrMV in cardamom using SYBR Green based RT-qPCR.

For initial standardization of RT-qPCR, known infected and healthy cardamom plants confirmed through RT-PCR using virus specific primers as described previously [1, 10] were used. Same plants were used for determining detection limits of the virus in plants and its comparison with conventional RT-PCR. To validate the RT-qPCR assay for the detection of CdMV, 17 symptomatic and 13 non-symptomatic cardamom samples collected from different regions of Kerala and Karnataka were used. Similarly to validate RT-qPCR assay for BBrMV, 9 symptomatic and 11 non-symptomatic cardamom samples collected from Wayanad District of Kerala were used. The non-symptomatic samples included the apparently healthy plants collected from infected clumps as well as from adjacent apparently healthy clumps.

Total RNA was isolated from infected and healthy plants following the procedure described previously [9]. Briefly, 100 mg of young tissue was ground in extraction buffer followed by addition of water saturated phenol and centrifugation. The aqueous phase containing RNA was transferred to a fresh tube and precipitated using equal volume of ice cold isopropanol. The yield of RNA was determined using a spectrophotometer (Eppendorf, Germany). The forward and reverse primers for the RT-qPCR assay were designed based on the coat protein gene sequences of BBrMV and CdMV deposited in the GenBank using the Primer 3 software (http://primer3.wi.mit.edu). The sequence specificity of the primers were checked by BLASTn programme of NCBI, secondary structure, intra and inter primer complementarity were checked through Oligo Calc (http://www.basic.northwestern.edu/biotools/oligocalc.html). The forward and reverse primers for CdMV (CdMV F-5′ TTTTTGCGTTCAAACGAATG 3′, CdMV R-5′ CTTGCTTCGCTGAACTTTCC 3′ with an expected amplicon length of 127 bp) and forward and reverse primers for BBrMV (BBrMV F-5′ CGTCAGCTC CATCTTCATCA-3′, BBrMV R-5′ TCGGTTTAATTCGA GGGATG 3′ with an expected amplicon length of 117 bp) were synthesized at Integrated DNA Technologies, Coralville, IA, USA.

RT-qPCR reaction for both the viruses were carried out separately in a final volume of 25 µl each containing: 12.5 µl of 2× QuantiFast^{TM} SYBR Green PCR Master mix (Qiagen, Hilden, Germany), 1.0 µl (1 µM/µl) of each forward and reverse primer and 1 µl template (about 120 ng) and 50 U of Revert Aid reverse transcriptase (Fermentas, Maryland, USA). Thermocycling conditions consisted of an initial cDNA synthesis at 42 °C for 45 min at the beginning and initial denaturation step at 95 °C for 10 min, followed by 35 cycles of 95 °C for 15 s and 60 °C for BBrMV and 56 °C for CdMV for 45 s. Total RNA from healthy cardamom and a water control (without template) were used as negative controls to monitor specificity of the primers and potential contamination within the real-time RT-qPCR reagents. In order to check the specificity of the RT-qPCR product, the amplicons were subjected to melt curve analysis from 60 to 95 °C. In melting curve analysis, the product was heated to 95 °C for 1 min, then decreased to 55 °C for 45 s (pre melt conditioning) followed by heating back to 95 °C at a rate of 0.5 °C increments. Apart from this, the RT-qPCR products were also verified using a 1.5 % agarose gel electrophoresis.

For developing a standard curve, known quantities of recombinant plasmid DNA of both viruses were used. The coat protein gene of both viruses was amplified through RT-PCR using total RNA as template and the products were gel purified using gel extraction kit (GenElute Gel Elution kit, Sigma-Aldrich, Bangalore, India). The products were then cloned into pTZ57R/T cloning vector (Fermentas, Glen Burnie, USA) and transformed into competent *Escherichia coli* strain DH5α using InsTAclone PCR cloning Kit (Fermentas) following manufacturer's instructions. Recombinant clones were selected and sequenced at the automated DNA sequencing facility available at Chromous Biotech, Bangalore, India. Yield of recombinant plasmid DNA was calculated using a spectrophotometer (Biophotometer plus, Eppendorf, Germany) and the copy number was calculated using the formula: copies/µl = (concentration in ng × 6.023 × 10^{23})/(genome length × 1 × 10^{9} × 650). The recombinant plasmid was then serially diluted tenfold (from 1 ng to 10^{-8}) using total RNA isolated from healthy cardamom as diluent and subjected to RT-qPCR as explained above in triplicates.

All of the output data were analyzed with the software available in Rotor Gene Q system (Qiagen) using preset parameters. Standard curve for each virus were constructed by plotting Ct values from the assays with standard dilution versus the logarithm of the copy number for the exponential phase of the reaction and fitting a straight line to these data by simple linear regression [13]. The slope of the standard curve was used to determine the efficiency (E) of

the PCR: $E = [10^{-1/\text{slope}}] - 1$ considering 100 % efficiency for a value of two [7]. Optimal PCR efficiency is achieved when a slope of −3.32 was reached. The coefficient (R^2) was calculated to determine the validity of the linear regression. For detection purposes, a quantification cycle of 35 was established as the cutoff for distinguishing positive from negative samples.

The detection limits of each RT-qPCR assay in plants were determined by using tenfold serial dilutions of total RNA (from 10^0 to 10^{-5}) diluted using total RNA from healthy plant and subjecting to RT-qPCR. The experiment was repeated a total of three times to check reliability of the assay. The detection limits and corresponding copy number for each dilution was then determined from standard curve developed above. The sensitivity of the assay was compared with conventional RT-PCR by using 1 µl of each of the dilutions using the same set of primers.

In order to validate the RT-qPCR assays for CdMV and BBrMV, 1 µl of total RNA isolated from symptomatic and non-symptomatic samples collected from different regions were subjected to respective RT-qPCR assay along with a known positive and negative controls. Prior to RT-qPCR assay, all the samples were subjected to direct antigen coated (DAC)-ELISA using polyclonal antiserum of CdMV (at 1:250 dilution) and BBrMV (at 1:1,000 dilution) and RT-PCR as per the procedure described previously [1, 2, 10]. Polyclonal antiserum for the detection of CdMV was produced in our lab while polyclonal antiserum for BBrMV was obtained from National Research Centre for Banana, Trichy, India.

SYBR Green RT-qPCR assays were carried out in one step and optimized for concentration of primers (40 nM for both the viruses), template RNA (about 120 ng) and annealing temperature (56 °C for CdMV and 60 °C for BBrMV). The RT-qPCR assay showed amplification only in positive samples that were previously confirmed by RT-PCR, whereas no amplification was seen in healthy and water control. The melt peak occurred at 83 ± 0.5 °C for CdMV and BBrMV and no evidence of nonspecific amplification or primer-dimerization. The healthy and water control showed no melting peak. The specificity of the product was further confirmed by agarose gel electrophoresis which showed a single band of the expected size in both cases.

The sensitivity and range of the SYBR Green RT-qPCR assays were evaluated from the standard curves for each virus. For the construction of the standard curve, 8 dilution steps of plasmid standards (from 2.3×10^8 to 2.3×10^0 plasmid copies for CdMV and from 2.4×10^8 to 2.4×10^0 for plasmid copies for BBrMV) were run in triplicates. The amplification of the standard dilution series yielded in linear relations with reliable results ($R^2 = 0.99906$ for CdMV and $R^2 = 0.99902$ for BBrMV) in the range of

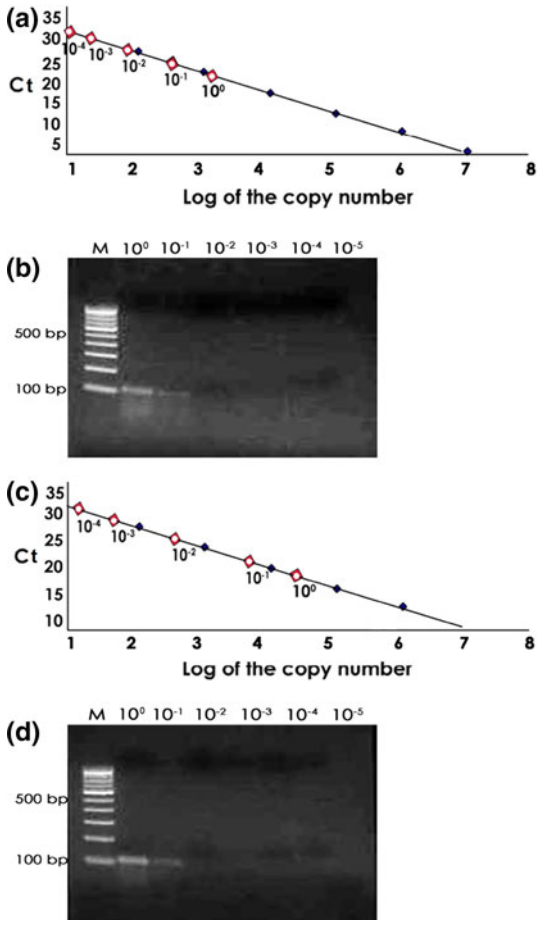

Fig. 1 Standard curve demonstrating the quantification and comparison of detection limits of *Cardamom mosaic virus* (CdMV) and *Banana bract mosaic virus* (BBrMV) in RT-qPCR and conventional RT-PCR. Development of standard curve and detection limits in plants through RT-qPCR for CdMV (**a**) and BBrMV (**c**). Cycle threshold (Ct) was plotted against the log of copy number of plasmid containing virus-specific amplicon as insert to get standard curve. (*Filled diamond*) show the Ct value obtained for different plasmid copy numbers used to develop standard curve. (*Open diamond*) show the Ct value obtained for different dilutions (10^0 to 10^{-5}) of total RNA from infected plant. Determination of detection limits of CdMV (**b**) and BBrMV (**d**) through conventional RT-PCR. *Lane* M shows 100 bp DNA ladder. *Lanes* 10^0 to 10^{-5} show different dilutions of total RNA from infected plant

$10–10^8$ copies of the target DNA locus per qPCR. The detection limit for CdMV was as low as 6 plasmid copies while it was 2 plasmids copies for BBrMV as estimated from the standard curve (Fig. 1a, c). The efficiencies of reactions were 0.60 for CdMV and 0.84 for BBrMV.

The detection limits of the viruses in plants were determined by using tenfold dilutions of total RNA from infected cardamom plant (10^0 to 10^{-5}dilutions, i.e. 12×10^1 to 12×10^{-4} ng total RNA). 1 µl of each dilution was used as template and was then subjected to RT-qPCR. By taking the Ct values for each dilution the detection limit (copy number) of CdMV and BBrMV was

determined from the standard curve (Fig. 1a, c). It was found that the detection limit was as low as 16 and 10 copies (10^{-4} dilution) for CdMV and BBrMV respectively in RT-qPCR while conventional RT-PCR could detect both the viruses up to 10^{-1} dilutions only (Fig. 1b, d). The results showed that RT-qPCR is 1,000 times more sensitive than conventional RT-PCR assays.

In order to validate the RT-qPCR assay for detecting CdMV, 17 symptomatic and 13 non-symptomatic field samples of cardamom collected from different agro-climatic regions were used. Similarly, 9 symptomatic and 11 non-symptomatic cardamom plants were used for validating RT-qPCR assay of BBrMV. In DAC-ELISA, 12 samples were negative for CdMV which included six symptomatic and six non-symptomatic samples. In the case of BBrMV, all the samples except three non-symptomatic samples showed positive reaction in DAC-ELISA. Total RNA isolated from these samples was then subjected to RT-PCR and RT-qPCR along with a known positive and negative control. In RT-PCR assay all the symptomatic samples and nine non-symptomatic samples were positive for CdMV while in the case of RT-qPCR, in addition to all the samples that were positive in RT-PCR, one more non-symptomatic sample showed positive reaction. The samples that were negative in RT-qPCR were from the apparently healthy clumps, which indicate that they are not infected by CdMV. In the case of BBrMV, all the samples except one non-symptomatic showed positive reaction in RT-PCR, whereas in RT-qPCR all the 20 samples were positive. Using Ct value obtained for each sample, the copy number of the virus in each of the samples was estimated from the standard curve. The Ct value ranged from 9 to 29 for CdMV samples with corresponding copy number ranging from 1.1×10^6 to 1.2×10^2 while Ct value ranged from 14–28 for BBrMV samples with corresponding copy number from 8.3×10^5 to 9.8×10^1. No correlation could be seen between Ct values and symptomatic or non-symptomatic samples used in the validation. This probably indicates that samples used for testing might have been infected by the virus at different times. However, the assay was not used to detect BBrMV from banana samples.

Sensitive, rapid and quantitative detection of CdMV and BBrMV infecting cardamom was developed using RT-qPCR. Primers were designed based on the conserved regions in the coat protein gene sequence. Consistent melt peak at 83 °C ± 0.5 by melt curve analysis, lack of cross reactivity between CdMV and BBrMV infecting cardamom and identification of amplicon as a single band of the expected size and absence of amplification signals in negative controls such as healthy cardamom and water confirmed the specificity of the reaction. The recombinant plasmids were used in qPCR for constructing a standard curve for calculating the absolute copy number [5] and tenfold serial dilutions of the total RNA were used to determine sensitivity and detection limit of the assay in plants. A minimum of 6 and 2 cDNA copies of the plasmid standard, and 16 and 10 copies of the virus in total RNA from infected plants for CdMV and BBrMV respectively could be detected through RT-qPCR. Comparative sensitivity study between RT-qPCR and the conventional RT-PCR assays in detecting CdMV and BBrMV in infected samples showed that RT-qPCR was 1,000 times more sensitive than conventional RT-PCR. Thus RT-qPCR can be used as an alternative to conventional RT-PCR assay for routine detection of viruses.

This is the first report of RT-qPCR assay for the detection of CdMV and BBrMV infecting cardamom. RT-qPCR based detection of BBrMV infecting banana using Taqman probe was reported, which was 1,000 times more sensitive than conventional RT-PCR [15]. The performance of the newly developed RT-qPCR assay for BBrMV and CdMV was evaluated by testing field samples of cardamom collected from different regions of Karnataka and Kerala states of India indicating its suitability to detect different isolates of both viruses. RT-qPCR could detect viruses in more number of non-symptomatic plants compared to ELISA and conventional RT-PCR indicating its higher sensitivity and suitability in identifying virus-free plants which can be used as source for production of virus-free planting materials through vegetative propagation.

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