Duplex PCR to detect both Papaya ring spot virus and Papaya leaf curl virus simultaneously from naturally infected papaya (Carica papaya L.)

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Papaya, a major fruit crop in India and worldwide, is affected by many fungal and viral diseases. A mixed infection of Papaya ring spot virus (PRSV), a linear single-stranded (+) RNA genome of approx 10 kb size, and Papaya leaf curl virus (PaLCV), a bipartite Gemini virus (component A & B) having circular single-stranded DNA (+) genome of about 5.2 kb, has hampered the production and productivity of papaya in many parts of world. Rapid detection techniques are important in prevention of spread of the disease in field conditions. In the present study, a rapid and reliable PCR based detection protocol has been standardized. Sets of primers were designed, based on the respective virus isolate sequence data available in GenBank, to obtain anticipated products of calculated size.

Keywords: Duplex PCR, Papaya ring spot virus (PRSV), Papaya leaf curl virus (PaLCV)

Papaya (Carica papaya L.; Family: Caricaceae) is an important fruit crop believed to be the native to Southern Mexico and neighbouring Central America. It is grown in many tropical and subtropical countries all over the world. The production is hampered by numerous fungal pathogens and viral diseases. Among the viruses, Papaya ring spot virus (PRSV), a definite member of the genus Potyvirus belonging to the family Potyviridae and having linear single-stranded (+) RNA genome of approximately 10 kb size, and Papaya leaf curl virus (PaLCV), a bipartite Gemini virus (component A & B) having circular single-stranded DNA (+) genome of about 5.2 kb in total, drastically reduce the yield and market quality of fruits either singly or in mixed infections.

PRSV is perhaps the most limiting factor in papaya production in many countries1-4. The virus was first reported from western India in 1958 and since then it has spread over many geographical locations causing severe crop losses of upto 85-90%5,6. The virions of PRSV are flexuous filamentous particles of about 760-800 nm × 12 nm and are capable of producing inclusion bodies in the cytoplasm of host cells. Natural transmission of PRSV occurs by various species of aphids in a non persistent manner and has a limited host range that consists mainly of cucurbits and papaya. The two serologically indistinguishable strains of PRSV have been recorded, viz., PRSV-P infecting papaya and cucurbits and PRSV-W infecting only cucurbits. PRSV causes an array of symptoms in papaya6. These include mottling and malformation of leaves, ringspots and streaking on fruits, stems and petioles. As a result of which the plant becomes stunted with fewer and smaller fruits, and eventually die.

Leaf curl disease of papaya, caused by PaLCV, is a devastating disease prevailing in several parts of India. This disease is characterized by severe curling, crinkling and deformation of leaves. Severity is more on young leaves, sometimes accompanied by vein clearing, inward rolling and thickening of veins. As the disease progresses plants become stunted and fruit yield is reduced. The virus measures 38 nm in length and 22 nm in diameter and is readily transmitted by grafting and by means of polyphagus white fly Bemisia tabaci Genn.9

Early infection of the cultivars by two different viruses results in complete loss in yield. In mixed infections of papaya with two or more viruses10,11, a synergistic interaction between two independent viruses in the same host can occur; it is typically characterized by a dramatic increase in symptoms and accumulation of one of the two coinfecting viruses12,13. Many synergistic diseases involve a member of the genus Potyvirus as one of the infecting viruses14,15. Although serological techniques can be deployed as a strategy to detect the mixed infections, it may be fatal at times to the low titre of one of the viruses of mixed infection. So, simple, sensitive and reliable tools for the early and simultaneous diagnosis need to be employed. RT-PCR and PCR is routinely used to detect ring spot and leaf curl virus of papaya16-19. In the present study, a sensitive and reliable technique has been devised to detect PRSV and PaLCV infections simultaneously in the same preparations employing PCR.
Papaya leaf sample naturally exhibiting mixed infection of both PRSV and PaLCV were collected from papaya orchard in Bihar, India (Fig. 1). Two sets of primers were designed to successfully amplify both viruses in simplex and duplex PCR preparations. The primers were forward primer PRS8617F: 5'-ATCACAATGATTACGCGCTGCG-3', and reverse primer PRSPATR: 5'-CTCTCATTCTAAGAGGCTCGAATAG-3' for PRSV coat protein plus partial Nib region; and forward primer MKBEGAF4: 5'-ATATCTGCAGGGNAARATHTGGATGGA-3' and reverse primer MKBEGAR5: 5'-TGGACTGCAGACNGGNAARACNATGTGGGC-3' for PaLCV coat protein plus partial AC2 protein (Figs 2 & 3). The mixed infected sample was individually amplified for PRSV and PaLCV in a simplex PCR preparation to confirm the presence of both viruses. Further, the same sample was subjected for simultaneous detection of both viruses in a duplex PCR preparation. Total RNA was extracted from mixed infected as well as PRSV infected papaya leaf sample (positive control) using TRI reagent following manufacturer’s protocol. Similarly DNA was extracted from mixed infected as well as from PaLCV infected papaya leaf sample (positive control) using CTAB method. 100 mg of healthy and PRSV infected papaya tissue was used for RNA extraction and 2 g of healthy and PaLCV infected papaya sample was used for DNA extraction. Simplex PCR for PRSV and PaLCV detection from single and mixed infected sample was first standardized. cDNA was synthesized using 3 µL of total RNA and 1 µL of antisense primer (100 pmole/µL), the mixture was centrifuged and incubated at 70°C for 5 min. A master mix was prepared separately, using 5x MMuLV buffer, 10 mM dNTP's, 25 U MMuLV RT enzyme (MBI, Fermentas), and then transferred into PCR tube containing template RNA and mixed gently. The PCR tube was incubated at 42°C for 1 h, followed by 70°C for 10 min in a thermal cycler (Applied Biosystems, GenAmp PCR system 9700). 3 µL of amplified cDNA (for PRSV) and DNA (for PaLCV) was used in 25 µL of reaction mixture containing 10× PCR buffer (5 µL), 2 mM dNTP's and 50 pmole/µL each of forward and reverse primers. PCR was performed with an initial denaturation step of 94°C (2 min), followed by 35 cycles of 94°C (45 sec), 54-58°C (60 sec) and 72°C (90 sec) and a final 20 min extension step at 72°C. PCR products were analyzed electrophoretically in 0.8% agarose gels in 0.5× TBE buffer and visualized on a UV-transilluminator by ethidium bromide staining.

In order to standardize the duplex PCR, two sets of primers were mixed in a 50 µL reaction mixture containing 10× PCR buffer (5 µL), 2 mM DNTP's (4 µL), 25 mM MgCl₂ (4 µL), 100 pmole/µL of PaLCV forward and reverse primer (1 µL each), 100 pmole/µL of PRSV forward and reverse primer (1 µL each), DNA (2 µL), cDNA (6 µL), Taq (0.6 µL). The cDNA was synthesized using same protocol followed for simplex PCR. PCR was performed using the following parameters: one cycle at 94°C for 2 min,
35 cycles at 94°C for 45 sec, 54-58°C for 60 sec and 72°C for 90 sec, followed by 72°C extension for 20 min to determine the annealing temperature for both the viruses. PCR products were analyzed electrophoretically in 0.8% agarose gels. Simplex and duplex PCR was simultaneously carried out for comparison.

In the present investigation, two different sets of primer pairs, designed to amplify coat protein plus partial AC2 protein coding region of PaLCV and coat protein plus partial Nib region of PRSV, successfully produced amplicons of 1.2 and 1.4 kb from the PaLCV and PRSV infected papaya leaf samples (positive control), respectively by simplex PCR, while no amplification products were observed in healthy plant samples (Figs 4 & 5). The same primers were successfully employed to amplify fragments of both viruses in a mixed infection in duplex PCR since they displayed commonality in terms of G+C content, melting temperature and length of primer pairs. Considerably good amount of DNA and RNA were extracted from conventional methods without using expensive kits for performing PCR. Various parameters, such as, primer concentration of forward and reverse primers (50-100 pmole/µL), annealing temperature (55-60°C), annealing time (45-60 sec) and number of cycles (25-35) used, were standardized for the simplex and duplex PCR reactions for PaLCV and PRSV detection. Better amplification was observed at 50 pmole/µL concentrations of each primer, 55°C annealing temperature for 60 sec and 35 cycles in case of simplex PCR for PaLCV and touchdown PCR at 58°C with 0.1°C decrease at each cycle. Duplex PCR successfully amplified both viruses at 55°C annealing temperature for 60 sec and 35 cycles with 100 pmole/µL primer concentration. Thus, the procedure can be effectively employed for validation of field samples with mixed infection.

The present optimized detection procedure was used for validating papaya samples collected from different geographical locations. Among the samples tested, few of them were found to be infected with both viruses, whereas others with either of the two. A representative gel picture showing detection of viruses in field samples tested is presented in Fig 6. Further, the sequence identity of amplicons was confirmed by sequencing in duplicate. The BLAST search revealed highest homology to AF120270 and AY738103 for PRSV and PaLCV, respectively.

In the present study, all reactions were carried out using conventional PCR mixture and the extraction procedures of RNA and DNA was also done without the use of expensive kits. Thus, here we report the simultaneous detection of RNA and DNA viruses.
infecting papaya using duplex PCR from India. Such fast and reliable detection techniques can be used to monitor disease at an early stage of crop growth in the field to forego losses. Moreover, there is a need to develop breeding lines/cultivars showing multiple resistances to economically important viruses. Transgenic technology, so far, is one of the effective strategies to control viruses of papaya and pyramiding genes into single cultivar to control more than one virus would sustain the productivity in papaya cultivation.

References