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SHORT REPORTS

Duplex PCR assay for detection of chickpea chlorotic dwarf virus and peanut witches' broom phytoplasma in chickpea

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Abstract

A duplex PCR assay was standardized by optimizing PCR reaction constituents and cycles for the simultaneous detection of chickpea chlorotic dwarf virus (CpCDV) and a peanut witches' broom (PnWB) phytoplasma associated with the chickpea stunt disease. Coat protein gene and *tuf* gene specific primers for CpCDV and phytoplasmas were used. Different concentrations of the PCR components such as *Taq* polymerase, primers and PCR annealing temperature were standardized for the identification of the two agents by a duplex PCR assay. Expected amplicons of 590 bp for CpCDV and 1090 bp for phytoplasmas were consistently amplified from the symptomatic chickpea tissues. That resulted in equally efficient and sensitive in detecting single or mixed infection of CpCDV and PnWB phytoplasma in 148 symptomatic chickpea stunt samples collected in two states of India. The results indicate the robustness in the detection of pathogens present in chickpea showing stunt disease and for theoretical use in epidemiological studies that would help the appropriate disease management strategies.

Keywords CpCDV · PnWB phytoplasma · Detection · Coat protein gene · *Tuf* gene

Chickpea (*Cicer arietinum* L.) is one of the prominent pulse crop grown in India. Although, India has the highest acreage, low productivity is reported due to the prevalence of biotic and abiotic stresses (Singh et al. 1993). Chickpea is challenged by several diseases among which chickpea stunt is an emerging disease caused by different groups of viruses like e.g. cucumber mosaic virus, chickpea chlorotic dwarf virus (CpCDV) and other poleroviruses, and is reported in many chickpea growing countries worldwide (Shreenath et al. 2020; Reddy et al. 2021). Chickpea chlorotic dwarf virus (CpCDV) was identified as the prevailing virus across Indian subcontinent, Middle East, North Africa and India (Kanakala and Kuria 2019; Shreenath et al. 2020; Reddy et al. 2021).

Phytoplasma enclosed in the 16SrII-D subgroup has been described as associated with CpSD and chickpea phyllody disease in Pakistan, Oman, Australia, Myanmar and Sudan (Reddy et al. 1991; Saqib et al. 2005; Al-Saady et al. 2006; Akhtar et al. 2008). The presence of phytoplasma infection has been reported throughout the major chickpea growing areas of India (Reddy et al. 2021).

Pathogen identification is crucial for implementing effective disease management measures, and it requires the use of various detection technologies, to distinguish distinct infections associated with identical symptoms is important. Plant viruses and phytoplasmas are still a serious concern pathogens in agriculture, causing significant economic losses in many crops. The nucleic acid-based detection technologies have allowed improving sensitivity and low titer limits for the sensitive and reliable detection of plant viruses and phytoplasmas (López et al. 2009; Pallás et al. 2018). Duplex and multiplex PCR are fast, consistent, and economical methods that have been used successfully for detecting mixed populations of plant viruses and viroids in a single PCR assay (Pallás et al. 2018). Duplex and multiplex PCR tests were also developed to detect different groups of plant viruses and phytoplasmas in important crops worldwide (Biswas et al. 2013; Majumder and Baranwal 2014; Swarnalatha and Reddy 2014; Malandraki et al. 2015).

In recent surveys of chickpea crops in two southern states of India (Andhra Pradesh and Telangana), both the CpCDV and phytoplasmas were detected in several chickpea

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commercial varieties (Reddy et al. 2021). A duplex PCR assay suitable for detecting the presence of both pathogens in chickpea crops simultaneously was therefore developed.

A roving survey was conducted during December-February of 2018–19 and 2019–2020 in different farmer's fields of Kadapa (56 samples) and Kurnool (50 samples) districts of Andhra Pradesh and Ranga Reddy (42 samples) district of Telangana for chickpea stunt and phyllody disease. The disease incidence was calculated in each chickpea field by counting the number of diseased plants with that of a total number of plants by using formula (C-T)/C × 100, where, C is the plant with no symptoms and T is the number of symptomatic plants in 5 × 5 m square area. Symptomatic and non-symptomatic chickpea plants showing suspected virus and/or phytoplasma symptoms were collected. All the collected plant samples were packed in polythene bags and kept in a deep freezer at − 80 °C for PCR analysis. Total genomic DNA was extracted from the 148 symptomatic and 6 asymptomatic plant samples using Qiagen DNeasy plant mini kit (Germany) as per manufacturer protocol and used as a template for PCR assays.

The extracted DNA was amplified using 16SrII-D specific *tuf* gene primer pair (TUF-II-F2/TUF-II-R1) (Al-Subhi et al. 2018) for the amplification of PnWB phytoplasma and partial coat protein gene (CP) specific primer pair (MCPF/MCPR) (Kanakala et al. 2013) for the detection of CpCDV. All the amplifications were carried out using a thermal cycler Eppendorf (Germany). The 50 µl reaction mixture contains 100 ng of template DNA (2 µl), 25 mM Mgcl2 (4 µl), 10 mM dNTPs (1 µl), 10 µM each of the forward and reverse primers (1 µl), 2.5 U/µl of *Taq* polymerase (0.5 µl), 10X PCR buffer (5 µl) and nuclease-free water up to 50 µl. The PCR assay cycling protocol used was as described (Al-Subhi et al. 2018; Kanakala et al. 2013).

The plant samples which were confirmed to be infected with virus and phytoplasma were used to optimize and evaluate the specificity of the duplex PCR assay.

PCR program cycles were confirmed with annealing temperatures ranging from 53 to 57 °C. The amplification cycle was programmed as pre-incubation at 94 °C for 2 min, followed by 35 cycles of melting at 94 °C for 1 min, annealing at (53 °C–57 °C) for 1 min, synthesis at 72 °C for 2 min and additional elongation at 72 °C for 10 min.

A preliminary PCR assay was carried out with primer concentrations of 0.2 µM at the annealing temperature (56 °C) determined from the experiment described above. However, the amplification was inconsistent, and some of the amplified products were faint and barely visible. To solve this difficulty, different combinations of primers' concentrations (0.15 µM, 0.2 µM, and 0.25 µM) for phytoplasma and CpCDV were tested.

PCRs were carried out using different *Taq* DNA polymerase concentrations of 1, 1.5, 2, 2.5, and 3U, with the optimum primer concentration determined by the preceding test. Based on the results of pilot experiments (Reddy and Rao 2019), the concentration of PCR buffer is increased from 1 to 2X, whereas $MgCl_2$ from 2 mM to 2.5 mM and dNTP from 0.2 to 0.3 mM.

The optimized duplex PCR was validated by performing it on 148 symptomatic samples of chickpea collected from different locations of AP and Telangana (Table 1). To evaluate repeatability, the duplex test was carried out three times. PCR products were electrophoresed in a 1% agarose gel stained with ethidium bromide and viewed with a UV transilluminator. The marker, a 1 kb DNA Ladder (Fermentas, Vilnius, Lithuania), was used for the estimation of the molecular weight of the PCR products.

To verify the specificity of the amplified product, PCR products from the representative chickpea samples from Andhra Pradesh and Telangana showing mixed infection were purified using a WizardR SV Gel Extraction Kit (Promega) and the resulting fragments were cloned into *Escherichia coli* DH5-α using pGEM®T vector PCR cloning kit (Promega) following the manufacturer's instructions. By using colony PCR, clones containing an insert were identified and sequenced in both directions at Agrigenome, Kerala, India. A BLAST search of the NCBI nucleotide database was used to confirm these sequences. (http://www.ncbi.nlm.nih.govt/blast).

Characteristic virus and phytoplasma symptoms of phyllody, leaf reddening, stunting and proliferation of axillary shoots were observed with average disease incidence ranging from 3 to 32% during 2018–2019 and 3–13% during 2019–2020 in different chickpea fields of Andhra Pradesh and Telangana states of India (Fig. 1, Table 1).

Out of 148 symptomatic samples tested by PCR, 102 were positive to CpCDV (73 from Andhra Pradesh and 29 from Telangana), 33 samples were positive to phytoplasmas (23 from Andhra Pradesh and 10 from Telangana) whereas 12 samples were positive both for CpCDV and phytoplasmas indicating mixed infection (10 from Andhra Pradesh and 3 from Telangana). PCR products of predictable size ~ 596 bp for CpCDV and ~ 1090 bp were amplified in all the symptomatic samples but not in any asymptomatic plant's samples (data not shown).

Amplifications were achieved with different annealing temperatures (53 °C, 54 °C, 55 °C, 56 °C and 57 °C) but clear and thick bands were achieved at 56 °C (data not shown) and therefore, this was further used as the optimal annealing temperature.

Best PCR product yields were achieved with lower primer concentration (0.15 µM) as compared to higher concentration (data not shown). The reaction conditions were used as one cycle of 94 °C for 2 min; 35 cycles of 94 °C for 1 min; 56 °C for 1 min; 72 °C for 2 min; 72 °C for 10 min; and a holding step at 4 °C.

Table 1 Detection of CpCDV and PnWB phytoplasma infecting chickpea by duplex PCR assay

State		District	Location	Total No of Samples	Symptomatic samples positive to			GenBank Acc.No	
					CpCDV	Phytoplasma	Both	CpCDV	Phytoplasma
Andhra Pradesh	2018–19	Kadapa	Proddatur	16	12	3	1	MT349389	MN634231
			Jammalamadugu	4	1	1	2		
			Yerraguntla	8	5	2	1		
			Mydukuru	4	2	1	1		
		Kurnool	Banaganapalle	8	6	2	–		
			Allagada	6	5	1	–		
			Nandyal	12	10	2	–		
	2019–20	Kadapa	Proddatur	12	9	3	–		
			Jammalamadugu	3	2	1	–		
			Yerraguntla	6	5	1	–		
			Mydukuru	3	2	1	–		
		Kurnool	Banaganapalle	9	5	2	1	MT339218	MT423392
			Allagada	5	3	1	1		
			Nandyal	10	6	2	2		
Telangana	2018–19	Ranga Reddy	Shankarpalle	14	11	3	–		
			Chevella	8	5	3	–		
	2019–20	Ranga Reddy	Shankarpalle	12	8	2	2	MT395665	MT423402
			Chevella	8	5	2	1		

The *Taq* DNA polymerase concentration did not influence the number of produced polymorphic bands, but a difference in band clarity was observed (data not shown) at 2.5U and it was therefore, determined to be optimal.

After the above optimization, the PCR reaction system was set as follows: 10 µl of 10X PCR buffer; 5 µl of 25 mM $MgCl_2$; 1.5 µl of 10 mM dNTP mix; 0.5 µl of DNA template; 0.75 µl each of the forward and reverse primers of CpCDV and phytoplasma; 1 µl of 2.5 U/ µl *Taq* in a total volume of 50 µl. PCR conditions for the experiment were as follows: 94 °C for 2 min; 35 cycles of 94 °C for 1 min, 56 °C for 1 min, and 72 °C for 2 min, followed by a final elongation step of 72 °C for 10 min and a final holding step at 4 °C.

Standardized duplex PCR was further tested with 148 symptomatic samples collected from different locations of Andhra Pradesh and Telangana states (Table 1) of which 12 samples were successfully detected with mixed infection of CpCDV and PnWB phytoplasma (Fig. 2) as amplified in case of normal PCR indicating duplex PCR was an efficient and highly reproducible method for detecting mixed infection of both the virus and phytoplasma associated with chickpea stunt disease in the study. However, no amplification of CpCDV and phytoplasma were detected from any of the asymptomatic chickpea samples collected from both the Telangana and the Andhra Pradesh.

As all the nine sequences of CpSD from different surveyed districts were found 100% identical, only one representative sequence from each district was submitted to NCBI database (Table 1). Pairwise sequence comparison of 1090 bp amplicon of three chickpea strains using *tuf* gene (Table 1) showed 99%–100% sequence identity with brinjal big bud (Acc. No. KX358596), sesamum phyllody (KX358587) and lime witches' broom (Acc. No. MG774510) phytoplasma strains identified in 16SrII (peanut witches' broom) group. Pairwise sequence comparison for CpCDV isolates using partial CP gene sequences of (Table 1) shared 99.08%–97.54% sequence homology with CpCDV isolates from *Spinacia oleracea* (Acc. No. MF178119), lentil (Acc. No. LN865162) from Pakistan, pea (Acc. No. KM229786) from Sudan and chickpea (Acc. No. MG913384) from India.

PCR is a widely used method for the molecular diagnosis of phytoplasmas and plant viruses. It is preferred over other detection methods for its precision and speed. It has been successfully used for plant virus, phytoplasmas and mixed infection diagnostics in different crops (Kakizawa 2019; Feng et al. 2020; Minutolo et al. 2020; Pappi et al. 2020). In this study, a duplex PCR protocol was developed and optimized for simultaneous identification of CpCDV and PnWB phytoplasma infecting chickpea crops from Andhra Pradesh and Telangana states of India associated with CpSD.

A perfect duplex/multiplex PCR system is not simply a mix of several single PCRs, and it also requires comprehensive authentication and validation. Specifically, the primer concentrations, annealing temperature and *Taq* DNA polymerase are key parameters that influence the final amplification results**.** In this study, these variables were optimized. Maximum PCR product yields were achieved with lower

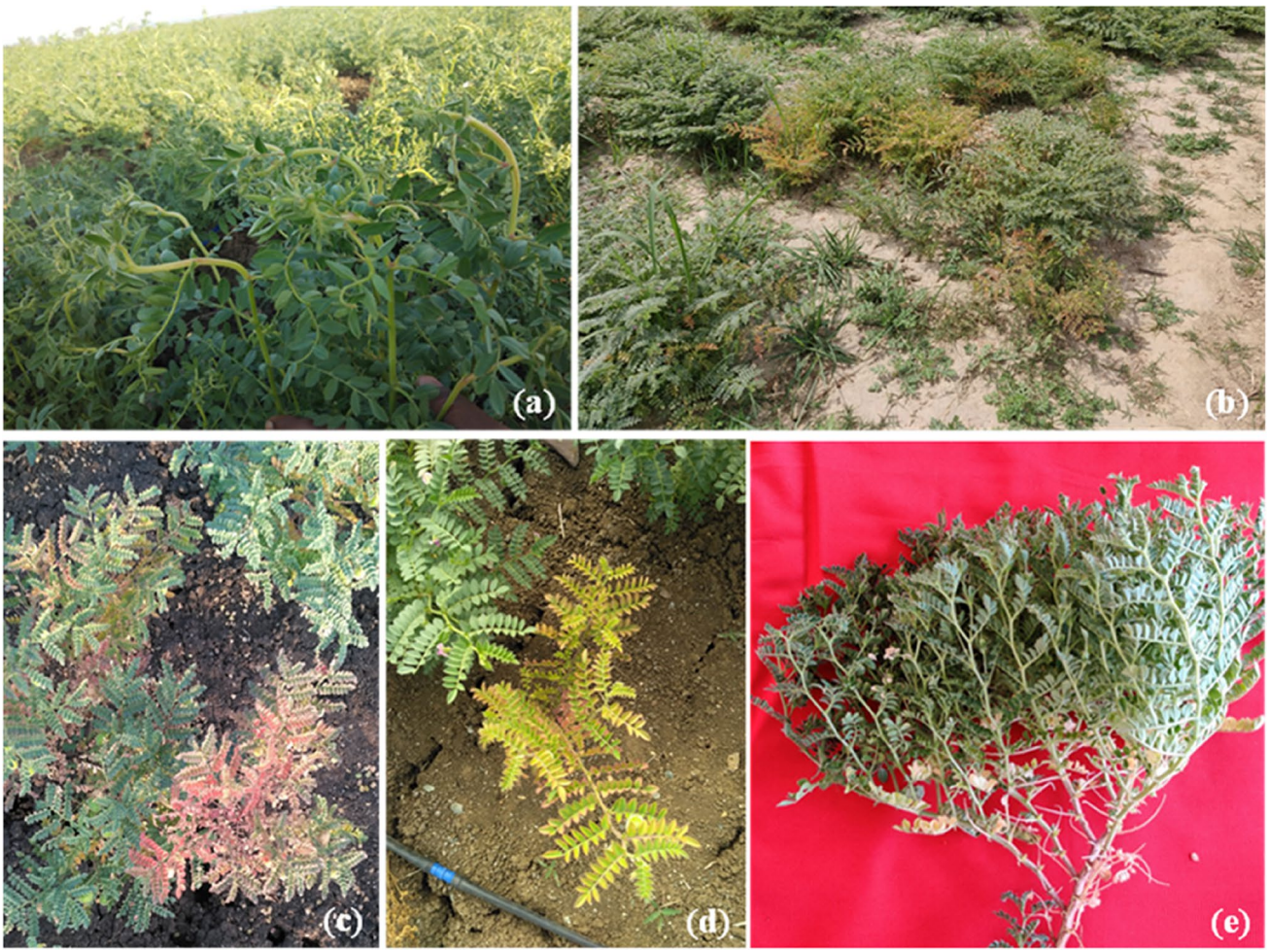

Fig. 1 Symptoms of chickpea stunt and phyllody disease at Andhra Pradesh (**a**–**b**) and Telangana (**c**–**e**): (**a**) Field view of phyllody and excessive proliferation of vegetative shoots (**b**) Field view of chickpea plants showing stunting, yellowing and leaf reddening (**c**) Stunting and typical leaf reddening (**d**) Stunting and yellowing (**e**) Phyllody and excessive proliferation of vegetative shoots

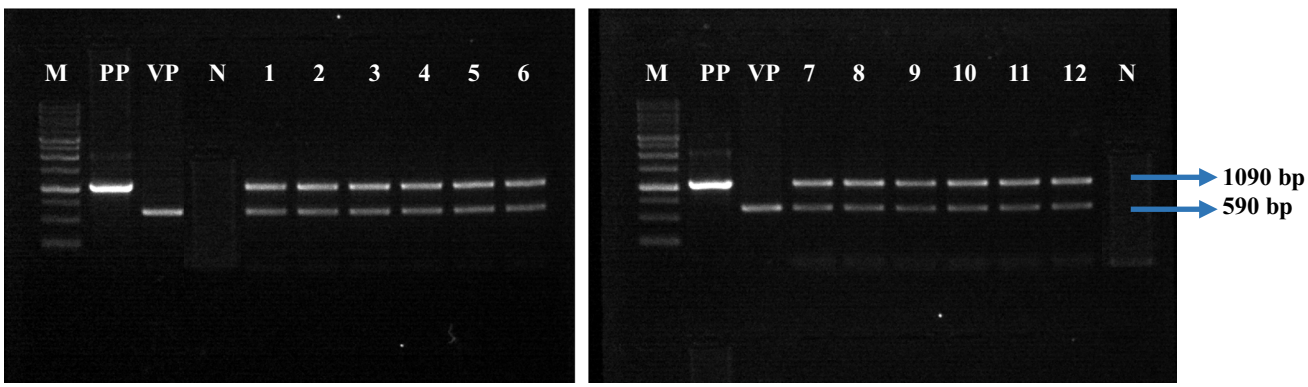

Fig. 2 Gel electrophoresis image for duplex PCR assay results of phytoplasma and CpCDV showing expected amplicons from symptomatic chickpea isolates; *Lane M* 1 kb ladder, *Lane PP* Phytoplasma positive control, *Lane VP CpCDV* positive control, *Lane N* Negative control, *Lane 1 to 12* Samples positive for both CpCDV and phytoplasma

primer concentration (0.15 µM) compared to higher concentration probably due to competition between primers during the PCR annealing step. In a duplex PCR system multiple DNA amplification reactions are simultaneously involved. The Taq DNA polymerase is competed by different steps in reactions. As a result, in a duplex PCR system, raising the quantity of Taq DNA polymerase properly may reduce the competition-induced inhibition; nevertheless, excessive Taq DNA polymerase can readily produce nonspecific amplifications, as previously documented in research (Chen et al. 2015).

So far, duplex PCR assays have been successfully applied for detection of mixed infections of viruses/phytoplasmas on wheat in China (Hongni et al. 2008; Tao et al. 2012), corchorus golden mosaic virus (CoGMV) and a phytoplasma in jute (Biswas et al., 2013) and tomato leaf curl virus and phytoplasma in tomato from India (Swarnalatha and Reddy 2014). In conclusion, the duplex PCR assay developed in the present study is useful for identification of mixed infection of virus and phytoplasma in chickpea.

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Author contributions MG Reddy carried out the survey, processed the samples, prepared the manuscript and standardized duplex PCR for the identification of phytoplasma and virus strains in chickpea. GPR carried out the survey, collected the samples and helped in editing the manuscript.

Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

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