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Research article

COMPARISON OF ELISA AND RT-PCR FOR THE DETECTION OF *PEANUT BUD NECROSIS VIRUS* IN ONION (*ALLIUM CEPA*.L)

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ABSTRACT: *Peanut bud necrosis* virus (PBNV) is an important re-emerging viral pathogen in onion (Allium cepa L.) in India. The virus transmitted by thrips vectors; it belongs to the genus Tospovirus and family Bunyaviridae. The onion crop infected by PBNV and it is a major problem in Southern India. This paper presents the comparison of DAC-ELISA and RT-PCR in the detection of PBNV infected onion samples from South India. The PBNV suspected onion samples (n=145) were collected in the major growing areas of Andhra Pradesh, Tamil Nadu and Karnataka states from South India. Among these collected onion samples, Seventy five samples (51.72%) were confirmed as PBNV infected by DAC-ELISA using the PBNV specific antiserum and in RT-PCR method one hundred twenty four samples (85.51%) were amplified (~800bp) by using the PBNV-CP gene specific primers. In comparison studies the RT-PCR method has added the advantage that it is more sensitive than the DAC-ELISA in the detection of PBNV in onion.
Key words: DAC-ELISA, Onion, Peanut bud necrosis virus, RT-PCR

INTRODUCTION

Onion (*Allium cepa.L.*) is one of the most important global horticultural crop cultivated in tropical and subtropical regions of the world and it is grown on 85.9 million tons from 4.44 million ha. In India it is grown in an area of 12.04 million ha with production of 19.40 million tonnes [1]. The onion productivity is affected by many biotic and abiotic stresses. Among the biotic stresses, viruses cause high qualitative and quantitative losses. More number of viruses reported to infect onion in worldwide belongs to the genera *Allexivirus*, *Carlavirus*, *Nepovirus, Potyvirus*, *Ilarvirus* and *Tospovirus* [2, 3, 4]. Most of the viruses incite more or less similar kind of disease symptoms i.e chlorotic mosaic streaks, spots, leaf striping and curling, stunting and necrotic tips on the leaves of the onion. Among the viral diseases, Peanut bud necrosis disease (PBND) is one of the threatened diseases in onion.

Peanut bud necrosis virus is the type species of the genus *Tospovirus* of the family *Bunyaviridae* consists of enveloped, quasi spherical particles, approximately 80–120 nm in diameter and has a tripartite, single-stranded, ambisense RNA genome. The RNAs are designated L (large), M (medium) and S (small) and have a size of c. 8.9, 4.8 and 2.9 kb, respectively. They are bounded by nucleocapsid (N) protein [5]. The L-RNA codes for the RNA-dependent RNA polymerase and is translated from the viral complementary sense RNA (vc). The m-RNA encodes a non-structural (NSm) protein in the viral (v) sense and the precursor for the glycoprotein's G1 and G2 in the VC sense. Among six serogroups of *Tospovirus*, only serogruop VI (*Iris yellow spot virus*) has been reported to infect the onion crop [6]. GBNV has a wide host range, infecting vegetable, fruit, oil seeds, ornamental crops and weeds with severe economic losses [7]. The incidence of groundnut bud necrosis disease ranges from 5 to 80% in different parts of the Indian subcontinent. GBNV is transmitted by thrips (*Thrips palmi*) in a persistent manner [8]. Only ten species of thrips were reported as vectors of tospoviruses recorded in the Worldwide [9]. Meena et al. [10] from India detected the presence of *Tospovirus* in *S.dorsalis.* Recently, natural infection of Jute and Taro [11, 12], Calotropis [13] (Bhaskara Reddy et al. 2011), Onion [4] and Jasmine [14] with PBNV was also reported.

The Peanut bud necrosis disease in onion is characterized by straw colored, mosaic and necrotic lesions were observed on the young leaves. The necrosis starts with the apical portion of young leaves and flower stalks and finally flower abortion and plant death.

The objective of the present study was to develop a reliable method to detect PBNV in the onion plants. A reverse transcriptase-polymerase chain reaction (RT-PCR) was developed to detect the presence of PBNV and the results were compared with those obtained using ELISA.

MATERIALS AND METHODS

Virus isolates

The *Peanut bud necrosis virus* suspecting onion plants (n=145) were collected from different places in Andhra Pradesh, Karnataka and Tamil Nadu states of India. The PBNV infected onion plants showed symptoms like straw colored, mosaic and necrotic lesions on the young leaves of onion stalks (Fig.1).

Enzyme linked immunosorbent assay (ELISA)

The onion samples were collected from the different places in South India subjected to direct antigen coating-ELISA (DAC-ELISA) [15] using specific polyclonal TSV antiserum. The suspected leaf samples were ground in carbonate buffer (pH 9.6) at 1:10 dilution (w/v) and crude leaf extracts were used as antigens. The healthy leaf tissue extract was used as a control. 100 µl of each sample extract was loaded into wells of ELISA plates (Nunc MaxiSorb; Denmark) and incubated at 37°C for 60 min. The antigen coated plates were washed three times with PBS-T buffer. The plates were then blocked with blocking buffer (PBS-TPO with 5% skimmed milk powder) and incubated at 37°C for another 60 min. The plates were then washed with PBS-T as described above. The polyclonal antisera of PBNV was used as primary antibodies at 1:5000 (v/v) dilutions in antibody buffer (PBS-TPO- 0.15 M NaCl in 0.1 M phosphate buffer pH 7.4, 0.05% Tween 20, 2% polyvinyl pyrrolidine, 0.2% ovalbumin), incubated for 60 min at 37°C and washed three times with PBS-T as above. Goat antirabbit-ALP conjugate (Sigma, Germany) at 1:10000 (v/v) dilution in antibody buffer was added and incubated at 37°C for 60 min. Para-nitrophenyl phosphate (PNPP) (Sigma, Germany) was used as a substrate at 5 mg / 10 ml of substrate buffer (Diethanolamine buffer, pH 9.8). Absorbance values were recorded in an ELISA plate reader (Bio-Rad, USA) at 405 nm after 15-30 min of substrate addition. Antigen buffer control was included along with leaf antigen samples. The reactions were terminated using 3 N NaOH (50µl/well). Only positive samples whose A405 values were twice or more greater than twice the value of healthy onion samples was considered as virus infected.

The ELISA positive samples were maintained in local lesion assay host as *Vigna unguiculata* (cv-C 152) cultivar was used following sub-culturing for three generations on the same host and later maintained on their natural hosts for further studies.

Preparation of total RNA and RT-PCR

Total RNA from 100 mg of healthy and GBNV infected onion leaf samples was isolated using RNeasy Plant Minikit according to the manufacturer's instructions (Qiagen, Germantown, MD, USA). The resulting total RNA was incubated with GBNV-CP gene-specific reverse primer at 65°C for 5 min and snap-chilled on ice for 2 min. cDNA was synthesized using M-MuLV reverse transcriptase (Fermentas, Ontario, Canada) at 42°C for 1 h. The genome sense primer, 5′ATGTCTAACGT(C/T) AAGCA (A/G) CTC 3′, and antisense primer, 5′TTACAATTCCAGCGAAGGACC 3, were used to amplify the complete CP gene of GBNV [16]. Two micrcolitre of cDNA was amplified in a 25 µl reaction volume containing 2.5 U of Taq DNA Polymerase (Fermentas), 10 pmol of forward (GBNV-CP-F) and reverse primer (GBNV-CP-R), 2.5 mM MgCl2 and 10 mM each dNTP's. PCR amplification conditions included an initial denaturation cycle of 5 min at 94°C, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 1 min at 56°C and extension for 1 min at 72°C with final extension for 60 min at 72°C. Amplified products were resolved following electrophoresis through a 1% agarose gel containing ethidium bromide (10 mg/ml).

RESULTS

The presence of PBNV was detected in 96 of the 145 onion plants from different places of the Andhra Pradesh, Tamil Nadu and Karnataka States from India. The symptoms of straw colored, mosaic and necrotic lesions were observed on the young leaves. The necrosis starts with the apical portion of young leaves and flower stalks and finally flower abortion and plant death (Fig. 1).

Fig. 1: Symptoms associated with natural occurrence of *Peanut bud necrosis virus* on onion: straw colored, mosaic and necrotic lesions were observed on the young leaves.

ELISA

Based on above symptomatology, the PBNV was confirmed by direct antigen coating (DAC)-ELISA (Clark and Joseph 1984) by using PBNV polyclonal antibodies (A405= 2.15). The sensitivity of ELISA for the detection of PBNV was evaluated using 145 onion plant leaf samples. Seventy five samples (51.72%) were confirmed as PBNV infected by ELISA by using the PBNV coat protein specific antiserum during the year 2010-2014 (Table.1).

Table 1. Collection and screening of *Peanut bud necrosis virus* (PBNV) infecting onion samples from different locations in South India.

S.No	Place	Crop	No. of Samples	ELISA	RT-PCR
	Andhra Pradesh				
1	Chittoor	Onion	15	5	12
2	Kadapa	Onion	9	7	8
3	Kurnool	Onion	12	10	11
4	Nellore	Onion	10	4	9
5	Guntur	Onion	4	1	3
6	Ananthapur	Onion	8	3	7
7	Medak	Onion	5	3	4
8	Karimanagar	Onion	6	4	5
9	Visakapatnam	Onion	5	2	4
	Tamil Nadu				
10	Perambalur	Onion	10	7	9
11	Dindugul	Onion	7	5	6
12	Coimbatore	Onion	9	4	7
13	Tiruchirappalli	Onion	10	5	9
14	**Karnataka**				
15	Dharwad	Onion	9	5	8
16	Kolar	Onion	11	6	10
17	Tumkur	Onion	7	2	5
18	Raichur	Onion	8	2	7
			145	75 (51.72%)	124 (85.51%)

The PBNV was easily sap transmitted to cowpea (Cv-c-152); both localized and systemic symptoms were observed on cowpea plants. After 3-5 days inoculation, initially chlorotic lesions were observed on cowpea leaves, which later turned into necrotic spots, followed by veinal necrosis. Finally, the pale yellow color was observed in leaves before senescence. The Emerging new leaves will be shown systemic symptoms which consisted of mild mosaic, chlorotic ring spots and necrotic spots. The virus-affected cowpea plants reacted with the PBNV polyclonal antiserum directed against the coat protein of PBNV.

RT-PCR

The total RNA was extracted from the infected onion tissue and subjected to RT-PCR by using the coat protein gene of PBNV resulted in an amplicon with the expected size of ~800 bp (Fig. 2) for 124 of the 145 onion samples (85.51%) during the 2011-2014 (Table 1). The amplified PCR product was sequenced and matched the PBNV coat protein gene sequence.

Comparative sensitivities of ELISA and RT-PCR:

The ELISA has been used to detect PBNV in onion plants, but gave fewer results when compared to RT-PCR. These results showed that RT-PCR is more sensitive than ELISA.

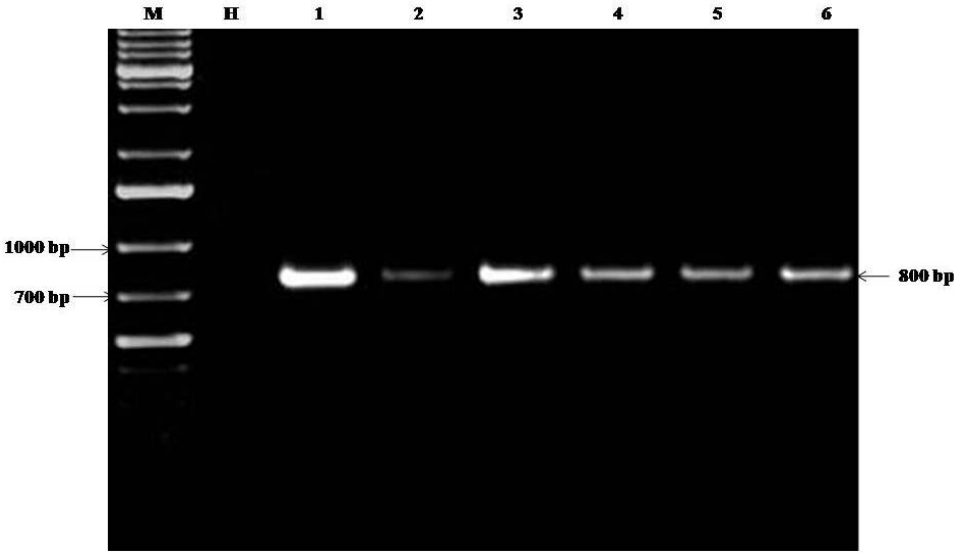

Fig. 2: Agarose gel electrophoresis of RT-PCR products. Lane M- 1Kb DNA ladder; Lane H- Healthy onion sample; Lane 1, 2, 3, 4, 5, 6- PBNV infected onion samples.

DISCUSSION

PBNV is easily transmissible to members of *Solanaceae*, *Leguminosae*, *Cucurbitaceae* and *Fabaceae* [17]. The PBNV has a wide host range and it infects several hosts such as cowpea, groundnut, mung bean, potato, soya bean, tomato, taro, calotropis, jute and jasmine [18, 19, 20, 12, 13, 11, 14]. This work presents the sensitivity comparison of two diagnostic methods, RT-PCR and ELISA in the detection of PBNV in onion. The symptoms of GBNV first appeared in the young leaflets such as straw colored, mosaic and necrotic lesions. If the PBNV infection occurs at a young age, it results in the death of the plant due to severe necrosis. The PBNV infected onion samples were confirmed by ELISA using the polyclonal antibodies of coat protein gene of PBNV. The confirmation of ELISA positive samples were maintained in cowpea (Cv-c-152). After five days sap inoculation, both localized and systemic infections were observed on cowpea plants. The PBNV affected cowpea plants reacted with the polyclonal antiserum directed against coat protein of PBNV (A405 = 1.89). Among these (n=145) collected onion samples, 75 samples (51.72%) were confirmed as PBNV infected by direct antigen coating enzyme linked immunosorbent assay (DAC-ELISA) by using the PBNV specific antiserum, where as 124 samples (85.51%) were confirmed by RT-PCR. A single band of the expected size of ~800 bp corresponding to the coat protein gene of PBNV was observed when total RNA extracted from infected tissue was used in RT-PCR.

CONCLUSION

The results indicate that this molecular technique (RT-PCR) is a more reliable assay for the detection of PBNV onion plants when compared to ELISA method.

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