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SEROLOGICAL TESTS FOR DETECTION OF SUNFLOWER NECROSIS TOSPO VIRUS CAUSING NECROSIS DISEASE OF SUNFLOWER *(HELIANTHUS ANNUS* L.)

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ABSTRACT
India occupies third place to produce oil seeds in the world. India has vast area of about 19 million hectares under oilseed crops In order to formulate management practices for the control of necrosis disease of sunflower; reliable tests are required for its detection. Such tests are lacking in India. Sunflower is widely cultivated in India. Sunflower crop is extensively cultivated in Rayalaseema region of Andhra Pradesh. Sunflower showed symptom of virus disease characterised by severe mosaic, necrosis of leaves, necrosis along the stems and floral parts, malformation of young leaves, markedly reduced leaves and stunting of plants. Hence, in this study, direct antigen coating-enzyme linked immunosorbent assay (DAC-ELISA), Double antibody sandwich-ELISA (DAS-ELISA) and Dot-ELISA tests were evaluated for the detection of sunflower necrosis a tospo virus (SfNV) causing necrosis disease of sunflower in Andhra Pradesh in India. In DAC-ELISA, Sunflower Necrosis Virus was detected up to 0.370μg/mL in purified virus preparation and up to 10^{-4} dilution in leaf sap extracted from virus infected sunflower leaves. In Dot-ELISA, Sunflower Necrosis Virus was detected up to 0.0370μg/mL in purified virus preparation and up to 10^{-5} dilution in leaf sap from infected sunflower leaves. Where as in DAS-ELISA the virus was detected up to 370μg/mL and 10^{-3} dilution in purified virus preparation and in leaf sap extracted from infected sunflower leaves, respectively. Hence, it is concluded that DAC-ELISA and Dot-ELISA were found to be the suitable tests for the detection of Sunflower necrosis virus.

INTRODUCTION

Sunflower, (*Helianthus annuus*) an oil seed crop grown in many countries for oil seed and for direct consumption as confection seed. The world wide cultivation of sunflower is more than 35MT in the year 2010 (FAO STAT, 2011). India ranks 12th position in sunflower production with the yield of 1.06MT (FAOSTAT, 2010). In India, sunflower is cultivated in around 18 lakh hectares (10%) of the world sunflower area) and production is around 12.52 lakh tons (4% of the world sunflower production). The major sunflower producing states are Karnataka (54.86%), Andhra Pradesh (20.83%) and Maharastra (14.58%) Anonymous, 2009.

Sunflower crop is affected by several fungal, bacterial and viral diseases. Among the viral diseases affecting the crop, sunflower necrosis virus disease caused by Tobacco streak virus belongs to the genus Ilarvirus and bud necrosis caused by tospovirus (Venkata Subbaiah *et al.,* 2000; Jain *et al.,* 2000; Ramaiah *et al.,* 2001; Bhat *et al.,* 2002; Lavanya *et al.,* 2005) are major production constraints. Among the virus diseases of Sunflower, necrosis disease has gained importance because of its heavy devastating nature in most of the sunflower growing states in India. It is a major threat to the economic and social well being of farmers in the Asian region. Necrosis disease is caused by a viruses namely, Tospo Virus *of* the family *Buniyaviridae* that belong to the genus Tomato spotted wilt virus of the family *Buniyaviridae* (Brunt et al 1996, Granoff and Webster, 1999). SfNV contains RNA as its genome. SfNV causes severe necrosis symptoms and is transmitted in nature by thrips species, primarily by the *T.palmi*. SfNV causes Chlorotic symptoms and severe mosaic and necrosis symptoms. The disease causes distinct stunting of plants, necrosis of leaves, stem and necrotic rings on floral heads and malformation of floral heads. These characteristics (particle type, genome type, virus-host and vector relationships) signify the importance of sunflower Necrosis in the field of plant virology, besides its economic importance. In India, this disease was first reported in 1997 (Nagaraju *et al.,* 1998) and from then onwards it has been reported from different sunflower growing states from time to time. Of the sixteen viruses infecting sunflower, 6 were reported from India. Tospoviruses have become a major problem for several leguminous and vegetable crops during the last decade (Jain *et al.,* 1999). Three tospovirus species, GBNV and groundnut yellow spot virus (GYSV) on groundnut (Reddy *et al.,* 1992, Satyanarayana *et al.,* 1996 and watermelon bud necrosis virus (WBNV) on watermelon, Jain *et al.,* 1998 and sunflower necrosis (Venkata subbaiah *et al.,* 2000; Jain *et al.,* 2000), have been established in India to date on the basis of host range, serology and sequence of data of *NP* gene. Among these, necrosis disease is economically most important one as it caused heavy losses from time to time. It is a highly erratic disease frequently reported from various sunflower growing states in India. Considerable yield losses were noticed from time to time in India. Even though the disease is economically important in India, till recently there are no reports on

purification and production of polyclonal antiserum required for widely used sensitive serological techniques like ELISA, Dot-ELISA and electroblot immuno assay (EBIA), to detect the viruses associated with necrosis disease. The disease diagnosis is mainly based on visual symptoms and transmission of the viruses from infected plants to susceptible sunflower varities using thrips vectors. So, sensitive techniques are required for characterization and detection of Sunflower necrosis virus isolate. Even though nucleic acid based techniques are widely used in other countries for detection of plant viruses, (Cortez *et al.*, 2001) have applied PCR for the detection of Tospo viruses in samples in Northern India (Jain *et al.*, 2001). The widely used ELISA has not been employed for its diagnosis. Hence, in this study antibody based ELISA detection tests were developed and compared for the detection of SfNV, for better understanding of disease epidemiology and to formulate effective disease management practices.

MATERIALS AND METHODS

Several necrosis disease suspected sunflower plants (cv. Morden, MHSF8, MHSF17) with chlorotic, mosaic and necrosis leaves were collected from commercial farmers fields around Tirupati (Chittoor district) of the state Andhra Pradesh. The virus was propagated in the wire mesh house by periodical mechanical inoculation onto 10-13 days old sunflower seedlings in earthen pots containing red soil and organic manure mixture (3:1). Sunflower Necrosis virus (SfNV) was purified according to the procedure of (unpublished data), characterized as a strain of Tospo virus belongs to serogroup IV and named as SfNV isolate (unpublished). Polyclonal antibodies were produced by intramuscular injection of purified virus (SfNV) into a New Zealand white inbred rabbit.

DAC-ELISA

DAC-ELISA was carried according to (Hobbs *et al.,* 1987) Leaf tissues were extracted in carbonate buffer (coating buffer) containing 0.01M DIECA (1g/9mL; w/v) and further dilutions were prepared using the same buffer as a diluents. The antiserum was diluted with PBS-TPO, and used at 1:2000 dilutions. Goat anti-rabbit antibodies labelled with alkaline phosphatase (GIBCO-BRL) were used at 1:5000.

DAS-ELISA

DAS-ELISA was carried according to (Clark and Bar-Joseph, 1984) The ELISA plate was coated with immunoglobulin's of Sunflower necrosis virus isolate as trapping antibodies (1:500). Purified virus, healthy and virus infected leaf antigens (extracted and diluted in PBS-TPO) were added to the IgG coated plates. Polyclonal antiserum 1:1000 is used both for DAC and DAS-ELISA systems the buffers were made according to the Clark and Bar-Joseph, 1984. Wells filled with coating buffer were considered as negative controls (blank).

Samples with absorbance values three times greater than healthy leaf extracts were considered as positives. Leaf antigens were used from $10^{-1}$to 10^{-6} dilution, purified virus was used from 370 - 0.00370μg/mL. 200uL/well of reagents were used and incubated for one and half an hour at each step. The plates were washed thrice with PBS-T after each incubation. Freshly prepared p-nitrophenyl phosphate (PNP) (Sigma) substrate (5mg/10mL) was used and incubated in dark for reaction to take place. The reactions were terminated by adding 2N NaOH (50μL/well) after 1h and the intensity of the colour was read at A_{405} nm in a Dynatech ELISA reader.

Dot-ELISA

Dot-ELISA was carried according to Berger *et al.*, 1985. Antigen samples were macerated in carbonate buffer and further dilutions were made with the same buffer. PVDF membrane (Millipore) was cut to the desired size. Samples were applied onto the nitrocellulose membrane using micropipette. Virus infected and healthy rice leaf extracts were used at 10^{-1} to 10^{-6} dilutions where as purified virus was used at 370-0.00370μg/mL. The membrane was blocked in blocking solution (5% Milk powder in TBS-TPO) for overnight at 4ºC. SfNV antiserum was used at1: 1000 diluted in TBS-TPO and kept for 1.5h at 37ºC. Goat anti rabbit IgG labeled with HRP conjugate (Genei, Bangalore) was used at 1: 5000 and incubated at 37ºC for 1.5h. The membrane was washed thrice with TBS-T for 3min each after every step. The membrane was placed in substrate solution until the reaction takes place to the desired level. Finally the membrane was kept in 3% sodium hypochlorite solution under shaking on a rocker for 30min to remove the background pigment colour and blotted in between tissue paper folds, dried and the results were recorded.

RESULTS AND DISCUSSION

Various tests were optimised by different workers for the detection of necrosis viruses, keeping in view testing the large number of samples with in shortest possible time, ease of performing the test, cost and expertise etc. Symptoms and transmission studies give a clue about the necrosis disease but these are time consuming and also unable to give final conclusive results. Electron microscopy and PCR are worthy techniques for the detection of the disease. But initial cost of these techniques is very high and moderately equipped labs cannot afford such costs. Among serological methods, some techniques like latex agglutination require higher quantities of

Table 1: Comparison of DAC-ELISA, DAS-ELISA and Dot-ELISA for the detection of SfNV

Nature of antigen and concentration	A_{405} reading of DAC-ELISA	DAS-ELISA	Dot-ELISA
Purified virus (μg/mL)			
370	3.53[a]	2.1	+
37	0.97	0.59	+
3.7	0.37	0.22	+
370	0.24	0.14	+
0.037	0.12	0.13	-
0.0037			
Infected leaf sap	1.49	1.57	
10^{-1}	0.57	0.27	+
10^{-2}	0.33	0.13	+
10^{-3}	0.13	0.09	+
10^{-4}	0.13	0.08	+
10^{-5}			-
Healthy leaf			
10^{-1}	0.22	0.4	-
10^{-2}	0.15	0.09	-
10^{-3}	0.15	0.09	-
10^{-4}	0.1	0.08	-
10^{-5}	0.11	0.01	-

[a]Values represent average of three A405 readings recorded after 1h incubation with at room temperature in dark

antisera and other reagents. Hence, ELISA based techniques are the preferable detection tools detect the necrosis disease (Venkata Subbaiah *et al.,* 2000; Jain *et al.,* 2001,) applied Dot-ELISA and tissue print ELISA to detect several viruses of vegetables crops (Krishna Reddy *et al.,* 1997). In this study, serological tests like DAC-ELISA, DAS-ELISA and Dot-ELISA tests were developed and compared for the detection of SfNV isolate. In DAC-ELISA, the virus was detected up to 0.370 ng/mL and 10^{-4} dilution in purified virus preparation and in leaf extract, respectively (Tabel 1). In this test, weak positive reactions with healthy leaf antigens were resulted by using low dilutions of crude antiserum. To minimize such reactions, the crude antiserum was used at higher dilutions. In Dot-ELISA, the virus was detected up to 0.0370 ng/mL and 10^{-5} dilution in purified virus preparation and leaf extract, respectively (Table 1). In Dot-ELISA also, there was a background problem with green pigments that were attached to the membrane while spotting antigens. These pigments were not completely removable while washing and are reacting with IgG-enzyme conjugates leading to false positive reactions or the pigment colour itself mimics the positive reactions. Hence the pigments were removed by treating the membrane with 3% sodium hypochlorite on a rocker for 30 min at room temperature. In DAS-ELISA, the virus was detected up to 370 ng/mL in purified virus preparation and up to 10^{-2} dilution in infected leaf material (Table 1). DAS-ELISA is comparatively less sensitive, requires high concentrations of antiserum but the background reactions were minimal.

By comparing the above tests, DAC-ELISA and Dot-ELISA were found to be more sensitive, requires very low concentrations of antiserum and can be used to test large number of samples at a time and hence it is concluded that DAC-ELISA and Dot-ELISA are superior for the detection of SfNV in Andhra Pradesh in India.

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