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Detection of *Tobacco streak* virus infecting sunflower by ELISA and RT-PCR method

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ABSTRACT

Sunflower necrosis disease caused by *Tobacco streak virus* (TSV) is major threat to sunflower crop. The presence of TSV in necrosis disease affected leaf samples collected from major sunflower growing areas of Andhra Pradesh, Karnataka, Maharashtra and Tamil Nadu was confirmed by direct antigen-coated enzyme linked immunosorbent assay using TSV specific polyclonal antiserum. All the isolates showed positive reaction in ELISA test using TSV specific polyclonal antiserum, indicating the presence of TSV. The ELISA values varied from 0.57 to 1.18 as compared to the positive control *i.e.* TSV infected cowpea (1.59) and healthy control (0.07). Disease reaction and symptomatology of three test plants *viz.*, cowpea cv. C-152, sunflower cv. MORDEN and *Nicotiana tabacum* cv. SAMSUN to these virus isolates were studied under artificial inoculated condition by sap transmission. Using primers specific to the coat protein region of TSV, RT-PCR was successful in amplifying TSV- CP gene from sunflower tissue. A DNA band of expected size (approximately 700 bp) was observed in all the necrosis disease affected sunflower samples collected from Maharashtra, Karnataka, Tamil Nadu and Andhra Pradesh. Characterization of the isolates of virus through cloning and sequencing may reveal the occurrence of pathotypes/serotypes from different sunflower growing locations of the country.

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INTRODUCTION

Among different diseases infecting sunflower (*Helianthus annuus* L.) world wide, sunflower necrosis caused by *Tobacco streak virus* (TSV), poses potential threat to the cultivation of sunflower in India. Various methods have been developed to detect TSV, including enzyme linked immunosorbent assay (ELISA), reverse transcription polymerase chain reaction (RT-PCR) and probe based technique. Of these RT-PCR has been used for the characterization of coat proteins and three un translated regions (UTR) regions of the TSV of infecting different host to define relationship of TSV isolate present in different crop and check the distribution and prevalence of strains and variants for different locations or regions. Ravi *et al.* (2001) reported that in RT-PCR, using oligonucleotide primers deduced from conserved sequences within TSV-RNA3 and flanking the entire coat protein region, an approximately 1000 bp dsDNA fragment was amplified from SNV-infected sunflower. The variability of nucleotide sequences retrieved from 3 sunflower isolates obtained from different locations was less than 3 per cent. Bhat *et al.* (2002b) had developed RT-PCR assay for the detection of TSV from sunflower using primers derived from CP gene.

Information on variability of TSV in natural population of various sunflower growing locations is very essential as this information will have important implications in the control of TSV by using resistant varieties. Studies on variation among the virus isolates from different locations is inadequate. Keeping this in view, work was initiated to ascertain the prevalence of TSV pathotypes /serotypes originating from different sunflower locations in the country.

MATERIAL AND METHODS

Collection of virus isolates :

Necrosis disease affected sunflower leaf samples were collected from different sunflower growing areas of Andhra Pradesh, Karnataka, Maharashtra, Tamil Nadu and Coimbatore during 2009-10 *Kharif* season. The presence of TSV in these samples was confirmed by DAC- ELISA (Hobbs *et al.*, 1987) using TSV specific polyclonal antiserum (source : ICRISAT). The isolates from the four states were maintained separately on cowpea cv. C-152 under glasshouse conditions. The virus isolates from Andhra Pradesh, Karnataka, Maharashtra and Tamil Nadu were designated as AP-1, AP-2, KA-1, KA-2, MH-1, MH-2, TN-1 and TN-2, respectively and used for further studies.

These isolates were sap inoculated separately to sunflower cv. MORDEN (at two leaf stage), cowpea cv. C-152 (at primary leaf stage) and tobacco cv. SAMSUN (at 6-8 leaf stage) to study the disease reaction and symptomatology under glass house conditions. For each cultivar, 10 plants were sap inoculated by each of the virus isolate. Observations on per cent disease incidence, type of symptoms and days taken for appearance of symptoms (incubation period) were recorded. Based on the per cent disease incidence, the disease reactions of cowpea cv. C-152, sunflower cv. MORDEN and tobacco cv. SAMSUN were recorded using 0-5 scale.

Detection of TSV from infected sunflower samples by Reverse transcription polymerase chain reaction (RT-PCR) :

Total RNAs extracted from ELISA positive necrosis affected sunflower leaf tissues from different locations in Andhra Pradesh, Karnataka, Tamil Nadu and Maharashtra were used as template for RT-PCR. In all, eight virus isolates *i.e.*, two from each state were used to study variation among the isolates.

Isolation of total RNA from leaf extract by TRIZOL method :

Total RNA was extracted from leaf samples by the method described by Chomczynski and Sacchi (1987).

Virus infected leaf sample (50-100 mg) was ground to powder in liquid nitrogen using pestle and mortar. The powder was homogenized with one ml TRIZOL reagent (Sigma, Aldrich) and incubated for 5 min at 15-30°C. 200 µl of chloroform was added for one ml of TRIZOL, vortexed vigorously for 15 sec and incubated for 2-3 min at 15-30°C. Then centrifuged at 10500 g for 15 min. Aqueous phase was transferred to a fresh tube and 0.5 ml isopropyl alcohol was added and mixed gently. Later, incubated at 15-30°C for 10 min. and centrifuged at 12000 g for 10 min. at 2-8°C. Supernatant was removed and the pellet was washed with 1ml of 75 per cent DEPC treated ethanol. Again centrifuged at 6500 rpm for 5 min and the pellet was dried and dissolved in RNase-free water or 0.5 per cent SDS solution. Sap extracted from non-infected tissues was used as control.

RT-PCR :

The procedure suggested by Bhat *et al.* (2002a) was followed for RT-PCR.

cDNA synthesis :

For cDNA synthesis, omniscript reverse transcriptase (Qiagen) was used. cDNA was synthesized using the reverse primer. The protocol followed for cDNA synthesis is given below :

cDNA was synthesized from RNA through the use of reverse transcriptase in a 10 µl reaction mixture containing 1 mg of total RNA isolated from infected sunflower leaf sample.

1.0 µl template RNA was mixed with 1.0 µl reverse primer (10uM) in a sterile Rnase-free tube (Table A). Template mix was heated at 70ºC for 10 min. Reaction was stopped at 4°C for the addition of premix (5×RT buffer, RNase inhibitor, 30mM dNTP mix, 0.1 M DTT, Reverse transcriptase, Water). The

Table A : Composition of RT-PCR mix

Reagents	Volume required per reaction
RNA	1.0 µl
Reverse Primer 10uM	1.0 µl
5×RT buffer	4.0 µl
RNase inhibitor	1.0 µl
30mM dNTP mix	2.0 µl
0.1 M DTT	1.0 µl
Reverse transcriptase	0.5 µl
Water	1.0 µl

Table B : Box 2 Composition of PCR mix

Reagents	Volume per reaction
Sterile water	6.3 µl
10×PCR buffer	1.0 µl
dNTP mix	0.6 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
Taq DNA polymerase	0.1 µl
Template (cDNA)	1.0 µl
Total	10.0 µl

reaction mix was continued with the PCR conditions at 45ºC for 50 min and at 70ºC for 15 min followed by final hold at 4°C for reverse transcription.

PCR amplification :

10µl cDNA was used as template for subsequent PCR reaction (Table B).

The amplification was carried out in an automated PCR machine (Applied biosystems) programmed for one cycle of initial denaturation at 94ºC for 5 min and 30 cycles of amplification with the following parameters : denaturation at 94ºC for 30s, annealing at 48ºC for 1 min, extension at 72ºC for 1 min followed by one cycle of final extension at 72ºC for 10 min.

TSV Primer Sequence (courtesy: IARI, New Delhi)

Forward primer (RKJ1) = 5' ATG AAT ACT TTG ATC CAA GG 3'

Reverse primer (RKJ2) = 5' TCA GTC TTG ATT CAC CAG 3'

Preparation of gels :

Gel plates (13×14 cm) were washed thoroughly with cleaning solution followed by distilled water and dried. The two open sides of the plates were sealed with cellophane tape. Gel solution (1.4 %) was prepared by mixing agarose powder (Molecular Biology Grade, SRL) with 1×TAE running buffer gel. The contents were boiled in microwave oven until a clear solution was obtained. 1µl of ethidium bromide (1mg/ml) was added to the agarose after cooling to around 50ºC. It was then poured into the casting tray for polymerization after inserting the Teflon comb.

Loading and running of gels :

The inserted comb was gently removed from the gel after polymerization. The gel plate was placed in horizontal electrophoresis apparatus and filled with 1×TAE buffer.

10 µl each of the RT-PCR product was mixed with 3 µl 6X loading dye. The samples were loaded in the wells with the help of micropipettes. An aliquot of 2 µl marker (1kb DNA ladder; DNA ladder mix, Fermentas) was electrophoresed to serve as molecular weight marker. After loading, the electrophoresis unit was connected with power pack with a regulated electric power supply of 90v for 90 min. After the run, the gel was observed under ultraviolet trans-illuminator (Biorad, USA) and photographed.

RESULTS AND DISCUSSION

Eight isolates of the virus causing necrosis disease were collected from four states *viz*., Andhra Pradesh (AP-1 and AP-2), Karnataka (KA-1 and KA-2), Maharashtra (MH-1 and MH-2) and Tamil Nadu (TN-1 and TN-2) during 2009-10 *Kharif* season. These isolates were studied for serological and molecular variability through DAC-ELISA and RT-PCR.

All the isolates showed positive reaction in ELISA test using TSV specific polyclonal antiserum, confirming the presence of TSV. The ELISA values varied from 0.57 to 1.18 as compared to the positive (TSV infected cowpea) control 1.59 and healthy (cowpea healthy) control 0.27 (Table 1).

Table 1 : Detection of TSV by DAC – ELISA in SND affected sunflower leaf samples from different states in India

Sr. No.	Isolate	Absorbance value at 405nm *
Andhra Pradesh		
1.	AP-1 (Rajendranagar)	1.18
2.	AP-2 (Kurnool)	1.01
Maharashtra		
3.	MH-1 (Akola)	0.95
4.	MH-2 (Akola)	0.76
Karnataka		
5.	KA-1 (Raichur)	1.05
6.	KA-2 (Bangalore)	0.7
Tamil Nadu		
7.	TN-1 (Ariyalur)	0.57
8.	TN-2 (Coimbatore)	0.62
	Infected cowpea (+ ve control)	1.59
	Healthy cowpea (- ve control)	0.27
	Healthy sunflower	0.19

Antigen and Antiserum at 1:10 and 1: 1000 dilutions, respectively;
*Average of two replications 1 hr after addition of substrate

The disease reaction and symptomatology of cowpea cv. C-152, sunflower cv. MORDEN and *N. tabacum* cv. SAMSUN to the eight virus isolates on artificial inoculation are presented in Tables 2, 3 and 4. The disease reaction of three test plants was susceptible to highly susceptible, of the eight virus isolates tested, the isolates AP-1, KA-1 and MH-1 were more virulent compared to other isolates as highly susceptible disease reaction was observed in cowpea cv. C-152, sunflower cv. MORDEN and tobacco cv. SAMSUN.

The symptoms produced by cowpea cv. C-152 were similar as chlorotic and necrotic lesions were recorded with all the isolates tested. Typical symptoms of white ring like line pattern and oak leaf pattern appeared on inoculated tobacco leaves and time taken for symptom development ranged from 6 days (AP-2, MH-1 and KA-1) to 8 days (MH-2 and TN-1). Disease incidence was 50 per cent with the isolates MH-2, TN-1 and TN-2, while it was 70 per cent with AP-1, MH-1 and KA-1 isolates. Infectivity assay of the eight virus isolates of SND collected from various locations on cowpea cv. C-152 and *N. tabacum* cv. SAMSUN showed no variation in symptom expression. On sunflower cv. MORDEN, the isolates produced mosaic (AP-2, MH-2, KA-2, TN-1 and TN-2) while necrotic

lesions were produced by AP-1, MH-1 and KA-1 within 6-7 days.

It is evident from the results that the isolates *viz.*, AP-1, KA-1 and MH-1 were more virulent than the other isolates. TN-2 isolates of the virus was least virulent compared to other isolates of the virus as less per cent infectivity was observed in the test plants *viz.*, cowpea cv. C-152, sunflower cv. MORDEN and tobacco cv. SAMSUN.

The difference in the infectivity of test plants found in the present study may be attributed to the genetic makeup of the cultivars used rather than the virulence of the virus isolates as 100 per cent similarity was detected among the virus isolates tested in RT-PCR.

Reverse transcription- polymerase chain reaction :

Using primers specific to the coat protein (CP) region of TSV, RT-PCR was successful in amplifying TSV CP gene from sunflower tissue. A DNA band of expected size (approximately 700 bp) was observed in all the necrosis disease affected sunflower samples collected from Maharashtra, Karnataka, Tamil Nadu and Andhra Pradesh. No such band was observed when healthy tissue was used as template indicating specificity of the PCR product. Molecular analysis studies showed uniform pattern of band and expressions (Plate 1).

The findings are in agreement with Bhat *et al.* (2002a) who employed the RT-PCR technique and reported that sunflower *Ilar virus* appearing in India was a strain of TSV

Table 2 : Infection of SND virus isolates collected from various locations on cowpea (cv. C-152) by sap inoculation

Isolates	Per cent transmission	Symptoms	Days taken to symptom development
AP-1	90	NL	5
AP-2	70	CL	4
MH-1	80	CL	4
MH-2	70	CL	5
KA-1	80	NL	3
KA-2	60	CL	4
TN-1	60	CL	5
TN-2	50	CL	4

Table 3 : Infection of SND virus isolates collected from various locations on tobacco (cv. SAMSON) by sap inoculation

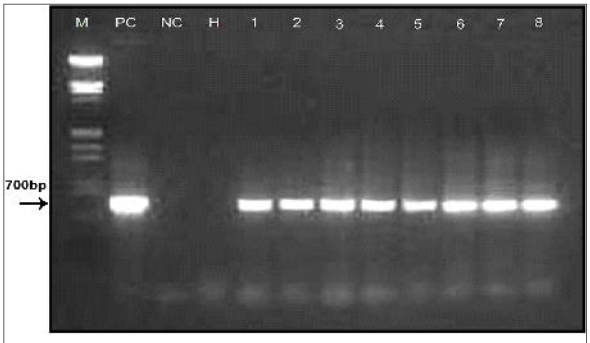
Isolates	Per cent transmission	Symptoms	Days taken to symptom development
AP-1	70	Wrlp	7
AP-2	60	Wrlp	6
MH-1	70	Wrlp	6
MH-2	50	Wrlp	8
KA-1	70	Wrlp	6
KA-2	60	Wrlp	7
TN-1	50	Wrlp	8
TN-2	50	Wrlp	7

CL -Chlorotic lesions, M – Mosaic, NL -Necrotic lesions, Wrlp-White ring like pattern

Table 4 : Reaction of sunflower cv. MORDEN on inoculation with SND virus isolates collected from various locations

Sr. No.	Isolates	Symptoms	Disease reaction*	Disease incidence (%)	Days taken to symptom development
1.	AP-1	NL	HS	70	6
2.	AP-2	M	S	40	7
3.	MH-1	NL	HS	60	6
4.	MH-2	M	S	50	7
5.	KA-1	NL, M	HS	70	6
6.	KA-2	M	S	50	7
7.	TN-1	M	S	50	7
8.	TN-2	M	S	40	7

M- Mosaic, NL - Necrotic lesions; * HS - Highly susceptible, S - susceptible



M–One kb ladder as size standard; PC–Positive control; NC–Negative control; H–Uninfected control (Healthy sunflower); Lane 1 to 8–RT - PCR product from diseased sunflower plant from Andhra Pradesh (AP-1, AP-2), Karnataka (KA-1), KA-2), Mahrastra (KH-1, MH-2) and Tamilnadu (TN-1, TN-2); Arrow head indicates the position of amplified product

Plate 1 : Agarose gel electrophoresis of reverse transcription polymerase chain reaction (RT-PCR) reaction products

belonging to subgroup I. Further, Bhat *et al.* (2002b) developed RT-PCR assay for the detection of TSV from sunflower using primers derived from CP gene and a DNA band of expected size (approximately 700 bp) was observed in all the necrosis disease affected sunflower samples collected from Maharashtra, Karnataka, Tamil Nadu and Andhra Pradesh. Similar findings are reported in cucumber and gherkins (Reddy *et al.*, 2003), soybean (Arunkumar *et al.*, 2008), groundnut and marigold (Reddy *et al.*, 2009 and Pankaja *et al.*, 2010) and sunflower, gherkin and pumpkin samples (Sarovar *et al.*, 2010) infected with TSV.

In the present study, CP gene of TSV isolates collected from different locations of four states, when compared showed similar banding pattern suggesting that TSV population is homogenous and perhaps has common origin. However, further studies are necessary to characterize the isolates of virus through cloning and sequencing so as to ascertain the occurrence of pathotypes / serotypes from different sunflower growing locations of the country.

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REFERENCES

Arunkumar, N., Laxmi Narasu, M., Zehr, U.B., and Ravi, K.S. (2008). Molecular characterization of tobacco streak virus causing soybean necrosis in India. *Indian J. Biotechno*., **7** (2) : 214-217.

Bhat, A.I., Jain, R.K. and Ramiah, M. (2002a). Detection of *Tobacco streak* virus from sunflower and other crops by reverse transcription polymerase chain reaction. *Indian Phytopatho.*, **55** (2) : 216-218.

Bhat, A.I., Jain, R.K., Kumar, A., Ramiah, M. and Varma, A. (2002b). Serological and coat protein sequence studies suggest that necrosis disease on sunflower in India is caused by strain of *Tobacco streak Ilarvirus*. *Arch. Viro.*, **147** (3) : 651-658.

Chomczynski, P. and Sacchi, N. (1987). Single step method of RNA isolation by acid guanidinium-thiocyanate -Phenol chloroform extraction. *Annu. Biochemis.*, **162** : 156-159.

Hobbs, H.A., Reddy, D.V.R., Rajeswari, R. and Reddy, A.S. (1987). Use of direct antigen coating method and protein-A coating ELISA procedures for detection of three peanut viruses. *Plant Dise*., **71** (8) : 747-749.

Pankaja, N.S., Babu, G.V. Harish and Nagaraju (2010). Serological diagnosis of crop and weed plants for the presence of Sunflower Necrosis Virus (SNV) through Direct Antigen Coated-Enzyme Linked Immuno Sorbent Assay (DAC-ELISA). *Internat. J. Plant Protec.*, **3** (2) : 213-218.

Ravi, K.S., Buttgereitt, A., Kitkaru, A.S., Deshmukh, S., Lesemann, D.E. and Winter, S. (2001). Sunflower necrosis disease from India is caused by an *Ilar* virus related to *Tobacco streak* virus. *New Dise. Reports*., **3** : 1-2.

Reddy, Bhaskar B.V., Sivaprasad, K., Rani, Rekha T., Rajeswari, R.P., Vasanthi and Reddy, Raja K. (2009). Cloning, sequencing and expression of coat protein gene of tobacco streak virus infecting groundnut in *Escherichia coli*. *J. Oilseeds Res*., **26** (Special issue): 220.

Reddy, Krishna M., Lakshmi, Devaraj R., Salil, J. and Samuel, D.K. (2003). Outbreak of *Tobacco streak* virus causing necrosis of cucumber (*Cucumis sativus*) and gherkin (*Cucumis anguria*) in India. *Plant Dise*., **87** (10) : 1264.

Sarovar, B., Prasad, Siva Y., Gopal and Sai, D.V.R. (2010). Detection of tobacco streak virus by immunocapture–reverse transcriptase–polymerase chain reaction and molecular variability analysis of a part of RNA3 of sunflower, gherkin and pumpkin from Andhra Pradesh, India. *Sci. Asia*, **36** (3) : 194-198.