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Effectiveness of higher dilutions of developed polyclonal antibodies in enzyme-linked immunosorbent assay for detection of *Citrus tristeza* virus in citrus.

Thakre RA[1*], Gahukar SJ[2], Akhare AA[3], Chungada AS[1], Surbhaiyya SD[1], Bodade SR[1]

[1]Department of Agriculture Botany, Biotechnology Center, Dr. Panjabrao Deshmukh Krishi Vidhypeeth, Akola, Maharashtra, India
[2]College of Food Technology, Yavatmal, Maharashtra, India
[3]Seed Technology Research Unit, Akola, Maharashtra, India

Abstract

DAS ELISA has revolutionized the detection test, making it feasible to test large number of samples. ELISA test can provide rapid, sensitive and economical detection of CTV in crude extracts from *citrus* trees. Polyclonal antibodies specific to CTV were produced in Rabbit using ultrapurified virus. Raised antiserum specificity was confirmed with Dot-blot. Plate ELISA at higher antibody dilutions was performed to check the reactivity at lowest concentration of antibody. No colour development observed in any combinations of antigen-antibody dilution. All OD readings were negative in the ELISA reader at 405 nm. Study revealed that dilutions of antibody beyond 1:32000, non-functional to detect CTV.

Keywords: ELISA, Polyclonal, Antiserum, Dot-blot, Beyond.

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Introduction

The global problem of *citrus* decline warranted special attention of agricultural scientists during the past few decades. In all over India the infestation of CTV in *citrus* orchards about 26.3-60 percent. In northeast region it is 47-56 percent, in south it is 36-60 percent. In northwest it is 16-60 percent and in central region it was approx. 20-40 percent. In Maharashtra highest *citrus* growing area i.e. Vidarbha is infected with CTV about 26.3 percent [1]. The *Tristeza* virus is genetically and biologically diverse and can cause field symptoms ranging from vein clearing, stem pitting, yellowing, slow decline and quick decline, or no symptoms depending on virus isolate, time of infection, root stocks, *citrus* cultivars and environmental conditions [2].

The biological-indexing is relatively less reliable and time consuming, but double antibody sandwich-enzyme-linked immuno-sorbent assay (DAS ELISA) has revolutionized the detection test, making it feasible to test large number of samples [3-5]. ELISA test can provide rapid, sensitive and economical detection of CTV in crude extracts from *citrus* trees.

Therefore in present investigation, effectiveness of higher dilutions of developed polyclonal antibodies in Enzyme-Linked Immunosorbent assay for detection of *Citrus tristeza* Virus in *citrus spp.* was studied.

Materials and Methods

Virus isolation and purification

Virus was isolated from the infected leaf samples and purified by using standardized protocol of Bar-Joseph et al. [2] with slight modifications.

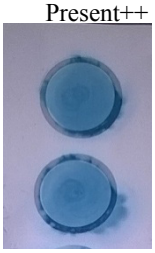
Electron microscopy

The virus particles obtained after purification were observed under transmission electron microscope for determining the shape and size. A drop of purified virus preparation was placed on the carbon-coated grids [6] and allowed to settle for 2-3 min. Excess sample was removed by using blotting paper. A small droplet of uranyl acetate was placed on it and allowed to stay for 2-3 min. The excess stain was drained by touching a blotting paper strip to the edge of the grid. The grids were dried for 15-30 min. in dessicator and examined under BIO-TECHNAI G-2 transmission electron microscope at various magnifications.

Raising of antiserum against purified CTV

Polyclonal antibodies were raised against CTV in Rabbit (New Zealand White Male) at Biotechnology centre, Department of Agricultural Botany, Dr. PDKV, Akola, as per the permission given by institutional animal ethics committee

Table 1. *Antiserum raising report of CTV.*

Days Interval in Injection	Sequence of Injection	Contents of Injection	No. of Bleed	Test Bleed	Control Bleed
1	First injection	0.5 ml antigen+0.5 ml complete adjuvant+0.5 ml PBS	First bleed	Present+	Absent
7	First booster	0.5 ml antigen+0.5 ml complete adjuvant+0.5 ml PBS			
14	Second booster	0.5 ml antigen+0.5 ml complete adjuvant+0.5 ml PBS			
21	Third booster	0.5 ml antigen+0.5 ml complete adjuvant+0.5 ml PBS			
30	Test bleed				
38	Fourth booster	0.5 ml antigen+0.5 ml complete adjuvant+0.5 ml PBS	Second bleed	Present++ 	Absent 
45	Fifth booster	0.5 ml antigen+0.5 ml complete adjuvant+0.5 ml PBS			
54	Final bleed				

of post graduate institute of Veterinary and animal sciences, Akola (IAEC, PGIVAS, Akola).

The rabbit was immunized following routine procedure followed Van Regenmortel [7].

DOT-ELISA for test bleed

Dot-blot technique was carried out for the test bleed sample collected from the rabbit to confirm the specificity of raised antibody against CTV.

Diagnosis of CTV in citrus spp. using higher dilutions of PAB in plate ELISA

The polystyrene ELISA plate was coated with suitably diluted antibody 1:64000, 1:128000, 1:256,000, 1:512,000 1:1024,000 and 1:2048,000 and healthy control in blocking buffer and the plate was covered under humid box and incubated at 4°C overnight. The plate was then washed with washing buffer (PBS-T) trice with 5 min interval by tapping or blotting plate on blotting paper to remove excess buffer. Blocking solution was added to the plate and was incubated at 37°C for 1 h and washing was repeated. Diluted antigen of 1:10, 1:20 and 1:50 in coating buffer was added and was incubated at 37°C for 1 hr and washed again. Diluted secondary antibody alkaline phosphataes conjugate (Goat antirabbit IgG) of 1:1000 and 1:2000 in blocking buffer was added and the plate was covered and incubated at 37°C for 1 hr and repeat the washing procedure. Enzyme substrate (para nitro phenyl phosphate) was added to each well and plate was covered and incubated at 37°C for colour development. Reaction was terminated with 5 N KOH. Visual observation and absorbance values using ELISA reader were taken at 405 nm [7].

Estimation of CTV positive sample

Samples were considered positive when the mean absorbance of duplicate well exceeded twice than the mean absorbance of appropriate healthy control.

Results and Discussion

Virus isolation and purification

Purification of crude CTV was done by ultracentrifugation method of cesium sulphate and sucrose gradient was reported by Gonsalves et al. [8]; Garnsey et al. [9] and Bar-Joseph et al. [10] with slight modifications. The ultra-purified virus was further used for development of serological diagnostic protocol.

Electron microscopy

To study the morphology and confirmation of CTV, before used in immunization for the production of CTV specific polyclonal antibodies, the electron microscopy was carried out; of the purified CTV. It showed the presence of flexuous rod shaped particles at different magnifications. The size of the virus particles measured was 2000 nm in length and 12-13 nm in width. Similar microscopic observations were reported by Bar-Joseph et al. [1,2,11-13].

Raising of antiserum against purified CTV

On completion of immunization schedule of experimental rabbit with CTV (ultrapurified antigen 12 mg/ml of concentration), antiserum was isolated from bleed (After fifth booster) of both treated and control rabbit (Plate 1).

The clear whitish fluid obtained after purification was

Plate 1. *A) Selection and maintainance of Rabbit B) Collection of antiserum after final booster.*

Plate 2. *Purified polyclonal antibodies from Rabbit.*

collected (Plate 2) and stored at -200°C deep freeze with pinch of sodium azide as a preservative.

DOT-ELISA for test bleed

After third booster antibody showed very faint blue colour on nitrocellulose membrane, indicates that, the concentration of raised antibody after third booster was very low and needed a more booster dose. After fifth and final booster again concentration of polyclonal antibody was checked on nitrocellulose membrane with CTV, dark blue colour appeared in test bleed whereas no coloure development was obsevered in control bleed (Table 1). These results at both stages revaled that, the antibody raised in the rabbit was specific to CTV and can be used in serological diagnosis of CTV.

Concentration of antibody leads to development of dark blue colour on Nitrocellulose blotting membrane (NCM) against CTV was earlier reported by Ozturk et al. [14] in rabbit and mice for detection assays.

Diagnosis of CTV in citrus spp. using higher dilutions of PAB in plate ELISA

The antibody dilutions used were 1:64000, 1:128000, 1:256,000, 1:512,000 1:1024, 000 and 1:2048,000. The dilutions of antigen used were 1:60, 1:70, 1:80 and buffer control with secondary antibody of 1:1000. No colour development was observed (Plate 3) in any combinations and all OD readings were negative (Table 2) in the ELISA reader at 405 nm.

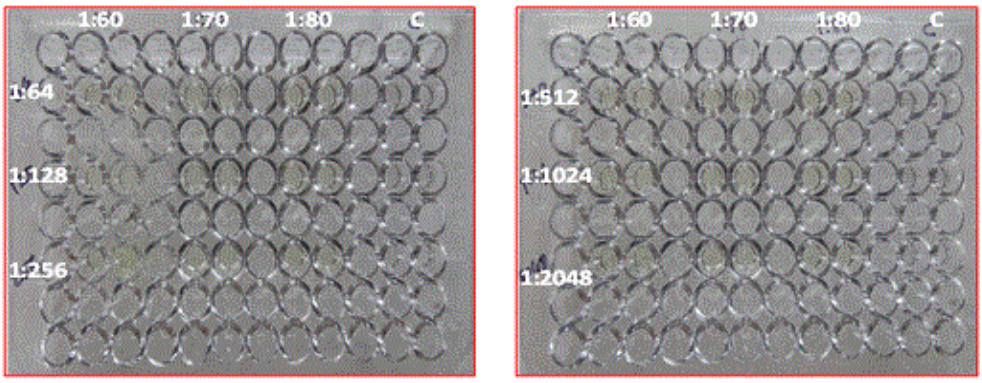


Plate 3. *Plate ELISA at higher antibody dilutions to check reactivity at lowest concentration.*

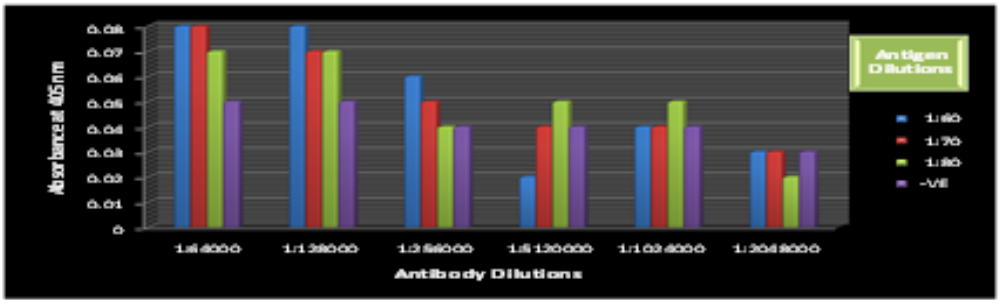


Figure 1. *Results showing the sensitivities of ELISA for diagnosis of CTV in infected tissues of orange at higher dilutions of primary antibody and antigen.*

Table 2. *Mean ELISA values (OD) at 405 nm of duplicate samples of leaves extract from orange infected with CTV and from healthy orange at higher antibody dilution.*

Primary Antibody Dilution	Antigen Dilution				Secondary Antibody Dilution 1:2000
		1:10	1:20	1:50	-VE
	1:1000	0.08	0.08	0.07	0.05
	1:2000	0.08	0.07	0.07	0.05
	1:4000	0.06	0.05	0.04	0.04
	1:8000	**0.02**	0.04	0.05	0.04
	1:16000	0.04	0.04	0.05	0.04
	1:32000	0.03	0.03	0.03	0.03

Conclusion

Graphical representation of mean value of OD reading in Figure 1 shows that the intensity of antigen and antibody interaction was very low due to higher dilutions of antibodies.

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***Correspondence to**

Ritesh A Thakre
Department of Agriculture Botany
Biotechnology Center
Dr. Panjabrao Deshmukh Krishi Vidhypeeth
Akola, Maharashtra
India