Meristem Culture of Mandarin Orange (*Citrus reticulata*) for Elimination of Citrus Tristeza Virus

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ABSTRACT

Mandarin trees grafted on trifoliate orange showing symptoms of citrus tristeza virus (CTV) such as yellowing of leaves, interveinal chlorosis and stem pitting were subjected to virus testing by double antibody sandwich enzyme-linked immuno sorbent assay (DAS-ELISA). Results with optical density (OD405) of more than 0.5 have confirmed the presence of CTV. Culture of apical meristems with few leaf primordia ranging from 0.2 to 0.5 mm in diameter has given rise to virus-free plantlets (OD405 of 0.16) after eight weeks. This technique of meristem culture may be useful to eliminate viruses from elite mandarin plants.

Key words: CTV, DAS-ELISA, Meristem Tissue Culture, In vitro

INTRODUCTION

Citrus Tristeza virus (CTV) is assumed to have originated in China quite a long time ago. Tristeza, also known as "quick decline" in the United States, is one of the destructive diseases of citrus especially in lime and sweet organge grafted on sour orange and has a world wide distribution. The climatic conditions of the midhills of Nepal favour the cultivation of high quality citrus fruits. Citrus cultivation is one of the main professions in the mid hills of Nepal. With the growing of citrus and the lack of knowledge of local farmers on virus infections, CTV and other citrus viral diseases are also rapidly growing. In 1981, the total world loss attributable to tristeza disease was estimated at 50 million trees (Bar Joseph *et al.,* 1981)

CTV belongs to the Closterovirus group. The causal virus is a filamentous, non-enveloped, and usually flexuous, with a clear modal length of 2000nm and 12nm wide. Its genome consists of single stranded RNA and has a total genome size of 17-20 kb (Brunt *et al.,* 1996). The virus particles are located in the phloem of the host plant and disturb its transportation systems. CTV is transmitted by several species of aphids. Among them, citrus brown aphid (*Toxoptera citricida*) is the most effective vector (Rocha-Pena *et al.*, 1995). The virus is also readily transmitted by grafting and budding. It is also transmitted by some species of dodder. However, it is not transmitted by seed and soil.

The severity of the disease depends on the virus strain, the species of citrus and the scion-rootstock combination. The most economically important symptom is the 'quick decline' or death of the affected trees such as lime. Together with Huanglongbing, CTV has been the main cause of decline of mandarin in Nepal (Ranjit and Khatri-Chhetri, 1997). A second symptom of the CTV is a 'slow decline' where the trees decline in health over a period of years. This decline is accompanied by a loss in productivity but the tree does not necessarily die. A third common symptom is 'stunting' where the virus does not kill the tree but the tree does not grow. Some CTV strains also cause 'stem-pitting' which may give a bumpy appearance to the trunk, because of the deep pits present under depressed areas of the bark. The virus may also have a latent form which gives no symptoms (Bennet and Costa, 1949).

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CTV can be detected by DAS-ELISA. It involves a capture step of the virus to a specific antibody in an ELISA, then the virus is further captured by another antibody conjugated with an enzyme, and a detection step uses a specific substrate of the used enzyme (Issa and Iraki, 2004). The developed color is finally read by an ELISA reader. For a virus-free plant no color development occurs.

Once an area becomes infested by the disease, it is impossible to eradicate because of the widespread distribution of the citrus aphid. However the manifestation of the disease can be minimized by planting citrus on tristeza resistant rootstocks such as *Poncirus trifoliate* (Mestre *et al.,* 1997). Complete eradication of virus from infected plant is practically impossible. *In vitro* culture has become the only effective technique to obtain virus-free plant from infected stock. Meristem culture technique has been applied to eliminate viruses from important cultivars. Apical meristems are generally either virus free or carry low number of viruses. The number of virus increases in the tissue as the distance from the meristem tip increases. Therefore it is generally accepted that smaller the size of the shoot tip cultured, greater are the chances of obtaining virus free plant (Stace-Smith and Mellor, 1968). For the elimination of CTV, a meristem of 0.2mm – 0.5mm is cultured *in vitro*. Another method is shoot-tip grafting where a very small portion of the shoot-tip, about 0.15mm is removed from the growth tip and grafted onto a rootstock seedling growing in a test tube. If the shoot tip removed is small enough then the disease is not present in the micro-grafted propagation (Navarro *et al.,* 1980).

Since Nepal is an agro economic based country, and citrus fruits have a wide economic impact, this research aims to explore the array of CTV infection within the plants and meristem culture technique to make the plant free from the causative virus. So the objectives of the research are;

To detect CTV in the suspected citrus plants by DAS-ELISA

To perform meristem culture of mandarin orange for elimination of CTV

To produce the meristem cultured plants free from CTV.

MATERIALS AND METHODS

Virus Testing

Collection and preparation of sample

Leaves were collected from four mandarin trees with CTV symptoms such as leaf yellowing, inter-veinal chlorosis and stem pitting from the garden of GREAT laboratory, Baneshwor. Under sterile conditions, the leaves were cleaned and about 0.5 gm of leaf midribs was ground in 3-5ml of Extraction buffer (PBST, 2% PVP, 0.02M Sodium sulfite, pH 7.4). The mixture was centrifuged at 2000rpm for 2-5min. The supernatant was then ready for use as the sample.

DAS ELISA

Coating with antibodies

The antibody is mixed with coating buffer (1.59gm Na_2CO_3 + 2.93gm $NaHCO_3$ + 0.20gm $NaNO_3$ per litre, pH 9.6) at 1:500 ratios. Coating antibody buffer (200µl) was poured into each well of the ELISA plate and incubated at 37°C for 4 hours. After the allotted incubation period, the plate was washed three times with washing buffer (PBS + 0.5 ml Tween 20 per litre).

Sample loading

Samples (200µl each) were loaded into different wells with a separate micropipette tip. After incubating at 4°C for overnight, the plates were washed three times with washing buffer.

Conjugate loading

The conjugate antibody (Conjugate Alkaline Phosphatase) was mixed with conjugate buffer (PBST + 2% PVP + 0.2% egg albumin) at 1:500 ratios. Conjugate antibody buffer (200µl) was poured into each well of the ELISA plate. After incubating at 37°C for 3-6 hours, the plate was washed three times with washing buffer.

Substrate loading

About 200µl of freshly prepared substrate (Alkaline Phosphate; 1mg/ml) was poured into each well of the ELISA plate and 30-60 minutes was allowed for the color reaction to take place.

Result reading

After the color formation in 60 minutes, the optical density (OD 405of each well was calculated with the help of an ELISA plate reader. The lens was set at 405 nm.

Stop reaction

The color reaction was stopped after plate reading. This was done by adding 50µl of 3M NaOH solution to each well.

Tissue culture

Sample collection

The shoot tips with meristem portion were collected from the mandarin plant in March. Fresh newly grown shoot tips were chosen. They were washed in tap water.

Disinfection

The meristems were washed in mild detergent three times and the soap was rinsed with tap water. Under laminar air flow, meristems were treated with 1% Sodium Hypochlorite solution for 5 minutes. Then the meristems were washed in sterile distilled water 3 times.

Meristem excision

The shoot tip was placed on Petri dish. Under a microscope of 30x magnification, the protective leaves on the tip were removed with a scalpel and the meristematic tip with less than 0.5mm in diameter was prepared..

Culture

1. The meristem was placed on a filter paper bridge in test tube containing the liquid MS media (Fig. 1). Incubation was done at light intensity of 2000 lux at 26°C for 8 weeks.
2. When the meristems grow to 2-3 mm they were transferred to MS solid shoot media and incubated at 25°C under diffused light.
3. After about 8 weeks the cultured plantlets were then ready for viral testing.

Virus testing on tissue cultured plantlet

The method was the same as described above for DAS-ELISA and the results were thus tabulated.

RESULTS AND DISCUSSION

Though the ELISA test for presence of CTV was performed on the four suspected mandarin samples, one was found to be highly infected and the others were mildly infected by CTV). Meristem culture was performed on all four samples to obtain a virus free plant. The plantlet derived from meristem culture technique had a decreased OD value at A405 nm, indicating successful elimination of CTV (Table 1).

Table 1: Optical Density readings by DAS-ELISA of the four Mandarin samples and four meristem-culture samples obtained from the garden (GREAT Laboratory, Baneshwor)

SN.	Sample	Symbol	OD Avg(A405)
1	Mandarin Sample 1	MS1	0.45
2	Mandarin Sample 2	MS2	0.37
3	Mandarin Sample 3	MS3	0.30
4	Mandarin Sample 4	MS4	0.56
5	T. culture Sample 1	TC1	0.17
6	T. culture Sample 2	TC2	0.16
7	T. culture Sample 3	TC3	0.17
8	T. culture Sample 4	TC4	0.18
9	Positive sample	Std +ve	0.47
10	In vitro –ve sample	Std –ve	0.16
11	Blank	Blank	0.14

The plate included four Mandarin samples from garden trees, four tissue culture plants as well as positive and negative indicators (Fig. 2). The OD of the sample as well as positive and negative indicators was taken. An OD value equal to or greater than 0.200 is considered CTV positive, while an OD value less than 0.200 is considered CTV negative.(Ranjit, 1999). The positive indicator used was an in house infected plant which was considered to be highly infected. The average OD calculated from six positive indicator samples was 0.507. Thus any results equal to or above this value were considered as being highly infected.

In the four given Mandarin samples, sample 4 had an average OD value of 0.562, so it is considered to be highly infected. Sample 1, 2 and 3 had an average OD value of 0.448, 0.365, and 0.276 respectively, whose value is greater than 0.200, so they are considered to be CTV positive.

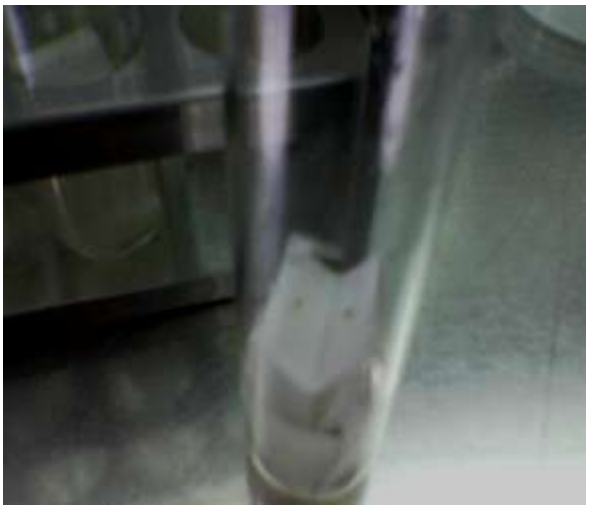

Fig.1:Meristem culture on filter paper bridge containing liquid MS media

However, the meristem cultured plants obtained from all four samples had an average OD value of 0.178, 0.154, 0.170, and 0.179 respectively, whose value is less than 0.200, so they are considered as being CTV negative.

Meristem culture is practiced to obtain virus free plants. This is an only effective method to produce virus free plantlets. Size of meristem is a critical

aspect in meristem culture. The virus infects the phloem vascular region of the plant; the apical meristem portion grows at a fast rate not allowing the virus to reach this region. So a meristem of 0.1mm-0.5mm will not contain virus particles (Stace-Smith and Mellor, 1968).

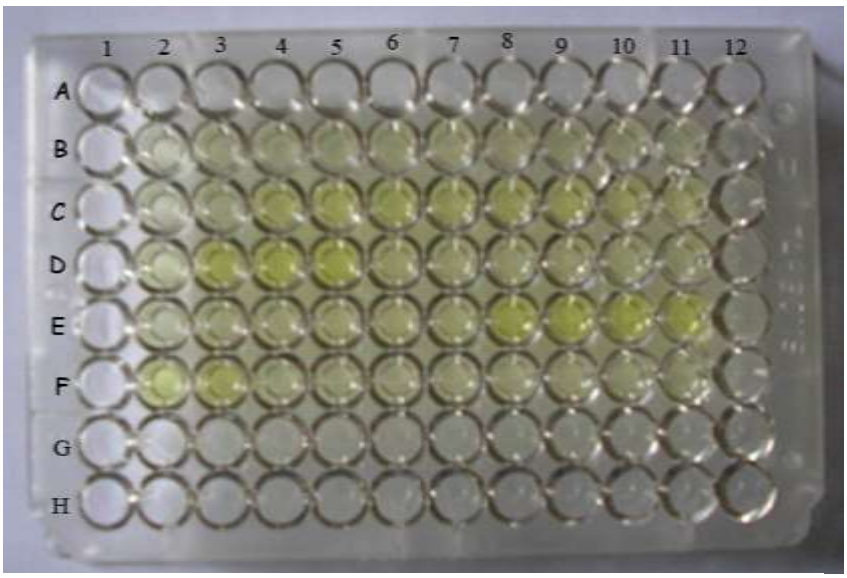

Fig. 2: ELISA plate for the test of CTV in four mandarin samples, where sample 1 (well no. C_4-C_6), sample 2 (well no. C_7-C_9), sample 3 (well no. C_{10},C_{11},D_2) and sample 4 (well no.D_3-D_5) are found to be CTV positive. The meristem tissue culture plantlet obtained from sample 1 (well no. D_6-D_8), sample 2 (well no. D_9-D_{11}), sample 3 (well no. E_2-E_4) and sample 4 (well no. E_5-E_7) were found to be CTV negative. An in house infected plant was used as Positive control (well no. E_8-E_{11}, F_2, F_3) and Tissue cultured Lime $Ao_{1\ (6)}$ was used as Negative control (well no. F_4-F_6).

The survival rate of the meristem cultured in this experiment was approximately 50% and all the plants that survived showed negative test for CTV. This shows that meristem tissue culture technique for virus elimination is fool proof. The main reasons why the other meristems did not survive may be (1) due to longer exposure to air, causing it to dry and (2) due to meristem falling into the liquid media depriving itself of oxygen. The smaller the size of the meristem, less are the chances of its survival.

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REFERENCE

Bar-Joseph M., Roistacher C.N., Garnsey S.M. and Gumpf D.J.(1981). A review of tristeza, an ongoing threat to citriculture. Proc. Int. Soc. Citricul. 1:419 – 423

Brunt, A.A, Crabtree, K, Dallwitz, M.J, Gibbs, A.J, Watson, L. and Zurcher, E.J. (eds.) (1996 onwards). Plant viruses online:Descriptions and Lists from the VIDE Database. Version: 20th August 1996

Bennet, C.W., and Costa, A.S. 1949. Tristeza disease of citrus. Jour. Agr. Res. 78(8) 207-237.

Issa and Iraki. (2004). Virus Detection: Citrus Tristeza Virus by DAS-ELISA for the detection of CTV. UNESCO, Biotechnology centre at Bethlehem University.

Mestre, P.F., M.J.Asins, J.A.Pina, E.A.Carbonell and I. Navarro. 1997. Molecular markers flanking citrus tristeza virus resistant genes from *Poncirus trifoliate* (L) Raf. Theor. Appl. Genet. 94:458-464.

Murashige, T., F. Skoog. (1962). A revised medium for rapid growth and bioassay with tobacco tissue culture. Physiologia Plantarum 15: 473-497

Murashige, T., Betters, W.P Rangan, *et al.* (1972). A technique of shoot Apex Grafting and its Utilization towards Recovering Virus free Clones. Hortscience, Vol. 7(2). April pp. 118-119.

Navarro, L., J. Juarez, J.F. Ballister and J.A. Pina (1980). Elimination of some citrus pathogen producing Psorosis-like leaf symptoms, by shoot tip grafting *in vitro*.

Ranjit, M., and G.BKhatri Chhetri (1997). Citrus Research and Development Action Plan. Agro Enterprises and Tech Systems Project. Pp 3.

Ranjit, M. (1999). Rapid Propagation and Transplantation of Disease-Free Mandarin Plants Grafted on Tissue Cultured Rootstocks of Trifoliate Orange in Gorkha and Lamjung District. In: Proc. Inter. Workshop BIO-REFOR, Nepal. pp 64-67.

Rocha-Pena, M.A., R.F. Lee, T.A. Permar, R.K. Yokomi, and S.M. Garnsey (1991). Use of enzyme-linked Immuno-sorbent and dot-immuno binding assays to evaluate two mild strains cross protection experiments after challenge with a severe citrus tristeza virus isolate. Proc 11th Conf. International Organization of Citrus Virologists, IOCV, I Riverside. pp.93-102.

Rocha-Pena, M.A., R.F. Lee, R. Lastra, C.L. Niblett, F.M. Ochoacorona, S.M. Gransey, and R.K. Yokomi (1995). Citrus tristeza virus and its aphid vector *Toxoptera citricida*: Threats to citrus production in the Carribean and Central and North America. Plant. Dis. 79: 437-443.

Stace-Smith, R. and F.C. Mellor (1968). Eradication of PVX and PVS by thermotherapy and auxillary bud culture. Phytopathology. 58: 199-203.