Turk J Agric For
26 (2002) 203-209

Application of Direct Tissue Blot Immunoassay in Comparison with DAS-ELISA for Detection of Turkish Isolates of Citrus Tristeza Closterovirus (CTV)

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Received: 27.11.2001

Abstract: In this study, the direct tissue blot immunoassay (DTBIA) procedure was tested for the detection of 10 different Turkish isolates of the citrus tristeza virus (CTV) by employing monoclonal and polyclonal antibodies. The DAS-ELISA and DTBIA procedures were compared using two different monoclonal antibodies (3E10 and MCA-13) for 10 CTV isolates. The storage effects of blotted membranes on the blot assay were also investigated by using different temperatures (4 and 25 °C) and storage intervals (1, 2, 3 and 4 weeks). In addition, 258 Satsuma trees in one orchard were tested using ELISA and DTBIA. All polyclonal (1212-1, 981-1 and 908-7) and monoclonal (ECTV-175, ECTV-176, 11B1-3 and 3E10-6) antibodies reacted to CTV isolates, except MCA-13, which only reacted to Igdir and Cyprus CTV isolates. DAS-ELISA was also positive for all CTV isolates that reacted to DTBIA. Membranes kept at 25 °C gave better results than those kept at 4 °C, whereas there were no clear differences between storage periods for the same treatment combinations. Assays of 258 Satsuma trees by ELISA and DTBIA indicated similar rates of CTV infection. Seven trees were infected and 251 trees were virus-free in both tests. DTBIA is a rapid, sensible and reliable procedure for the detection of CTV. It requires little sample preparation and tissue blots can be stored at 25 °C for at least 4 weeks prior to the assay. Moreover, blotted membranes can be sent safely to another place for testing.

Key Words: Tissue blot, nitrocellulose membrane, citrus tristeza virus, ELISA

Direk Doku Bastırma ve DAS-ELISA Yöntemleriyle Türkiye'de Yetiştirilen Turunçgillerde Hastalık Oluşturan Tristeza Closterovirüsü (CTV) İzolatlarının Saptanması

Özet: Bu çalışmada Türkiye'deki turunçgil tristeza virüsünün 10 izolatı, farklı poliklonal ve monoklonal antibadi'ler kullanılarak direk doku bastırma (DTBIA) yöntemiyle test edilmişlerdir. DAS-ELISA ve DTBIA yöntemleri iki farklı monoklonal (3E10 ve MCA-13) antibadi kullanılarak 10 CTV izolatı için kıyaslanmıştır. Doku bastırılan membranlar farklı sıcaklık (4 ve 25 °C) ve farklı zaman süreleri (1, 2, 3 ve 4 hafta) kullanılarak test edilmişlerdir. Ayrıca bir Satsuma bahçesindeki 258 ağaç hem ELISA ve hem de direk doku bastırma yöntemleri kullanılarak testlenmiştir. Tüm poliklonal (1212-1, 981-1 ve 908-7) ve MCA-13 dışında tüm monoklonal (ECTV-175, ECTV-176, 11B1-3 ve 3E10-6) antibadi'ler CTV izolatlarına pozitif reaksiyon gösterirken, MCA-13 yalnızca Iğdır ve Kıbrıs izolatlarına pozitif reaksiyon göstermiştir. İki farklı monoklonal antibadi kullanılarak test edilen CTV izolatından DTBIA testinde pozitif reaksiyon gösterenler ELISA testinde de pozitif reaksiyon göstermiştir. İki farklı sıcaklıkta test edilen doku bastırılmış örnekler 25 °C'de 4 °C'ye göre daha iyi sonuç verirken aynı sıcaklıkta farklı sürelerle test edilen örnekler arasında bir fark görülmemiştir. ELISA ve DTBIA yöntemiyle test edilen 258 Satsuma ağacı her iki yöntemde de benzer sonuçlar vermiştir. Her iki test sonuçlarında toplam 7 ağaç infekteli bulunurken 251 ağaç sağlıklı bulunmuştur. Direk doku bastırma turunçgil tristeza virüsünün tanısı için hızlı, duyarlı ve güvenilir bir yöntemdir. Yöntem için küçük miktarda örnekler yeterlidir ve örnek bastırılan membranlar 25 °C'de en az 4 hafta süreyle bozulmadan saklanabilir. Ayrıca membranlar test için diğer bölgelere güvenli bir şekilde gönderilebilir.

Anahtar Sözcükler: Doku bastırması, nitroselüloz membran, turunçgil tristeza virüsü, ELISA

Introduction

The citrus tristeza virus (CTV) is phloem-limited and a member of the closterovirus group. It is one of the most widespread and economically important virus diseases for citrus in the world (Bar-Joseph and Lee, 1990). The virus was first observed in the Orient and was spread by man to most citrus production regions. There were epidemics in the Western Hemisphere, in Brazil and Argentina, in the 1930s (Zeman, 1931). CTV and its vector, *Aphis gossipii*, Glover, have been known in Turkey for many years without causing any serious losses in citrus plantations; however, it remains a potential threat to the

citrus industry of Turkey because of the use of sour orange as a rootstock (Cinar *et al.*, 1993).

Several serological procedures have been developed to diagnose CTV such as SDS-immunodiffusion techniques (Garnsey *et al.*, 1979; Gonsalves *et al.*, 1978), in situ immunoflorescence (Brlansky *et al.*, 1984), serologically specific electron microscopy (Garnsey *et al.*, 1980; Brlansky *et al.*, 1984) and several enzyme-linked immunosorbent assay (ELISA) procedures (Bar-Joseph *et al.*, 1979; Bar-Joseph and Malkinson, 1980) including the use of the biotin-avidin complex (Irey *et al.*, 1988) to increase the sensitivity of detection. Each of these methods has different advantages and sensitivity levels. Therefore, they have been selectively chosen on the bases of purposes and applications (Rocha-Pena and Lee, 1991).

The ELISA tests, particularly the double-antibody sandwich (DAS-ELISA) (Bar-Joseph *et al.*, 1979) and the DAS-indirect system (Bar-Joseph and Malkinson, 1980; Garnsey and Cambra, 1991), are the most widely preferred techniques and serological methods developed for virus detection (Bar-Joseph *et al.*, 1989; Garnsey *et al.*, 1981; Rocha-Pena and Lee, 1991). Both the DAS-ELISA and DAS-I ELISA are relatively easy to perform and are highly sensitive. However, they do require some special equipment, in addition to being laborious and time consuming for large-scale indexing and need large amounts of antibodies and different buffers in each test.

A simple and rapid serological method known as direct tissue blot immunoassay (DTBIA) has been developed and applied for the detection of several plant virus including cucumovirus, luteovirus, potexvirus, and tomato spotted wilt virus group (Lin *et al.*, 1990; Hsu and Lawson, 1991). Garnsey *et al.* (1993) also applied this technique to detect Florida and Spanish isolates of citrus tristeza virus.

The objective of this research was to establish DTBIA for the detection of Turkish isolates of CTV using monoclonal and polyclonal antibodies in comparison with the DAS-ELISA method.

Materials and Methods

CTV isolates: Ten citrus tristeza virus isolates representing different citrus growing areas of Turkey were used in this study. CTV isolates were numbered based on the name of their isolation origin as follows: Guzelyurt (G), Denizkuyusu-1 (DK-1), Denizkuyusu-2 (DK-2), Igdir-1 (I-1) Igdir-2 (I-2), Dortyol-1 (D-1), Dortyol-2 (D-2), Karacailyas-1 (KI-1), Karacailyas-2 (KI-2), and Karacailyas-3 (KI-3). These isolates were maintained in Madam Vinous on Troyer citrange rootstock and kept in temperature controlled greenhouse conditions in the Subtropical Fruit Research and Experimental Centre of Çukurova University. All isolates gave typical CTV reactions in Mexican lime (vein clearing) indicator plants. Additionally, Guzelyurt isolate showed only stem pitting and stunting symptoms in Mexican lime and Madam Vinous sweet orange. Some properties of these isolates are given in Table 1.

Tissue blotting technique: This technique was described by Lin *et al.* (1990) and Garnsey *et al.* (1993). Blots were prepared from stem pieces of new growing young flushes taken from greenhouse or field samples. A smooth fresh cut was made with a razor blade and the cut surface was pressed gently and evenly onto the nitrocellulose membrane. Sometimes, two blots were made continuously from the same cut if tissue was succulent. To compare different antibodies, blots were made from the same tissue piece on separate membranes. Disposable gloves and tweezers were used during the blotting process to prevent non-specific background. Blotted membranes were left to dry for 30 min. In some cases blotted membranes were kept for longer periods at 4 and 25 °C at 65% relative humidity to test storage effects on the blot assay.

Bio-Rad Trans-Blot nitrocellulose membranes (Bio-Rad Laboratories, CA) were used in this study. The 10-cm^2 membranes were cut into a suitable size for the number of samples to be blotted. Then, membranes were premarked with a pen of 1-cm^2 size (Figure 1A) so the position of individual samples on a membrane could be marked (Figure 1B). After a membrane was imprinted and dried, it was placed in a solution of 1% BSA in PBS and incubated for 30 min at room temperature, with gentle shaking in a plastic dish on a shaker. Membranes were incubated 1 to 2 h at room temperature for the virus-specific antibodies and 1 h for streptavidin conjugates. Substrate was prepared from Sigma Fast BCIP (5-bromo-4-chloro-3-indoly phosphate) / NBT (nitroblu tetrazolium) tablets (Sigma Chemical Co., St. Louis, MO). Membranes were incubated in the substrate solution for 5 to 20 min. The reaction was stopped by

Table 1. Biological properties of Turkish isolates of citrus tristeza virus (CTV) used for direct tissue blot immunoassay (DTBIA).

Isolates	Original tree	Location (Province)	Indexing results	
			Psorosis	Exocortis
Güzelyurt	Shaumoti SwO	Cyprus	+	+
Denizkuyusu-1	Washington Navel SwO	Adana	+	+
Denizkuyusu-2	Washington Navel SwO	Adana	+	+
Iğdır-1	Shaumoti SwO	Içel	+	-
Iğdır-2	Shaumoti SwO	Içel	+	-
Dörtyol-1	Clemantine Mandarin	Hatay	-	+
Dörtyol-2	Clemantine Mandarin	Hatay	-	+
Karacailyas-1	Satsuma Mandarin	Içel	-	+
Karacailyas-2	Satsuma Mandarin	Içel	-	+
Karacailyas-3	Satsuma Mandarin	Içel	-	+

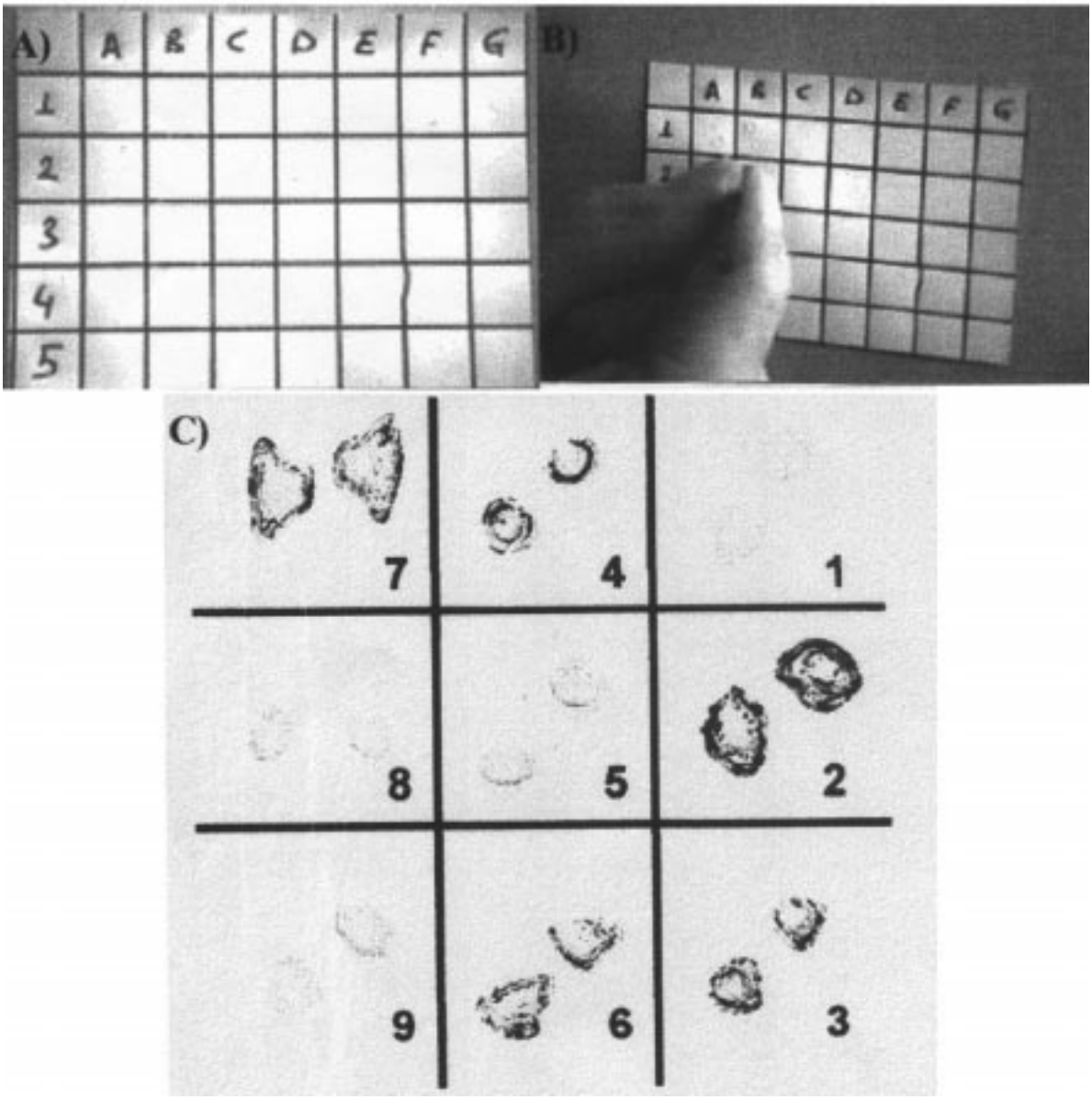

Figure 1. Direct tissue blot immunoassay for Turkish isolates of citrus tristeza virus. A) Nitrocellulose membrane, B) Blotted freshly cut surface of stem samples on a nitrocellulose membrane, C) The results of direct tissue blot immunoassay for some citrus tristeza virus isolates used in this study: 1) Healthy, 2) Guzelyurt-1, 3) Igdir-1, 4) Dortyol-1, 5) Healthy, 6) Denizkuyusu-1, 7) Karacailyas-1, 8) Healthy, 9) Healthy.

washing the membranes in distilled water. The membranes were washed three times, shaken gently during process, between steps in PBS-Tween (Clark and Bar-Joseph, 1984) for 5 min. The results were examined in a dissecting scope at 10 to 20X magnifications.

Polyclonal and monoclonal antibody source: Several different polyclonal and monoclonal antibodies (PABs and MABs) were used for DTBIA. Polyclonal antibodies (1212-1, 981-1 and 908-7) were prepared against Florida CTV isolates and have been successfully used for ELISA (Garnsey, personal communication). Monoclonal antibodies (ECTV-175, ECTV-176, 11B1-3 and 3E10-6) are reactive to most isolates of CTV (Garnsey *et al.*, 1989). The CTV MCA-13 is a MAB reacting with only virulent isolates of CTV, but not with the mild isolates from Florida and/or CTV from other countries (Permar *et al.*, 1990).

Immunoblots: Two procedures were used. The first was a direct method where the blotted membranes were exposed to CTV-specific antibodies conjugated to alkaline phosphatase (Permar *et al.*, 1990). The second procedure was an indirect method where the blotted membrane was exposed first to unlabeled CTV-specific antibodies and then to commercially prepared alkaline phosphatase-labeled secondary antibodies (goat anti-rabbit for monoclonals and goat anti-mouse for polyclonal) (Garnsey *et al.*, 1991). The source of commercial alkaline phosphatase-labeled secondary antibodies was Boehringer Mannheim Bio., IN, USA. Concentration of IgG varied with the different sources and applications. Commercially labelled secondary antibody and conjugates were used based on the procedure described by the manufacturer (Sigma Chemical Co., St. Louis, MO).

To test storage effects on the blot assay, blots were made of healthy and I-1, I-2, DK-1 and DK-2 infected Madam Vinous sweet orange plants. Each sample set consisted of two blots each of healthy tissue and four sources of (I-1, I-2, DK-1 and DK-2) CTV infected tissue. Blotted membranes were stored at 4 and 25 °C at 65% relative humidity. Assays were accomplished at 1, 2, 3 and 4 weeks after the initial blots were made.

ELISA: ELISA was carried out by a double antibody sandwich indirect procedure (DAS-I) as previously described (Garnsey *et al.*, 1989). The 1212-1 PAB was used as a coating antibody for DAS-I. The secondary antibody was a monoclonal antibody (MAB) 3E10, which was made of an isolate of CTV-D, is reactive to most isolates of CTV tested previously (Garnsey *et al.*, 1989; Vela *et al.*, 1988). IgG conjugated to alkaline-phosphates, a commercial source of goat anti-mouse, was used to detect bound CTV-specific MAB. Substrate (p-nitrophenol phosphate) was added to the ELISA plate and incubated for 30 min at room temperature. Absorbance at 405 nm was measured on a microtiter plate reader (Stat Fax-2100, Awareness Tech. Ins. (USA)).

Comparison of DTBIA and ELISA for field assays: To compare both serological procedures, a total of 258 Satsuma mandarin trees, which were 25 years old in a field planting, were tested with ELISA and DTBIA. Previous observations indicated that some Satsuma trees were infected with CTV in this orchard. Shoots of new flush growth were collected and a 5-10 cm stem section was selected. Each shoot tip was freshly cut and blotted to a nitrocellulose membrane. An extract of a 1 g sample from a remaining stem piece was prepared and tested by DAS-I ELISA.

Results

Under 10X magnification, the outline of the young stem imprint was clearly visible (Figure 1C) and strong areas of deep purple staining were present in the imprinted area that corresponded to the phloem of CTV-infected stems. Otherwise, these strongly stained areas were absent in blots of comparable healthy tissue (Figure 1C). When antibody concentration and incubation time were optimal, the healthy tissue imprint was pink, and the remaining membrane was white or a faint pink. The pink background was clearly distinguished from the intensely stained areas in the phloem of virus-infected tissue. The best results were obtained when the tissue was pressed to the membrane just firmly enough to allow a faint green image of tissue without a strong imprint in the membrane. Non-specific background increased when imprints were made too firmly onto the nitrocellulose paper.

Generally, a lot of intensely stained areas were present and these sometimes blended to form a ring of staining corresponding to the phloem region. In most cases, positive blots were directly and easily identified, even when only one or two small areas of intense staining were present.

The reactivity of the different polyclonal and monoclonal antibodies for the 10 selected CTV isolates in DTBIA is given in Table 2. All polyclonal antibodies (1212-1, 981-1 and 908-7) showed a strong positive reaction with all CTV isolates tested by DTBIA. Monoclonal antibodies, ECTV-175, ECTV-176, 11B1-3 and 3E10-6, also reacted similarly to all CTV isolates in both direct and indirect procedures. The MAB MCA-13 gave a strong reaction with only Cyprus isolates and a slightly positive reaction with Igdir-1 and Igdir-2 isolates, but no reaction with the other isolates tested in this study.

To compare the sensitivity of ELISA and DTBIA procedures, 10 CTV isolates were tested using a broadly reactive 3E10 and severe strain-selective MCA-13 MABs. These results are summarized in Table 3. MAB 3E10 gave a positive reaction to all CTV isolates tested in ELISA and DTBIA. However, among the 10 CTV isolates, Guzelyurt, which causes stem pitting and stunting on Mexican lime showed a strong reaction to MCA-13 in both ELISA and DTBIA. Igdir-1 and Igdir-2 were slightly positive, but the remaining isolates that did not cause any stem pitting or stunting symptoms on Mexican lime did not react with MCA-13 in ELISA or DTBIA. 3E10 MAB had a strong reaction with all the isolates tested.

The results from the experiment looking at storage effects on blot assays showed that membranes kept at 25 °C gave better results than those kept at 4 °C. On the other hand, there were no clear differences between storage periods (1, 2, 3 or 4 weeks) for the same treatment combination.

A total of 258 Satsuma mandarin trees were tested with ELISA and DTBIA to compare both serological procedures. The results obtained from both methods showed that 7 trees were infected and 251 trees were virus-free.

Table 3. Comparison of enzyme-linked immunosorbent assay (ELISA) and direct tissue blot immunoassay (DTBIA) for differential detection of Turkish isolates of citrus tristeza virus.

	3E10-6 MAB		MCA-13 MAB	
Isolates	ELISA	DTBIA	ELISA	DTBIA
Cyprus	+	+	+	+
Denizkuyusu-1	+	+	-	-
Denizkuyusu-2	+	+	-	-
Iğdır-1	+	+	+	+
Iğdır-2	+	+	+	+
Dörtyol-1	+	+	-	-
Dörtyol-2	+	+	-	-
Karacailyas-1	+	+	-	-
Karacailyas-2	+	+	-	-
Karacailyas-3	+	+	-	-
Healthy	-	-	-	-

Discussion

DTBIA was successfully used for the detection of Turkish isolates of CTV. Several polyclonal and monoclonal antibodies tested gave a strong reaction to CTV isolates, except for MCA-13 MAB. This result confirmed the findings of Garnsey *et al.* (1991) and Permar *et al.* (1992). They reported that MCA-13 MAB

Table 2. Reaction of different monoclonal and polyclonal antibodies to citrus tristeza virus isolates in direct tissue blot immunoassays (DTBIA).

		Different CTV isolates*									
Antibodies	Healthy	G	DK-1	DK-2	D-1	D-2	I-1	I-2	KI-1	KI-2	KI-3
ECTV-175	0/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
ECTV-176	0/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
11B1-3	0/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
3E10-6	0/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
MCA-13	0/2	2/2	0/2	0/2	0/2	0/2	2/2	2/2	0/2	0/2	0/2
1212-1	0/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
981-1	0/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2
908-7	0/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2	2/2

*Number of positive reactions/Number of total tested samples

reacts with only virulent isolates of CTV, but does not react to mild isolates from Florida and other areas (Permar *et al.*, 1990). In contrast, Igdir isolate of CTV are known mild Turkish isolates and also gave a slightly positive reaction MCA-13 with MAB. This observation suggested that MCA-13 might have serologically positive reaction with mild strains of CTV except for the Florida strains.

In this study, Madam Vinous sweet orange was used as a source plant for DTBIA and it gave good results. Permar *et al.* (1992) also used numerous citrus hosts including Hamlin, Valencia, and Navel sweet oranges, Marsh and Red Blush grapefruit, Mexican lime, alemov, *Citrus hystrixs*, pummelo and rough lemon. They found no evidence of host-associated non-specific reactions with any of the varieties tested.

CTV blotted membranes stored at 25 °C and 65% relative humidity for up to 4 weeks worked perfectly. This result is in agreement with previous works. Garnsey *et al.* (1993) found no obvious differences between the 1-day and the 15- or 30-day storage periods for the same treatment combination, even after blotted membranes were stored for six months. Lin *et al.* (2000) also obtained similar results. They stored samples in plastic bags at 4 °C or in a frozen state for 4 weeks and obtained good results.

The fieldwork indicated that DTBIA was as sensitive as ELISA. This result is in accordance with work conducted by Garnsey *et al.* (1991) in Spain. They tested 560 trees as 5 composites and the composites' samples with infected trees and they were identified equally well by DTBIA and ELISA.

DTBIA provides direct information about the distribution of the virus within host plants. Even samples with weak positive reactions to ELISA or other immunoblots generally give clear results with DTBIA, since only one infected cell group is needed to give a clear signal (Garnsey *et al.*, 1993; Mestre *et al.*, 1997; Lin *et al.*, 2000).

Our results showed that DTBIA procedures are reliable for the detection of CTV under field or greenhouse conditions. It is a very convenient method for shipping a sample for testing from one place to other. No live tissue is necessary and the possible introduction of other pests or diseases is eliminated. The sample is stable on the membrane, therefore, refrigeration or protection of the sample is not required, and shipping costs are minimized. Garnsey *et al.* (1991) suggested that DTBIA is extremely convenient for field survey work in remote sites. All an investigator needs is to carry several sheets of nitrocellulose membrane, a few razor blades and disposable gloves.

The tissue blotting technique was also applied to detect viruses in cucumoviruses, luteoviruses, potexvirus, potyvirus, and tomato spotted wilt virus group and Phytoplasma. Passion fruit woodiness, papaya ring spot, sweet potato feathery mottle, bean yellow mosaic, cucumber mosaic and tomato spotted wilt viruses were detected in blots of infected tissue (Lin *et al.*, 1990).

DTBIA has several advantages over conventional DAS-ELISA or DAS-indirect ELISA for detection of CTV. It was rapid and easy to perform and it was as sensitive as either ELISA procedure for CTV detection. It does not require the preparation or extraction of the samples, eliminating the need for tubes and containers to store extracts prior to testing. It provides precise delivery of the sample to the membrane without need for manifolds or other loading devices (Garnsey *et al.*, 1991; Rocha-Pena *et al.*, 1991; Lin *et al.*, 2000).

As pointed out by Lin *et al.* (1990) and Garnsey *et al.* (1991), the major disadvantages of DTBIA is that it is not convenient to precisely perform quantitative results. DTBIA can be used reliably for routine diagnostic work when no quantitative measurements are needed. For quantitative studies, ELISA is still a preferable assay.

Acknowledgments

The author is grateful to Dr. Stephen M. Garnsey, Orlando Horticulture Research Laboratories, FL, USA, for supplying all antibodies and nitrocellulose membranes used.

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