REACTIVITY OF SOME HAWAIIAN BANANA BUNCHY TOP DISEASED SAMPLES USING DAS-ELISA AND A DIGOXIGENIN LABELLED DNA PROBE

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Abstract

Some Hawaiian infected banana with BBTD gave positive reaction using DAS-ELISA and Dot-Bolt, a digoxigenin labelled DNA probe. However, DAS-ELISA, was more sensitive with dilution of crude sap than the digoxigenin labelled DNA probe (Dot-blot) but both assays were similar at 1/5 dilution infected banana sap in all cases.

Thereby, a digoxigenin labelled DNA probe could be used as well as DAS-ELISA for routine diagnosis of BBTV in suspicious banana tissue for phytosanitary purpose.

INTRODUCTION

Banana bunchy top virus, BBTV, is the most devastating virus disease on banana in many countries i.e. Fiji, Egypt, Ceylon, Australia, Ellice Islands, Wallis Islands; Bonin Island; Western Samoa, Sabah, India (Magee 1953); South Vietnam, Cambodia, Laos (Vakili 1969); Taiwan (Sun 1961); Congo (Wardlaw 1961) and Hawaii (Nagata *et al* 1989). This systemic disease has spread to new areas by planting infected banana plants (including those derived from tissue culture, corms, suckers). Moreover, the virus is persistently transmitted by the banana aphid, *Pentalonia nigronerosa*. Diseased plants have yellowed leaves and damaged phloem (Magee 1940). It has been found that direct ELISA was 16 times more sensitive than indirect ELISA in detecting BBTV; however, PTA ELISA could not detect BBTV in crude extract of diseased rissues (Wu and Su 1990). BBTV c DNA clones were used in Dot-Blot analysis for detection of BBTV using both radioactive and non-radioacive la-

belled probes (Xie *et al.* 1994).

Detection of BBTV in some samples of diseased banana tissue collected from Oahu Island using DAS-ELISA and Dot-blot (non-radioactive labelled DNA probe) was invesigated.

MATERIALS AND METHODS

Samples :

The youngest and second youngest leaves and midrib (cvs tall Brazillian; dwarf Brazillian and William) were collected from different localities in Oahu Island i.e. Kailua, Maunawilli, Scott and Stanley Kawabita. Besides, samples of banana, artificially inoculated, were collected from greenhouse of Dept. Plant Path., UHM, at Oahu Island. Moreover, some aphid samples were collected from courtyard and the greenhouse and tested to confirm results of artificial inoculation.

DAS-ELISA:

ELISA was used with polystyrene microtitre plates. The double antibody sandwich (DAS) form of direct ELISA was employed as described by Clark and Adams (1977) and Su's ELISA assay (Personal communication). Coating was done with Taiwanese cell line 2 H6 at 0.5 ug/ml. Banana leaf tissues and aphid samples were prepared in extraction buffer (1:10)PBS with 0.1% DIECA. For routine detection, banana tissue was extracted at rate of 0.1 g/1 ml and aphid at rate of 1,3 or 5 aphids/ 100 ul extraction buffer. BBTV MAB 2H6 conjugate (1:2000) was incubated at 37^{o}C for 4 hrs. P-nitrophenyl phosphate substrate solution (1 tab. /5 ml) was incubated for 1 hr, 2 hrs and overnight at room temperature. ELISA reaction was considered positive when the A 405 value was greater than the mean plus two standard deviations of appropppriate control sample.

Extraction buffer :

It consisted of 176 m M NaCl; 176 mM Glycine; 20 mM EDTA; 24 mM NaOH and 2% SDS, pH 9.0.

Dot-blot :

Some infected banana samples infected with BBTV were tested with Dot-Blot

as well as ELISA to compare sensitivity of the two methods to detect BBTV in plant.

DNA probe, cloned BBTV c DNA (pBT 338) was provided by W.S. Xie (3 rd author).

Preparation and extraction of samples :

These followed procedures used by SU and Wu (1989) and Hull *et al.* (1986). Working sheet papers were provided by Dr. John (2 nd author), and pulverising banana tissue frozen in liquid nitrogen, were followed by additional grinding with a mortar and pestle and acid-washed sand. The squeezed tissue was thawed in 2 ml nucleic acid extraction buffer (pH 9.0) and mixed well with 1 ml phenol plus 1 ml chlorofrom in plastic tube. Mixture was centrifuged at 8500 rpm for 10 min.; supernatant taken into new tube, then mixed well with 0.2 ml 3 M Na Ac (pH, 5.2) and 5 ml ethanol 95%. After one hour at-20^{o}C, tubes of different samples were centrifuged at 14000 rpm for 10 min., then supernatant was carefully poured and tubes were dried under vacuum for 10 min. Pellets were resuspended in 500 ul TE (banana tissue was exracted at the rate of 0.5 g/ sample), vortexed and centrifuged for a few seconds. Samples were kept at -20^{o}C. After a while, 25 ul from each sample was mixed well with 25 ul 20 X SSC (final concentration of denaturing solution 10 X SSC) and heated for 5min. at 100^{o}C, followed by quick chilling on ice and 100 ul 10 X SSC with blue stain (bromophenol blue 0.1 %).

The pieces of Whatman paper (3 mm) and Zeta-probe nylon membrane were soaked in 10 X SSC without stain and placed in Dot-Blot apparatus, vacuum connected. Wells rinsed 1st with 10 X SSC, then samples were loaded, 100 ul each, and rinsing was repeated again. The membrane was taken out, then soaked in 1.5 M NaCl/0.5 M Na OH, denaturing solution, for 5 min.; 1.5 M Na Cl/0.5 M tris HCl/I nM EDTA (pH 7.0), neutralising solution, for 2 min. Rinsing with distilled water was repeated between the aformentioned steps. DNA was fixed by 5 min. exposure to UV light.

To prepare DIG-Labelled DNA probes, pBt 338 in *E.coli* JM 109, was digested with restriction enzymes Eco RI and Pst 1 and labelled with DIG by random priming as recommended by the supplier (Boehringer, Mannheim biochemica).

Pre-hybridization and hybridization :

Membrane was prehybridized in 0.02 % sodium dodecyl sulphate (SDS), 0.1% N. Lauroylsarcosine, 5 X SSC and 1 % blocking reagent for 1-2 hrs at 65^{o}C. Steril-

ized deionized water was used to adjust the forementioned concentrations of the same buffer containing DIG labelled DNA as recommended by the supplier for 16 hrs at 65°C. The hybridization solutions was saved and stored at - 20°C. for reuse.

Immunological detection :

The membranes were washed in buffer 1 (0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5) for 1 min and then incubated for 30 min with the blocking solution (0.1 % blocking reagent in buffer 1). Anti-digoxigenin alkaline phosphatase was diluted with blocking solution, buffer 2 (15 ml of diluted antibody conjugate solution/membrane) and incubated for 40 min, 1 hr and 2 hrs. The unbound antibody conjugate was removed washing twice for 15 min in washing buffer and the membrane was equilibrated for 2 min. with 15 ml of buffer 3 (0.1 M Tris-HC1, 0.1 M NaCl, 0.05 M MgC12, pH 9.5).

On a piece of clingfilm dropped about 1.5 ml chemiluminescent solution. Membrane face was gently placed on chemiluminescent solution drop and clingfilm was folded to cover membrane; then placed into cassette at the same time for 1-3 hrs to print reaction on Kodak-X OMAT (13 x 18 cm) film. Reaction test of Dot-Blot was remarked as follows : +++, spot on Sigma film was very dark; ++; spot on Sigma film was dark; +, spot on Sigma film was light dark; + -, spot on Sigma film had a faint color (weak positive) and - , for negative reaction).

RESULTS AND DISCUSSION

1- DAS - ELISA TEST :

Some samples, collected from outside and inside the greenhose, were tested with direct ELISA. Data in Table (1) with 30 samples of different material, plant and aphid were suspected to be infected with BBTV. However, some samples, collected from Maunawilli and Kailua Residential areas with unclear symptoms, showed negative reaction in DAS-ELISA test, but positive control from the greenhouse had very positive reaction. Moreover, some plant material collected from the greenhouse with unclear symptoms showed negative reaction, test 2, but positive check, clear sympoms, from the greenhouse gave a positive reaction. Besides, some aphid samples, collected from (healthy looking plants) the greenhouse or courtyard showed negative reaction, except one sample had a positive reaction. Nymph or adult aphids, which

Table 1. Reaction of different samples collected from outside and inside the greenhouse to DAS ELISA .

Serial No. of sample	Material	Location	A 405	Reaction
Test 1				
1	Midrib	Maunawilli	0.045	-
2	Leaf	Maunawilli	0.049	-
3	Midrib	Kailua Residential	0.045	-
4	Leaf	Kailua Residential	0.052	-
5	Midrib	The greenhouse-ck	0.043	-
6	Leaf	The greenhouse-ck	0.07	-
7	Midrib	The greenhouse + ck	0.414	+
8	Leaf	The greenhouse + ck	0.789	+
Test 2				
9	Leaf	The greenhouse	-0.005	-
10	Leaf	The greenhouse	-0.003	-
11	Leaf	The greenhouse	0.009	-
12	Leaf	The greenhouse	-0.004	-
13	Leaf	The greenhouse	0.007	-
14	Leaf	The greenhouse - ck	0.013	-
15	Leaf	The greenhouse - ck	0.006	-
16	Leaf	The greenhouse + ck	0.065	+
Test 3 (Adult or nymph)				
17	Aphid	The greenhouse or courtyard	0.002	--
18	Aphid	The greenhouse or courtyard	0.009	--
19	Aphid	The greenhouse or courtyard	0.005	--
20	Aphid	The greenhouse or courtyard	0.003	--
21	Aphid	The greenhouse or courtyard	0.005	--
22	Aphid	The greenhouse or courtyard	0.158	+
Aphids from non-infective colony				
23	Aphid	The greenhouse or courtyard	0.003	--
24	Aphid	The greenhouse or courtyard	0.006	--
25	Aphid	The greenhouse or courtyard	0.005	--
26	Aphid	The greenhouse or courtyard	0.010	--
Aphids from infective colony				
27	Aphid	The greenhouse	0.364	+
28	Aphid	The greenhouse	0.314	+
29	Aphid	The greenhouse	0.243	+
30	Aphid	The greenhouse	0.118	+

a) Values of absorbance readings recorded 60 min. after substrate addition.

were collected from banana plants infected with BBTV, gave positive reaction with DSA-ELISA. Thereby, positive correlation was noted between plant with clear symptoms or aphid from infective colony and ELISA assay.

DAS - ELISA has been tested on a large number of BBTV infected material and both are reliable and sensitive technique for verifying the presence of virus in samples (Drew *et al.* 1992; Wu and Su 1990 and Thomas and Dietzgen 1991). Moreover, sampling was done according to Sam Raj *et al.* 1970) who stated that the first sign of systemic infection in plants appears on the youngest leaves far away from the point of inoculation (using the banana aphid). In addition, 1,2,3 and 5% triphenyl tetrazolium chloride has been tested (Arvind *et al.* 1982) to distinguish BBTV in midrib and leaves from diseased plants, but results (data not shown) were not confirmed in most cases.

2- Comparison of Dot-blot and DAS-ELISA assays of some banana samples :

a- Without dilution of stock extracted tissue : Forty samples of banana were collected from some areas of Oahu (Scott Chun; Stanley Kawabita and Zimmers). The procedures of both assays were as mentioned previously and data were recorded (Table 2). Moreover, samples marked wo/s (without symptom), and w/s (with symptom). Data proved that, DAS-ELISA test or a DIG labelled DNA probe, dot-blot, was confirmed with external symptoms except one sample (No. 36) where both assays were positive. Also, a DIG labelled DNA probe was confirmed with DAS-ELISA test except in one sample (No. 39), whereas dot-blot reaction was weak positive. Detecting viruses, viroids and MLOs by nucleic acid spot hybridization is now a well established procedure. Therefore, developing methods of Dot-blot (non-radioactive method) for detecting pathogens in plant tissue, as routine diagnosis, with the objective of keeping a good balance between simplicity and sensitivity and avoiding risk of radioactive method, is a good objective (Borkhardt *et al* 1994). Nucleic acid of BBTV was studied by Su and Wu (1989) with isolates from Taiwan, Thomas and Dietzgen (1991) with isolates from Gabon, Taiwan and Australia and Xie *et al.* (1994) with isolates from Hawaii. The last authors reported that BBTV, Leteovirus, is a single stranded circular DNA. Detection of BBTV by nucleic acid spot hybridization, Dot-blot using P32 labelled probe has been used effectivelly in plant virus lab., UHM, as routine diagnosis, and the same procedure of preparation and clarification of banana sap to immobilize the target sequence DNA of BBTV on membrane has been carried out with nonradioactive material. No special protective

Table 2. Comparison of Dot-blot and ELISA assays of some banana samples collected from field .

Sample No.	Location	Symptom	OD 405 @	Dot-blot
1	Scot Chun	W/S	1.431*	+++
2	Scot Chun	WO/S	0.046	-
3	Scot Chun	WO/S	0.044	-
4	Scot Chun	WO/S	0.040	-
5	Scot Chun	WO/S	0.046	-
6	Scot Chun	WO/S	0.046	-
7	Scot Chun	WO/S	0.031	-
8	Scot Chun	WO/S	0.030	-
9	Stanley Kawabita	WO/S	0.519*	+
10	Stanley Kawabita	WO/S	0.046	-
11	Stanley Kawabita	WO/S	0.042	-
12	Stanley Kawabita	WO/S	0.003	-
13	Stanley Kawabita	WO/S	0.049	-
14	Stanley Kawabita	WO/S	0.047	-
15	Stanley Kawabita	WO/S	0.043	-
16	Stanley Kawabita	WO/S	0.047	-
17	Stanley Kawabita	WO/S	****	++
18	Stanley Kawabita	WO/S	0.022	-
19	Stanley Kawabita	WO/S	0.007	-
20	Stanley Kawabita	WO/S	0.032	-
21	Stanley Kawabita	WO/S	0.044	-
22	Stanley Kawabita	WO/S	0.046	-
23	Stanley Kawabita	WO/S	0.045	-
24	Stanley Kawabita	WO/S	0.010	-
25	Stanley Kawabita	WO/S	0.049	-
26	Stanley Kawabita	WO/S	0.048	-
27	Stanley Kawabita	WO/S	0.045	-
28	Stanley Kawabita	WO/S	0.048	-
29	Zimmers	WO/S	1.808*	++
30	Zimmers	WO/S	1.030*	+-
31	Zimmers	WO/S	0.014	-
32	Zimmers	WO/S	0.033	-
33	Zimmers	WO/S	0.050	-
34	Zimmers	WO/S	0.048	-
35	Zimmers	WO/S	0.45	+
36	Zimmers	WO/S	0.700*	-
37	Zimmers	WO/S	0.046	-
38	Zimmers	WO/S	0.048	-
39	Zimmers	WO/S	0.045	-+
40	Zimmers	WO/S	0.046	-
+ck	The greenhouse	WO/S	****	+++
-ck	The greenhouse	WO/S	0.049	-

* ELISA test positive for BBTV.
@ Values absorbance readings recorded 60 min after substrate addition.

equipment is required. Thus, the hybridization solution containing the non-radioactive probe can be saved and re-used several times without any risk.

b- With diluted stock extracts :

Some banana samples from the geenhouse proved to be infected with BBTV were selected and tested with Dot-Blot (non-radioactive material) and DAS-ELISA assays. Different dilutions were made from stock extracted tissue with extraction buffer (DAS-ELISA, 0.1 g/1 ml) i.e. 1 X; 1/5 X; 1/25 X, 1/125 X; 1/625 X; 1/3125 X, 1/15625 X and 1/781125 X.

Besides, the forementioned dilutions were made from stock denatured DNA with 10 X SSC (Dot Blot, 0.1 g./100 ul TE) followed by quick chilling on ice. Data of both tests till 1/625 X were recorded in Table (3) because all data beyond the abovementioned dilutions were clearly negative. Dilution of plant sap 1/25 X gave a clear positive reaction with DAS, ELISA assay. However, 2/5 of the samples gave a positive reaction with ELISA assay till 1/125 X. In case of Dot-Blot, dilution of denatured DNA till 1/5 a clear psoitive reaction was noted. Only 2/5 of the samples gave a positive reaction till 1/25 X of denatured DNA. However, in another test, data not shown, Dot-Blot with a DIG labelled DNA probe, reaction was positive till 1/125 of denatured DNA.

The present results proved that ELISA assay is more sensitive than Dot-blot, a DIG, labelled DNA probe, and the last test might have been be inore sensitive than recorded if the materials were handled better. However, lower concentration of BBTV, DNA could be detected with P^{32} labelled DNA probe than a DIG labelled DNA probe (personal communication) but our results showed that BBTD was confirmed with Dot-blot as well as DAS-ELISA till 1/5 X of diluted sap (number of virus particles or DNA concentration). Moreover, serological detection of BBTV was discussed by Thomas and Dietzgen (1991), who reported that using Taiwanese MAb (3 D12 at 0.5 ug/ml; MAb 3 D 12 conjuate 1 : 4000 and banana tissue at rate of 0.25 g/1 ml), a total of 21 Hawaiian BBTD specimens were positive when tested by State of Hawaii. Whereas, MAb 2 H6 used in PTA-ELISA (indirect method) and positive reaction was obtained from cv. Cavendish and cv. Lady finger showing BBTD symptoms when extracts concentrations were at least 10-fold, but no BBTV was detected in extract diluted four folds. Besides Wu and Su (1990) found that ATA-ELISA could detect the virus even when extracts were diluted 1/512. Therefore, sensitivity of methods for detection of the virus is very important to discover the in tissues as early as

Table 3. Comparison of Dot-blot and DAS, ELIS Assays of some diluted banana samples collected from the greenhouse.

Plant	1 X		1/5 X		1/25 X		1/5 X		1/5 X	
	ELISA	Dot-blot	ELISA	Dot-blot	ELISA	Dot-blot	ELISA	Dot-blot	ELISA	Dot-blot
Healthy leaf	0.015	-	0.003	-	0.002	-	0.008	-	0.002	-
Healthy, midrib	0.004	-	0.008	-	0.008	-	0.009	-	0.012	-
Dis. 3, leaf	0.446*	+	0.139*	+-	0.037*	-	0.004	-	0.007	-
Dis. 4, leaf	0.960*	+++	0.623*	++	0.212*	+-	0.039*	-(n)	0.004	-
Dis. 6, leaf	0.181*	++	0.061*	+-	0.017*	+-	0.001	-	0.003	-
Dis. 3, midrib	1.014*	+	0.278*	+-	0.051*	-(n)	0.009	-	0.006	-
Dis. 4, midrib	1.163*	++	0.688*	+-	0.229*	-(n)	0.079*	-(n)	0.011	-

* ELISA test positive for BBTV (Values absorbance readings recorded 60 min. after substrate addition).

(n) Another infected sample (BBTV) collected from the greenhouse showed reaction to dot-blot test till 1/125 x as follows : 1/25 X midrib, +-; 1/125 X leaf, +- and 1/125 X midrib, +-.

possible. However, dilution of infected sap with healthy sap is more real than dilution with other solutions.

Conclusion :

DAS-ELISA as well as Dot-blot (a DIG labelled DNA probe) could be used effecively for routine diagnosis of BBTV in infected tissues and both assays were able to detect the virus even when extract was diluted 1/5 X in all cases.

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استجابة بعض عينات الموز المصابة بمرض تورد القمة فى هاواى (اواهو) لاختبارى الاليزا المباشرة وتهجين الحمض النووى للفيروس باستخدام دعائم غير مشعة (a DIG Labelled DNA probe)

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أعطت بعض عينات الموز المصابة بأعراض مرض تورد القمة نتائج ايجابية تدلل على وجود الفيروس بها وذلك باستخدام طريقتى الاليزا المباشرة وتهجين الحمض النووى للفيروس مع مجسمات معلمة بمواد غير مشعة : (a DIG Labelled DNA probe) .

وبالرغم من أن طريقة الاليزا المباشرة أكثر حساسية من الطريقة الأخرى وذلك لاستجابة تخفيفات عصير النباتات المصابة المحتوية على الانتيجينات أو جزيئات الفيروس للتفاعل مع الأجسام المضادة للفيروس بدرجة أكبر من استجابة تخفيفات ال DNA للفيروس نفسه للاتحاد مع المجسمات المعلمة بمواد غير مشعة. ولكن كلا الاختبارين تساويا فى الحساسية عند تخفيف جزئيات الفيروس بمقدار الخمس فى النباتات المصابة وهذا يدل على أن طريقة التهجين باستخدام المجسمات المعلمة بمواد غير مشعه يمكن أن تستخدم فى الفحص الروتينى للشتلات أو الأجزاء النباتية المستخدمة فى التكاثر ، المشتبه فى اصابتها خصوصا اذا تعذر الحصول على الاجسام المضادة الخاصة بالفيروس.