Tomato Spotted Wilt Virus in Papaya and Detection of the Virus by ELISA

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ABSTRACT

Gonsalves, D., and Trujillo, E. E. 1986. Tomato spotted wilt virus in papaya and detection of the virus by ELISA. Plant Disease 70:501-506.

A new disease of papaya caused by the tomato spotted wilt virus was observed in Hawaii on the island of Kauai in 1962. Disease symptoms on plants 2–3 mo old were spotting, chlorosis, and necrosis of the top leaves; water-soaked lesions of the petioles and stems; and crooks at the stem apexes. These young plants were usually killed, although some survived, producing healthy axillary shoots. Fruit-bearing infected plants produced deformed fruit, which at ripening, had prominent green rings on a yellow background. The disease was invariably associated with orchards that had numerous *Emilia fosbergii* (formerly *E. sonchifolia*) weeds infected with tomato spotted wilt virus (TSWV). The host range and physical properties of the papaya virus were identical to those of TSWV. The disease was reproduced by mechanically inoculating papaya seedlings with leaf extracts from infected papaya plants and from TSWV-infected lettuce. The lettuce isolate of TSWV was purified and antiserum produced to it. The antiserum was effective in detecting TSWV in leaf tissue by sodium dodecyl sulfate agar gel immunodiffusion tests and by direct and indirect ELISA (enzyme-linked immunosorbent assay). Serology proved useful in diagnosing TSWV infections in plants.

Additional key words: papaya viruses, Trinidad mosaic, Waialua disease

Papaya (*Carica papaya*) is among Hawaii's leading fruit crops, only exceeded by macadamia and pineapple according to *Statistics of Hawaiian Agriculture 1983*. Although virus diseases of papaya were reported in Hawaii before the 1950s (14), they attained significant economic importance in 1959, when the papaya mosaic virus was first observed in Hawaii on the island of Oahu, where it has since eliminated large-scale commercial papaya production (14). This virus, now referred to as papaya ringspot virus (10), is also found in the Kona and Hilo districts of the island of Hawaii but does not occur on the islands of Maui, Molokai, and Kauai.

In 1962, a virus disease of papaya unlike papaya ringspot was observed on the island of Kauai. Diseased plants developed severe chlorosis and necrosis of the apical leaves and defoliated prematurely. Young infected plants usually had a pronounced bend at the stem apex. Since originally detected, the disease has occurred sporadically and occasionally causes significant damage in newly planted orchards. Surveys conducted in 1966 on three Kauai papaya farms showed infection of 50–90% in 3-mo-old orchards.

Two observations suggested that the disease was caused by tomato spotted wilt virus (TSWV). First, the symptoms of the disease were very similar to those caused by TSWV on tobacco and tomato, especially the tip necrosis and the pronounced bending of the apical shoot (9,12). Second, papaya orchards infected with the virus invariably had high populations of *Emilia fosbergii* Nicolson (= *E. sonchifolia* (L.) DC. = *E. javanica* (Burm.) Rob.) (8,20) with symptoms typically caused by TSWV infection (16,24).

A number of laboratories have purified TSWV and have determined its biochemical properties (9); however, there are few reports on the use of serology to identify infected plants (2,7,15,21,25,27). In a recent review, Francki and Hatta (9) stated that the conspicuous lack of serology for diagnosis of TSWV was

Journal Series Paper 2988, Hawaii Agricultural Experiment Station.

Accepted for publication 15 December 1985 (submitted for electronic processing).

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almost certainly due to the difficulty in obtaining sufficient amounts of virus for use as immunogen. Tas et al (25) also showed that virus preparations that were stringently purified still reacted to healthy host antigens.

The objectives of this study were to identify the causal agent of the papaya disease and to purify and produce an antiserum to a known source of TSWV. In this report, we show that the papaya disease is caused by TSWV. An antiserum to TSWV was produced and used to detect the virus in several hosts by enzyme-linked immunosorbent assay (ELISA) and immunodiffusion tests. A preliminary report on certain aspects of this work was presented previously (26).

MATERIALS AND METHODS

Virus isolates. Three isolates were used in this work. The papaya virus was originally obtained from leaves of an infected papaya growing on Kauai. TSWV-E was from TSWV-infected *E. fosbergii* plants found in the above papaya orchard, and TSWV-L was from TSWV-infected lettuce (*Lactuca sativa*) from the Kula district on the island of Maui. They were maintained under greenhouse conditions in papaya, *E. fosbergii*, and *Nicotiana benthamiana*, respectively. All isolates were transferred to test plants by grinding leaf extracts in 0.1 M potassium phosphate, pH 7.0, containing 0.01 M sodium sulfite (solvent 4) and rubbing extracts on leaves of plants previously dusted with 600-mesh Carborundum or corundum.

Virus distribution and movement. Tests for virus distribution were done with three papaya plants with 15 open leaves and no abscised leaves. Relative virus titer in each leaf was determined by macerating 2.5 g of tissue in 10 ml of solvent 4 buffer and inoculating the extract to the lowest eight half-leaves of *N. tabacum* cv. White Burley, which developed local lesions on inoculated leaves in addition to systemic infection.

Two-month-old plants in an orchard with 80% infection were used for testing the downward translocation of the virus in papaya. In one experiment, trees showing the same degree of widespread necrosis on the upper leaves were topped at four levels: 1 in. from the apical shoot, 1 in. above the lowest infected leaf or shoot, and 1 and 6 in. below the lowest symptomatic leaf or shoot. Five plants were used per treatment. In another test, 16 papaya plants showing severe and five plants showing mild necrosis on the foliage were cut off 6 in. above the soil level. Axillary shoots that grew out 1 mo after the stem was cut were observed for symptoms.

Virus purification and physical properties. The lettuce isolate (TSWV-L) was purified according to the method originally described by Black et al (4) and modified by Mohamed et al (19). Inoculated and uninoculated *N. benthamiana* leaves with prominent symptoms were ground with a Waring Blendor in solvent 4 (1 g/3 ml). After filtering through cheesecloth, the extract was centrifuged at 10,000 *g* (maximum) for 15 min and the resulting pellets were thoroughly dispersed with a Brinkmann homogenizer set at low speed in a volume (in milliliters) of 0.01 M Na_2SO_3 numerically equal to the original weight of tissue (in grams) and allowed to set at 4 C for 30 min. After clarification (8,000 *g* for 15 min), the supernatant was centrifuged at 100,000 *g* for 30 min. The pellets were resuspended in a volume (in milliliters) of 0.01 M Na_2SO_3 numerically equal to 1/10 the original tissue weight (in grams) and again dispersed and incubated at 4 C as described. After clarification (9,000 *g* for 10 min), the preparation was further purified by density-gradient centrifugation in 10–40% sucrose dissolved in 0.01 M Na_2SO_3. The opalescent zones containing virus were collected, concentrated by centrifugation (100,000 *g* for 25 min), and resuspended in 0.01 M Na_2SO_3. Dilution end point and thermal inactivation point of the virus isolated from papaya were tested using papaya tissue as inoculum and *N. glutinosa* as a local lesion host.

Serology. A white New Zealand rabbit was initially injected with purified TSWV-L mixed 1:1 (v/v) with Freund's complete adjuvant and subsequently with TSWV-L mixed 1:1 with Freund's incomplete adjuvant. Injections were administered twice the first week and periodically over a period of 5 mo. Antiserum was fractionated from blood samples collected at weekly intervals starting 3 wk after the first injection.

Direct and indirect ELISA were done as described by Clark and Adams (6) and Lommel et al (18), respectively. Antiserum was absorbed with healthy antigens from *N. benthamiana* as described recently by Gonsalves et al (11). SDS (sodium

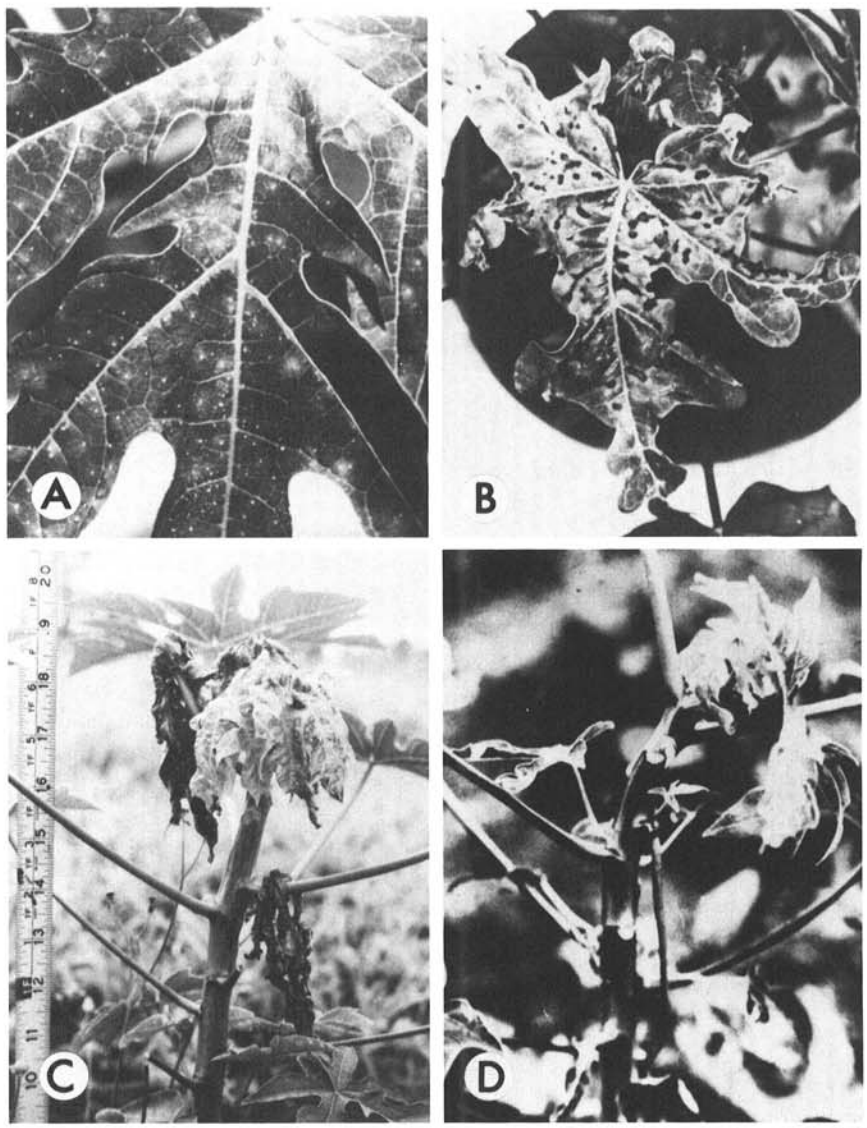

Fig. 1. Sequential symptoms in papaya plants 2–3 mo old infected with the papaya virus under field conditions. At initial stages, the lower leaves develop **(A)** small white spots that become **(B)** necrotic in the younger leaves. **(C)** Severely infected plants develop extensive necrosis in the young leaves that gives the top a whithered appearance. **(D)** Stems usually have a distinct bend at the apex.

dodecyl sulfate) immunodiffusion tests were done by the method of Purcifull and Batchelor (23). SDS-agar plates contained 0.8% Ionagar, 0.5% SDS, and 1.0% sodium azide. Test samples contained 0.5% SDS.

RESULTS

Symptomatology and viral nature of the papaya disease. Under field conditions, young papaya plants 2–3 mo old showed the most severe symptoms. Generally, initial symptoms appeared on lower leaves as white necrotic spots 1 mm in diameter (Fig. 1A). Subsequently, similar white spots appeared on younger leaves followed by necrosis spreading around the spots (Fig. 1B). In some instances, the leaves and stem at the apex became necrotic and appeared shriveled (Fig. 1C). Infected leaves below the apex turned yellow and abscised prematurely, except for the bottom three or four leaves, which usually had no symptoms. Water-soaked lesions developed on the stem and petioles during the initial stages of disease development. On young plants, the upper apical portion of the stem bent sharply to one side (Fig. 1D) and longitudinal cracks developed at the bend. Leaves on the inner arc of the bend showed severe necrosis, whereas those on the opposite side had mild necrosis or were symptomless. Young plants were usually killed by the virus. Older plants were stunted. Leaves below the apex abscised (Fig. 2A) and numerous axillary shoots developed at the base of the stem that were usually without disease symptoms. Fruits from infected trees were deformed (Fig. 2B) and ripe fruits had dark green rings connected, giving a mosaic pattern on a yellow background (Fig. 2C). Hard, brown necrotic spots and white lumps were scattered in the fruit pulp. These fruits were harder than healthy ones of the same age.

The disease symptoms were reproduced on papaya by mechanically inoculating young papaya seedlings with extracts of leaves from naturally infected field-grown papaya plants. Plants mechanically infected at a very early stage (3–4 in. tall) often died after extensive development of necrosis at the shoot tip. Those that survived, however, often recovered and subsequently produced symptomless side shoots. Plants developed a crook near the apex similar to that observed in the field.

Virus host range and physical properties. We observed that young papaya orchards with a high incidence of the disease were invariably infested with *E. fosbergii* showing symptoms identical to those caused by TSWV (Fig. 3A). This suggested that the disease on papaya was caused by TSWV that came from infected *E. fosbergii*. Host range, physical properties, and inoculations of papaya with known sources of TSWV demonstrated that the papaya disease was in fact caused by TSWV. The host range of the papaya virus was similar to that of TSWV-E and TSWV-L (Table 1). These isolates produced similar symptoms of local and systemic infection on tobacco, *Datura stramonium*, and tomato (*Lycopersicon esculentum*). However, inocula of the papaya virus from tobacco, but not from papaya, infected tomato. Similarly, inocula from infected *E. fosbergii* (TSWV-E) collected from infected papaya orchards failed to infect papaya in a limited trial (six plants inoculated), but inocula from TSWV-L did infect papaya on numerous occasions and incited symptoms similar to those produced by the papaya virus. All isolates produced similar symptoms on *E. fosbergii* (Table 1, Fig. 3B). Inoculations with six other TSWV isolates from lettuce, tomato, and chrysanthemum also induced the disease on papaya.

Infectivity of extracts from infected papaya was completely destroyed at 47 C but not at 40, 44, 45, and 46 C. Infectivity was detected in inocula diluted 1/10,000 but not at 1/100,000 and higher.

Virus distribution in field-infected papaya plants. Data on the distribution of the papaya virus in leaves of field-infected plants are shown in Table 2. Leaves were numbered 15 to 1 from the youngest to the oldest. Virus infectivity

Fig. 2. Symptoms on fruiting papaya trees infected with the papaya virus. Bearing tree **(A)** with leaves abcised below the stem apex and **(B)** with deformed fruit. **(C)** Ripe fruit with broken green rings in a yellow background.

Table 1. Comparative host range of the papaya virus and known isolates of tomato spotted wilt virus (TSWV)

Test plants	Virus isolates[a]		
	Papaya virus	TSWV-E	TSWV-L
Tobacco cultivars White Burley, H423	LL, S[b]	LL, S	LL, S
Nicotiana glutinosa	LL	–[c]	–
Papaya cultivar Solo	S	NI[d]	S
Datura stramonium	S	S	S
Tomato cultivars Pritchard, Foremost 21, line 80-2-1	S	–	S
Petunia hybrida	LL	LL	–
Emilia fosbergii	S	S	S
Chenopodium amaranticolor	LL	LL	–
C. quinoa	–	–	LL

[a]Inoculum sources for the isolates: papaya virus = tobacco, *E. fosbergii*, or field- or greenhouse-grown papaya; TSWV-E = *E. fosbergii*, and TSWV-L = *N. benthamiana*.
[b]LL = local lesion and S = systemic infection.
[c]– = Not tested.
[d]NI = no infection (six plants inoculated).

Table 2. Relative infectivity of tomato spotted wilt virus in leaves of infected papaya in orchards

Locations of leaves	Virus infectivity in leaves[a]		
	Plant 1	Plant 2	Plant 3
15 (top)	0	0	0
14	83	19	5
13	40	24	8
12	129	106	19
11	219	86	12
10	1,066	136	8
9	192	10	48
8	200	0	0
7	128	59	8
6	14	12	12
5	0	0	11
4	33	0	5
3	0	0	3
2	0	0	0
1 (bottom)	0	0	0

[a]Total number of lesions produced on eight inoculated half-leaves of *Nicotiana tabacum* 'White Burley.'

Table 3. Symptomatology of axillary shoots that developed after papaya trees infected with tomato spotted wilt virus were topped at different levels

Topping level	No. recovered/ no. tested[a]
Experiment 1	
1 in. Below stem apex	0/5
In relation to lowest symptomatic leaf or shoot:	
1 in. Above[b]	2/5
1 in. Below	1/5
6 in. Below	1/2
Experiment 2	
6 in. Above soil level	6/21

[a]Number of plants that developed healthy-appearing axillary shoots per number of plants tested.
[b]In this treatment, plants were topped in relation to symptomatic leaf.

was highest in leaves 14 to 7, and no infectivity was detected in the uppermost leaf. The highest infectivity was in the sixth leaf from the top (leaf 10 in Table 2).

Attempts to eliminate the virus by topping field-infected trees. The development of apparently healthy axillary shoots in the lower part of diseased plants suggested that the virus was primarily confined to the upper portions of the tree. Furthermore, previous data showed that the lowest three leaves had no virus detectable by bioassay (Table 2). Thus, it appeared that the disease might be controlled by cutting off the stem of infected plants and encouraging the development of one or more healthy axillary shoots. In one experiment, infected trees were topped at various levels in relation to the lowest symptomatic axillary shoot or leaf (Table 3). Shoots from four of 17 test plants did not show

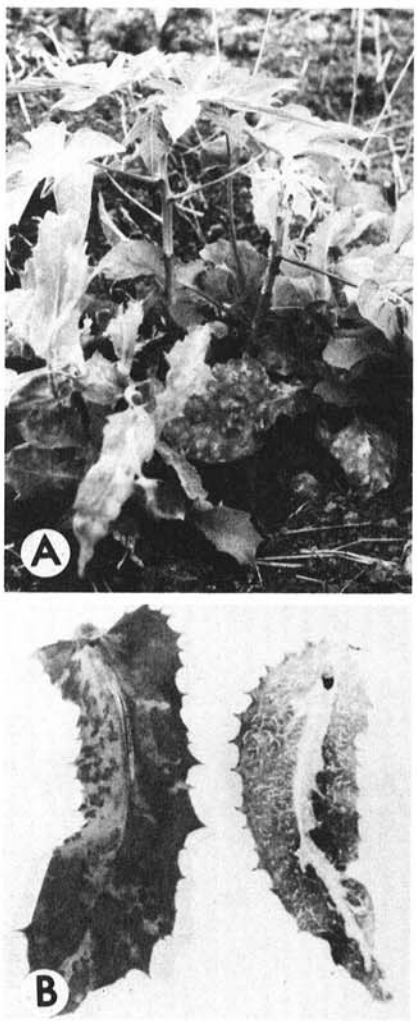

Fig. 3. Symptoms of tomato spotted wilt on *Emilia fosbergii*. **(A)** Symptomatic *E. fosbergii* in a young papaya orchard that is infected with the papaya virus. **(B)** Symptoms on *E. fosbergii* inoculated with extracts of (left) tobacco and (right) papaya infected with the papaya virus. Note similarity of symptoms in A and B. Experimental data showed that the papaya virus disease is caused by tomato spotted wilt virus.

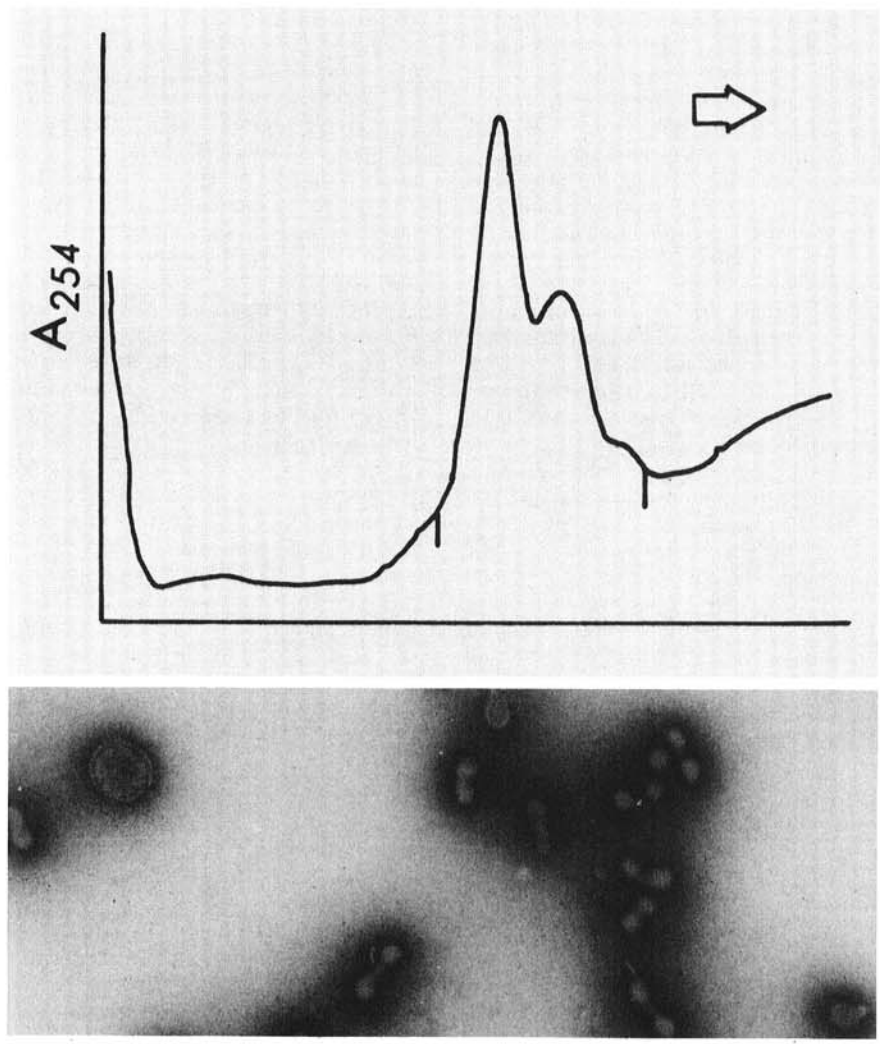

Fig. 4. A typical UV profile of a tomato spotted wilt virus (lettuce isolate) preparation centrifuged in **(top)** sucrose density gradients and **(bottom)** electron micrographs of particles isolated from the viral zones. Virus isolated from between each of the two hash marks were examined under the electron microscope and used to immunize rabbits for antiserum production. The two prominent sedimenting zones were consistently observed in virus preparations; the third one, which formed a small shoulder on the right side of the middle zone, was usually not present. Preparations were centrifuged through 10–40% sucrose gradients at 27,000 rpm for 25 min in an SW 28 rotor. Arrow indicates direction of sedimentation. Virus particles were fixed with 3% glutaraldehyde and stained with 2% aqueous phosphotungstic acid before examination with the electron microscope (×50,000).

symptoms. In another experiment where plants were topped 6 in. above ground level (and below symptomatic shoots or leaves), six of 21 plants produced healthy shoots (Table 3). Topped plants with healthy shoots grew normally and produced edible fruits within a year, but those with infected shoots remained stunted and unproductive.

Virus purification and serology. TSWV-L was easily purified from leaves of *N. benthamiana* that were at the proper stage of infection. Inoculated plants that showed pronounced symptoms (chlorotic spots and mottle) tended to collapse very quickly, sometimes even overnight. It was important to harvest the tissue just before the leaves collapsed, which was 5–8 days after inoculation. After centrifugation in sucrose gradients, virus preparations normally showed two opalescent bands that were absent in healthy preparations. Material collected from below these UV-absorbing peaks had numerous dumbell-shaped particles and a few oval to round ones (Fig. 4). Such particles have been reported from TSWV preparations by others (3,9). From measurements taken beneath the absorbance curves of the virus zones (Fig. 4), about 10 optical density units (254 nm) of virus were typically purified from 100 g of leaf tissue.

Antiserum was produced to TSWV-L collected from the two sedimenting zones in the sucrose gradients. Antiserum reacted specfically to homologous virus antigens from purified preparations and from infected tissue. Titers (= reciprocal of dilution) as high as 32 were obtained in SDS-immunodiffusion tests.

TSWV-L was easily detected in infected tissue by both direct and indirect ELISA. Strong reactions were obtained even when ELISA plates were coated with γ-globulin at 1 μg/ml and the conjugate used at 1/8,000 dilution (Fig. 5). With indirect tests, the antirabbit γ-globulin conjugate could be diluted at least 1/2,000 when the γ-globulin was used at 1 μg/ml. A high healthy background reaction appeared with indirect ELISA unless the γ-globulin was absorbed with healthy extracts of *N. benthamiana*. This background was much less obvious with direct ELISA. Because absorption with healthy *N. benthamiana* antigens was very simple and fast, it was routinely used in both types of ELISA. By ELISA, TSWV was detected in inoculated papaya, *Gomphrena globosa, Vinca rosea*, tobacco, tomato, and lettuce.

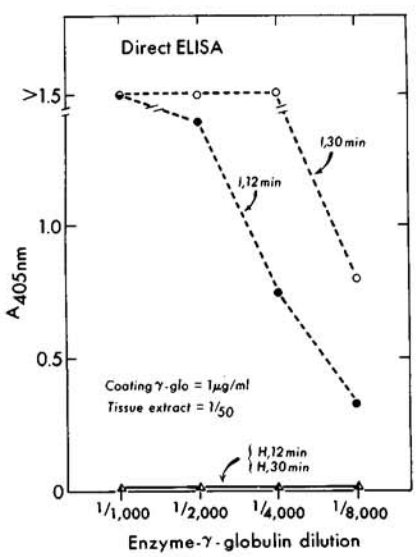

Fig. 5. Detection of tomato spotted wilt virus (TSWV) in leaf extracts by direct ELISA (enzyme-linked immunosorbent assay) at various dilutions of the enzyme-conjugated γ-globulin. Alkaline phosphatase was the enzyme source and *Nicotiana benthamiana* the virus source. The minutes refer to the times when the reactions were read after pouring the substrate in the wells.

DISCUSSION

Data on host range, symptomatology, serology, and the infection of papaya with known isolates of TSWV (3,9) lead us to conclude that the disease affecting papaya on the island of Kauai is caused by TSWV.

The failure of leaf extracts from infected papaya to infect tomato is probably due to the low virus titer in the leaves at the time of inoculation. Also, low virus titer or host conditioning of the virus are possible reasons for the failure of leaf extracts of TSWV-E-infected *E. fosbergii* to infect papaya. We have had similar problems in infecting lettuce with TSWV when *E. fosbergii* is the inoculum source (*unpublished*). Sakimura (24) stated that TSWV titer in *E. fosbergii* is generally low and fluctuates with time. On the other hand, *E. fosbergii* is easily infected by TSWV from almost any source. Nevertheless, our data clearly show that the papaya virus disease is caused by TSWV.

It is surprising that TSWV infection in papaya has not been reported in other areas because this virus is widespread throughout the world. However, two papaya virus diseases, Trinidad Mosaic (1) and Waialua disease (22), have symptoms similar to those induced by TSWV. Trinidad mosaic shows a dying back of the stem tip while the lower leaves remain healthy. Also, infected trees produce healthy shoots when they are cut off at the stem base. Symptoms of the Waialua disease are stunting and premature defoliation. Similarly, some trees when cut off at the base recover from Waialua disease (22). Photographs of the Waialua disease published by Parris (22) resemble TSWV on papaya. On the basis of symptomatology, it would appear that the Waialua disease is caused by TSWV. However, we were unable to experimentally compare the Waialua disease with TSWV of papaya because the former has not been observed on Oahu for many years.

The sporadic occurrence of TSWV on papaya is similar to that of TSWV on pineapple in Hawaii (13,16). In fact, Linford (16) showed that *E. sagittata* was one of the most important virus sources for TSWV infections of pineapple. Likewise, our observations indicated that *E. fosbergii* is the major virus reservoir for TSWV on papaya. On the island of Maui, tomato spotted wilt is now the most destructive disease of lettuce and tomato.

The uneven distribution of TSWV in papaya and the development of symptomless axillary shoots near the base of infected papya plants suggested to us that topping trees below symptomatic leaves or shoots would free the plants of TSWV. Our tests showed that a number of axillary shoots from topped plants were symptomless and produced healthy fruit. However, given the sporadic occurrence of TSWV and the relatively short period before a papaya tree bears fruit, this practice does not seem economically practical.

We have not seen any reports on the use of ELISA for detecting TSWV. Thus, one of our primary objectives was to produce an antiserum to TSWV that could be used with ELISA. We have shown that our TSWV-L antiserum can be used in both direct and indirect ELISA for detecting several isolates of TSWV in plant tissue. Both types of ELISA worked well although the indirect test showed a higher healthy reaction. However, this was easily removed by absorbing the γ-globulin with healthy antigens of *N. benthamiana*, the host from which TSWV-L was purified. The absorption step in the ELISA, which was originally reported by Lister (17), is a simple way to eliminate background reaction without having to absorb large amounts of crude serum. Thus serology (ELISA in particular) should be a very easy and economical way to index for this virus. ELISA is now being used to determine which weeds are important reservoir hosts for TSWV in Hawaii (5).

ACKNOWLEDGMENTS

We wish to thank D. K. Hummer and E. D. Williams for technical assistance. This study was supported in part with funds from the Governor's Agriculture Coordinating Committee, State of Hawaii.

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