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Characterization of a new virus isolated from pineapple

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CHARACTERIZATION OF A NEW VIRUS

ISOLATED FROM PINEAPPLE

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By

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ABSTRACT

Mealybug-wilt of pineapple is one of the serious diseases in pineapple. Severe mealybug infestation may result in large yield losses in commercial plantations. The cause of this disease has been linked to a toxin secreted by mealybugs during their feeding upon pineapple. The biological evidence supporting a viral etiology began to emerge in recent years. In this study I investigated the possible involvement of a virus with this disease by studying the association of dsRNA with infected plants, developing purification protocols for the closterovirus-like particles associated with mealybug-wilt infected pineapple, conducting detailed electron microscopic studies of the virus leading to the determination of its physical properties such as length, width, and buoyant density. A polyclonal antiserum was produced to the virus isolated from pineapple and agar diffusion tests and serologically specific electron microscopic studies were conducted on this virus. The viral coat protein was analyzed by SDS-polyacrylamide gel electrophoresis. That the antiserum produced was specific to the viral coat protein was shown by western blotting studies.

Complementary DNA (cDNA) probes were developed and were used for the detection of virus in pineapple plants, mealybugs, and other plant species found commonly near

commercial pineapple plantations. cDNA probes also were used in Northern blot analysis to detect subgenomic RNAs of viral origin in plant extracts obtained from diseased pineapple plants. cDNA probes developed were sensitive and virus was detected in wilt-affected pineapple plants as well as symptomless pineapple plants in the commercial plantations. The presence of virus also was detected in mealybugs taken from diseased pineapple plants and in a common grass species around pineapple plantations.

The results of this study confirm the presence of a virus associated with "mealybug-wilted" pineapple plants. Based on the virion morphology and its physical properties this virus may tentatively be assigned to the closterovirus group. The role of the virus in the etiology of wilt disease has yet to be determined.

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INTRODUCTION

Pineapple (Ananas comosus L.) is one of the world's most popular fruits. Probably originally cultivated in Brazil, wild pineapple was developed by the indians of tropical America who named a number of varieties of pineapple which they selected because of the fruit size, and absence of seeds. The pineapple was unknown to the old world before 1492 until Columbus discovered the new world (25). Since then, pineapple has gained importance in the world's agriculture where it is grown worldwide as a cash crop. At present, major pineapple growing areas include the Hawaiian islands, South Africa, South and Central America, South East Asia, and Australia. Major industries based upon pineapple cultivation are the fresh fruit and canning industries.

In the Hawaiian Islands the most popular cultivar grown is Smooth Cayenne with the total area under cultivation about 36,000 acres. Even though this is only 1.8% of the total agricultural lands, the market value of the pineapple produced was $242 million in 1986 thus making pineapple cultivation the major source agricultural income in the state (1).

Several pest and diseases have been reported on pineapple (25). Those caused by fungi and nematodes are important. The fungal root rots caused by Pythium spp.,

wilt caused by Fusarium spp., heart rot caused by Phytophthora spp. and leaf diseases caused by Thielaviopsis spp. are important. Among the nematode diseases, root-knot nematodes (Meloidogyne spp.) and root-lesion nematodes (Tylenchus spp.) are important and cause severe damage to pineapple if not controlled.

HISTORY OF MEALYBUG-WILT RESEARCH

Mealybug-wilt is the most widely distributed pineapple disease and probably one of the most severely damaging, particularly to plants of the variety Cayenne. In 1910 Larsen first reported pineapple wilt disease in Hawaii (39). Since then it has been reported from all over the world (15). It is especially severe in Jamaica, Central America (10), Puerto Rico (43), Mauritius, Florida, and the islands of Loocho, Palau, Saipan and Bonin (15). It has also been reported from South Africa, East Africa, Malaya, Java, Bali, Borneo, the Philippine Islands, Australia, Fiji (15) and South America (18). Singh et al, have reported this disease in India (45). At the time when this disease was first reported the cause was unknown. The symptoms described were loss of rigidity of leaves followed by loss of crispiness and wilting of plants (18). During the last 70 years, the causal organism of the disease has not been isolated, and still remains controversial.

Higgins in 1912 reported the sporadic spread of wilt disease in pineapple fields in Hawaii (33). Illingworth

(1931) first established the relationship between mealybugs and the wilt disease of pineapple (35). In his studies he was able to transmit wilt disease to healthy plants via mealybugs. Carter (1932), proposed that the cause was a toxin secreted by mealybugs while feeding upon pineapple plants. In his early work Carter reported that green spotting of pineapple is different from wilt (5). In that study he observed two forms of mealybugs, that cause chlorotic and green spots on pineapple. The mealybugs were varied in their capacity to produce green or chlorotic spots according to the host plant from which they were taken. Those from the roots of red top Natal and panicum grass had the least green or chlorotic spotting ability. Carter reported that the infestation of new fields with mealybugs was partly due to the movement of mealybugs with planting materials (6). In the same study he reported that in newly planted fields infestation occurs first on the outer edge and moves in gradually. The rate of movement was influenced by the size of the initial population. In new fields bounded by wild vegetation or old pineapple plantings, heavy populations of mealybugs were established in a few months. Fields that were not bounded by wild vegetation or in old pineapple plantings had a lower incidence of disease. This suggested that the mealybugs were moving from wild vegetation and old plantings. In 1933 Carter proposed that an insect secretion was the cause of pineapple wilt (8). In

this study he reported the occurrence of sudden wilt following infestation by high populations of mealybugs. Carter correlated the loss of green spotting ability of mealybugs to disappearance of a rod-like symbiont from the mycetone of the insects when the insects fed on panicum grass (11). The relationship between the symbiont of mealybugs and the phytotoxic secretion of the insect was unsubstantiated.

In Carter's experiments on the dosage effect of mealybugs and wilt disease it was shown that in some cases the increased number of mealybugs increased the incidence of wilt (9, 13). However,the increase of wilt was not always directly proportional to the number of mealybugs. In some cases plants wilted following infestation by a single mealybug.

In extensive studies on the etiology of mealybug wilt, Carter divided symptom expression into 4 classes (16, 20). Stage 1 is the preliminary reddening of leaves. In stage 2, a definite color change from red to pink occurs as well as the curling of the leaf margins. In stage 3, the affected leaves lose turgor. In stage 4, the affected leaves dry up along most of their length. At later stages wilt symptoms are associated with root death. If plants recover after the 4th stage, the oldest affected leaves senesce. The youngest affected leaves, having then grown out, wilt at the tip only. New central leaves are apparently normal. Carter

indicated that this recovery may be due to effects of nitrogen fertilization, but the age of the plant at the time of infestation was also a factor. It was also noted that frequent mealybug infestations adversely affected the recovery by inducing severe wilt symptoms. However, these recovered plants were susceptible to later mealybug infestations and wilted for a second time, showing typical wilt symptoms. The influence of plant nutrition on the susceptibility of pineapple plants to mealybug wilt has been investigated (17). Reduction of susceptibility to mealybug wilt was observed with high nitrogen applications.

The semipersistent relationship between the transmissible factor and mealybugs was shown by Carter (21). Intermediate feeding of mealybugs on solid agar medium just before transferring from wilted plants to healthy plants resulted in elimination of wilt-inducing capacity. Mealybugs transferred from wild hosts did not produce any wilt symptoms.

The resistance of some pineapple clones to wilt disease has been reported (19, 24). The value of these resistant clones as sources of the mealybug wilt has been evaluated. They were found to be very good sources for mealybugs to acquire the wilt-inducing factor (23). Some of the clones tested by Carter showed the lowered susceptibility in older plants, indicating that the age of the plant may be an important factor in mealybug wilt.

After the establishment of mealybugs as an important factor in wilt of pineapple, one species of mealybugs (Pseudococcus brevipes (CKL.) was implicated as the major wilt-causing species (36). During this time pink and grey mealybugs observed on pineapple were considered to be forms of P. brevipes (5). Latter, it was established that these two forms are different species, namely, Dysmicoccus neobrevipes (grey mealybugs) and Dysmicoccus brevipes (pink mealybugs) (2). These two species are the most prevalent species on pineapple in the Hawaiian islands. Ants are responsible for establishing active mealybug colonies on pineapple by moving the mealybugs to new plantings (3, 35). In return the ants use honey dew secreted by mealybugs as a food source. The most prevalent ant species in Hawaii is the big headed ant Pheidole megacephala (Fab.) (3). The wind can also affect the movement of mealybugs within pineapple fields. (personal communication with Neil J. Reimer).

CONTROL STRATEGIES

After mealybug wilt was reported in Hawaii, various control methods were suggested. Most methods concentrated on control of the spread of mealybugs. Planting several rows of pineapple plants as buffer zones parallel to the edge of field and separated from the main plantation by a cleared zone has been used successfully to control mealybug spread (7). These borders served as guard rows against

rapid movement of mealybugs into newly planted fields. Localized infestations within these borders were easily controlled by spraying with insecticides.

The control of ants has been tried by using insecticides such as Heptachlor and Mirex (3, 41, 46). These insecticides are no longer in use because of high toxicity to humans and beneficial insects. The current practice is application of Amdro outside the field to control ants.

The feasibility of biological control of mealybugs has been investigated using two species of chalcid parasites, Hambletonia pseudococci Compere, and Anagyrus coccidiovorus Dozier (12, 14). Under laboratory conditions these parasites effectively controlled mealybug populations. But under field conditions no control was achieved because of low survival rates of predators in the field. Biological control of big-headed ants has been tested and no economically feasible practices have been adopted (34).

EVIDENCE OF VIRAL ETIOLOGY EMERGING FROM EARLY STUDIES

It is interesting to note that most of the investigations done with this devastating disease suggest some evidence of the involvement of a latent factor, possibly a virus, transmitted by mealybugs (19, 21, 22, 23, 35, 37).

The early studies of Illingworth proved that the disease was transmitted to healthy plants by mealybugs

transferred from diseased plants but not from healthy plants (35). If a toxin was responsible for the disease, no matter what the original source of mealybugs, all mealybugs should have been able to produce wilt disease. Based on the data obtained from a series of field studies, Illingworth reported that wilt creeps slowly into the sides of the fields. He reported that wilt usually comes in especially along the edges that border old wilt infected patches, from dumps of old wilted plants and other refuse from plant failures or along margins of weedy gulches and stone piles. Based on experimental data over several years, Illingworth stated that "we accept the hypothesis that mealybugs transmit a disease, the known facts fit together perfectly" (35). According to his explanation, a virus was moving from infested fields to the new plantings via mealybugs.

The sequential feeding studies of Carter proved that mealybugs taken from diseased plants lost their wilt-causing ability after feeding upon artificial media (21). With other insect vectors it has been shown that the non-persistent and semi-persistently transmitted viruses are lost during feeding on virus-free hosts (31, 32).

Ito has provided evidence that a virus may be involved with wilt disease of pineapple (37). He found that wilt-recovered plants, and all vegetative progenies of them, were chronically diseased and that these plants were positive sources of mealybug wilt. These positive sources were

resistant to further wilting when they were subjected to mealybugs from the same progeny sources and these healthy plants were highly susceptible to the disease when mealybugs were fed on them if taken from any other disease source. From these results, Ito concluded that the reaction of pineapple clones which showed resistance to wilting after feeding by mealybugs from the same progeny plants is parallel to the phenomena of acquired immunity. He thought that the same strain of a virus is perpetuated in vegetatively propagated clones and therefore these plants did not produce wilt symptoms when they were subjected to feeding by mealybugs from the same progeny plants. But mealybugs taken from these plants were able to produce wilt symptoms when feeding upon different pineapple plants derived from different progeny. Ito's transmission studies, together with other information, led him to conclude that mealybugs are not toxicogenic and that mealybug wilt of pineapple is a virus disease which is perpetuated in the vegetative progenies of wilt-recovered plants. The virus is transmitted from these chronically diseased plants to healthy plants by the insect vector, Pseudococcus brevipes (CKL.) The results of Carter's experiments on wilt resistant clones support the viral etiology suggested by Ito (19, 23, 24). Singh et al. also concluded, on the basis of transmission experiments, that pineapple wilt disease is a virus disease transmitted by mealybugs (45).

Carter was not ready to give up the toxin theory until 1951. In his paper on the reappraisal of pineapple wilt, Carter states that his toxin theory had to be modified because he has recognized that some field grown pineapple plants were positive sources and some are not; seedling pineapple plants that had never been infested by mealybugs were all negative sources as were the wild host plants such as sisal, yucca, and panicum grass (22). The most consistently positive sources were found to be resistant hybrids and clones of the standard smooth Cayenne variety. Based on these data Carter proposed a new hypothesis stating that some transmissible latent factor was involved, separate and distinct from the actual wilt-inducing secretion of the mealybugs. The experimental evidence described above strongly supports the viral etiology of mealybug wilt of pineapple.

Even though it has been a long time since the emergence of a latent factor theory, no reports on the isolation of a virus have been published. Therefore isolation of a virus associated with this disease and the determination of a possible role of this virus in causing wilt symptoms will support the biological evidence observed so far from other experiments and it will be the most logical first step in understanding and controlling this important disease.

DEVELOPMENT OF HYPOTHESIS

The hypothesis, formulated based on biological data obtained from previous studies, is that a virus may be the causal agent of mealybug-wilt of pineapple. If a virus is present, it should be possible to isolate it from diseased plants. The determination of physical and biological properties of this virus and development of detection techniques will make it possible to investigate the role of the virus in inducing wilt disease of pineapple. This study involves the searching for the preliminary evidence of presence of a virus, development of purification protocols and development of detection techniques. The various aspects of this work will be dealt with individually in the following section.

PRELIMINARY STUDIES

Most plant viruses are single stranded RNA. These viruses replicate by forming an intermediate replicative form (RF) which is double stranded RNA (26, 49). In this study CF 11 chromatography was used to isolate these replicative forms (24, 25, 26). Purified dsRNA was analyzed by polyacrylamide gel electrophoresis (PAGE) (24). For the virus purification, salt precipitation, PEG precipitation, differential centrifugation techniques were used (30). For the determination of virus density $Cs2_{SO4}$ centrifugation was used (40). The optical characters were determined using spectrophotometry.

The electron microscopic studies were conducted to study the virus morphology and ultra-structure. The viral coat protein was analyzed by SDS-PAGE (38).

VIRUS DETECTION

A polyclonal antiserum was produced to the virus isolated from pineapple by injecting purified virus to New Zealand rabbits. This antiserum was used in agar diffusion tests and serologically specific electron microscopy (26, 44). Western blotting studies were conducted to investigate the specificity of the antiserum to viral coat protein (4).

The cDNA probes were used in virus detection. The random priming method was used in the synthesis of cDNA probes from dsRNA isolated from diseased plants (47). A Dot-blot assay was used in detecting virus in pineapple plants, mealybugs, and other alternative hosts (42). Northern blot analysis was used in analysis of total RNA isolated from diseased pineapple (48). Nitrocellulose papers were used as a solid matrix to transfer nucleic acid in Northern blot analysis and to blot nucleic acid samples in dot-blot assay (48).

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CHAPTER II

ISOLATION OF DOUBLE STRANDED RNA (dsRNA), PURIFICATION, SEROLOGY AND PARTIAL CHARACTERIZATION OF A VIRUS FROM MEALYBUG-WILT AFFECTED PINEAPPLE

ABSTRACT

Double stranded RNA (dsRNA) was consistently isolated from wilt affected pineapple plants by CF 11 chromatography. This dsRNA was disease specific and was never isolated from pineapple plants which were not exposed to mealybugs. Purification protocols were developed for a clostero-like virus associated with diseased pineapple plants. The virus particles were determined to have a width of 12 nm and length of about 1200 nm. The coat protein of the virus was analyzed by SDS-PAGE. The molecular weight of the coat protein was 23,800 daltons. The A_{260}/A_{280} ratio of the purified virus was 1.8. The density of the virus particles determined by Cs_2SO_4 density gradient centrifugation was 1.5 g/cc. A polyclonal antiserum was produced to the virus purified from pineapple by injecting partially purified virus into New Zealand rabbits. This polyclonal antiserum was used for virus detection in agar diffusion tests. Purified IgG was used in serologically specific electron microscopic studies. The virus was specifically decorated with this IgG. Western blotting studies confirmed that the

protein specific to virus infected plants was viral coat protein and that purified IgG was specific to the viral coat protein.

INTRODUCTION

The investigation of the association of a virus with mealybug-wilt of pineapple involves searching for preliminary evidence indicating the presence of a virus and development of purification procedures. Once a virus has been isolated, the role of this virus in the disease can be determined. Biological and physical properties of this virus can be investigated and the virus may be accommodated into a described virus group or can be described as a new type. Many plant viruses are recognized by the disease they cause in plants and are named for the disease (31). Viruses also can be grouped based upon their morphology and genome that they carry either DNA or RNA (29, 30, 31). Most plant viruses are of single stranded RNA. These viruses replicate by forming an intermediate replicative form (RF) which is double stranded (56). RNA-dependent RNA polymerase uses viral RNA as a template and catalyzes the synthesis of both positive and negative strands (56, 43). Base-paring of positive and negative strands results in formation of replicative forms in virus-infected tissues. Since no process occurs in normal or uninfected cells which result in the production of dsRNA, its presence is strong evidence for RNA virus infection. In recent years techniques for

isolating these dsRNAs have been developed and used as a diagnostic indicator for RNA viruses (16, 17, 37). A considerable catalog of dsRNA profiles has been assembled for several groups of plant viruses and dsRNA has been associated with some diseases of uncertain etiology (34, 42, 11). Extraction protocols for isolating dsRNA from diseased and healthy plant tissue have been developed and the association of dsRNA with mealybug-wilt infected pineapple has been shown.

Isolation of virus from diseased plants enables the study of the detailed structure of virus as well as of its biological and physical properties. In this study several purification schemes were tried, and one was selected for a virus associated with pineapple in Hawaii. Using partially purified virus, transmission electron microscopic studies were performed and some optical properties such as A_{260}/A_{280} and UV scanning profile of purified virus were determined by spectrophotometry. A polyclonal antiserum was produced to the isolated virus and used in several serological tests such as Western blotting, serologically specific electron microscopy and agar diffusion test. Using this polyclonal antiserum an ELISA technique has been developed in collaboration with Dr. Diane Ullman.

MATERIALS AND METHODS

Isolation of dsRNA

The method used for dsRNA isolation from pineapple

utilized the method of Dale et al, (11) for isolation of dsRNA from banana with bunchy top disease, which they developed from the method of German, et al. (18) and Morris, et al. (36). Leaf tissues used for isolation of dsRNA were taken from pineapple plants showing typical wilt symptoms. Most of the old leaves of these plant were wilted and younger leaves in the middle whorls were only wilted at the tip. Care was taken to select only green parts of younger leaves. Leaves taken from these plants were cut into small pieces and 100 g was ground to powder in liquid nitrogen using a mortar and pestle. The powdered tissue was shaken for 1 hr at 4 ^{0}C in 200 ml of 50 mM Tris-HCl pH 7.0, 100 mM NaCl and 1 mM EDTA (STE), 100 ml water-saturated phenol containing 0.1% 8-hydroxyquinoline, 100 ml chloroform, 25 ml 10% sodium dodycel sulfate (SDS) and 2.5 ml 2-mercaptoethanol. The mixture was centrifuged at 7000g for 15 min in GSA rotor. The aqueous phase was collected and ethanol was added slowly while stirring to give a final concentration of 15%. Whatman CF-11 cellulose (Whatman International Ltd., Maidstone, Kent, UK) was then added (0.15 g/g of original tissue) and the suspension was stirred under vacuum for 15 min at room temperature. The suspension was then poured into a 50 cc disposable syringe the bottom of which was blocked with a piece of miracloth disk (Chicopee Mills, Inc.. 1450 Broadway, New York, NY 10018). The column was washed with STE containing 16.5% ethanol

until the A_{254} of the elute was equal to that of STE containing 16.5% ethanol. Double stranded RNA was then eluted by washing with 1 X STE and precipitated by adding 2.5 volumes of ethanol and 1/20th volume of 3 M sodium acetate (pH 5.5) and the mixture was kept at -20 ^{0}c overnight. The precipitated dsRNA was collected by centrifugation at 10,000g for 30 min in a GSA rotor and dried under vacuum. The dried dsRNA pellet was suspended in 1/5th X STE and magnesium chloride was added to a final concentration of 10 mM and incubated at 37 ^{0}c with deoxyribonuclease (DNase, RNase free, Sigma) (10 μg/ ml) for 1 hr. Equal volumes of phenol and chloroform were added to the mixture which was vortexed and placed in ice for 20 min. The mixture was then centrifuged in a microfuge for 4 min, the aqueous phase was collected and nucleic acids were precipitated with 2 1/2 volumes of ethanol and 1/20th volume of 3 M sodium acetate and stored at -20 ^{0}c overnight. dsRNA was then analyzed by electrophoresis using 6% polyacrylamide: 0.15% Bis acrylamide in a slab gel (7 cm X 10 cm X 0.75 mm) system run at 30 mA for 10 hr at room temperature in electrophoresis buffer consisting of 40 mM tris, 20 mM sodium acetate, and 1 mM disodium ethylenediamine tetraacetate (EDTA), pH 7. Gels were stained with 25 ng/ml ethidium bromide in electrophoresis buffer and photographed with UV light (310 nm) using a LP-3 Polaroid land camera and type 665 Polaroid film. The

molecular weight of dsRNA isolated from pineapple was estimated by comparing its electrophoretic mobility to dsRNA isolated from citrus tristeza virus (CTV) infected Citrus paradisi Macf. cv. Marsh, tobacco mosaic virus (TMV) infected Nicotiana tabacum L. cv. Xanthi and brome mosaic virus (BMV) infected Hordeum vulgare L. Healthy pineapple leaves were obtained from the Maui Pineapple and Land Company which had been maintained mealybug-free from seeds. dsRNA from these plants was isolated by using the same procedure as for diseased plants and analyzed by gel electrophoresis.

That the nucleic acids isolated from wilt infected pineapple plants were dsRNA was proven by incubating dsRNA gels with ribonuclease A (1 μg/ ml) in high salt and low salt conditions. High salt conditions were achieved by incubating dsRNA gels in 2 x SSC (1 X SSC = 150 mM Nacl, 15 mM sodium citrate, pH 7.4) for 10 hr and low salt conditions were achieved by incubating the gels in 0.1 X SSC for 4 hr at room temperature (24 ^{0}c). After incubation with RNase A, gels were restained and visualized as above.

Virus Purification

After the observation that dsRNA was consistently associated with mealybug-wilt affected pineapple plants, procedures were developed to investigate the association of virus particles. The first method tried was precipitation by ammonium sulfate followed by differential centrifugation.

The method adopted was a modification of the methods used for cocoa mottle leaf virus and cocoa swollen shoot virus (7, 24,). During the initial purification attempts, a very few long virus particles were observed in the electron microscope. Therefore several standard purification procedures for the purification of long, flexuous, rod-shaped viruses were tried. Purification procedures used for bamboo mosaic virus and papaya ringspot virus were tried (20, 27). In the first method pineapple was ground in liquid nitrogen and thawed in citrate buffer (pH 8) followed by chloroform extraction. The virus in the aqueous phase was precipitated using 8% polyethylene glycol (MW 8000). In the method adopted from bamboo mosaic virus purification, pineapple leaf tissue was ground in liquid nitrogen, thawed in phosphate buffer (pH 7.5) and clarified by centrifugation after straining through cheese cloth. Virus in the supernatant was pelleted by adding PEG to a final concentration of 8% followed by centrifugation at 10,000g for 30 min in a SS 34 rotor. Virus was further purified by differential centrifugation or a second cycle of PEG precipitation. In both of these methods very few virus particles were observed, especially in the chloroform extraction. In the second method, in which chloroform was not used in the initial clarification, more particles were observed and preparations using this method were used to inject rabbits to produce polyclonal antiserum.

In the purification procedures describe above, the virus particles observed resembled closteroviruses. Therefore, a few procedures described for closteroviruses were tried to improve the yield of virus. The purification procedures reported for citrus tristeza virus (CTV) and grape leaf-roll virus were tried (2, 19, 26, 57). Leaves were ground in liquid nitrogen and thawed in 100 mM Tris-HCl buffer with or without 1 mM EDTA. After straining through cheese cloth, sap was clarified by low-speed centrifugation (5,000-6,000g) and virus was precipitated by adding 5-8% PEG and .8-1% sodium chloride. Precipitated virus was pelleted by centrifugation at 10,000g for 20 min. The resulting pellet was resuspended in 40 mM potassium phosphate buffer (pH 8.0) and clarified by low-speed centrifugation (5,000g). In some cases virus was re-precipitated by PEG and pellets from these preparations were resuspended in 15 mM potassium buffer (pH 8).

Although it was possible to obtain higher virus yields from these methods, particles seen in the electron microscope were highly aggregated and broken. Various methods were investigated to obtain intact virus particles for measurement of the dimensions of the particles. The process finally adopted was a modification of the purification procedure described for grape leaf-roll virus by Zee et al. (57). Two hundred grams of pineapple leaves obtained from wilt affected pineapple were frozen in liquid

nitrogen and ground in a mortar and pestle. Tissue was thawed in 400 ml of 500 mM Tris-HCl (pH 8.2) with 2% (v/v) Triton X-100 (Sigma) and .2% (v/v) 2-mercaptoethanol (Sigma). The slurry was stirred for 30 min to 1 hr at 4 ^{0}C and strained through 4 layers of cheese cloth. The resulting suspension was clarified by centrifugation at 5,300g for 20 min in a GSA rotor. The retained supernatant was centrifuged at 200,000g for 35 min in a Ti 70 rotor (Beckman instrument) and the pellet was dissolved in 100 mM Tris-Hcl (pH 8.5) and 10 mM $MgCl_2$ (TM buffer) using 1/8 th volume of extraction buffer and stirred for 2-4 hr at 4 ^{0}C. The suspension was clarified by centrifugation at 5,000g for 10 min in a SS 34 rotor. The resulting supernatant was layered over 5 ml of 0.48 molal Cs_2SO_4 in TM buffer and centrifuged at 200,000g in a Ti 70 rotor for 16 hr at 8 ^{0}C. Other samples were layered over 5 ml of 1.5 M sucrose in TM buffer and centrifuged at 150,000g for 2 hr at 4 ^{0}C in the Ti 70 rotor. Resulting pellets were dissolved in 200 μl of TM buffer and subsequently used for electron microcopy and coat protein analysis.

Sucrose and Cesium Sulfate Gradient Centrifugation

Partially purified virus from the methods described above was subjected to sucrose or Cs_2SO_4 density gradient centrifugation. Sucrose gradients were made using a gradient generating mixer or by layering sucrose solutions with concentrations of 10%, 20%, 30%, 40%, and 50% (W/V) in

TM buffer. Sucrose gradients were centrifuged at 25,000 rpm in a SW 25 rotor for 35 min-1 hr at 4 ^{0}C.

Cs_2SO_4 gradients were made by layering solutions with concentrations of 10%, 15%, 22.5%, 35%, and 40% (W/V) and storing overnight at 4 ^{0}C. The virus suspension (1 ml) was layered over the gradient and centrifuged in the SW 50.1 rotor for 16 hr at 8 ^{0}C. In some cases 1.25 g of Cs_2SO_4 was dissolved in 5 ml of virus solution and centrifuged in a VTi 60 rotor at 100,000g for 16 hr at 8 ^{0}C. After centrifugation, sucrose and Cs_2SO_4 gradients were fractionated by using an ISCO model 640 gradient fractionator. The gradient fractions were examined in the electron microscope for the presence of virus. The density of the Cs_2SO_4 gradients was determined by weighing 100 μl of each gradient fraction.

Electron Microscopy

Virus in final pellets from purification procedures was viewed using either a Hitachi model HS-8-1 or Zeiss 10A transmission electron microscope. Samples were prepared by placing 5 μl of a virus suspension on a carbon stabilized, formvar coated grid (200 mesh) (Ted Pella, Inc. Irvine, California). Excess liquid was carefully drained from the grid using filter paper wicks. Grids were then washed with bacitracin solution in water (300 μg/ml) and stained by floating either for 5 min on a drop of 2% phosphotungstic acid (PTA, pH 6.7) in 250 μg/ml bacitracin or 2 min on a

drop of saturated solution of uranyl formate in methanol. When uranyl acetate was used as negative stain, grids were floated for 4 min on a drop of 2% uranyl acetate in water. Photographs including TMV were used to calibrate the microscope using the width of TMV particles as a standard for length and width measurements of virus obtained from pineapple. One hundred particles randomly selected were measured to obtained the modal length of virus particles.

Optical Properties of Purified Virus

The A_{260}/A_{280} ratio of purified virus was measured by spectrophotometry (Turner, model 350). Ultraviolet spectra of the purified virus was determined with a Perkin Elmer 552 spectrophotometer.

Viral Coat Protein Analysis

Coat protein was analyzed by SDS polyacrylamide gel electrophoresis (SDS-PAGE). For coat protein analysis, virus purification started with 100 g of wilt infected leaf tissue. Virus was purified as described above by differential centrifugation followed by centrifugation through 1.5 M sucrose cushions. The final virus pellet from this preparation was dissolved in 200 µl of electrophoresis sample buffer (62 mM Tris-Cl, pH 6.7, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.004% bromophenol blue). One microliter of this was equivalent to 1/2 g of leaf tissue. From this, 5, 10, and 15 µl were boiled for 2 min and applied to a discontinuous slab gel (10 cm X 7 cm X 0.75

mm) system (Laemmli) (25). The same amount of leaves from healthy pineapple plants were processed in parallel with diseased tissue and the final pellet was dissolved in sample buffer and 10 μl was analyzed by gel electrophoresis with diseased tissue. Coat protein prepared from purified TMV and molecular weight markers (Sigma) were also run to estimate the molecular weight of coat protein of the virus isolated from pineapple. Electrophoresis buffer was 25 mM Tris, 192 mM glycine, and 0.1% SDS. Electrophoresis was at 180 volts for 60 min at room temperature. Gels were stained with coomasie blue and destained with 10% methanol and 15% acetic acid. Destained gels were visualized on fluorescent light. Gels were dried on a gel dryer and stored.

Serology

Polyclonal antiserum was produced in a New Zealand white rabbit against partially purified pineapple virus. Virus for this purpose was purified by PEG precipitation followed by differential centrifugation. The virus purified from 100 g of infected leaf tissue was dissolved in 1 ml of 15 mM potassium phosphate buffer (pH 8.0), mixed with 1 ml of Freund's incomplete adjuvant (Sigma) and injected intramuscularly at weekly intervals. Titers of the antiserum were determined using the microprecipitant test. After the 17th week, rabbits were bled by heart puncture, the blood allowed to clot and serum was recovered by centrifugation.

Immunoglobulin (IgG) Preparation

Procedures of Clark & Adam (10) and Zee et al, (57) were used for immunoglobulin preparation from crude serum. Crude antiserum was cross absorbed with healthy antigen before immunoglobulin fractionation. Healthy leaf extractions were prepared by grinding 5 g of healthy pineapple tissue in liquid nitrogen and thawing in 10 ml of 100 mM Tris-Cl (pH 8.2) with 10 mM $MgCl_2$, 4% polyvinylpolypyrrolidone (PVP) (Sigma) and 0.2% 2-mercaptoethanol, agitating for 30 min at room temperature and centrifuging at 5,000g for 10 min in the SS 34 rotor. Crude antiserum was then mixed with 2 volumes of healthy leaf extract and incubated for 16 hr at 6 ^{0}C and centrifuged at 8,000g for 15 min in the SS 34 rotor. For separation of immunoglobulin, 1 ml of cross-absorbed antiserum was diluted to 10 ml with distilled water and 10 ml of saturated ammonium sulfate solution was added. After 30 min at room temperature the precipitant was collected by centrifugation at 10,000g for 20 min in a SS 34 rotor. The resulting pellet was dissolved in 2 ml of half-strength PBS (1 X PBS = 136 mM NaCl, 2 mM KCl, 4 mM Na_2HPO_4, and 1 mM KH_2PO_4) and dialyzed against 1/2 strength PBS (at least three changes of 500 ml each). IgG was further purified by passage through a column of DEAE cellulose (Whatman Ltd.) and collecting the unabsorbed fractions while monitoring optical density at A_{280} (fig 1). The DEAE cellulose was poured in a glass tube

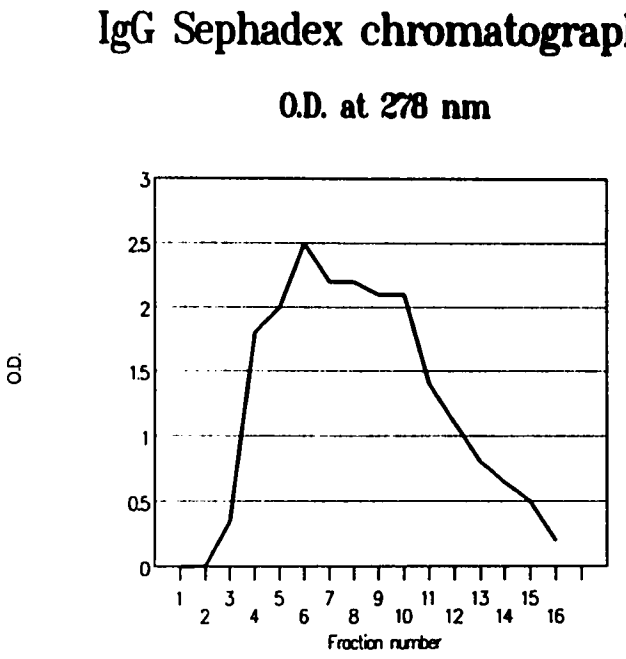


Fig. 1. Optical density at 278 nm of unabsorbed fractions from Sephadex chromatography. Fractions 4-13 were pooled and protein concentration was adjusted to 1 mg/ml with 1/2 X PBS.

1/2 X PBS. Antiserum was added to the column dropwise and washed through with 1/2 X PBS and 1 ml fractions were collected. The fractions with high OD were pooled (4- 13 in fig. 1), their concentration was adjusted to 1 mg/ml (E_{278} = 1.4) and 1 ml aliquots were stored at 4 ^{0}C with 0.01% sodium azide.

Immunodiffusion Test

Ouchterloney double-diffusion assays were performed using agar plates with or without SDS (41). Agar plates were made as follows. Four grams of Noble agar (Difco) was added to 300 ml of distilled or deionized water and autoclaved for 5 min at 250 ^{0}F. After autoclaving, 5 g of sodium azide was added and dissolved by stirring. To this mixture, 2.5 g of SDS dissolved in 150 ml of hot water was added and the volume was brought to 500 ml with distilled water. Plates were also prepared which were identical except for omission the SDS. Plastic petri dishes were placed on a level surface and 12 ml of agar media was added per plate. After the medium was solidified, plates were wrapped in aluminum foil and stored at 4 ^{0}C. Under these storage conditions plates with SDS became opaque due to precipitation of SDS but became clear upon warming to room temperature. Sample wells were cut using a cork borer and cut portions were removed using house vacuum and a pasteur pipette. One well was cut in the center of the plate and 6 peripheral wells were cut around it in a circle using a template made on a piece of paper as guide. Two grams of

leaf tissue were ground in a mortar with a pestle in 2 ml of water and 2 ml of 3% SDS (w/v) was added. The sap was expressed through cheese cloth and 50 µl was added to each well. Extracts from healthy pineapple, virus purified from diseased pineapple, extracts from diseased pineapple, and grape leaves infected with leaf-roll virus were tested.

Serologically Specific Electron Microscopy (SSEM)

The technique used for SSEM was adopted from Derrick (12, 13, 14) and Milne, et al (33). Virus preparation from diseased pineapple was placed on a 200 mesh carbon stabilized, formvar coated copper grid (Ted Pella) and incubated 5 min at room temperature. Excess liquid was drained using filter paper wicks and grids were washed with 30 drops of bacitracin (300 µg/ml in water). Grids were floated on a drop of either purified IgG or crossed-absorbed antiserum and incubated for 30 min at either 37 ^{0}C or room temperature. After incubation, grids were washed with 30 drops of bacitracin and dried by wicking with filter papers. Grids were then floated for 5 min either on 2% PTA made in bacitracin (300 µg/ml) or on 2% uranyl acetate made in water and dried by wicking with filter papers. Uranyl formate was used as a saturated solution in methanol. Grids were floated for 2 min on uranyl formate solution. After staining, grids were viewed in a Hitachi model HS-8-1 transmission electron microscope. Purified TMV was included in SSEM as a negative control.

Western Blotting with Immunodetection

The specificity of antiserum produced to virus was further tested by western blotting followed by immunodetection. The method used to run the protein gels was the same as described under coat protein analysis. The electroblotting procedure was adopted from Beisiegl, (3), Walker (52), Schleicher & Schuell (S&S) (46), and Towbin, et al. (50). Usually, gels were run in pairs, and transferred as pairs. One of the transferred blots was used for immunodetection and the other was stained with amidoblack. After running, the gels were equilibrated with transfer buffer for 15 min. The transfer buffer used was a low ionic strength buffer containing 10 mM Tris-Cl, pH 8.6, 15% methanol and 0.01% SDS. Nitrocellulose (S&S) used as the solid matrix was cut to the size of the gels to be transferred and soaked in water, then in transfer buffer. After soaking the gels and NC paper in transfer buffer, a transfer apparatus was set up as follows. Starting from the cathode side, layered in order, were a foam pad, a piece of 3MM filter paper, the gel, two nitrocellulose papers, a piece of 3MM paper and a foam pad. All these were assembled in transfer buffer and care was taken to avoid trapping any air bubbles in the sandwich. For transfer, gels and nitrocellulose were immersed in the tank with transfer buffer and exposed to 150 volts for 1 hr in the cold room at 4 ^{0}C. After the transfer, gels were removed from the

apparatus and one gel was stained with 0.1% amidoblack in 25% isopropanol and 10% acetic acid for 15 min, and destained with the same solution without amidoblack. The blot to be immunodetected was stored in blocking buffer (4% Bovine Serum Albumin (BSA) in PBS) at 4 ^{0}C overnight until used. On the following day, blots were agitated 30 min at room temperature, and then washed 3 times, each for 10 min in about 25 ml of PBS containing 1% Tween-20 (Fisher Bio tech). After washing, the blots were incubated with IgG purified from polyclonal antiserum produced against virus isolated from pineapple. IgG concentration was 4 µg/ml, in PBS with 1% BSA. Incubation was for 1-3 hr at 37 ^{0}C with gentle agitation. After completing the incubation, blots were washed 3 times for 10 min each in PBS containing 1% tween-20. After washing, blots were incubated with goat anti-rabbit conjugated to alkaline phosphatase (Cappel, Cochranville, PA 19330) used at a dilution of 1:1,000 in PBS containing 1% BSA. Incubation lasted 1 hr at room temperature with gentle agitation. Blots were then washed as after incubation with first antibody. The substrate used was described by Pratt, et al. (39). After washing, the blots were immersed in substrate buffer (pH 9.6) which contained 150 mM sodium bicarbonate, and 4 mM $MgCl_2$. Substrate solutions were made as follows. To 45 ml of substrate buffer, 0.5 ml of bromochloroindolyl phosphate (Sigma) stock solution (5 mg/ml in dimethyl sulfoxide,

stored in 4 ^{0}C) and 5 ml of nitroblue tetrazolium (Sigma) stock solution (1 mg/ml in substrate buffer, stored at 4 ^{0}c in darkness, replaced every 2 weeks) was added. After leaving the blots in substrate buffer about 15 min, substrate solution was added. Color development began in about 5 min and was completed in 3-6 hr. After the color development, substrate solution was removed, blots were washed with distilled water several times and dried between two filter papers.

RESULTS

Isolation of dsRNA

Polyacrylamide gel electrophoresis reveals the presence of several dsRNAs in mealybug-wilt infected pineapple tissues (Fig. 2a). These bands were not observed in healthy plant tissues obtained from Maui Land and Pine Company. In all diseased samples a high molecular weight dsRNA was present. The occurrence of low molecular weight dsRNA was variable (see fig. 2a P1-P4). Occasionally only the large dsRNA was present, suggesting that this is the major replicative form of viral RNA. These dsRNA bands were disease specific. The RNA bands were also resistant to RNase in high salt conditions, suggesting that these are dsRNA. The relative electrophoretic mobilities of standards were plotted versus the logarithm of their molecular weight (fig. 3). These plots were used to determine the molecular weight of dsRNA isolated from diseased pineapple.

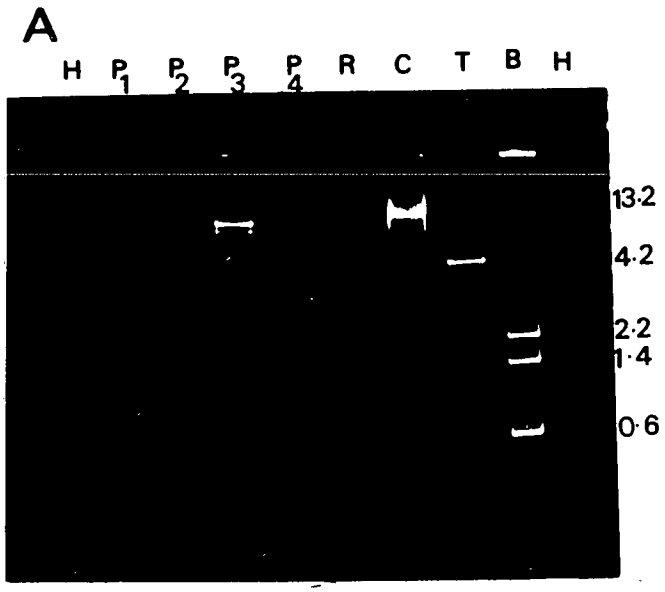


Fig. 2A. Polyacrylamide gel electrophoresis of dsRNA. Lane H, healthy pineapple; lane P1-P4, dsRNA isolated from 4 different diseased pineapple plants; lane R, dsRNA isolated from diseased roots of pineapple; (dsRNA from 20 g of plant tissue was loaded in each lane); lane C, dsRNA isolated from CTV infected citrus; lane B, dsRNA from BMV infected barley; lane T, dsRNA isolated from TMV infected tobacco. Numbers on the right of the figure are molecular weights of CTV, TMV, and BMV dsRNAs in millions of daltons.

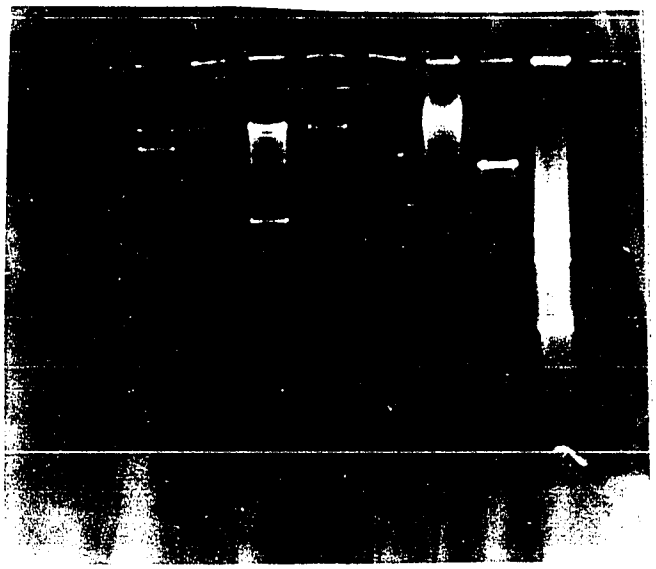


Fig. 2B. After incubation of the gel in fig. 2A with RNase A in 2 X SSC (high salt) for 10 hr.

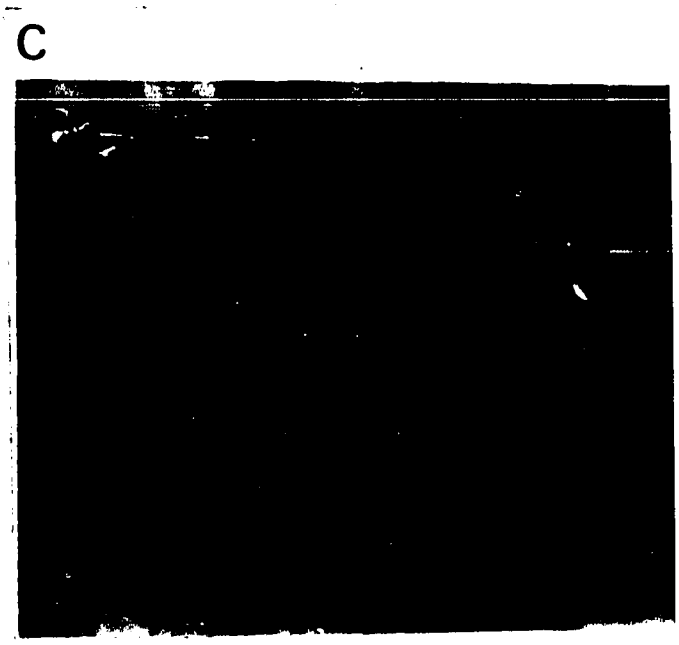


Fig. 2C. Same gel as in B after incubation with RNase A in 0.1 X SSC (low salt) for 4 hr. After each incubation the gel was restained with ethidium bromide before visualizing with UV light.

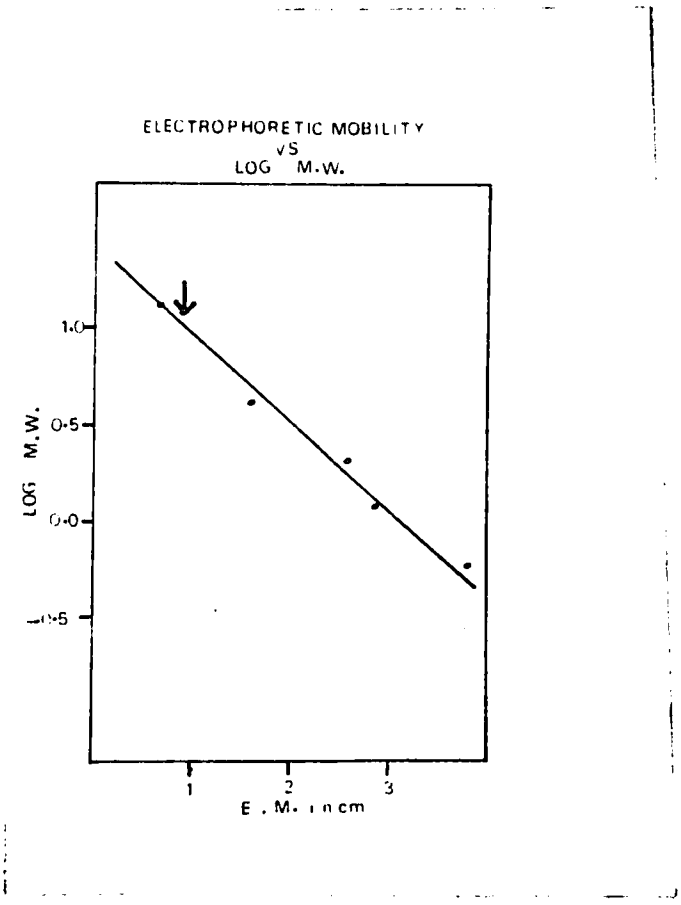


Fig. 3. A plot of the electrophoretic mobilities of molecular weight standards (dsRNA) versus the logarithm of their molecular weights. Arrow indicates the mobility of dsRNA from pineapple.

Using these data the molecular weight estimated for dsRNA isolated from pineapple was 8.35 X 10^6 daltons.

Virus Purification

Preliminary methods were unsuccessful in yielding useful quantities of virus. Virus purified from PEG precipitation was always aggregated, and was not suitable for any physical measurements such as width and length (fig 4). In the initial extraction procedure, freezing pineapple tissue in liquid nitrogen followed by grinding in a mortar with a pestle was suitable for virus extraction. If a commercial blender was used instead of liquid nitrogen, longer blending time was needed to grind pineapple tissue because these tissues were thick and hard to grind but the grinding resulted in shearing of virus particles. Among the buffers tried for virus extraction, 500 mM Tris-Cl at pH 8.2 was the best. The virus yield was much lower when low ionic strength buffers were used. The most suitable pH range was pH 8.0-8.5 and other pH range (pH 6.0-7.5) were not effective in virus extraction. In most filamentous virus purification procedures the pH range of 7.2-9.0 was successfully used.

The purification procedure finally adopted was modified from Zee et al, (57). This procedure was modified by centrifugation through sucrose or Cs_2SO_4 cushions instead of Cs_2SO_4 gradients because these gradients were not successful. Centrifugation through sucrose cushions

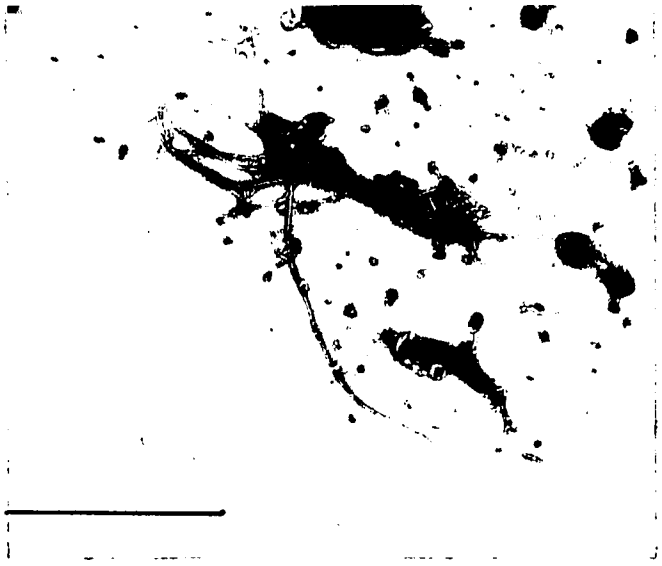


Fig. 4. Electron micrograph of the virus isolated from pineapple by PEG precipitation method. The virus particles were negatively stained with 2% uranyl acetate made in water. Scale bar represents 500 nm.

resulted in less aggregation of virus particles than direct high speed centrifugation. This purification procedure resulted in a high yield of virus particles and made possible the measurement of length and width of the virus particles. Virus particles obtained from this method are shown in fig. 5. Measurements of the length of 100 particles were made and a histogram (fig. 6) was constructed. The modal length of the virus isolated from pineapple was 1200-1500 nm. However, a considerable amount of the measured particles were 600-800 nm long. The reason for this was breakage of virus particles during purification. The estimated width of 25 measured particles was 12 nm.

Sucrose and Cs_2SO_4 Gradient Centrifugation

It was not possible to obtain virus purification using sucrose gradient centrifugation. The virus was never observed as a distinct band in sucrose gradients. In Cs_2SO_4 gradients, occasionally, 1 or 2 distinct bands were observed (fig. 7) and when these gradients were fractionated 1 or 2 small peaks (A= 254) were observed. All fractions were observed in the electron microscope and virus peaks were identified. The virus particles recovered from these gradients were degraded in a few days upon storage. The density of the virus particles was measured using data obtained by weighing gradient fractions (fig. 7).

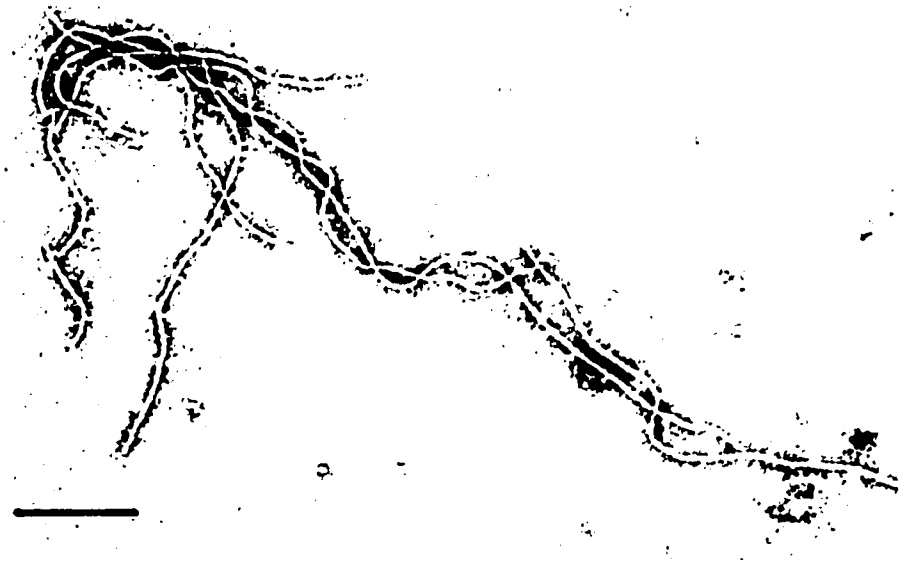


Fig. 5. Electron micrograph of the virus isolated from pineapple by the sucrose cushion centrifugation method. Virus particles were negatively stained with 2% PTA in water containing 300 µg/ml bacitracin. These particles were less aggregated and more flexuous in nature than virus particles purified by PEG precipitation. Scale bar represents 200 nm.

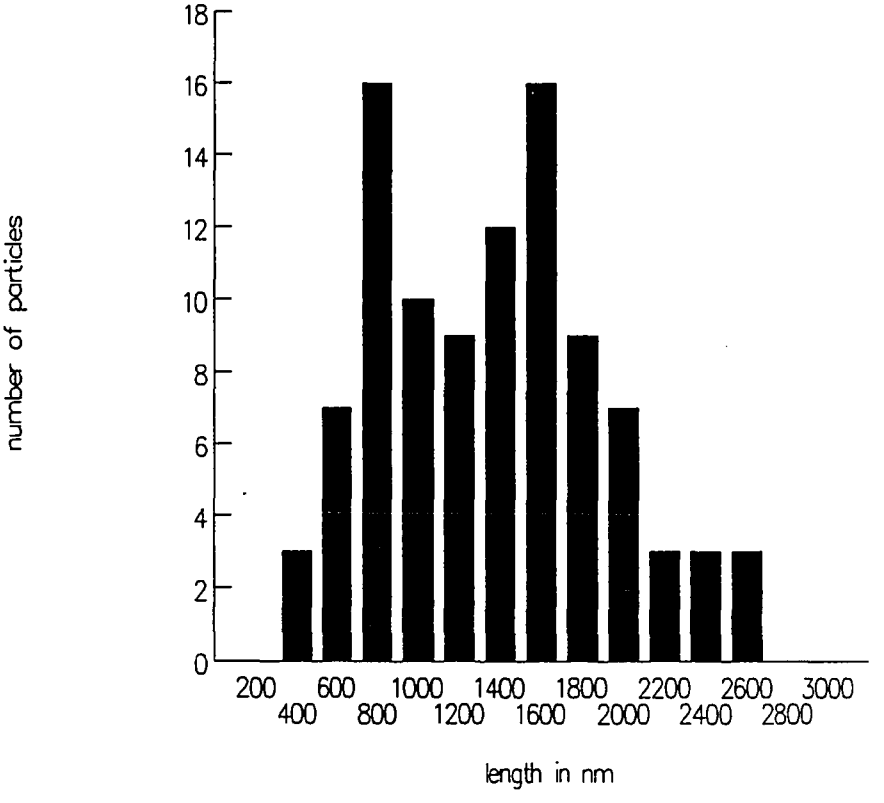


Fig. 6. Distribution of lengths of 100 virus particles isolated from diseased pineapple.

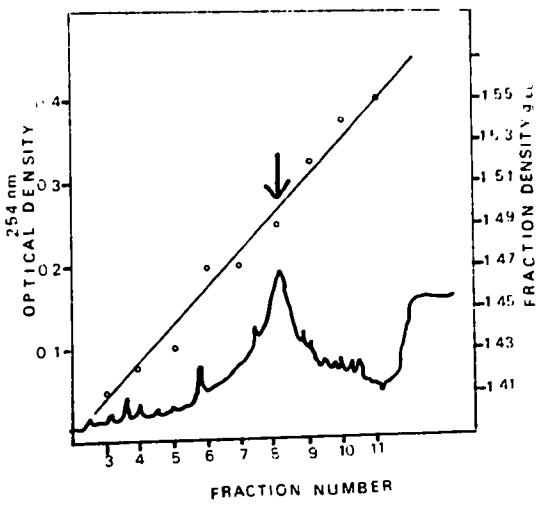


Fig. 7. Absorption at 254 nm of Cs_2SO_4 gradient after 18 hr centrifugation at 150,000g in a VTi 70 rotor. Density of the fractions is plotted on the right side. The virus peak occurs at 1.5 g/cc density (arrow).

Electron Microscopy

Long, flexuous rod-shaped virus particles from diseased pineapple are shown in fig. 5. Virus purified by PEG precipitation always tended to aggregate as observed in the electron microscope (fig. 4). When uranyl acetate (2%) was used as a negative stain, virus particles were always aggregated and of a less flexuous nature. Also, the precipitation of stain and resultant damage of formvar resulted with uranyl acetate. When PTA made in water (pH 6.7) was used as a negative stain, less aggregation of virus particles was observed and virus was of a more flexuous nature. However, better results were obtained when PTA made with 200 μg/ml bacitracin was used as negative stain and 300 μl/ml bacitracin made in water was used in all washing steps in grid preparation for electron microscopy. The virus particles were less aggregated and no stain precipitation was observed when this procedure was followed. Uranyl formate gave greater detail of virus particles at high magnification allowing the arrangement of protein subunits of virus particles to be observed (fig. 8a). Saturated uranyl formate solution made in methanol was superior to uranyl formate in water. The open structure characteristic for closteroviruses is shown in fig 8a. The rope-like structure was also characteristic for closteroviruses when stained with uranyl acetate. The virus isolated from pineapple also shows this type of structure when visualized

Fig. 8a. Electron micrograph of virus particles stained with saturated uranyl formate in methanol. The open structure characteristic of closteroviruses can be seen. The bending of the filaments suggests that this virus isolated from pineapple is flexible. Scale bar represents 50 nm.

Fig. 8b. Electron micrograph of a virus particle stained with 2% uranyl acetate in water. The rope-like structure characteristic of closteroviruses can be seen. Scale bar represents 100 nm.

at high magnification in the electron microscope (fig 8b).

Optical Properties of Purified Virus

The ultraviolet absorption spectrum is shown in fig 9. The A_{260}/A_{260} ratio of virus was 1.8. This high ratio has been reported for some closteroviruses.

Coat Protein Analysis

Polyacrylamide gel electrophoresis revealed the presence of one species of protein associated with the virus isolated from wilt-infected pineapple plants (fig. 10). The electrophoretic mobility of this protein band was compared to the mobility of known standards (fig. 11). The molecular weight determined for the coat protein from virus associated with mealybug-wilt infected pineapple plants was 23,800 daltons. This band was never observed when healthy plants were subjected to the same purification procedure. This suggests that the protein species is disease specific and must be the coat protein of the virus isolated from pineapple.

Serology

The titer of the antiserum from New Zealand rabbits increased very slowly during weekly injections of purified virus as monitored by microprecipitant tests. At the seventeenth week titers were 1:8-1:16. The serum obtained at this time was used for ouchterloney double diffusion tests and as a source of immunoglobulin for use in western blotting and serological specific electron microscopy. When

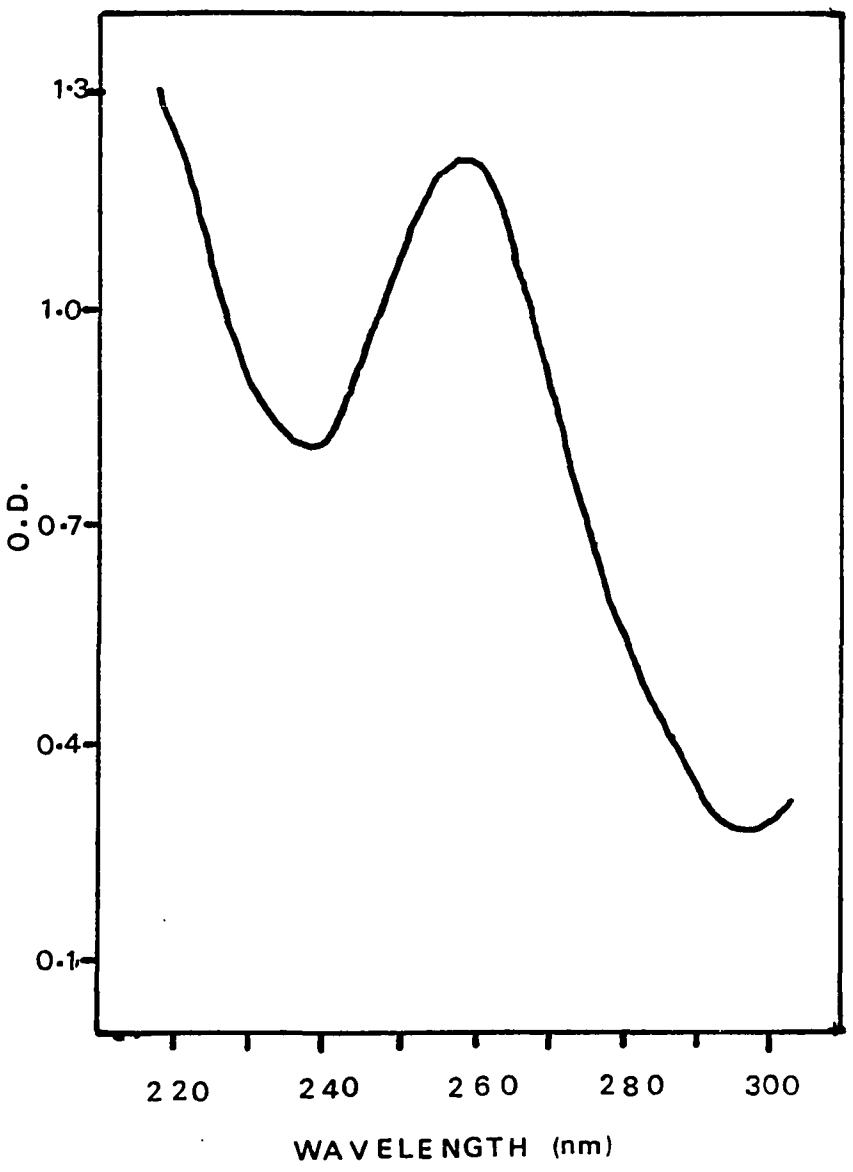


Fig. 9. UV absorption spectrum of virus suspension purified from pineapple.

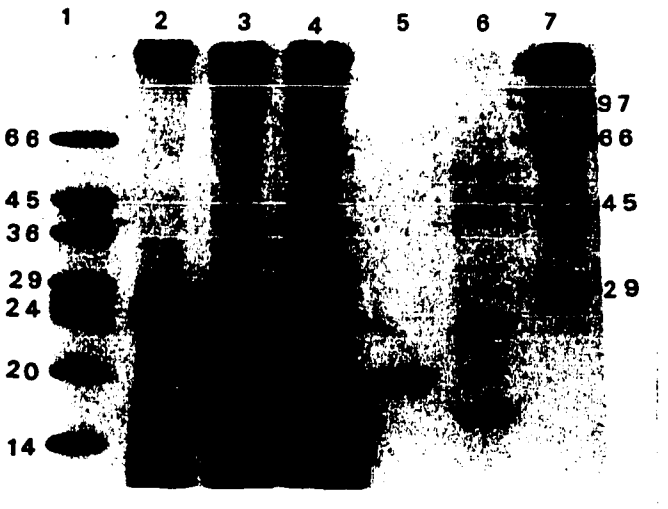


Fig. 10. SDS-polyacrylamide gel electrophoresis of protein from pineapple. Lane 1, molecular weight markers (numbers on left side indicate the molecular weight in thousands of daltons). Lanes 2, 3, 4, virus isolated from diseased pineapple by the sucrose cushion method. Aliquots representing 2.5 g, 5.0 g, and 7.5 g of tissue were loaded in these lanes respectively. Lane 5, coat protein of TMV. Lane 6, an aliquot representing 7.5 g of healthy tissue prepared along with diseased tissue. Lane 7, molecular weight markers (numbers on the right side represent the molecular weight in thousands of daltons).

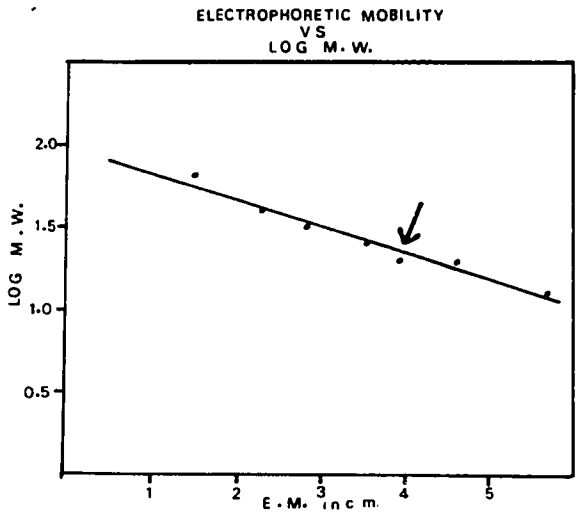


Fig. 11. Electrophoretic mobility of molecular weight markers versus logarithm of their molecular weights. Molecular weight determined for coat protein of the virus isolated from pineapple is 23,800 (arrow).

cross-absorbed crude serum was used in immunodiffusion tests, a single virus-specific precipitin band was observed with SDS disrupted purified virus particles. When crude plant samples were used with SDS, 2 precipitant bands were observed. The extra band was very close to the antiserum well and could be due to some viral specific protein in infected tissue or some degradation product (fig. 12). The plates made with agar medium with SDS were not successful in immunodiffusion test. At no time were precipitant bands observed with these plates.

Serologically Specific Electron Microscopy

IgG preparations from cross-absorbed antiserum gave specific decoration of virus isolated from pineapple as observed in the electron microscope (fig 13a, 13c). TMV was never decorated with the IgG produced against pineapple virus. when crossed-absorbed crude serum was used highly decorated particles were observed (fig. 13b). In these procedures, good results were obtained when 300 µg/ ml bacitracin solution was used in each washing step. This may be due to the bacitracin helping to spread the virus particles on the grid, preventing aggregation. In addition, decorated particles were clearly observed when 200 µg/ml bacitracin was used with PTA in water as negative stain. Virus particles were aggregated and it was not possible to distinguish individual decorated virus particles when UA or UF was used as negative stain.

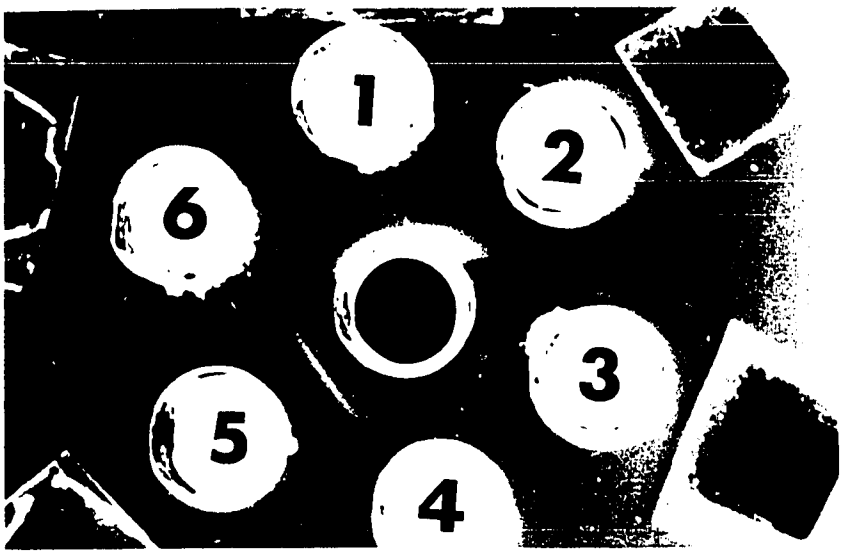


Fig. 12. An immunodiffusion test showing reaction of antiserum prepared to the virus isolated from pineapple. Samples tested were leaf extracts of diseased pineapple cv. Smooth Cayenne (well 1); Sample buffer (well 2); leaf extract of healthy grapevine (well 3); leaf extract of healthy pineapple cv. Smooth Cayenne (well 4); partially purified virus from pineapple (well 5); and leaf extract of grapevine leaf-roll virus infected tissue (well 6). Arrow indicates precipitant reaction to partially purified virus and leaf extract from diseased pineapple. All the samples were mixed with 1% SDS before being applied to sample wells.

Fig. 13a. Electron micrograph of decorated virus particles with purified IgG. The grid was incubated with IgG at room temperature for 30 min. Scale bar represents 200 nm.

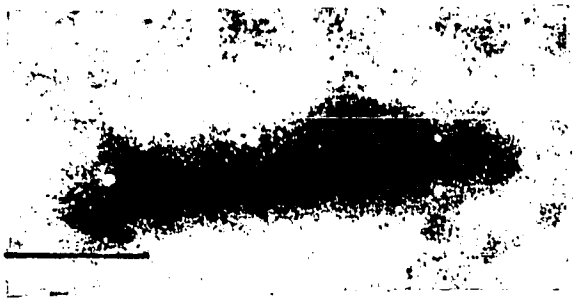
13b. Electron micrograph of a decorated virus particle with cross-absorbed whole serum. Scale bar represents 200 nm.

13c. Electron micrograph of virus particles decorated with purified IgG. Incubation with IgG was at 37^0 C for 30 min. Scale bar represents 200 nm.

a

b

c



The effect of the temperature also was important in the decoration procedure. After virus suspension was applied to the grids as described in materials and methods, grids were incubated on IgG at either room temperature or at 37 ^{0}C. The virus particles were heavily decorated when incubated at 37 ^{0}C (fig. 13a, 13c).

Western Blotting with Immunodetection

The buffer system used in protein transfer was developed in our laboratory by Dr. Douglas Rice. This low ionic strength buffer gave nearly complete transfer of all the protein. (fig. 14b).

IgG against pineapple virus was used at a concentration of 4 μg/ml in western blotting. At this concentration a specific reaction with viral coat protein transferred to nitrocellulose was observed (fig. 14a). Incubation time with first antibody was 2-3 hr and best results were obtained when incubation was at 37 ^{0}C.

DISCUSSION

The use of dsRNA as a diagnostic tool in plant virology has become more useful in recent years (11, 15, 42, 52). Diseases with uncertain etiology have had viruses implicated using this technique (11). In this study it was found that dsRNA was specifically associated with mealybug-wilt affected pineapple plant tissue. Healthy plants obtained from breeding stock of Maui Land and Pine Company which were

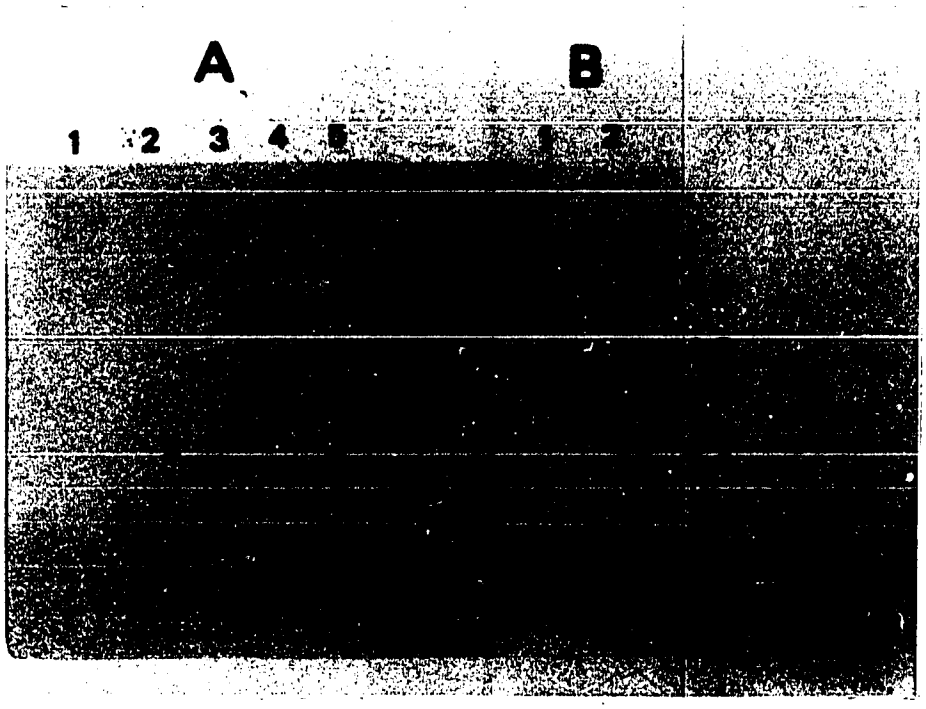


Fig. 14. Western-blot of viral coat protein and immunodetection with IgG prepared to viral protein.

A. A western-blot after incubation with substrate. Lane 1, healthy preparation representing 7.5 g of plant tissue; lane 2, coat protein of TMV; lane 3 & 4, preparation from diseased pineapple. Aliquot representing 7.5 and 5.0 g tissue were loaded in these two lanes respectively. Arrow shows the color development of viral coat protein band.

B. Protein transferred to nitrocellulose stained with amido black. Lane 1, preparation from diseased pineapple; Lane 2, molecular weight standards. Numbers on the right side represent the molecular weight of markers in thousands of daltons.

never exposed to mealybugs, were carefully maintained to avoid any mealybug infestation. At no time were we able to detect dsRNA from these healthy plants, suggesting that they were virus free. The resistance of this RNA to RNase in high salt conditions proves that these RNAs are dsRNA. Also the resistance of these nucleic acids to DNase indicates that these are not DNA. This leads us to conclude that there is a virus in diseased plants and that this virus is ssRNA because it produces typical replicative forms which could be detected by PAGE.

It has long been recognized that filamentous virus particles are difficult to purify. Because these particles are often absorbed to normal cell constituents, tend to fragment in vitro, and tend to aggregate side to side or end to end (5, 9, 23, 32, 40, 48, 53). This was also the case with the virus isolated from pineapple. Virus purified by PEG precipitation always aggregated and made any size measurement of virus particles very difficult. None of the attempts to purify virus from pineapple plants using chloroform extraction followed by PEG precipitation were successful. This virus may not be stable in the presence of chloroform. Although organic solvents such as chloroform and carbon tetrachloride have been used to purify some viruses (22, 35, 49,), these solvents have deleterious effects on many viruses including sugarcane mosaic virus (45) and tulip breaking virus (55). However, chloroform

extraction is not suitable in the virus purification from pineapple. Based on the results of this study, the purification procedure we have developed (from Zee, et al.) can be recommended for purifying virus from mealybug-wilt infected pineapple.

It has been reported that some filamentous virus particles form a single band with a density of 1.2-1.3 g/cm^2 after centrifugation in preformed gradients for 8 hr at 181,000g in a swinging bucket rotor. The virus isolated from pineapple also formed a single band with a density of 1.4 g/cm^2. But virus purified from this procedure degraded faster than virus purified from centrifugation through a sucrose cushion. Apparently cesium salts are harmful to this virus.

The particle morphology as observed by transmission electron microscopy indicates that the virus isolated from pineapple is a closterovirus. The viruses in this group are difficult to purify, and it is difficult to measure the length of particles because they are very flexuous, often become entangled, break easily and may assume different dimensions in different negative stains (1, 4, 44). With the virus isolated from pineapple all the problems mentioned above were observed. When UA was used as a negative stain, virus particles were aggregated and they were in a less flexuous state. The best electron micrographs of virus were obtained when PTA (pH 6.8) or uranyl formate in methanol was

used. Furthermore when bacitracin was included in PTA at the concentration of 300 μg/ml, virus particles were less aggregated and more flexuous. The electron micrographs with less entangled virus particles were used in all width and length measurements. The width measured for virus isolated from pineapple was 12 nm. Most viruses in the closterovirus group have widths of about 12 nm. The modal length of the virus particles ranged from 1200-1500 nm. The viruses included in the type 2 closterovirus group fall into this approximate length range (38, 54). It has also been reported that viruses in the closterovirus group varied in appearance according to its purification and preparation for electron microscopy (44, 45). With the virus isolated from pineapple this was true. Virus isolated through PEG precipitation was entirely different in appearance to the virus purified through sucrose cushion centrifugation. The virus particles obtained through PEG precipitation were highly aggregated compared to virus obtained through sucrose cushion centrifugation. It has also been reported that virus particles in the closterovirus group had no fine structure when stained with PTA, while in uranyl acetate or uranyl formate a cross banding appearance could be observed (44, 45). The cross banding pattern of the virus isolated from pineapple was clearly visible at high magnification when uranyl formate was used. The A_{260}/A_{280} ratio of virus isolated from pineapple is 1.82. This value is

characteristic for each virus group. For the closterovirus group this value ranged from 1.16-1.85 (53, 54). This value also can be used in estimating the percentage of nucleic acid present in the virion (47).

Polyacrylamide gel electrophoresis of viral coat from pineapple revealed a single protein with a molecular weight of 23,800 daltons. Most of the viruses in the closterovirus group have a single protein of molecular weight ranging from 23,000 - 24,000 daltons (1).

The serological studies show that polyclonal antiserum produced against purified virus gave specific reactions after cross-absorbing with healthy plant extract. However, the titer of the antiserum was very low. The western blotting results indicate that the protein specific to preparations from diseased plants is viral coat protein since IgG specifically bound to this protein. Also these results prove that the antiserum is virus specific and does not detect host component. Therefore it is reasonable to say that the coat protein of the virus isolated from pineapple consists of a single capsid protein with a molecular weight of 23,800 daltons.

The virus isolated from pineapple can tentatively be assigned to the type 2 closterovirus group for the following reasons. The virus morphology, (especially the cross-banding patterns observed in uranyl formate), and the length and width are similar to the closterovirus group. The

molecular weight of the coat protein, the dsRNA, and the dsRNA patterns observed in polyacrylamide gels all closely resembles type 2 closteroviruses. No other characterized virus group can accommodate a virus with these properties.

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CHAPTER III

VIRUS DETECTION AND FURTHER CHARACTERIZATION USING cDNA PROBES

ABSTRACT

Complementary DNA (cDNA) was synthesized to viral RNA using dsRNA isolated from pineapple as the template. The specificity of probes produced was enhanced when dsRNA was treated with RNase A under high salt conditions. Using these probes, viral nucleic acid sequences were detected in pineapple plants from commercial plantations and also in a species of grass commonly found around pineapple plantations. cDNA probes were also used to detect viral nucleic acids in mealybugs taken from wilt-diseased pineapple plants. Northern-blot analysis revealed that RNA isolated from diseased plants specifically hybridized to cDNA synthesized from dsRNA.

INTRODUCTION

The vast majority of plant viruses contain single-stranded RNA genomes. In many cases the genomes are split among two or three RNA segments (5, 15). The relationship of these RNA segments as well as relationships between virus groups and strains have been established by molecular hybridization analysis (10, 20, 23). This technique is also very useful in virus detection in different host species and for detecting subgenomic RNAs in nucleic acids extracted

from infected tissue (7, 14, 20). There are a number of methods available for preparing cDNA probes to viral RNAs. Any particular method is dependent upon the physical properties of the viral RNA (eg. molecular weight, structure, 3'-polyadenylate sequences) and the type of information to be obtained by using such probes.

The preparation of cDNA involves four steps; 1) the cDNA synthesis reaction, 2) the separation of the cDNA from the template and enzyme; 3) the separation of the cDNA from unincorporated radioisotopes and other components of the reaction mixture and 4) the concentration of the cDNA.

The preparation of cDNA requires a template (the viral RNA), a primer (oligonuleotides complementary to sequences within the template), and reverse transcriptase (a retrovirus RNA-dependent DNA polymerase).

Three types of primers can be used in cDNA synthesis. Oligo dT can be used as a primer for RNAs with 3'-polyadenylated sequences (22, 7, 11). Random primers can be used if there are no 3' polyadenylated sequences (29). Specific or synthesized primers can be used when cDNA is to be synthesized to a target sequence (21).

In this study, dsRNA isolated from diseased pineapple (Ananas comosus L.) plants were used as the template to synthesize cDNA using random primers. This cDNA was then used in dot-blot assays to detect viruses in mealybugs, pineapple plants and wild hosts.

MATERIALS AND METHODS

Extraction of dsRNA and Preparation of dsRNA for Synthesis of cDNA Probes

dsRNA was isolated and treated with DNase as described in chapter 2. After the DNase treatment, nucleic acids were precipitated with ethanol and sodium acetate. Precipitated nucleic acids were centrifuged and resulting pellets were dried under vacuum and dissolved in 2 X SSC and RNase A (Sigma) was added to a final concentration of 1 μg/ml. The reaction mixture was incubated at 37^0 C for 30 min. Proteinase K was added to a final concentration of 20 μg/ml and incubation was continued for another 30 min. The reaction mixture was extracted with phenol:chloroform (1:1) and centrifuged to separate the phases. The aqueous phase was collected and nucleic acids were precipitated by adding 1/20 volume of 3 M sodium acetate and 2 1/2 volume of cold 95% ethanol and storing at -20^0 C overnight.

Synthesis of cDNA Using Random Primers

The method used for cDNA synthesis was as described by Taylor, et al. (29). The random primers used were obtained from Sigma Chemical Company. These were produced by DNase 1 digestion of salmon sperm DNA (29) and were 10-20 nucleotides in length. The final reaction mixture for cDNA synthesis contained 50 mM Tris-Cl (pH 8.3) 5 mM $MgCl_2$, 70 mM KCl, 2 mM DDT (dithiothritiol) 0.5 mM each of three dNTPs (dATP, dTTP, dGTP) , 10 μM of (Radiolabled) dCTP, 8-10 units

of reverse transcriptase (Sigma), template RNA and random primers. The reaction buffer was prepared as 500 mM tris (pH 8.1), 50 mM $MgCl_2$ and 700 mM KCl, adjusted to pH 8.1 at 40^0 C and autoclaved. Before buffer was used DTT was added to a final concentration of 20 mM. The reaction volume of 50 µl was made as follows. In a microfuge tube, 2-5 µg of dsRNA from diseased pineapple in 20 µl of 1 mM EDTA (pH 7.5) was mixed with 6 µl of random primers (5 mg/ml), boiled for 5 min and quench cooled on ice. To this mixture, the following components were added sequentially: reaction buffer (5 µl), 10 mM dATP (2 µl), 10 mM dGTP (2 µl), 10 mM dTTP (2 µl), 10 mM dCTP (1 µl), water (4 µl), alpha ^{32}P dCTP (5 µl) (SA= 800 Ci/mmM), and reverse transcriptase (3 µl). The mixture was incubated at 40^0 C for 1-3 hr. The reaction was stopped by adding 1 µl of 500 mM EDTA. Six µl of 3 M NaOH was then added and the mixture was incubated at 60^0 C for 1 hr to hydrolyze the RNA template. The reaction mixture was then neutralized by adding 6 µl of 3 M HCl and 5 µl of 1 M tris-Cl. cDNA was precipitated by adding 1/20 volume of 3 M sodium acetate (pH 5.5) and 2 1/2 volume of cold 95% ethanol and storing at -20^0 C overnight. Precipitated cDNA was collected by centrifugation for 30 min in a microfuge and the pellet was dried under vacuum. The unincorporated radioisotopes were removed by subjecting the cDNA to several cycles of ethanol precipitation.

The cDNA synthesis reaction was monitored by spotting 1

µl of the reaction mixture onto DEAE cellulose filters before the addition of reverse transcriptase (2 filters), and after incubation with reverse transcriptase (1 filter). One of the DEAE cellulose filters with reaction mixture at 0 time (before addition of enzyme) and one with reaction mixture after incubation with reverse transcriptase were washed 5 times (each 5 min) with 500 mM phosphate buffer (pH 7.5) and once (1 min) with distilled water and were then counted in water using a scintillation counter using a Cherenkov program. The unwashed 0 time filter represented the total counts in the reaction and the washed filter from the same time represented the efficiency of removal of unincorporated radiolabled nucleotides. The number of counts on the washed filter from step 2 minus the number of count on the filter from time 0 represented the total counts incorporated into polynucleotide.

Plant Tissue and Mealybugs for Dot-blot Assay

Diseased pineapple tissues were obtained from Dole pineapple plantation on Oahu and Lanai. Plants were harvested from fields with mealybug infestations. Healthy pineapple tissues were obtained from a breeding stock of Maui Land and Pineapple Company originating from seeds and maintained mealybug-free. Some weed species around the Dole pineapple plantation in Lanai were tested as possible secondary hosts of the virus in pineapple. The most prominent grass species around these fields were collected.

These included Sour grass (Andropogon insularis L.) and Vassi grass (Paspalum urvullei L.). Diseased mealybugs were obtained from pineapple plants showing typical wilt symptoms and control mealybugs were obtained from agave plants. These agave plants also were tested in dot-blot assay.

Isolation of Nucleic Acids from Plant Tissue and Mealybugs for Dot-blot Assay

The isolation of nucleic acids from plant tissue and mealybugs was performed according to the method described by Garger and Turpen (9). Three leaf disks (9 mm) from plant tissue were ground in 100 µl of TE buffer in a 1.5 ml Eppendorf tube. To this mixture equal volumes of phenol and chloroform were added and vortexed thoroughly. The homogenate was centrifuged and 50 µl of aqueous supernatant was added to 150 µl 6.15 M formaldehyde solution in 10 X SSC (made by mixing equal volumes of 20 X SSC and 37% formaldehyde). The nucleic acids in formaldehyde and SSC were incubated at 65 ^{0} C for 15 min and blotted directly onto nitrocellulose paper or stored at -20^{0} C for future use. For isolation of RNA from mealybugs, 2-3 mature individuals were crushed in a microfuge tube in TE buffer followed by phenol-chloroform extraction as above.

Dot-blot Assay

Dot-blot hybridization was performed using standard procedures (19, 30). The solid matrix support used to blot RNA samples was nitrocellulose (S & S) (26). The

nitrocellulose paper was cut to the size of a dot-blot apparatus (Schleicher and Schuell, Inc., (Keem, N. H.) and soaked in water until the paper was completely saturated. The nitrocellulose paper was then transferred to 20 X SSC and soaked for about 1/2-1 hr before use. The samples in formaldehyde were denatured by incubating at 65^0 C for 15 min. Two-fold dilutions were made in 10 X SSC in 6.15 M formaldehyde. The wells of the dot-blot apparatus were washed with 200 µl of 20 X SSC before samples were loaded. Samples were loaded (200 µl/well) and a vacuum was applied to the apparatus. Samples wells were then washed with 200 µl of 20 X SSC, the apparatus was dissembled and the nitrocellulose paper removed and dried under a heat lamp for 10 min, followed by baking at 80^0 C for 30 min in a vacuum oven. The baked blot was either used directly or stored at -20 ^{0}C for future use.

Isolation of Total RNA from Plant Tissue

The procedure used for isolating RNA from plant tissue was described by Chomczynski and Sacchi (3). The denaturing solution (solution D) contained 4 M guanidium thiocyanate, 25 mM sodium citrate, pH 7; 0.5% sarcosyl, 0.2% 2-mercaptoethanol. A stock solution of guanidium thiocyanate solution was prepared by dissolving two hundred fifty grams of guanidinium thiocyanate (Fisher Scientific) in 293 ml of water (whole manufacture's bottle without weighing), 17.6 ml of 0.75 M sodium citrate pH 7, and 26.4 ml 10% (w/v)

sarcosyl at 65^0 C. Solution D was prepared when needed by adding 0.36 ml of 2-mercaptoethanol to 50 ml of stock solution. To isolate total RNA from plant tissue, 10 grams of either healthy or diseased tissue were ground to powder in liquid nitrogen and thawed in a baked-glass beaker with 20 ml of solution D. To the thawed mixture, 1 ml of 2 M sodium acetate (pH 5.5), 20 ml phenol, and 2 ml of SEVAG (SEVAG = 24:1 Chloroform:Isoamyl alcohol) were added sequentially. The slurry was then stirred 1/2 hr at room temperature, and incubated on ice for 15 min and centrifuged at 10,000g for 20 min in a SS 34 rotor. After centrifugation, the resulting aqueous phase was transferred to a baked-glass flask, mixed with 20 ml of isopropanol and incubated at -20^0 C for 1 hr. Precipitated nucleic acids were collected by centrifugation at 10,000g for 20 min in the SS 34 rotor. Pellets were dissolved in 3 ml of solution D and precipitated by adding isopropanol and storing at -20^0 C for 1 hr. Precipitated nucleic acids were pelleted by centrifugation at 10,000g for 20 min in a SS 34 rotor and the resulting pellet was washed with 70% cold ethanol and centrifuged for 10 min at 10,000g. The RNA pellet was dried under vacuum and dissolved in 2 mM EDTA (DEPC treated). This RNA was used in dot-blot analysis and Northern blot analysis.

Denaturing Agarose Gel Electrophoresis of RNA

ssRNA and dsRNA was analyzed by denaturing agarose gel

electrophoresis (28). The procedure used was as described by Faurney, et al. (12). The electrophoresis buffer was MOPS/EDTA buffer at pH 7. A stock solution of 10 X MOPS buffer was prepared by mixing 0.2 M MOPS [3-(N-morpholino) propanesulfonic acid], 50 mM sodium acetate, 10 mM EDTA, the pH was adjusted to 7.0 and the solution autoclaved. Electrophoresis sample buffer was made by mixing 0.75 ml deionized formamide, 0.15 ml 10 X MOPS, 0.24 ml formaldehyde, 0.1 ml deionized, RNase free, water, 0.1 ml glycerol and 0.08 ml 10% (w/v) bromophenol blue. Formamide (Fisher Scientific) was deionized by mixing with analytical grade-ionexchange bed resin (AG 501- X8 CD, 20-50 mesh, fully regenerated) (Bio Rad), until the pH was near 7.0. Deionized formamide was stored at -20^0 C. Agarose gels (1%, w/v) were prepared by melting 0.5 g of agarose (SeKem) in 5 ml 10 X MOPS and 42 ml DEPC treated distilled water. The agarose was melted in a microwave oven and the total volume was adjusted to 47.5 ml with water, allowed to cool to about 50^0 C and 2.5 ml formaldehyde was introduced and mixed well. The gels were poured in 10 cm X 6 cm mini gel trays. After pouring, gels were kept for 1 hr at room temperature. The gels were pre-electrophoresed for 1/2 hr before samples were loaded. RNA samples were prepared by mixing 5 μl RNA in 2 mM EDTA with 25 μl electrophoresis sample buffer and heating at 65^0 C for 15 min. After heat denaturing, RNA was mixed with 1 μl 1 mg/ml ethidium bromide, loaded onto the gel and

electrophoresed at 50V for 3-4 hr at room temperature. Total RNA isolated from CTV infected Citrus paradisi cv. Mash and BRL RNA ladder was used as markers.

Northern Blotting of RNA

After electrophoresis, gels were visualized on UV light (310 nm) and photographs were taken using a LP 3 Land camera with type 660 polaroid film. The gels were then soaked in 20 X SSC for 10 min with slight agitation. The gels were then placed on a piece of 3 MM paper which had been pre-soaked with 20 X SSC. The transfer of RNA onto nitrocellulose (26) was performed by capillary blotting (27) in 20 X SSC buffer. Nitrocellulose paper was cut to the size of the gel, soaked in sterile water for 10 min and transferred to 20 X SSC for 30 min. The nitrocellulose paper was placed on the gel and filter papers and a stack of paper towels were placed on top of it and 500 g weights were placed on the top. The transfer was continued for 20 -24 hr.

Prehybridization and Hybridization of Northern Blots and Dot-blots

Prehybridization and hybridization was in the presence of 50% formamide. Hybridization buffer contained 5 X Denhardt's solution, 50% formamide (v/v), 0.1% SDS (w/v), 100 µg yeast tRNA and 5 X SSPE or SSC. Formamide was deionized as described above. Denhardt's solution (1 X) contained 0.02% (w/v) ficol, 0.02% (w/v)

polyvinylpyrrolidone and 0.02% (w/v) BSA. A stock solution of 50 X Denhardt's (6) solution was made and stored at -20^0 C. Prehybridization was performed for 1 hr at 42^0 C in a Seal- A-Meal bag containing hybridization solution without the probe. Hybridization solution was filtered through 0.2 μm pore size filter before use. About 5 ml of hybridization solution was used for hybridization of a 10 cm X 6 cm nitrocellulose blot. After pre-hybridization, the solution was replaced with fresh hybridization solution containing the cDNA probe. The cDNA probe was heat denatured in boiling water for 5 min and quench cooled on ice before mixing with hybridization solution. The Seal-A-Meal bag containing probe, hybridization solution, and nitrocellulose blot was heat sealed after carefully removing air bubbles. Hybridization was carried out for 24 hr at 42^0 C with slight agitation. After hybridization the solution was removed from the bag and stored at -20^0 C for reuse. Blots were placed in a plastic container for washing. The first 2 washes were in 2 X SSC with 0.1% SDS (w/v), each for 10 min at room temperature followed by two washes in 0.1 X SSC and 0.1% SDS (w/v), each 10 min at room temperature. The fifth wash was in 0.1 X SSC and 0.1% SDS (w/v) at 42^0 C for 1 hr. The final wash was the same as the fifth but with a temperature of 60^0 C.

Autoradiography

After the washing steps, nitrocellulose blots were put

into Seal-A-Meal bags and placed in film cassettes between Cronex (Dupont) lighting plus intensifying screens and X-ray film. Film cassettes with blots and X-ray films were store at -80^0 C. Exposure time was determined by the counts detected in the blot by the Geiger counter. After appropriate exposure time, films were taken to the darkroom and developed.

RESULTS

Detection of Virus Using cDNA Probes

cDNA probes synthesized using dsRNA as a template gave specific reactions in virus detections. Specificity of the probes was increased when dsRNA was treated with RNase under high salt conditions before use as a template for cDNA synthesis (fig 15). Using such a probe, it was possible to detect the presence of viral sequences in mealybugs taken from diseased plants, and in symptomless pineapple plants and of one grass species. Virus was not detected in healthy pineapple plants obtained from breeding stocks of Maui Land and Pine Company or in agave plants (another mealybug colonizing plant) (fig. 16). Among the wild grass species tested for the presence of viral sequences, only Paspalum urvullei L. gave positive reactions (fig 17). In the field, mealybugs were observed on these plants. The presence of virus in mealybugs taken from diseased plants, but not in mealybugs taken from agave was shown in the dot-blot assay. The cDNA probe was sensitive enough to detect virus in a

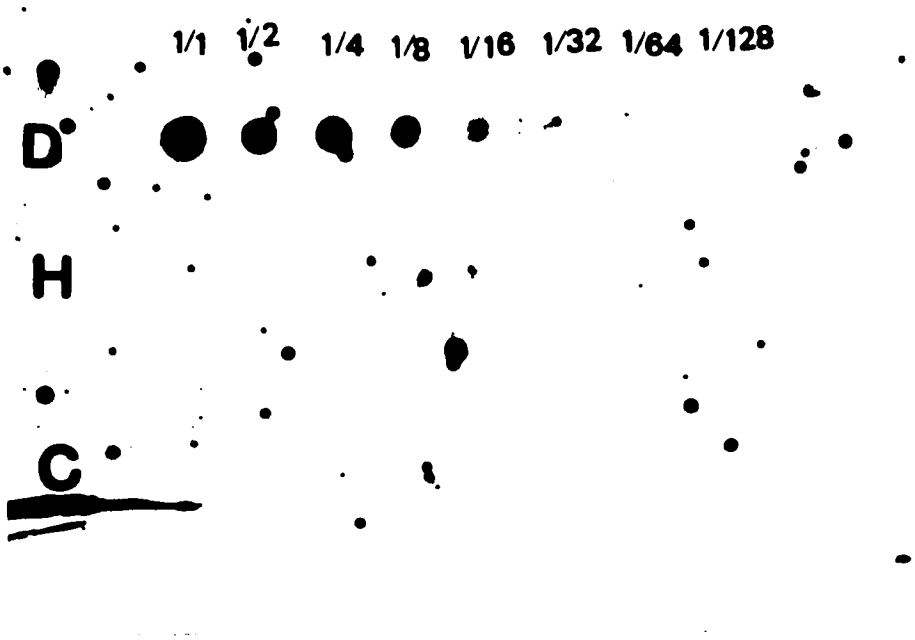

Fig. 15. Dot-blot assay using total RNA isolated from diseased pineapple (D), total RNA isolated from healthy pineapple (H), and total RNA isolated from CTV infected citrus (C). RNA samples blotted onto nitrocellulose were hybridized to cDNA synthesized from RNase A treated dsRNA.

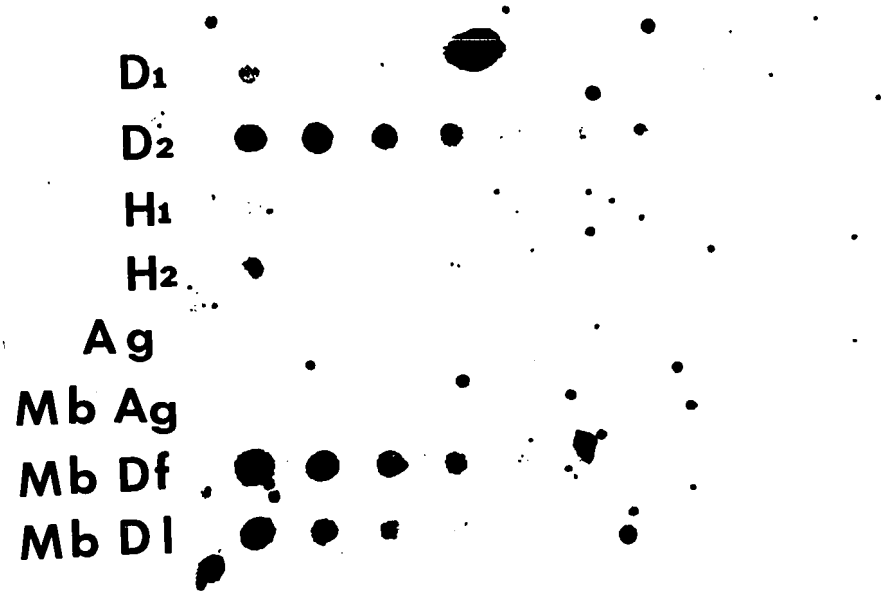

Fig. 16. Dot-blot hybridization with cDNA probe synthesized from RNase A treated dsRNA isolated from diseased pineapple plants. D1 & D2; leaf extract from different diseased plants. H1 & H2; leaf extract from two different healthy plants. Ag; leaf extract from Agave spp. MbAg; extract of 2 mealybugs taken from agave plants. MbDf; extract of 2 mealybugs taken from diseased pineapple fruits. MbDl; extract of 2 mealybugs taken from diseased pineapple leaf.

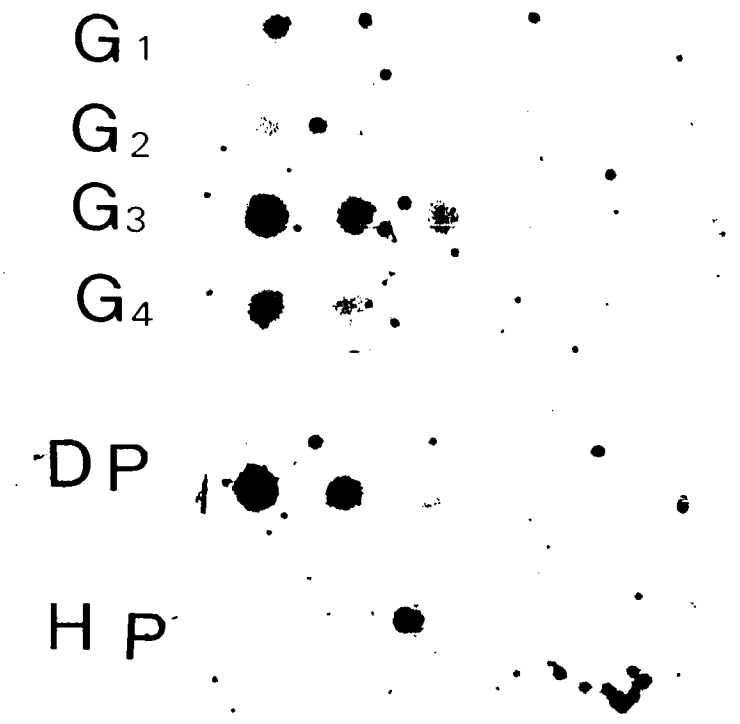

Fig. 17. Dot-blot hybridization of cDNA probe synthesized from dsRNA to different plant samples. G1; leaf extract from Andropogon insularis. L., G2; leaf extract from Paspalum urvullei. L., G3; extract from root of G2 sample, G4; leaf extract from Paspalum urvillei. L., DP; leaf extract of diseased pineapple, and HP; leaf extract of healthy pineapple plant.

single mealybug. In some cases, mealybugs taken from diseased plants and fed 2 days on squash gave a positive reaction for virus, (data not shown) suggesting that the virus could be retained in mealybugs for a few days.

Northern Blot Analysis

Following the virus isolation procedure, attempts were made to isolate ssRNA from virus. Formaldehyde gel electrophoresis revealed that RNA isolated from virus was degraded, and ran as a smear in gels. When this type of a gel was transferred onto nitrocellulose, and hybridized with a cDNA probe synthesized from RNase treated dsRNA the probe was hybridized to a degraded RNA smear on the lane where ssRNA isolated from purified virus was loaded and gave a strong signal where dsRNA loaded (fig. 18). The size of denatured dsRNA was similar to ssRNA because denatured dsRNA runs as ssRNA. These results show that viral RNA is homologous to dsRNA and therefore the major dsRNA observed in polyacrylamide gel electrophoresis is the replicative form of viral RNA. Attempts were made to isolate total RNA from diseased and healthy plants in order to estimate the size of the viral RNA and possibly to detect subgenomic RNAs produced in infected plants. Northern blot analysis revealed that the viral component of total RNA from diseased plants ran in the formaldehyde gels as a smear and hybridized to the cDNA probe synthesized from dsRNA. The largest RNA species corresponded to half the size of dsRNA

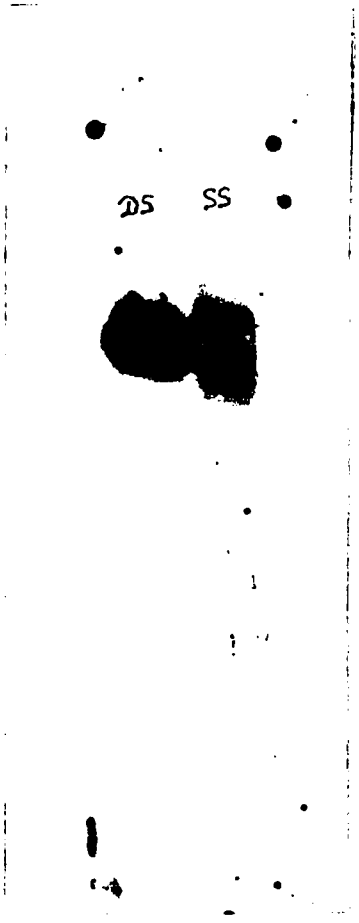

Fig. 18. Northern blot hybridization using a cDNA probe synthesized from dsRNA; dsRNA (DS), and viral RNA (SS).

and could be the viral RNA (fig. 19, lane D2). It was also observed that several small species of RNA are present (shown by arrow in fig. 19) in the total RNA isolated from diseased plants and that these species also hybridized with cDNA synthesized from dsRNA. These low molecular weight bands could be subgenomic RNAs produced by viral RNA upon infection. In the lanes loaded with total RNA isolated from healthy plants, a few low molecular weight bands were observed but they did not hybridize to cDNA probes. It was also observed that total RNA extracted from diseased pineapple degraded upon storage in buffers. As shown in the fig. 19, lane D1, a weak signal was detected at the end of the lane suggesting that RNA has been degraded. RNA extracted from purified virus also seems to degrade upon storage. In fig. 19, lane V shows the hybridization of cDNA probe to degraded viral RNA.

DISCUSSION

The most important factor in the characterization of any new virus and defining its epidemiology and host-plant interaction is the availability of a sensitive diagnostic technique. In this study I have investigated the use of dsRNA as a template for the synthesis of radiolabeled cDNA probes specific to viral RNA. The cDNA probes, synthesized using dsRNA without pretreatment with RNase under high-salt conditions, gave non-specific reactions under low-stringency conditions, possibly because of contamination of viral

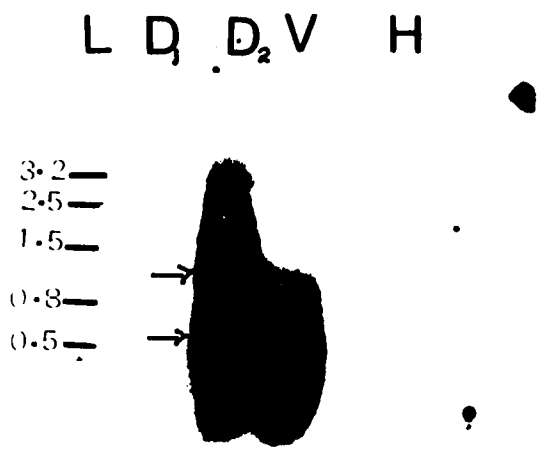

Fig. 19. Northern blot hybridization of cDNA probe with RNA extracts. Lane L, BRL ladder (numbers on the right side indicate the molecular weight of RNA ladder); lane D1, total RNA isolated from diseased pineapple and stored for more than 2 weeks; lane D2, total RNA isolated from diseased pineapple by guanidium thiocyanate method; lane V, RNA isolated from purified virus; and lane H, total RNA isolated from healthy pineapple.

dsRNA with small amounts of plant RNA during CF-11 chromatography of dsRNA. However, this problem was overcome by treatment of dsRNA with RNase A under high salt conditions before using it as a template for cDNA synthesis. Under these conditions, ssRNA was digested and remaining nucleic acids contained dsRNA only, which was specific for virus. The use of such a probe resulted in a specific positive reaction in virus diagnosis. In future studies these probes can be effectively used.

The detection of viral sequences in mealybugs suggests that during their feeding on pineapple plants they can acquire virus and they may be involved in the transmission of this virus to pineapple plants in plantations. Mealybugs taken from agave gave negative reactions for the presence of viral sequences but single mealybugs taken from diseased pineapple plants showed a positive reaction with cDNA probes.

The presence of virus in most field-grown pineapple shows that the virus exists in symptomless plants. Pineapple plants in commercial plantations in the Hawaiian Islands have been vegetatively propagated for more than 100 years. It is possible that a mild strain of this virus can perpetuate among vegetatively propagated clones. Mealybugs may transmit a severe strain of the virus from another host resulting in the expression of wilt symptoms upon infestation. The presence of virus in grass species

supports this hypothesis. It has been previously reported that it is not possible to produce wilt symptoms upon feeding of mealybugs taken from some sources (1, 2, 17). In Ito's studies it was suggested that another strain introduced into plants already infected with an existing mild strain can cause severe wilt symptoms (17). This phenomenon has been reported for citrus tristeza virus (CTV) in California (13, 14, 18), another closterovirus, and the presence of several strains has been documented in molecular hybridization studies (25). Based on this study and evidence from extensive biological studies on mealybug-wilt it is reasonable to speculate that this disease is caused by a severe strain of the virus transmitted from alternate outside hosts via mealybugs.

Northern-blot analysis shows that dsRNA is complementary to viral RNA. Total RNA isolated from diseased plants revealed the presence of several small RNAs in diseased plants. Investigation of these small RNAs may lead to an understanding of the genome expression of this virus. CTV, another closterovirus, produces subgenomic RNAs and molecular hybridization studies have been used to understand the genome expression of this virus (25). The dot-blot procedure and cDNA probe procedure reported in this study can be successfully used in investigations of the presence of any strains, presence of alternate hosts, and understanding of the etiology of this important disease.

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CHAPTER IV

CONCLUSIONS

An understanding of the etiology of pineapple mealybug-wilt includes the investigation of the possible involvement of a virus with the disease. Because reported data suggest that a virus may be involved in the etiology of this disease (2, 3, 10), the isolation and characterization of a virus from diseased pineapple is another step toward understanding the disease. This study the hypothesized that a virus is involved with pineapple plants infected with mealybug wilt. In chapter two of this dissertation evidence was presented for the association of dsRNA with infected plants. Evidence is also presented documenting the occurrence of long, flexuous, rod-shaped virus particles in infected plants. The role of this virus in the etiology of the disease has yet to be determined. Based on morphology, dsRNA patterns and molecular weight of the coat protein, the virus isolated may be assigned to the type II closterovirus group. Purification protocols developed for the isolation of this virus may also be useful in the isolation of other flexuous, rod-shaped viruses. The cDNA probes developed for virus detection are sensitive and can be used in studies of the etiology of this disease. In this study, virus was detected in mealybug-wilt infected pineapple plants, mealybugs taken from diseased pineapple plants and in one

species of grass commonly found around commercial pineapple plantations. The virus was not detected in pineapple plants which had never been exposed to mealybugs.

Early workers on this disease have suggested that the wilt-inducing factor may exist in symptomless plants and these plants can serve as a positive sources for wilt inducing factor (1, 4). Ito suggested that a mild strain of some virus may exist in field-grown pineapple plants and that a severe strain of the virus may be transmitted by mealybugs, producing severe symptoms (8). The results of this study confirm that a clostero-type virus is present in most field grown-pineapple plants with severe wilt symptoms. It is possible that a mild strain of this virus may be perpetuated among pineapple plants in Hawaii because these plants have been vegetatively propagated for more than 100 years.

The virus was also shown to be present in a grass species surrounding pineapple fields. It may be possible that mealybugs can acquire a severe strain of the virus from outside the fields and introduce it into new plantations resulting in production of severe wilt symptoms in those plants. This type of a interaction has been reported to occur in citrus plantations in California and Texas with citrus tristeza virus, another closterovirus.(5, 6, 7, 9).

The results presented in this dissertation can be correlated with the biological data accumulated on this

disease. The next logical step towards understanding the etiology of this important disease is to document the presence of other strains of the virus using DNA technology and monoclonal antibody techniques. The purification protocols and diagnostic techniques developed in this study are of great value in understanding virus-plant interactions, virus-vector interactions and in completing Koch's postulates for this disease.

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