

Serology of a Closteroviruslike Particle Associated with Mealybug Wilt of Pineapple

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ABSTRACT

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Antiserum was produced to a closteroviruslike particle consistently associated with pineapple plants (*Ananas comosus* 'Smooth Cayenne') affected with mealybug wilt of pineapple. Purified immunoglobulins (IgG) specific to pineapple virus and goat anti-rabbit IgG were used to develop an indirect enzyme-linked immunosorbent assay (ELISA) for detection of pineapple virus in crude pineapple leaf extracts. Specific reactions between IgG specific to pineapple virus and virus from pineapple plants

were shown by serologically specific electron microscopy, Ouchterloney double diffusion tests, and ELISA. The etiological significance of this pineapple virus remains to be determined. Detection of virus in crude leaf extracts with ELISA will enable identification of potentially virus-free plants for use in testing Koch's postulates, determination of infection in pineapple before symptom expression, and study of the plant host range of the virus.

Additional keywords: diagnosis, *Dysmoccocus brevipes*, *Dysmoccocus neobrevipes*, immunology, serology, toxin.

A wilt disease of pineapple was first described in Hawaii in the early 1900s (19) and since that time has been reported as a serious problem in most areas of the world where pineapple is cultivated (2,3,6,8,18). The disease has been associated consistently with the presence of mealybugs (1,2,17,24), thus earning the name mealybug wilt of pineapple. The wilt disease syndrome was first thought to be caused by toxins present in salivary secre-

tions from feeding mealybugs (2-7,10). Later, biological data (8,9) suggested that mealybug wilt of pineapple was not caused solely by toxins in mealybug salivary secretions, but that an unidentified "latent transmissible factor" also was associated with the disease.

Recently, a long, flexuous, rod-shaped virus was isolated from pineapple plants with symptoms of mealybug wilt (14,15). Based upon virus morphology, molecular weight of the coat protein (23,000 Da), and the approximate size of the genomic RNA, this pineapple virus has been assigned tentatively to the type II closterovirus group. The consistent association of these closterovirus-

like particles with pineapple plants showing symptoms of mealybug wilt (14,15) and reports of mealybug transmission of other closteroviruses (16,23) implicates pineapple virus as the possible causal agent of mealybug wilt of pineapple. Conclusive demonstration of the etiological significance of pineapple virus requires completion of Koch's postulates and as yet remains to be determined.

A major factor hindering completion of Koch's postulates for pineapple virus has been the lack of a rapid diagnostic test. Pineapple virus is not mechanically transmissible by sap inoculation or grafting. Inoculation of virus with mealybugs is laborious, and symptoms of mealybug wilt cannot be seen for 4-18 mo after inoculative feedings. Furthermore, symptomology is not always an accurate diagnostic tool because plants infected late in growth and development often produce symptomless vegetative growth, fruit, and crowns (the vegetative portion of the pineapple fruit) (8,22). Commercial pineapple clones have been propagated from crowns for more than 100 yr, and crowns from recovered mealybug-wilt plants produce symptomless plants that can serve as "positive sources" of the disease agent (8). Thus, a diagnostic test that does not rely on symptomology is critical for development of a large stock of known virus-free plants to be used in testing Koch's postulates, for evaluation of transmission experiments, and for use in epidemiological studies. The purpose of this investigation was to develop rabbit immunoglobulins (IgG) specific to pineapple virus, to use this IgG to develop an enzyme-linked immunosorbent assay (ELISA) to be used as a rapid diagnostic tool, and to test the reliability of this ELISA as a tool for sampling plants for pineapple virus presence.

MATERIALS AND METHODS

Plant material and virus purification. Approximately 330 commercially cultivated pineapple plants (*Ananas comosus* (L.) Merr. 'Smooth Cayenne'), some exhibiting severe symptoms of mealybug wilt and some symptomless but potentially infected, were collected on the Hawaiian islands of Maui, Oahu, and Lanai and used for virus purification and for serologically specific electron microscopy (SSEM), immunodiffusion, and ELISA. Virus-free control plants (shown to be free of double-stranded RNA and virus particles) were obtained from the breeding stock collection of Maui Pineapple Company, Ltd. The methods of Gunasinghe and German (14,15) were used for all purifications of pineapple virus from pineapple. Tobacco mosaic virus (TMV) for use in SSEM was purified from *Nicotiana benthamiana* Domin using the procedure of Gooding and Hebert (13).

Antiserum production. A polyclonal antiserum was produced in a New Zealand white rabbit against preparations of pineapple virus purified as described (14) from 200 g of diseased pineapple tissue. After the second polyethylene glycol (PEG) precipitation, the pellet was dissolved in 0.015 M phosphate buffer (pH 8.0), clarified by low-speed centrifugation, and mixed thoroughly with 1 ml of Freund's incomplete adjuvant. Intramuscular injections were performed weekly for 17 wk. The titer was monitored by microprecipitin test after 5 wk. After week 17, the rabbit was bled by heart puncture. The blood was allowed to clot, and serum was recovered after centrifugation. Serum was adjusted to 0.01% sodium azide and stored at 4 C.

Immunodiffusion. Ouchterlony double diffusion assays were performed using agar without sodium dodecyl sulfate (SDS) (21). Tissues (1 g) were ground in 1 ml of water and mixed with 1 ml of 1% SDS (in double distilled water) immediately before loading in the antigen wells. Plates were stored for 24 hr and then read over transmitted fluorescent light. Antiserum was tested in this manner with extracted mealybug wilt-diseased pineapple tissue, partially purified pineapple virus, extracted healthy pineapple tissue, tissue infected with grapevine leafroll virus, and healthy grapevine tissue (grape tissues obtained from Francis Zee, National Germplasm Repository, Hilo, Hawaii).

Immunoglobulin preparation. The techniques of Clark and Adams (11) and Zee et al (25) were used to purify IgG. Whole pineapple virus antiserum was preabsorbed with virus-free plant

material before IgG fractionation using the methods of Gonsalves et al (12). Cross-absorbed material then was used for IgG fractionation. After fractionation, the same procedure was used to preabsorb IgG with 2 ml of virus-free plant extract.

Serologically specific electron microscopy. Formvar and carbon-coated copper grids (200-mesh, Type B, Ted Pella, Inc., Redding, CA) were used for all preparations. One drop (5 μ l) of purified pineapple virus was placed on the carbon side of the grid for 5 min, wicked away with filter paper, washed with 100 μ l of bacitracin (300 μ g/ml of double distilled water), and then wicked away with filter paper. Grids prepared in this way were floated for 30 min on 5 μ l of cross-absorbed pineapple virus antiserum at 37 C, washed with 100 μ l of bacitracin, and stained with phosphotungstic acid (2%, pH 6.8). Using a similar procedure, control grids were prepared with pineapple virus, TMV, and IgG specific to TMV (obtained from the American Type Culture Collection).

ELISA protocol. An indirect ELISA technique was used (11,20) in which plates were coated with test antigens from crude leaf extracts (pooled samples from one young and one mature leaf, 114 mg of plant tissue/1 ml of buffer), suspended in coating buffer (pH 9.6, 1.59 g of Na_2CO_3 , 2.93 g of NaHCO_3 , 0.2 g of NaN_3 suspended in 1 L of H_2O), and incubated for 3 hr. Plates then were washed four times with phosphate-buffered saline plus Tween (PBS-Tween, pH 7.4, 8.0 g of NaCl , 0.2 g of KH_2PO_4 , 2.9 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.2 g of KCl , 0.2 g of NaN_3 suspended in 1 L of H_2O plus 0.5 ml of Tween-80 per liter), loaded with 1% powdered nonfat milk suspended in coating buffer, and incubated for 1 hr. After incubation, plates were washed four times with PBS-Tween, loaded with fractionated pineapple virus IgG suspended in enzyme buffer (PBS-Tween, 2% water-soluble polyvinylpyrrolidone-40, 0.2% ovalbumin) that had been preabsorbed with extracts of healthy pineapple tissue as described by Gonsalves et al (12), and placed in a refrigerator overnight (12-14 hr, 4 C). Plates then were washed four times with PBS-Tween, loaded with goat anti-rabbit IgG conjugated to alkaline phosphatase (A-8025, Sigma Chemical Co., St. Louis, MO) suspended in enzyme buffer, and incubated for 3 hr. All incubations were done at 30 C.

Plates were washed again four times with PBS-Tween, loaded with phosphatase substrate (104-105, Sigma Chemical Co.) suspended in substrate buffer (pH 9.8, 97 ml of diethanolamine, 800 ml of H_2O , 0.2 g of NaN_3 suspended in 1 L), and allowed to develop for 45 min to 1 hr. Absorption was measured at 405 nm with an EIA EL-307 reader (Bio-Tek Instruments, Inc., Burlington, VT). Controls with buffer, virus-free, and known

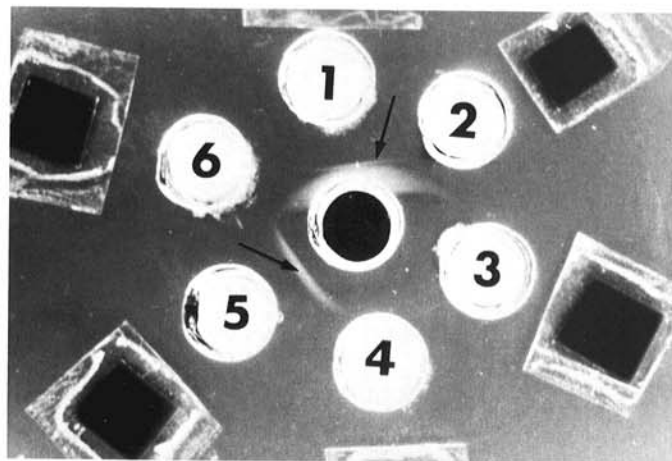


Fig. 1. Reactions of antiserum prepared to pineapple virus against leaf extracts from: well 1, mealybug wilt-diseased pineapple (cultivar Smooth Cayenne); well 2, buffer; well 3, leaf extracts from healthy grapevine; well 4, leaf extracts from healthy pineapple (cultivar Smooth Cayenne); well 5, partially purified pineapple virus; and well 6, leaf extracts from grapevine leafroll virus-infected tissue. Arrows indicate precipitin reactions to partially purified pineapple virus and leaf extract from diseased pineapple.

virus-infected plant samples were included in all ELISA tests. Virus-free and known infected control plants were frozen in liquid nitrogen, powdered, and maintained frozen for use as standard controls. This assay was performed with 330 pineapple plants.

RESULTS

Precipitin reactions specific to partially purified pineapple virus and to crude leaf extracts from pineapple with symptoms of mealybug wilt were visible in immunodiffusion tests (Fig. 1). Two precipitin bands occurred when crude leaf extracts from pineapple with symptoms of mealybug wilt were used, whereas only one precipitin band occurred when partially purified pineapple virus was used. No reaction was observed between pineapple virus antiserum and grapevine leafroll virus-infected tissues or healthy grapevine tissues. Specificity between pineapple virus and pineapple virus IgG is further supported by SSEM (Figs. 2A and B). Figure 2A shows that pineapple virus particles purified from diseased pineapple were decorated with IgG prepared to pineapple virus. In reciprocal tests, IgG prepared to TMV decorated purified TMV particles but did not decorate pineapple virus (Fig. 2B).

When indirect ELISA was used, IgG from pineapple virus antiserum reacted to pineapple virus antigens from crude leaf extracts but not with buffer controls or crude leaf extracts from virus-free pineapple plants. Optimum reactivity was obtained

using crude leaf extracts from 114 mg of plant tissue/ml of buffer, 1/1,000 (v:v) dilution of purified pineapple virus IgG, and 1/2,000 (v:v) dilution of goat antirabbit IgG enzyme conjugate (Fig. 3). When buffer, uninfected, and infected standards from 12 different ELISA plates were compared, final absorbance was found to be very consistent: 0.084 ± 0.01 , 0.086 ± 0.007 , and 0.94 ± 0.08 for buffer, uninfected, and infected controls, respectively ($n = 24$, per standard). Background reactions were minimized by preabsorbing IgG with healthy leaf extracts before and after fractionation and again before use. The reliability of the assay was further demonstrated by purifying pineapple virus from ELISA-positive pineapple plants and viewing virus particles with SSEM (Fig. 2A and B). Virus was purified from plants tested with ELISA and having optical density readings greater than 0.4. The standard deviation around mean optical density of positive standards was ± 0.08 ; therefore plant samples resulting in optical density readings greater than 0.3 with this ELISA were considered to be positive. Strong specific reactions were observed to infected plant samples kept frozen for more than 6 mo. In contrast, reactivity decreased in plant samples taken from infected pineapple plants held at room temperature in low light for more than 2 wk.

Our ELISA results demonstrate that cultivated pineapple in the Hawaiian islands is widely infected with pineapple virus. When plants from the field were randomly sampled, final absorbance

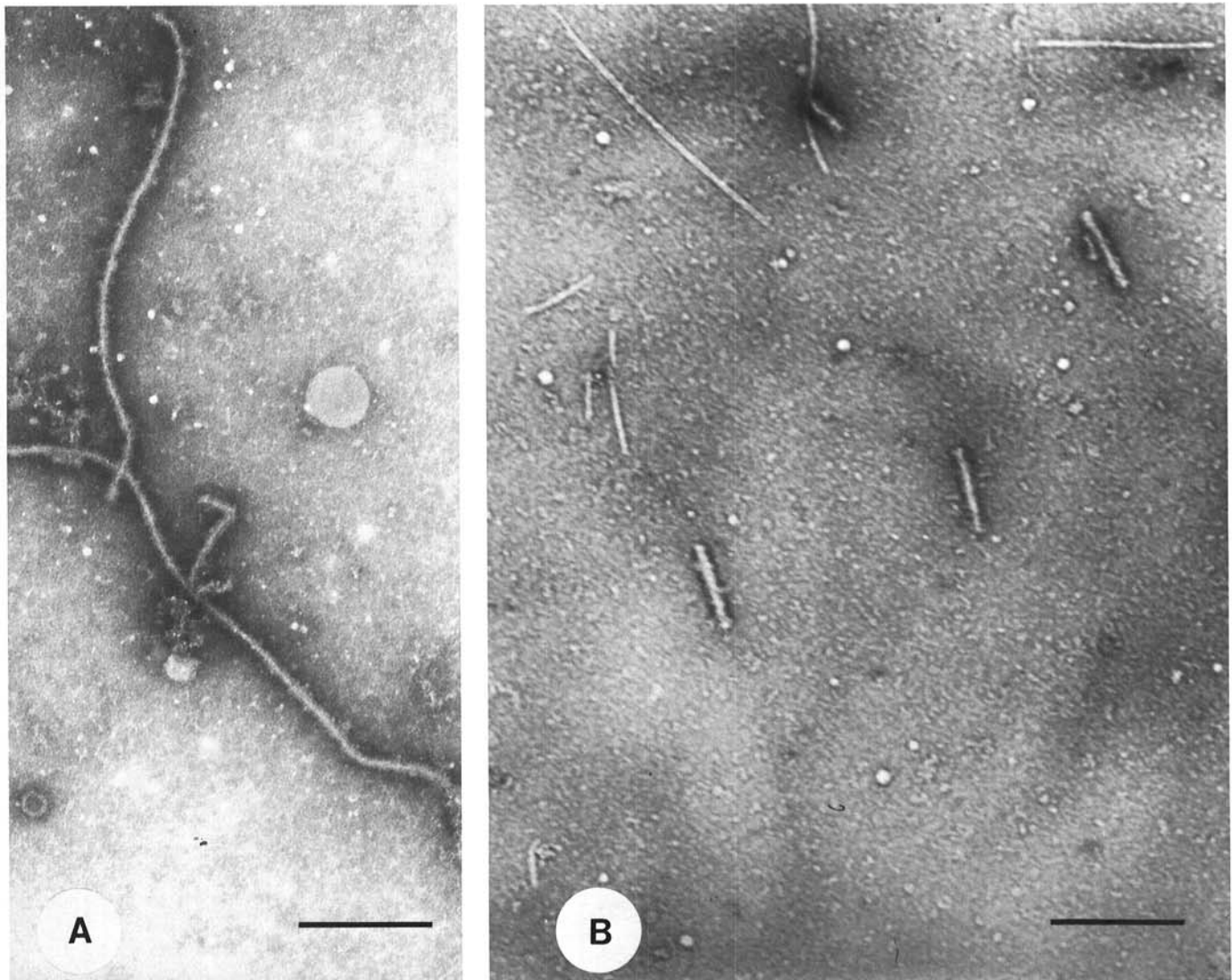


Fig. 2. A, Serologically specific electron micrograph showing decoration of pineapple virus particles with antiserum prepared to pineapple virus. Bar = 457 nm. B, In reciprocal tests, antiserum to tobacco mosaic virus decorated tobacco mosaic virus particles and not pineapple virus. Bar = 475 nm.

of plant samples varied, presumably because of differences in virus titer or the presence of virus strains (Fig. 4).

DISCUSSION

Data from SSEM, immunodiffusion, and ELISA indicate that pineapple virus IgG is specific for partially purified or purified pineapple virus and for pineapple virus in crude pineapple leaf extracts. In immunodiffusion tests (Fig. 1), the two precipitin bands forming in response to crude leaf extracts from diseased pineapple suggest that virus in these preparations is partially degraded, thus diffusing at different rates through the agar. In the same immunodiffusion test, the single precipitin band forming in response to partially purified virus indicates that purified virus preparations contain predominantly whole virus. The lack of precipitin bands when grapevine leafroll virus (a mealybug-transmitted plant closterovirus) was used demonstrates that the two viruses are not serologically related.

The ELISA protocol we report here is well adapted for use as a diagnostic screening tool for large numbers of plant samples because it permits use of crude leaf extracts, produces very low background readings, and gives consistent results from plate to

plate. As a further demonstration of the reliability of this assay, ELISA-negative plants have been shown to be free of double-stranded RNA and pineapple virus, whereas pineapple virus has been consistently purified from ELISA-positive pineapple and detected with the transmission electron microscope (Fig. 2A and B).

Our results suggest that cultivated pineapple (cultivar Smooth Cayenne) in the Hawaiian islands is widely infected with pineapple virus (Fig. 4). Pineapple plants that recover from mealybug wilt usually produce symptomless crowns. Carter (8) found that plants grown from these crowns were symptomless, positive sources of mealybug wilt. He postulated that a hypothetical "latent transmissible factor" was passed through vegetative propagation. All of the pineapple produced in Hawaii is Smooth Cayenne, and propagation of this clone has been through vegetative propagation of crowns since 1820, when the Cayenne variety came to Europe from French Guiana. Thus, it is not surprising that pineapple virus is widespread in cultivated pineapple. The variation in reactivity of samples from different plants that we observed may be attributed to variation in virus titer in different plants or potentially to the existence of different strains of the same virus in pineapple. Future research will be directed toward detection of possible strain differences in pineapple virus from plants with and without mealybug-wilt symptoms.

Although we found a consistent association between pineapple virus and pineapple affected with mealybug-wilt disease (14,15), the etiological significance of these closteroviruslike particles remains to be determined. Several factors have prevented conclusive determination of the etiology of mealybug wilt of pineapple. Namely, there is no local lesion host, the virus cannot be mechanically transmitted, the virus apparently has a long incubation period, and dependence upon symptom expression is not always a reliable means of identifying infected plants. The indirect ELISA protocol we have reported here is a critical first step toward determining whether pineapple virus is the causal agent of mealybug wilt of pineapple. Detection of virus in crude pineapple leaf extracts will enable us to identify virus-free plants to be used in transmission tests and to determine whether infection has occurred in inoculated pineapple before symptom expression. In addition, the ELISA reported herein will be invaluable as a tool for studying the epidemiology and plant host range of this virus.

LITERATURE CITED

1. Beardsley, J. W., Su, T. H., McEwen, F. L., and Gerling, D. 1982. Field investigations on the interrelationships of the big-headed ant, the gray pineapple mealybug, and pineapple mealybug wilt disease in Hawaii. *Proc. Hawaii. Entomol. Soc.* 24:51-67.
2. Carter, W. 1932. Studies of populations of *Pseudococcus brevipes* (Ckl.) occurring on pineapple plants. *Ecology* 13:296-304.
3. Carter, W. 1933. The pineapple mealy bug, *Pseudococcus brevipes*, and wilt of pineapples. *Phytopathology* 23:207-242.
4. Carter, W. 1935. Studies on the biological control of *Pseudococcus brevipes* (Ckl.) in Jamaica and Central America. *J. Econ. Entomol.* 28:1037-1041.
5. Carter, W. 1945. Some etiological aspects of mealybug wilt. *Phytopathology* 35:305-315.
6. Carter, W. 1949. Insect notes from South America with special reference to *Pseudococcus brevipes* and mealybug wilt. *J. Econ. Entomol.* 42:761-766.
7. Carter, W. 1951. The feeding sequence of *Pseudococcus brevipes* (Ckl.) in relation to mealybug wilt of pineapples in Hawaii. *Phytopathology* 41:769-780.
8. Carter, W. 1963. Mealybug wilt of pineapple; a reappraisal. *Ann. N. Y. Acad. Sci.* 105:741-764.
9. Carter, W. 1973. Pages 274-308 in: *Insects in Relation to Plant Disease*. 2nd ed. John Wiley & Sons, New York.
10. Carter, W., and Ito, K. 1956. Study of the value of pineapple plants as sources of the mealybug wilt factor. *Phytopathology* 46:601-605.
11. Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* 34:475-483.
12. Gonsalves, D., Trujillo, E., and Hoch, H. C. 1986. Purification and some properties of a virus associated with cardamom mosaic, a new member of the potyvirus group. *Plant Dis.* 70:65-69.

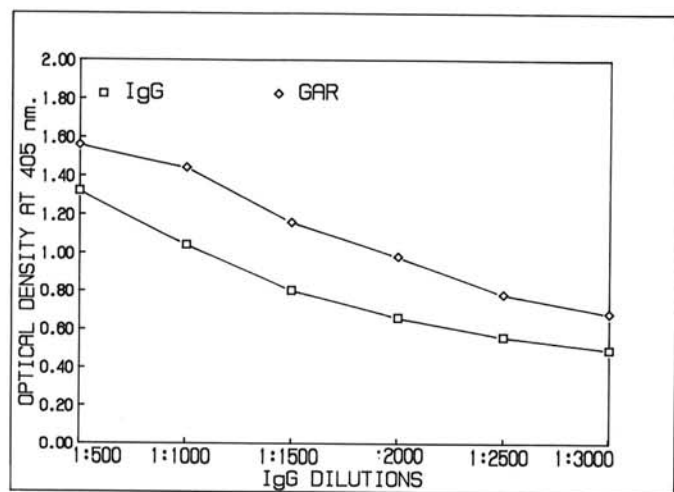


Fig. 3. The line denoted by the diamond-shaped symbol represents reactivity of pineapple leaf extracts positive for pineapple virus when immunoglobulin (IgG) to pineapple virus was held constant at 1/1,000 and goat anti-rabbit IgG (GAR) was used at various dilutions. The line denoted by the square symbol represents reactivity of pineapple leaf extracts when GAR was held constant at 1/2,000 and pineapple virus IgG was used at various dilutions.

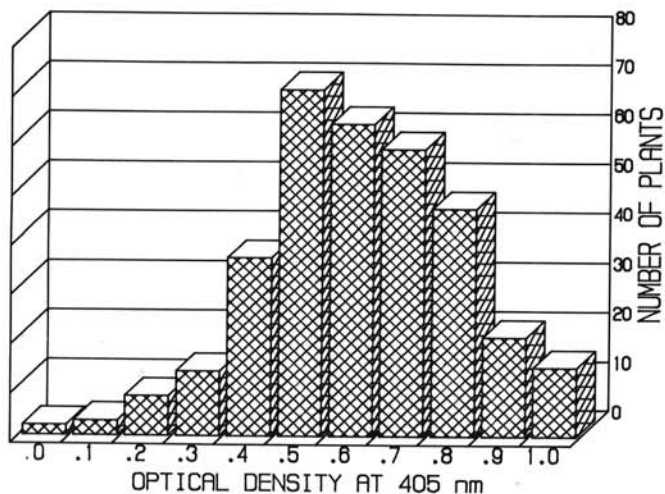


Fig. 4. Variation in the reactivity (expressed as optical density at 405 nm) of crude leaf extracts from 330 pineapple plants tested with enzyme-linked immunosorbent assay.

13. Gooding, G. V., and Hebert, T. T. 1967. A simple technique for purification of tobacco mosaic virus in large quantities. *Phytopathology* 57:1285.
14. Gunasinghe, U. B., and German, T. L. 1987. Further characterization of a virus associated with mealybug wilt of pineapple. (Abstr.) *Phytopathology* 77:1776.
15. Gunasinghe, U. B., and German, T. L. 1989. Purification and partial characterization of a virus from pineapple. *Phytopathology* 79:1337-1341.
16. Hewitt, W. B. 1968. Viruses and virus diseases of the grapevine. *Rev. Appl. Mycol.* 47:433-455.
17. Illingworth, J. F. 1931. Preliminary reports on evidence that mealybugs are an important factor in mealybug wilt. *J. Econ. Entomol.* 24:877-889.
18. Ito, K. 1962. Additional immunological evidence supporting the virus nature of mealybug wilt. *PRI News* 10:158-162.
19. Larsen, L. D. 1910. Diseases of pineapple. *Hawaii Sugar Plant. Assoc. Pathol. Physiol. Ser. Exp. Stn. Bull.* 10:1-72.
20. Lommel, S. A., McCain, A. H., and Morris, T. J. 1982. Evaluation of indirect enzyme-linked immunosorbent assay for detection of plant viruses. *Phytopathology* 72:1018-1020.
21. Purcifull, D. E., and Batchelor, D. L. 1977. Immunodiffusion tests with sodium dodecyl sulfate (SDS)-treated plant viruses and plant virus inclusions. *Bull. Agric. Exp. Stn. Fla.* 88.
22. Rohrbach, K. G., Beardsley, J. W., German, T. L., Reimer, N. J., and Sanford, W. G. 1988. Mealybug wilt, mealybugs, and ants on pineapple. *Plant Dis.* 72:558-565.
23. Rosciglione, B., Castellano, M. A., Martelli, G. P., Savino, V., and Cannizzaro, G. 1983. Mealybug transmission of grapevine virus A. *Vitus* 22:331.
24. Sastry, K. S. M., and Singh, S. J. 1974. Wilt of pineapple. A new virus disease in India. *Indian Phytopathol.* 27:298-303.
25. Zee, F., Gonsalves, D., Goheen, A., Kim, K. S., Pool, R., and Lee, R. F. 1987. Cytopathology of leafroll-diseased grapevines and the purification and serology of associated closteroviruslike particles. *Phytopathology* 77:1427-1434.