# Closterovirus Infection and Mealybug Exposure Are Necessary for the Development of Mealybug Wilt of Pineapple Disease

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## ABSTRACT

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The roles of *Pineapple mealybug wilt-associated virus*es (PMWaVs) and mealybug (*Dysmicoccus* spp.) feeding in the etiology of mealybug wilt of pineapple (MWP) were evaluated. Container-grown pineapple (*Ananas comosus*) plants from five commercially grown Hawaiian proprietary selections and a field study utilizing a randomized complete block design were used to test four treatments for induction of MWP: PMWaV-1-free and PMWaV-1-infected plants maintained mealybug-free, and PMWaV-1-free and PMWaV-1-infected plants that received monthly applications of nonviruliferous mealybugs. A second PMWaV, PMWaV-2, was identified in some of the test plants during the course of these studies and was shown to be an integral factor in MWP etiology. Typical MWP symptoms developed only in plants infected with PMWaV-2 and exposed to mealybugs. MWP did not develop in PMWaV-1-free or PMWaV-1-infected plants that were exposed to mealybugs, or in mealybug-free plants infected with PMWaV-1, PMWaV-2, or both viruses. Plants from all five Hawaiian proprietary selections developed MWP when PMWaV-2 infected plants were exposed to mealybug feeding. A PMWaV-2-specific monoclonal antibody was produced that decorated the particles in immunosorbent electron microscopy and detected the virus in tissue blot immunoassays. PMWaV-2 was acquired and transmitted by pink and gray pineapple mealybugs (*Dysmicoccus* spp.) to pineapple plants, and these plants subsequently developed MWP symptoms while sustaining mealybug populations.

*Additional keywords*: badnavirus, *D. brevipes*, *D. neobrevipes*, insect transmission, pineapple closterovirus, virus vector.

Mealybug wilt of pineapple (MWP), one of the most serious diseases of pineapple (*Ananas comosus* (L.) Merr.), is present in all major pineapple-growing areas of the world (4,7,8,19,27,28, 37). MWP is characterized by severe tip dieback, reddening of leaves, and leaf wilting that can lead to total collapse of mature plants (5,8). A recovery phase, characterized by the absence of symptoms in new leaves, frequently is observed in Hawaii (6,8). Historically, MWP has been associated with mealybug feeding (5, 9,10,23,27). Several hypotheses have been proposed to explain the etiology of MWP, including mealybug salivary toxins (6), latent transmissible factors (8,9,10) or viral infection (11–15,20,30,31). Flexuous rod-shaped virus particles have been isolated from pineapple plants with MWP in Hawaii, Australia, and Cuba (4,12,18, 21,22,36,37). Formerly referred to as pineapple closterovirus (PCV) (19,21,22), this virus is currently referred to as *Pineapple mealybug wilt-associated virus* (PMWaV) (1,20,26,31,34,35). However, PMWaV is actually a complex of closteroviruses (26, 33). Based on particle morphology (15,19,22,35) and nucleic acid characteristics (26), PMWaVs have been placed in the family *Closteroviridae* (1,24).

PMWaV-1, which can be detected with monoclonal antibody (MAb) 35-6-5 (22), is not sap transmissible but can be acquired and transmitted by gray and pink pineapple mealybugs (*D. neobrevipes* Beardsley and *D. brevipes* Cockerell, respectively) (34). It is phloem limited and can be detected in roots, leaves, stems, fruit cores, and crowns of infected plants (21,22). PMWaV-1 has been detected in pineapple plants with and without symptoms throughout Hawaii (21) and the world (33), as well as in pineapple accessions maintained at the U.S. Department of Agriculture-Agricultural Research Service (USDA-ARS) National Clonal Germplasm Repository (NCGR) in Hilo, HI (22,33). The presence of this virus in symptomless plants correlates with reduced growth (29) and fruit yield (32). In field samples, there is a higher incidence of PMWaV-1 infection in plants with MWP symptoms than in symptomless plants (21). However, many PMWaV-1-infected plants do not show MWP symptoms. Therefore, PMWaV-1 infection is not consistently associated with MWP. These facts suggest that another factor or factors, such as mealybug feeding or another virus, may be involved in MWP symptom development.

A study with container-grown pineapple plants from five Hawaiian-grown commercial proprietary selections and a simultaneous field study conducted on the island of Maui, HI, were used to evaluate the involvement of PMWaV-1 and feeding by gray and pink pineapple mealybugs in MWP etiology. During the course of these studies, a second PMWaV, PMWaV-2, was found to be involved in MWP. PMWaV-2 has since been characterized (26) and surveys of commercially grown pineapple throughout the world and the Hawaiian Islands have shown a 99 to 100% correlation of MWP symptoms and the presence of PMWaV-2 (33). Here, we show that PMWaV-2 is mealybug transmitted and present evidence for the roles of PMWaV-2 and mealybug feeding in the etiology of MWP.

## MATERIALS AND METHODS

**Container-grown plant study.** Crowns used to establish plants were collected from fields of mature pineapple fruits of an *A. comosus* clonal selection (referred to here as selection 5), and four other proprietary clonal selections (selections 1 to 4) of *A. comosus* cv. Smooth Cayenne (21,33). Agronomic selections are referred to by number only due to the proprietary nature of the commercially grown selections in Hawaii. The PMWaV-1 infection status of each crown was determined using PMWaV-1-specific MAb 35-6-

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5 (22) in a tissue blot immunoassay (TBIA) as previously described (21). Crowns were tested for PMWaV-1 infection twice prior to planting and subsequently at monthly intervals. The PMWaV-2 infection status at the time of planting was unknown because a specific assay for this virus was not yet available. Crowns were planted in 12-liter plastic pots and grown outdoors. Irrigation and fertilization (20 g of Osmocote Plus 15-9-12 per pot; Scotts-Sierra Horticultural Products Co., Marysville, OH) were applied as needed. Plants were divided into four treatment groups as follows: PMWaV-1-free and PMWaV-1-infected plants maintained free of mealybugs, and PMWaV-1-free and PMWaV-1-infected plants that received monthly applications of nonviruliferous mealybugs, reared as previously described (34). The study was conducted from July 1998 to January 2000, with 10 plants from each of the five selections per treatment. The PMWaV-1-free treatment group consisted only of selections 1 to 4 because all crowns of selection 5 were PMWaV-1 infected. Each plant in mealybug-exposed treatments received 25 to 50 mixed-aged, nonviruliferous *D. brevipes* placed near the stem base and 50 to 100 mixed-aged, nonviruliferous *D. neobrevipes* placed in the center leaf whorl of plants at monthly intervals. Inoculations began 3 months after planting of crowns and continued for 12 months. Amdro ant bait (American Cyanamid Co., Parsippany, NJ) was used according to manufacturer's instructions to prevent ant infestations. Treatment groups were separated with cloth barriers to eliminate cross-contamination of mealybugs between treatments. Mealybugs could move from plant to plant within a treatment group. Plants in mealybug-free treatments were sprayed at 40-day intervals with Prentox Diazinon 50W (Prentiss Inc., Floral Park, NY).

Reverse transcription-polymerase chain reaction (RT-PCR) assays, capable of detecting and distinguishing PMWaV-1 and PMWaV-2, were conducted on all plants beginning 4 months after planting and thereafter at monthly intervals on plants not showing symptoms of MWP.

**Field study.** A study using a randomized complete-block design with four treatments arranged factorially and replicated four times was established in a commercial pineapple field on the island of Maui, HI. Treatments were crowns with and without PMWaV-1 and the presence and absence of mealybugs. Pineapple crowns were collected from the plant crop fruit of 12,000 pineapple plants from a proprietary clonal selection and assayed for PMWaV-1 with MAb 35-6-5- in TBIAs. Soil was fumigated with methyl bromide, drip irrigation lines were installed down the middle of each bed, and beds were covered with black plastic mulch. Each treatment plot consisted of 130 crowns planted on 28-cm centers in four rows (two beds). The 16 plots were separated by a minimum of two buffer beds of PMWaV-1-free plants on two sides and 7.7-m plantings of PMWaV-1-free plants on the ends. Plots that were maintained mealybug-free received applications of diazinon according to manufacturer's instructions. Amdro ant bait was applied as a broadcast treatment twice during the course of the study to control ants. Mealybug inoculations began 45 days after planting of the crowns and continued through the plant crop (18 months) at 6-week intervals. Each inoculation consisted of approximately 10,000 pink and gray pineapple mealybugs dispersed evenly throughout a plot. Presence or absence of MWP symptoms were noted at monthly intervals. PMWaV-1- and PMWaV-2-specific RT-PCR assays were conducted beginning 4 months after planting. Duplicate sets of leaf blots for use in PMWaV-1- and PMWaV-2-specific TBIA were made from each plant 6, 10, and 14 months after planting and at time of harvest.

**Mealybug transmission of PMWaVs and symptom induction.** Plants used for mealybug transmission experiments were derived from proprietary selection 1 and propagated by tissue culture. Prior to mealybug exposure, plants were screened for PMWaV-1 and PMWaV-2 with virus-specific TBIA and RT-PCR assays. Plants were established in soil for 40 to 80 days prior to exposure to mealybugs. Transmission experiments with PMWaV-1 and PMWaV-2 were conducted with mixed-aged populations of *D. brevipes* and *D. neobrevipes* in separate studies. Mealybugs were given acquisition access periods (AAPs) of 2 to 4 days on PMWaV-1-infected, PMWaV-2-infected, or PMWaV-free detached leaves. A minimum of 10 mealybugs then were transferred to each of the 142 pineapple plants free of PMWaV-1 and PMWaV-2 for inoculation access (IA). *D. brevipes* and *D. neobrevipes* mealybugs that were given AAP to PMWaV-2 were given IA to 72 and 30 plants, respectively; groups of *D. brevipes* and *D. neobrevipes* given AAP to PMWaV-1 each were given IA to 10 plants; and groups of *D. brevipes* and *D. neobrevipes* given AAP to PMWaV-free plants were given IA to 10 plants each. Mealybug populations were allowed to develop on these plants. Plants were grown in a greenhouse at 22 to 30°C and monitored for infections with PMWaV-1- and PMWaV-2-specific TBIAs beginning 30 days after initial mealybug exposure. To maintain mealybug populations in the absence of ants, additional inoculations of 10 to 20 nonviruliferous mixed populations of *D. brevipes* and *D. neobrevipes* mealybugs were made to the plants beginning 45 days after IA and, subsequently, at 45-day intervals for up to 5 months.

**Virus purification and immunosorbent electron microscopy.** The basal white portions of leaves from entire pineapple plants were ground to a fine powder in liquid nitrogen, combined (1:5) with purification buffer (0.5 M Tris-HCl, pH 8.4; 4% [vol/vol] Triton X-100; 0.5% $Na_2SO_3$; and 10 mM $Mg_2SO_4$) (37), and stirred overnight at 40°C. Clarified extract was layered over 5-ml cushions of 20% sucrose in resuspension buffer (RB) (100 mM Tris-HCl, pH 8.4; 0.5% $Na_2SO_3$; and 10 mM $Mg_2SO_4$) and centrifuged at 55,000 × *g* for 4.25 h at 40°C. Resulting pellets were either resuspended in RB and stored at –80°C for subsequent immunosorbent electron microscopy (ISEM) studies as previously described (22) or resuspended in RB with 10% sucrose (wt/vol). Resuspensions with sucrose were applied to a $Cs_2SO_4$-sucrose cushion step gradient (3) in RB and centrifuged at 100,000 × *g* for 3 h. Fractions (0.4 ml) were collected using a density gradient fractionator (ISCO, Lincoln, NE) with a chase solution of Fluorinert FC-40 (ISCO) and stored at –80°C for MAb production or ISEM evaluation.

**PMWaV-2-specific MAb production and screening.** Virus was purified from plants that contained closterovirus-like particles that were not decorated by MAb 35-6-5 but were PMWaV-2 positive with virus-specific RT-PCR. After purification through two $Cs_2SO_4$-sucrose cushion step gradients as previously described, the virus suspension was dialyzed against RB and then concentrated using a Centricon-10 microconcentrator (Millipore Corp., Bedford, MA). Six-week-old BALB/c mice were immunized (17) and hybridoma cell lines secreting antibodies were screened using TBIA. Leaves from pineapple plants infected with PMWaV-1, PMWaV-2, both viruses, or neither virus were sectioned transversely through the basal white tissue and blotted onto 1-by-1-cm squares of Nitro ME nitrocellulose membranes (Micron Separations Inc., Westborough, MA). Membranes containing the four leaf blots were placed in siliconized microfuge tubes, blocked with 1 ml of 2% (wt/vol) powdered Carnation nonfat dry milk (Nestle Food Corp., Glendale, CA) in phosphate-buffered saline (PBS) (wt/vol), and rinsed with PBS. Membranes then were incubated with hybridoma cell supernatants in PBS (1:25) for 3 h at 37°C, washed once with PBST (PBS + 0.5% Tween 20), and incubated with goat anti-mouse immunoglobulin G alkaline phosphatase conjugate (Sigma A5153) in PBS (1:1,500) for 3 h at 37°C. Membranes were removed from the microfuge tubes, washed three times with PBST, and incubated with Sigma Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (Sigma B5655) according to the manufacturer's instructions. Positive reactions were identified using a stereomicroscope as previously described (21) and the corresponding cell lines were cloned and subcloned by the limiting dilution method (2).

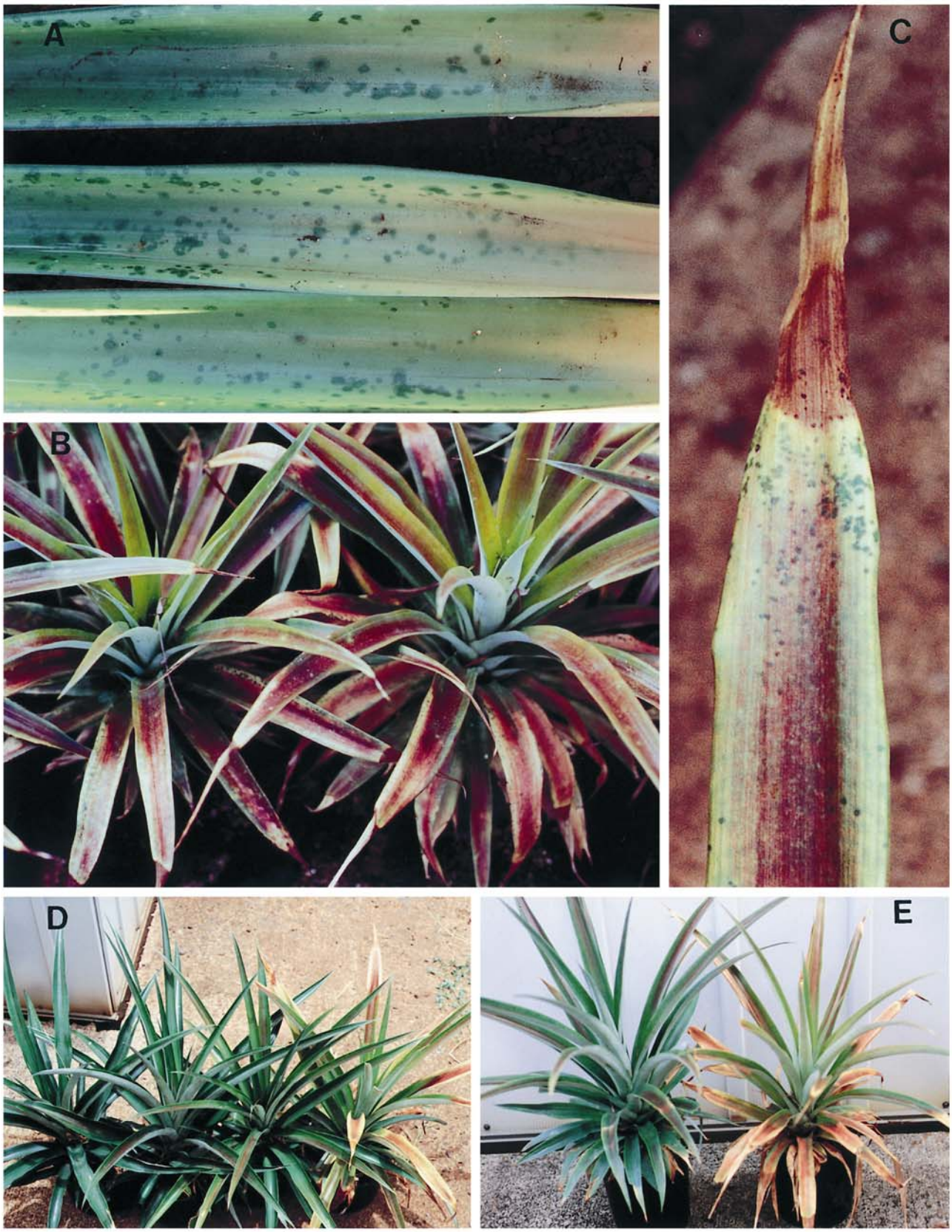


**Fig. 1. A,** Pineapple leaves exhibiting green spotting caused by the feeding of gray pineapple mealybugs (*Dysmicoccus neobrevipes*). **B,** Example of typical mealybug wilt of pineapple (MWP) symptoms expressed by *Pineapple mealybug wilt-associated virus* (PMWaV)-infected plants exposed to mealybug feeding. **C,** Typical leaf of an MWP-symptomatic plant showing early stages of tip dieback, reddening, and downward curling of leaf margins, and raised, green bumps caused by direct mealybug feeding. **D,** Typical symptoms of mealybug wilt disease are expressed only in the plant with PMWaV-2 and mealybug exposure, others appear healthy. Left to Right: PMWaV-free plant exposed to mealybugs; PMWaV-1 and -2-infected plant not exposed to mealybugs; PMWaV-2-infected plant not exposed to mealybugs; and PMWaV-2 infected plant exposed to mealybugs; **E,** Pineapple plants exposed to mealybug feeding that were infected with PMWaV-1 (left) and PMWaV-2 (right). Both plants were infected with badnavirus.

**RT-PCR.** Total RNA was extracted from pineapple tissue using RNeasy Plant Mini Kits, Cat. 74704 (Qiagen, Valencia, CA) and were stored at –80°C. RT-PCR assays which could differentiate between PMWaV-1 and PMWaV-2 were used as previously described (33). Primer sequences used for detection of PMWaV-1 were 5′-ACAGGAAGGACAACACTCAC-3′ and 5′-CGCACAAACTT-CAAGCAATC-3′ and for PMWaV-2 were 5′-CATACGAACT-AGACTCATACG-3′ and 5′-CCATCCACCAATTTTACTAC-3′.

## RESULTS

**Container-grown plant study.** No MWP symptoms developed in the plants that were maintained mealybug-free regardless of proprietary selection. Of the 40 PMWaV-1-free plants, 8 were infected with PMWaV-2, and 20 of the 50 PMWaV-1-infected plants were infected with PMWaV-2. No PMWaV-1 infections were detected with PMWaV-1-specific TBIA, ISEM, or RT-PCR assays in any plants in the PMWaV-1-free treatment groups during the course of this study. All plants in all selections that were exposed to mealybugs developed 1- to 6-mm-diameter dark green spots (Fig. 1A) that, over time, became raised bumps on most of the leaves. MWP symptoms developed in 80 of 90 plants exposed to mealybugs. These MWP-symptomatic plants occurred in both the PMWaV-1-free and PMWaV-1-infected treatment groups (Table 1). There was a temporal disparity of MWP symptom expression; 15 of 90 plants showed symptoms 2 months after mealybug exposure began, whereas an additional 65 of 90 plants became symptomatic over the next 8 months (Table 1). Plants from all five of the proprietary selections tested developed MWP (Table 1). Typical symptoms included severe tip dieback that spread 5 to 30 cm in an inverted "V" pattern down newly mature leaves (Fig. 1B). Areas proximal to the dieback zone often became white. Leaf

TABLE 1. *Pineapple mealybug wilt-associated virus* (PMWaV) infection status and presence or absence of mealybug wilt of pineapple (MWP) symptoms over time for plants from five Hawaiian-grown *Ananas comosus* selections exposed to monthly inoculations of mealybugs

| | Months after mealybug inoculations began[y] | | | | | | | No. with symptoms/no. infected[z] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Sel, status[x] | Initial | 2 | 4 | 6 | 8 | 10 | 12 | 1 or 2 | 1 | 2 | 1 + 2 |
| 1 | | | | | | | | | | | |
| Virus | CCCCC | CCCCC | XXXXX | XXXXX | X22XX | X22XX | X222X | 0/2 | … | 7/8 | … |
| Virus | CCCCC | CCCCC | XXXXX | X22XX | 2222X | 22222 | 22222 | … | … | … | … |
| Symptoms | HHHHH | HHHHH | HHHHH | HHHHH | HHHHH | HHHHH | HSSHH | … | … | … | … |
| Symptoms | HHHHH | HHHHH | HHHHH | HHSHH | HHSSH | SSSSH | SSSSS | … | … | … | … |
| 1 | | | | | | | | | | | |
| Virus | AAAAA | AAAAA | B1BB1 | BBBBB | BBBBB | BBBBB | BBBBB | … | … | 10/10 | … |
| Virus | AAAAA | AAAAA | 11BBB | BBBBB | BBBBB | BBBBB | BBBBB | … | … | … | … |
| Symptoms | HHHHH | SHHHH | SHSSH | SSSSS | SSSSS | SSSSS | SSSSS | … | … | … | … |
| Symptoms | HHHHH | HHHHH | HHSSH | HHSSS | SSSSS | SSSSS | SSSSS | … | … | … | … |
| 2 | | | | | | | | | | | |
| Virus | CCCCC | CCCCC | 2222X | 22222 | 22222 | 22222 | 22222 | … | 10/10 | … | … |
| Virus | CCCCC | CCCCC | XX222 | 22222 | 22222 | 22222 | 22222 | … | … | … | … |
| Symptoms | HHHHH | SHHSH | SHHSH | SHSSH | SSSSH | SSSSS | SSSSS | … | … | … | … |
| Symptoms | HHHHH | HHSHH | HHSSS | HHSSS | HHSSS | SSSSS | SSSSS | … | … | … | … |
| 2 | | | | | | | | | | | |
| Virus | AAAAA | AAAAA | 1BBBB | BBBBB | BBBBB | BBBBB | BBBBB | 0/1 | … | 8/9 | … |
| Virus | AAAAA | AAAAA | 11BBB | 11BBB | 11BBB | 11BBB | 1BBBB | … | … | … | … |
| Symptoms | HHHHH | HSHSS | HSHSS | SSSSS | SSSSS | SSSSS | SSSSS | … | … | … | … |
| Symptoms | HHHHH | HHSHH | HHSSH | HHSSS | HHSSS | HHSSS | HHSSS | … | … | … | … |
| 3 | | | | | | | | | | | |
| Virus | CCCCC | CCCCC | XXXX2 | 2XXX2 | 2XX22 | 2X222 | 22222 | … | … | 8/10 | … |
| Virus | CCCCC | CCCCC | 2XX2X | 22X2X | 22X2X | 22222 | 22222 | … | … | … | … |
| Symptoms | HHHHH | HHHHH | HHHHS | HHHHS | HHHHS | SHHSS | SHHSS | … | … | … | … |
| Symptoms | HHHHH | HHHHH | HHHSH | SHHSH | SSHSH | SSHSH | SSSSS | … | … | … | … |
| 3 | | | | | | | | | | | |
| Virus | AAAAA | AAAAA | 11BB1 | 1BBBB | BBBBB | BBBBB | BBBBB | … | … | 10/10 | … |
| Virus | AAAAA | AAAAA | 1BBBB | 1BBBB | BBBBB | BBBBB | BBBBB | … | … | … | … |
| Symptoms | HHHHH | HHHHH | HHSSH | HSSSS | SSSSS | SSSSS | SSSSS | … | … | … | … |
| Symptoms | HHHHH | HSSHS | HSSSS | HSSSS | SSSSS | SSSSS | SSSSS | … | … | … | … |
| 4 | | | | | | | | | | | |
| Virus | CCCCC | CCCCC | XXXXX | X22X2 | XX222 | XX222 | XX2220/2 | … | 8/8 | … | … |
| Virus | CCCCC | CCCCC | X222X | 22222 | 22222 | 22222 | 22222 | … | … | … | … |
| Symptoms | HHHHH | HHHHH | HHHHH | HHHHH | HHHHS | HHSSS | HHSSS | … | … | … | … |
| Symptoms | HHHHH | HSHSH | HSSSH | SSSSH | SSSSS | SSSSS | SSSSS | … | … | … | … |
| 4 | | | | | | | | | | | |
| Virus | AAAAA | AAAAA | BBBB1 | BBBB1 | BBBB1 | BBBB1 | BBBB1 | 0/2 | … | 8/8 | … |
| Virus | AAAAA | AAAAA | 1BBB1 | BBBB1 | BBBB1 | BBBB1 | BBBB1 | … | … | … | … |
| Symptoms | HHHHH | HSHHH | SSSSH | SSSSH | SSSSH | SSSSH | SSSSH | … | … | … | … |
| Symptoms | HHHHH | HHHHH | HHHSH | HSSSH | SSSSH | SSSSH | SSSSH | … | … | … | … |
| 5 | | | | | | | | | | | |
| Virus | AAAAA | AAAAA | 1B111 | 1BBB1 | 1BBB1 | 1BBBB | 1BBBB | 0/4 | … | 6/6 | … |
| Virus | AAAAA | AAAAA | 1111B | B111B | B111B | B111B | B111B | … | … | … | … |
| Symptoms | HHHHH | HSHHH | HSHHH | HSSSH | HSSSH | HSSSH | HSSSS | … | … | … | … |
| Symptoms | HHHHH | HHHHH | HHHHS | SHHHS | SHHHS | SHHHS | SHHHS | … | … | … | … |
| Total | … | … | … | … | … | … | … | 0/4 | 0/7 | 33/36 | 42/43 |

[x] Sel = proprietary selections grown in Hawaii. Each plant is designated by a character; virus status of each plant is shown in the two upper rows as X = PMWaV-1 and PMWaV-2 free; A = infected with PMWaV-1, status of PMWaV-2 unknown; B = infected with PMWaV-1 and PMWaV-2; C = PMWaV-1 free, status of PMWaV-2 unknown; 2 = infected with PMWaV-2 only; or 1 = infected with PMWaV-1 only. MWP symptom status of each plant is shown in the two lower rows as H = symptom-free and S = showing typical MWP symptoms. Typical symptoms included severe tip dieback, reddening, inflexing, and wilting of the leaves.

[y] Monthly inoculations of *Dysmicoccos brevipes* and *D. neobrevipes* began 3 months after crowns were planted.

[z] Based on status 12 months after mealybug inoculations began.

margins and tips of mature leaves developed a downward curl and the youngest mature leaves developed bright red coloration, which was often mottled with pale green and pink (Fig. 1C). The 6 to 12 oldest living leaves at the bases of plants became red with white margins and curled downward at the tip and along the margins.

RT-PCR assays that could detect and differentiate PMWaV-1 and PMWaV-2 were used at monthly intervals beginning the fourth month after mealybug exposure began. PMWaV infection status and the occurrence of symptoms were monitored over time (Table 1). RT-PCR analyses showed that MWP symptomatic plants were always infected with PMWaV-2, whereas infection with PMWaV-1 was not consistently associated with development of MWP (Table 1). Mealybugs could not move between treatment groups, but could move between plants and selections within a treatment group. New infections of PMWaV-2, but not PMWaV-1, were detected over the course of the study among plants in the PMWaV-1-free and PMWaV-1-infected treatment groups exposed to mealybugs (Table 1). MWP symptoms generally appeared 30 to 90 days after the initial detection of PMWaV-2-infection with RT-PCR (Table 1). Mealybug-exposed, PMWaV-1-infected plants that did not become infected with PMWaV-2 over the course of the study did not develop MWP symptoms (Table 1). After 12 months of mealybug exposure, 79 of 90 plants were infected with PMWaV-2, and 75 of these plants had developed MWP symptoms. The remaining four PMWaV-2-infected plants developed MWP symptoms over the next several months.

The majority of the plants that developed MWP symptoms entered a recovery phase 2 to 5 months after initial symptoms appeared, even though mealybug inoculations continued months after recovery was observed. The recovery phenotype was characterized by newly emerging leaves showing no MWP symptoms (i.e., severe tip dieback, reddening, downward curling of margins, or wilting), whereas older leaves remained symptomatic. New leaves did show dark-green spotting from mealybug feeding.

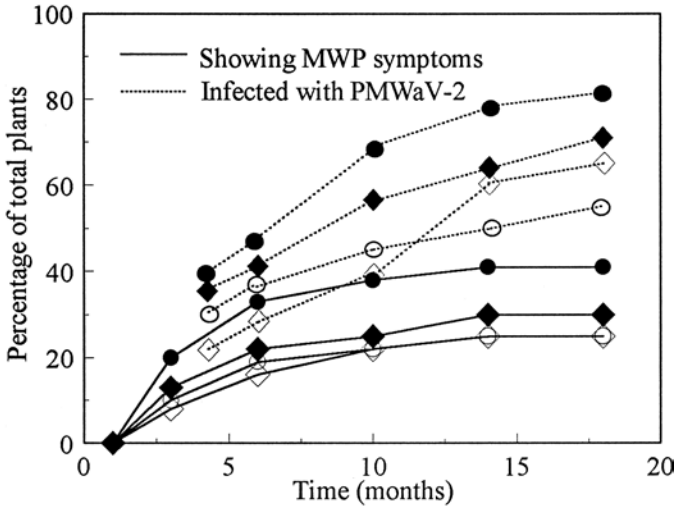


**Fig. 2.** Incidence of mealybug wilt of pineapple and *Pineapple mealybug wilt-associated virus* (PMWaV)-2 in four replicate plots (●, ○, ♦, ◊) of PMWaV-1-infected plants exposed to mealybugs over a 15-month period.

**Field study.** MWP symptoms did not develop in any plants growing in plots that were maintained mealybug free, regardless of PMWaV status. PMWaV-1- and PMWaV-2-specific RT-PCR assays, used 4 months after planting, showed that PMWaV-2-infected plants were present in the treatment plots. Leaf blots made 6, 10, and 14 months after mealybug inoculations commenced and at time of harvest were screened in TBIA with PMWAV-2-specific MAb 63-2-2. TBIA results also confirmed the presence of PMWaV-2 in the mealybug-free plots. Incidence of PMWaV-2 among the four plots of PMWaV-1-free plants maintained mealybug free ranged from 1 to 4%. Incidence of PMWaV-2 in the four plots of PMWaV-1-infected plants maintained mealybug free ranged from 15 to 27%. These plants were left in place and no new PMWaV-2 infections were detected in these plots during the course of the study.

Typical MWP symptoms, characterized by severe tip dieback, leaf reddening, downward curling along the leaf margins, and wilting of symptomatic leaves, developed only in some plants in plots exposed to mealybugs. MWP symptoms began appearing on some plants 2 to 3 months after mealybug exposure began, whereas other plants developed symptoms over the next 15 months. Symptomatic plants were observed in all four plots of PMWaV-1-infected plants that were exposed to mealybugs. PMWaV-1- and PMWaV-2-specific RT-PCR assays revealed that these symptomatic plants were infected with PMWaV-2 as well as PMWaV-1. Two months after mealybug inoculations commenced, the incidence of MWP ranged from 5 to 20% among replicate plots of PMWaV-1-infected plants and increased over the next 15 months (Fig. 2). TBIA with PMWaV-2-specific MAbs of blots made at 6, 10, and 14 months after planting and at the time of harvest showed that PMWaV-2 incidence had increased in these four plots (Fig. 2), presumably due to transmission by mealybugs. There was generally a 1- to 3-month interval between time of PMWaV-2 detection and the development of MWP symptoms. At 2 to 5 months after MWP symptoms appeared, the majority of the plants entered a recovery stage even though mealybug inoculations continued. At the time of plant crop fruit harvest, MWP incidence among replicate plots of PMWaV-1-infected plants that were exposed to mealybugs ranged from 25 to 41%. PMWaV-2 incidence in these plots ranged from to 50 to 79% (Fig. 2). None of the plants in the mealybug-inoculated plots that became infected with PMWaV-2 at least 10 months after planting developed MWP during the study.

Seven plants in the PMWaV-free plots exposed to mealybugs showed MWP symptoms 3 months after mealybug inoculations

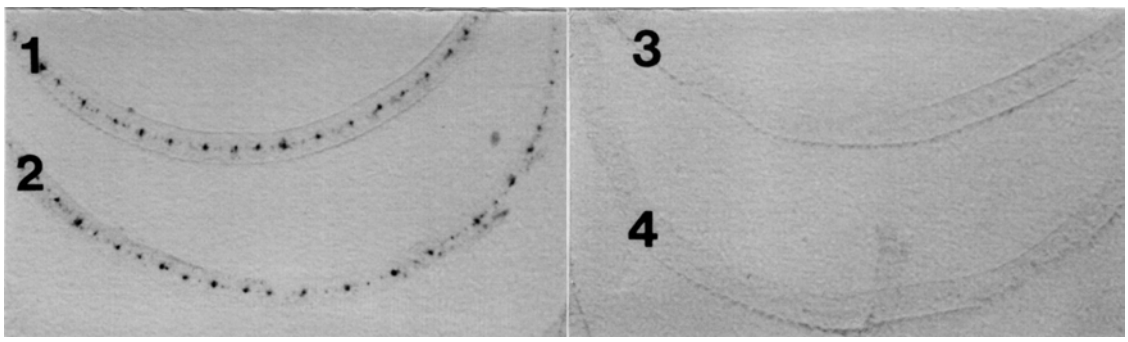


**Fig. 3.** Detection of *Pineapple mealybug wilt-associated virus* (PMWaV)-2 with monoclonal antibody 63-2-2 in a tissue blot immunoassay. Blots are from **1,** PMWaV-2-infected plant; **2,** PMWaV-1- and PMWaV-2-infected plant; **3,** PMWaV-1-infected plant; and **4,** PMWaV-free plant.

commenced. These plants were infected with PMWaV-2 based on RT-PCR assays. A total of 20 PMWaV-2-infected plants, including the 7 MWP symptomatic plants, were removed from the four PMWaV-1-free plots that were exposed to mealybugs to prevent secondary spread of PMWaV-2 and subsequent MWP development in these plots. This was done to maintain the PMWaV-free status of these mealybug-exposed plots. Upon removal of PMWaV-2-infected plants from these plots, no additional plants developed MWP symptoms despite monthly inoculations of nonviruliferous mealybugs. Many plants in these plots sustained populations of hundreds of mealybugs for the duration of the experiment and did not develop any symptoms of MWP, although green spotting of the leaves was observed.

**Production of PMWaV-2-specific MAb.** PMWaV-specific RT-PCR assays were used to identify plants infected with only PMWaV-2 for virus isolation and subsequent monoclonal antibody production. The cell line 63-2-2 was selected using a TBIA-based method of screening. MAb 63-2-2 detects PMWaV-2 in blots made from plants infected with PMWaV-2 only or in mixed infections with PMWaV-1 (Fig. 3). Positive signals were localized to the vascular bundles of leaf blot imprints. MA 63-2-2 does not detect PMWaV-1 in plants infected with only PMWaV-1, nor does it react with pineapple plant proteins (Fig. 3).

**ISEM of container-grown plants.** Analysis of $Cs_2SO_4$-sucrose fractions purified from PMWaV-1-free pineapple plants with MWP symptoms revealed two types of particles: badnavirus-like particles and closterovirus-like particles that could not be decorated by MAb 35-6-5 specific for PMWaV-1 (Fig. 4A), but that could be decorated by MAb 63-2-2 specific for PMWaV-2 (Table 2). Extracts from PMWaV-1-infected plants with MWP symptoms contained three types of particles: badnavirus-like particles, long flexuous rod-shaped particles decorated by MAb 35-6-5, and long flexuous rod-shaped particles that could not be decorated with MAb 35-6-5 (Fig. 4B) but that could be decorated with MAb 63-2-2 (Fig. 4C; Table 2). Using both MAbs together, 99 to 100% of the closterovirus-like particles isolated from plants with MWP symptoms were labeled. ISEM analysis of extracts from plants in the treatment groups not exposed to mealybugs showed that 8 of 40 PMWaV-1-free plants were infected with PMWaV-2 (Table 2). These plants remained symptomless for the duration of the study. Badnaviruses were found in all samples evaluated with ISEM (Table 2).

**Mealybug transmission of PMWaVs and symptom induction.** *D. brevipes* mealybugs that were allowed acquisition access to PMWaV-2-infected pineapple leaf material acquired and transmitted PMWaV-2 to 54 of 72 PMWaV-free pineapple plants (75%) when groups of 10 mealybugs were used. *D. neobrevipes* under the same conditions transmitted PMWaV-2 to 28 of 30 PMWaV-free pineapple plants (93%). Infections were detected 30 to 45 days after mealybug exposure by PMWaV-2-specific TBIA, and confirmed with RT-PCR for 10 randomly chosen plants. Plants that were infected with PMWaV-2 and sustained mealybug populations developed leaf-tip dieback and yellow and red mottling of leaves beginning approximately 20 to 40 days after PMWaV-2 detection with TBIA. Oldest leaves curled downward along margins and shriveled after 60 days. PMWaV-1-specific TBIA tests on these plants were negative. Plants that were only exposed to nonviruliferous mealybugs did not develop mottling, tip dieback, or other characteristic MWP symptoms. All plants exposed to *D. neobrevipes* developed dark green spots. Plants that were exposed to *D. brevipes* (7/10) and *D. neobrevipes* (10/10) mealybugs given acquisition access to PMWaV-1-infected leaves developed PMWaV-1 infections, but did not develop MWP symptoms despite sustaining populations of mealybugs. The 20 plants exposed to mealybugs that were allowed acquisition access to PMWaV-1- and PMWaV-2-free leaves remained negative for both closteroviruses, and did not develop any MWP symptoms after sustaining mealybug populations for over 5 months.

## DISCUSSION

Typical symptoms of MWP (8), including severe tip dieback, bright red coloration of the leaves, downward curling of leaf margins, and wilting of mature and older leaves, were induced in container- and field-grown pineapple plants. In these experiments, mealybug exposure and the presence of PMWaV-2 were correlated with development of MWP, suggesting a role for both factors in the disease. In our studies, infection with PMWaV-1, PMWaV-2, or both viruses did not result in MWP in the absence of mealybugs. Also, MWP symptoms did not develop in PMWaV-free or PMWaV-1-infected plants exposed to mealybugs. PMWaV-2-free plants sustained populations of hundreds of mealybugs for up to 12 months with no expression of MWP symptoms. Only plants that were infected by PMWaV-2 and exposed to mealybugs developed MWP. Symptoms typically developed in younger, mealybug-exposed plants 4 to 7 weeks after PMWaV-2 was detected by RT-PCR. MWP did not always appear in mealybug-exposed plants that had been planted for over 10 months prior to becoming infected with PMWaV-2. This suggests that older plants may exhibit tolerance to one or both factors.

Ants typically are associated with mealybugs in commercial pineapple fields. The ants consume the honeydew produced by the mealybugs and can interfere with natural enemies (27). One of the MWP management strategies currently utilized by pineapple in-

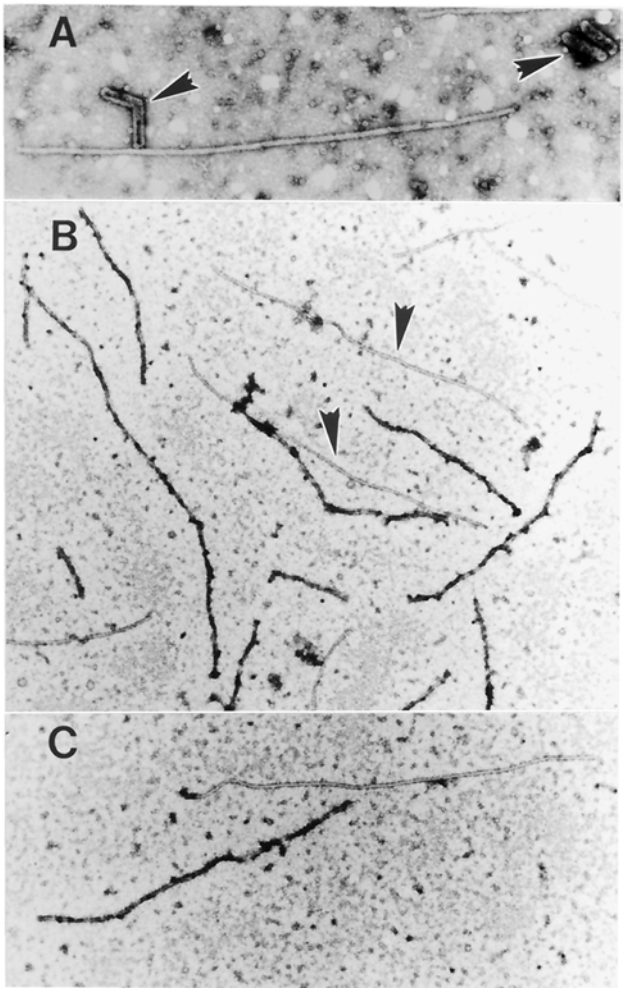


**Fig. 4. A,** Particles of *Pineapple mealybug wilt-associated virus* (PMWaV)-2, not decorated by specific monoclonal antibody (MAb) 35-6-5, and a badnavirus (arrows) from a mealybug wilt of pineapple (MWP)-symptomatic plant. **B,** Decorated with MAb 35-6-5 and undecorated (arrows) particles from MWP-symptomatic plants infected with PMWaV-1 and PMWaV-2. **C,** Decorated with MAb 63-2-2 and undecorated particles from MWP-symptomatic plants infected with PMWaV-1 and PMWaV-2.

TABLE 2. Virus detection in preparations from selected pineapple plants with or without mealybug wilt of pineapple (MWP) symptoms using either *Pineapple mealybug wilt-associated virus* (PMWaV)-specific tissue blot immunoassays (TBIA), reverse transcription-polymerase chain reaction (RT-PCR), or immunosorbent electron microscopy (ISEM)

| | | TBIA[w] | RT-PCR[x] | | ISEM[y] | | |
|---|---|---|---|---|---|---|---|
| Plant status[u] | Mealybugs[v] | PMWaV-1 | PMWaV-1 | PMWaV-2 | PMWaV-1 | PMWaV-2 | Badnavirus |
| No symptoms | – | 0/40 | 0/40 | 8/40 | 0/40 | 8/40 | 40/40 |
| No symptoms | – | 50/50 | 50/50 | 20/50 | NT | NT | NT |
| No symptoms | + | 8/15 | 8/15 | 4/15[z] | 2/4 | 0/4 | 4/4 |
| Symptoms | + | 42/75 | 42/75 | 75/75 | 5/10 | 10/10 | 10/10 |

[u] No symptoms = pineapple plants that did not exhibit typical symptoms of MWP any time during the study. Symptoms = pineapple plants with typical symptoms of MWP including severe tip dieback, reddening, inflexing, and wilting of mature leaves. NT = not tested.

[v] – = plants maintained mealybug free by applying diazinon every 40 days for the duration of the study; + = plants were inoculated at monthly intervals with 20 to 50 mixed-aged nonviruliferous *Dysmicoccos brevipes* and 50 to 100 mixed-aged nonviruliferous *D. neobrevipes.*

[w] Number of PMWaV-1-infected plants/total number screened as determined by TBIA with PMWaV-1-specific monoclonal antibody (MAb) 35-6-5.

[x] Number of infected plants/number tested by RT-PCR assays for PMWaV-1 and PMWaV-2.

[y] Number of plants having PMWaV-1 particles decorated with MAb 35-6-5/total number of plants assayed; number of plants having PMWaV-2 particles decorated with MAb 63-2-2/total number of plants assayed.

[z] These four plants subsequently developed MWP symptoms.

dustry is to control ants. This effectively reduces mealybug populations. Because all of the studies presented here were conducted in the absence of ants, our results demonstrate that ants are not required for induction of MWP. However, frequent reinoculations of mealybugs to the pineapple plants were required to maintain the constant presence of the mealybugs necessary for the spread of PMWaV-2 and the induction of MWP. This suggests that ants may have an indirect role in facilitating the spread of PMWaV-2 and in the induction of MWP symptoms. Mealybug populations flourish in the presence of ants; thus, more mealybugs are available for acquisition and transmission of PMWaV-2 and for establishing the populations necessary for induction of MWP.

Secondary spread of PMWaV-2 was detected in the mealybug-inoculated field plots. New infections likely arose from mealybugs acquiring the virus from an infected pineapple plant, derived from a PMWaV-2-infected crown, and subsequently transmitting it to other plants. Pineapple is propagated vegetatively, usually by crowns or slips. PMWaV-2-infected propagules planted to the field are the primary virus sources for mealybug acquisition of the virus (33). The results of our transmission tests with PMWaV-2 establish that it can be acquired and transmitted by *D. brevipes* and *D. neobrevipes* mealybugs. Both of these species are prevalent in pineapple worldwide. No alternate plant hosts of the PMWaVs have been identified (33); thus, the elimination of either the PMWaV-2-infected propagules or mealybugs in and around commercial fields could significantly reduce the incidence of MWP.

The prevalence of PMWaV-1 in commercially grown selections of Hawaii (21,29) and its transmissibility by mealybugs (34) contribute to its frequent detection in MWP-symptomatic plants, even though it is not directly involved in MWP symptom development. In Hawaii, the incidence of PMWaV-2 in healthy-appearing, field-grown selections is much lower than that of PMWaV-1 (33). Plants with MWP often produce a small fruit and crown, a fruit that ripens out of cycle with the majority of the fruit in the field, or no fruit at all. First ratoon or sucker production also may be reduced, and subsequent fruit produced by the ratoon also may be small or out of cycle (32). Thus, plants with MWP may contribute fewer crowns for the next planting cycle than healthy-appearing plants, thereby reducing the incidence of PMWaV-2.

Our working hypothesis for development of MWP symptoms is that pineapple plants are tolerant to PMWaV-2 infection and to mealybug feeding and only in the presence of both factors will MWP symptoms develop. This is consistent with PMWaV-2-infected plants appearing healthy if kept mealybug free. Likewise, other than green spotting caused by *D. neobrevipes* feeding, PMWaV-2-free pineapple plants do not develop symptoms in the presence of pink or gray pineapple mealybug populations consisting of hundreds of insects per plant. As the symptomatic plants aged, a recovery phenomenon characterized by lack of symptoms on the newest leaves (8,10) was observed in the majority of plants. Recovery occurred while mealybugs were still being applied to the plants, and PMWaV-2-infections still could be detected with virus-specific TBIA. This suggests the plant may have a mechanism that is activated during symptom expression that leads to a silencing of symptom expression. Plant age, dosage or mealybug number, virus titer, or other factors may play a role in this recovery phenomenon.

MAb 63-2-2, specific for PMWaV-2, was produced and is useful for PMWaV-2-specific TBIA and ISEM. The use of a TBIA-based system for screening large numbers of MAb clones has proven to be a durable and reliable method for clone selection. Although this method is only qualitative, it provides a means of selecting specific MAbs for exposed epitopes. Because of the lack of cross-reactivity between MAbs 35-6-5 and 63-2-2, mixed PMWaV infections now can be detected and distinguished by preparation of duplicate tissue blots and incubation with the appropriate MAbs. ISEM analysis of PMWaV-1-free, MWP-symptomatic plants with MAb 63-2-2 revealed labeling of virtually all closterovirus-like particles. However, this does not preclude the possibility that other MWP-associated viruses exist.

The role of the badnavirus in MWP remains unclear. Badnavirus infection previously has been detected in pineapple plants with MWP symptoms as well as in plants without symptoms in Australia (37) and Hawaii (18). Badnavirus-specific PCR and ISEM showed that all pineapple plants tested from the eastern coast of Australia were infected with a badnavirus (35). That study and our findings that badnavirus-infected plants that are PMWaV-2 free and exposed to mealybugs did not develop MWP suggest that this badnavirus is not a primary cause of mealybug wilt. However, interactions between the badnavirus, PMWaV-2, and mealybug feeding, such as the synergy observed in the rice tungro disease complex (16,25), cannot be ruled out. Until badnavirus-free pineapple plants are identified, the precise role of badnavirus infection in MWP will remain unclear. We previously have found that PMWaV-1 can be eliminated through the use of bud tissue culture (33), and studies are underway to determine if badnavirus infection can be eliminated through similar techniques.

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## LITERATURE CITED


1. Agranovsky, A. A. 1996. Principles of molecular organization, expression and evolution of closteroviruses: Over the barriers. Adv. Virus Res. 47:119-158.
2. Alvarez, A. M., Benedict, A. A., and Mizumoto, C. Y. 1985. Identification of Xanthomonads and grouping of strains of *Xanthomonas campestris* pv. *campestris* with monoclonal antibodies. Phytopathology 75:722-728.
3. Bar-Joseph, M., and Hull, R. 1974. Purification and partial characterization of sugar beet yellows virus. Virology 62:552-562.
4. Borroto, E. G., Cintra, M., Gonzalez, J., Borroto, C., and Oramas, P. 1998. First report of closterovirus-like particle associated with pineapple plants (*Ananas comosus* cv. Smooth Cayenne) affected with pineapple mealybug wilt in Cuba. Plant Dis. 82:263.
5. Carter, W. 1933. The pineapple mealy bug, *Pseudococcus brevipes*, and wilt of pineapples. Phytopathology 23:207-242.
6. Carter, W. 1939. Injuries to plants caused by insect toxins. Bot. Rev. 5:273-326.
7. Carter, W. 1942. Geographic distribution of mealybug wilt with some other insect pests of pineapple. J. Econ. Entomol. 35:10-15.
8. Carter, W. 1945. Some etiological aspects of mealybug wilt. Phytopathology 35:305-315.
9. Carter, W. 1951. The feeding sequence of *Pseudococcus brevipes* (Ckl.) in relation to mealybug wilt of pineapples in Hawaii. Phytopathology 41:769-780.
10. Carter, W. 1963. Mealybug wilt of pineapple; a reappraisal. Ann. N.Y. Acad. Sci. 105:741-764.
11. German, T. L., Ullman, D. E., and Gunasinghe, U. B. 1992. Mealybug wilt of pineapple. Adv. Dis. Vector Res. 9:241-259.
12. Gunasinghe, U. B., and German, T. L. 1986. Association of virus particles with mealybug wilt of pineapple. (Abstr.) Phytopathology 76:1073.
13. Gunasinghe, U. B., and German, T. L. 1987. Further characterization of virus associated with mealybug-wilt of pineapple. (Abstr.) Phytopathology 77:1776.
14. Gunasinghe, U. B., and German, T. L. 1988. Use of cDNA probes to characterize and detect virus in mealybug wilt affected pineapple plants. (Abstr.) Phytopathology 78:1586.
15. Gunasinghe, U. B., and German, T. L. 1989. Purification and partial characterization of a virus from pineapple. Phytopathology 79:1337-1341.
16. Hibino, H. 1983. Transmission of two rice tungro-associated viruses and rice waika virus from doubly or singly infected source plants by leafhopper vectors. Plant Dis. 67:774-777.
17. Hsu, H. T., Aebig, J., and Rochow, W. F. 1984. Differences among monoclonal antibodies to barley yellow dwarf viruses. Phytopathology 74:600-605.
18. Hu, J. S., Barry, K., Borth, W., Sether, D., Wu, Z. C., and Wang, M. 1995. Detections of plant viruses in Hawaii. Page 102 in: Proceedings: Hawaii Agriculture: Positioning for Growth. Hawaii Farm Bureau Federation and the College of Tropical Agriculture and Human Resources, Honolulu.
19. Hu, J. S., Gonsalves, A., Sether, D., and Ullman, D. E. 1993. Detection of pineapple closterovirus, a possible cause of mealybug wilt of pineapple. Acta Hortic. 334:411-416.
20. Hu, J. S., and Sether, D. M. 1999. Etiology of mealybug wilt of pineapple. Page 321 in: Abstr. Xth Int. Congr. Virology. Sydney, Australia.
21. Hu, J. S., Sether, D. M., Liu, X. P., Wang, M., Zee, F., and Ullman, D. 1997. Use of a tissue blotting immunoassay to examine the distribution of pineapple closterovirus in Hawaii. Plant Dis. 81:1150-1154.
22. Hu, J. S., Sether, D. M., and Ullman, D. E. 1996. Detection of pineapple closterovirus in pineapple plants and mealybugs using monoclonal antibodies. Plant Pathol. 45:829-836.
23. Illingworth, J. F. 1931. Preliminary report on evidence that mealybugs are an important factor in pineapple wilt. J. Econ. Entomol. 24:877-889.
24. Karasev, A. 2000. Genetic diversity and evolution of closteroviruses. Annu. Rev. Phytopathol. 38:293-324.
25. Koganezawa, H. 1998. Present status of controlling rice tungro virus. Pages 459-469 in: Plant Virus Disease Control. A. Hadidi, R. K. Khetarpal, and H. Koganezawa, eds. The American Phytopathological Society, St. Paul, MN.
26. Melzer, M. J., Karasev, A. V., Sether, D. M., and Hu, J. S. 2001. Nucleotide sequence, genome organization, and phylogenetic analysis of pineapple mealybug wilt-associated virus-2. J. Gen. Virol. 82:1-7.
27. 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.
28. Sastry, K. S. M., and Singhe, S. J. 1974. Wilt of pineapple. A new virus disease in India. Indian Phytopathol. 27:298-303.
29. Sether, D. M., and Hu, J. S. 1998. Corollary analyses of the presence of pineapple mealybug wilt associated virus and the expression of mealybug wilt symptoms, growth reduction, and/or precocious flowering of pineapple. (Abstr.) Phytopathology 88(suppl.):S80.
30. Sether, D. M., and Hu, J. S. 1999. Mealybugs and pineapple mealybug wilt associated virus are both necessary for mealybug wilt. (Abstr.) Phytopathology 89(suppl.):S70.
31. Sether, D. M., and Hu, J. S. 2000. A closterovirus and mealybug exposure are both necessary components for mealybug wilt of pineapple symptom induction. (Abstr.) Phytopathology 90(suppl.):S71.
32. Sether, D. M., and Hu, J. S. 2001. The impact of pineapple mealybug wilt-associated virus-1 and reduced irrigation on pineapple yield. Australas. Plant Pathol. 30:31-36.
33. Sether, D. M., Karasev, A. V., Okumura, C., Arakawa, C., Zee, F., Kislan, M. M., Busto, J. L., and Hu, J. S. 2001. Differentiation, distribution, and elimination of two different pineapple mealybug wilt associated viruses found in pineapple. Plant Dis. 85:856-864.
34. Sether, D. M., Ullman, D. E., and Hu, J. S. 1998. Transmission of pineapple mealybug wilt-associated virus by two species of mealybug (*Dysmicoccus* spp.). Phytopathology 88:1224-1230.
35. Thomson, K. G., Dietzgen, R. G., Thomas, J. E., and Teakle, D. S. 1996. Detection of pineapple bacilliform virus using the polymerase chain reaction. Ann. Appl. Biol. 129:57-69.
36. Ullman, D. E., German, T. L., Gunasinghe, U. B., and Ebesu, R. H. 1989. Serology of a closteroviruslike particle associated with mealybug wilt of pineapple. Phytopathology 79:1341-1345.
37. Wakman, W., Teakle, D. S., Thomas, J. E., and Dietzgen, R. G. 1995. Presence of a clostero-like virus and a bacilliform virus in pineapple plants in Australia. Aust. J. Agric. Res. 46:947-958.