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RESEARCH ARTICLE

Effect of banana bunchy top virus infection on morphology and growth characteristics of banana

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Abstract

Field experiments were conducted in Oahu, Hawaii, to investigate the effects of banana bunchy top virus (BBTV) infection on growth and morphology of banana (*Musa acuminata*). The time interval between aphid inoculation of BBTV and the initial appearance of disease symptoms (i.e. incubation period) was also determined. Plants infected with BBTV showed a significant reduction in petiole size (i.e. length and distance), plant canopy and height, leaf area, pseudostem diameter and chlorophyll content compared with control plants. Growth differences between virus-infected and control plants were not observed until 40–50 days after the plants were inoculated with viruliferous aphids. Other growth parameters such as petiole width and leaf production were not statistically different between infected and control plants. The incubation period of banana bunchy top disease or appearance of symptoms ranged from 25 to 85 days after aphid inoculation. However, PCR assays provided earlier detection of BBTV in banana plants compared with visual symptoms.

Introduction

Banana is one of the most important fruit crops in many tropical areas in terms of production and consumption (FAO, 2003). Hawaii ranks first in the USA in banana production (*Musa* sp.), and it is among the states' top crop (National Agricultural Statistics Service, 2006). Commercial banana production occurs on all major Hawaiian Islands with 80% of the production concentrated on the islands of Hawaii and Oahu (Constantinides & McHugh, 2003).

Banana bunchy top disease (BBTD) caused by banana bunchy top virus (BBTV) (*Babuvirus*, Nanoviridae) is one of the most economically important diseases of bananas (*Musa* spp.) in many production regions, including Asia, Africa and the South Pacific (Dale, 1987). The virus is transmitted in a persistent manner by the banana aphid, *Pentalonia nigronervosa* (Magee, 1927). BBTV is also spread through infected plant suckers and other plant tissues used in banana propagation (Magee, 1927). Plants infected early with BBTV do not bear fruit, and fruits of later infected plants are typically stunted and unmarketable. Additionally, the virus spreads to suckers through the rhizome and thus the entire banana mat eventually becomes infected (Dale & Harding, 1998). Magee (1927) reported that the first symptom of the disease was the appearance of dark-green streaks on the undersurface of the leaf. As the disease progresses, infected leaves become progressively stunted and malformed and have a more upright bearing than usual, eventually resulting in a 'bunchy' display.

Banana bunchy top virus was first observed in Hawaii in 1989 (Conant, 1992). Despite the economical and cultural importance of bananas, limited field research has been conducted on BBTV and its associated vector, *P. nigronervosa* (Hemiptera, Aphididae), in Hawaii. Currently, the recommended strategy for controlling BBTV includes identifying and removing symptomatic plants as early as possible and replanting with virus-free plants (Dale & Harding, 1998). This is because infected plants

are a likely source of secondary infection and spread of the pathogen (Magee, 1967). However, BBTD will persist indefinitely within plantations because inspectors are unable to identify all infected plants during a single inspection and non-symptomatic infected plants that remain between inspections may serve as a source of inoculum for virus spread (Allen, 1978*b*). As such, an effective management strategy is dependent on rapid detection of infected plants so that potential source plants of BBTV can be destroyed promptly.

Earlier studies conducted on BBTD focused mainly on the physical appearance of infected plants. Magee (1927) provided detailed descriptions of the progress of disease symptoms, and Allen (1978*a*) provided a summary of estimates of BBTD incubation period made by other authors. However, their work was conducted in regions such as Australia, where temperature and other biotic factors differ from Hawaii. Further, in these studies, observations were made on banana plants propagated by suckers. However, an increasing number of growers are using micropropagated banana plantlets as part of an integrated disease management programme targeting BBTV. Additionally, there is little quantitative knowledge from earlier studies on how BBTV infections affect various growth, morphological or physiological features such as chlorophyll content of infected banana plants. In this study, we addressed the following questions. (a) Are aspects of banana growth, morphology and physiology different between infected and healthy micropropagated plants? (b) If BBTV infection changes banana growth and morphological features, when do these changes occur after infection? (c) What is the range of time between aphid inoculation of BBTV and the appearance of BBTD symptoms?

Materials and methods

Aphids, plants and banana bunchy top virus sources

A *P. nigronervosa* colony was started from a single aptera collected from a healthy banana plant from a population in the Kahuku district, Oahu, and reared on Cavendish banana (*Musa acuminata* cv. Williams). Banana suckers infected with BBTV were also obtained from a banana (cv. dwarf Brazilian; *Musa* spp. AAB genome) plantation located in Kahuku. The infected suckers displayed the characteristic symptoms of BBTV, and their infection was confirmed with the use of molecular techniques (i.e. PCR) as described below. These suckers were used later as virus-infected source plants, and aphids placed on these source plants were used to infect additional banana plants, which served as sources of the virus for the field trials.

Experimental layout and planting time

Three field trials were conducted to assess the impact of BBTV on banana growth and determine BBTD's incubation period. In 2005, field experiments were conducted from April to July (trial 1), June to September (trial 2) and September to December (trial 3), respectively. The trials were conducted at the University of Hawaii, Poamoho Research Station (elevation 265 m) on Oahu, Hawaii. This area was chosen because there were no known banana plants in the vicinity and thus, this would limit the likelihood of infection occurring from external sources. All banana plantlets used for the field experiments were micropropagated from pathogen-free banana plants (Robson *et al.*, 2007). For trial 1, 140 banana plants [cv. Williams; AAA genome (derived exclusively from *M. acuminata*)] were transplanted on 13 April in a 26 × 24 m plot. The spacing between rows and plants was 2.4 and 1.8 m, respectively. For trials 2 and 3, 160 and 144 healthy transplants were planted on 15 June and 16 September, respectively. The plot sizes for trials 2 and 3 were 34 × 27 m and 34 × 30 m, respectively, and spacing between rows and plants were 2.4 and 2.1 m for each plot.

Field trials

Plants at the five to six leaf stages were inoculated in each trial. For trial 1, 32 test plants were randomly selected and subjected to one of two treatments: (a) virus (inoculated with viruliferous aphids) or (b) non-treated control (no aphids). Groups of five apterous aphids were removed from a single BBTV-infected source plant on which they were reared, using a fine-haired brush and placed in the 'throat' of the pseudostem at the third youngest fully expanded leaf. Five aphids were used to increase the likelihood of successful virus transmission (Hu *et al.*, 1996). Sixteen plants were inoculated with viruliferous aphids, and another 16 plants were randomly chosen as non-treated controls. Because virus transmission was low during the initial trial, the number of infected aphids were increased by five for the remaining trials. Thus, for trials 2 and 3, 10 aphids were collected from infected source plants in the laboratory and placed in the throat of the pseudostem at the third and fourth youngest fully expanded leaves (total of 10 aphids per plant), respectively. A total of 24 and 48 plants were inoculated for trials 2 and 3, respectively. An equivalent number of test plants were used as non-treated controls. Immediately after placing the aphids on the plants, a sleeve cage constructed of a 36-mesh cm^{-1} transparent fabric (Super Poly Organza; Hyman Hendler and Sons, Los Angeles, CA, USA) was carefully placed over all the test plants.

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The sleeve cages were used to protect aphids from natural enemies and restrict them to inoculated plants. After 5 days, the sleeve cages were removed, and the plants sprayed with imidacloprid (Provado 1.6F®; Bayer Crop Science Inc., Research Triangle Park, NC, USA) at a rate of 0.7 mL L^{-1} of H_2O using a hand-pumped backpack sprayer.

Plant growth parameters

Plant growth and chlorophyll measurements for all trials were initiated at 5 days after termination of the aphid inoculation period and taken every 5 days for trial 1 and every 10 days for trials 2 and 3, respectively. The measurements were terminated at 90 days after insect inoculation.

Determination of leaf area, leaf production, plant height, pseudostem diameter and canopy development

Plant height, pseudostem diameter and leaf area and emergence were recorded on each sampling occasion. Leaf area was estimated by measuring the leaf length and maximum width and multiplying the results by a conversion factor of 0.83 (length × width × 0.83) (Robinson & Neil, 1985). The number of leaves were counted for all test plants at each date. Leaf production rates were determined as measured by Turner (1971). As such, a leaf was regarded as emerged if the ventral surface of the midrib was fully exposed and at least half the leaf was unfurled. Plant height was measured as the distance from the ground to the fork created by the petioles of the uppermost fully emerged leaf (Smith *et al.*, 2000). The diameter of the pseudostem was measured at the area immediately below the fifth most recently fully expanded leaf.

Determination of petiole morphology

The morphology of the petiole was evaluated using the protocol of Ennos *et al.* (2000). This involved measuring the length, maximum width and distance of attachment from the base of the petiole to where it joins the pseudostem to the leaf. These measurements were taken from the third youngest fully expanded banana leaf on each sampling date.

Measurements of chlorophylls *a* and *b* and total chlorophyll content

Leaf chlorophyll content can be directly related to stress physiology. As such, the relative chlorophyll content of banana leaves was compared between infected and check plants using a Minolta SPAD-502® chlorophyll meter (Minolta Corporation, Ramsey, NJ, USA). The SPAD (Special Products Analysis Division) meter determines the greenness of the leaf and the interaction of thylakoid chlorophyll with incident light (Jifon *et al.*, 2005). Six readings representative of the entire leaf length were taken from the edge of the most recently matured fully unfolded leaf. The average of the six readings was recorded from each test plant. Afterwards, three 6.3-mm diameter discs representing the entire lamina length were punched along the same leaf area where the SPAD readings were taken and used to determine chlorophylls *a* and *b* content. Chlorophylls *a* and *b* are two of the most important pigments in photosynthetic processes, and low concentrations of these chlorophylls can directly limit photosynthetic activity (Taiz & Zeiger, 1991). Individual leaf discs collected for chlorophyll extraction were immediately placed in 1.5-mL microcentrifuge tubes containing dimethyl sulphoxide (DMSO) and placed in a brown paper bag. A standard chlorophyll extraction protocol was used (Richardson *et al.*, 2002). The absorbance was measured with a Genesys 5 spectrophotometer (Spectronic Unicam, Rochester, New York, USA) at 663 and 645 nm (Richardson *et al.*, 2002). The absorption spectra of chlorophyll (between 600 and 680 nm) extracted in 90% acetone or DMSO are very similar (Hiscox & Israelstam, 1979), allowing Arnon's equations (Arnon, 1949) to be used to calculate the chlorophyll content.

Virus testing

All test plants were inspected for disease (symptoms) at 5-day intervals, commencing 10 days after aphid inoculation (e.g. margin of leaves faintly chlorotic) until 90 days after planting. The dates banana plants were first observed displaying symptoms were recorded. For each trial, PCR was used to test plants for BBTV 10 days after aphid inoculation. Another sample was taken 5 days later and conducted thereafter at 15-day intervals for trials 1 and 2, and at 20-day intervals for trial 3, respectively. A 6.3-mm diameter cork borer was used to obtain a disc sample near the mid-vein of the most recently developed fully open leaf. A fire torch was used to sterilise the borer in between samples; preliminary work showed no transfer of virus from sample to sample using this approach. Plant tissue was ground with sterile plastic mortars and the DNA extracted from the leaf samples with a Qiagen DNeasy kit following manufacturer's instructions (Qiagen Inc., Valencia, CA, USA). A BBTV-specific primer set was designed by aligning sequences of its DNA 1 component [=master Rep component (mRep)] available in GenBank and selecting two conserved regions to prime an 815-bp amplicon. Primers designed were DNA1f (5′-GGCGCGATATGTGGTATGC-3′) and

DNA1r (5′-CCTTCGGAAGGAAGTTAGCC-3′). The following PCR cycles were used: 2 min for 94°C, followed by 30 cycles of 1 min for 94°C, 1 min for 60°C, 1 min for 72°C and 5 min for 72°C. The annealing temperature was optimised with a thermal gradient block. The Promega mastermix #M7505 (Promega Corp., Madison, WI, USA) was used for PCR reactions and agarose gels stained with ethidium bromide for detection of amplicons following standard protocols.

Statistics

The incubation periods for BBTD were similar for each trial, and there were no significant trial or trial × treatment effect for the various growth parameters. As such, the results from all three trials were pooled and the analysis was performed on measurements taken at 10-day intervals. To determine if the various growth, physiological and morphological parameters differed among treatments, the data were subjected to a repeated measure analysis of variance (Proc Mixed SAS Institute, Cary, NC, USA) with trial designated as a random factor.

Results

Symptom development, virus detection and chlorophyll content

During the three field trials, 43.2% (38 of 88) of the banana plants inoculated with aphids reared on BBTV source plants became infected; based on an infection efficiency of 43.8%, 33.3% and 47.9% for trials 1, 2 and 3, respectively. Virus presence was confirmed by PCR analysis for all plants (Table 1). BBTD incubation period ranged between 25 and 85 days for the three trials (Fig. 1). Most infected plants (71%) were symptomatic between 25 and 50 days after inoculation (DAI). The initial symptoms were characterised by the appearance of slightly chlorotic margins along newly developing leaves. The earliest time period a plant was diagnosed with BBTV using PCR was 15 DAI.

Table 1 Mean difference in the number of days it took banana plants to be diagnosed with banana bunchy top virus (BBTV) using PCR methods compared with visual symptoms

Trial[a]	Number of Infected Plants	Mean Difference in Time (days)[b]	SE
1	7	10.71	1.30
2	8	5.63	2.58
3	23	0.0	2.74

[a]Initial PCR samples were taken 10 days after viruliferous aphid inoculation for each trial. For trials 1 and 2, additional samples were collected 5 days later and thereafter at 15-day intervals. For trial 3, samples were collected at 20-day intervals after the initial sampling.
[b]Average of how many days earlier PCR methods diagnosed BBTV-infected plants prior to visual diagnosis. Visual inspections were conducted at 5-day intervals following viruliferous aphid inoculation.

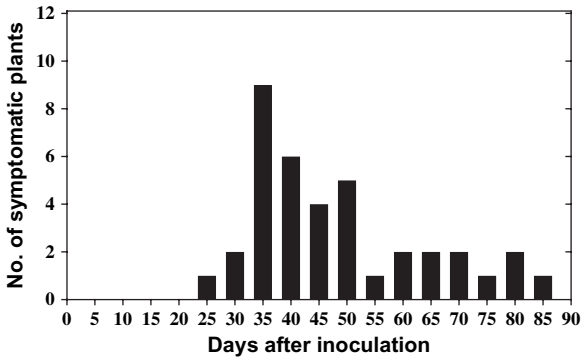

Figure 1 Banana bunchy top disease incubation period after inoculation of banana plants with viruliferous aphids (combined results of three experiments).

There was a significant difference in chlorophyll content between control and infected plants ($F = 182$, $P < 0.0001$). The chlorophyll content of infected plants was lower compared with the control beginning 40 DAI (Fig. 2). Chlorophyll content measured with the spectrophotometer also differed significantly between control and infected plants, and these distinctions became more pronounced, 40 DAI (Fig. 3). Both chlorophylls *a* and *b* and total chlorophyll were significantly reduced in infected plants compared with control ($F = 16.3$, $P < 0.0001$; $F = 17.4$, $P < 0.0001$; $F = 16.9$, $P < 0.0001$, respectively). For most samples, the ratio of chlorophyll *a* to *b* was typically in the range of 3.0 to 3.4 (Fig. 3D).

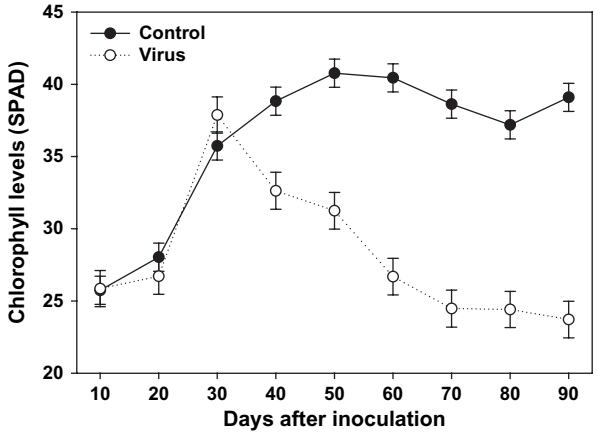

Figure 2 Estimate of chlorophyll levels in banana bunchy top virus-infected (virus) and healthy (control) banana plants at different times after inoculation. Chlorophyll estimates were obtained using a SPAD-502 [SPAD (Special Products Analysis Divison) units] chlorophyll meter.

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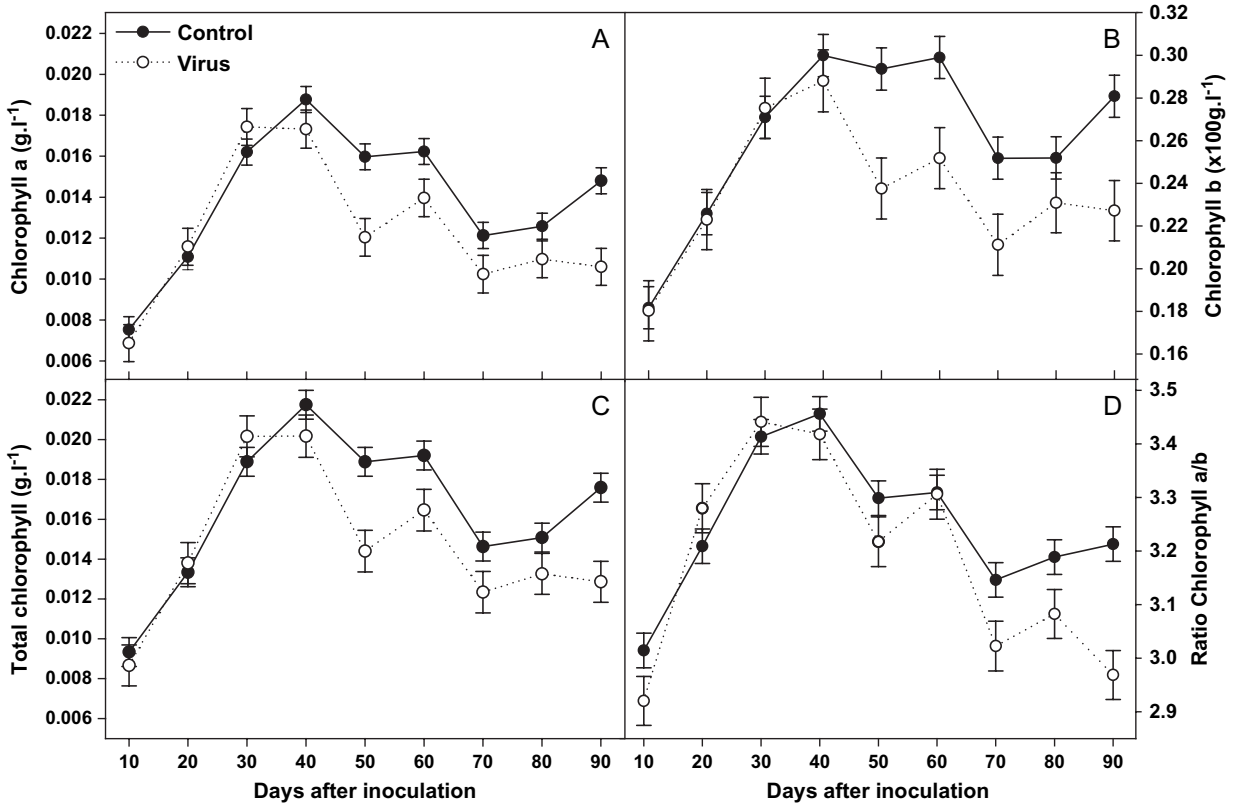

Figure 3 (A) Chlorophyll *a*, (B) chlorophyll *b*, (C) total chlorophyll content and (D) chlorophyll *a*/*b* of infected (virus) and healthy (control) banana plants at different times after inoculation as measured by a spectrophotometer.

Petiole morphology, pseudostem diameter, height and canopy

Petiole length and distance of infected plants were significantly less than those of control plants ($F = 5.7$, $P = 0.0174$; $F = 8.4$, $P = 0.0038$; respectively; Fig. 4A and Fig. 4B). However, petiole width was similar among treatments on most sampling dates ($P \geq 0.05$; Fig. 4C). Mean petiole width increased for both treatments until 50 DAI. Pseudostem diameter, plant height and canopy size of infected plants were all significantly smaller than those of control plants ($F = 5.63$, $P = 0.0179$; $F = 76.96$, $P \leq 0.0001$; $F = 147.0$, $P = 0.0001$, respectively, Fig. 5).

Leaf area and production

Leaf areas of infected plants were significantly smaller than control plants ($F = 143.8$, $P < 0.0001$). This difference in leaf size between control and infected plants became apparent at 50 DAI (Fig. 6A). Despite the significant size difference in leaf area between infected and control plants, each treatment produced new leaves at a similar rate (Fig. 6B). As such, no significant differences were detected in the number of leaves produced by infected and control plants ($P \geq 0.05$).

Discussion

Earlier studies on BBTV-infected banana plants focused on visual attributes of symptomatic plants and factors influencing virus spread (Magee, 1927; Allen, 1978*a*,*b*). This study was conducted to investigate the effects BBTV has on banana growth and morphology and the time interval between vector inoculation of BBTV and the onset of disease symptoms and virus detection by PCR. BBTV infection affected several banana growth and morphological features, while other parameters were uninfluenced. These findings are in agreement with the results of other studies showing that certain aspects of plant growth may be affected by virus infection, while others remained similar to healthy plants (Guo *et al.*, 2005). These differences in banana growth characteristic were generally significant approximately 50 DAI. The incubation period of BBTD was found to range from 25 to 85 DAI. Although we did not compare virus transmission

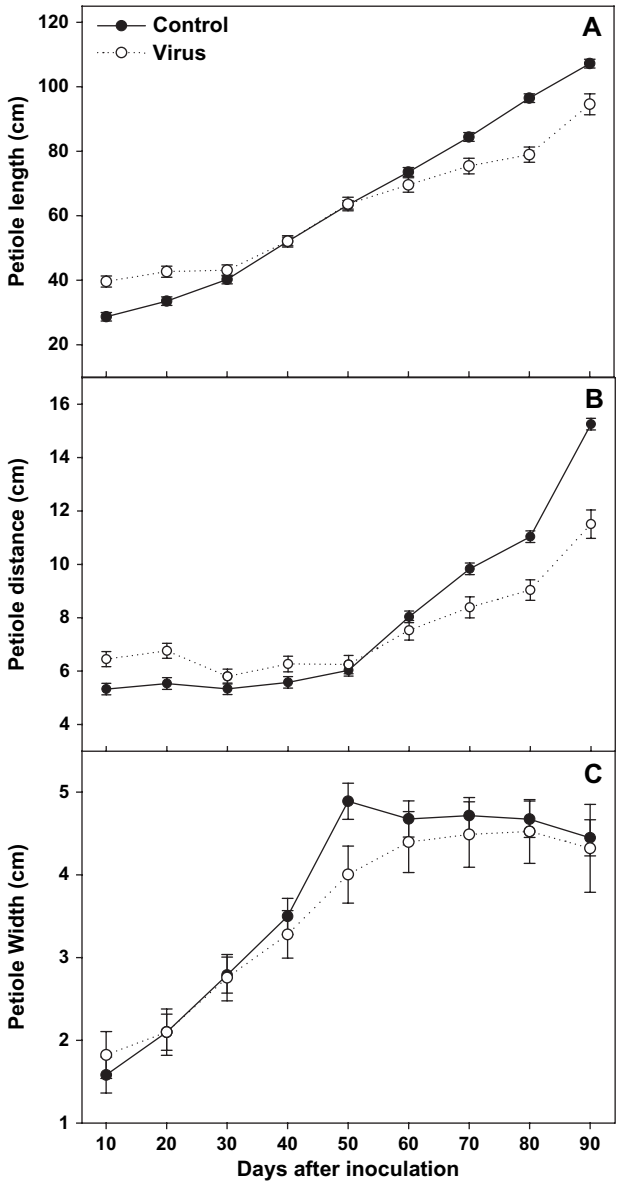

Figure 4 (A) Leaf petiole length, (B) distance and (C) width of infected (virus) and healthy (control) banana plants at different times after inoculation.

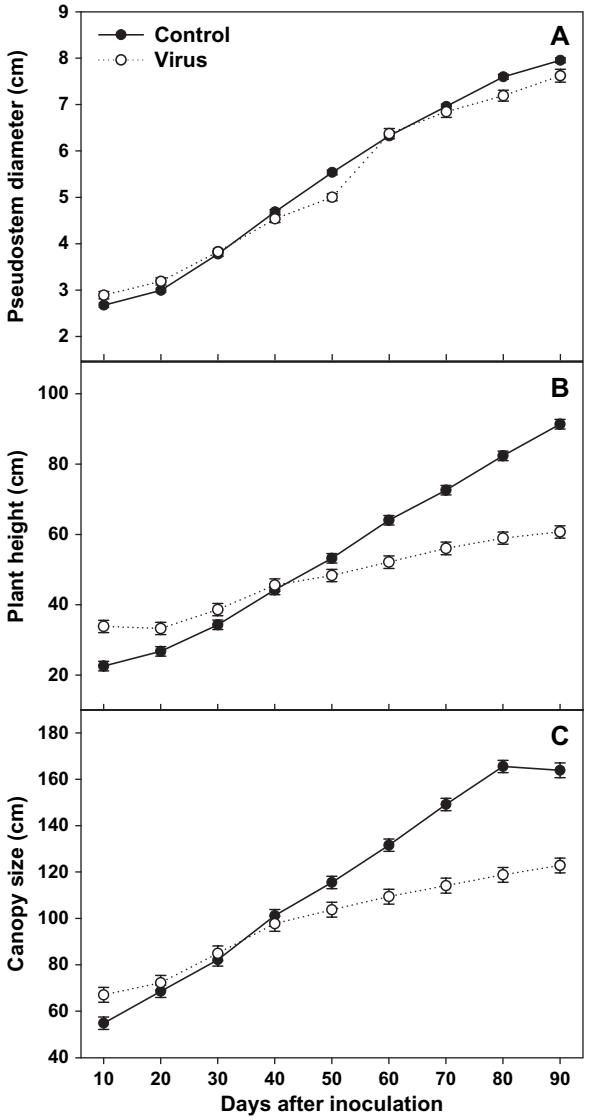

Figure 5 (A) Diameter, (B) height and (C) canopy size of infected (virus) and healthy (control) banana plants at different times after inoculation.

rates under field and laboratory conditions, aphids transmitted BBTV in this study less efficiently (43%) than in a greenhouse study that used the same number of individuals per plant (100% transmission efficiency; Hu *et al.*, 1996). Because of the importance of vector numbers on disease management and decision making, determining BBTV's transmission efficiency under field conditions compared with laboratory experiments is warranted. Furthermore, once a plant is infected, the outcome is generally no fruit production or the plant being rogued. Thus, if vectors are less successful in transmitting BBTV in a field environment, their overall impact on yield could significantly differ from what would be predicted from laboratory findings.

It is not uncommon for virus infection to have a negative impact on plants by limiting their growth (Wilfert Eckel & Lampert, 1993; Miteva *et al.*, 2005). However, to our knowledge, this is the first study to compare morphological and growth characteristics between BBTV-infected and healthy control banana plants. In the present study, there were several banana growth parameters significantly different between BBTV-infected and control plants. Infected plants showed a significant reduction in petiole length and distance, pseudostem diameter, plant height and canopy and leaf area. One of the

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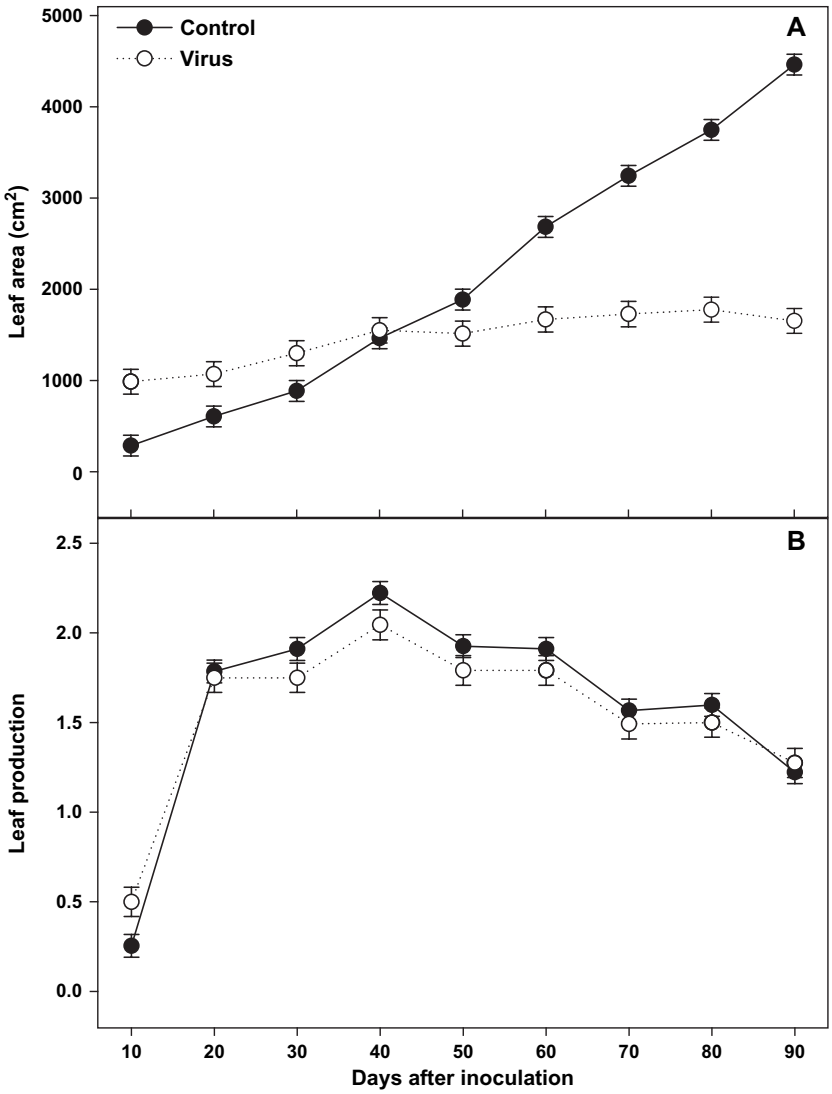

Figure 6 (A) Leaf area and (B) leaf production of infected (virus) and healthy (control) banana plants at different times after inoculation.

explanations for reduced leaf size in plants infected with pathogens or infested by pests is reductions in the amount of available assimilate (Ayres, 1992). During this study, aphids were killed after the inoculation access period and no new aphids colonised the test plants. Thus, reduction in plant growth was not because of assimilate removal by the banana aphid. However, it is uncertain whether BBTV infection induces a reduction in plant assimilates or whether BBTV exerts a direct effect on leaf expansion and other growth parameters. Although there was a substantial effect on several growth and morphological parameters in plants infected with BBTV, these effects are not unambiguous to the naked eye, until several weeks after inoculation, subsequently visual symptoms (e.g. chlorotic leaf margins) are readily apparent at that time. As such, it may be plausible to assume that the low productivity of infected banana plants is partly attributable to physiological stress associated with low photosynthesis rate of chlorotic leaves (Chia & He, 1999).

Growth features such as petiole width and leaf production were similar between diseased and control plants. New leaves appeared at a similar rate (every 5–6 days), regardless of whether the plants were infected or healthy. This may have important implications in the management of this disease for several reasons. For example, the number of symptomatic leaves might indicate the length of time since the end of the incubation period. If future research determines the spatial and temporal spread of BBTV from a symptomatic source plant over time, coupling the

number of symptomatic leaves (as an indication of time post-infection) would allow the estimation of the area surrounding this plant that must be rogued to eliminate infected, but asymptomatic, banana plants. Additionally, as noted during this experiment, BBTV symptoms are most apparent in the newly developing leaves. As such, if BBTV infection slowed leaf production, the potential to overlook infected plants during inspections would increase, enhancing the likelihood that newly infected plants could serve as a secondary source for continuous virus spread.

Chlorophylls *a* and *b* and total chlorophyll decreased significantly in infected plants. The differences between mean chlorophyll levels of infected and control plants are noteworthy as the appearance of slightly pale, chlorotic margins was the most obvious early characteristic present for diagnosing infected plants. All plants identified as being infected using these characteristic were verified with PCR, and there was no plant falsely identified as being infected.

Although the study was not designed to compare the time interval by which PCR could be used to detect BBTV opposed to visual detection, the PCR detection of BBTV dataset suggests that diseased plants can be detected 5–10 days prior to the appearance of visual symptoms. For the trials, disc extracts were taken from the most recently matured leaves (i.e. leaves developing after inoculation) on each sampling date, and PCR detection of BBTV may be possible earlier if samples are taken from the inoculation site (Hafner *et al.*, 1995). Conversely, studies in Taiwan showed that BBTV titre was higher in younger leaves and decreased with increasing leaf age (Hwang & Su, 1998). Findings did, however, indicate that PCR methods can provide earlier detection of BBTV in banana plants compared with visual symptoms. However, the economic feasibility of using rapid and sensitive PCR diagnostic tools for BBTV detection should be considered, given that banana is a staple or low cash crop in most production regions. Diagnostic procedures such as PCR and serology tests are also time consuming because additional work is required in the field and laboratory. Further, banana orchards typically contain thousands of plants and if PCR methods only yield a 10-day advance warning system, this may reduce its serviceable use as a presymptomatic indicator of infection. In addition, PCR tests are considerably more expensive when compared with visual detection of symptoms. Finally, as implicated in this study, unless samples for PCR diagnosis are collected timely and routinely, they would offer no advantage over visual diagnostics.

The incubation period for BBTD observed during this study differed from earlier reports. Allen (1978*a*) summarised data from Magee (1927) and Sun (1961), reporting that the incubation period for BBTD ranged from 19 days in the summer to 125 days in the winter. Our findings indicated that the incubation period for BBTD ranged from 25 to 85 days in Hawaii. Magee (1927) and Sun (1961) used the appearance of dark-green streaks referred to as the 'first symptom' as an indicator of infected plants, and in some instances, this may have allowed them to report symptoms earlier (approximately 1 leaf in advanced) compared with this study. However, this would further imply that BBTD has a shorter incubation period in Hawaii. This shorter incubation period may be attributed to banana varieties used, climatic and abiotic factors or genetic differences between the BBTV strains present elsewhere and Hawaii. Su *et al.* (1998) reported differences among strains of BBTV collected from bunchy top-infected banana plants in Hawaii (USA), Australia, and other countries. This difference further suggests that BBTV findings from field studies conducted in one area should not be accepted as a truism for all regions. During this study, the incubation periods of BBTV were found to be similar during each field experiment and thus the wide range could not be attributable to different environmental conditions associated with each trial. However, future studies directed at determining the cause of the wide range of incubation periods associated with BBTV could be helpful in understanding the epidemiology of this disease. In addition, determining the relationship between BBTV's incubation and latent periods within banana would have important implications for the development of management practices that incorporate roguing of non-symptomatic plants neighbouring symptomatic plants.

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