Trop. Agr. Develop. 57 (1) : 1 − 7, 2013

Cross-Transmission and New Alternate Hosts of *Banana bunchy top virus*

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Abstract Cross-transmission of bunchy top virus from and to banana (*Musa* sp.) cv. Lakatan and abacá (*Musa textilis* Nee) cv. Tinawagan Pula was achieved. Bunchy top virus (BTV) from abacá showed a 10% rate three months post-inoculation when aphid-inoculated to banana, whereas a 30% infection rate was obtained when BTV from banana was transmitted to abacá in the same manner. We also confirmed plant species outside the Musaceae family, namely *Alpinia zerumbet* (shell ginger), known as "gettou" in Japanese, *Colocasia esculenta* (satoimo) and *Canna indica*, were alternate hosts of *Banana bunchy top virus* (BBTV) isolates from banana in Japan and the Philippines. *C. indica* was found to be a host of BBTV isolates from Japan and the Philippines in which the latter inoculum source could induce severe bunchy top symptoms at a high incidence (100%). Interestingly, *A. zerumbet* served for the first time as a silent host of Japanese BBTV isolates with a low detection frequency (2/8) yet was conducive for aphid, *Pentalonia nigronervosa*, multiplication at a rate comparative to varieties of *C. esculenta* (satoimo, gabi and taimo). Moreover, *C. esculenta* (satoimo) was found to be an alternate host of Japanese BBTV isolates (11%) for the first time. These findings partly explain the naturally occurring specificity of BBTV isolates towards cultivated and wild plant species as a component of plant-aphid-virus etiology.

Key words: *Alpinia zerumbet,* Aphids, Taro, Abacá, Polymerase chain reaction (PCR)

Introduction

Banana (*Musa* sp.) and abacá (*Musa textilis* Nee) share common diseases including bunchy top, bract mosaic and mosaic diseases. These are known to be caused by viruses and transmitted by insect vectors. Banana bunchy top disease (BTD), which was first recorded in Fiji in 1889 by Magee, has been inadvertently introduced to Southeast Asia, the South Pacific, (Magee, 1927) and Hawaii (Conant, 1992). The virus causing BTD known as *Banana bunchy top virus* (BBTV) is a Nanovirus possessing isometric virion (18-20 nm diameter) and has a genome of 6 separately encapsidated cssDNAs (Thomas and Dietzgen, 1991; Karan *et al.*, 1997). Transmission of BBTV is solely through aphids in a circulative, nonpropagative and nontransovarial manner (Hu *et al.*, 1996).

Insects as vectors play a vital role in the quick spread or dissemination of numerous plant viruses in adjacent and neighboring areas and also from one geographic location to another. Due to their minute size, huge numbers, ability to fly and high reproduction rate, they are the most efficient animated transmitters of several plant viruses infecting particularly high-valued crops. Species of aphids (Aphididae) can transmit over 197 plant viruses (28%) from different virus groups (Hogenhout *et al.*, 2008). Among these the black banana aphid (*Pentalonia nigronervosa* Coq.) is a known efficient vector of BBTV and *Banana bract mosaic virus* (BBrMV) (Anhalt and Almeida, 2008; Pinili *et al.*, 2011). This insect inhabits the inside of the pseudostems and bases aboveground in plants like *Musa.* This plant is also widely distributed and found in tropical and subtropical regions worldwide including Japan. There are 17 plants known as hosts of black banana aphids (CABI, 2011). However, their capability to transmit BBTV has been reported primarily to genus *Musa* including abacá. Previous reports; however, showed that only bunchy top virus from banana is capable of cross-transmission to abacá. The infected abacá on the contrary could not be transmitted by aphid to some Philippine banana cultivars (cvs.) (Bajet and Magnaye, 2002). Moreover, the identity of the virus infecting abacá, *Abacá bunchy top virus* (ABTV), has been reported to be related but different in one DNA component, DNA-R (Sharman *et al.*, 2008).

Although other potential hosts have been tested for BTV transmission including *Colocasia esculenta* (ornamental gabi), *Heliconia psittacorum* (false bird of paradise) and *Hedychium coronarium* (camia), no symptoms were observed nor were virus particles

Communicated by C. Tanaka
Received Apr. 18, 2012
Accepted Sep. 14, 2012
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detected (Bajet and Magnaye, 2002). Other reports show specific BBTV isolates can induce infection among plant species outside the Family Musaceae (Dela Cueva *et al.*, 2011; Hu *et al.*, 1996; Geering and Thomas, 1997; Ram and Summanwar, 1984).

Chemical control is the most common means to eradicate this insect vector and the inoculum source. Biological controls such as other species of aphids or predators have been tested but failed to reduce the aphid's population. Vector-resistant crops have been developed and used as counter measures in controlling aphid populations and thus the spread of the virus (Wang *et al.*, 2005). The etiology of the virus, its vector and plant host species play an important role in developing an effective and holistic approach to combat virus and disease spread.

This study was conducted to: (i) determine whether cross-transmission of bunchy top virus can be occur between banana and abacá, (ii) assess plant species outside the genus *Musa* as potential alternative hosts of BBTV and reservoirs of banana aphids, and (iii) evaluate the capability of BBTV isolates from the Philippines and Japan to infect cultivated and wild species as potential alternative hosts.

Materials and Methods

Plant Material and Aphids

Tissue-cultured banana and abacá were obtained from the Plant Cell and Tissue Culture Laboratory, Institute of Plant Breeding (IPB), University of the Philippines – Los Baños. Individual plantlets were acclimatized and potted-out in sand, soil and coir dust mixture (1:1:0.5). Whereas plant species for the alternative host evaluations including *Alpinia zerumbet* and *Canna indica* were obtained from seeds and germinated in growth chamber at 27°C with a 24h photoperiod. The other test plants were planted from viable rhizomes under greenhouse condition. All plants were found virus-free prior to inoculation.

Aphids, *P. nigronervosa*, were collected separately from bananas in Okinawa, Japan and both bunchy top infected bananas and abacás in Laguna, Philippines.

Cross-transmission of BBTV within Musa

Comparative BBTV inoculation experiments were conducted under screen house conditions. Cross-inoculations using Philippine BBTV isolates were done in two *Musa* species, namely banana cv. Lakatan and abacá cv. Tinawagan Pula at IPB in the Philippines. Inocula came separately from banana and abacá showing severe bunchy top symptoms. Around 30 to 35 aphids (adult and nymph) per plant were transferred to 1 month old tissue-cultured banana and abacá using a camel hair brush after a 24h acquisition feeding period (AFP). Test plants were replicated 10 times and kept in two groups according to the inoculum source under screen house conditions for symptom development. Uninoculated plants were also maintained as negative controls. Plants were evaluated at three months post-inoculation for symptom development and BBTV infection.

Alternate hosts experiment

Plant species outside the genus *Musa* (Table 1) were tested as potential virus and aphid reservoirs. Black banana aphids were used in both comparative artificial inoculation experiments conducted in Japan and the Philippines. In Japan, two varieties of *Colocasia esculenta*, satoimo and taimo, and *Anthurium andraeanum* were inoculated with BBTV from Okinawa using 15 to 20 adult aphids per plant. These aphids were reared from BBTV infected banana and allowed to transmit the virus to healthy test plants for a one month inoculation feeding period (IFP). All plants were kept in the laboratory at 25°C for symptom development. To increase the efficiency of virus transmission, a number of aphids were added aside from the initial count. Unequal replications were employed on each test plant with a varying frequency of inoculation. Uninoculated plants were also

Table 1 List of potential alternate host of *Banana bunchy top virus* isolates used in the study.

English or common name	Local name*	Scientific name	Place where plant species collected
Taro	Satoimo	*Colocasia esculenta* (L.) Schott	Tokyo, Japan
Taro	Taimo	*C. esculenta* (L.) Schott	Okinawa, Japan
Taro	Gabi	*C. esculenta* (L.) Schott	Laguna, Philippines
Canna	Canna	*Canna indica* L.	Tokyo, Japan
Anthurium	Anthurium	*Anthurium andraeanum* (L.) Schott	Tokyo, Japan
Arrowroot	Uraro	*Maranta arundinacea* L.	Laguna, Philippines
Ginger	Luya	*Zingiber officinale* Roscoe	Laguna, Philippines
Shell ginger	Gettou	*Alpinia zerumbet* (Pers.) B.L. Burtt & R.M. Smith	Okinawa, Japan

*: Name of the varieties is not specified.

kept as negative controls.

A. zerumbet (shell ginger) and *C. indica* (canna) were inoculated at the 3 to 4 leaf-stage with Okinawa BBTV using 40 viruliferous aphids (24h AFP). Both plants were replicated 8 times and kept in an incubator at 26°C.

Plant species outside of the Family Cannaceae and Araceae were also tested as potential virus/aphid alternate hosts. These included *Maranta arundinacea* (arrowroot) of the family Marantaceae and *Zingiber officinale* (ginger) of the family Zingiberaceae. These plants were artificially inoculated with BBTV from the Philippines (banana and abacá isolates) using 30 to 35 aphids (adult and nymph) per plant after a 24h AFP following the methods of the cross-transmission experiment. All test plants were unequally replicated (6 to 10 replications) including uninoculated healthy controls. All plants were maintained in the screen house for three months for symptom development, aphid multiplication and virus detection.

Cannas were inoculated with Philippine BBTV using varying aphid counts (0, 5, 10, 15 and 20) per plant with five replications each. All inoculated plants were kept under the same screen house condition for observation.

Virus detection and analysis

Samples were processed from fresh leaf or leaf impregnated into a Whatman FTA™ Plant Card (GE® Amersham Place, Little Chalfont, UK). FTA plant cards were used for leaf samples with soft tissues such as *C. esculenta* (gabi) to avoid sample deterioration during handling and transit to Japan prior to virus detection.

For fresh samples, total nucleic acid was extracted using the PhytoPure™ Plant DNA Extraction kit (GE® Amersham Place, Little Chalfont, UK) following the manufacturer's instructions. Amplification of the target DNA-R genome of BBTV was then conducted using primer pairs D11/D12 by Karan *et al.* (1994). PCR reactions of 25µl volumes with TaKaRa ExTaq™ (TaKaRa, Shiga, Japan) as the polymerase enzyme were run under the following PCR conditions: 94°C for 4 min; 29 cycles of 94°C for 1 min, 61°C for 1 min and 72°C for 2 min; and an extension of 72°C for 10 min. PCR products were gel electrophoresed (2% agarose) and then viewed and photographed under a UV transilluminator (EDAS 290 KODAK, Japan).

For samples impregnated into FTA Plant Cards, total nucleic acid was extracted from eight punched discs eluted in processing buffer following the extraction procedures of Ndunguru *et al.* (2005). cDNA was synthesized using a first-strand cDNA synthesis kit (ReverTra Ace™ TOYOBO, Osaka, Japan). DNA amplification of BBTV was done following the methods described above.

An enzyme-linked immunosorbent assay (ELISA) was used for virus detection from cannas inoculated in the Philippines. A specific BBTV antibody (Agdia, Elkhart, USA) was used following the manufacturer's methods. Samples having three times higher absorbance values than healthy uninoculated controls were considered positive to BBTV. Two readings were done for each inoculation pattern.

Results and Discussion

Cross-transmission of BBTV within the genus Musa

Comparative BBTV transmissibility experiments using aphids were performed on banana cv. Lakatan and abacá cv. Tinawagan Pula. Individual experiments showed apparent vein-clearing and bunchy top symptom development in some plants three months post-inoculation (Fig. 1). The incidence of infection was recorded at a low frequency based on PCR analysis. The BBTV-

Fig. 1 Severe bunchy top symptom on two cross inoculated banana and abacá through aphid inoculation compared to healthy control (left to right).

abacá isolate only infected one out of 10 inoculated banana seedlings (10%), whereas the BBTV-banana isolate was detected from three inoculated abacá plants (33.3%). Other inoculated seedlings, either from banana or abacá, were found to be negative for BBTV and did not show typical bunchy top infections even after high aphid infestations.

Aphids and BBTV alternative hosts

As early as 10 days after aphid transfer, species of *Colocasia* and *A. zerumbet* were highly infested with aphids. These insects colonized underneath the leaf surface down to petiole (Fig. 2). Aphids noticeably refused to settle on the upper leaf lamina. Nymphal molts of various stages were observed from all inoculated plantlets. *C. indica* inoculated using aphids obtained from banana (Okinawa); however, did not exhibit high insect infestation and multiplication.

High aphid infestation was also observed from inoculated *C. esculenta* (gabi) under Philippine conditions regardless of the BBTV source. When most of the *C. esculenta* (gabi) plants died due to fungal infections, aphids continuously colonized on newly-emerged shoots. The aphids; however, did not show any signs of colonization on *M. arundinacea* and *Z. officinale* throughout the experiment.

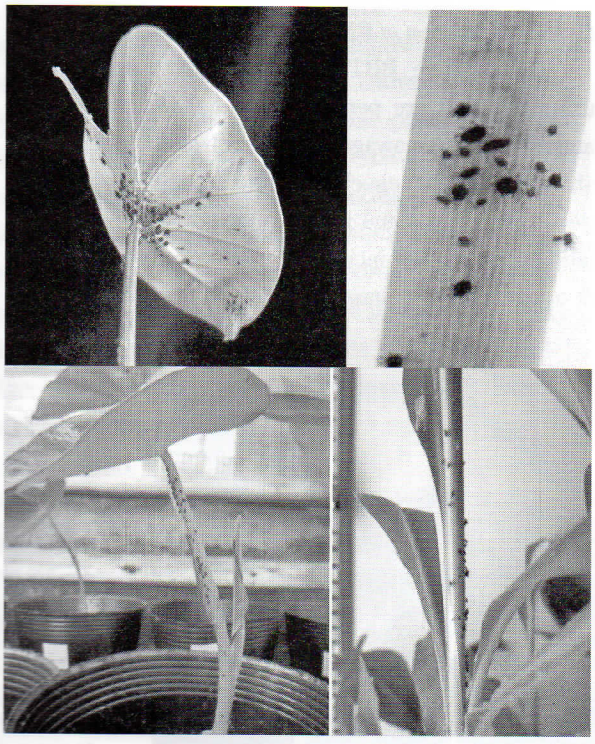

Fig. 2 High aphid infestation occurring in *Colocasia esculenta* (satoimo, gabi) (upper and lower left) and *Alpinia zerumbet* (gettou) (lower right).

The evaluation of potential alternate hosts of BBTV was done on the same plant species using the same aphids as vectors. Among the tested plant species only *C. indica* showed severe bunchy top after being inoculated with Philippine BBTV isolated from banana (Fig. 3). Increasing the number of aphids from 5 to 20 resulted in an increased severity of bunching on plants and stunted growth. Serological detection using a specific monoclonal antibody supported these results. Inoculated *Canna* sp. showed high mean absorbance values, ranging from 0.586 to 0.813 with increasing numbers of aphids (data not shown), compared to the mean absorbance value obtained from the healthy uninoculated plants (0.263). This is in contrast to the BBTV isolate from Okinawa that did not express any initial symptoms on inoculated cannas.

As shown in Table 2, PCR analyses confirmed the incidence of BBTV on inoculated *C. esculenta* (satoimo) after two months post-inoculation with Okinawan BBTV isolates. Although low virus detection (1/9) has been recorded, bands of up to 1.1kbp were visibly amplified (Fig. 4). *C. esculenta* (taimo) and *A. andraeanum* were both negative for BBTV infection when inoculated with the Okinawan BBTV isolate despite a high aphid infestation. *M. arundinacea* and *Z. officinale* were also found negative for BBTV after transmission.

C. indica, which was inoculated with two virus isolates, one from Okinawa and one from the Philippines, responded positively. A single plant out of eight total was found positive for Okinawan BBTV, whereas all (100%) plants were positive when inoculated with the Philippine banana isolate.

Two plants out of eight *A. zerumbet* inoculated with the Okinawan BBTV isolate were found to be infected.

Fig. 3 *Canna indica* showing severe bunchy top infection 3 months post-inoculation with *Banana bunchy top virus* (BBTV) Philippine banana isolate.

Table 2 Plant reactions to *Banana bunchy top virus* isolates and frequency of detection after artificial inoculation using banana aphids *Pentalonia nigronervosa*.

Test plant	BBTV isolates		
	Okinawa-banana	Philippines-banana	Philippines-abacá
Satoimo	1/9*	nt	nt
Taimo	0/5	nt	nt
Gabi	nt	0/4	0/3
Canna	1/8	20/20	nt
Anthurium	0/5	nt	nt
Arrowroot	nt	0/8	0/8
Ginger	0/1	0/5	0/5
Shell ginger	2/8	nt	nt

nt: not tested

*: number of infected plant over the total number of inoculated test plants.

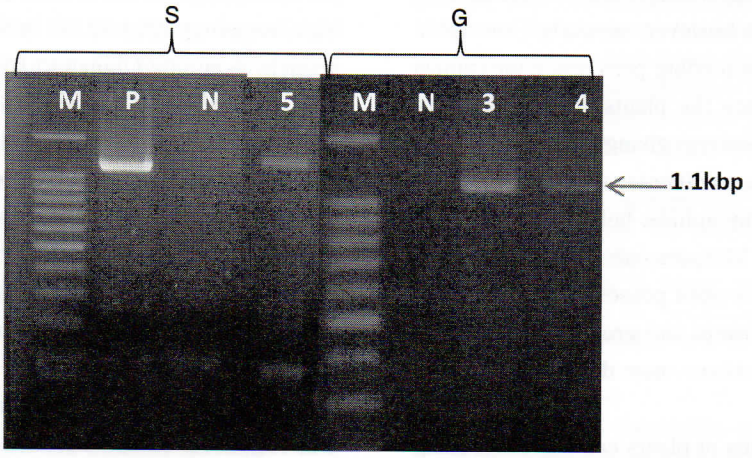

Fig. 4 Gel electrophoretic analysis (2% agarose) on inoculated *Colocasia esculenta* (S-satoimo) and *Alpinia zerumbet* (G-gettou) showing expected band size (arrowed) amplified from the DNA-R genome of *Banana bunchy top virus* (BBTV). A 100-bp ladder (M) was included as size marker; P-positive and N-negaitve.

Although no apparent symptoms were observed, the expected PCR band for the virus (up to 1.1kbp) was consistently amplified after two detection assays (six and eight months post-inoculation).

In this study, the transmission of BBTV was demonstrated successfully between banana and abacá via the black banana aphid, *P. nigronervosa*. The virus was detected as early as 20 days post-inoculation from both abacá and banana. This is in contrast to previous investigations that indicated bunchy top virus of abacá can infect only abacá and not banana cvs. such as Lakatan (AA/AAA), Bungulan (AAA), Latundan (AAB), Dwarf Cavendish (AAA), Morong Princesa (AA) and Cardaba (BBB). Although in this study, the bunchy top virus infecting abacá was BBTV and not ABTV as reported earlier by Sharman *et al.* (2008), the virus particles can be transmitted between these two *Musa* species. This study also validated the work of Eusebio and Bajet (1994) who studied *Musa* spp. (Sexy Pink), *Musa textilis* 51 and 52 and banana cvs. Giant Cavendish (AAA), Lakatan and Latundan.

Species outside the Musaceae family evaluated both as potential hosts of BBTV and as aphid reservoirs belong to the order Zingeberales except for *Colocasia* spp. and *Anthurium andraeanum* (order Alismatales). These plant species are propagated vegetatively using rhizomes or seeds, and they grow all year round in the tropics or semi-tropics. *C. esculenta* (gabi) is a delicacy and is cultivated and grown side by side with abacá and banana in the Philippines along with arrowroot and other *Colocasia* sp. (ornamental taro). The latter harbors a huge number of aphids throughout the growing season of *Musa*. We found most species of *Colocasia* such as taimo and satoimo were capable of harboring banana aphids regardless of the insect's location of origin. These species of *Colocasia* are distinct for their heart-

shaped leaves with a waxy lamina on the upper layer and a soft and succulent stem or petiole providing ambiance suitable for insect feeding and colonization. Although both *Colocasia* and *Anthurium* belong to the family Araceae, the latter did not show any aphid infestation probably due to its waxy and hard leaves and petioles.

Among the order Zingiberales *A. zerumbet* could also attracts aphids as a breeding ground. *A. zerumbet*, locally known as gettou in Okinawa, Japan, is commonly used as a food wrapping material due to its aromatic characteristic. In this species, aphids were noticeably found scattered on the lower leaf surface and pseudostem down to above ground level. Aphids could multiply but in low numbers compared to *C. esculenta* probably due to its distinct odor. *C. indica*, which resembles plantlets of *Musa* at its early stages; however, supported low aphid colonization. The aphids feeding preference for cannas most likely changes once the plants start to elongate and extends their pseudostems giving the insects a wide exposure to light. *M. arundinacea*, which is the least colonized or preferred by aphids, belongs to the same order under the family Marantaceae. Locally known as 'uraro' or arrowroot, this plant possess a narrow, waxy leaf lamina, thin pseudostem and grows in clusters. Its semi-hard waxy pseudostem may deter aphids from colonization.

These varied species of plants can sustain feeding of *P. nigronervosa* and seem to mimic *Musa* spp. in providing an environment conducive for aphid growth and multiplication.

Hosts of BBTV have been reported and limited only to *Musa* species including abacá. In this study we found that *C. esculenta* (satoimo) serves as potential host of BBTV isolates from Okinawa, Japan. This is in agreement with the report of Ram and Summanwar (1984) on *C. esculenta* inoculated using aphids carrying an Indian BBTV isolate. However, *C. esculenta* (taimo) failed to maintain a systemic infection of the Okinawan BBTV isolate. Although both satoimo and taimo were native to Okinawa, the difference in cultivar may potentially affect the pathogenicity. The finding of Geering and Thomas (1997) on cultivated plants and some wild plant species in Australia including *C. esculenta* also failed to show any systemic BBTV infections when artificially inoculated using black banana aphids. In the Philippines, *C. esculenta* has not been recorded as a host of BBTV but *Caladium bicolor* (family Araceae), commonly known as 'elephant ear', and *Heliconia* sp. were found positive for the virus upon aphid inoculation (Dela Cueva *et al.*, 2011). This is similar to *A. zerumbet* collected from Okinawa that has been shown to serve as a silent host of BBTV for the first time. *A. zerumbet* did not show any symptoms even 10 months post-inoculation but was capable of BBTV multiplication. This is in contrast to *A. zerumbet* and other species of *Alpinia* inoculated with a BBTV isolate from Australia (Geering and Thomas, 1997).

In the case of *C. indica*, both the BBTV isolate from Japan and from the Philippines are known to be infectious but to a different level. The Philippine isolate was found to be more virulent and could induce severe bunchy top symptoms with increasing aphid counts (minimum of 5 viruliferous aphids). The Okinawa isolate used in this study represented the BBTV isolates previously sequenced by Furuya *et al.* (2005). With a high homology (99.1-99.9%) in the DNA-R full-length nt sequences among Okinawan BBTV isolates (Accession nos. AB108452 –AB108458) and between the Philippine isolates (92.0-99.5%) (Furuya *et al.*, 2005; Pinili *et al.*, 2011a) the pathogenicity of *C. indica* observed in Japan was not due to the BBTV isolates but most likely by the experimental conditions. Since the experiment was carried out in two different conditions (screen house and controlled chamber), the aphids behavior during virus acquisition, transmission and varying temperatures upon inoculation and in the incubation period may have a strong effect on the infection efficiency. As reported by Anhalt and Almeida (2008), external factors including temperature fluctuations have significant effects on both aphid behavior and virus infection.

These findings show that speciation occurs in relation to BBTV isolates. Isolates tested in this study that all belong to the Asian group share about 92 to 99.5% homology in the DNA-R genomic nt sequence (Furuya *et al.*, 2005; Pinili *et al.*, 2011a). Isolates from Japan and India are capable of infecting *Colocasia* spp. particularly *C. esculenta* although the isolate belongs to the South Pacific group. However, the Philippine isolate, which also belongs to the Asian group, failed to infect *C. esculenta* but caused infection to *Caladium*, which is a closely related genus. The *C. esculenta* (gabi) used in this study was different from cultivated taro samples in Japan, thus it may partly explain its host-virus non-compatibility. Several varieties and landraces exist among *C. esculenta* possessing morpho- agronomical, physio-chemical characteristic variations. Furthermore, genetic diversity (allelic and isozyme) has been observed among Southeast Asian and Pacific groups of *C. esculenta* (Irwin *et al.*, 1998; Lebot *et al.*, 2004; Lebot and Aradhya, 1991). These features may have an effect

on their reactions to BBTV infection.

We often depend on the notion that the spread of BBTV is mainly due to transporting infected planting materials, and secondarily to an abundance of aphids. The role of alternate hosts is significant in BBTV etiology, as is knowing the speciation between cultivated and wild plants that host isolates of BBTV from the Asian and South Pacific groups. Controlling BTD and its rapid spread geographically would rely on identifying potential alternative hosts of both the virus and the vectors. For a plant to become a host of the virus it must be suitable for the vector to sustain its feeding activity. Cultivated and even wild plant species, particularly those not showing typical symptom, are therefore vital components in both disease and virus spread aside from being the vectors' reservoir. Their capability to propagate vegetatively should also be considered as one of the risk factors. Furthermore, other wild species including noxious weeds, which cover much of our natural vegetation, must be evaluated. On the other hand, the virulence of BBTV isolates may vary towards different plant species and should be taken into consideration. Collective efforts pertaining to this etiological study could help us in designing an effective and holistic control strategy.

Acknowledgment

The Ministry of Education, Culture, Sports, Science and Technology (Monbukagakusho) is highly acknowledged for the first author's full financial assistance. Authors also thank Ms. Rie Koide and Dr. Noriko Furuya for the support during the conduct of some parts of the experiment.

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