

PCR and ELISA-based virus surveys of banana, papaya and cucurbit crops in Vietnam

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Abstract. Between 1998 and 2002, we conducted PCR-based plant virus disease surveys throughout Vietnam, on banana, papaya, and several cucurbit crops, namely pumpkin, cucumber, gourds and loofa. *Banana bunchy top virus* in banana and *Papaya ringspot virus* in both papaya and cucurbits were widespread. *Squash leaf curl virus-Vietnam* and *Cucumber mosaic virus* were also widespread, predominantly infecting pumpkin and cucumber respectively.

Keywords. BBTv, begomovirus, CMV, survey, PCR, PRSV.

INTRODUCTION

Plant diseases including those caused by viruses are a major constraint to the production of agricultural crops in Vietnam, however a lack of suitable diagnostic techniques has limited their detection. In 1998, we commenced a project to characterise viruses infecting some of the major agricultural crops in northern Vietnam, namely banana, papaya, and several cucurbit crops (pumpkin, cucumber, gourds and loofa).

Prior to the commencement of this project, the only advanced diagnostic capacity for plant viruses in northern Vietnam was ELISA for some viruses of potato, and electron microscopy. Based on symptomology, Vietnamese scientists suspected that many crops were infected with a range of viruses, but it was not possible to determine the causal agents, primarily due to the lack of resources and training.

In the Red River Delta region of northern Vietnam, most cucurbit crops are grown from April to October and banana and papaya are generally grown throughout the year in small plantations or in backyards. In the northern highlands, crops are grown in small plots, with the particular crop dependent on the season. Based on visual inspections prior to the commencement of the surveys, we perceived that some of the most important viruses of crops in Vietnam were *Banana bunchy top virus* (BBTV) in bananas, *Papaya ringspot virus* (PRSV) in papaya, and probably PRSV and geminiviruses in cucurbits. BBTV causes banana bunchy top disease (BBTD), the most serious virus disease of bananas worldwide and although the disease was first reported in Vietnam in the 1960s (Vakali, 1969), the distribution and impact of the virus throughout the country has not been thoroughly investigated. PRSV is the major pathogen of papaya (Purcifull *et al.*, 1984) and an important pathogen of cucurbits (Tomlinson, 1987). The virus is aphid-transmitted and has two biotypes; PRSV-P that

only usually infects papaya, and PRSV-W that infects cucurbits and is unable to infect papaya. However, there is considerable evidence that PRSV-P evolves from PRSV-W by mutation (Bateson *et al.*, 1994; 2002), indicating that PRSV-W infected cucurbits are a potential reservoir of new strains that may infect neighbouring papaya.

Geminiviruses are a major constraint on agricultural production in tropical and subtropical regions, with viruses such as *Tomato yellow leaf curl virus* (Rochester *et al.*, 1994), and *Cotton leaf curl virus* (Zhou *et al.*, 1998) having a major impact. At the commencement of this study in 1998, the only geminivirus reported in cucurbits in South East Asia was *Chinese squash leaf curl virus* (SLCV-Ch) (Hong *et al.*, 1995), infecting Chinese squash (*Cucurbita pepo*) crops. This virus was distinct from *Squash leaf curl virus* (SLCV) that had been previously reported from the USA (Lazarowitz and Lazdins, 1991). A number of other begomoviruses have subsequently been reported infecting South East Asian cucurbit crops, including Squash leaf curl Yunnan virus from China (Xie and Zhou, 2003), and two viruses from Vietnam identified during the virus surveys reported in this paper, namely Squash leaf curl virus-Vietnam (SLCV-Vn) and Loofa yellow mosaic virus-Vietnam (LYMV-Vn) (Revill *et al.*, 2003).

The main aims of this study were to identify some of the most common viral pathogens present in banana, papaya, and cucurbit crops, initially in northern Vietnam but the survey was subsequently expanded to include some southern and central provinces. It was not feasible to test for all potential virus pathogens of each crop and it is important to note that

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this was not an incidence survey. The virus variability and characterisation studies for BBTv, PRSV, SLCV-Vn and LYMV-Vn, have been reported elsewhere (Bell *et al.*, 2002; Bateson *et al.*, 2002; Revill *et al.*, 2003) and herein we report our survey results, and discuss the ramifications of our findings for plant health in Vietnam.

METHODS

Surveys. Seven surveys were conducted between November 1998 and May 2001; four in the north of Vietnam, and three in southern and central regions, with over 280 banana, papaya and cucurbit samples collected from 36 provinces (Table 1). Samples were collected from representative crops and cropping areas, as well as from backyards in villages and along roadsides.

Sample collection and storage. Plant material was collected and stored in a cool box before being diced and placed into vials half filled with silica gel. The silica was changed until it remained blue in colour. This material was then transported back to Australia, where it was analysed using methods described below. In most instances, a duplicate sample set was also stored at Hanoi Agriculture University (HAU). GPS reading was taken for most samples collected after June 2000.

Nucleic acid extraction. Total nucleic acids were extracted from plant tissue using RNeasy and DNeasy kits (QIAGEN) and used as templates in RT-PCR or PCR respectively.

RT-PCR from papaya. RNA extracted from samples exhibiting PRSV symptoms was used as template in an RT-PCR with PRSV specific primers designed to amplify a fragment of the PRSV capsid coding region (Bateson *et al.*, 1994).

PCR from banana. Forty six samples exhibiting typical BBTv symptoms were collected and analysed for BBTv infection by PCR using the methods described in Bell *et al.* (2002).

RT-PCR, PCR and ELISA from cucurbits. Cucurbit samples were tested for PRSV (n = 117) as described above, and geminiviruses (n = 85) using the primers and protocols described by Revill *et al.* (2003). Sixty four samples were tested for both PRSV and geminiviruses. Seventy three samples were also tested for *Cucumber mosaic virus* (CMV) using indirect ELISA (antiserum supplied by Greg Hafner, Queensland University of Technology). Briefly, leaf tissue was extracted in carbonate buffer (18 mM Na₂CO₃, 40 mM NaHCO₃, 1/20 w/v) and 100µl of clarified sap was loaded per well. Following overnight incubation at 4 °C, plates were washed three times with PBS-Tween (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄·7 H₂O, 1.4 mM KH₂PO₄, 0.05 % Tween 20) and incubated for 2 hrs with 100µl of CMV antiserum (1/1000).

Table 1. The regions and provinces surveyed for BBTv, PRSV, CMV and begomoviruses in Vietnam.

Region	Province	Region	Province	
Northern	Ha Noi	Central	Da Nang	
	Bac Ninh		Hue	
	Bac Giang		Quang Binh	
	Ninh Binh		Ha Tinh	
	Hung Yen		Nghe An	
	Phu Tho		Thanh Hoa	
	Hai Duong		Hoi An	
	Hoa Binh		Hoa Binh	
	Son La			
	Lang Son		Southern	Phan Rang
	Cao Bang			Tien Giang
	Bac Kan			Dong Nai
	Thai Nguyen			Binh Thuan
	Tuyen Quang			Dac Lac
Hai Phong	Nha Trang			
Yen Bai	Ho Chi Minh City			
Vinh Phuc				
Dong Nai				
Lai Chau				
Hai Phong				
Lao Cai				

Plates were washed, incubated with anti-rabbit IgG-alkaline phosphatase conjugate (1/7500) at room temperature for 1hr, washed, and 100µl of K-Gold substrate (Neogen) was added to each well. A positive ELISA result was recorded when optical densities were at least 2.5 times the reading obtained from a healthy cucurbit control. Forty nine samples were tested for all four virus types.

Cloning and analysis. PCR products were electrophoresed through 1% (w/v) agarose gels and viewed with a UV transilluminator. To confirm sequences were viral, DNA was excised from the gel and purified by the QIAX II (Qiagen) procedure and ligated into the plasmid vector pGEM-T Easy (Promega), as recommended by the manufacturer. Plasmids were transformed into *E. coli* strain JM109 (Promega), purified and cloned inserts were sequenced.

RESULTS

A summary of all samples tested is presented in Table 2. In all, 282 samples exhibiting virus-like symptoms were tested by PCR and/or ELISA, consisting of 46 banana, 93 papaya, and 143 cucurbit samples. Of these, 87 % were positive for virus infection.

Banana. The most common virus-like symptoms observed in Vietnam were those typically attributed to BBTv infection, ranging from mild yellowing of leaf margins and interveinal

Table 2. The number of samples that tested positive for virus infection. The number of samples tested for each crop is shown in parentheses. PRSV = *Papaya ringspot virus*, BBTV = *Banana bunchy top virus*, SLCV-Vn = *Squash leaf curl Vietnam virus*, LYMV-Vn = *Loofa yellow mosaic Vietnam virus*, CMV = *Cucumber mosaic virus*.

Crop	PRSV	BBTV	SLCV-Vn	LYMV-Vn	CMV
Papaya	93 (93)				
Banana		46 (46)			
Cucumber	10 (16)		1 (13)	0 (13)	12 (12)
Pumpkin	54 (68)		17 (51)	0 (51)	2 (42)
Green Cucurbit	7 (15)		1 (8)	0 (8)	3 (6)
Loofa	6 (13)		0 (8)	2 (8)	2 (8)
Bottle Gourd	2 (3)		1 (3)	0 (3)	0 (3)
Zucchini	2 (2)		1 (2)	0 (2)	0 (2)

flecking, to plants with leaves forming a tight upright bunched growth pattern. BBTVD was observed throughout Vietnam in the following cultivars, Chuoi Tieu, Chuoi Ngu, Chuoi La Nang Tien, Chuoi Sung Bo, Chuoi Chau and Chuoi Bom (Chuoi = banana in Vietnam). BBTVD was observed in both mature plants and newly emerged suckers. All 46 plants tested exhibiting typical symptoms were positive for BBTV using PCR and/or Southern hybridisation. Symptomless plants were often observed growing adjacent to infected plants and PCR confirmed that all symptomless plants tested were not infected with BBTV (data not shown). Generally the proportion of BBTV-infected bananas was small; however we did observe one very severe outbreak of BBTV, with virtually every plant in the crop exhibiting strong BBTV symptoms, in a number of small plantations of the cultivar Chuoi Tieu growing near Sa Pa in northern Vietnam. At least in one instance, the plants had been produced in tissue culture and imported from China.

Symptoms of BBTV infection were most commonly observed in Chuoi Tieu, which is a synonym for members of the Cavendish subgroup. Interestingly, BBTV was not observed or detected in the Vietnamese cultivar Chuoi Tay, even when Chuoi Tay plants were surrounded by BBTV-infected Chuoi Tieu. Although BBTV was endemic throughout Vietnam, we did visit large banana plantations in southern Vietnam that were not infected with BBTV. These large plantations of Chuoi Bom and Chuoi Su cultivars were north-east of Ho Chi Minh City. We did detect BBTV in Chuoi Bom in northern Vietnam, although we did not determine if these plants were the same cultivar as those grown in the plantations in southern Vietnam. Chuoi Su was not observed in northern Vietnam, although a small number of BBTV-negative plants were surveyed near Hoi An in central Vietnam.

Although we did observe many hundreds of plants with BBTVD symptoms throughout Vietnam, four banana plants were also observed with virus-like yellow streak symptoms and a further 10 plants were observed with mosaic symptoms. These symptoms were observed on plants in northern and southern Vietnam. PCR confirmed that these plants were not

infected with BBTV (data not shown) and it remains to be determined if these or any other samples were infected with another virus(es) such as *Banana streak virus* (BSV) or *Cucumber mosaic virus* (CMV).

Papaya. PRSV was detected in all 93 papaya plants exhibiting typical PRSV symptoms. Symptoms varied, but generally consisted of yellow leaf spots, green streak marks on the petiole, and ringspots on fruit. In severe cases, strap like leaves, stunting, and death of the plant were observed.

PRSV was endemic in papaya throughout most of the areas surveyed in Vietnam, and although the incidence of virus infection was not quantified, we estimated that approximately 80% of papaya in the areas we surveyed were infected with PRSV. However, we did visit two regions where we did not observe virus symptoms. One region was in the southern highlands between Nha Trang and Buon Ma Thuot and the first infected papaya in this region was not observed until 55 km south east of Buon Ma Thuot town, a distance of approximately 60 km from the last infected plant observed. In the second region near Dien Bien Phu in northwest Vietnam, the last infected papaya was observed 12 km west of Son La township (GPS: N 21.58317, E 103.35542) and we did not observe any PRSV-infected papaya between this point and Dien Bien Phu, a distance of approximately 80 km. Interestingly, PRSV was detected in cucurbits between Son La and Dien Bien Phu and in the Dien Bien Phu region itself.

Cucurbits. Virus symptoms including mosaics, yellowing, mottling and vein clearing were most commonly observed in pumpkin (*Cucurbita sp.*) and cucumber (*Cucumis sativa*). Virus symptoms were less frequently observed on green cucurbit/waxy gourd (*Benicasa hispida*), bottle gourd (*Lagenaria leucantha*), and loofa (*Luffa acutangula*) (in southern Vietnam only). Zucchini (*Cucurbita pepo* var *Pepo*) is not commonly grown in Vietnam, but in the only crop examined, stunting, twisting and plant death was observed. The most common viruses detected in cucurbits were PRSV (47%), followed by CMV (27%) and geminiviruses (26%). The most common

geminivirus detected in cucurbits was SLCV-Vn, with LYMV-Vn only detected in loofa in southern Vietnam. PRSV symptoms generally consisted of a chlorotic mottle on the leaves, whereas geminivirus symptoms consisted of a bright yellow leaf mosaic. CMV symptoms in cucumber consisted of a mild yellow leaf mosaic.

Viruses were detected in a number of different cucurbit species, with PRSV detected in pumpkin, green cucurbit/waxy gourd, bottle gourd, zucchini, cucumber, and loofa. SLCV-Vn was detected in pumpkin, zucchini, and green cucurbit throughout Vietnam, and LYMV-Vn was only detected in loofa in southern Vietnam. CMV was detected in cucumber, pumpkin, green cucurbit, and loofa, however it was most common in cucumber, with 100% of samples exhibiting virus symptoms infected with CMV. Virus symptoms were never observed on bitter melon (*Momordica charantia*) or choko (*Sechium edule*) and viruses were not detected by PCR or ELISA (data not shown). A small number of cucurbit samples had mixed virus infections, with 9% of those tested infected with PRSV and SLCV-Vn, 10% infected with PRSV and CMV, and 3% infected with CMV and SLCV-Vn. No samples were infected with all three viruses.

DISCUSSION

Our PCR-based virus surveys have confirmed that viruses infect banana, papaya and cucurbit crops throughout Vietnam. BBTV was widespread in Vietnam, probably due to a lack of adequate control measures, as we consistently observed many old infected plants growing among newly planted suckers. Removal or burial of these infected plants, after first killing the aphid vector, would greatly reduce the virus inoculum and spread. One measure used to control virus diseases in many countries is the use of tissue culture to provide virus-tested propagating material. However, we received two independent reports suggesting that this approach may not be suitable for control of BBTV in Vietnam. Growers in both the Sa Pa district in northern Vietnam and the Nui Thanh District in southern Vietnam stated they prefer to use vegetatively propagated suckers instead of tissue cultured bananas, as tissue cultured bananas were more susceptible to disease than suckers. This was despite the presence of a tissue culture laboratory in the Nui Thanh District that produced virus-tested planting material. It is unlikely that the tissue culture plantlets were infected with BBTV prior to release from tissue culture, as farmers stated that symptoms developed only after a period of time. The reasons for this apparent increased susceptibility of tissue culture banana plantlets to BBTV infection are unknown, however Whiley *et al.* (1998) reported increased susceptibility to *Fusarium oxysporum* subtropical race 4 in micropropagated Cavendish bananas. Further studies are required to investigate if there is

any relationship between susceptibility to BBTV and tissue culture propagation.

Another interesting feature of the BBTV infections in Vietnam was the presence of many healthy banana plants surrounding mature BBTV-infected plants. The only crops where we observed high incidence of BBTV were plants propagated in tissue culture. The reasons for this are unknown. The reasons for not detecting BBTV in the local Vietnamese cultivars Chuoi Tay and Chuoi Su are also unclear. Chuoi Tay is reportedly a synonym for Pisang Awak (Vu, pers. comm.), a cultivar known to be susceptible to BBTV infection. However, preliminary RAPD analysis in our laboratory has shown that many of the plants designated Chuoi Tay in Vietnam are genetically diverse, being different to both Pisang Awak and to each other (data not shown). There is no known resistance to BBTV and it is unclear whether the Chuoi Tay and Chuoi Su cultivars in Vietnam are resistant to BBTV, or whether other factors such as vector transmission are playing a role. BBTV is transmitted by the banana black aphid *Pentalonia nigronervosa* and although we observed similar levels of aphid infestation on both healthy and BBTV-infected plants, the efficiency of vector transmission in Vietnam is unknown. South East Asia is a center of origin for edible bananas (Simmonds, 1962) and Bell *et al.* (2002) showed that the level of BBTV sequence variability in Vietnam was almost double that previously reported for BBTV isolates in Asia. These observations suggest that BBTV has been present in Vietnam for a very long time and it is possible that some level of resistance to BBTV may have evolved. BBTV transmission experiments and the genotyping of Vietnamese banana cultivars are required, to determine if BBTV-resistant banana cultivars are present in Vietnam. The incidence of other important viral pathogens of banana in Vietnam, such as *Banana streak virus*, *Banana bract mosaic virus* and CMV, also requires investigation.

PRSV was detected in both papaya and cucurbits throughout Vietnam. Most papaya plants we observed in Vietnam were infected with PRSV. PRSV-P is a major limiting factor in papaya production worldwide (Purcifull *et al.*, 1984), and in some regions of Vietnam such as the foot-hills surrounding Ha Noi, PRSV-P infection had rendered papaya production uneconomic and papaya had been replaced with lychee. Papaya is no longer grown in large plantations in Vietnam, mainly due to the effects of PRSV-P. Interestingly, we did visit two regions of Vietnam where we did not observe nor detect PRSV-P in papaya, although PRSV was detected in cucurbits in these areas. Bateson *et al.* (1994) proposed that PRSV moves from cucurbit into papaya following mutation of an as yet unidentified host-range determinant in the PRSV-W genome. The absence of PRSV-P in papaya in areas containing PRSV-infected cucurbits suggests that mutation of PRSV-W to PRSV-P is a relatively rare event and is most likely due to the isolation of these regions from other infected papaya. The only effective measure for control of PRSV in papaya is transgene-mediated resistance (Tennant *et al.*, 1994),

which has been used to great effect to restore the papaya industry in Hawaii.

In addition to PRSV, approximately 25% of all cucurbits analysed were infected with the geminivirus SLCV-Vn, although it did not infect loofa. Generally, infected plants exhibited an intense yellow mosaic, and in the only crop of zucchini examined stunting, twisting and plant death was observed. A different begomovirus, LYMV-Vn, was isolated from loofa but only in Southern Vietnam. Revill *et al.* (2003) showed that the nucleotide sequence of the SLCV-Vn and LYMV-Vn DNA-A molecules were 80% similar, yet they shared almost identical coat protein coding regions, suggesting a recombinant origin. Recently the DNA-A component of a new begomovirus infecting cucurbits has been identified in China (Xie and Zhou, 2003). This virus has been provisionally named Squash leaf curl Yunnan virus (SLCYNV) and is only 63% and 72% similar to the DNA-A molecules of SLCV-Vn and LYMV-Vn respectively. SLCYNV also appears to have had recombinant origins, with sequence similarity to both *Tomato yellow leaf curl Thailand virus* and *Pepper leaf curl virus*. The identification of recombinant geminiviruses in cucurbits is not surprising, as recombinant geminiviruses have been identified in many crops, including cotton (Zhou *et al.*, 1998), tomato (Navas-Castillo *et al.*, 2000) and cassava (Berrie *et al.*, 2001; Fondong *et al.*, 2000; Pita *et al.*, 2001). It remains to be determined whether SLCV-Vn and LYMV-Vn are the only geminiviruses infecting cucurbits in Vietnam.

CMV was detected in 25% of cucurbit samples. CMV is one of the most widespread viral plant pathogens, infecting over 1000 species (Hull, 2002), although its effect on fruit quality and yield in Vietnam is unknown. No viruses were detected in 13% of cucurbit samples exhibiting virus-like symptoms. This indicates the possibility of other viruses that were not identified using these PCR-based detection techniques. All survey samples are stored dried on silica gel at the Queensland University of Technology and many are duplicated at Hanoi Agriculture University. This repository has also been catalogued into a database which contains information on the type of sample, location, symptoms, and indexing results. These samples are an invaluable resource, as they will enable us to test for additional viruses using alternative approaches such as double-stranded RNA analysis, or PCR as additional sequence information becomes available. The tools described here could also be used to determine the incidence of these viruses in the respective crops, which in conjunction with yield studies would provide valuable information on their impact on agriculture in Vietnam.

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