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Distribution of *Potato spindle tuber viroid* in New Zealand glasshouse crops of capsicum and tomato

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Abstract. An initial investigation of *Potato spindle tuber viroid* (PSTVd) in capsicum revealed the presence of the viroid at two sites in the Auckland area. Subsequently, a delimiting survey of PSTVd was conducted in glasshouse capsicum and tomato crops in New Zealand from April to June 2002. The viroid was not detected in any of the 59 tomato sites surveyed. PSTVd was found at a further three of 41 capsicum sites surveyed in the Auckland region. Prior to the delimiting survey, three tomato sites where PSTVd was detected in April 2001 were visited and PSTVd was still present in tomato plants at one of these sites. Seeds and flesh from fruit of diseased capsicum and tomato plants tested PSTVd positive at two sites prior to the delimiting survey. The viroid was not detected in 8-, 16- and 27-week-old seedlings grown from seeds of infected capsicum and tomato crops. The PSTVd isolate from capsicum had 100% nucleotide identity to a tomato isolate from New Zealand (GenBank accession AF369530), 98.3% to a tomato isolate from the Netherlands (GenBank accession X17268) and 96.3% to a *Solanum* isolate from Australia (GenBank accession U51895). As PSTVd has never been reported in New Zealand before, capsicum and tomato seeds are considered to be the most likely source of PSTVd infection.

Additional keyword: *Capsicum annuum*, *Lycopersicon esculentum*, survey.

Introduction

Potato spindle tuber viroid (PSTVd, genus *Pospiviroid*) is an unencapsidated, single-stranded, circular RNA consisting of 356–360 nucleotides (Herold *et al*. 1992). PSTVd was originally reported in North America but has since spread to Africa, Asia, Europe and South America (Smith *et al*. 1997). The viroid has continued to spread and incursions have been reported in both New Zealand (Elliott *et al*. 2001) and Australia (Mackie *et al*. 2002).

PSTVd is primarily a pathogen of potato and may cause severe yield losses in this crop (Pfannenstiel and Slack 1980). However, the viroid also naturally infects other solanaceous species including aubergine (*Solanum melongena*), pepino (*Solanum muricatum*), and tomato (*Lycopersicon esculentum*) (Diener and Raymer 1971; Salazar 1989; Putcha *et al*. 1990; Shamloul *et al*. 1997). The pathogen experimentally infects at least 48 species in eight families (Singh *et al*. 2003*a*) with many of these hosts, including capsicum (*Capsicum annuum*), apparently having few or no disease symptoms (O'Brien and Raymer 1964).

In potato, the viroid is mainly transmitted vegetatively through infected tubers but it is also easily mechanically transmissible during normal agronomic practices. PSTVd is also transmitted through the seed and pollen of infected plants, at least in potato and tomato (Singh 1970; Grasmick and Slack 1986; Kryczyński *et al*. 1988). The rate of seed transmission varies depending on whether the male, female, or both parents are infected but figures of ~10% are typically reported (Singh 1970; Grasmick and Slack 1986). In addition, the viroid is transmitted by aphids such as *Myzus persicae* when trans-encapsidated with the coat protein of *Potato leafroll virus* (Querci *et al*. 1997).

Tomatoes and capsicums are important crops in New Zealand and the industries are worth $52.8 million and $28.1 million, respectively (K. Robertson, New Zealand Vegetable and Potato Growers' Federation, personal communication). PSTVd was first recorded in New Zealand in May 2000 when characteristic symptoms were detected in glasshouse tomatoes in South Auckland (Elliott *et al*. 2001). These symptoms were restricted to the top of affected plants and included leaf interveinal chlorosis, epinasty and

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brittleness. A subsequent delimiting survey conducted on 50 New Zealand tomato glasshouse facilities from February to March 2001 concluded that PSTVd was present at a further two sites in South Auckland and one site in Nelson (Elliott *et al*. 2001). The viroid was detected at one additional site in South Auckland in late 2001. Since PSTVd can cause significant yield losses in tomato, the New Zealand tomato industry has instituted management procedures to control and eventually eradicate the viroid from affected glasshouses.

In November 2001, a diseased capsicum plant was received for diagnosis. Due to the recent incursion of PSTVd in tomatoes in New Zealand, the plant was tested for the viroid. This paper reports the initial investigation of PSTVd in capsicum by the Plant Environmental Laboratory (PEL), the subsequent research to characterise the PSTVd capsicum isolates and to determine the distribution of the viroid in New Zealand through capsicum and tomato surveys. Survey results were used to make disease management decisions.

Methods

Initial diagnoses of PSTVd infection of capsicum

In November 2001, a diseased capsicum plant (*Capsicum annuum* cv. Special) from a glasshouse in North Auckland was submitted for diagnosis. A sample from this plant was sent to a commercial laboratory (Central Science Laboratory, York, England) to be tested for PSTVd. In March 2002, a second capsicum sample (*C. annuum* cv. Special) from a site 80 km distant to the first, was tested for PSTVd by the PEL.

Isolates of PSTVd and host plant observations

To determine whether PSTVd-infected capsicum plants could be reliably identified by sight during surveys, 15 healthy and 25 symptomatic leaf samples were collected from a North Auckland glasshouse and tested using RT-PCR as described below. Capsicum flowers were also collected and sent to the PEL-Entomology for the identification of arthropods.

Prior to the delimiting survey, three tomato sites in South Auckland where PSTVd was detected in April 2001 were visited. Tomato plants showing typical PSTVd symptoms including epinasty, plant stunted with bushy appearance, and leaf yellowing and necrosis (Kryczyński *et al*. 1988) were collected and tested by RT-PCR as described below.

Survey of capsicum and tomato glasshouses

The delimiting survey was carried out at 100 properties (41 capsicum and 59 tomato) throughout New Zealand between April and June 2002 (Table 1).

Sampling strategy

To prevent mechanical transmission during sampling, disposable overalls, gloves and boots were worn. Leaf samples were collected from up to ten symptomatic plants per glasshouse unit. For each plant, five leaves were collected from the youngest middle growth. In addition, leaf samples were taken from 30 randomly selected plants in six rows of each glasshouse unit. Five plants were sampled at 1 m intervals along a row, starting at the central pathway. For the randomly sampled plants, only one leaf was taken per plant and these were pooled in groups of five for later testing.

Table 1. Number of capsicum and tomato sites surveyed to determine the distribution of *Potato spindle tuber viroid* throughout New Zealand from April to June 2002

Regions	Capsicum sites	Tomato sites
North Island		
Auckland	13	24
Bay of Plenty	1	6
Gisborne	2	1
Hawke's Bay	4	3
Northland	3	3
Taranaki	2	1
Waikato	3	2
Wairarapa	0	2
Wanganui	2	2
Wellington	2	1
South Island		
Dunedin	3	6
Marlborough	2	1
Mid Canterbury	2	1
Nelson	1	3
North Canterbury	1	0
South Canterbury	0	2
Southland	0	1
Total	41	59

Total nucleic acid extractions

During the initial investigation, total RNA was extracted from 200 mg of leaf tissue from naturally infected capsicum and tomato plants and from mechanically inoculated indicators as described by Clover and Henry (1999). During the delimiting survey, a rapid total RNA extraction method was adapted from Thomson and Dietzgen (1995) with the following modifications. A 3 mm^2 piece of leaf was removed from each of the five leaves using a clean scalpel blade. The leaf pieces were then placed in an ice-cold microfuge tube containing 100 µL of extraction buffer (100 mM Tris–HCl pH 8.4, 1 M KCl and 10 mM EDTA). The leaf tissues were ground thoroughly using a pipette tip and a further 400 µL of extraction buffer was then added. The tubes were vortexed, incubated for 10 min at 95°C, then chilled and briefly centrifuged. The supernatant was diluted 1/10 (v/v) in sterile distilled water and 2 µL of this dilution was used for RT-PCR as described below.

Seeds and flesh of 13 capsicum and three tomato fruit collected from PSTVd-infected plants were tested for the presence of the viroid. RNA was extracted from one seed or 2 mm^2 of flesh using 100 µL of extraction buffer, and tested as described above.

RT-PCR

RT-PCR was performed using the primers and thermocycling conditions described by Shamloul *et al*. (1997). The RNA was denatured at 65°C for 10 min and cooled immediately on ice. Reverse transcription (RT) was carried out in a 10 µL volume using 1 µL of heat-denatured total nucleic acid, 2 µL of 5× reverse transcriptase reaction buffer, 1 µM reverse primer (5′-CCC TGA AGC GCT CCT CCG AG-3′), 1 mM dNTPs, 10 units of RNase inhibitor and 100 units of MMLV-reverse transcriptase (Promega, Wisconsin, USA) at 37°C for 1 h. Polymerase chain reaction (PCR) was performed in a 20 µL volume containing 2 µL of cDNA, 2 µL of 10× *Taq* polymerase reaction buffer, 1.5 mM $MgCl_2$, 0.2 µM forward primer (5′-ATC CCC GGG GAA ACC TGG AGC GAA C-3′), 0.2 mM dNTPs and 1 unit of *Taq* DNA polymerase (Promega,

Wisconsin, USA). The amplified products of 358 bp were analysed by agarose gel electrophoresis and stained in ethidium bromide.

Sequencing

Amplified products were sequenced directly using an ABI PRISM automated DNA sequencer at the School of Biological Sciences, University of Auckland, New Zealand. The nucleotide (nt) sequences were analysed and compared with other PSTVd isolates using Clustal V method from the MegAlign package (DNA star, Madison, USA).

Transmission studies

Symptomatic leaves of tomato and capsicum plants were ground in extraction buffer [84 mM Na_2HPO_4, 16 mM $NaHPO_4$, pH 7.5, containing 5% (w/v) polyvinylpyrrolidone and carborundum powder] at a rate of 1 g of leaf per 4 mL of buffer. The separate tomato and capsicum homogenates were then mechanically inoculated onto *Chenopodium amaranticolor*, *C. quinoa*, *Cucumis sativum*, *Gomphrena globosa*, *Capsicum annuum*, *L. esculentum* cv. Rutgers, *Nicotinana benthamiana*, *N. clevelandii*, *N. glutinosa*, *N. occidentalis* and *N. tabacum* cv. White Burley. The plants were grown in a glasshouse at ~25°C and symptom development was observed over the following 4 weeks.

Twenty five seeds were collected from each capsicum and tomato plant that was confirmed PSTVd-infected by RT-PCR. The 50 seeds were sown in sterilised peat and pumice sand mix (50 : 50 w/w) and germinated at ~25°C. The seedlings were tested for PSTVd by RT-PCR at 8-, 16- and 27-weeks after germination.

Results

Initial diagnoses of PSTVd infection of capsicum

In November 2001, a capsicum plant from a glasshouse in North Auckland tested positive for PSTVd by the Central Science Laboratory, making this the first published report of PSTVd naturally infecting capsicum. In March 2002, a second capsicum sample from a site 80 km distant from the first was found to be infected with PSTVd when tested by the PEL.

Isolates of PSTVd and host plant observations

Symptoms of PSTVd infection in capsicum plants in a North Auckland glasshouse were subtle. The only obvious symptom was a wavy margin to the leaves near the top of the plant (Fig. 1*B*). Arthropods that may cause similar symptoms were not detected in any crop. RT-PCR tests were negative for 14 of the 15 healthy samples (flat leaf margins; Fig. 1*A*) and positive in 22 of 25 symptomatic samples suggesting that by targeted sampling of symptomatic plants, the majority of PSTVd infections were detected.

Prior to the delimiting survey, three tomato sites where PSTVd was detected in April 2001 were visited and PSTVd was still present in tomato plants at one of these sites. All the seeds and fruit from PSTVd-infected tomato plants tested positive using RT-PCR.

Survey of capsicum and tomato glasshouses

During the delimiting survey in April–June 2002, a total of 1468 samples was collected from 100 glasshouses with tomato and capsicum crops. PSTVd was not detected in any of the 801 tomato samples (745 random and 56 symptomatic samples). However, the viroid was detected in five random and 11 symptomatic capsicum samples (cvv. Fiesta, Special, Spirit, and an unknown cultivar) out of the 667 samples

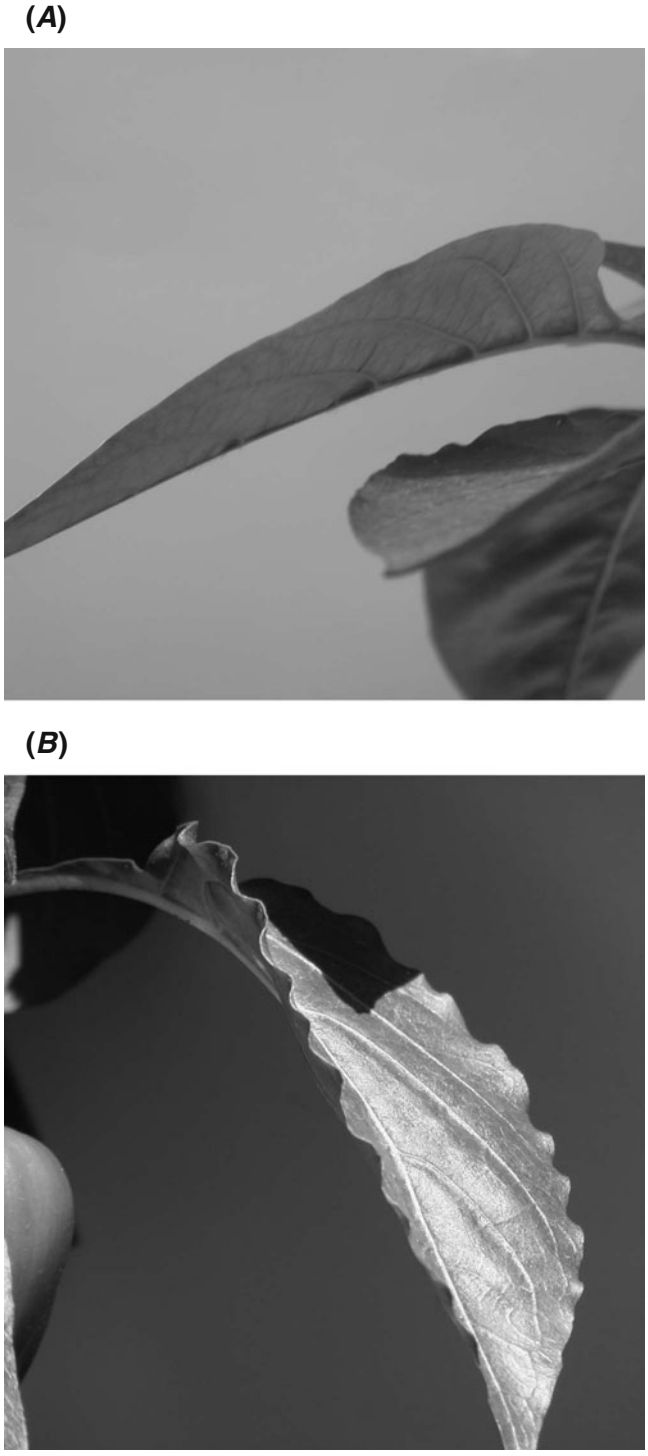

Fig. 1. Flat-edged leaf of a healthy (*A*) and wavy-edged leaf of a PSTVd-infected (*B*) capsicum plant.

collected (449 random and 218 symptomatic samples), with bands of the expected size amplified by RT-PCR. These samples were from two glasshouses on one property and a single glasshouse on a second property, both located near the initial outbreak in the Auckland area. All the seeds and fruit from PSTVd-infected capsicum plants tested positive using RT-PCR.

Sequence analyses

The complete nucleotide sequences of four isolates of PSTVd from *C. annuum* cvv. Fiesta, Special, Spirit, and an unknown cultivar were determined and deposited in GenBank (accessions AY532801–AY532804). These sequences were identical and their alignment produced a consensus sequence of 358 nt in length, which was identical to that previously determined for a New Zealand tomato isolate (GenBank accession AF369530). A BLAST analysis indicated that these sequences had the closest nucleotide identity to a Dutch tomato isolate (98.3%, GenBank accession X17268) and an Australian *Solanum* isolate (96.3%, GenBank accession U51895). These sequences also shared 84.7–88.5% nt identity with other tomato isolates, 84.5–91.1% nt identity to other potato isolates and 89.7% nt identity to a tobacco isolate.

Transmission studies

No symptoms were observed on *Chenopodium* spp., *Cucumis sativum*, *Gomphrena globosa* or *Nicotiana* spp. mechanically inoculated with an extract from PSTVd-infected tomato and capsicum plants. Typical PSTVd symptoms were observed on three out of four tomato plants inoculated with an extract from PSTVd-infected tomato plants but were not observed on inoculated capsicum plants. Mechanical transmission from PSTVd-infected capsicum was successful in only four out of 18 tomato plants and one out of five capsicum plants. PSTVd infection was detected by RT-PCR, but no symptoms were observed on either the tomato or capsicum plants. PSTVd was not detected by RT-PCR in 50 seedlings grown from 25 seeds produced by each PSTVd-infected capsicum or tomato plant at 8-, 16- or 27-weeks after germination, nor were any symptoms observed.

Discussion

This paper presents clear molecular and biological evidence that PSTVd naturally infects capsicum. Although this host has been previously demonstrated to be an experimental host (O'Brien and Raymer 1964), this is the first report of natural infection. During the initial investigation, PSTVd was detected in two capsicum glasshouse sites in the Auckland area. Subsequently, PSTVd was detected at a further three capsicum sites in the Auckland area but not at tomato sites during the delimiting survey. Prior to the delimiting survey, three tomato sites where PSTVd was detected in April 2001 were visited and PSTVd was still present in tomato plants at one of these sites. This would indicate that the viroid is still present in New Zealand tomato glasshouses, but its distribution appears limited. The PSTVd isolates from capsicum and tomato in New Zealand had 100% nucleotide identity, suggesting that the viroid may have a common origin. At some of the affected sites, both crops were grown and it is possible that the viroid may have spread between crops by mechanical inoculation. Although we were unable to demonstrate seed transmission of the isolate of PSTVd found in New Zealand, we cannot discount a very low rate of seed transmission, which would provide a means by which the viroid could be introduced to a property, given the large volumes of seed involved.

No arthropods or other pathogens from infected plants were identified by light microscopy. A wavy leaf margin was the only symptom that appeared to be related to infection. However, in experimental inoculations of capsicum, this symptom was not reproducible using the same PSTVd isolate. O'Brien and Raymer (1964) also noted that 3–5-week-old capsicum plants infected with PSTVd were symptomless. Viroid multiplication and symptom expression probably depend on environmental conditions such as temperature and light intensity (Singh *et al*. 2003*b*). Symptom expression may also depend on the age of the plant at inoculation and the plant's tolerance to infection (Kryczyński *et al*. 1988).

Seeds and flesh of PSTVd-infected tomato and capsicum plants tested positive for PSTVd by RT-PCR, as previously reported in tomatoes and potatoes by Kryczyński *et al*. (1988). However, seedlings grown from PSTVd-infected seeds were symptomless and negative for PSTVd by RT-PCR. This suggests infection of the seed coat but not the embryo. Kryczyński *et al*. (1988) were able to transmit PSTVd from tomato seeds but with variable results giving transmission rates from 0 to 100%. PSTVd in potatoes has never been reported in New Zealand and it was not detected in potato fields surrounding the initial PSTVd outbreak in tomato glasshouses (BSM Lebas, unpublished results). Therefore, infected seeds are still considered to be the most likely source of infection. Our experiment was conducted using a small sample size (50 seeds) and this work should be repeated using a larger sample size to confirm the PSTVd transmission rate from capsicum and tomato seeds.

The effect of PSTVd on the yield of capsicum was not studied, although there were no obvious effects on plant vigour and fruit production. However, in other crops such as tomato and potato, severe yield losses have been reported (Pfannenstiel and Slack 1980; Kryczyński *et al*. 1988) and the extent of these losses varies dramatically according to the viroid isolate and the cultivar. For example, Singh *et al*. (1971) reported that in a field trial, three mild strains of

PSTVd reduced potato yield by 1–24% whereas a severe strain decreased yield by 64%.

Asymptomatic plants are a potential source of infection. Symptoms on capsicums are subtle and hard to detect and symptoms on tomato appear late in plant development. Thus, by the time symptoms are observed, PSTVd may be distributed throughout a glasshouse. Continued monitoring of glasshouses where the viroid has been found is important for determining how successful the industry has been in eradicating the pathogen. Another delimiting survey will be required to monitor the spread of PSTVd in tomato and capsicum glasshouses.

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