Graft-transmissible agents in oriental persimmons (*Diospyros kaki* L) in the southeastern USA.

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Abstract

The “Sudden Death Syndrome” of oriental persimmon (*Diospyros kaki* L) described from the southeastern USA almost 30 years ago has been associated with infection by *Xylella fastidiosa* using PCR. The association between symptoms and the etiological agent is not absolute. Moreover, several viroids (Apple fruit crinkle viroid [AFCVd], Persimmon viroid [PEVd], Persimmon viroid 2 [PeVd 2], Citrus viroid VI [CVd VI - formerly referred to as Citrus viroid OS]) and viruses (Persimmon virus A [PeVA], Persimmon virus B [PeVB], Persimmon cryptic virus [PeCV], and Persimmon latent virus [PeLV]) have also been detected both in persimmon germplasm showing the “Sudden Death Syndrome” and in trees that displayed no distinct symptoms typical of infection by viruses or viroids.

Keywords *Xylella fastidiosa* · Persimmon · *Diospyros kaki* · Viroids · Viruses

The oriental persimmon is native to China, where it has been cultivated for centuries and more than two thousand different cultivars exist. The species was transported to Korea and Japan many years ago where additional cultivars were developed. The plant was introduced to California in the mid 1800’s and the state is today the largest producer of persimmon fruit in the USA with production based in 3 counties: Fresno, Tulare, and San Diego (Kong and Zalom 2017). On more than one occasion cultivation of persimmon has been suggested as an alternative crop in other areas of the USA (Georgia 1980s, Mississippi 1990), but with little success. A trial involving some 19 cultivars located at the Southeastern Fruit and Tree Nut Research Laboratory of the USDA-ARS, Byron, Georgia begun in the 1980s was abandoned after 5 years because of the death of 90% of the trees as a result of a disease that caused a rapid decline with veinal necrosis, premature defoliation, bud death, and the death of individual scaffold limbs (Fig. 1) (Scott and Payne 1988). Trees propagated on rootstocks of *D.*

virginiana using scionwood from the planting at Byron developed the same symptoms 2–4 years after grafting. This “Sudden Death Syndrome” (Reighard and Payne 1991) has appeared intermittently in plantings of kaki persimmon in the southeastern USA over the past 30 years but no association with a specific pathogen has been described. Cohen et al. (1991) documented a decline of persimmon associated with the incompatibility of trees of *D. kaki* on rootstocks of *D. virginiana* and suggested the involvement of a “transmittable biotic factor”. However, there were inconsistencies between the symptoms they described and the symptoms of “Sudden Death”.

Whenever material symptomatic of the “Sudden Death Syndrome” (Figs. 2 and 3) became available, attempts to identify a specific etiological agent (fungus, bacterium or virus) were made without success. On each occasion tissue samples and total nucleic acid (TNA) extracts were stored at –80 °C. Most recently (2016) a few trees in a planting of approximately 500 trees of cv. Fuyu in middle Georgia (Telfair County) began to display the characteristic symptoms described in earlier reports. In some of these most recent samples, cross sections of shoots showed discolored xylem similar to that associated with cross-sections of peach trees diagnosed as displaying symptoms of phony peach (Giesbrecht and Ong 2012). The symptomatic trees were restricted to the perimeter of the planting suggesting that an infectious agent was moving in from surrounding vegetation. This distribution of symptomatic trees mimicked distributions observed in peach orchards

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Fig. 1 A 4 year-old persimmon tree displaying symptoms of “Sudden Death Syndrome”. This tree was recorded in the trial at the Southeastern Fruit and Tree Nut Research Laboratory of the USDA-ARS, Byron Georgia that was begun in the mid1980s and abandoned after 5 years due to the disease

in the southeastern USA when infections with diseases that have leafhopper vectors (such as peach rosette caused by a phytoplasma and phony peach caused by *X. fastidiosa*) had been mapped (Scott, unpublished). The coincidence of the distribution, the discolored xylem, and the incidence of the disease in an area of the USA in which *X. fastidiosa* is endemic (Mizell et al. 2015), led us to test both the most recent samples and those in storage for the presence of *X. fastidiosa* using PCR and Real-time PCR protocols (Table 1).

A number of viroids and viruses have been detected in persimmon in the past decade, using RT-PCR techniques (Ito et al. 2013a, b, 2015; Nakaune and Nakano 2008) and next generation sequencing (Morelli et al. 2015). We chose to test all the samples from Telfair County for the presence of these agents by RT-PCR using the primers and cycling conditions described in published manuscripts as appropriate (Table 1). Wherever possible we also tested the materials collected over the past 30 years for these viroids and viruses.

Fig. 2 Leaf and stem symptoms of “Sudden Death Syndrome” seen on trees growing in Clemson, South Carolina in 2008. Blackening of segments of the veins and die-back and curving of the shoot tips are visible. The curving and die-back of newly developing shoots is typical of the disease. Similar symptoms were observed in trees of cv. Fuyu growing in Telfair County, Georgia in 2016

Total nucleic acid (TNA) samples were extracted from the material collected in Telfair County, Georgia in 2016 using the method of Li et al. (2008). TNA extracted from petioles of a peach tree infected with *X. fastidiosa* was used as a positive control in PCR. TNA extracted from petioles of a peach seedling (*Prunus persica* cv. Nemaguard) that was maintained in an insect-proof screenhouse was used as a negative control for

Fig. 3 Trunk of young persimmon tree cv. Fuyu displaying “Sudden Death Syndrome” collected in Telfair County Georgia in 2016: A. Asymptomatic tree. B. Symptomatic tree showing dark patches moving down from apex of the tree toward the root. C. Blackening does not appear to spread below the graft union and indeed some rootstocks continue to grow after the scion has died

PCR. We did not have positive controls for the viruses and viroids. The quality of the TNA preparations was verified using spectrophotometry. Internal control primer pairs (Nad5 for RNA – Menzel et al. 2002) and (COX for DNA- Weller et al. 2000) were included in reactions to avoid false negative results. Multiple detection procedures were employed as we had no idea of the possible identity of any potential *X. fastidiosa* isolate that might be involved. In all cases where an amplicon of the size described in the original publication was produced in PCR or RT-PCR, the product was cloned and sequenced in both directions (Scott et al. 2003). Sequences were submitted to a Basic Local Alignment Search Tool (megablast) search at the National Center for Biotechnology Information (NCBI) website and relationships to bacteria, viruses, and viroids described previously submitted to GenBank

were identified. The sequences of the amplicons were deposited in GenBank (Table 1). Comparison of the sequences of the amplicons we obtained from persimmon with those of *X. fastidiosa* held in GenBank showed 100% nucleotide identity with many of the curated accessions.

X. fastidiosa was detected in all the samples from the planting in Telfair County, Georgia displaying the symptoms of “Sudden Death” (Table 2). In addition, the viroid (PeVd2) and 4 viruses (PeVA, PeVB, PeLV, and PeCV) were detected in trees from this planting. However, this viroid and these viruses were also detected in trees within the planting that showed neither symptoms of “Sudden Death” nor symptoms typical of any other viral infection. The bacterium (*X. fastidiosa*) was also detected in almost all (16 out of 18) of the TNA extracts from samples showing symptoms of the

Table 1 A listing of the PCR, RT-PCR and real time PCR techniques used to detect pathogens in samples of *Diospyros kaki*. The primer pairs used for the PCR assays are described and the accession numbers for the products that were amplified, sequenced, and deposited in GenBank, are presented. The 2 real-time assays for *X. fastidiosa* each were completed using a probe labelled with FAM and which had a BHQ-1 quencher. C_T values for the real-time reactions of positive controls typically had values of >20

Pathogen	Assay	Citation	5' primer sequence 3'	Amplicon size (bp)	GenBank Accession #
<i>Xylella fastidiosa</i>	PCR	Rodrigues et al. 2003	CGGCAGCACATTGGTAGTA(F) CTCCTCGCGTTAAGCTAC(R)	1349	KY776470
	PCR	Rodrigues et al. 2003	CAGTTAGGGGTGTCAGCG(F) CTCAATGTAATTACCCAAGGT(R)	429	MF581192
	PCR	Minsavage et al. 1994	GCGTTAATTTTCGAAGTGATTCGATT GC(F) CACCATTTCGTATCCCGGTG(R)	721	MF581193
	Real-Time PCR	Schaad et al. 2002	AAAAATCGCCAACATAAACCC(F) CCAGGCGTCCTCACAAGTTAC (R)	71	
		Probe	ACCTATGCCAACATCAAACCCTGAAT GCA		
	Real Time PCR	Harper et al. 2010	CACGGCTGGTAACGGAAGA (F) GGGTTGCGTGGTGAAATCAAG (R) TCGCATCCCGTGGCTCAGCC	70	
Apple fruit crinkle viroid	RT-PCR	Nakaune and Nakano 2008	GCCCGGTCTGTGGATACCTAG (F) GCCCCGAGGGAAAAATAGGA (R)	373	MF804513
Persimmon viroid	RT-PCR	Nakaune and Nakano 2008	AGCTCGGGCTGTGAGCTTGG (F) CGGCAGGGAGCCTTGCGAAC (R)	394	MF804515
Persimmon viroid 2	RT-PCR	Ito et al. 2013a	CTCCGTGTGGTTCCTGTGTG (F) GACTCCACTCTCCCTAGGCT (R)	358	MF804512
Citrus viroid VI	RT-PCR	Nakaune and Nakano 2008	TCGTCGACGAAGGCATGTGA (F) CGACAGGTGAGTCTCCTTGC (R)	326	MF804514
Persimmon Cryptic Virus	RT-PCR	Morelli et al. 2015	TCCACGACCGATTGTGC (F) ACGAAGACACGTAACCACGCAGTGG (R)	141	MF804510
	RT-PCR	Morelli et al. 2015	AGTTTCGAGTCGTCTTCGGCGG (F) GTGTTGTTACACGGTGTAGTAACT (R)	144	MF804511
Persimmon latent virus	RT-PCR	Ito et al. 2013b	CGATCCACTGACCTGATCAAC (F) TAGAGCACGCGCAAATACTC (R)	250	MF804509
Persimmon virus A	RT-PCR	Ito et al. 2013b	GGATGGCTTCTGATGTGTGG (F) TTGACCCGAATATGTGCGAT (R)	296	MF581191
Persimmon virus B Variant 1	RT-PCR	Ito et al. 2015	CAGTCACATTGGTGAAATGA TACCGGA (F) TGTGCCTCGAGATGGCCACAA (R)	No amplicon detected	
Variant 2	RT-PCR		CGAAGGACGACGGTCCTG (F) TATTTGGAGCGTGGACACGA (R)	209	MF782854
Variant 3	RT-PCR		ACTTCTCTTCGTCCATATCTG (R) CTACTTTCACAGTAAATGCTGC (F)	246	MF782854
Variant 4	RT-PCR		TCCAACCGAATACGATCTCC (F) GTAGGTAGCTAGCACCGCAG (R)	245	MF782855

Table 2 Results of PCR testing of samples collected from Telfair County, Georgia, USA in 2016. All trees were of the cultivar “Fuyu”

Sample ID	SDS	Xf	AFCVd	PeVd	PEVd 2	CVd-VI	PeVA	PeVB	PeLV	PeCV
Telfair 1	+	+	+	+	+	+	+	+	+	+
Telfair 2	+	+	+	+	+	+	+	+	+	+
Telfair 3	+	+	+	+	+	+	+	+	+	+
Telfair 4	+	+	+	+	+	+	+	+	+	+
Telfair 5	+	+	+	+	+	+	+	+	+	+
Telfair 6	+	+	+	+	+	+	+	+	+	+
Telfair 7	+	+	+	+	+	+	+	+	+	+
Telfair 8	+	+	+	+	+	+	+	+	+	+
Telfair 9	as	nd	+	+	+	+	+	+	+	+
Telfair 10	as	nd	+	+	+	+	+	+	+	+

disease that had been collected, extracted and stored at -80°C in previous years. Three other viroids (AFCVd, PeVd and CVd VI) were detected in some of these samples (Table 3). We were unable to conduct a complete screening of this material collected over the years as we had a limited amount of TNA and had to select the agents against which we should test.

This is the first report worldwide of *X. fastidiosa* in persimmon of which we are aware. However, symptoms similar to those observed for “Sudden Death” had been reported on persimmons from Italy in the late 1940s (Mezzetti 1947, 1950, 1956 and 1957 – See images). Mezzetti concluded that the graft-transmissible nature of the disease suggested viral or bacterial etiology but did not identify the agent.

This is also the first report of various viroids (AFCVd, PeVd, PEVd 2, CVd VI) and viruses (PeVA, PeVB, PeLV, and PeCV) being detected in persimmon germplasm in the southeastern USA. However, the detection of these viruses and viroids is not surprising as persimmon germplasm has been moved world-wide from Japan where these viruses and viroids were initially detected. Recently, PeCV has been detected in Italy (Morelli et al. 2015) and Spain (Ruiz-García et al. 2017).

Although the association between the “Sudden Death Syndrome” and the presence of *X. fastidiosa* is not absolute, the PCR results suggest a relationship between this bacterium and the disease. Despite previous research this is the first time that a potential etiological agent has been identified for this syndrome and clearly, pathogenicity studies are necessary to demonstrate Koch’s postulates and to validate the relationship between “Sudden Death Syndrome” and infection with *X. fastidiosa*.

In the original report of the “Sudden Death Syndrome” (Scott and Payne 1988) crystals of isometric viruses were revealed by electron microscopy of fixed and embedded tissue from diseased leaves, and concentrated leaf dip preparations showed a few isometric particles. In addition, comparison of dsRNA banding patterns in asymptomatic and symptomatic

trees detected the presence of two bands of dsRNA (MW 1.2 and 1.03×10^6 , respectively) in some, but not all, infected cultivars. Although supporting the idea of a graft-transmissible agent being associated with the symptoms, no definitive identification was made. The detection in this work of the previously unknown PeCV (a possible cryptovirus) in

Table 3 Results of PCR testing of samples of persimmon cultivars from which samples were collected between 1988 and 2008

Cultivar	SDS	Xf	AFCVd	PeVd	PEVd2	CVd-VI
Fuyu(*)	+	+	+	+	+	+
Matsumoto	+	+	+	+	+	+
Ichikikei jiro	+	+	+	+	+	+
Korean	+	nd	+	+	+	+
Izu	+	+	nd	nd	nd	+
Sheng	+	+	nd	nd	+	+
Jiro	+	+	nd	nd	nd	+
Hanagosho	+	+	+	+	+	+
Siajo	+	+	+	+	+	+
Eureka	+	+	nt	nt	nt	nt
Tamopan	+	+	nt	nt	nt	nt
Aizumishirazu	+	+	nt	nt	nt	nt
Giombo,	+	+	nt	nt	nt	nt
Hana fuyu(*)	+	nd	nt	nt	nt	nt
Shogatsu	+	+	nt	nt	nt	nt
Tanenashi	+	+	nt	nt	nt	nt
Hiratanenashi	+	+	nt	nt	nt	nt
Hachiya	+	+	nt	nt	nt	nt

Key Tables 2 and 3. Agent detected using PCR (+). The presence of “Sudden Death Syndrome” (SDS) *Xylella fastidiosa* (Xf), Apple fruit crinkle viroid [AFCVd], Persimmon viroid [PEVd], Persimmon viroid 2 [PEVd 2], Citrus viroid VI [CVd -VI] and viruses (Persimmon virus [PeVA], Persimmon virus B [PeVB], Persimmon cryptic virus [PeCV]), and Persimmon latent virus [PeLV])

as = asymptomatic for SDS, nd = not detected by PCR, nt = not tested.

*PeCV detected in this sample in Table 3

two samples collected between 1988 and 2008 support the evidence of the isometric particles and small dsRNA molecules in the original report.

The fact that the disease reduced a trial of 238 trees comprising 17 cultivars of persimmon to 22 trees within 5 years would indicate that this is a serious problem, and potentially a limiting factor in the development of persimmon as an alternative crop in the southeastern USA, particularly when *X. fastidiosa* is endemic to the region.

Lyophilized tissues from *Diospyros kaki* displaying symptoms of “Sudden Death Syndrome”, and which has been shown to be positive for *X. fastidiosa* in real-time PCR, is freely available from the corresponding author at Clemson University. The material has been stored at -80°C since collection and lyophilization.

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