Eur J Plant Pathol (2009) 124:691–694
DOI 10.1007/s10658-009-9440-4

Citrus exocortis viroid transmission through commercially-distributed seeds of *Impatiens* and *Verbena* plants

Rudra P. Singh · Andrea D. Dilworth · Xiaoping Ao · Mathuresh Singh · Virendra K. Baranwal

Received: 29 September 2008 / Accepted: 29 January 2009 / Published online: 12 February 2009

Abstract Surveys of *Impatiens* and *Verbena* species in local nurseries in Fredericton, Canada and *Verbena* species in New Delhi, India showed widespread infection of *Citrus exocortis viroid* (CEVd) in vegetatively-propagated and seed-grown plants. To determine viroid seed transmission, samples of eight varieties of *Impatiens* and 11 varieties of *Verbena* were obtained from four commercial sources. All 19 samples collected contained viroid infection irrespective of variety. The presence of viroid in non-germinated seed was 21%, while the transmission rate in seedlings was 66% in *Impatiens walleriana* in 2006. Following 2 years of seed storage, the respective figures were 6% and 26%. Similarly, in *Verbena* x *hybrida* the presence of viroid in seed was 13% in 2006 with a seed-transmission rate in seedlings of 28%, while the respective figures after 2 years of storage were 5% and 45%.

Keywords Ornamental plants · Seed transmission · Spread of viroids

R. P. Singh (✉) · A. D. Dilworth
Potato Research Centre,
Agriculture and Agri-Food Canada,
850 Lincoln Road,
Fredericton, NB, Canada E3B 4Z7
e-mail: singhr@agr.gc.ca

X. Ao
Faculty of Horticulture and Landscape,
Hunan Agricultural University,
Changsha, Hunan Province 410128, China

M. Singh
Agricultural Certification Services,
1030 Lincoln Road,
Fredericton, NB, Canada E3B 8B7

V. K. Baranwal
Plant Virology Unit, Division of Plant Pathology,
Indian Agricultural Research Institute,
New Delhi 110012, India

Introduction

Citrus exocortis viroid (CEVd), probably one of the most ancient of viroid diseases (Bar-Joseph 2003), has the widest reported naturally occurring host-range among the pospiviroids. It consists of 371 to 375 nucleotides (nt) and is a species within the genus *Pospiviroid* belonging to the family *Pospiviroidae* (Flores et al. 2000). A relatively large number of CEVd sequence variants (>170, NCBI database, June 2008) have been reported, which is regarded as evidence of the ability to incorporate mutations and changes without impairing transmission and replication (Duran-Vila and Semancik 2003).

CEVd causes exocortis (bark shelling) disease in citrus (Fawcett and Klotz 1948). In vegetatively-propagated citrus and related species, the main means of CEVd spread is by bud-grafting and/or use of contaminated tools during pruning operations (Garnsey

and Jones 1967). Symptomatic diseases caused by CEVd in nature are not restricted to citrus. CEVd sequence variants have been reported to cause bunchy-top or leaf chlorosis symptoms in tomato under field conditions (Mishra et al. 1991; Verhoeven et al. 2004) in India and the Netherlands, respectively. In contrast, in several other host plants in nature such as grapevines (García-Arenal et al. 1987), vegetable crops such as broad bean (Fagoaga et al. 1995), eggplant, turnip and carrot (Fagoaga and Duran-Vila 1996), CEVd has been shown to be carried symptomlessly. Considering that most of these vegetables are annual seed crops, the route of CEVd entry is currently unknown.

Recent surveys to determine the prevalence of pospiviroids in ornamental plants have shown that CEVd is carried symptomlessly in vegetatively-propagated plants of *Impatiens* and *Verbena* species (Bostan et al. 2004; Nie et al. 2005; Singh et al. 2006a). The finding of CEVd infection in seed-grown seedlings, in moss *Verbena* (*Glandularia puchella*; Singh et al. 2006a) in India and *Impatiens* (*Impatiens walleriana;* unpublished observation) in Canada in 2006 pointed to the possibility of seed transmission of CEVd from *Impatiens* and *Verbena* seeds.

To determine the possibility of seed transmission in both ornamental species, seeds were obtained from four commercial companies, which were either available in local nurseries or from the seed companies themselves. Eight varieties of *Impatiens* (*I. walleriana*), and 11 varieties of *Verbena* (*V.* x *hybrida*) were tested for viroids from seed and seedlings (Table 1). Seeds set aside for later testing were stored at 4°C. To facilitate grinding, individual seeds were soaked in 30 µl of extracting solution (50 mM NaOH, 2.5 mM EDTA) in a micro centrifuge tube, incubated overnight and then ground using a pellet pestle. For seedling assays, another seed sample from the same seed source was germinated and grown for six weeks in a greenhouse. The leaves and vine samples from seedlings were passed through a tissue grinder (Electrowerk, Behncke and Co. Hanover, Germany) and about 150–200 µl of sap was collected in a micro centrifuge tube containing 300 µl of extracting solution. The resulting extracts from seed or seedlings were centrifuged at 12,000×*g* at 4°C for 15 min. From the supernatant, RNA was precipitated with 1 vol of isopropanol in the presence of 0.1 vol of 3 M sodium acetate (−20°C overnight). The precipitate was collected by centrifugation (12,000×*g* at 4°C), washed with 70% ethanol, vacuum dried, and dissolved in 20 µl of sterile distilled water for seed or 1,000 µl for leaves (Singh et al. 2006b; Singh and Dilworth 2008).

RT-PCR was performed as described (Singh et al. 2006b) using 2.5 µl RNA for reverse transcription. For PCR amplification, 2 µl cDNA was added to 23 µl solution mix as described (Singh et al. 2006b) with 0.1 µg of *Pospiviroid* specific primer pair consisting of (5′-ATTAATCCCCGGGGAAACCTGGAG-3′ and 5′-AGCTTCAGTTGTTTCCACCGGGT-3′; Bostan et al. 2004) and the full-length CEVd primer pair consisting of two contiguous segments (5′-GGAAACCTGGAG GAA GTCGAG-3′ and 5′-CCGGGGATCCCTGAAG GACTT-3′; Ito et al. 2002). Amplified products were analysed by electrophoresis in a 2% agarose gel containing 0.5 µg ml^{-1} ethidium bromide and photographed under UV illumination with an imaging system (FluorChem, Alpha Innotech Inc. San Leandra, CA).

CEVd isolates identified in *Impatiens* (acc. # EU877742) and *Verbena* (acc. # EU877745) species in this study do not differ significantly in their nt sequence compared to those identified from the

Table 1 Detection of *Citrus exocortis viroid* in commercially distributed seeds and resulting seedlings of *Impatiens* and *Verbena* varieties in 2006 and 2008

Plant species	Samples[a]	Number of infected samples			
		Seed		Seedlings	
		2006	2008	2006	2008
Impatiens walleriana	No. positive	84	9	106	23
	Total no. tested	400	140	160	90
	% Positive	21	6	66	26
Verbena x *hybrida*	No. positive	72	9	18	9
	Total no. tested	550	180	64	20
	% Positive	13	5	28	45

[a] Seeds were obtained from four different commercial seed producers. Eight varieties of *Impatiens* and 11 varieties of *Verbena* were used

original host *Impatiens* and *Verbena* in an earlier study (Nie et al. 2005).

From each seed sample of *Impatiens* and *Verbena* varieties, two tests were carried out: one in 2006 using 50 seeds from each sample and the other in 2008 using 20 seeds after the seeds were stored for 2 years. The number of seedlings varied in both tests, because germination was poor in most seed samples. The presence of viroid in non-germinated seed was 21%, while the transmission rate in seedlings was 66% in *Impatiens walleriana* in 2006 and after storage of seed for 2 years the respective figures were reduced to 6% in seeds and 26% in seedlings. Similarly, in *Verbena* x *hybrida* the presence of viroid in seed was 13% in 2006 with a seed transmission rate in seedlings of 28%, while the respective figures after storage for 2 years were 5% and 45%. The viroid detection rate in seed may be lower than in seedlings due to the small size of the seeds and the resulting lower yield of viroid from the seed tissue compared to the seedlings.

To further confirm the presence of CEVd in the seedlings, CEVd-positive and CEVd-negative seedlings of *Impatiens* (50 seedling of each) and *Verbena* (18 seedlings of each) were grown in separate plots in the summer of 2006. Infected and healthy plants of both ornamental plants did not show any difference in plant growth and there were no visible symptoms on any plant irrespective of their viroid status. In three RT-PCR tests during July through to September, the CEVd was detected in leaves and petals of *Impatience* and *Verbena* from all CEVd-positive plants but not from healthy plants.

This study provides strong evidence for the presence of CEVd in commercially distributed seeds and its seed transmission in two widely grown ornamental plants. Earlier reports in the literature about the possible seed transmission of CEVd do exist. Initially, CEVd was reported to be seed-transmitted in citrus (Salibe and Moreira 1965) but later this observation was interpreted as natural transmission rather than via seed (Semancik 1980), while more recently it has been stated that seed transmission has not been demonstrated in citrus (Duran-Vila and Semancik 2003). However, CEVd has been claimed to be seed-transmitted through the seed of tomato cv. Rutgers (Semancik 1980).

Widespread movement and infection of a viroid is greatly enhanced through seed transmission. The finding of two ornamental plants as carriers of a seed-borne viroid (Table 1) raises speculation of seed-borne CEVd in earlier outbreaks of the viroid in vegetable crops.

Acknowledgements We gratefully acknowledge the critical reading and editorial comments of Avinash Singh (AgraPoint International Inc., Truro, Nova Scotia).

References

Bar-Joseph, M. (2003). Natural history of viroids—historical aspects. In A. Hadidi, R. Flores, J. W. Randles, & J. S. Semancik (Eds.), *Viroids* (pp. 246–251). Collingwood: CSIRO.

Bostan, H., Nie, X., & Singh, R. P. (2004). An RT-PCR primer pair for the detection of *Pospiviroid* and its application in surveying ornamental plants for viroids. *Journal of Virological Methods*, *116*, 189–193. doi:10.1016/j.jviromet.2003.11.014.

Duran-Vila, N., & Semancik, J. S. (2003). *Citrus viroid*. In A. Hadidi, R. Flores, J. W. Randles, & J. S. Semancik (Eds.), *Viroids* (pp. 178–194). Collingwood: CSIRO.

Fagoaga, C., & Duran-Vila, N. (1996). Naturally occurring variants of *Citrus exocortis viroid* in vegetable crops. *Plant Pathology*, *45*, 45–53. doi:10.1046/j.1365-3059.1996.d01-104.x.

Fagoaga, C., Semancik, J. S., & Duran-Vila, N. (1995). A *Citrus exocortis viroid* variant from broad bean (*Vicia faba* L.): Infectivity and pathogenesis. *The Journal of General Virology*, *76*, 2271–2277. doi:10.1099/0022-1317-76-9-2271.

Fawcett, H. S., & Klotz, L. J. (1948). Exocortis of trifoliate orange. *Citrus Leaves*, *28*, 8–9.

Flores, R., Randles, J. W., Bar-Joseph, M., & Diener, T. O. (2000). Viroids. In M. H. V. van Regenmortel, C. M. Fauquet, D. H. L. Bishop, E. B. Carstens, M. K. Estes, S. M. Lemon, et al. (Eds.), *Virus taxonomy 7th Report of the International Committee on Taxonomy of Viruses* (pp. 1009–1024). San Diego, CA: Academic.

García-Arenal, F., Pallás, V., & Flores, R. (1987). The sequence of a viroid from grapevine closely related to severe isolates of *Citrus exocortis viroid*. *Nucleic Acids Research*, *15*, 4203–4210. doi:10.1093/nar/15.10.4203.

Garnsey, S. M., & Jones, J. W. (1967). Mechanical transmission of exocortis virus with contaminated budding tools. *Plant Disease Reporter*, *51*, 410–413.

Ito, T., Ieki, H., & Ozaki, K. (2002). Simultaneous detection of six citrus viroids and *Apple stem grooving virus* from citrus plants by multiplex reverse transcription polymerase chain reaction. *Journal of Virological Methods*, *106*, 235–239. doi:10.1016/S0166-0934(02)00147-7.

Mishra, M. D., Hammond, R. W., Owens, R. A., Smith, D. R., & Diener, T. O. (1991). Indian bunchy top disease of tomato plants is caused by a distinct strain of *Citrus exocortis viroid*. *The Journal of General Virology*, *72*, 1781–1785. doi:10.1099/0022-1317-72-8-11781.

Nie, X., Singh, R. P., & Bostan, H. (2005). Molecular cloning, secondary structure, and phylogeny of three pospiviroids

from ornamental plants. *Canadian Journal of Plant Pathology*, *27*, 592–602.

Salibe, A. A., & Moreira, S. (1965). Reaction of types of citrus as scion and as rootstock to xyloporosis virus. In W. C. Price (Ed.), *Proceedings of the 3rd Conference of the International Organization of Citrus Virologists* (pp. 70–75). Gainesville, FL: University of Florida Press.

Semancik, J. S. (1980). *Citrus exocortis viroid. CMI/AAB descriptions of plant viruses*, *226*, 4.

Singh, R. P., & Dilworth, A. D. (2008). *Tomato chlorotic dwarf viroid* in the ornamental plant *Vinca minor* and its transmission through tomato seed. *European Journal of Plant Pathology*, *123*, 111–116. doi:10.1007/s10658-008-9344-8.

Singh, R. P., Dilworth, A. D., Baranwal, V. K., & Gupta, K. N. (2006a). Detection of *Citrus exocortis viroid*, *Iresine viroid*, *and Tomato chlorotic dwarf viroid* in new ornamental host plants in India. *Plant Disease*, *90*, 1457. doi:10.1094/PD-90-1457A.

Singh, R. P., Dilworth, A. D., Singh, M., & Babcock, K. M. (2006b). An alkaline solution simplifies nucleic acid preparation for RT-PCR and infectivity assays of viroids from crude sap and spotted membrane. *Journal of Virological Methods*, *132*, 204–211. doi:10.1016/j.jviromet.2005.09.007.

Verhoeven, J. T. J., Jansen, C. C. C., Willemen, T. M., Kox, L. F. F., Owens, R. A., & Roenhorst, J. W. (2004). Natural infections of tomato by *Citrus exocortis viroid*, *Columnea latent viroid*, *Potato spindle tuber viroid* and *Tomato chlorotic dwarf viroid*. *European Journal of Plant Pathology*, *110*, 823–831. doi:10.1007/s10658-004-2493-5.