

1 **Recently Accepted**

2

3 **BRIEF REPORT**

4

5 **Citrus Bent Leaf Viroid Present in Citrus in South Africa**

6 .

7

8 C. Steyn¹, J. H. J. Breytenbach¹ and G. Cook¹

9

10

11 ¹ Citrus Research International, Mbombela 1200, South Africa

12 Correspondence to: glynnis@cri.co.za

13

14 **Citation:** Steyn, C., Breytenbach, J. H., & Cook, G. (2023). Citrus Bent Leaf Viroid Present in
15 Citrus in South Africa. *Journal of Citrus Pathology*, 10. <http://dx.doi.org/10.5070/C410160582>
16 Retrieved from <https://escholarship.org/uc/item/14h9q8rk>.

17

18

19

20

21

22

23

24

25

26

27

28

29

30

31

32

33

34

35 Abstract

36 Currently six viroid species are recognised which infect the genera *Citrus* and *Poncirus*, with
37 an additional tentative new species reported. Citrus bent leaf viroid (CBLVd) has been reported
38 from various citrus growing regions world-wide, but has not been formally documented from
39 South Africa. CBLVd was detected in field samples in various citrus growing regions in South
40 Africa during routine diagnostic analyses conducted since 2011. The detection and sequence
41 verification of CBLVd from field samples is reported in this study. Biological confirmation of
42 CBLVd presence was done for one sample that was shown to contain a single viroid infection.
43 Bent-leaf symptom expression was observed after slash inoculation of sample RNA to the
44 ‘Etrog’ citron indicator host. This study was a retrospective analysis, of previously identified
45 CBLVd-positive samples, to document the long-standing presence of CBLVd in South Africa.

46 Keywords

47 *Pospiviroidae*, Apscaviroid, detection, RT-PCR

48

49 Introduction

50 Citrus viroids are single-stranded, circular RNA species that can infect all citrus types and
51 rootstocks of citrus. Citrus viroid species belong to the family *Pospiviroidae* and four genera
52 including *Pospiviroid* (*Citrus exocortis viroid*), *Cocadviroid* (*Citrus bark cracking viroid*),
53 *Hostuviroid* (*Hop stunt viroid*) and *Apscaviroid* (*Citrus bent leaf viroid*, *Citrus dwarfing viroid*,
54 *Citrus viroid V*, *Citrus viroid VI*) (Di Serio et al. 2021). A further tentative species of the genus
55 *Apscaviroid*, *Citrus viroid VII*, was reported from Australia (Chambers et al. 2018).

56 Citrus viroids are mechanically and graft transmissible but are not transmitted by seed in citrus.
57 Infected budwood, and not fruit, is how citrus viroids are widely transmitted (Duran-Vila and
58 Semancik 2003).

59 The natural host range of citrus bent leaf viroid (CBLVd) is rutaceous hosts (Duran-Vila and
60 Semancik 2003). CBLVd, previously designated CVd-Ia and CVd-Ib, was shown to induce a
61 leaf bend on the indicator, 'Etrog' citron Arizona 861-S-1 (*Citrus medica* L.) (Duran-Vila et
62 al. 1988; Ashulin et al. 1991). As a single infection this viroid appears to be latent and its most
63 significant effect on the citrus host is through synergistic or antagonistic interactions with other
64 citrus viroid species. The predominant, commercial impact has been the reduction in tree
65 canopy volume, but only in combination with other citrus viroids (Vernière et al. 2006;
66 Vidalakis et al. 2010). Significant nucleotide variability is reported for this viroid species in
67 addition to genome size differences ranging from 318 to 330 nucleotides (Ashulin et al. 1991;
68 Semancik et al. 1997).

69 Although CBLVd has been reported in numerous citrus producing countries, its distribution is
70 likely wider but under-reported due to the mild and synergistic disease association. CBLVd
71 was detected using RT-PCR in South Africa, but the presence of this viroid was not verified or
72 formally documented. Therefore, this study was done to confirm the presence of CBLVd in
73 South Africa.

74

75 **Materials and Methods**

76 Molecular diagnostics of field samples, submitted for pathogen screening, have been conducted
77 at Citrus Research International, Nelspruit since 2011. Analysis of field samples included RT-
78 PCR testing for citrus viroids including CBLVd, citrus dwarfing viroid (CDVd), citrus
79 exocortis viroid (CEVd), hop stunt viroid (HSVd), citrus bark cracking viroid (CBCVd) and
80 citrus viroid V (CVd-V). Samples were commonly obtained as budwood and total RNA was
81 extracted from green bark using an acid-phenol method previously described (Cook et al.
82 2016). Random primed reverse transcription was done as detailed in Cook et al. (2019) and

83 viroid PCRs were done as in Cook et al. (2016). For CBLVd PCR detection the CBLVd-F2
84 and CBLVd-R2 primer pair was used (Wang et al. 2008). Three samples that had tested positive
85 for CBLVd and for which RNA, stored at -20°C, was still available included samples R130821-
86 3, R130902-1 and R160707-4. Sample R130821-3 was obtained in 2013 from a navel orange
87 tree (*Citrus sinensis* cultivar ‘Rustenburg’ Navel) in the Harry Gwala district of KwaZulu-
88 Natal Province. Sample R130902-1 (*C. sinensis* cultivar ‘Bennie’ Valencia) was collected in
89 the Mopani district of Limpopo Province in 2013 and sample R160707-41 (*C. sinensis* cultivar
90 ‘Turkey’ Valencia) was collected in 2016 in the Vhembe district, a northern region of the
91 Limpopo Province. Sample R160707-41 was the only sample that tested positive for a single
92 viroid species, namely CBLVd.

93 Full genome nucleotide sequencing of CBLVd from the three selected samples was done from
94 the stored total RNA extracts by RT-PCR and using two overlapping primer pairs as previously
95 detailed (Steyn et al. 2016), but using GoTaq G2 Hot Start Green Master Mix (Promega Corp.,
96 Madison, WI, USA). PCR amplicons were gel-purified using the Zymoclean gel DNA
97 Recovery kit (Zymo Research, CA, USA) and direct Sanger sequencing was performed in both
98 orientations. Overlapping sequences were aligned and low-quality bases removed using
99 BioEdit (Hall 1999). BLAST was used to determine closest sequence identity (Altschul et al.
100 1990).

101 In order to biologically confirm the presence of CBLVd in the singly infected viroid sample,
102 R160707-41, a droplet of total RNA extract was slash inoculated to six ‘Etrog’ citron plants as
103 previously described (Steyn et al. 2016). Plants were maintained in a temperature-controlled
104 glasshouse with temperatures ranging between 28°C and 32°C. Transmission success was
105 confirmed with RT-PCR, seven months post inoculation (pi). A full genome consensus
106 sequence was obtained from a CBLVd-positive ‘Etrog’ host plant as described above. The

107 'Etrog' plants were cut back and regrowth was monitored for symptom development for a
108 period of five months.

109 **Results and Discussion**

110 CBLVd has been detected since 2011 in South Africa with the implementation of RT-PCR
111 screening for citrus viroids. Various disease investigations indicated the presence of CBLVd,
112 in orchards as mixed infections with other citrus viroids and citrus tristeza virus which is
113 endemic in southern Africa. These detections were primarily in older Valencia and Navel
114 orchards which were established prior to the use of shoot-tip grafted budwood supply through
115 the South African Citrus Improvement Scheme. The purpose of this study was not to document
116 each detection over a prolonged period, but to retrospectively verify detection of CBLVd in a
117 few samples.

118 CBLVd was detected in combination with other citrus viroids in sample R130821-3 (HSVd,
119 CDVd, CBCVd) and sample R130902-1 (HSVd, CDVd), but was found as a single viroid
120 infection in sample R160707-41.

121 Transmission to one of six 'Etrog' indicator plants, after slash inoculation of sample R160707-
122 41 RNA, was confirmed with RT-PCR seven months post inoculation (pi) and designated as
123 sample R220811-8. The 'Etrog' plants were cut back and regrowth was monitored for symptom
124 development. The single CBLVd positive plant first showed leaf bend ten months pi and no
125 symptoms were noted on the five plants that tested negative for CBLVd. The bent leaf symptom
126 was observed on further growth flushes of this plant (Figure 1).



127

128 **Figure 1.** ‘Etrog’ plant (R220811-8) showing leaf bend symptoms typical for citrus bent leaf
129 viroid infection (indicated by black arrows), post slash-inoculation with total RNA extract of
130 sample R160707-41.

131

132 Full genome consensus sequences for CBLVd were obtained for the three field samples and
133 for the positive ‘Etrog’ seedling. These nucleotide sequences were deposited in GenBank under
134 accession numbers OP616802, OP616803, OP616804 and OP616805 for samples R130821-3,
135 R130902-1, R160707-41 and R220811-8, respectively. The genomes of the field samples
136 differed. Sequence OP616802 showed 100% sequence identity to Genbank accession
137 AF428053 from Uruguay. Sequence OP616803 showed closest sequence identity (99.06%) to
138 accessions GQ260200, AB006736, AF428056 from Iran, Japan and Uruguay, respectively and
139 sequence OP616804 showed closest identity (99,69%) to accession AF428057 from Uruguay.
140 The consensus sequence, OP616805, of sample R220811-8 from ‘Etrog’ citron showed two
141 base pair changes compared to sequence OP616804 of sample R160707-41, from which it was
142 derived. These changes may have been induced by the host change as previously reported for
143 CEVd (Bernard et al. 2009). Alternately, a CBLVd variant within the original sample was
144 transmitted by the slash inoculation, rather than the sequence variant OP616804.

145 The detection and sequence verification of CBLVd from field samples from South African
146 orchards are reported in this study. Biological confirmation of CBLVd presence was
147 demonstrated by symptom expression in ‘Etrog’ citron for a single viroid infected sample. This
148 study was a retrospective analysis of previously identified CBLVd-positive samples to
149 document the long-standing presence of this citrus viroid in South Africa.

150 Acknowledgments

151 This work was funded by Citrus Research International (Pty) Ltd.

152 References

153 Altschul SF, Gish, W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search
154 tool. *Jnl Mol. Biol.* 215(3): 403–410. [https://doi.org/10.1016/s0022-2836\(05\)80360-2](https://doi.org/10.1016/s0022-2836(05)80360-2).

155 Ashulin L, Lachman O, Hadas R, Bar-Joseph M. 1991. Nucleotide sequence of a new viroid
156 species, citrus bent leaf viroid (CBLVd) isolated from grapefruit in Israel. *Nucl. Acids Res.*
157 19 (17):4767.

158 Bernad L, Duran-Vila N, Elena, SF. 2009. Effect of citrus hosts on the generation, maintenance
159 and evolutionary fate of genetic variability of citrus exocortis viroid. *J. Gen. Virol.* 90: 2040-
160 2049. <https://doi.org/10.1099/vir.0.010769-0>.

161 Chambers, G. A., Donovan, N. J., Bodaghi, S., Jelinek, S. M., and Vidalakis, G. 2018. A novel
162 citrus viroid found in Australia, tentatively named citrus viroid VII. *Arch. Virol.* 163: 215-
163 218.

164 Cook G, van Vuuren SP, Breytenbach JHJ, Steyn C, Burger JT, Maree HJ. 2016.
165 Characterization of citrus tristeza virus single-variant sources in grapefruit in greenhouse
166 and field trials. *Plant Dis.* 100: 2251-2256. <https://doi.org/10.1094/PDIS-03-16-0391-RE>.

167 Cook G, Kirkman W, Clase R, Steyn C, Basson E, Fourie PH, Moore SD, Grout TG, Carstens
168 E, Hattingh V. 2019. Orchid fleck virus associated with the first case of citrus leprosis-N in

- 169 South Africa. Eur. J. of Plant Pathol. 155:1373-1379. <https://doi.org/10.1007/s10658-019->
170 [01854-4](https://doi.org/10.1007/s10658-019-01854-4).
- 171 Di Serio F, Owens RA, Li, S-F, Matoušek J, Pallás V, Randles JW, Sano T, Verhoeven JTJ,
172 Vidalakis G, Flores R, ICTV Report Consortium. 2021. ICTV Virus Taxonomy Profile:
173 *Pospiviroidae*. Journal of General Virology 102. <https://doi.org/10.1099/jgv.0.001543>.
- 174 Duran-Vila N, Roistacher CN, Rivera-Bustamante R, Semancik JS. 1988. A Definition of
175 citrus viroid groups and their relationship to the exocortis disease. J. Gen. Virol. 69:3069-
176 3080. <https://doi.org/10.1099/0022-1317-69-12-3069>.
- 177 Duran-Vila N and Semancik JS. 2003. Citrus Viroids. In: Hadidi A, Flores R, Randles JW,
178 Semancik, JS, editors. Viroids. CSIRO Publishing. Australia. p 178-194.
- 179 Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis
180 program for Windows 95/98/NT. Nucleic Acids Symposium Series. 41: 95-98.
- 181 Semancik JS, Rakowski AG, Bash JA, Gumpf DJ. 1997. Application of selected viroids for
182 dwarfing and enhancement of production of 'Valencia' orange. J. Hort. Science 72:563-570.
- 183 Steyn C, Cook, G, Burger, JT, Maree, H J. 2016. Construction and application of infectious
184 citrus viroids for biological indexing. J. Citrus Pathol. 3(1).<https://doi.org/10.5070/C431>
185 [037092](https://doi.org/10.5070/C431037092).
- 186 Vernière C, Perrier X, Dubois C, Dubois A, Botella L, Chabrier C, Bové J M, Duran-Vila N.
187 2006. Interactions between citrus viroids affect symptom expression and field performance
188 of clementine trees grafted on trifoliolate orange. Phytopathology 96:356-368.
189 <https://doi.org/10.1094/PHYTO-96-0356>.
- 190 Vidalakis G, Pagliaccia D, Bash JA, Semancik JS. 2010. Effects of mixtures of citrus viroids
191 as transmissible small nuclear RNA on tree dwarfing and commercial scion performance on
192 Carrizo citrange rootstock. Ann. Appl. Biol. 157:415-423.

193 Wang, X-f, Zhou C-y, Tang, K-z, Lan, J-q, Zhou Y, Li Z-a. 2008. Preliminary studies on
194 species and distribution of citrus viroids in China. Agric. Sciences in China. 7: 1097-1103.
195 [https://doi.org/10.1016/S1671-2927\(08\)60152-2](https://doi.org/10.1016/S1671-2927(08)60152-2).

196

197