Association of a Viroid With Gum Pocket Disease of Trifoliate Orange

L. J. Marais, R. F. Lee, J. H. J. Breytenbach, B. Q. Manicom, and S. P. van Vuuren

ABSTRACT. Buds cut from Marsh grapefruit trees exhibiting gum pocket symptoms in their trifoliate orange rootstocks were used to inoculate healthy Etrog citron plants. Polyacrylamide gel electrophoresis (PAGE) on non-denaturing 5% gels was conducted 8 wk later on extracts from the infected citron plants using citrus exocortis viroid (CEVd) as a marker. Each extract from gum pocket-inoculated citrons contained a putative viroid-like band which was cut from the gel, resuspended in buffer and slash inoculated into healthy citron plants. A total nucleic acid extract was also prepared from the inoculated citron plants and slash inoculated into healthy citron plants. Buds from the citron plants inoculated with the field source of gum pocket disease, the total nucleic acid extracts and viroid-like band were used to inoculate healthy Marsh grapefruit on trifoliate rootstocks in 1985. All three inoculum sources induced gum pocket disease symptoms and contained only one viroid-like band which co-migrated with a group III citrus viroid standard when assayed by sequential PAGE. No bark scaling typical of CEVd was observed. Gum pocket disease of trifoliate orange appears to be associated with a viroid of the same apparent size as group III viroids.

A disease characterized by gum pockets in the bark and wood tissues of trifoliate orange rootstocks and severe stunting of affected sweet orange scions was described in South Africa by Schwarz and McClean in 1969 (10). This disease is graft transmitted and resembles disorders reported by researchers in Argentina (5, 6), Australia (7) and Italy (1). In 1983, a similar disorder occurring in Marsh grapefruit on trifoliate orange rootstocks was reported from Swaziland. The affected trees had been propagated from buds cut from Marsh grapefruit parent trees on Rough lemon rootstocks. These parent trees had been inoculated with buds from a Cecily grapefruit tree to introduce a mild protecting isolate of tristeza stem pitting. Schwarz and McClean (10) suspected that Cecily grapefruit was contaminated with the causal agent of gum pocket disease. Growers in Swaziland confirmed these suspicions when this disorder was discovered in 3-yr-old Marsh grapefruit trees in 1983. This paper reports research started in 1985 which suggests the viroid nature of gum pocket disease.

MATERIALS AND METHODS

Preparation of inoculum. To prepare inoculum for inoculating viroid-free Marsh grapefruit trees on trifoliate orange rootstocks, eight budlings of 861-S1 Etrog citron on rough lemon rootstock were graft inoculated with blind buds from 4-yr-old gum pocket-affected Marsh grapefruit trees on trifoliate orange rootstocks. Approximately 8 wk after inoculation leaf tissue was collected from the new growth on the citron plants for polyacrylamide gel electrophoresis (PAGE) analysis (2) on non-denaturing 5% gels using citrus exocortis viroid (CEVd) infected and healthy citron tissue as viroid positive and negative controls, respectively. The putative viroid-like band obtained from each of the unknown samples was cut from the PAGE gel using the CEVd band as a guide, and homogenized in 0.5 ml STE buffer (0.05 M Tris, 1 mM EDTA and 0.1 M NaCl, pH 6.9) in a cold sterile mortar. Etrog citron budling receptor plants were inoculated by slashing the bark 40 times with a hanging droplet of this inoculum. Another set of Etrog citron budlings receptor plants were

slash-inoculated with a total nucleic acid extract prepared by commencing the viroid extraction step in the above procedure (2) but instead of adding STE buffer plus ethanol to bind to CF11 powder, the total nucleic acids were precipitated by adding 2.5 volumes of ethanol and stored overnight at -20°C. The pellet was collected by centrifugation at 10,000 *g* for 10 min. at 4°C, then resuspended in 1 ml of 1 x STE, centrifuged at 10,000 *g* for 5 min. The supernatant was transferred to a clean sterile centrifuge tube and re-ethanol precipitated overnight at -20°C. The pellet was collected by centrifugation as above and resuspended in glycine-phosphate buffer (0.02 M glycine, 0.2 M K_2HPO_4, pH 9.0).

Inoculation of viroid-free Marsh grapefruit on trifoliate orange rootstock. The experimental trees were propagated from "Nartia" CTV mild isolate protected Marsh grapefruit budwood (8), budded onto 10-mo-old virus and viroid-free trifoliate orange seedlings in October 1986. Eight months later, these plants were graft-inoculated with blind buds from the citron receptor plants used for preparing the inoculum and with buds cut from the original donor Marsh grapefruit tree on trifoliate orange rootstock exhibiting gum pocket symptoms. The following sources of inocula were used: 1) blind buds from the original citron receptor plants inoculated with the field source of gum pocket; 2) blind buds from the field source itself, and uninoculated controls; 3) blind buds cut from citron receptor plants slash inoculated with the putative viroid-like band from PAGE; and 4) blind buds cut from citron receptor plants slash inoculated with total nucleic acid extracts. Each treatment was replicated seven times. The experimental trees were transferred from the nursery at Inyoni Yama in Swaziland to the field in October 1987. Rootstocks were examined annually for symptoms. In 1993, 1994 and 1995, trunk circumferences were measured 10 cm above the budunion, height and canopy diameters were measured to determine canopy volumes and yield and fruit size were measured. Statistical significances were determined according to Fisher's LSD Comparison (P=0.05).

Detection of gum pocket viroid using sequential PAGE. To determine the viroid status of the trees, budwood was collected from the trees in each treatment in July 1995. Four budsticks were collected from each tree and four buds (one from each budstick) graft-inoculated to 861-S1 Etrog citron budlings on Rough lemon rootstock. Beginning 3 to 4 mo after inoculation, the young flush tissue of the citron plants was harvested and processed for nucleic acid analysis by sequential PAGE (sPAGE) (3, 9).

RESULTS

Symptom expression in citron. Citron inoculated by inoculum sources 1 to 4 all induced similar symptoms. Occasional mild bent leaf and droop occurred on several but not all of the receptor plants whereas petiole wrinkle and browning was evident on the lower leaves of the majority of plants (Fig. 1). Only a slight reduction in size was observed. No symptoms were observed in the uninoculated citron.

Symptoms in the trifoliate rootstock. After passage of the eluted viroid-like band from PAGE into citron and then grafting from the citron to grapefruit on trifoliate rootstock, symptoms started to appear in the grapefruit trees approximately 4 yr later. Four distinct symptoms appeared in the trifoliate rootstocks of all trees associated with all of the four sources of inoculum used: (i) Deep pitting in the wood; (ii) with an accumulation of gum in the pits and bark; (iii) accompanied by fissuring;

A

B

C

Fig. 1. Symptoms induced on 861-S1 citron inoculated with the gum pocket agent. 1A) Healthy control is on the left, and inoculated plant on the right showing stunting. 1B) Typical symptoms of petiole wrinkle, browning and leaf droop shown on citron indicator plants. 1C). Bent leaf symptom on citron indicator plants.

and (iv) flattening on the side of the rootstock facing west (Fig. 2). No bark cracking, budunion crease or scaling was observed. All uninoculated control trees showed no symptoms of fissuring, flattening, pitting or gum pockets in wood or bark, and all trees appeared vigorous and were generally larger than the inoculated trees (Table 1). Seven out of seven trees inoculated with the nucleic acid extract (Inoculum 4) exhibited symptoms, and five out of seven of all the other inoculated trees (Inocula 1, 2 and 3) showed symptoms.

Tree size and growth. Table 1 shows the effect of the gum pocket inducing agent on the trunk circumference of the scion, tree height and canopy volume. At the termination of the experiment in 1995, there were no significant differences between the trunk circumference, height and canopy volumes of the inoculated trees from all four inocula, regardless of whether the source was from the bud-inoculated trees or from the eluted viroid-like band from PAGE. The presence of the gum pocket inducing agent caused an 11% reduction of trunk circumference, a 25% reduction in height and a 29% reduction in canopy volume compared with uninoculated control trees. The canopies of the inoculated trees still appeared vigorous at the time of assessment though slightly sparser than the non-inoculated controls.

Fruit size and yield. Table 2 shows the effect of gum pocket on the cumulative yield and fruit size on the Marsh grapefruit non-inoculated and inoculated trees. Gum pocket apparently had no significant effect on yield or fruit size in spite of the significant reduction in canopy volume and trunk circumference.

sPAGE analysis of gum pocket-inducing agent. sPAGE was performed several times recently on the citron plants inoculated from the experimental trees. A single viroid band which migrates to the same position in the denaturing gel was present in all four sources of gum pocket inocula (Fig. 3). The viroid band from each treatment co-migrates with CV-III marker. CEV, CV-III and CV-IV (from California), used as size markers, resolved into three distinct bands when run in the same gel lane (Fig. 3, lane 1). Avocado sun blotch viroid (ASBV) migrated as a single viroid band in lane 8 (Fig. 3).

DISCUSSION

The field symptoms of gum pocket, which resembled cachexia in its indicator plants, suggested involvement of viroid-like RNAs in the disease, especially the tendency of the disease to be more severe on the west part of the rootstock as the western exposure of the tree is usually the warmest side of the tree. Baksh et al. (2) previously had developed an extraction procedure where viroid-like RNAs could be extracted and purified from 5 gm fresh weight of young flush tissue and applied to one gel lane of a non-denaturing PAGE gel. When utilized with RNase digestion in high salt buffer to digest single-stranded RNA, this is a sensitive diagnostic tool for identification of viroid-like RNAs. We had discovered that use of this procedure would result in viroid-RNA like bands on PAGE gels from extracts made from "graft transmissible dwarfing agents of citrus" which did not index as positive for CEV in citron indicator plants (van Vuuren and Lee, unpublished data). We used this approach to test our hypothesis that a viroid may be the cause of gum pocket disease.

When this experiment was initiated, the sPAGE procedure was not yet widely adapted for the study of viroid-like RNAs. The sPAGE procedure has only been applied for analysis of this experiment recently, and these results suggest that only one

Fig. 2. Symptoms on *P. trifoliata* rootstock associated with the gum pocket agent. 2A) Fissuring of the rootstock. 2B) Gum pockets and pitting in wood on the flattened western side of the rootstock. 2C) Deep pits on flattened side of stock near ground level.

TABLE 1
EFFECT OF GUM-POCKET INDUCING AGENT ON THE STEM CIRCUMFERENCE, HEIGHT AND CANOPY GROWTH OF MARSH GRAPEFRUIT TREES ON TRIFOLIATE ORANGE ROOTSTOCKS

Year	Treatment[z]	Trunk circumference (cm)[y]	Height (m)[y]	Canopy volume (m^3)[y]
1989	Control	17.2 a	2.27 a	—
"	1	16.0 ab	1.86 b	—
"	2	15.3 b	1.84 b	—
"	3	15.9 b	1.98 b	—
"	4	15.1 b	1.87 b	—
1992	Control	26.0 a	2.81 a	11.4 a
"	1	24.6 ab	2.13 b	5.73 b
"	2	22.9 bc	2.00 bc	5.15 b
"	3	24.1 b	2.15 b	6.20 b
"	4	22.3 c	1.90 c	5.01 b
1993	Control	29.8 a	2.81 a	14.96 a
"	1	27.8 ab	2.35 b	10.22 b
"	2	26.4 b	2.24 b	8.09 b
"	3	27.7 ab	2.41 b	10.42 ab
"	4	26.9 b	2.38 b	7.49 b
1995	Control	32.4 a	3.30 a	7.60 a
"	1	28.9 b	2.44 b	5.20 b
"	2	28.3 b	2.45 b	5.25 b
"	3	29.6 b	2.65 b	6.00 b
"	4	29.2 b	2.51 b	5.42 b

[z]Treatments are: 1) blind buds cut from the original citron receptor plants inoculated with the field source of gum pocket; 2) blind buds from the field source itself; 3) blind buds cut from citron receptor plants slash inoculated with the PAGE viroid-like band; and 4) blind buds cut from citron receptor plants slash inoculated with total nucleic acid extracts.
[y]Values in the columns with different letters within a year indicate significant differences (P=0.05) based on Fisher's LSD comparison.

TABLE 2
EFFECT OF GUM-POCKET INDUCING AGENT ON CUMULATIVE YIELD AND FRUIT SIZE OF MARSH GRAPEFRUIT ON TRIFOLIATE ORANGE ROOTSTOCKS (1993-1995)

		Fruit size (kg/tree)[y]						
Treatment	Yield (kg)[y]	64	56	48	40	36	32	27
Control	247.4 a	93.6 a	37.4 a	42.8 a	31.3 a	11.3 a	7.3 a	23.4 a
1	236.2 a	109.4 ab	31.6 ab	37.2 a	30.8 a	16.9 ab	6.2 a	14.5 a
2	199.6 a	64.2 a	28.3 a	46.6 a	43.4 a	18.7 ab	6.1 a	4.9 a
3	252.0 a	75.0 a	32.9 a	50.9 a	39.3 a	16.2 ab	15.7 b	18.8 a
4	246.4 a	56.3 a	24.8 a	57.4 a	50.3 a	19.1 ab	11.3 ab	21.3 a

[z]Treatments are: 1) blind buds cut from original citron receptor plants inoculated with the field source of gum pocket; 2) blind buds from the field source itself; 3) blind buds cut from citron receptor plants slash inoculated with the PAGE viroid-like band; and 4) blind buds cut from citron receptor plants slash inoculated with total nucleic acid extracts.
[y]Values in columns of a fruit size category with different letters indicate significant differences (P=0.05) based on Fisher's LSD comparison.

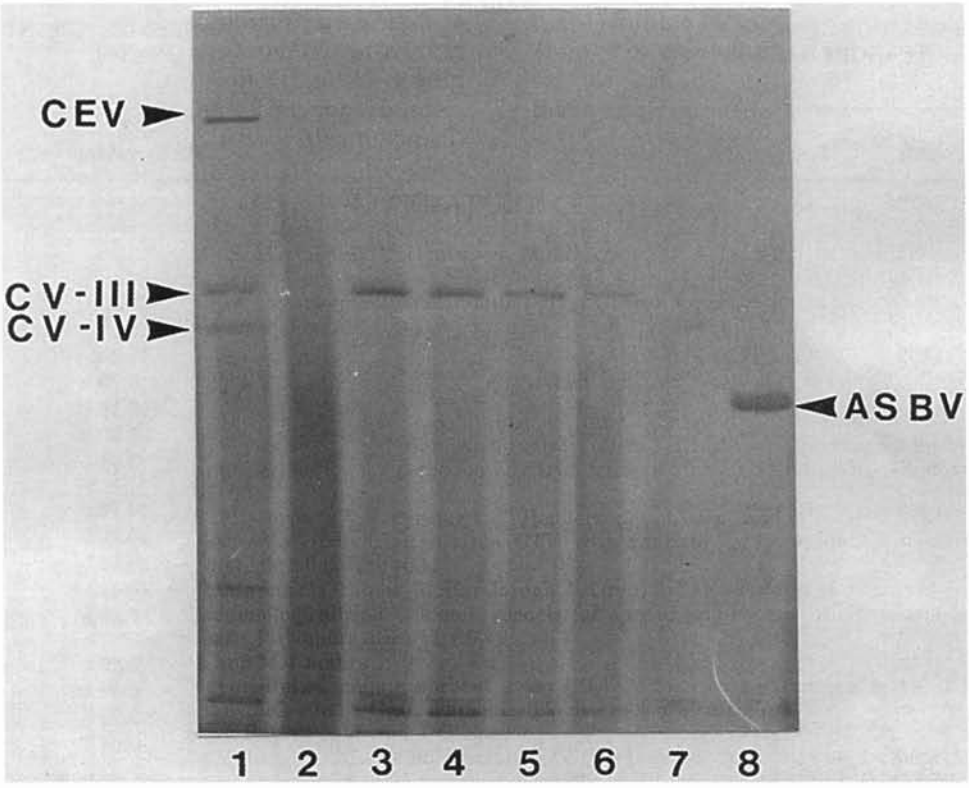

Fig. 3. Sequential polyacrylamide gel electrophoresis (sPAGE) analysis of extracts from citrons inoculated with gum pocket inoculum and uninoculated controls. Lane 1 contains citrus exocortis viroid and citrus viroids (CV) -III and -IV as size markers. Lane 2 contains extracts from uninoculated control inoculum. Lane 3 contains extracts from treatment 1 (blind buds cut from the original citron receptor plants inoculated with the field source of gum pocket). Lane 4 contains extracts from treatment 2 (blind buds from the field source itself). Lane 5 contains extracts from treatment 3 (blind buds cut from citron receptor plants slash inoculated with the PAGE viroid-like band); Lane 6 contains extracts from treatment 4 (blind buds cut from citron receptor plants slash inoculated with total nucleic acid extracts). Lane 7 contains extracts from healthy citron. Lane 8 contains avocado sun blotch viroid (ASBV) as a size marker.

viroid-like RNA band of the same size is present in all the treatments. Symptoms induced in both citron indicator plants and grapefruit on trifoliate rootstock trees are the same for all treatments. However, the exact size and identification of the viroid group has not been determined. Further molecular characterization of the viroid associated with gum pocket disease is under way.

Duran-Vila et al. (3, 4) classified the citrus viroids into five groups, CEV, CV-I, CV-II, CV-III and CV-IV based on the electrophoretic mobility of their nucleic acids and sequence homology defined by molecular hybridization studies. They observed an association of the various groups to specific reactions in citron as well as alternate hosts. The symptoms expressed in citron by the gum pocket agent are very similar to those induced by the citrus viroid group CV-III (3, 9), but the evidence of deep pits in the trifoliate rootstock is not typical of CV-III and suggested that a viroid similar to CV-Ia may also be present (9). van Vuuren (pers. comm.) has found that the CV-III group of viroids can induce symptoms similar to gum pocket in trifoli-

ate rootstocks. Based on symptomatology in citron indicator plants, it is hard to predict which viroid group the viroid-like RNA associated with gum pocket belongs to. While it migrates as a single band on sPAGE, it still could be a mixture of viroids. Further molecular characterization should resolve this.

Data presented here and as reported previously (6, 7, 10) show a significant reduction in growth of the trees affected by the gum pocket agent. This type of stunting is also associated with group CV-I and CV-III viroids and mixtures of the two (9). In spite of the stunting effect, there were no significant differences in yield and fruit size between the non-inoculated and inoculated trees. The decline observed in gum pocket affected sweet orange trees as reported by other researchers (5, 6, 10) was not evident here. Fraser (7) reported that in Australia the onset of the disease was later and severity less under Marsh grapefruit scions than Valencia orange. The vigorous condition of the canopies of the inoculated trees in this experiment may also be attributed to the fact that the trees were cross protected against severe stem pitting isolates of CTV. The deep pitting in the trifoliate rootstocks of the inoculated trees obviously is not caused by CTV as this rootstock is immune to CTV, and non-inoculated trees did not exhibit this symptom. The symptoms described by Foguet et al. (6), viz. wood pitting, fissuring and flattening of the rootstock, are almost identical to those observed in South Africa. The appearance of the fissuring and flattening on the side of the stock facing west, the hottest sector of the trunk, concur with the results presented here. Bark scaling, bark shelling and budunion crease have not been associated with gum pocket disease in South Africa.

Evidence presented suggests that gum pocket disease of trifoliate orange in South Africa is caused by an infectious agent of viroid nature.

ACKNOWLEDGMENT

We wish to acknowledge the expert technical assistance of M. Luttig.

LITERATURE CITED

1. Cartia, G., Rosa La Rosa, and A. Catara
   1984. A gummy pitting of trifoliate orange in Italy, p. 184-187. *In*: Proc. 9th Conf. IOCV., IOCV, Riverside.
2. Baksh, Nadeer, R. F. Lee, and S. M. Garnsey
   1984. Detection of citrus exocortis viroid by polyacrylamide gel electrophoresis, p. 343-352. *In:* Proc. 9th Conf. IOCV., IOCV, Riverside.
3. Duran-Vila, N., J. A. Pina, J. F. Ballester, J. Juarez, C. N. Roistacher, R. Rivera-Bustamante, and J. S. Semancik
   1988. The citrus exocortis disease: A complex of viroid-RNAs, p. 152-164. *In*: Proc. 10th Conf. IOCV., IOCV, Riverside.
4. Duran-Vila, N., C. N. Roistacher, R. Rivera-Bustamante, and J. S. Semancik
   1988. A definition of citrus viroid groups and their relationship to the exocortis disease. J. Gen. Virol. 69: 3069-3080.
5. Fernandez Valiela, M. V., C. Fortugno, and F. Corizzi
   1965. Incidence of bud-union crease in citrus trees grafted on trifoliata rootstock in the Delta del Paraná and San Pedro areas of Argentian, p. 182-186. *In:* Proc. 3rd Conf. IOCV., IOCV, Gainesville, Florida.
6. Foguet, J. L. and C. A. Oste
   1968. Disorders of trifoliate orange rootstock in Tucuman, Argentina, p. 183-189. *In:* Proc. 4th Conf. IOCV., IOCV, Gainesville, Florida.
7. Fraser, L. R., P. Broadbent, and J. E. Cox
   1976. Gummy pitting of *Poncirus trifoliata*: Its association with dwarfing of citrus in New South Wales, p. 147-151. *In*: Proc. 7th Conf. IOCV., IOCV, Riverside.
8. Marais, L. J. and J. M. Kotzé
   1985. Stem pitting of grapefruit - An evaluation of cross protection trials using the "Nar-

tia" mild strain of citrus tristeza virus. Citrus and Subtrop. Fr. J. 619: 6- 11.

9. Roistacher, C. N., J. A. Bash, and J. S. Semancik
   1993. Distinct disease symptoms in *Poncirus trifoliata* induced by three citrus viroids from three specific groups, p. 173-179. *In:* Proc. 12th Conf. IOCV., IOCV, Riverside.
10. Schwarz, R. E. and A. P. D. McClean
    1969. Gum-pocket, a new virus-like disease of *Poncirus trifoliata*. Plant Dis. Reptr. 53: 336-339.