Interactions Between Citrus Viroids Affect Symptom Expression and Field Performance of Clementine Trees Grafted on Trifoliate Orange

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ABSTRACT

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Citrus exocortis viroid (CEVd), *Citrus bent leaf viroid* (CBLVd), a noncachexia variant of *Hop stunt viroid* (HSVd), *Citrus viroid III* (CVd-III), and *Citrus viroid IV* (CVd-IV) were co-inoculated as two-, three-, four-, and five-viroid mixtures to Clementine trees grafted on trifoliate orange to evaluate their effect on symptom expression, tree growth, and fruit yield. Most trees infected with CEVd-containing viroid mixtures developed exocortis scaling symptoms, as did CEVd alone, whereas most trees infected with HSVd- or CVd-IV-containing mixtures developed bark-cracking symptoms. Trees infected with mixtures containing both CEVd and CVd-IV revealed the existence of antagonism between these two viroids in terms of the expected bark-scaling and cracking symptoms. Synergistic interactions also were identified in trees infected with certain viroid combinations that, in spite of lacking CEVd, expressed exocortis-like scaling symptoms. Viroid interactions also affected the expected response of trees in terms of vegetative growth and fruit yield. Trees infected with viroid combinations containing CEVd or CVd-III were smaller and produced less fruit than trees infected with mixtures not containing these viroids. Viroid interactions on scion circumference and cumulative fruit yield, in terms of additivity of their effects, were statistically confirmed using a factorial analysis of variance model with two mean estimation approaches. In single-viroid infections, CEVd, CVd-III, and, to a lesser extent, CBLVd consistently and significantly reduced tree size and fruit yield. Conversely, HSVd and CVd-IV slightly increased fruit yield and reduced scion circumference. Rare and not consistent significant interactions were detected with the five-, four-, and three-viroid combinations. Antagonistic interactions between CEVd and CVd-III or CBLVd and CVd-III were revealed over the years with consistent significance. The antagonistic interaction between CEVd and CVd-IV was highly significant over the years when additional viroids were present; however, this antagonism appeared much later in the case of an exclusive interaction. HSVd and CVd-IV showed a consistent and significant synergistic interaction on yield only when both viroids were exclusively present. These results demonstrate antagonistic or synergistic relationships between citrus viroids depending on the viroid mixtures present in the host.

Additional keyword: dwarfing.

Viroids are small (246 to 401 nucleotides), covalently closed single-stranded RNAs that replicate independently in their hosts, in which they may act as phytopathogenic agents. They are classified into two families, *Pospiviroidae*, composed of species with a central conserved region (CCR) and without hammerhead ribozymes, and *Avsunviroidae*, composed of only three members having hammerhead ribozymes but lacking CCR (9). Citrus trees are natural hosts of several viroid species of the family *Pospiviroidae*: *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus viroid III* (CVd-III), and *Citrus viroid IV* (CVd-IV) (5,6). These viroids have been found to be widespread in all citrus-growing areas. Recently, another viroid, named Citrus viroid-original sample (CVd-OS), sharing only 68% sequence identity with CVd-III, has been proposed in Japan as a new species (17).

Each viroid species induces specific symptoms on inoculated Etrog citron plants (6), but only CEVd and variants of HSVd containing a specific six-nucleotide motif are agents of two well-known diseases of citrus in the field, exocortis and cachexia, respectively (28,29,34). Exocortis disease was described as a bark-shelling or -scaling disorder associated with dwarfing of trees grafted on trifoliate orange (*Poncirus trifoliata* (L.) Raf.) and some of its hybrids (8). The cachexia disease is a gumming and wood-pitting disorder, described on Orlando tangelo (*Citrus paradisi* Macfad. × *C. reticulata* Blanco), which also affects other citrus species and cultivars (4).

Citrus field trees are naturally infected with various combinations of viroids and, for many years, the effect of each viroid on symptom expression and tree performance remained undetermined. The first exhaustive study on the effect of individual viroids was carried out on Clementine trees grafted on trifoliate orange (36). This work confirmed that (i) only CEVd and specific variants of HSVd were the causal agents of exocortis and cachexia, respectively; (ii) bark cracking on trifoliate orange was induced by

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all the variants of HSVd as well as CVd-IV; and (iii) all viroids induced various degrees of stunting and reduced fruit yield, as described previously (16,32).

Biological indexing revealed mixed infections resulting in complex interactions among citrus viroids. In some instances, the severe response of Etrog citron indicator plants co-inoculated with CBLVd, HSVd, and CVd-III indicated a synergistic effect (6). Conversely, co-inoculation of cachexia sources of HSVd with other viroids or viroid combinations masked the symptom expression of cachexia on the Parson's special mandarin indicator (25), suggesting an antagonistic effect that later was demonstrated to occur between cachexia and noncachexia variants of HSVd (31). However, this type of antagonism was not observed when different viroid species co-infected a single indicator plant (13).

The above observations on viroid interactions were based on the response of indicator plants inoculated with viroid mixtures. However, a report from Japan indicating that trees grafted on trifoliate orange rootstock showed bark scaling in the absence of CEVd (18) suggested that viroid interactions also could occur in field-grown trees.

In this study, we documented viroid interactions in field trees experimentally co-inoculated with two, three, four, or five viroid species. Viroids effects on symptom expression, growth characteristics, and fruit production of these trees are presented here, in comparison with the response of noninoculated trees as well as trees inoculated with single viroid species.

MATERIALS AND METHODS

Viroid sources. Isolates of five viroid species were selected from the viroid collection maintained at IVIA on inoculated citron plants. The isolates chosen were CEVd (CEVd-117) (12), CBLVd (CVd-Ia-117) (10), HSVd (CVd-IIa-117) (23), CVd-III (CVd-IIId) (10), and CVd-IV (CVd-IV-Ca) (11). The individual effect of these isolates on Clementine trees grafted on trifoliate orange has been reported recently (36).

Plant materials and inoculation. In May 1989, Pomeroy trifoliate orange seedlings were graft inoculated (six plants per treatment and six noninoculated controls) with the five viroid species (treatments 1 to 5) and all possible combinations of two (treatments 6 to 15), three (treatments 16 to 25), four (treatments 26 to 30), and five viroids (treatment 31) (Table 1). These inoculations, as well as budding the inoculated trifoliate seedlings with the Commune Clementine SRA85 selection from the Corsican budwood registration program, were performed as described previously (36). The following year, the young trees were transplanted to a field plot of the Station de Recherches Agronomiques INRA/CIRAD (SRA) located at San Giuliano (Corsica, France) in a randomized block arrangement. This plot was adjacent to, but completely independent of, a similar plot planted with an identical experimental design in the same orchard. In the first plot, inoculated trees carried only one viroid species, and were used to compare the biological and pathogenic properties of different

TABLE 1. Symptoms observed on Pomeroy trifoliate orange rootstocks 13 years after infection with several viroid species

	Viroids[a]					Bark symptoms				Wood symptoms
						Scaling		Cracking		Bumps
Treatment	CEVd	CBLVd	HSVd	CVd-III	CVd-IV	Intensity	No. of trees[b]	Intensity	No. of trees[b]	No. of trees[b]
Control										
0	–	–	–	–	–	…	0/6	…	0/6	0/6
Single viroids										
1	+	–	–	–	–	Severe	6/6	Mild	3/6	6/6
2	–	+	–	–	–	…	0/6	…	0/6	0/6
3	–	–	+	–	–	…	0/6	Mild	3/6	0/6
4	–	–	–	+	–	…	0/6	…	0/6	0/6
5	–	–	–	–	+	…	0/5	Severe	5/5	0/5
Two viroids										
6	+	+	–	–	–	Severe	6/6	Mild	1/6	6/6
7	+	–	+	–	–	Severe	5/5	Mild	3/5	5/5
8	+	–	–	+	–	Severe	4/4	…	0/4	4/4
9	+	–	–	–	+	Severe	3/5	Severe	2/5	5/5
10	–	+	+	–	–	…	0/5	Mild	3/5	0/5
11	–	+	–	+	–	…	0/5	…	0/5	0/5
12	–	+	–	–	+	…	0/4	Severe	4/4	0/4
13	–	–	+	+	–	…	0/5	Mild	2/5	0/5
14	–	–	+	–	+	…	0/6	Severe	6/6	0/6
15	–	–	–	+	+	…	0/5	Severe	5/5	0/5
Three viroids										
16	+	+	+	–	–	Severe	5/5	Mild	2/5	5/5
17	+	+	–	+	–	Severe	6/6	…	0/6	6/6
18	+	+	–	–	+	Mild	1/4	Severe	4/4	4/4
19	+	–	+	+	–	Severe	6/6	Mild	1/6	6/6
20	+	–	+	–	+	Mild	4/6	Severe	6/6	4/6
21	+	–	–	+	+	Mild	5/5	Severe	4/5	5/5
22	–	+	+	+	–	…	0/6	Mild	4/6	0/6
23	–	+	+	–	+	…	0/6	Severe	6/6	0/6
24	–	+	–	+	+	Mild	1/5	Severe	5/5	1/5
25	–	–	+	+	+	…	0/5	Severe	5/5	0/5
Four viroids										
26	+	+	+	+	–	Severe	6/6	Mild	3/6	6/6
27	+	+	+	–	+	Mild	4/4	Severe	4/4	1/4
28	+	+	–	+	+	Mild	6/6	Severe	5/6	4/6
29	+	–	+	+	+	Mild	6/6	Severe	4/6	5/6
30	–	+	+	+	+	Mild	2/6	Severe	6/6	1/6
Five viroids										
31	+	+	+	+	+	Mild	5/5	Severe	4/5	2/5

[a] CEVd = *Citrus exocortis viroid*, CBLVd = *Citrus bent leaf viroid*, HSVd = *Hop stunt viroid*, CVd-III = *Citrus viroid III*, and CVd-IV = *Citrus viroid IV*.
[b] Number of trees showing symptoms/total number of trees.

isolates of viroid species (36). No data from this plot was used in this study. Two pruning operations were conducted in 1990 and 1991 to shape the trees. Since 1996, as in commercial orchards, the trees were pruned yearly to open the canopy. During these operations, tools were disinfected with a sodium hypochlorite solution from one tree to the next. Fertilization and mineral-oil-based pesticide treatments were carried out following normal procedures in the region. Irrigation was applied by a microjet system. All trees were indexed at least twice during a 10-year period by biological indexing on Etrog citron 861-S1, followed by nucleic acid extraction and sequential polyacrylamide gel electrophoresis (sPAGE) analysis, as well as by sPAGE and slot-blot hybridization analysis of nucleic acid extracts of Clementine samples, as described previously (36).

Symptom evaluation. Outer-bark symptoms (scaling and cracking) were recorded annually. In 2002, when the experiment was terminated, the trees were cut 50 cm above the bud union. Bark below the cut was completely peeled off for a final evaluation of symptoms on the wood above and below the bud union of the stumps.

Tree growth and fruit yield. Tree height, trunk circumferences of scion and rootstock (10 cm above and below the bud line), and fruit yield were measured annually. Fruit were harvested manually when the ratio of sugar content/acidity was greater than 7.0 and fruit coloration greater than 80%.

Statistical analysis. Data were analyzed by analysis of variance (ANOVA) using the GLM procedure of SAS (SAS Institute, Inc., Cary, NC). Nonconform trees were found to lack one of the inoculated viroids or be infected with an additional viroid after repeated indexing. Out of 32 viroid treatments, 16 viroid treatments were completely conform, 12 treatments included only 5 conform trees instead of 6, and 4 treatments had 4 conform trees. The occurrence of nonconform trees resulted in having less than six replicates for some treatments, leading to the use of adjusted mean values (least squares means [lsm]). Hence, 192 trees were analyzed in the complete design.

A first ANOVA model with one factor (viroid treatment) and 32 levels was used to compare all the viroid treatments. Whenever F values were significant at $P < 0.05$, each adjusted mean was compared with the noninoculated control mean using Dunnett's test. The one-factor model is simple but provides limited information concerning viroid interactions. In this model, the treatments are considered as different modalities but the model does not acknowledge that different treatments may share common viroids. A second ANOVA factorial model, with five factors (or viroids) and two levels per factor (presence or absence), permitted evaluation of interactions. The concept of control disappears in the factorial model, and the control represents only one combination among others. With this model, principal effects and interaction effects between viroids were measured by two approaches on the basis of different estimations of the adjusted means, which were either independent of the presence of additional viroids in the host (i.e., the total mean approach) or exclusive (i.e., the treatment mean approach).

In the total mean approach, all trees containing one or several viroids were considered for estimating the mean, whatever the status of the other viroids. In other words, for each viroid, the mean of all the trees that received this viroid was tested against the mean of those that did not have this viroid. In this approach, the healthy control is regarded as a combination without the given viroid. The principal effect for a single viroid CVd-X then was estimated by

$$\text{Effect(CVd-X)} = \text{lsm(CVd-X)} - \text{lsm(no CVd-X)} \quad (1)$$

In this case, both lsm (CVd-X) and lsm (no CVd-X) are adjusted means from 96 trees. Interaction effects between viroids CVd-X and CVd-Y can be expressed by

$$\begin{aligned}\text{lsm(CVd-X, CVd-Y)} &= \text{lsm(no CVd-X, no CVd-Y)} + \\ &\text{effect(CVd-X, no CVd-Y)} + \text{effect(no CVd-X, CVd-Y)} + \\ &\text{interaction effect(CVd-X, CVd-Y)}\end{aligned} \quad (2)$$

Considering equation 1, we obtain

$$\begin{aligned}\text{interaction effect(CVd-X, CVd-Y)} &= \text{lsm(no CVd-X, no CVd-Y)} + \\ &\text{lsm(CVd-X, CVd-Y)} - \text{lsm(CVd-X, no CVd-Y)} - \\ &\text{lsm(no CVd-X, CVd-Y)}\end{aligned} \quad (3)$$

In the case of the two-viroid interaction, each adjusted mean was from 48 trees.

In the treatment mean approach, only viroids that are in question were considered, other viroids being absent. The healthy control corresponds to the absence of all the viroids, and each adjusted mean was estimated from six trees. The principal effect then was estimated by

$$\text{effect(CVd-X)} = \text{lsm(CVd-X)} - \text{lsm(control)} \quad (4)$$

This estimated principal effect is similar to that obtained by the one-factor model and given by the Dunnett's test. The model of interaction of two viroids is expressed by

$$\begin{aligned}\text{lsm(CVd-X, CVd-Y)} &= \text{lsm(control)} + \text{effect(CVd-X)} + \\ &\text{effect(CVd-Y)} + \text{interaction effect(CVd-X, CVd-Y)}\end{aligned} \quad (5)$$

Equation 5 also can be written using equation 4 as

$$\begin{aligned}\text{interaction effect(CVd-X, CVd-Y)} &= [\text{lsm(CVd-X, CVd-Y)} - \\ &\text{lsm(control)}] - [\text{effect(CVd-X)} + \text{effect(CVd-Y)}]\end{aligned} \quad (6)$$

Principal effects and interaction effects were tested with a Fisher test or by contrast analysis with a t test. The level of significance, whatever the statistical tests, was defined as nearly significant ($0.05 < P < 0.10$), significant ($0.01 < P < 0.05$), or highly significant ($P < 0.01$). The number of trees retained to estimate the means was only 6 for the treatment mean approach of the factorial model, as for the one-factor model, whereas it amounted to 96 for the total mean approach, which made the statistical analyses more powerful. In both mean estimation approaches, the interaction effect of two viroids represents the deviation from the additivity of principal effects estimated for each viroid. A positive deviation indicates that the estimated effect of the two viroids in combination is greater than the sum of the two individual effects. A "greater effect" has to be translated here as a less depressive biological effect, and will be interpreted as antagonism. Conversely, a negative deviation will be interpreted as synergism. Interactions between more than two viroids were estimated similarly by these two approaches.

RESULTS

Rootstock bark symptoms. *Single-viroid treatments.* Bark-scaling symptoms were first observed in 1993, 4 years after inoculation. As reported earlier (36), only CEVd-infected trees showed the bark-scaling characteristic of the exocortis disease. In 2002 (13 years after inoculation), all CEVd-infected trees presented severe scaling of the rootstock from the soil level up to the bud union (Table 1, treatment 1). Bark-cracking symptoms were observed in the rootstocks of HSVd-infected trees and those infected with CVd-IV, but were more intense and more consistently found in trees infected with CVd-IV (five of five) (Table 1, treatment 5) than in trees infected with HSVd (three of six) (Table 1, treatment 3). In 1995, small bark cracks also were observed in CEVd-infected trees but later were masked by intense scaling. In 2002, the cracks remained perceptible in only some of the trees (three of six) (Table 1, treatment 1). No symptoms were found in trees infected with CBLVd or CVd-III (Table 1, treatments 2 and 4).

Two-viroid treatments. Bark scaling was observed only in CEVd-containing treatments (Table 1, treatments 6 to 9; Fig. 1A). Bark cracking was observed in trees of all treatments which contained either HSVd, CVd-IV, or both, being more intense and more consistently found in treatments containing either CVd-IV (Table 1, treatments 9, 12, and 15) or both HSVd and CVd-IV (Table 1, treatment 14; Fig. 1B) than in those containing only HSVd (Table 1, treatments 7, 10, and 13; Fig. 1C). It must be noted that only some of the trees (three of five) co-infected with CEVd and CVd-IV showed the bark scaling characteristic of CEVd (Fig. 1D), whereas the remaining trees (two of five) showed only the severe bark cracking characteristic of CVd-IV infection (Table 1, treatment 9; Fig. 1E). This last observation suggests the existence of interference between symptom expression due to CEVd and CVd-IV. Trees co-infected with CBLVd and CVd-III were symptomless (Fig. 1F).

Three-, four-, and five-viroid treatments. Bark scaling was observed in all CEVd-containing treatments (Table 1, treatments 16 to 21, 26 to 29, and 31) and was severe in the absence of CVd-IV (Table 1, treatments 16, 17, and 19) but mild in the presence of CVd-IV (Table 1, treatments 18, 20, and 21), suggesting interference between CEVd and CVd-IV. Bark cracking was observed in trees of all treatments containing either HSVd, CVd-IV, or both, being more intense and more consistently found in treatments containing CVd-IV. In treatments containing both CEVd and CVd-IV (Table 1), trees showed either mild bark scaling, severe bark cracking, or both. The identification of trees with scaling in the absence of cracking (treatments 9, 21, 28,

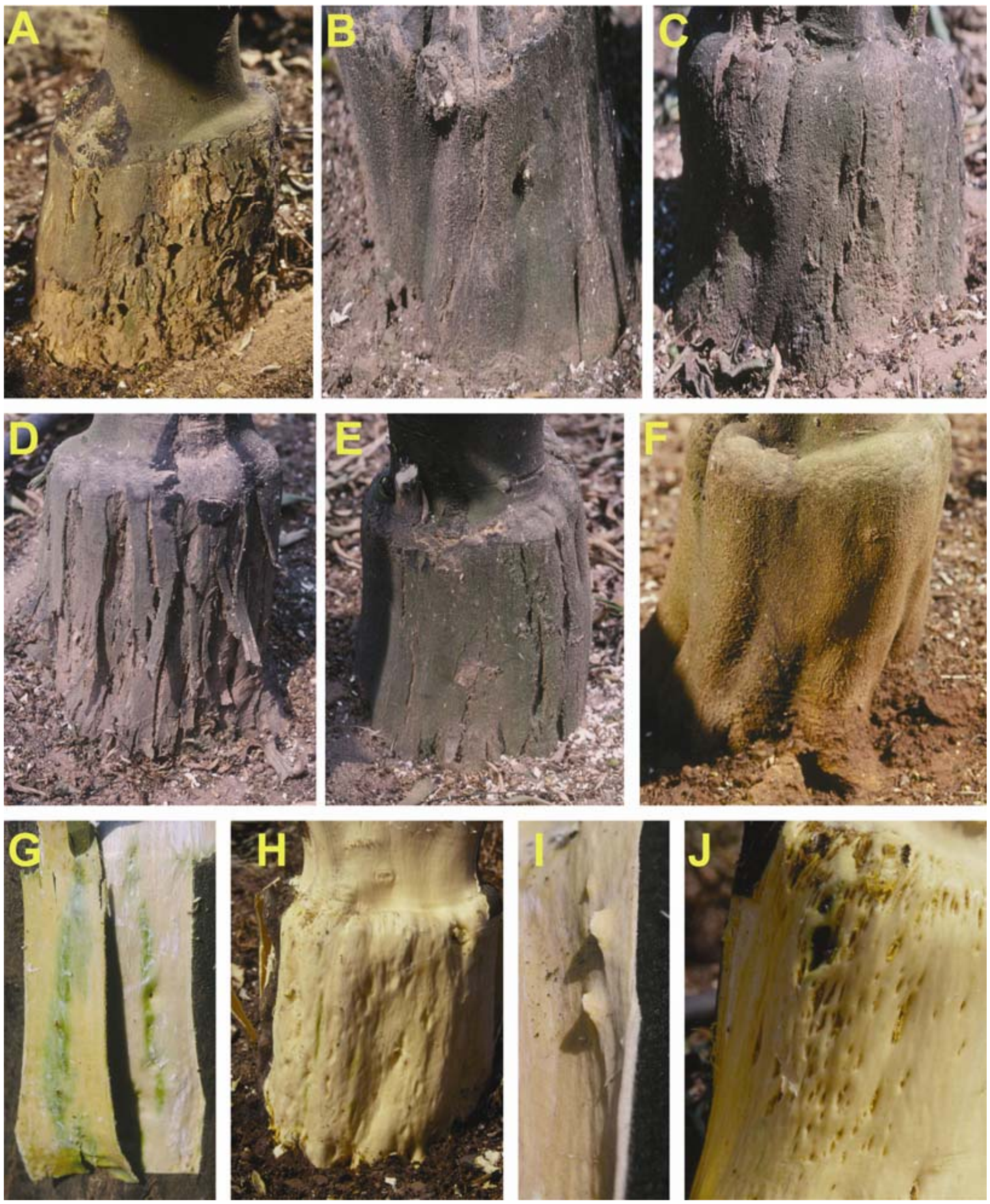

Fig. 1. Symptoms and abnormalities observed in Commune Clementine grafted on trifoliate orange rootstock. **A,** Bark scaling characteristic of trees infected with *Citrus exocortis viroid* (CEVd)-containing viroid combinations. **B,** Mild bark cracking characteristic of trees infected with *Hop stunt viroid* (HSVd)-containing treatments. **C,** Severe bark cracking characteristic of trees infected with viroid combinations containing *Citrus viroid IV* (CVd-IV) or HSVd + CVd-IV. **D,** Severe bark scaling observed in some trees co-inoculated with CEVd and CVd-IV. **E,** Bark cracking observed in some trees co-inoculated with CEVd and CVd-IV. **F,** Lack of symptoms in trees co-inoculated with *Citrus bent leaf viroid* and *Citrus viroid III*. **G,** Green streaks associated with bark cracking symptoms. **H,** Wood bumping associated with bark-scaling symptoms. **I,** Vertical crests not associated with viroid infection. **J,** Gummy pitting not associated with viroid infection.

29, and 30), or cracking in the absence of scaling (treatments 9, 18, and 20), further suggests the existence of interference. Interestingly, a few trees of treatments 24 (one of five) and 30 (two of six), showed bark scaling in the absence of CEVd infection (Table 1).

Inner rootstock abnormalities. *Symptoms associated with viroid infection.* After removing the bark, additional symptoms were observed on the trifoliate orange rootstock: (i) green streaks associated with cracks on the cambial face of the bark (Fig. 1G) and (ii) bumping on the wood associated with bark scaling (Fig. 1H). Cracking as a result of HSVd or CVd-IV infection alone or in combination with other viroids (data not shown) was more perceptible on the cambial side of the bark due to the presence of green streaks with protuberances that fit into depressions in the wood (Fig. 1G), as described by Vernière et al. (36) in trees infected only with HSVd or CVd-IV. Bumps on the wood were observed in all CEVd-containing treatments (Fig. 1H), confirming its association with scaling symptoms induced by CEVd alone (36). However, in some trees of treatments containing CEVd and CVd-IV, deviations from the expected pattern of symptoms were observed: (i) bumping in the absence of scaling (Table 1, treatments 9 and 18) and (ii) scaling in the absence of bumping (Table 1, treatments 27, 28, 29, and 31). Bumps also were observed on two of the three trees showing bark scaling in the absence of CEVd (Table 1, treatments 24 and 30).

Other abnormalities not associated with viroid infection. As described by Vernière et al. (36), small vertical crests or pegs on the cambial side of the trifoliate orange bark (Fig. 1I), corresponding to pits on the cambial side of the wood, were observed in almost all the treatments as well as on the noninoculated controls (data not shown). Many trees showed pronounced pitting on the bottle neck shoulder of the rootstock/scion union (Fig. 1J). These pits were observed in trees of all the treatments as well as in the noninoculated controls. In a few trees, round gummy pits also were visible along the wood of the trifoliate orange rootstock (data not shown).

Effect of viroid infection on vegetative growth. Highly significant *F* values ($P < 0.01$) among the treatments were observed consistently since 1994 for tree height and rootstock and scion circumferences (Table 2). Rootstock circumference differed significantly as early as 1990. Some block effect was regularly observed after 1992 for all growth parameters (Table 2). The mean values for vegetative growth parameters of each treatment compared with the control by the Dunnett's test are shown in Table 3.

All single-viroid infection treatments induced reduction of tree height in 1996 (except treatment 3) and rootstock and scion circumferences in 2001 (Table 3). The effect was highly significant ($P < 0.01$) in the case of CEVd and, to a lesser extent, with CVd-III (Table 3). A significant reduction ($P < 0.05$) also was observed with CVd-IV but only on scion circumference in 2001. Growth of trees infected with combinations of viroids containing CEVd, CVd-III, or both were most affected for tree height and trunk circumference and generally were significantly smaller than the control except for treatments 11, 18, 20, and 22 (Fig. 2). For instance, all treatments containing CEVd or CVd-III showed a similar range of scion circumference reductions: 23.6 to 37.7% for CEVd, and 25.2 to 34.3% for CVd-III; however, in treatments with both viroids, scion circumference reductions were much greater, ranging from 34.8 to 50.5%. All treatments without CEVd or CVd-III (treatments 10, 12, 14, and 23) were not significantly different from the control for tree height and rootstock circumference and differed significantly from the control for scion circumference only in 2001 (Table 3). All treatments with four viroids, including CEVd, and five viroids were significantly different from the control at $P < 0.001$ (Table 3). In the case of multiple-viroid infections, significant reductions of growth occurred much sooner than for single-viroid infection. In addition,

TABLE 2. Growth and yield parameters of Commune Clementine grafted on Pomeroy trifoliate orange co-infected with several viroids in 1989: source of variation and significance[a]

Parameters	Years 1990	1992	1994	1996	1998	2001
Tree height (m)						
General mean[b]	0.71	1.26	2.09	2.55	…	…
Root mean square error[b]	0.14	0.21	0.21	0.22	…	…
Residual error (CV%)[b]	20	16	10	8.5	…	…
Mean amplitude[c]	0.28	0.38	0.53	0.86	…	…
Treatments: *P* > *F*	+	NS	***	***	…	…
Blocks: *P* > *F*	NS	NS	**	+	…	…
Rootstock circumference (cm)						
General mean	3.5	11.1	22.5	33.6	43.6	60.0
Root mean square error	0.5	1.2	1.9	2.6	3.1	4.3
Residual error (CV%)	15	11	9	8	7	7
Mean amplitude	1.2	3.6	9.4	15.2	19.6	29.2
Treatments: *P* > *F*	***	***	***	***	***	***
Blocks: *P* > *F*	NS	*	**	+	NS	NS
Scion circumference (cm)						
General mean	1.7	7.5	16.4	24.4	30.3	38.0
Root mean square error	0.5	1.0	1.6	2.3	2.9	3.7
Residual error (CV%)	31	13	10	9	9	10
Mean amplitude	1.1	2.5	7.0	13.3	17.7	26.8
Treatments: *P* > *F*	+	+	***	***	***	***
Blocks: *P* > *F*	NS	NS	***	*	+	*
Cumulative weight (kg)						
General mean	…	4.4	37.9	105.3	194.2	288.0
Root mean square error	…	3.1	13.3	27.8	45.8	65.1
Residual error (CV%)	…	71	35	26	24	23
Mean amplitude	…	5.9	47.9	150.4	254.1	335.4
Treatments: *P* > *F*	…	NS	***	***	***	***
Blocks: *P* > *F*	…	NS	**	**	*	**

[a] Level of significance: + = $0.05 < P < 0.10$; *, **, and *** = $P < 0.5$, 0.01, and 0.001, respectively; NS = no significance at 10%.
[b] Estimated values from all the 32 treatments in a complete randomized block design with six replicates. CV = coefficient of variance.
[c] Mean amplitude is the difference between the smallest and the largest mean.

as illustrated in Figure 2 for rootstock circumference, it was observed over the years that reduction in growth correlated with the number of viroids in a tree.

Effect of viroid infection on yield. Fruit were harvested first in 1992 and then each year thereafter. Highly significant differences ($P < 0.001$) in cumulative yields among treatments have been observed since 1994 (Table 2). Treatments 26 and 3 induced the lowest and highest cumulative weights in 1996 and 2001, respectively (Table 3; Fig. 2). Trees with single-viroid infections showed no significant reduction of cumulative yields in 1996 (Table 3). Some treatments with a combination of viroids containing CEVd gave yields significantly different from those of the control. In 2001, cumulative yields ranged from 122.5 to 457.9 kg for treatments 26 and 3, respectively (Table 3). The lowest cumulative yields were recorded from treatments containing CEVd, all below 300 kg, except treatment 20. The cumulative yields of these treatments were significantly different from the noninoculated control except for a few treatments including both CEVd and CVd-IV. When CEVd and CVd-III were absent, no significant differences in the cumulative yields were observed in comparison with the control. The cumulative yields of a few treatments (treatments 3, 5, 10, and 12) were higher than those of the control in both 1996 and 2001 (Table 3) but they were not significantly different, except for treatment 3 in 1996. None of these treatments contained CEVd or CVd-III. Multiple-viroid infections induced significant differences in cumulative yields earlier than single-viroid infections. In addition, it was observed that reduction in cumulative yields correlated also with the number of viroids within a tree (Fig. 2).

Viroid interaction and vegetative growth. Scion circumferences were highly correlated with rootstock circumferences and heights, with $P < 0.0001$ from 1993 to 2001 (data not shown). Because of these high correlations and the partial redundancy of these vegetative traits, scion circumference was chosen as the growth variable for further analyses to evaluate interactions among viroids.

The difference among the adjusted means of treatments with or without a given viroid illustrates the raw effect of this viroid (Table 4). All viroids had a significant negative effect on scion circumference in 2001 compared with the control, except HSVd. The addition of CEVd or CVd-III to other viroids consistently caused a decrease of scion circumference, which was significant in most cases even after a Bonferroni's correction of the *t* test (Table 4). Addition of CBLVd, HSVd, or CVd-IV to combinations of viroids did not significantly modify this effect except for a few cases. Interestingly, addition of CVd-IV to seven of eight combinations, including CEVd, increased scion circumference in 2001 (Table 4) and, similarly, rootstock circumference (Fig. 2).

Analyses with the factorial ANOVA model first were done using the total mean approach. With this approach, when estimating the principal effect, each mean takes into account 96 trees of the complete design. As shown by the Fisher test in Table 5, trees

TABLE 3. Mean comparisons of growth parameters and yield of Commune Clementine grafted on Pomeroy trifoliate orange co-infected in 1989 with several viroid species[a]

	Height (m)	Rootstock circumference (cm)		Scion circumference (cm)		Cumulative weight (kg)	
Treatment	1996	1996	2001	1996	2001	1996	2001
Control							
0	2.97	38.3	71.0	28.4	53.1	136.7	403.3
Single viroids							
1	2.45**	33.5*	57.5***	25.1	38.0***	102.6	232.0***
2	2.78	36.7	66.6	28.4	48.1	130.5	315.4
3	3.03	39.2	70.0	30.9	50.1	192.6*	457.9
4	2.58+	35.2	61.7*	23.6*	36.4***	105.4	287.9+
5	2.77	38.3	68.5	30.0	45.8*	157.9	441.7
Two viroids							
6	2.42***	30.6***	58.2***	22.8**	36.2***	78.4**	224.5***
7	2.54*	31.5***	60.4**	23.4*	37.0***	93.2	271.1+
8	2.25***	30.1***	55.4***	20.9***	33.7***	71.9*	226.9**
9	2.36***	30.5***	58.5***	24.6+	40.6***	91.2+	284.0+
10	2.87	38.6	71.0	28.5	45.7*	165.5	457.3
11	2.67	37.0	65.2	26.0	39.7***	121.5	368.1
12	2.92	40.5	73.5	30.1	47.5	164.3	443.5
13	2.41**	34.5	60.5**	23.2*	36.8***	98.4	277.8+
14	2.88	37.6	68.6	28.8	44.4**	130.1	384.2
15	2.59+	35.7	59.4***	24.1*	36.2***	99.9	256.6*
Three viroids							
16	2.31***	30.1***	56.9***	22.8**	38.1***	86.6+	237.6**
17	2.17***	28.8***	51.8***	20.5***	31.4***	51.5***	155.0***
18	2.65	33.0*	63.8	24.4	38.5***	115.3	282.4
19	2.35***	29.5***	50.7***	20.1***	30.5***	72.5**	189.1***
20	2.73	34.6	63.7+	25.0	38.2***	131.7	349.3
21	2.39***	33.4*	54.4***	23.3**	34.6***	81.9*	245.7**
22	2.74	35.4	62.6*	25.6	38.1***	131.1	339.3
23	2.82	36.9	65.5	26.6	42.2***	127.8	362.1
24	2.57*	34.3	60.5**	23.9*	37.0***	98.6	267.1*
25	2.47**	32.8*	56.0***	23.6*	35.8***	106.6	303.4
Four viroids							
26	2.17***	25.3***	44.3***	17.6***	26.3***	42.2***	122.5***
27	2.37***	29.4***	55.9***	21.4***	33.1***	90.8	258.9*
28	2.37***	31.6***	55.3***	22.1***	32.3***	75.0**	198.1***
29	2.22***	31.0***	52.0***	21.9***	32.2***	84.0*	212.0***
30	2.58*	34.4	60.4**	23.8*	34.9***	99.3	299.5
Five viroids							
31	2.31***	29.0***	51.6***	20.9***	30.6***	80.9*	213.1***

[a] Means within columns differ significantly from the control with the level of significance + = $0.05 < P < 0.10$; and *, **, and *** = $P < 0.5$, 0.01, and 0.001, respectively, according to the Dunnett's test.

infected with CEVd, HSVd, CVd-III (treatments 1, 3, and 4), or to a lesser extent, CBLVd (treatment 2) were significantly different than those lacking these viroids. These negative effects were consistently highly significant ($P < 0.01$) since 1992 for treatments 1 or 4 and significant ($P < 0.05$) since 1995 for treatments 2 or 3 (Table 5). No significant difference on scion circumference was observed in the case of CVd-IV-infected trees (treatment 5) over the years (Table 5). When using the treatment mean approach, significant negative effects on scion circumference appeared later for treatments 1, 2, and 4 than in the case of the total mean approach (Table 5). In addition, no significant difference was observed for HSVd-infected trees contrary to what was found using the total mean approach. Single infection of CVd-IV reduced scion circumference highly significantly only in 2001 (Table 5).

The factorial model made it possible to study the interactions among viroids by estimating the deviation from additivity of principal effects. Few interactions among more than two viroids were significant on scion circumference between 1990 and 2001, whatever the approach used to estimate the means (data not shown). Moreover, these interactions were isolated and may have been due to uncontrolled factors and mainly observed only during the first few years. For viroid pairs, significant interactions were observed consistently over the years for two sets of viroids (CEVd/CVd-III and CEVd/CVd-IV) when estimated from equation 3 of the total mean approach (Table 5). The steady increase in

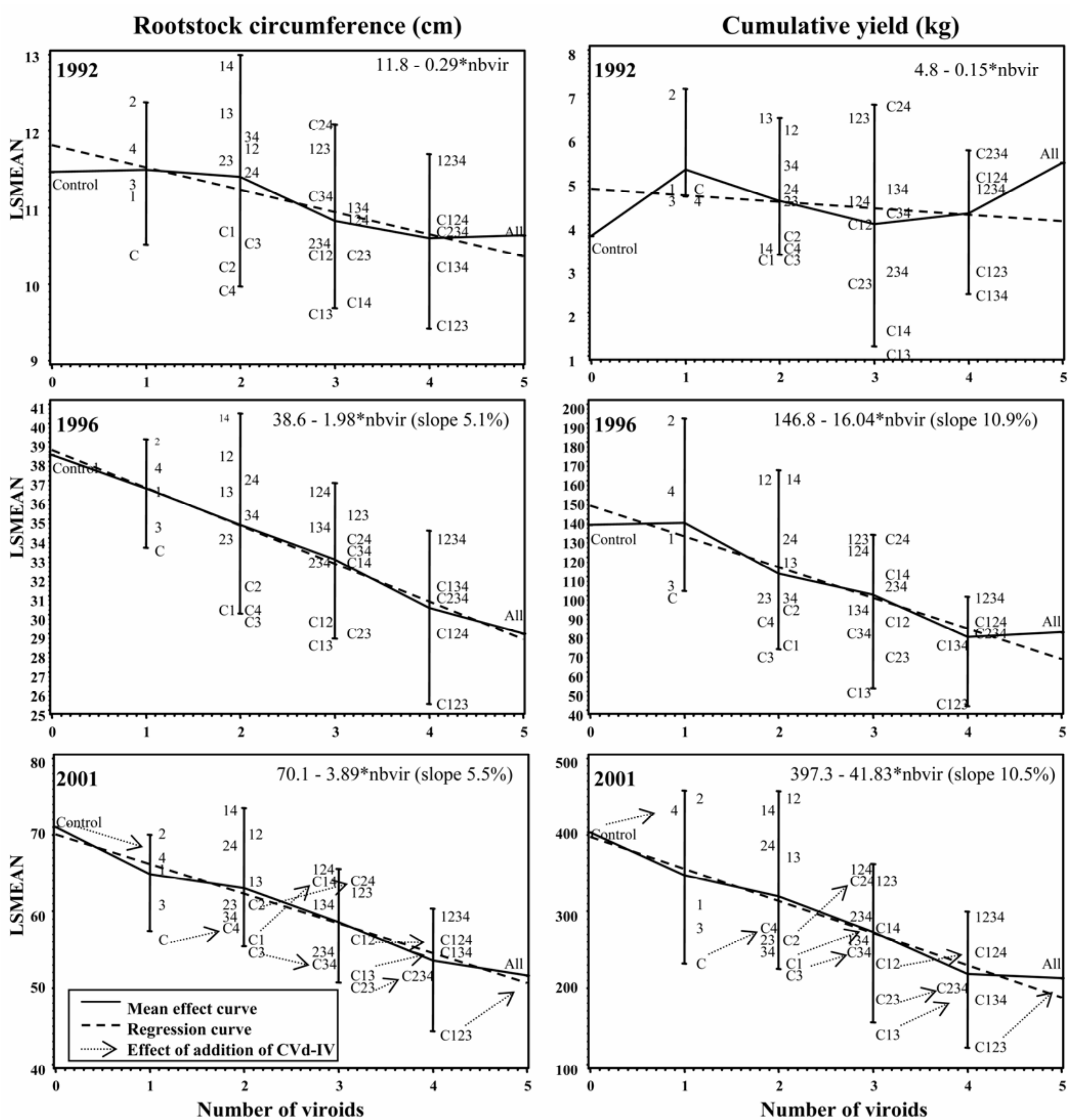

Fig. 2. Effect of each combination of viroid and global effect of viroid infection on rootstock circumference and cumulative weight of Commune Clementine grafted on Pomeroy trifoliate orange infected in 1989. Combinations of viroids are indicated by C = *Citrus exocortis viroid*, 1 = *Citrus bent leaf viroid*, 2 = *Hop stunt viroid*, 3 = *Citrus viroid III*, 4 = *Citrus viroid IV*, and All = C1234; for instance, 13 represents the association of CBLVd and CVd-III.

positive interactions was similar for both pairs and indicated the presence of antagonism (Fig. 3B and C). Positive deviation from additivity between CEVd and CVd-III increased because infection with either viroid alone had a strong effect over time. Interaction effects between CEVd and CVd-III estimated from the mean treatment approach were similar to those estimated from total means, but significant antagonism was observed only since 1999 (Table 5). Deviation to additivity strongly increased after 1998 due to important principal negative effects for each viroid (Fig. 3C).

In the case of antagonism between CEVd and CVd-IV, the deviation from additivity by the total mean approach increased, whereas CEVd induced a strong negative effect. No CVd-IV effect was detected until 1999 (Fig. 3B); however, during later years (1999 to 2001), scion circumference of trees co-infected with CEVd and CVd-IV was larger than that of trees infected with CEVd alone. Interaction between CEVd and CVd-IV estimated by treatment means first was negative until 1997 (Table 5). The interaction became positive after 1998, revealing antagonism, when CVd-IV started to induce an effect on scion circumference (Fig. 3C). CVd-IV-infected trees behaved similarly to the control trees until 1998, when scion growth was affected (Fig. 3A). The growth of co-infected trees was similar to that of CEVd-infected trees until 1998, but increased afterward.

Two additional pairs of viroids showed significant interactions. Synergism consistently was observed between CEVd and CBLVd when estimated from total means with significance from 1995 to 1998 (Table 5). When estimated from treatment means, no statistical significance was shown for this interaction (Table 5). Deviation to additivity was consistently positive for CBLVd and CVd-III and significant antagonism was observed after 1995 and 1997 with total and treatment mean approaches, respectively (Table 5). Three viroid pairs that contained HSVd (treatments 7, 10, and 14) gave negative interaction estimates with rare statistical significance, except after 1999, when estimated from treatment means (Table 5).

Viroid interaction and yield. Cumulative yields tended to decrease as the number of viroids present in multiple infections increased, as observed in 1996 and 2001 (Fig. 2). When CEVd or CVd-III was added to a healthy tree or a tree infected with a combination of viroids, the raw effect on the cumulative weight, as estimated in 2001, was always negative except when added to CBLVd-infected trees (Table 4, treatment 2). The effect of CEVd or CVd-III was nearly too highly significant for most of the treatments. Addition of CBLVd to different viroid combinations tended to decrease the cumulative weights of Clementine fruit in most of the cases; however, a noticeable increase was observed in two combinations containing CVd-III (treatments 4 and 13). Infection of trees with HSVd or CVd-IV did not affect significantly the control or most of the infected trees (Table 4). Interestingly, addition of CVd-IV to treatments containing CEVd systematically resulted in an increase of cumulative yield in 2001, which was already noticeable in 1996 (Fig. 2). These observations suggest that viroid interactions affected yield.

TABLE 4. Effect of the addition of a viroid to different viroid combinations on scion circumference and cumulative weight in 2001[a]

	Scion circumference (cm)					Cumulative weight (kg)				
Treatment	+ CEVd	+ CBLVd	+ HSVd	+ CVd-III	+ CVd-IV	+ CEVd	+ CBLVd	+ HSVd	+ CVd-III	+ CVd-IV
Control										
0	−15.1***	−5.0*	−3.0 NS	−16.8***	−7.4**	−171.3***B	−87.9*	54.6NS	−115.4**	38.4 NS
Single viroids										
1	…	−1.8 NS	−1.1 NS	−4.4+	2.5 NS	…	−7.5 NS	39.1 NS	−5.1 NS	52.0 NS
2	−11.9***B	…	−2.4 NS	−8.4***B	−0.7 NS	−90.9*	…	141.9**	52.7 NS	128.1**
3	−13.2***B	−4.4+	…	−13.4***B	−5.7*	−186.8***B	−0.6 NS	…	−180.1***B	−73.7+
4	−2.7 NS	3.4 NS	0.4 NS	…	−0.2 NS	−61.0 NS	80.2+	−10.1 NS	…	−31.3 NS
5	−5.2*	1.7 NS	−1.4 NS	−9.6***B	…	−157.7***B	1.8 NS	−57.5 NS	−185.1***B	…
Two viroids										
6	…	…	1.9 NS	−4.8*	2.3 NS	…	…	13.1 NS	−69.5+	57.9 NS
7	…	1.1 NS	…	−6.4**	1.2 NS	…	−33.5 NS	…	−82.0+	78.2+
8	…	−2.3 NS	−3.1 NS	…	1.0 NS	…	−71.9+	−37.8 NS	…	18.8 NS
9	…	−2.1 NS	−2.4 NS	−5.9*	…	…	−1.6 NS	65.3+	−38.3 NS	…
10	−7.6**	…	…	−7.6**	−3.5 NS	−219.7***B	…	…	−118.0**	−95.2*
11	−8.3***	…	−1.6 NS	…	−2.7 NS	−213.1***B	…	−28.8 NS	…	−101.0*
12	−9.0**	…	−5.2*	−10.4***B	…	−161.1**	…	−81.4+	−176.4***B	…
13	−6.2**	1.3 NS	…	-	−1.0 NS	−88.7*	61.5 NS	…	…	25.6 NS
14	−6.2**	−2.2 NS	…	−8.6***	…	−34.9 NS	−22.1 NS	…	−80.8+	…
15	−1.5 NS	0.8 NS	−0.4 NS	…	…	−10.9 NS	10.5 NS	46.8 NS	…	…
Three viroids										
16	…	…	…	−11.9***B	−5.0*	…	…	…	−115.1**	21.3 NS
17	…	…	−5.2*	…	0.9 NS	…	…	−32.5 NS	43.1 NS	…
18	…	…	−5.4+	−6.2*	…	…	…	−23.5 NS	−84.3+	…
19	…	−4.3+	…	…	1.7 NS	…	−66.6+	…	22.9 NS	…
20	…	−5.1*	…	−6.0**	…	…	−90.4*	…	−137.3***	…
21	…	−2.3 NS	−2.4 NS	…	…	…	−47.6 NS	−33.7 NS	…	…
22	−11.9***B	…	…	…	−3.2 NS	−216.8***B	…	…	…	−39.8 NS
23	−9.1***B	…	…	−7.3**	…	−103.2*	…	…	−62.6 NS	…
24	−4.7+	…	−2.1 NS	…	…	−69.0+	…	32.4 NS	…	…
25	−3.6 NS	−0.9 NS	…	…	…	−91.4*	−3.9 NS	…	…	…
Four viroids										
26	…	…	…	…	4.4+	…	…	…	…	90.6*
27	…	…	…	−2.5 NS	…	…	…	…	−45.8 NS	…
28	…	…	−1.7 NS	…	…	…	…	15.0 NS	…	…
29	…	−1.6 NS	…	…	…	…	1.1 NS	…	…	…
30	−4.3+	…	…	…	…	−86.4*	…	…	…	…
Five viroids										
31	…	…	…	…	…	…	…	…	…	…

[a] CEVd = *Citrus exocortis viroid*, CBLVd = *Citrus bent leaf viroid*, HSVd = *Hop stunt viroid*, CVd-III = *Citrus viroid III*, and CVd-IV = *Citrus viroid IV*. $P > |t|$, *t* test for each pair (with and without the given viroid), with the level of significance + = $0.05 < P < 0.10$; *, **, and *** = $P < 0.5$, 0.01, and 0.001, respectively; and NS = no significance at 10%. B: after Bonferroni's probability correction, significant differences between two means at 5%.

Analysis by the factorial model showed significant negative effects whatever the mean estimation approach for treatments 1, 2, and 4 (i.e., CEVd, CBLVd, and CVd-III, respectively) (Table 6; Fig. 3E and F). Significant effects for treatments 1 and 4 appeared sooner and with a higher level of significance when the total mean approach was used than with the treatment mean approach (Table 6). Treatments 3 (HSVd) and 5 (CVd-IV) consistently induced positive effects on yield over the years; however, significant to highly significant effects were observed only during a few years for HSVd using the treatment mean approach (Table 6).

Significant interaction effects between two viroids were observed for different pairs of viroids (Table 6). A synergistic effect was observed consistently between HSVd and CVd-IV (treatment 14) whatever the mean estimation approach used, but with significant and greater effects using the treatment mean approach (Table 6). Deviation to additivity was also consistently negative for treatments 7, 13, and 15; however, a significant synergistic effect was observed only for treatment 7 between 1995 and 1998. In contrast, deviation to additivity was positive over the years between CEVd and CVd-III, expressing nearly significant antagonism only in 2001. Both principal effects of CEVd and CVd-III were strongly negative, giving a positive interaction effect whatever the mean estimation approach (Fig. 3E and F). Consistent positive interactions also were observed between CBLVd and CVd-III, with a significant antagonism after 1997 only when both viroids were considered (Table 6). Patterns of interactions changed according to the mean estimation approach for some pairs of viroids. A consistent antagonism was detected between CEVd and CVd-IV (treatment 9) on cumulative weight over the years using the total mean approach. In fact, the interaction effect on cumulative weight was much greater than the positive CVd-IV effect in spite of a strong negative CEVd effect (Fig. 3E). However, a nonsignificant negative interaction was observed using the treatment mean approach until 2000. Trees infected with CEVd and CVd-IV behaved like CEVd-infected trees until 1998; then, the interaction effect decreased while the CEVd effect increased, and the CVd-IV effect did not change (Fig. 3D and F). Negative interactions were detected between CEVd and CBLVd or CBLVd and HSVd (treatments 6 and 10) when estimated by the total mean approach, with significant synergism after 1995 for treatment 6. However, when estimated by the treatment mean approach, these negative interactions became positive after 1997 for both treatments. Positive interaction was expressed between CBLVd and CVd-IV (treatment 12), which became negative in 2001 with the total mean approach.

DISCUSSION

Previously, laboratory experiments demonstrated antagonism and cross-protection between closely related viroids and between variants of the same viroid (3,7,19,22,24,31), whereas synergism has been noticed only in indicator plants co-inoculated with different viroid species (6). In the field, viroids occur as mixtures within their citrus hosts (1,2,6,14,18,20). Therefore, possible interactions among certain partners of the mixture, leading to antagonism or synergism, may have an effect on symptom expression and tree performance.

As demonstrated earlier (36), the CEVd isolate used in the present study induced severe tree stunting and bark scaling of the trifoliate orange rootstock, both characteristic of the exocortis

TABLE 5. Estimates of principal effects and interaction effects between two viroids on scion circumference by the factorial model using two approaches for mean estimation and associated *F* statistics[a]

Treatment[b]	Mean[c]	Scion circumference (cm)[d]										
		1990	1991	1992	1993	1994	1995	1996	1997	1998	1999	2001
1 CEVd	Total	–0.02	–0.08	–0.52**	–1.44***	–2.62***	–3.33***	–4.28***	–5.05***	–5.74***	–7.06***	–7.54***
	Treatment	0.25	–0.13	–0.35	–2.02**	–2.03*	–2.45*	–3.30*	–4.85***	–6.90***	–11.19***	–15.10***
2 CBLVd	Total	–0.20*	–0.10	–0.21	–0.32	–0.28	–0.60+	–0.73*	–0.90*	–1.26**	–1.54**	–1.47*
	Treatment	–0.17	–0.30	–0.48	–0.42	0.55	0.38	0.00	–0.60	–1.92	–4.53*	–5.00*
3 HSVd	Total	–0.06	–0.07	0.26	–0.24	–0.38	–0.72*	–0.89*	–1.25**	–1.69***	–2.07***	–2.21***
	Treatment	0.01	–0.02	0.72	0.43	1.29	2.36+	2.51	1.96	0.83	–0.91	–3.01
4 CVd-III	Total	–0.08	–0.03	–0.46**	–0.87***	–1.90***	–2.87***	–3.75***	–4.73***	–5.54***	–6.26***	–8.13***
	Treatment	0.00	0.38	–0.18	–1.12	–2.47**	–3.53**	–4.80***	–6.55***	–8.62***	–12.11***	–16.77***
5 CVd-IV	Total	–0.05	0.13	0.17	0.16	0.25	0.28	0.40	0.24	0.21	–0.12	–0.96
	Treatment	–0.50+	0.23	0.58	0.85	1.18	2.12+	1.58	1.07	–0.30	–2.69	–7.35**
6 CEVd/CBLVd	Total	0.21	0.10	–0.22	–0.47	–0.71	–1.24*	–1.50*	–1.56*	–1.81*	–1.69	–1.64
	Treatment	–0.42	0.35	0.28	0.70	–1.53	–1.87	–2.28	–1.92	–0.97	1.86	3.17
7 CEVd/HSVd	Total	–0.04	0.17	0.24	0.06	–0.40	–0.65	–0.92	–0.76	–0.75	–0.29	–0.45
	Treatment	–0.66	–0.16	–0.61	–0.04	–2.07	–3.77*	–4.19*	–3.70+	–2.80	–0.58	1.94
8 CEVd/CVd-III	Total	0.14	–0.02	–0.05	0.33	0.79	1.89**	1.99**	2.49**	2.98**	3.51**	4.26***
	Treatment	–0.50	–0.59	0.38	1.22	–0.66	0.40	0.62	2.12	3.52	7.41*	12.41***
9 CEVd/CVd-IV	Total	0.21	0.36*	0.57	1.07**	1.37**	1.39*	1.77*	1.91*	2.58**	3.42**	4.15***
	Treatment	–0.58	–0.45	–0.93	–0.37	–1.40	–2.00	–2.10	–1.25	0.78	3.78	9.89**
10 CBLVd/HSVd	Total	–0.04	0.23	0.16	–0.21	–0.56	–0.80	–0.99	–1.29	–0.98	–1.00	–1.04
	Treatment	–0.25	0.27	0.46	0.49	–1.23	–2.01	–2.43	–2.14	–1.64	0.01	0.57
11 CBLVd/CVd-III	Total	–0.18	–0.11	0.05	0.06	0.73	0.99	1.38+	1.75*	1.96*	2.57*	1.52
	Treatment	–0.58	–0.57	0.66	1.36	1.82	1.73	2.35	3.18	4.30+	7.85**	8.38*
12 CBLVd/CVd-IV	Total	0.33*	–0.12	–0.02	–0.43	–1.17*	–1.08	–0.56	–0.45	–0.16	0.10	0.06
	Treatment	0.69	0.44	0.21	0.35	–1.28	–0.47	0.05	0.57	2.07	4.80	6.69*
13 HSVd/CVd-III	Total	0.16	0.07	–0.19	0.09	–0.38	–0.03	–0.15	–0.29	0.08	0.25	0.35
	Treatment	–0.21	–0.48	–0.79	–0.34	–1.06	–2.35	–2.93	–2.38	–1.69	0.42	3.40
14 HSVd/CVd-IV	Total	0.18	–0.17	–0.26	–0.78*	–0.53	–1.00	–0.85	–0.98	–1.01	–1.08	–0.87
	Treatment	0.16	–0.41	–1.20	–1.65	–1.97	–3.84*	–3.73+	–3.26	–2.75	–2.05	1.62
15 CVd-III/CVd-IV	Total	0.19	0.10	0.15	–0.10	0.46	0.38	0.73	0.44	0.73	1.23	2.12+
	Treatment	0.29	–0.43	–0.47	–0.37	–0.07	–1.40	–1.14	–0.52	0.26	2.41	7.16*

[a] In a complete block design with 192 trees.

[b] CEVd = *Citrus exocortis viroid*, CBLVd = *Citrus bent leaf viroid*, HSVd = *Hop stunt viroid*, CVd-III = *Citrus viroid III*, and CVd-IV = *Citrus viroid IV*.

[c] Mean estimation. Total = effects estimated from equation 1 for single-viroid and from equation 3 for two-viroid treatments by contrast analysis of principal effects and interaction effects (=deviation from additivity) of rank 2. Treatment = effects estimated from equation 4 for single-viroid and from equation 6 for two-viroids treatments by contrast analysis of principal effects and interaction effects (=deviation from additivity) of rank 2.

[d] Level of significance: + = 0.05 < P < 0.10; *, **, and *** = P < 0.05, 0.01, and 0.001, respectively.

disease as first described by Fawcett and Klotz (8). In the present study, co-inoculations of CEVd with either one, two, or three of the viroids CBLVd, HSVd, and CVd-III also resulted in severe stunting and bark-scaling symptoms. However, when the above viroid mixtures also contained CVd-IV, scaling symptoms were milder or even suppressed. Thus, it seems that CVd-IV and CEVd act as antagonists for symptom expression, even though these two viroids are not classified as being closely related taxonomically and phylogenetically (9). However, the primary and secondary structure of the right half of the CEVd and CVd-IV molecules are nearly identical (27), suggesting a closer relationship than that indicated by taxonomy (30,33).

On the other hand, as shown earlier (36), plants inoculated with single viroid species other than CEVd did not show exocortis scaling on the trifoliate orange rootstock. As expected, in the present work, most of the trees inoculated with the 11 viroid combinations that lacked CEVd did not show exocortis bark-scaling symptoms. Surprisingly, a few plants inoculated with the viroids of 2 of these 11 CEVd-lacking combinations (treatments 24 and 30) did exhibit exocortis-like scaling symptoms. These two treat-

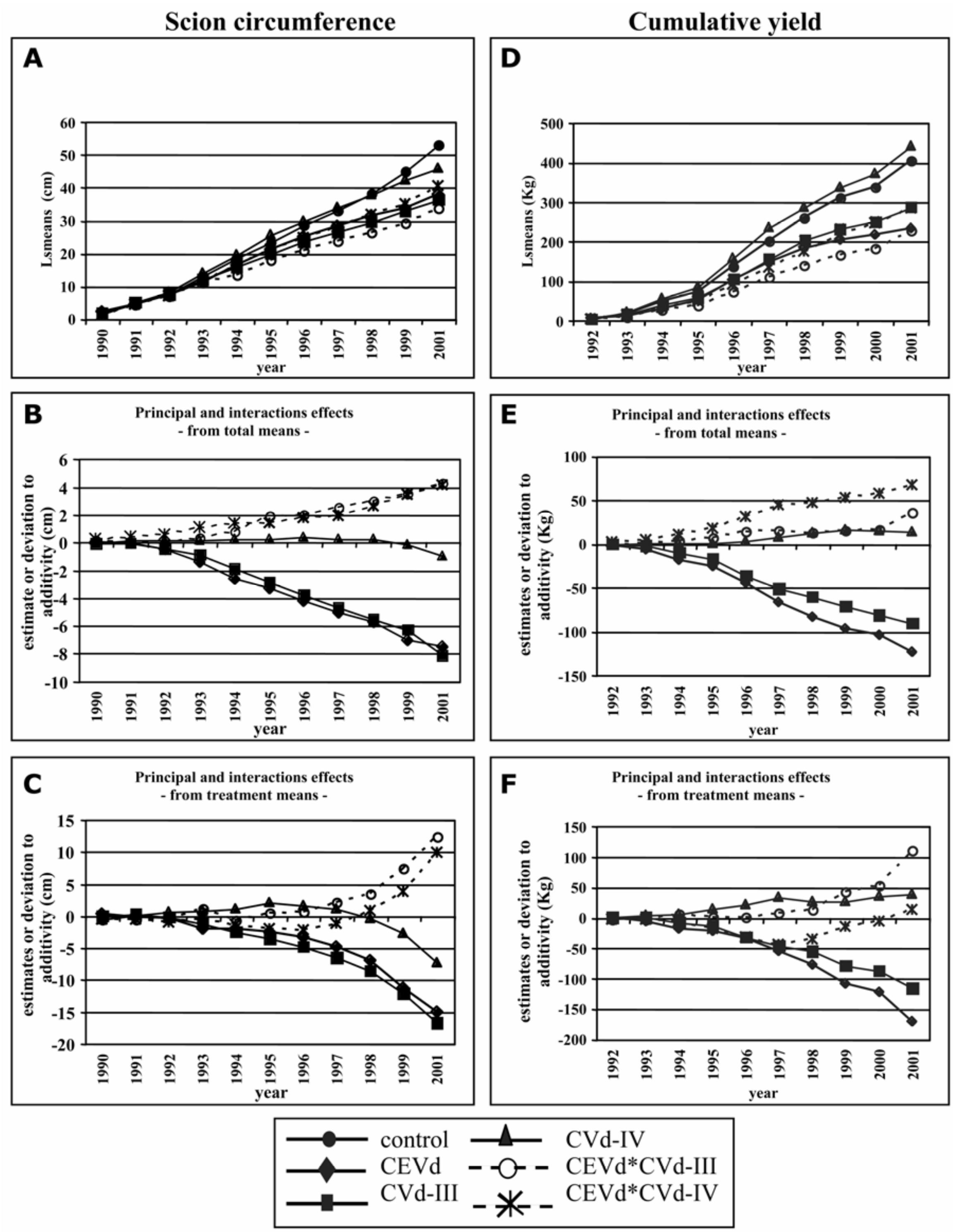

Fig. 3. Effect of viroids on growth and yield of Commune Clementine grafted on Pomeroy trifoliate orange infected in 1989. Influence on **A,** scion circumference and **D,** cumulative yield, and evolution of their principal and interactions effects according to different mean estimations **B** and **E,** from the total mean approach and **C** and **F,** from the treatment mean approach. CEVd = *Citrus exocortis viroid*, CVd-III = *Citrus viroid III*, and CVd-IV = *Citrus viroid IV*.

ments lack CEVd (as confirmed by sPAGE and molecular hybridization) but contain CBLVd, CVd-III, and CVd-IV, suggesting that synergism among these three viroids is responsible for the exocortis-like symptoms. Exocortis-like symptoms on the trifoliate orange rootstock already had been reported in three field-grown trees from Japan which also lacked CEVd but were infected with mixtures of other viroids (18). Similar to the results reported here, two of the trees showing exocortis-like symptoms were infected with viroid combinations that contained CBLVd, HSVd, CVd-III, and CVd-IV (18). However, according to our data, HSVd probably plays no role. Ito et al. (18) also have observed exocortis-like symptoms in a single Tsutsumi navel tree infected only with HSVd and CVd-III. This viroid combination did not induce exocortis-like symptoms in our study.

Cachexia is caused by variants of HSVd containing a specific five- or six-nucleotide motif (23,28). However, because little was known about the effect of non-cachexia-inducing variants of HSVd, such a variant was included in the present study. We have confirmed that no other viroid or viroid combinations induce cachexia, including those containing HSVd variants lacking the five- or six-nucleotide motif specific for cachexia.

As reported earlier (36), CVd-IV and HSVd induce bark cracking on the trifoliate orange rootstock. These bark-cracking symptoms were not influenced by viroids other than CEVd. In some instances, the severe bark scaling induced by CEVd masked the bark cracking induced by HSVd and CVd-IV.

Australian field trials have shown that inoculation of trees with CVd-III alone or in combination with a non-cachexia source of HSVd reduces tree size of selected sweet orange and grapefruit scions on trifoliate orange and citrange rootstocks (16). Also, in California, South Africa, Israel, France, and Italy, citrus viroid sources which contained CEVd or CVd-III significantly reduced tree size and fruit yields of trees on trifoliate orange, Troyer citrange, and sour orange when compared with control trees (15, 21,26,32,35,36). However, in none of these studies were the interactions among citrus viroids and their effect on tree performance systematically evaluated.

The purpose of the present work was to evaluate the effect of all possible viroid combinations on Clementine trees grafted on trifoliate orange and to identify putative interactions. These results were subjected to statistical analysis using two ANOVA models. The one-factor model was simpler than the factorial model, but limited information about viroid interaction was obtained. However, statistical analyses were more powerful with the factorial model using the total mean approach due to the number of trees retained for mean estimations.

With the one-factor model, it was demonstrated that trees infected with viroid combinations containing CEVd, CVd-III, or both were, in general, significantly smaller than the noninoculated control trees in terms of tree height, rootstock and scion circumferences, and cumulative yield, whereas combinations without CEVd or CVd-III were not significantly different than the control. Reductions in growth and yield for multiple viroid infections occurred sooner than those obtained with single viroids. It seemed that the greater the number of co-infecting viroids, the more dramatic the effect on growth and yield.

With the factorial model, certain combinations of viroids were compared with combinations that did not have these viroids, using two different approaches for mean estimation which were either exclusive (i.e., no other viroid) or not. Concerning single-viroid

TABLE 6. Estimates of principal effects and interaction effects between two viroids on cumulative yield by the factorial model using two approaches for mean estimation and associated *F* statistics[a]

Treatment[b]	Mean[c]	Cumulative weight (kg/tree)[d]									
		1992	1993	1994	1995	1996	1997	1998	1999	2000	2001
1 CEVd	Total	–1.1*	–5.9***	–18.1***	–25.5***	–44.8***	–66.0***	–83.7***	–96.2***	–103.6***	–122.7***
	Treatment	1.1	–5.6	–18.8*	–20.8+	–34.1*	–54.5*	–77.6**	–108.2***	–121.9***	–171.3***
2 CBLVd	Total	–0.3	0.2	1.2	–0.8	–6.1	–10.2+	–12.0	–12.9	–14.0	–17.4+
	Treatment	1.2	–0.1	–2.3	–2.8	–6.2	–18.7	–43.7	–63.5*	–66.1+	–88.0*
3 HSVd	Total	1.0*	0.3	0.7	3.4	3.2	2.2	2.8	3.7	8.0	6.4
	Treatment	3.4	2.0	12.9	35.9**	55.9**	72.6**	69.5*	57.2	56.5	54.5
4 CVd-III	Total	–0.3	–2.1*	–9.9***	–17.0***	–35.9***	–51.4***	–60.4***	–70.9***	–81.0***	–90.2***
	Treatment	0.9	0.6	–8.3	–13.2	–31.4+	–45.1*	–55.4*	–78.0*	–87.3*	–115.4**
5 CVd-IV	Total	0.0	0.7	0.6	1.3	3.4	8.0	12.9+	16.6*	15.9+	14.8
	Treatment	0.9	5.3	6.8	15.6	21.2	33.3	27.6	27.6	35.1	38.4
6 CEVd/CBLVd	Total	–1.5	–3.0	–5.7	–4.8	–14.9+	–28.0*	–34.0*	–40.5*	–46.1*	–44.7*
	Treatment	–2.7	1.8	3.1	–3.3	–18.1	–13.6	7.3	34.9	44.9	80.4
7 CEVd/HSVd	Total	1.1	0.8	2.7	–0.4	–2.8	–10.1	–9.1	–10.4	–10.6	–11.6
	Treatment	–4.3	–3.3	–13.7	–40.3*	–65.3**	–82.9*	–74.1+	–44.2	–31.5	–15.5
8 CEVd/CVd-III	Total	–0.8	0.5	3.5	6.9	14.3+	15.5	12.8	15.7	16.6	36.0+
	Treatment	–2.5	–1.8	5.7	1.2	0.6	7.9	13.3	42.6	53.6	110.2+
9 CEVd/CVd-IV	Total	2.1*	4.9*	10.6*	17.9**	31.1***	44.0***	46.9**	52.7**	57.6**	66.7**
	Treatment	–2.2	–2.5	–5.2	–18.4	–32.6	–44.8	–35.3	–13.7	–5.7	13.5
10 CBLVd/HSVd	Total	1.0	3.0	–1.3	–3.6	–9.1	–11.3	–12.4	–9.2	–10.4	–3.8
	Treatment	–2.1	6.8	4.7	–12.9	–20.9	–19.0	8.4	45.9	61.2	87.4
11 CBLVd/CVd-III	Total	0.7	–1.4	–1.3	3.9	7.0	10.6	13.6	16.4	20.8	25.6
	Treatment	0.5	2.0	14.1	16.8	22.3	40.0	69.0+	105.2*	123.4*	168.2**
12 CBLVd/CVd-IV	Total	–0.8	–2.6	0.5	1.3	4.3	11.1	17.4	12.5	4.0	–3.2
	Treatment	–2.2	–3.2	4.5	–0.2	12.6	18.4	67.9+	91.0*	82.7	89.8
13 HSVd/CVd-III	Total	–1.1	0.3	–1.3	–1.6	–4.0	–5.1	–9.4	–15.6	–17.4	–25.0
	Treatment	–3.4	-5.3	–16.6	–39.7*	–62.8*	–84.5*	–83.5*	–63.9	–63.0	–64.6
14 HSVd/CVd-IV	Total	0.5	–1.0	–3.0	–7.6	–14.6+	–18.7	–22.3	–26.1	–21.4	–22.0
	Treatment	–3.3	–11.9*	–31.0**	–57.8***	–83.6***	–125.0***	–115.5**	–105.3*	–102.0*	–112.1+
15 CVd-III/CVd-IV	Total	0.9	0.3	1.9	1.8	1.1	–1.9	–9.1	–16.3	–21.1	–22.2
	Treatment	0.0	-5.8	–10.2	–19.0	–26.6	–51.2	–45.5	–44.3	–63.8	–69.7

[a] In a complete block design with 192 trees.

[b] CEVd = *Citrus exocortis viroid*, CBLVd = *Citrus bent leaf viroid*, HSVd = *Hop stunt viroid*, CVd-III = *Citrus viroid III*, and CVd-IV = *Citrus viroid IV*.

[c] Mean estimation. Total = effects estimated from equation 1 for single-viroid and from equation 3 for two-viroid treatments by contrast analysis of principal effects and interaction effects (=deviation from additivity) of rank 2. Treatment = effects estimated from equation 4 for single-viroid and from equation 6 for two-viroid treatments by contrast analysis of principal effects and interaction effects (=deviation from additivity) of rank 2.

[d] Level of significance: + = $0.05 < P < 0.10$; *, **, and *** = $P < 0.05$, 0.01, and 0.001, respectively.

infection, CEVd or CVd-III always gave a highly significant negative effect on scion circumference and cumulative yield whatever the presence or absence of additional viroids. The same was true for CBLVd, but with lower significance. HSVd and CVd-IV did not significantly affect growth and yield, except for HSVd on scion circumference in the presence of additional viroids.

Conclusions with CVd-IV varied according to the different mean approaches used in the factorial model analysis. A negative effect on scion circumference was significant only when comparing the CVd-IV-infected trees with the control, and no significance was observed with the total mean approach. Interaction effects with CVd-IV were observed for two-viroid (treatment 9) and four-viroid (treatment 28) combinations and may have masked a global effect of CVd-IV alone. Such interactions at a higher rank have to be taken into account to interpret discrepancies. For instance, CVd-IV had a significant negative effect on scion circumference when CEVd was absent, but not when it was present (data not shown). Thus, the limit of the factorial model is reached as interactions at higher ranks reveal a risk of erroneous conclusions in interpreting more global effects with one or several of those factors. What can be concluded for CVd-IV? Is there no effect, or are there some effects only in specific situations (without either CEVd or CVd-III)? The results obtained by the treatment mean approach suggest the latter possibility.

Examination of interaction effects should begin with the analysis of the highest rank interaction, then progressing down to the rank 1 interaction (i.e., principal effect). Additionally, only significant effects that lasted over years should be considered, as in the case of single-viroid infections or a few two-viroid interactions. Significant effects observed only in certain years (e.g., when the titer of viroids in the trees was still low) probably have no real biological meaning and could be an effect only of statistical hazard. No significant interaction was detected for the five-viroid combination and only a few exceptional significant interactions were found with the four- and three-viroid combinations for both scion circumference and cumulative yield. Hence, it seems difficult to give them some biological meaning. Considering two-viroid combinations, significant interactions could be found consistently only within a few treatments: CEVd and CVd-III, CEVd and CVd-IV, CEVd and CBLVd, and CBLVd and CVd-III for growth and yield; and, to a lesser extent, HSVd and CVd-IV for yield only. There was no additivity of the effects of each viroid over the years between pairs CEVd and CVd-III, CEVd and CVd-IV, and CBLVd and CVd-III, which could be interpreted as antagonism. A continuous increase of positive effects values between CEVd and CVd-III or CBLVd and CVd-III may indicate that this antagonistic effect became greater over the years, once the trees were fully systematically infected.

As previously shown (36), CVd-IV did not significantly affect growth and yield. However, the interaction between CEVd and CVd-IV is antagonistic. CVd-IV appeared to decrease or suppress the CEVd effect on growth, yield, and symptom expression. As suggested by Semantic and Vardoulakis (33), the antagonism observed between these two viroids supports the existence of a close biological relationship. Synergism was detected for two pairs of viroids. In trees co-infected only with CVd-IV and HSVd, synergism was observed between those viroids but the presence of additional viroids limited this synergistic effect. In contrast, synergistic effects between CEVd and CBLVd were favored by the presence of additional viroids.

In conclusion, a number of interactions among viroids have been identified. The most clear-cut interaction occurs between CEVd and CVd-IV, resulting in attenuation of bark-scaling or bark-cracking symptoms, suggesting antagonism between these two viroids. This also was supported by a limitation of the negative effects of CEVd on tree performance when CVd-IV was present. Synergistic effects also have been observed in certain viroid combinations that caused exocortis scaling symptoms in the absence of CEVd. The reduction of tree size and fruit yield occurred mainly in trees infected with combinations containing CEVd or CVd-III and, to a lesser extent, those containing CBLVd. However, such effects were stronger and appeared sooner in trees infected with multiple-viroid combinations rather than in trees infected with single viroids. However, their effects on tree performance were not additive, thus revealing an antagonism. Finally, it was shown that the nature of interaction between two viroids can be interpreted differently whether additional viroids are present or not.

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