Performance of Navelina Sweet Orange on Five Rootstocks Inoculated with Citrus Viroids

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ABSTRACT. A marked variation in tree size and bark scaling was observed on Navelina ISA 315 sweet orange infected by different viroids and grafted on Rubidoux trifoliate orange, Swingle CPB 4473 citrumelo, Carrizo CES 2863 and BA-300 citranges and Thomasville citrangequat rootstocks. Two-year-old rootstock seedlings were inoculated with a complex of three citrus viroids (CVds) and 6 mo later grafted with Navelina ISA 315 sweet orange. The trees were planted in 1994 and yield, fruit quality and size were evaluated every year. Bark scaling and gumming symptoms were also assessed on each rootstock. Each scion/rootstock combination inoculated with CVds showed a smaller tree size compared to the non-inoculated controls. The smallest canopy volume was observed in plants grafted on Rubidoux trifoliate orange and BA-300 citrange. Typical viroid symptoms (bark scaling, dieback and canopy yellowing) were observed on Rubidoux trifoliate orange, whereas BA-300 citrange and other rootstocks did not show any symptoms 10 yr after inoculation.

Index words. Tree size, bark scaling, yield, fruit quality.

The continuous spread of *Citrus tristeza virus* (CTV) in new citrus growing areas of the world, the new CTV foci found in Italy (3, 4) and the serious economic crisis of Italian citriculture, have pressed researchers to find new rootstocks to replace sour orange. These rootstocks must be resistant or tolerant to CTV and also increase fruit quality and production (9, 10).

About 200,000 Ha of citrus are grown in Italy, of which 90% is grafted on sour orange, with the remainder on Troyer citrange, Carrizo citrange, Volkamer lemon and Alemow. Sour orange is the rootstock best adapted to the environmental and soil conditions of Italy. However, it shows a high sensitivity to CTV and although the production is good, it never reaches the qualitative and quantitative standards of trees grafted on Troyer and Carrizo citranges. As an example, the production of Tarocco nuc. 57-1-E1 sweet orange grafted on citranges is larger (160-170 Kg/plant) than that of sour orange (100-120 Kg/plant) (9).

Numerous studies have been conducted recently on high density plantings, prompted by the possibility of such plantings to reduce the period of non-productivity and to increase the yield per unit area. Another advantage of high density planting is the potential reduction of canopy size. To date, the techniques used to achieve this purpose have been: (i) the use of dwarfing viroids; (ii) the use of interstocks selected from related genera of plants; (iii) the use of dwarfing rootstocks; and, (iv) the use of specific agronomic techniques (15). Earlier studies (1) have shown that Flying dragon trifoliate orange is the only available dwarfing rootstock capable of reducing growth by 75% as compared to standard rootstocks.

The goal of the present study was to verify the behavior of Navelina ISA 315 sweet orange grafted on five rootstocks infected with three sources of citrus viroids (CVds). In particular the effect of exocortis and other viroids, which are widely dispersed in Sicilian citrus orchards, was tested

on a selection called BA-300, which was selected from a citrange seedbed, and which is characterized by zig-zag growth similar to that of Flying dragon trifoliate orange (12).

MATERIALS AND METHODS

Plant material and viroid sources. The rootstocks used in this experiment were Rubidoux trifoliate orange, BA-300 and Carrizo CES 2863 citranges, Thomasville citrangequat and Swingle CPB 4473 citrumelo. All rootstocks were propagated from seeds derived from single tree sources which were free of detectable virus and virus-like pathogens.

Three isolates of citrus viroids (inocula 1, 2 and 3) were utilized in the trial. Inoculum 1 had been collected from a high density planting in Israel, whereas inocula 2 and 3 were collected respectively from Monreal and Comune Clementine trees growing in Sicily. They were tested by biological indexing on Etrog 861-S-1 citron and showed slightly different symptoms.

Inoculation and propagation procedures. The three inocula were graft transmitted in the nursery to 2-yr-old rootstock seedlings using bark tissue from the source trees. Three bark chips per tree were used, and a tree was considered infected when at last two bark chips remained alive 2 mo after inoculation.

Six mo after inoculation all rootstocks were grafted with a budstick of virus-free Navelina ISA 315 sweet orange recovered by *in vitro* culture of undeveloped ovules. The trees were transplanted to the field in a randomized block arrangement of four trees with five repetitions.

Data collection and statistical analysis. Tree size and fruit yield and quality were recorded annually. Fruit quality was determined at the same date each year by collecting ten representative fruits from each tree and using standard laboratory practices for analysis. Statistical analysis was carried out on mean values for three fruiting years. All data were subjected to simple variance analysis and the averages were compared with Tukey's test. The parameters analyzed were yield (Kg/tree), canopy volume (m^3, calculated according to Turrel's formula), fruit weight (g), juice content (% by weight), peel-thickness (mm), percentage total soluble solids (TSS), percentage total acidity (TA) and ripening ratio (TSS/TA). Observations on tree condition and bark scaling below the bud union were made periodically.

Identification of viroids in the inoculum sources. Ten years after inoculation the inoculum sources were graft transmitted to citron and three months after tissue from the inoculated citrons was subjected to nucleic acid analysis to identify the viroids present.

For viroid identification, citron tissues (equivalent to a combination of 5 g of fresh leaves and young stems) were homogenized in one volume of extraction medium (0.4 M Tris-HCl, pH 8.9, 1% SDS (w/v); 5 mM EDTA, pH 7.0, 4% mercaptoethanol (v/v)) and three volumes of water saturated phenol (14). The total nucleic acids were partitioned in 2 M LiCl and the soluble fraction was concentrated by ethanol precipitation and resuspended in TKM buffer (10 mM Tris-HCl, 10 mM KCl, 0.1 mM $MgCl_2$, pH 7.4).

For sequential polyacrylamide gel electrophoresis (sPAGE) analysis, aliquots (20 µl) of nucleic acid preparations (equivalent to 300 mg of fresh tissue) were first subjected to non-denaturing PAGE at 60 mA for 2.5 h and stained with ethidium bromide as previously described (7). A segment of the gel (comprising the region between the host 7S RNA and 1 cm above this) was excised and electrophoresed for 4 h on a second gel (containing 8 M urea) at 16 mA (13, 11). The viroid bands were viewed by silver staining (5).

To confirm the identity of viroids based on their expected mobility

under sPAGE, aliquots (10 µl) of the same nucleic acid preparations (equivalent to 150 mg of fresh weight tissue) were subjected to slot-blot hybridization analysis. The samples were pretreated in 6× SSC and 8% formaldehyde for 15 min at 60°C and blotted onto positively charged Nylon membranes (Boehringer Mannheim) using a Hybri-slot filtration manifold (BRL), immobilized by UV crosslinking and hybridized to DIG-labeled probes specific for *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd) (ex CVd-I), *Hop stunt viroid* (HSVd, ex CVd-II), *Citrus viroid III* (CVd-III) and *Citrus viroid IV* (CVd-IV). DIG-labeled DNA probes were synthesized by PCR amplification of cloned viroid sequences as described (8). Prehybridization and hybridization were carried out in 50% formamide and 6× SSPE as described by Maniatis et al. (6). The membranes were prehybridized at 42°C for 2-4 h and hybridized overnight at 50°C. After hybridization, membranes were washed twice in 2× SSC containing 0.1% SDS (w/v) for 60 min at 60°C. The DIG-labeled hybrids were detected with an anti-DIG-alkaline phosphatase conjugate (Fab fragments, Roche Diagnostics GmbH, Mannheim, Germany) and visualized with the chemiluminescent substrate CSPD (Roche Diagnostics).

RESULTS

Effect on tree growth, yield and fruit quality. Measurements of canopy volume, yield and fruit weight of Navelina ISA 315 sweet orange grafted on Rubidoux trifoliate orange, BA-300 and Carrizo citranges, Thomasville citrangequat and Swingle citrumelo infected with citrus viroids are reported in Table 1.

All the inoculum sources caused a marked reduction of canopy volume compared to the non-inoculated control plants, except for trees grafted on Thomasville citrangequat (Table 1). The most marked effect was observed in trees grafted on 'Rubidoux' trifoliate orange, on which all the inoculum sources caused significant reductions of canopy volume (70% for inocula 1 and 2; 77% for inoculum 3), annual yield (70% for inoculm 1; 62% for inocula 2 and 3) and cumulative yield (72% for inoculum 1; 63% for inocula 2 and 3) with no significant differences among the three inoculum sources assayed (Table 1). The reduction of canopy volume was less marked in trees grafted on BA-300 and Carrizo citranges. All the inoculum sources induced a significant effect in trees grafted on BA-300 citrange, but in trees grafted on Carrizo citrange only inocula 2 and 3 caused a significant effect. Yield and cumulative yield were also affected but only inoculum 3 caused a consistent and significant effect on trees grafted on BA-300 citrange. Trees grafted on Thomasville citrangequat remain generally unaffected, with inoculum 2 causing a significant effect on yield. Trees grafted on Swingle citrumelo were only slightly affected with inocula 2 and 3 causing a significant reduction on canopy volume and yield (Table 1).

Statistical differences in fruit weight were not constant during the trial. The only consistent effect was observed in fruits from trees grafted on Carrizo citrange inoculated with inoculum 3 which were significantly smaller than those of the non-inoculated controls (Table 1). Fruit quality was only slightly affected (Table 2). Fruits from trees grafted on 'Rubidoux' trifoliate orange contained less juice than the uninoculated control, with inoculum 3 being the only one than induced a statistically significant effect. Fruits from trees grafted on BA-300 citrange had similar (inoculum 1) or lower TA (inocula 2 and 3) than the controls, the difference being statistically significant only in the case of inoculum 2. These fruits presented similar (inocula 2 and 3) or lower (inoculum 1) ripening ratios relative to the controls.

TABLE 1
VIROID EFFECTS ON THE GROWTH AND YIELD OF 'NAVELINA ISA 315' SWEET ORANGE GRAFTED ON FIVE ROOTSTOCKS

Rootstock	Inoculum	Cumulative yield (Kg)	Yield (Kg/tree)			Canopy volume (m^3)		Average fruit weight (g)		
			2002	2003	2004	2003	2004	2002	2003	2004
'Rubidoux' trifoliate orange	1	45.03 A*	9.77 A	14.03 A	21.20 A	1.88 A	2.35 A	204.2 ns	216.1 B	180.8 A
	2	60.48 A	17.70 A	15.72 A	27.06 A	2.15 A	2.43 A	201.0 ns	177.8 A	208.4 AB
	3	60.95 A	18.56 A	16.64 A	26.68 A	1.93 A	1.81 A	197.3 ns	189.3 AB	197.3 AB
	uninoculated	162.63 B	43.59 B	48.45 B	70.59 B	6.34 B	8.00 B	189.2 ns	189.4 AB	215.5 B
'BA-300' citrange	1	83.15 AB	24.18 A	26.23 AB	32.74 AB	3.10 B	3.19 A	160.8 A	174.8 ns	195.0 ns
	2	87.28 BC	26.02 AB	28.91 AB	32.29 AB	2.67 AB	3.27 A	205.9 BC	146.8 ns	193.4 ns
	3	61.09 A	17.78 A	19.66 A	23.03 A	1.76 A	2.02 A	208.7 C	193.0 ns	184.2 ns
	uninoculated	111.52 C	36.06 B	31.43 B	44.04 B	4.41 C	4.72 B	177.8 AB	154.6 ns	190.2 ns
'Carrizo' citrange	1	131.70 B	35.19 ns**	39.83 B	56.67 ns	5.65 BC	6.71 BC	188.5 AB	170.5 B	206.6 AB
	2	108.98 AB	30.18 ns	33.04 AB	45.76 ns	4.28 AB	5.29 AB	192.3 B	187.3 B	192.6 AB
	3	95.37 A	29.17 ns	24.62 A	42.59 ns	3.65 A	4.45 A	166.8 A	144.5 A	177.0 A
	uninoculated	138.47 B	36.93 ns	43.50 B	58.02 ns	7.39 C	8.39 C	198.7 B	172.9 B	216.2 B
'Thomasville' citrangequat	1	91.78 AB	26.32 ns	27.92 AB	37.53 AB	3.55 AB	4.35 ns	178.0 A	163.2 AB	192.4 AB
	2	87.74 A	25.07 ns	29.36 AB	33.31 A	3.10 A	4.00 ns	200.8 B	179.6 B	181.1 A
	3	90.25 AB	26.32 ns	26.00 A	37.93 AB	3.44 AB	4.19 ns	177.2 A	149.6 A	191.3 AB
	uninoculated	110.51 B	29.85 ns	35.08 B	45.58 B	4.11 B	5.06 ns	187.9 A	178.6 B	211.3 B
'Swingle' citrumelo	1	200.25 B	53.24 B	60.66 B	86.35 B	7.61 BC	10.47 BC	189.1 B	170.2 A	216.4 B
	2	146.68 A	36.90 A	45.25 A	64.51 A	5.52 A	7.16 A	171.7 A	172.9 AB	179.4 A
	3	158.73 A	43.58 AB	48.60 AB	66.66 A	6.04 AB	8.16 AB	184.4 AB	161.4 A	198.3 AB
	uninoculated	193.73 B	45.98 AB	58.96 B	88.22 B	9.11 C	12.05 C	173.4 A	196.7 B	215.5 B

*Capital letters apply to P = 0,01; **not significant.

TABLE 2
VIROID EFFECTS ON FRUIT QUALITY (MEAN VALUES FOR THREE FRUITING YEARS, 2002-2004) OF 'NAVELINA ISA 315' SWEET ORANGE GRAFTED ON FIVE ROOTSTOCKS

Rootstock	Inoculum	Juice (%)	Peel thickness (mm)	TSS (%)[z]	TA (%)[y]	TSS/TA[x]
'Rubidoux' trifoliate orange	1	45.71 AB*	5.02 ns	10.51 A	0.67 ns	15.82 ns
	2	45.85 AB	4.94 ns	11.42 B	0.68 ns	17.08 ns
	3	42.96 A	5.17 ns	11.03 AB	0.66 ns	16.89 ns
	uninoculated	46.10 B	5.05 ns	11.18 AB	0.68 ns	16.86 ns
'BA-300' citrange	1	43.47 ns**	4.84 ns	11.33 ns	0.76 A	15.25 B
	2	45.09 ns	5.17 ns	11.06 ns	0.61 B	18.46 A
	3	46.56 ns	5.03 ns	11.17 ns	0.65 AB	17.54 A
	uninoculated	44.13 ns	5.09 ns	11.72 ns	0.73 A	17.22 A
'Carrizo' citrange	1	46.27 ns	4.64 ns	11.08 ns	0.69 ns	16.64 ns
	2	43.56 ns	4.96 ns	11.13 ns	0.60 ns	19.21 ns
	3	45.58 ns	4.61 ns	11.35 ns	0.73 ns	16.12 ns
	uninoculated	46.48 ns	4.55 ns	11.54 ns	0.80 ns	15.48 ns
'Thomasville' citrangequat	1	45.57 ns	4.66 ns	11.38 ns	0.77 ns	15.36 ns
	2	45.39 ns	4.89 ns	10.94 ns	0.66 ns	16.77 ns
	3	46.24 ns	5.00 ns	11.07 ns	0.70 ns	16.44 ns
	uninoculated	45.87 ns	4.78 ns	10.74 ns	0.70 ns	16.04 ns
'Swingle' citrumelo	1	48.21 A	4.97 ns	10.60 ns	0.73 ns	15.00 ns
	2	43.81 B	5.11 ns	11.29 ns	0.74 ns	15.91 ns
	3	46.19 AB	4.74 ns	10.72 ns	0.69 ns	15.84 ns
	uninoculated	46.79 AB	4.85 ns	10.75 ns	0.79 ns	14.30 ns

[z]TSS = Percentage total soluble solids.
[y]TA = Percentage total acidity.
[x]TSS/TA = Ripening ratio.
*Capital letters apply to P = 0.01; **not significant.

Viroids present in the inoculum sources. No trees showed viroid symptoms two years after inoculation. The following year two trees grafted on Rubidoux trifoliate orange showed symptoms of bark scaling on the rootstock and dieback and severe yellowing of the leaves, characteristic of the exocortis disease (Fig. 2). After 10 yr all the trees grafted on Rubidoux trifoliate orange showed symptoms with leaf yellowing more evident in the summer. The other rootstocks did not show any symptoms 12 yr after inoculation (Fig. 3).

sPAGE analysis showed that the three inoculum sources contained viroid-like RNAs with electrophoretic mobilities of the circular forms of viroids (Fig. 1). The identity of the bands with those of CEVd, CBLVd, HSVd and CVd-III controls was confirmed by slot-blot hybridization (data not shown). As expected, inoculum 2 gave a positive signal with CEVd, HSVd and CVd-III probes and inoculum 3 with CEVd, CBLVd, HSVd and CVd-III probes. Although the presence of

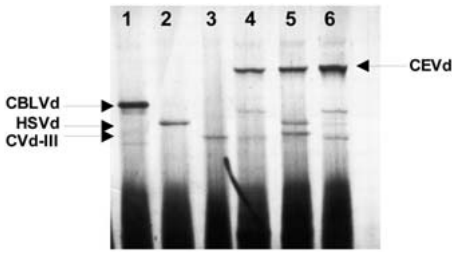

Fig. 1. sPAGE analysis of citrus viroids. Lanes 1-3 show the positions of CBLVd, HSVd and CVd-III respectively. Lanes 4-6 show positions of viroids extracted from citrons inoculated with inoculum sources 1, 2 and 3 respectively.

Fig. 2. Symptoms on 'Rubidoux' trifoliate orange inoculated with a citrus viroid complex (inoculum 3). A) Severe bark scaling symptoms, and B) yellowing of leaves.

HSVd in inoculum 2 was not perceptible in sPAGE, it gave a positive signal with CEVd, CBLVd, HSVd and CVd-III probes. None of the inoculum sources was positive for CVd-IV. It should be noted that the intensity and mobility of HSVd in inoculum 2 differs from that of HSVd in inoculum 3. Similarly, CVd-III in inoculum 2 presents a lower mobility than CVd-III in inocula 1 and 3. These differences suggest that they differ in size and/or nucleotide composition.

DISCUSSION

As the results showed, the three viroid isolates tested reduced significantly the canopy volume of the inoculated plants. Reduced development was mainly observed on plants grafted on Rubidoux trifoliate orange, BA-300 and Carrizo citranges. The reduction of canopy size correlated with reduced yield, which was most significant in trees grafted on 'Rubidoux' trifoliate orange. Whereas the trees on Rubidoux trifoliate orange were similarly affected regardless of the inoculm source used, the effect on those grafted on BA-300 and Carrizo citranges varied depending on the inoculum source. This observation indicates that viroid sources

Fig. 3. Trunk of BA300 citrange rootstock inoculated with a citrus viroid complex (inoculum 3). No bark scaling symptoms are visible on this rootstock.

must be adequately tested on specific scion/rootstock combinations before being used as dwarfing agents.

The fruit quality parameters did not show major differences among the rootstocks and inoculum sources used in this trial. Some indication of adverse effects with some inoculum sources were observed in the juice content of fruits from trees grafted on Rubidoux trifoliate orange Swingle citrumelo and in total acid and ripening ratio of fruits from trees grafted on 'BA-300' citrange. The average fruit weight of plants, which is directly correlated to yield and number of fruit per plant, was not consistently influenced by the three different viroid complexes. Only in the case of trees grafted on Carrizo citrange did inoculum 3 cause a significant reduction of average fruit weight. In general, the effect on fruit quality and average fruit weight was not consistently observed and should be further study before introducing any type of inoculum for commercial dwarfing.

It should be noted that the plants grafted on Rubidoux trifoliate orange showed, starting the third year after planting, severe symptoms of decline as indicated by reduction in canopy volume, severe bark scaling and yellowing of leaves caused by CEVd, whereas trees on the other rootstocks did not show any symptoms. In spite of the absence of bark scaling symptoms, plants grafted on BA-300 and citrange Carrizo were remarkably dwarfed. Therefore, these rootstocks may be used for high density planting since viroid infection influenced the tree size without causing any negative effect on plant longevity. The lower productivity could be balanced by the higher density of trees planted. Similar results have been obtained in other researches (2).

The results reported here illustrate the dwarfing effect of citrus viroids on sweet orange grafted in different rootstocks. The effect depended on the rootstock and the inoculum source. The assay was initiated before the inoculum sources had been characterized and further research is needed to understand the role of each viroid on the observed effects. Additionally, HSVd and CVd-III in inoculum 2 presents differences in intensity and mobility suggesting differences in their nucleotide composition, an issue that should be investigated further.

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LITERATURE CITED

1. Bitters, W. P., D. A. Cole, and C. D. McCarty
1979. Facts about dwarf citrus trees. Citrograph 64: 54-56.
2. Caruso, A., S. Davino, M. Guardo, G. Sorrentino, and M. Davino
2005. Performance of "Comune" Clementine infected with exocortis disease on seven rootstocks. In: *Proc. 16th Conf. IOCV*, 320-324. IOCV, Riverside, CA.
3. Davino, M. and G. Terranova
1999. Il virus della "tristezza degli agrumi" e i criteri di prevenzione e controllo. Frutticoltura 61 (1): 18-25.
4. Davino, M., M. Barba, A. Caruso, S. Davino, M. Guardo, A. D'Onghia, and V. Savino
2004. Citrus tristeza (CTV): a serious threat to the Italian citrus groves. Proc. Int. Soc. Citricult. (in press).
5. Igloi G. L.
1983. A silver stain for the detection of nanogram amounts of tRNA following two dimensional electrophoresis. Analyt. Biochem.134: 184-188.
6. Maniatis, T., E. F. Fritsch and J. Sambrook
1989. *Molecular Cloning: A Laboratory Manual, 2nd ed.* Cold Spring Harbor Laboratory, NY.

7. Morris, T. J., and N. S. Wright
1975. Detection by polyacrylamide gel of a diagnostic nucleic acid from tissue infected with potato spindle tuber viroid. Amer. Potato J. 52: 57-63.
8. Palacio, A., X. Foissac and N. Duran-Vila
2000. Indexing of citrus viroids by imprint hybridization. Eur. J. Plant Pathol. 105: 897-903.
9. Reforgiato-Recupero, G. and M. Davino
1988. I portinnesti degli agrumi alla luce delle più recenti acquisizioni. In: I portinnesti delle piante da frutto. Ferrara, 15-16 dicembre 1988: 253-258.
10. Reforgiato-Recupero, G., A. Caruso, and G. Russo
1992. The Flying Dragon trifoliate orange and BA-300 citrange: Effects on scion performance. Proc. Int. Soc. Citriculture 1: 286-290.
11. Rivera-Bustamante, R., R. Gin, and J. S. Semancik
1986. Enhanced resolution of circular and linear molecular forms of viroid and viroid-like RNA by electrophoresis in a discontinuous pH system. Analyt. Biochem. 156: 91-95.
12. Russo, F. and A. Starrantino
1988. The citrange mutants discovered in Italy. Proc. Int. Soc. Citriculture 1: 201-205.
13. Sänger, H. L., K. Ramm, H. Domdey, H. J. Gross, K. Henco, D. Riesner
1979. Conversion of circular viroid molecules to linear strands. FEBS Lett. 99: 117-121.
14. Semancik, J. S., T. J. Morris, L. G. Weathers, G. F. Rordorf, and D. R. Kearns
1975. Physical properties of a minimal infectious RNA (viroid) associated with the exocortis disease. Virology 63: 160-167.
15. Semancik, J. S.
2003. Considerations for the introduction of viroids for economic advantage. In: *Viroids*, A. Hadidi, R. Flores, J. W. Randles and J. S. Semancik (eds.), 357-362. CSIRO, Australia.