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RESEARCH PAPERS

Viroid infection and rootstocks affect productivity and fruit quality of the Tunisian citrus cultivar Maltaise demi sanguine

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Summary. In Tunisia, sweet orange citrus cultivars are usually grafted on sour orange rootstock. However, this rootstock is susceptible to *Citrus tristeza virus* (CTV). A trial was established in 2005 to evaluate the performance of newly introduced rootstocks compared to sour orange, using the sweet orange cultivar 'Maltaise demi sanguine' as the grafted scion. The effect of single or mixed viroid infections were monitored over 12 years. Once established, tree growth, cumulative yield, tree performance and fruit quality of 'Maltaise sweet orange' were assessed from 2008. Mixed viroid infections caused significant decreases (39 to 60%) in the canopy volume of 'Maltaise' grafted on Carrizo citrange, Swingle citrumelo, Cleopatra mandarin, Rangpur lime, Volkamer lemon and Trifoliate orange. The cumulative yield of trees grafted on'Alemow' (*Citrus macrophylla*) and inoculated with *Hop stunt viroid* (HSVd) was 76% less than the healthy control. Mixed infections caused production decreases of 56% from trees grafted on Swingle citrumelo and 69% from those grafeted on Trifoliate orange. When quality parameters of fruit from trees infected with each viroid, and combinations of viroids were compared, no significant differences were recorded.

Key words: vegetative growth, yield, canopy, decrease.

Introduction

The genus *Citrus* of the family Rutaceae includes several species producing fruit such as oranges, mandarins, limes, lemons, sour oranges, and grapefruits. *Citrus* is one of the most important horticultural crops, with worldwide fruit production of over 110 million tons per year.

In Tunisia, citrus is a traditional crop with cultivation covering about 24,000 ha and annual production is over 350,000 t (DGPA, 2016). Production is for fresh fruit, food industry and export. Sour orange (*Citrus aurantium* L.) is the most common rootstock used in Tunisia (about 95%), which is well adapted to calcareous and other common soil types, and is widely used in the Mediterranean basin (Yildirim *et al.,* 2010). However, sour orange has the disadvantage of high susceptibility to *Citrus tristeza virus* (CTV), the agent of Tristeza disease, and this limits the use of this rootstock in many citrus growing countries. Following this problem, researchers and citrus growers devoted efforts to find alternative rootstocks. Selection of suitable rootstocks to control CTV has been successful in many citrus growing countries, but in some cases sensitivity to other diseases has been reported, such as those caused by viroids (Roistacher, 1983). Viroids are small, circular single stranded-RNA pathogens that replicate in their hosts, causing diseases of economic importance. Eight viroids have been reported to infect *Citrus* spp., and these belong to four genera of the fam-

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ily Pospiviridae (Duran Vila and Semancik, 2003; Serra *et al.*, 2008; Chambers *et al.*, 2017): *Citrus exocortis viroid* (CEVd), *Citrus bent leaf viroid* (CBLVd), *Hop stunt viroid* (HSVd), *Citrus dwarfing viroid* (CDVd), *Citrus bark cracking viroid* (CBCVd), *Citrus viroid V* (CVd-V), *Citrus viroid VI* (CVd-VI) and *Citrus viroid VII* (CVd-VII). CVd-VI has only been reported in Japan (Ito *et al.* 2003) and CVd-VII only in Australia (Chambers *et al.*, 2017). Viroids were identified and found to be widespread in citrus growing areas of Tunisia (Najar and Duran-Vila, 2004; Najar *et al.*, 2005; Hamdi *et al.*, 2015). However, some promising rootstocks that give rootstock/scion combinations tolerant to CTV, including Carrizo citrange (*C. sinensis* x *Poncirus trifoliata* (L.) Raf.) and Swingle citrumelo (*C. paradisi* × *P. trifoliata*), are known to be sensitive to viroid infections. Investigating possible Tristeza disease prevention, a rootstock trial was implemented in 2005 with the aim to compare the performance of the locally important cultivar Maltaise demi sanguine sweet orange grafted on newly introduced rootstocks with that of the commonly used sour orange. The trial was carried out within a period of 12 years, after inoculation with the most important viroids in single or mixed infections.

Materials and methods

Viroid sources

Single viroid sources CEVd, CBLVd, HSVd, CDVd and CBCVd were selected from the viroid collection maintained at National Institute of Agricultural Research of Tunisia (INRAT). These had been characterized from survey conducted in 2001 in Tunisian citrus orchards (Najar and Duran-Vila, 2004). Each source had been maintained in Etrog citron plants grafted on Volkamer lemon.

Plant material and viroid inoculation

The rootstocks selected for the trial included sour orange (SO) sensitive to CTV but tolerant to viroids, Alemow (*C. macrophylla* Webster) (CM) sensitive to CTV stem pitting and cachexia disease, and six CTV tolerant rootstocks [Carrizo citrange (CC), Volkamer lemon (*C. Volkameriana* Ten. and Pasq.) (CV), Cleopatra mandarin (*C. reshni* Hort. ex Tan.) (MCL), Swingle citrumelo (Citru), Rangpur lime (*C. limonia* Osb.) (LR) and trifoliate orange (*Poncirus trifoliata* Raf.) (PT). Two of these rootstocks (CV and MCL) are considered to be viroid tolerant, three (CC, Citru and PT) are considered to be sensitive to CEVd but tolerant to the cachexia, and one (LR) is considered sensitive to both CEVd and cachexia.

In March 2003, buds of certified material of pre-basic category of "Maltaise demi-sanguine", that tested negative for the graft-transmissible citrus diseases (CTV, Citrus psorosis virus, Concave gum, Blind pocket, Impietratura, Stubborn and viroids) were grafted on seedlings of the eight rootstocks (species or hybrids) and inoculated under glasshouse conditions with five viroid treatments (single: CEVd, HSVd(variant CVd-IIb), CBCVd and/or mixed infections: CEVd+CBCVd, CBLVd+HSVd+CDVd+CBCVd), or left as viroid-free non-inoculated controls. Six plants per rootstock and inoculation treatment were used, giving a total of 288 plants. In June 2005, the young trees were transplanted, in a field plot of the INRAT Gobba station (Cap Bon region), in a randomized block design field trial. The field plot was protected against unwanted intrusions by means of a wire fence. An initial pruning operation was conducted in 2007 to shape the trees. Since 2011, the trees were pruned annually as in commercial orchards, to develop open canopies. During these operations, tools were disinfected with a sodium hypochlorite solution from one tree to the next. Fertilization and pesticide treatments were carried out following normal procedures in the region. Irrigation was applied by a microjet system. All plants were tested for viroids detection 6 months after inoculation to verify pre-transplanting positive viroid transmission to the inoculated plants. In the 2010-2011 season, all the trees were tested again to verify that they remained infected with the viroid isolates, and that the non-inoculated controls remained viroid-free.

Viroid indexing

Viroid indexing was performed using Etrog citron Arizona 861-S-1 (*Citrus medica* L.) grafted on Volkamer lemon (*C. jambhiri* Lush.) as the biological indicator species. These plants were maintained in a greenhouse under controlled temperature conditions (28–32°C) (Roistacher *et al.*, 1977). Two to 6 months after inoculation, nucleic acid preparations from the inoculated citrons were obtained following the nucleic acid extraction protocol described by Semancik *et al*. (1975). Nucleic acids recovered from 5 g of bark tissue were resuspended in 300 µL TKM buffer (10 mM Tris, 10 mM KCl, 0.1 mM $MgCl_2$, pH 7.4), and 20 µL of the purified nucleic acids were analyzed by sequential polyacrylamide gel electrophoresis (sPAGE) and silver staining (Duran-Vila *et al*., 1993).

To confirm the identity of the viroid bands observed by sPAGE analysis, the nucleic acid preparations were further subjected to either dot-blot or slot-blot hybridization, using viroid-specific probes for CEVd, CBLVd, HSVd, CDVd and CBCVd. Ten µL of nucleic acid preparations as well as the corresponding controls were denatured with 7.4% formaldehyde in 6 × SPPE (3 M NaCl, 0.2 M NaH_2PO_4, 0.02 M EDTA) at 60°C for 15 min and loaded on positively-charged nylon membranes, which were further hybridized with digoxigenin (DIG)-labeled specific probes generated by PCR. The DIG-labelled hybrids were detected with an anti-DIG-alkaline phosphatase antibody, and visualized with the CSPD chemiluminescent substrate (Roche Diagnostics) (Palacio *et al*., 2000).

Tree growth

Tree height (m) was measured in 2015 after fruit harvesting. Canopy volume (m^3) was estimated from the canopy tree radius (m) and height (m), assuming that the canopy was ellipsoidal.

Estimation of cumulative fruit yield

Fruit were manually harvested each season from 2008, usually during the first week of March when the ratio of sugar content/acidity was greater than 7.0. The fruit from each tree was weighed and recorded. Average cumulative fruit yield was evaluated annually until 2015.

Organoleptic parameters

To assess fruit quality, ten fruit were collected from the four sides of the canopy of all the trees of each treatment. Orange juice samples were extracted from the fruit, filtered to remove pulp and seeds, and stored in labeled plastic tubes at 4°C until processing.

Juice yield (%)

The juice content, expressed as percentage of total mass, was calculated according to the formula:

$$\text{Juice yield} = \frac{\text{Extract juice weight}}{\text{Total weight of the fruit extracts}} \times 100$$

Juice pH

The pH of the juices was measured using a digital pH meter at 27ºC.

Total soluble solids (TSS)

Total soluble solids were determined using an Abbe refractometer, and corrected to the equivalent reading at 20ºC (AOAC, 1990), density (d20), using the densimetric method, and were expressed as percent according to the formula:

$$\text{TSS} = \frac{\text{L ref} \times \text{P jus (g)}}{100 \times \text{V jus (mL)}}$$

L ref: reading at the refractometer (°Brix)
P jus (g): weight of the fruit juice
V jus (ml): volume of the juice

Total acids (TA)

Total acids were determined by titrating the anhydrous citric acid, which is the dominant acid in orange juice. The base used for titration was 0.1 N sodium hydroxide (NaOH) containing a few drops of phenolphthalein indicator. TA of each juice was determined according to the formula:

$$\text{TA} = \frac{\text{Nb} \times \text{Vb X M}}{\text{Va} \times \text{P}}$$

Va: volume of the juice (10 ml)
Vb: volume (in ml) of NaOH
Nb: normality of 0.1 N NaOH solution
M: molar mass of citric acid = 192g
P: number of protons (H^+) in citric acid

Statistical analyses

Data were analyzed by ANOVA using the GENSTAT program, and mean comparisons were made by the Duncan's multiple range test at $\alpha = 0.05$.

Results

Vegetative growth tree height

Data from non-inoculated plants showed rootstock effects on the Maltaise tree heights. The tallest trees were those grafted on Volkamer lemon (mean = 2.8 m) followed by Cleopatra mandarin, sour orange and Rangpur lime. The effect on this parameter was significant (*P*<0.05) for Swingle citrumelo, Carrizo citrange, Alemow and trifoliate orange, for which the mean tree heights were, respectively 20, 21, 27 and 36% less than that for Volkamer lemon (Figure 1).

Single viroid infection

CEVd effects were observed mainly on trees grafted on trifoliate orange, with a significant tree size reduction of 25% compared to the non-inoculated controls. HSVd affected both trifoliate orange and Alemow, with tree height reduced, respectively, by 26 and 33%. CBCVd gave no significant effect in any of the scion/rootstock combinations.

Mixed viroid infections

A marked effect (*P*<0.05) on tree height was observed especially from the multiple viroid infection treatment (CBLVd + HSVd + CDVd + CBCVd). The mean decrease in height reached 21% for trees grafted on Cleopatra mandarin, 23% on Volkamer lemon and 31% on trifoliate orange. There were also non-significant reductions observed in trees grafted on Carrizo citrange (17%) and Swingle citrumelo (19%).

Canopy volume

Statistical analysis showed significant effect (*P*<0.05) of the rootstock on canopy volumes. The Maltaise cultivar grafted on Volkamer lemon had the greatest canopy volume (20.3 m^3), followed by trees on Cleopatra mandarin and Rangpur lime. The sour orange was part of an homogeneous group together with Carrizo citrange, Alemow and Swingle citrumelo (Figure 2).

Single viroid infection

CEVd induced significant reductions in mean canopy volume of trees grafted on Volkamer lemon (33%) and trifoliate orange (49%) compared to the controls. Rangpur lime and Cleopatra mandarin did not show statistically significant decreases in canopy volumes (respectively, 28 and 25%). The most rele-

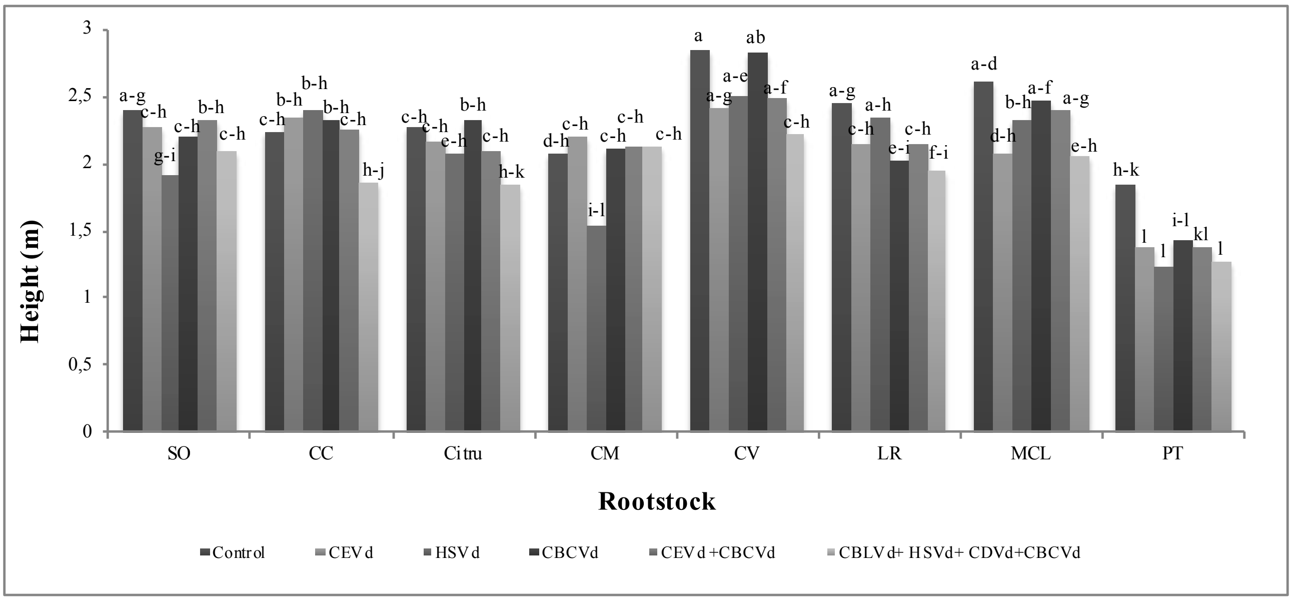

Figure 1. Mean heights (m) of trees of 'Maltaise demi sanguine' sweet orange propagated on eight rootstocks; Cleopatra' mandarin (MCL), *Trifoliate orange* (PT), *Swingle citrumelo* (Citru), 'Rangpur' lime (LR), Volkamer lemon(CV), Sour orange (SO), Alemow (CM) and Carrizo citrange (CC) non-inoculated (control), or inoculated with; *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), *Citrus bent leaf viroid* (CBLVd), *Citrus dwarfing viroid* (CDVd) and *Citrus bark cracking viroid* (CB-CVd) in single or mixed infections. Means followed by the same letters are not significantly different at α= 0.05.

vant effect of the cachexia inducing variant of HSVd (CVd-IIb) was observed in trees grafted on Alemow, which showed a mean reduction of 77%. The other two rootstocks adversely affected by this viroid were Volkamer lemon (30% reduction) and trifoliate orange (45%. No statistically significantly effect was registered in the case of CBCVd.

Mixed viroid infections

A significant decrease (P<0.05) around 35 and 50% of the canopy volume was recorded respectively in the case of trees grafted on Volkamer lemon and trifoliate orange infected with CEVd + CBCVd. The cumulative effect induced by multiple infection with four viroids (CBLVd + HSVd + CDVd + CBCVd) was the reduction of the canopy volume of cv. Maltaise grafted on most rootstocks. This reduction was rather relevant, ranging from 40 to 60% for Carrizo citrange, Swingle citrumelo, Cleopatra mandarin, Rangpur lime, Volkamer lemon and trifoliate orange (Figure 2).

Cumulative fruit yields

Mean cumulative yields from trees grafted on different rootstocks infected with citrus viroids are shown in Figure 3. Based on the data collected over the years, the non-inoculated trees differed significantly (P<0.05) for the rootstock effects. Based on the average data, four rootstock types could be differentiated. The first group, including Volkamer lemon, Alemow and Rangpur lime, gave the most productive trees (average mean yield greater than 176 kg per tree). In the second group the yields from trees grafted on Swingle citrumelo and Carrizo citrange gave similar and intermediate yields (respectively, 155 and 132 kg per tree). The third group were trees on sour orange and Cleopatra mandarin, which produced average yields of 100 kg per tree. The fourth group were trees on trifoliate orange, which grew slowly and fruited poorly as compared to the other rootstock/scion combinations. 'Maltaise demi sanguine' grafted on Volkamer lemon, Rangpur lime and Alemow produced significantly (P<0.05) greater higher yields, so these rootstocks produced approx. twice the yield of those for trees grafted on sour orange, which is the most common rootstock used in Tunisia.

Single viroid infection

Aside from trifoliate orange (with 46% yield loss), CBCVd did not give significant yield effects

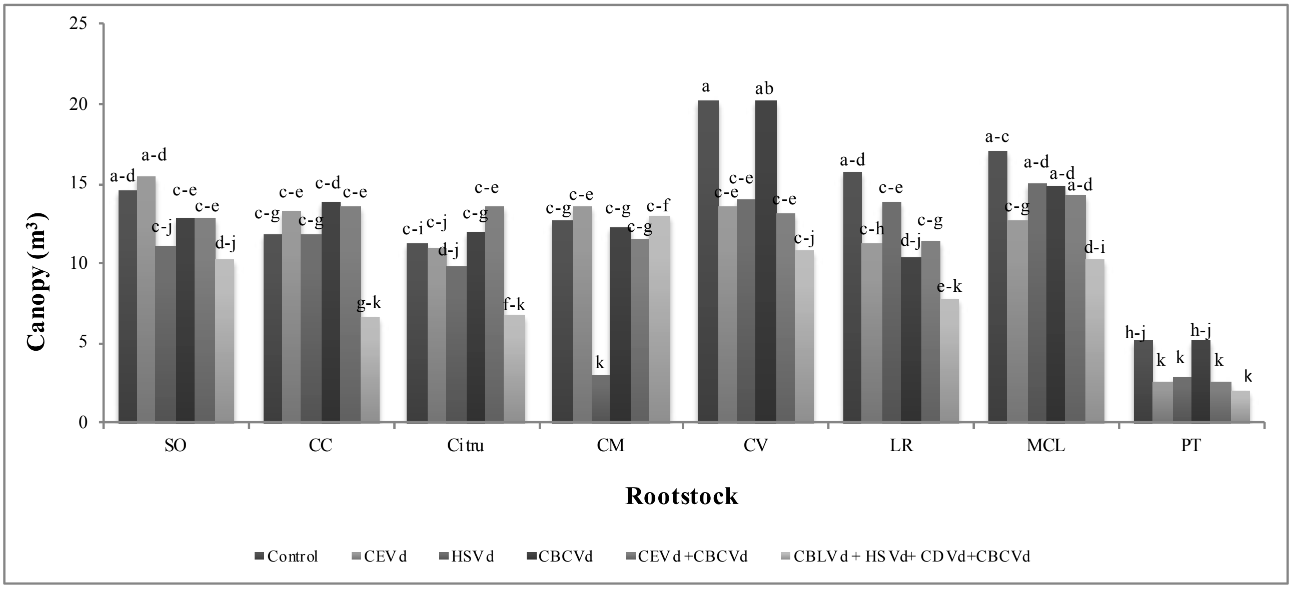

Figure 2. Mean canopy volumes (m³) of 'Maltaise demi sanguine' sweet orange trees propagated on eight rootstocks; Cleopatra' mandarin (MCL), *Trifoliate orange* (PT), *Swingle citrumelo (*Citru), 'Rangpur' lime (LR), Volkamer lemon(CV), Sour orange (SO), Alemow (CM) and Carrizo citrange (CC) non-inoculated (control), or inoculated with; *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), *Citrus bent leaf viroid* (CBLVd), *Citrus dwarfing viroid* (CDVd) and *Citrus bark cracking viroid* (CBCVd) in single or mixed infections. Means followed by the same letters are not significantly different at α= 0.05.

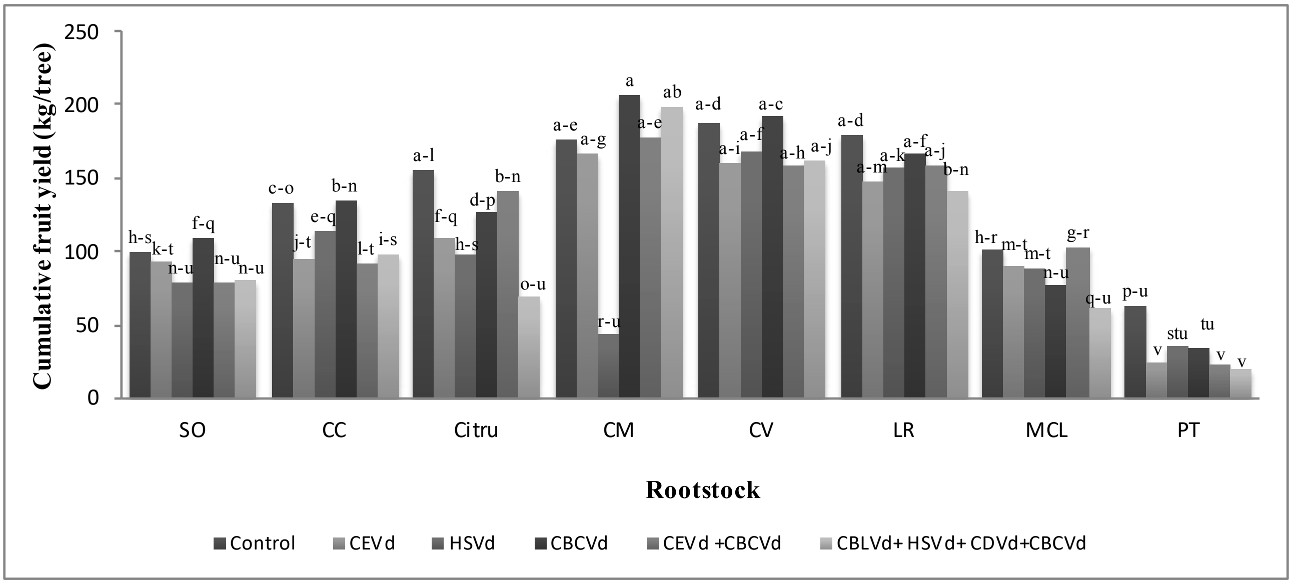

Figure 3. Mean cumulative yields (kg per tree) of 'Maltaise demi sanguine' sweet orange trees propagated on eight rootstocks; Cleopatra' mandarin (MCL), *Trifoliate orange* (PT), *Swingle citrumelo (*Citru), 'Rangpur' lime (LR), Volkamer lemon(CV), Sour orange (SO), Alemow (CM) and Carrizo citrange (CC) non-inoculated (control), or inoculated with; *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), *Citrus bent leaf viroid* (CBLVd), *Citrus dwarfing viroid* (CDVd) and *Citrus bark cracking viroid* (CBCVd) in single or mixed infections. Means followed by the same letters are not significantly different at $\alpha = 0.05$.

from trees grafted on the other rootstocks. CEVd and HSVd did not affect yields from trees grafted on sour orange, Volkamer lemon, Rangpur lime and Cleopatra mandarin (Figure 3), even though the yield decreases were approx. 20%. The mean cumulative yield of trees grafted on Alemow infected with HSVd were significantly less (76%) than that of the control. This viroid also reduced the production of trees grafted on Swingle citrumelo (36%) and trifoliate orange (66%). CBCVd did not significantly affect yield.

Mixed viroid infections

The cumulative yields of trees grafted on trifoliate orange were significantly less (63%) than the control in the case of the CEVd + CBCVd infection. The combination of four viroids (CEVd + HSVd + CDVd + CBCVd) showed that Swingle citrumelo (56% reduction) and trifoliate orange (69%) were particularly susceptible, Alemow, Volkamer lemon, Rangpur lime and Carrizo citrange were not significantly different from the controls, illustrating the tolerance of these rootstocks to mixed viroid infection (Figure 3). Although the differences were not statistically significant, the infection with these four viroids reduced the cumulative yield of trees grafted on Cleopatra mandarin by 29% compared to the non-inoculated controls (Figure 3).

Organoleptic parameters

Juice yield

For the viroid-free controls, the new rootstocks did not significantly affect the juice yields compared to trees grafted on sour orange (Figure 4). Similarly, viroid infections did not affect the juice yields of trees grafted on the majority of the rootstocks tested. Only trifoliate orange affected juice production, for the multiple-viroid infections CEVd + CBCVd, which decreased mean juice yield by 16%.

pH of juice

The statistical analysis of juice pH measurements showed no statistically significant differences among fruits collected from trees grafted on the different rootstocks (Figure 5). Viroid infections did not affect

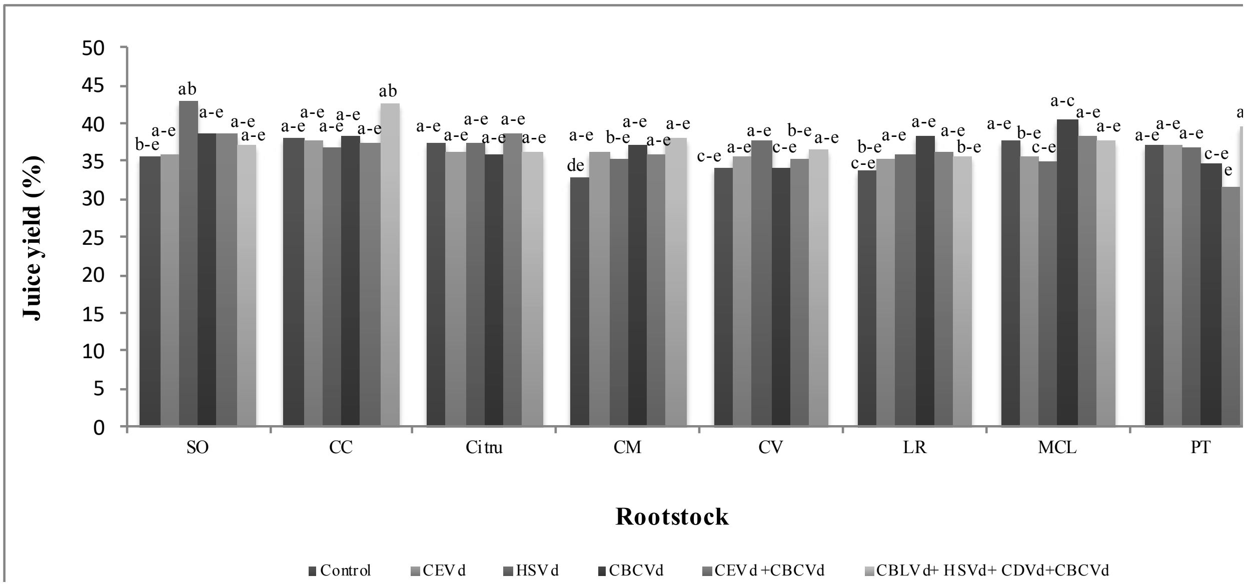

Figure 4. Mean juice yields (%) of fruits from 'Maltaise demi sanguine' sweet orange grafted on eight rootstocks; Cleopatra' mandarin (MCL), *Trifoliate orange* (PT), *Swingle citrumelo* (Citru), 'Rangpur' lime (LR), Volkamer lemon(CV), Sour orange (SO), Alemow (CM) and Carrizo citrange (CC) non-inoculated (control), or inoculated with; *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), *Citrus bent leaf viroid* (CBLVd), *Citrus dwarfing viroid* (CDVd) and *Citrus bark cracking viroid* (CBCVd) in single or mixed infections. Means followed by the same letters are not significantly different at α= 0.05.

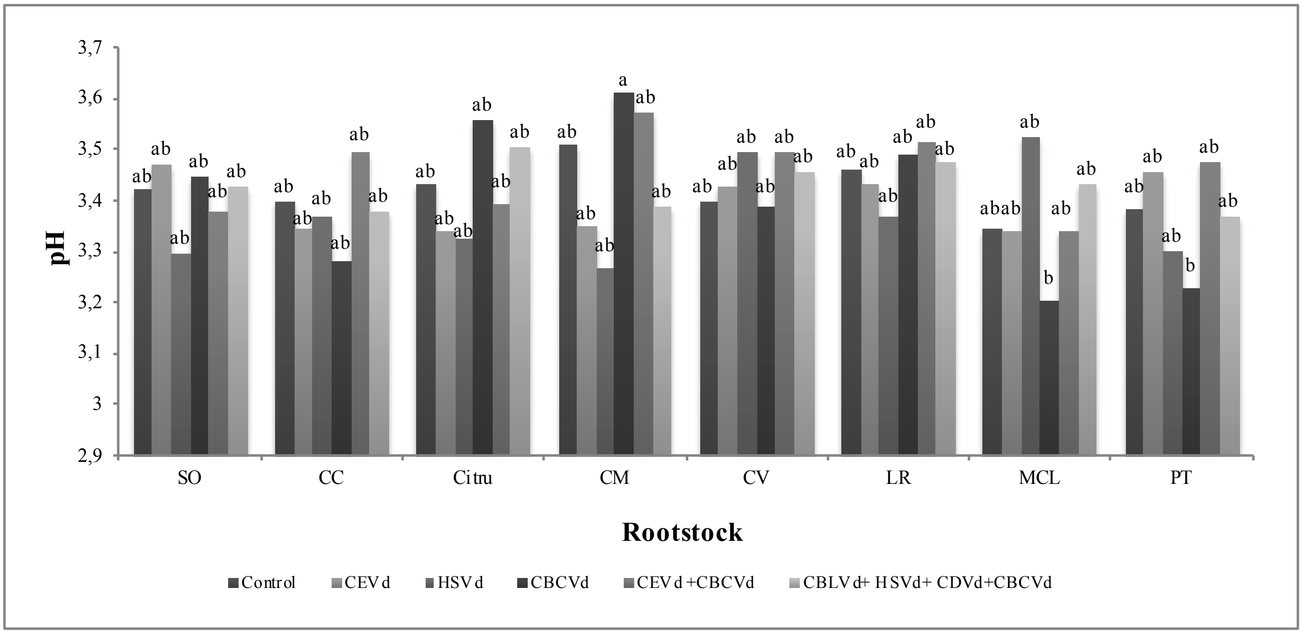

Figure 5. Mean pHs of fruits from 'Maltaise demi sanguine' sweet orange trees propagated on eight rootstocks; Cleopatra' mandarin (MCL), *Trifoliate orange* (PT), *Swingle citrumelo* (Citru), 'Rangpur' lime (LR), Volkamer lemon(CV), Sour orange (SO), Alemow (CM) and Carrizo citrange (CC) non-inoculated (control), or inoculated with; *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), *Citrus bent leaf viroid* (CBLVd), *Citrus dwarfing viroid* (CDVd) and *Citrus bark cracking viroid* (CBCVd) in single or mixed infections. Means followed by the same letters are not significantly different at α= 0.05.

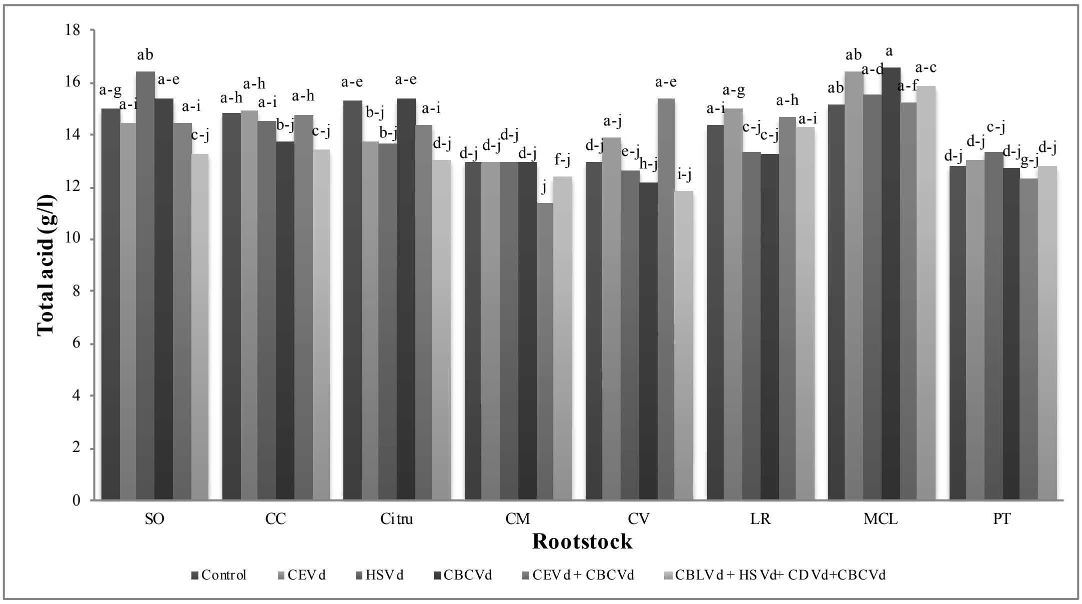

Figure 6. Mean total acid contents (g L^{-1}) of juice from fruits from 'Maltaise demi sanguine' sweet orange trees propagated on eight rootstocks; Cleopatra' mandarin (MCL), *Trifoliate orange* (PT), *Swingle citrumelo* (Citru), 'Rangpur' lime (LR), Volkamer lemon(CV), Sour orange (SO), Alemow (CM) and Carrizo citrange (CC) non-inoculated (control), or inoculated with; *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), *Citrus bent leaf viroid* (CBLVd), *Citrus dwarfing viroid* (CDVd) and *Citrus bark cracking viroid* (CBCVd) in single or mixed infections. Means followed by the same letters are not significantly different at α= 0.05.

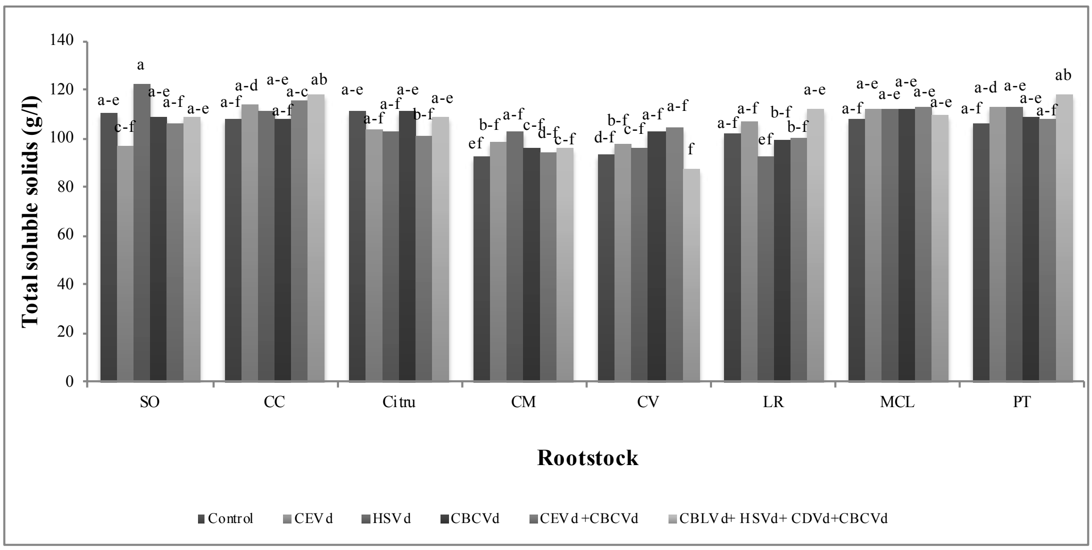

Figure 7. Mean total soluble solids (g L^{-1}) of the juice from fruits from of 'Maltaise demi sanguine' sweet orange trees propagated on eight rootstocks; Cleopatra' mandarin (MCL), *Trifoliate orange* (PT), *Swingle citrumelo* (Citru), 'Rangpur' lime (LR), Volkamer lemon(CV), Sour orange (SO), Alemow (CM) and Carrizo citrange (CC) non-inoculated (control), or inoculated with; *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), *Citrus bent leaf viroid* (CBLVd), *Citrus dwarfing viroid* (CDVd) and *Citrus bark cracking viroid* (CBCVd) in single or mixed infections. Means followed by the same letters are not significantly different at α= 0.05.

mean juice pH, which ranged from 2.94 to 3.83. The fruit collected from trees grafted on Alemow had the greatest mean pH value of 3.83.

Total acid

Total acid of citrus juice is an important factor of the overall juice quality, as well as a determinant for harvesting time in most citrus producing countries. In this study, the statistical analysis showed that the acidity of the juice of Maltaise fruits was not significantly (P<0.05) influenced by viroid infections. However, mean TA was affected by the different rootstocks (Figure 6), being greatest for Maltaise grafted on Swingle citrumelo (15.3) followed by Cleopatra mandarin, sour orange, Carrizo citrange and Rangpur lime. The lowest TA was from fruit from trees grafted on Alemow, Volkamer lemon and trifoliate orange.

Total soluble solids

In the absence of viroid infection, fruit from trees grafted on Alemow and Volkamer lemon had the lowest levels of total soluble solid (TSS), of respectively, 92.3 g L^{-1} and 94 g L^{-1} (Figure 7). In the case of fruit from trees grafted on other rootstocks, the values were similar and approx 110 g L^{-1}.

There was a non-significant decrease (13%) of TSS for fruit from trees grafted on sour orange and inoculated with CEVd, compared to the non-inoculated controls. A similar result (10% reduction) was recorded for fruit from trees grafted on Rangpur lime infected with HSVd, and from trees grafted on Volkamer lemon as affected by multiple-viroid infections (CBLVd+ HSVd+ CDVd+CBCVd) (6% reduction).

Discussion

Vegetative growth

Results of a 10 year field experiment have demonstrated effects of rootstock and viroid infection on citrus tree height and canopy volume.

In the case of non-inoculated rootstocks, the greatest yield and growth effects were observed for trees grafted on Volkamer lemon and the least were from those grafted on trifoliate orange. The remaining rootstocks studied showed similar/comparable effects to each other. These results agree with the findings of Bassal (2009), who reported that Marisol clementin trees grafted on sour orange, Carrizo citrange and Swingle citrumelo had similar heights and canopy volumes. Filho *et al.* (2007) also found that canopy volumes of Sunburst mandarin trees grafted on Cleopatra Mandarin and Swingle citrumelo were comparable. Our results also confirm the sensitivity of trifoliate orange to soils with alkaline pH (Loussert, 1989), as is generally the case in the Cap Bon region of Tunisia.

In the case of viroid infections, CEVd and HSVd caused slight reductions in tree size when the trees were grafted on Cleopatra Mandarin, Swingle citrumelo, Volkamer lemon, sour orange and Rangpur lime. However, for trees grafted on trifoliate orange, tree size and canopy volume were significantly reduced compared to the non-inoculated trees. These results agree with those reported by Vernière *et al.* (2004; 2006), who observed severe stunting of clementine trees grafted on the trifoliate orange rootstock. The greatest effect resulting from HSVd infection was observed on tree height and canopy volume of trees grafted on Alemow, which were significantly smaller and in a state of general decline. The sensitivity of Alemow was reported by Aubert and Vullin (1998).

Infection with CBCVd as a single viroid infection did not significantly affect growth and yield of any of the scion/rootstock combinations tested. This is in agreement with the study of trifoliate orange conducted in Corsica (France) by Verniére *et al*. (2004), but contradicts the results of Murcia *et al*., (2015) from Valencia (Spain). These authors recorded strong vegetative growth and yield reduction effects from CBCVd. This could be explained by the climatic conditions that are warmer in Valencia compared to the Cap bon region in Tunisia or to Corsica.

Under mixed viroid infections, trees infected by four viroids (CBLVd + HSVd + CDVd + CBCVd) showed large reductions in canopy volume for trees grafted on Carrizo citrange, Volkamer lemon, Rangpur lime, Cleopatra mandarin or trifoliate orange. Effects of multiple infection were greater than from single or double viroid infections. This suggests that synergism among the four viroids may cause important reductions of canopy volume. As reported by Verniére *et al.* (2006) for clementine trees grafted on trifoliate orange, the greater the number of co-infecting viroids, the greater were the effects on tree growth. In the present study, the statistical analyses demonstrated that trees infected with the two viroids CEVd + CBCVd were smaller than the non-inoculated controls, particularly for canopy volume, when they were grafted on Volkamer lemon or trifoliate orange. CBCVd did not decrease or suppress the effect of CEVd, because the same level of stunting was also recorded from single infection with CEVd. This agrees with results obtained in California, South Africa, and Italy, that showed that CEVd-infected sources reduced vegetative growth of trees grafted on trifoliate orange and Troyer citrange when compared with non-infected control trees (Polizzi *et al.*, 1991; VanVuuren and Da Graça, 1997).

Cumulative yields

Non-inoculated trees were more productive when grafted on Volkamer lemon, Alemow or Rangpur lime. This result was consistent with those of several studies conducted in different citrus growing regions, where cumulative yields were greater from trees grafted on Volkamer lemon, Rangpur lime or Alemow than those grafted on sour orange, Swingle citrumelo, Carrizo citrange or Cleopatra mandarin (Fallahi *et al.*, 1989; Monteverde *et al.*, 1989; Zekri 2000; Al-Jaleel and Zekri, 2003 Ramin and Alirezanezhad, 2005; Shafieizargar *et al.*, 2013).

Results from the present study indicate that under the growing conditions of Tunisian citriculture, viroid infections did not affect the cumulative yields of trees grafted on Volkamer lemon, Alemow, Rangpur lime or sour orange. For trees grafted on the other rootstocks tested, the measured cumulative yields showed specific tendencies, as follows: For trees grafted on Alemow, the only effect was due to infection with HSVd, which reduced cumulative yield by more than 70%. In contrast, deviation from a cumulative effect was observed in the case of the combinations of HSVd with CBLVd, CDVd and CBCVd, which showed significant antagonism. The cumulative yields from mixed-infected trees were almost similar to those from the non-inoculated trees. This indicates there may be cross protection effects or interference among the different viroid species, as has been previously reported by Niblett *et al.* (1978) and Semancik *et al.* (1992).

Trees grafted on Carrizo citrange infected with CEVd or viroid mixtures showed yield reductions of approx. 30% compared to non-inoculated trees, although these differences were not statistically significant. Similar results were obtained by Bani Hashemian *et al.* (2009), who reported that the cumulative yields of infected sweet orange trees were 67% of that of non-infected trees. In the present study, Swingle citrumelo rootstock was very susceptible, particularly to the mixed viroid infection of CBLVd + HSVd + CDVd + CBCVd, which induced a significant cumulative yield reduction of 55% as compared to the non-inoculated controls. To our knowledge, this is the first record of a viroid infection affecting yield of Swingle citrumelo.

Infected trees on trifoliate orange showed large reductions of productivity of approx. 45% for HSVd and CBCVd infections, and nearly 65% in for trees infected with CEVd or with either of the two combinations citrus viroids that were assayed. These reductions were comparable to the canopy reductions reported previously (Broadbent *et al.*,1991; Stuchi *et al.*, 2007). Similarly, Vernière *et al.* (2004) reported that losses of cumulative yields of clementine grafted on trifoliate orange were 48% in the case of trees infected with two CEVd isolates and 34% in the case of trees infected with one of the cachexia variants of HSVd (CV-IIc).

Fruit quality

Significant rootstock effects ($P<0.05$) on TA of cv. Maltaise were determined in non-inoculated trees. TA was greatest in the fruits from trees grafted on Cleopatra mandarin. Verdu (1993) reported that fruits of Clemenules mandarin on Cleopatra mandarin rootstock had greater acidity than those on sour orange. The lowest TA found in the present study was from fruits from trees grafted on Volkamer lemon. These results are in agreement with previous reports (Stover *et al*. 2004; Ramin and Alirezanezhad, 2005), in which the same trend was observed for Volkamer lemon related the TSS parameter, but the differences were not significant.

Under viroid infections, only differences in soluble solids content were found in the fruit from trees grafted on Volkamer lemon and infected with the mixed viroid combination of CBLVd + HSVd + CDVd + CBCVd).

In summary, results from the present study indicate that under the growing conditions in which the experiment was performed, viroid infections reduced height and canopy volume of inoculated trees without reducing yields or fruit quality, particularly when the high producing Volkamer lemon or Rangpur lime were used as rootstocks.

Trees grafted on Swingle citrumelo and Carrizo citrange registered lower performance, mainly affected by combination viroid infection. In the field citrus trees are usually exposed to several viroids, as previously reported (Malfitano *et al*., 2005; Mohamed *et al*., 2009; Najar *et al*., 2017), and mixed viroid infections have been shown to affect the field performance of Nules clementin and Navelina sweet orange grafted on Carrizo citrange (Bani Hashemian *et al*., 2009).

According to our results, use of Volkamer lemon and Rangpur lime are the most promising rootstocks for the main Tunisian citrus cultivar Maltaise demi sanguine, as a strategy to combat the deleterious effects of diseases caused by viruses and viroids.

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