Preliminary Evaluation of the Sensitivity of Alemow Rootstock to *Citrus tristeza virus* in Spain

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ABSTRACT. *Citrus tristeza virus* (CTV) epidemics in Spain caused decline and death of trees propagated on sour orange rootstock. This situation forced progressive replacement of sour orange by tristeza-tolerant rootstocks, mainly citranges and Cleopatra mandarin. However, problems encountered with these rootstocks in soils with high salinity or lime content recently triggered the use of alemow. This rootstock has good tolerance to both abiotic stresses and induces early bearing, high productivity and excellent fruit size, particularly in mandarins, but it is sensitive to CTV stem pitting. Inoculation of alemow seedlings in the greenhouse showed that CTV isolate T385 induced very mild symptoms whereas isolates T402 and T405 induced stem pitting and stunting. Groups of nine CTV-free plants of Nova mandarin propagated on alemow rootstock were planted in a field plot covered with an insect-proof screenhouse to avoid aphid-inoculation with uncontrolled CTV sources. One group was left as non-inoculated control and the others were graft-inoculated with isolates T385, T402 or T405 at planting time, or after 1, 2 or 3 yr. Five years after planting, plants inoculated with T385 were similar in size to the non-inoculated controls, whereas plants inoculated with T402 or T405 showed average growth reductions that ranged from 19.2 to 33.7% in height, from 8.6 to 31.6% in trunk circumference, and from 39.9 to 62.2% in canopy volume. Size reduction was generally more important in early than in late infections. These results suggest that: i) in areas with high inoculum pressure and moderate to severe CTV strains, plantings on alemow rootstock may grow poorly, and ii) nursery plants on this rootstock should be produced under plastic houses with strict aphid control.

In 1959 *Citrus tristeza virus* (CTV) became epidemic in Spain and since then it has caused decline and death of more than 40 million trees propagated on sour orange rootstock (5). This situation forced progressive replacement of sour orange by tristeza-tolerant rootstocks, mainly citranges and Cleopatra mandarin (6, 7). Presently, more than 90% of the Spanish citrus industry comes from certified pathogen-free budwood propagated on those tolerant rootstocks (13). However, the new rootstocks are not as tolerant to pathogens and abiotic stresses as sour orange was. Particularly, citranges do not perform well in heavy soils and are sensitive to high lime or salt content and also to soil fungi (*Phytophthora* spp. and *Armillaria mellea*), and Cleopatra mandarin induces slow bearing and is also sensitive to soil-borne fungi. These problems associated with the new rootstocks recently have triggered the use of alemow as an alternative rootstock. This rootstock shows good tolerance to high lime and salinity and induces early bearing, high productivity and excellent fruit size, particularly in mandarins, but it is sensitive to CTV stem pitting (6, 16). In 2003-2004, about 14% of the 4.5 million citrus plants produced by authorized citrus nurseries were propagated on alemow. The philosophy behind the increasing use of this CTV-sensitive rootstock is that, even if citrus trees are severely affected by the virus and have to be replanted after 12-15 yr, the high economic returns derived from early bearing (fruit set usually starts the second or third year after planting), heavy fruit set, and marketable fruit size, would pay back the investment. However, available

data on field performance of trees propagated on alemow under high CTV pressure are scarce and essentially restricted to lemons (2, 3). Also, since experiments were performed in the open field, the characteristics of the CTV isolates infecting different plants could not be controlled.

Here we have compared the effects of three CTV isolates of known pathogenicity characteristics on plants propagated on alemow rootstock, grown in an insect-proof screenhouse and graft-inoculated at different plant ages.

MATERIALS AND METHODS

Virus isolates and inoculations. The CTV isolates T385, T402 and T405 used in this study belong to the IVIA collection and were obtained from infected field trees in Orihuela, Alicante (T385) (12), or from the Ribera Alta del Júcar area in Valencia (T402 and T405) (10). They were aphid transmitted to free them from other graft transmissible pathogens and are maintained in an insect proof screenhouse. Isolate T385 is asymptomatic in most host species and only causes faint vein clearing in Mexican lime, and T402 and T405 cause moderate vein clearing, stem pitting and stunting in Mexican lime and alemow, but not seedling yellows or stem pitting in grapefruit or sweet orange.

Ninety-nine buds of Nova mandarin were propagated on alemow seedlings grown in the greenhouse and transplanted 10 months later to a field plot completely covered with an insect-proof screen, carefully sealed and provided with double doors to avoid insect entry. The plants were arranged in nine rows separated by 4.25 m, with trees planted 3.40 m apart within each row. The following 11 treatments were used: 1-2) inoculation with isolates T385 or T405 at year 0 (3 mo before planting in the field), 3-8) inoculation with isolates T385, T402 or T405 at years 1 or 2 after planting, 9-10) inoculation with T385 or T405 at year 3 after planting, and 11) non-inoculated control. CTV infection was checked by direct tissue print ELISA (8) using a mixture of monoclonal antibodies 3DF1 and 3CA5 (4, 17). Each treatment was composed of nine plants, one in each row.

Fertilizer was applied by a drip system and pests, particularly aphids, were strictly controlled by careful inspection of individual plants twice a week and spraying with insecticides when necessary.

Growth measurement. Plant growth was evaluated by measuring trunk circumference, tree height and canopy volume. Trunk circumference was the average of two measurements taken with a metric tape 5 cm above and below the bud union. Tree height and canopy diameter were measured with a 3 m-long telescopic rule, the latter value being the average of two perpendicular measurements along and across rows. The canopy volume was estimated assuming that tree shape could be assimilated to a cylinder plus a semi-sphere and using the formula:

$$V = 0.7854 \times D2 \times (H - 0.1667 \times D)$$

where V = the estimated canopy volume, D = mean canopy diameter and H = tree height (1). For each parameter and treatment, data were averaged and expressed as the mean value and as the percentage relative to the mean value obtained for the corresponding parameter in the control treatment.

Statistical analyses. Data were subjected to three different statistical analyses. First, considering all 11 treatments, a one-way analysis of variance followed by a comparison with the control using the Dunnett test was conducted. Then, disregarding the control, the remaining 10 treatments were considered as a two factor (three isolates and 4 yr) factorial design with two missing cells. One analysis had all three isolates

but only years 1 and 2, and another analysis included all years for the T385 and T405 isolates. Given that, in some cases, the isolate by year interaction was significant, the analysis within isolates and within years was also calculated. Contrast among years was obtained by comparing successive years.

RESULTS

Tree growth was evaluated the fifth year after planting (2 yr after inoculating the last group of plants) and the results are summarized in Tables 1 to 3. Statistical analyses showed significant differences ($p < 0.0001$) between treatments for the three parameters measured. When compared with the control, the effect of CTV on growth parameters depended upon the isolate inoculated and the year of inoculation. Values for tree height (Table 1), trunk circumference (Table 2) or canopy volume (Table 3) in treatments involving isolate T385 did not significantly differ from the corresponding values in non-inoculated controls. Conversely, a significant reduction of these measurements was observed in trees infected with T402 or T405.

Comparison among isolates showed that growth of plants infected with T385 was significantly greater than that of plants infected with T405 or with T402 for all years and traits analyzed. The effects of inoculation with T402 or with T405 on the three growth parameters did not differ significantly, except for trunk circumference (Table 2) of plants inoculated in year 2, in which size reduction caused by T402 was greater than that caused by T405.

The age of the plant at inoculation time was not important for tree growth of plants infected with either T385 (four years data) or T402 (two years data), but it had significant effects on the growth of trees infected with T405 (Tables 1-3). Growth reduction caused by T405 was not significantly different between plants inoculated in years 2 or 3, nor between plants inoculated in years 0 or 1, except for trunk circumference in the latter case (Table 2). The most dramatic effects on growth were observed between trees inoculated two years after planting, and trees inoculated at time of planting or one year after planting. This trend suggests that tree size is most affected if infection occurs within the first 2 yr after planting, whereas later infections may have a more moderate effect on tree growth.

In addition to size reduction, CTV infection affected the appearance of Nova trees on alemow rootstock. Table 4 summarizes results of an

TABLE 1
AVERAGE TREE HEIGHT IN METERS (H) OF NOVA MANDARIN TREES PROPAGATED ON ALEMOW ROOTSTOCK EVALUATED 5 YR AFTER PLANTING AND INOCULATION AT THE INDICATED TIMES WITH THREE ISOLATES OF *CITRUS TRISTEZA VIRUS*

	Inoculation year after planting.							
	Year 0		Year 1		Year 2		Year 3	
Isolate	H[x]	%[y]	H	%	H	%	H	%
Non-inoculated	2.61 a	100.0	—	—	—	—	—	—
T385	2.51 aA	96.2	2.50 aA	95.8	2.46 aA	94.3	2.37 aA	90.8
T402	—	—	1.91 bA	73.2	2.06 bA	78.9	—	—
T405	1.73 bA	66.3	1.83 bA	70.1	2.11 bB	80.8	2.08 bB	79.7

[x]Values of H in the same column followed by the same lower case letter, or in the same row followed by the same capital letter, are not significantly different ($P < 0.05$).
[y]Percentage of H calculated for uninoculated control trees.

TABLE 2
AVERAGE TRUNK CIRCUMFERENCE IN CENTIMETERS (TC) OF NOVA MANDARIN TREES PROPAGATED ON ALEMOW ROOTSTOCK EVALUATED 5 YR AFTER PLANTING AND INOCULATION AT THE INDICATED TIMES WITH THREE ISOLATES OF *CITRUS TRISTEZA VIRUS*

	Inoculation year after planting.							
	Year 0		Year 1		Year 2		Year 3	
Isolate	TC[x]	%[y]	TC	%	TC	%	TC	%
Non-inoculated	20.9 a	100.0	—	—	—	—	—	—
T385	18.9 aA	90.4	20.1 aA	96.2	20.5 aA	98.1	20.2 aA	96.7
T402	—	—	16.2 bA	77.5	16.7 bA	79.9	—	—
T405	14.3 bA	68.4	16.2 bB	77.5	18.5 cC	88.5	19.1 bC	91.4

[x]Values of TC in the same column followed by the same lower case letter, or in the same row followed by the same capital letter, are not significantly different ($P < 0.05$).
[y]Percentage of TC calculated for uninoculated control trees.

evaluation of the foliage density carried out by two persons using a 0 to 3 scale (0, no foliage, and 3, normal dense foliage). Again, the foliage of non-inoculated controls and of trees infected with isolate T385 was essentially indistinguishable, whereas trees infected with T402 or T405 had thinner foliage. The effect of the two latter isolates was similar.

DISCUSSION

CTV epidemics in Spain forced the creation of a mandatory certification program based on the use of virus-free budwood propagated on CTV tolerant rootstocks (13). The use of CTV-sensitive rootstocks such as sour orange or alemow in new plantings was forbidden, except for propagation of lemons. Thirty years after starting the certification program, a substantial percentage of lemon trees have been grown on alemow rootstock without apparent damage from CTV, contrasting with the experience in other countries where this rootstock/scion combination was seriously affected by local CTV strains (2). Several reasons may have contributed to this situation: i) common CTV strains in Spain are relatively mild, ii) the incidence and rate of spread of CTV is low in traditional lemon growing

TABLE 3
AVERAGE CANOPY VOLUME IN CUBIC METERS (CV) OF NOVA MANDARIN TREES PROPAGATED ON ALEMOW ROOTSTOCK EVALUATED 5 YR AFTER PLANTING AND INOCULATION AT THE INDICATED TIMES WITH THREE ISOLATES OF *CITRUS TRISTEZA VIRUS*

	Inoculation year after planting.							
	Year 0		Year 1		Year 2		Year 3	
Isolate	CV[x]	%[y]	CV	%	CV	%	CV	%
Non-inoculated	9.462 a	100.0	—	—	—	—	—	—
T385	8.549 aA	90.4	8.862 aA	93.7	8.808 aA	93.1	7.808 aA	82.5
T402	—	—	4.515 bA	47.7	4.963 bA	52.4	—	—
T405	3.581 bA	37.8	3.824 bA	40.4	5.686 bB	60.1	5.372 bB	56.8

[x]Values of CV in the same column followed by the same lower case letter, or in the same row followed by the same capital letter, are not significantly different ($P < 0.05$).
[y]Percentage of CV calculated for uninoculated control trees.

TABLE 4
FOLIAGE DENSITY[z] OF NOVA MANDARIN TREES PROPAGATED ON ALEMOW ROOTSTOCK EVALUATED 5 YR AFTER PLANTING AND INOCULATION AT THE INDICATED TIMES WITH THREE ISOLATES OF CTV

	CTV isolate			
Inoculation year	Non-inoculated control	T385	T405	T402
0	2.9	2.6	1.1	—
1	2.9	2.8	1.4	1.9
2	2.9	2.9	1.4	1.3
3	2.9	3.0	1.6	—

[z]Estimated in a 0 to 3 scale (0 = no foliage and 3 = dense foliage). Each value is the mean of two independent observations of nine individual trees.

areas in Spain (9), and iii) aphid transmission of CTV to lemon plants is usually very inefficient (11, 15). The excellent performance of lemon trees on alemow rootstock in Spain, even on sites surrounded by CTV foci, and the problems and limitations of citrange and Cleopatra mandarin rootstocks in many soils have fostered the increasing use of alemow as rootstock for sweet orange and mandarins. However, while accumulation of mild or moderate CTV strains in grafted lemons is usually low, these strains may accumulate to high levels in sweet oranges and mandarins, with the potential risk of damage to alemow rootstock limiting development of the root system.

In our experiments we observed that the effects of CTV on Nova mandarin trees propagated on alemow were isolate dependent. Thus, while size and foliage density of trees infected with the isolate T385 were essentially indistinguishable from those of non-inoculated controls, trees infected with isolates T402 or T405 were clearly stunted and showed thin foliage, indicating unthrifty growth. In previous experiments in the greenhouse, alemow seedlings graft-inoculated with T385 showed mild vein clearing or remained symptomless, whereas seedlings inoculated with T402 or T405 showed moderate to severe stunting, vein clearing and stem pitting. T402 and T405 originated from two adjacent locations in Southern Valencia where tristeza first appeared, and likely represent a common CTV type, suggesting a potential risk for using alemow rootstock in this particular area.

The CTV effect on tree size was also dependent on the plant age at time of inoculation, size reduction being more dramatic with earlier infections. This effect was observed only in plants inoculated with T405, since T402, the other isolate causing stunting and thin foliage, was inoculated only in years 1 and 2. No significant difference was observed between trees inoculated in years 0 and 1, or between trees inoculated in years 2 and 3, but size reduction was clearly more dramatic in the groups of trees inoculated at a younger age than in those inoculated when they were older. These data suggest that the most severe effects are caused by CTV infection within the first two years after bud propagation, whereas infections at later ages have a less pronounced effect. Further observations will be necessary to assess potential effects on production and/or fruit quality.

Previous studies on the effect of CTV on trees propagated on alemow rootstock were carried out in the open field, without any control of aphid transmission of CTV strains, and these studies were restricted to lemons (2, 3). Our experiment was performed with Nova mandarin, using virus-free budwood inoculated

with biologically characterized CTV isolates, and it was planted in an insect-proof screenhouse with careful pest control to avoid contamination with unknown CTV sources. The results obtained indicate that the use of alemow as rootstock for mandarins in Spain may have risks in certain locations, since the stunting effects will depend on the local CTV isolates and on the age at which infection occurs. In areas like the Ribera Alta del Júcar, where CTV incidence is close to 100% and common isolates cause moderate to severe symptoms on alemow seedlings, it is likely that plants will be infected soon after planting in the field and that many trees will be stunted. In other places with lower CTV incidence and/or transmission rates (for example, lemon growing areas), and with milder CTV strains, the stunting effect may be limited or insignificant.

The idea of short-cycle citrus cropping is becoming increasingly popular in Spain. Under certain conditions, it is possible that the advantage of CTV tolerant rootstocks with a longer cropping period will be economically counter-balanced by early bearing, heavy fruit set and excellent fruit size realized on alemow rootstock, even if some trees are stunted and have to be replaced earlier than usual.

Alemow seedlings and young budded trees produced by citrus nurseries on this rootstock may be easily infected by CTV in the field (14) and later show poor growth if the infecting strain is moderate to severe. Although the Spanish certification program requires citrus nurseries to be located at least 2 km away from the closest CTV focus, it is highly recommended that plants propagated on alemow are produced under plastic houses with strict aphid control to avoid potential stunting of these plants.

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