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Cross Protection Against the Severe *Citrus tristeza virus* Stem Pitting in Peru

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ABSTRACT. During the 1970s and early 1980s the commercial production of oranges, specifically the Washington navel orange, was virtually terminated in the coastal citrus growing regions of Peru. This was due to extremely severe stem pitting isolates of the *Citrus tristeza virus* (SP-CTV) which affected scions regardless of rootstock. These severe and destructive isolates were introduced into Peru in the 1950s with the known importations of satsuma mandarin budwood from Japan. A search was initiated in the mid 1980s for productive surviving trees of the popular Washington navel orange. Thirty promising budlines of Washington navel were identified and extensively tested. Five of them were finally selected as protective navel orange sources. In addition, the search also identified two other protective sources. One was a highly productive Mexican (Key) lime tree that was growing under the cool Mediterranean climatic conditions of coastal Peru. This tree was fruitful with large fruit and showed no stem pitting. The other sources were imported scion budsticks of Duncan grapefruit and Madam Vinous sweet orange containing attenuated cross-protective isolates of codes 37 and 40 derived by vector passage of CTV through *Passiflora* and introduced from California in 1990. Over the years, all of these native and introduced protective budlines were severely screened by challenge with vector-transmitted local inoculum. Some of them have proven their ability to protect citrus under open field conditions. Adjacent trees of similar but non-protected susceptible cultivars showed tree decline, with typical severe stem pitting symptoms and with few and very small fruit. Washington navel oranges and Mexican (Key) limes carrying these protective isolates have been planted with commercial success since the early 1990s This paper will relate the early results on the cross protective ability of some of these native and introduced budlines which were used in 1998 to pre-immunize CTV-free Fukumoto, Navelina, Cara Cara, Navelate and Lane Late navel oranges, as well as Star Ruby, Flame, Marsh and Oro Blanco grapefruit and thornless Key lime.

The Peruvian citrus industry has suffered two devastating declines of citrus due to *Citrus tristeza virus* (CTV). First, in the 1950s CTV-induced quick decline of sweet orange on sour orange rootstock (QD-CTV) virtually destroyed the citrus industry (1). This same QD-CTV decline had already killed millions of trees throughout South America on sour orange rootstock. This problem was overcome by the use of a number of CTV tolerant rootstocks. However, in the 1960s trees again began to decline and various reports on this decline suggested that the problem was due to nutrition (J. M. Wallace, unpublished report), a new disease "X" (S. M. Garnsey, unpublished report), or possibly a severe CTV isolate similar to the Capão Bonito isolate found in Brazil (http://ecoport.org/ep?SearchType=earticleView&earticleId=834).

The Washington navel orange industry had virtually disappeared from these once productive coastal valleys of Peru when observations on this new decline of oranges were made in 1987 (6). At that time, the Washington navel was the only citrus grown and nearly all trees in all orchards had few and very small fruits while some trees were chlorotic and showed frenching with upright growth. Many branches had thickened, cheesy bark and broke readily at the nodes. When the bark was peeled, classic CTV stem pitting was evident. Most trees were non-productive and needed to be replaced. The cause of this decline was thought to be a severe stem pitting strain of

CTV (SP-CTV) that was being transmitted to navel oranges by the brown citrus aphid, *Toxoptera citricida* (Kirkaldy), from satsuma and other mandarins which had been imported earlier from Japan. Growers had top-worked their trees with mandarins or replanted with various mandarin varieties which were tolerant to the severe SP-CTV.

Since the early 1980s, the senior author has searched continually for navel orange and Mexican lime trees that survived the ravages of severe SP-CTV. Several surviving trees were selected, propagated and extensively tested. A small number maintained their horticultural superiority over time and remained productive, showing virtually no symptoms of SP-CTV. Since the early 1990s these superior trees have been used as sources of budwood to establish new commercial Washington navel and Mexican lime orchards.

These superior native budlines of Washington navel and Mexican lime trees were then tested as potential source trees to cross protect other citrus cultivars that were susceptible to SP-CTV under Peruvian coastal conditions. Introduced cross protective isolates of CTV derived via passage through *Passiflora* spp. (4, 5, 7) were also tested. The cross protection work was initiated in 1998 and early results became available in 2004. This paper describes the methods used to find cross protection by some of the sources that were tested against the severe SP-CTV in Peruvian Washington Navel oranges and Mexican limes and presents results of highly successful protection by these three sources.

MATERIALS AND METHODS

Origin of Washington Navel budwood source orchards: Source Orchard "B". This source was from the University of California foundation block at Lindcove, CA. The senior author obtained budwood of the old line parent Washington navel from the University of California Citrus Variety Improvement Program in the mid 1970s and used it to establish an increase mother block at the Topara nursery. Initial growth and productivity were excellent and this budline was propagated commercially. One of the first plantations that ware established with this budline was source orchard B. It was planted in the early 1980s on rough lemon and Cleopatra mandarin rootstocks in the Chincha Valley south of the city of Lima.

Trees in source orchard B were reaching bearing age when SP-CTV symptoms were observed. Trees in the Topara nursery increase mother block also showed similar symptoms at that time. Budwood from this increase block was eliminated in 1989. The ten selected trees of Source B that had provided buds for the Topara nursery increase block also declined, and were eliminated.

Source Orchard "L". The origin of this source was the original old line parent Washington navel orange tree in Riverside, California. Budwood from this tree was sent from the University of California at Los Angeles to the La Molina Experiment Station in Lima, Peru in the late 1920s. Many orchards were planted in Peru with this budwood source on sour orange rootstock until the early 1950s. However, most of the trees displayed classic symptoms of CTV decline of sweet orange on sour orange rootstock. The senior author supervised one of those orchards in the 1960s where he selected ten superior surviving trees out of approximately 10,000 trees. Buds from these ten selected trees were used during the 1970s to plant new orchards on the CTV tolerant rough lemon and Cleopatra mandarin rootstocks. One of these orchards became source orchard L, and orchards of this selection were planted in the Cañete valley south of the city of Lima in the late 1970s. The trees of source orchard L were observed to be uniform in their initial growth and productivity. Initially, symptoms of SP-CTV were absent but gradually spread throughout the orchard with symptoms varying from mild to very severe. Buds were obtained from the ten superior selected trees in orchard L in 1987 and propagated at the Topara Nursery for further testing and observation. The original source orchard L was eliminated in 1989.

Source Orchard "R". Willits and Newcomb (Thermal, California) provided budwood of Frost nucellar Washington navel to Hacienda Hualcará in the Cañete Valley south of the city of Lima in the late 1950s. These buds were used to establish new orange orchards on Cleopatra mandarin rootstock. Trees in these orchards displayed severe SP-CTV symptoms in the early 1970s. The senior author identified a few superior trees at that time and used them as budwood sources for new orchards established on the same Hualcará farm in the late 1970s and early 1980s on Cleopatra mandarin rootstock. These became source orchard "R".

The trees in source orchard R remained free of SP-CTV symptoms and were uniform in their initial growth and productivity. However, again, SP-CTV symptoms, varying from mild to severe gradually spread throughout the entire orchard. Buds were selected from ten superior trees in 1987 and propagated at the Topara Nursery. The original source orchard R was eliminated in 1989.

Procedure for evaluation and selection of superior protective Washington navel bud sources. Beginning in 1984, approximately 10,000 Washington navel trees in three different source orchards, B, L and R (code names for orchard owners) were critically observed for uniform large fruit sizes and high yields. Thirty trees in each of the three citrus orchards were flagged in 1985 followed by an evaluation in 1986 which reduced the flagged trees from 30 to 20 in each orchard. An additional follow up evaluation in 1987 reduced the flagged trees from 20 to10 in each orchard. The surviving 10 trees in each orchard were numbered one to ten. Fig. 1 shows a superior tree in source orchard R which is flagged with a blue banner for further observation. If all of the trees had been of uniform performance in fruit size and yield, the yield of this Washington navel orchard would have been in the range of 40 T/ha.

A nursery increase block of 6000 field grown UCLA rough lemon seedlings were budded with material from the three orchards and planted at the Topara nursery in 1986. Two hundred rough lemon seedlings were grafted with budwood from each of the 10 surviving trees from the three source orchards (200 seedlings × 10 surviving trees × 3 source orchards = 6,000 trees). The planting design consisted of 30 individual rows with 200 trees in

Fig. 1. Photo taken in 1987 of a superior tree in source orchard R flagged with a blue banner for further observation.

each row (one row for each of the ten surviving trees from each of the three source orchards). This nursery increase block was then surrounded by severely pitted trees planted simultaneously to serve as a source of continuous challenge inoculum. Aphids were not controlled in order to facilitate vector transmission from the challenge source trees.

All trees of this nursery increase block were carefully evaluated twice a year. Stunted or weak growing trees were eliminated during each evaluation. When a given row had lost more than half of its trees, the entire row was discarded. When trees started producing fruit by the year 1989, small fruit size became an additional reason for elimination of trees or rows.

Discovery of a symptomless small fruited lime tree in a cool and humid location. In the late 1980s a nursery client informed the senior author of two small fruited seedling lime trees that were growing at his grandmother's home close to the ocean in an area optimal for expression of severe CTV symptoms. He was told that one of the trees produced large fruit. The author visited the grandmother's home and observed two neglected lime trees growing side by side. One tree showed extreme stem pitting and miniature fruit and the other tree was vigorous with large fruit. They were named *C. aurantifolia* cv. XXX and *C. aurantifolia* cv. Topara, respectively. Buds from both trees were grafted on field grown UCLA rough lemon seedlings. Six trees from each of both selections were planted in 1988 for further testing at the Topara nursery SP-CTV trial block.

Introduction of protective CTV isolates from California. In 1986-1987 a new approach was initiated at the Citrus Research Center in Riverside, California for finding protective isolates of CTV by passing severe SP-CTV isolates through *Passiflora* species via transmission by *Aphis gossypii.* Progeny virus derived from passage of CTV through *Passiflora* could attenuate pitting symptoms in grapefruit and sweet orange (4). These attenuated isolates were shown to have potential for cross protection (4, 5, 7) and the senior author perceived a need to test the protective character of these isolates in Peru. The objective was to test the long term stability of these California protective introductions under the severe challenge of SP-CTV strains present in coastal Peru. Despite the risk of having exotic California CTV strains spread at the Topara nursery and beyond, the perceived benefits of stopping the destruction by SP-CTV in Peruvian coastal areas justified the risks.

Buds of the *Passiflora*-derived protective isolates coded 37A (in a Madam Vinous sweet orange seedling) and 37B, 37C and 40A (all in Duncan grapefruit seedlings) were grafted at the Topara Nursery on field grown UCLA rough lemon seedlings in December, 1989. The resulting 16 trees, (4 per coded source) were planted at the Topara nursery SP-CTV trial block adjacent to the navel orange and to the *C. aurantifolia* selections previously described, and surrounded by trees infected with severe SP-CTV isolates.

Protective character of source orchard B. The initial performance of this source in the Topara mother block was excellent. However, after a period of time, trees displayed severe SP-CTV symptoms and all ten rows from the source orchard B were eliminated from the Topara nursery increase block by 1989.

RESULTS AND DISCUSSION

Protective character of source orchard L. In 2004, two of the ten superior budlines (L1 and L2) selected from source orchard L had reached 17 yr of age at the Topara nursery with normal fruit size and productivity. The L-1 and L-2 orchards planted since the mid 1990s in different locations in Peru also produced satisfactorily. The observation that the trees in source orchard L displayed no SP-CTV symptoms when planted also in other locations suggested the possibility that the QD-CTV isolates infecting these lines prevented expression of SP-CTV symptoms. The L -2 selections appear true to type with a small navel as described for the old line parent Washington navel. L-2 is now the recommended selection for new Washington navel plantings in the Peruvian coastal citrus growing areas. Even though they are effective in Washington navel, the L-1 and L-2 selections have not protected other citrus cultivars (grapefruit, tangelos etc.) against SP-CTV under Peruvian coastal conditions.

The L-1 and L-2 selections carry a weak strain of Citrus vein enation virus (2) which can be eliminated by shoot tip grafting. However, it is believed this virus causes no economic damage.

Protective character of source orchard "R". Two of the 10 superior budlines (R-1 and R-2) that were selected from source orchard R and grown at the Topara nursery have remained productive after 17 yr. The R-1 and R-2 source navels are of nucellar origin and show a large and irregular protuberant navel at the stylar end, a character not ascribed to the original old line parent Washington navel orange. For this reason, the R selections are not being recommended for new Washington navel plantations. However, as mentioned, the R selections contain CTV isolates that protect other susceptible navel orange cultivars (Fukumoto, Navelina, Cara Cara, Navelate and Lane Late) from SP-CTV under Peruvian coastal conditions. Time has proven R-2 to be the better protective source for protecting other varieties of navel orange (Fig. 2). The fact that the trees in source orchard R resisted early symptom expression of SP-CTV again suggests that since its introduction into Peru in the late 1950s, the older QD-CTV isolates present in this selection were protective against newer SP-CTV isolates.

Protective character of *C. aurantifolia* sources. The *C. aurantifolia* cv. XXX trees developed severe stem pitting, stunted growth and vein corking as early as the first flush of growth after budding (Fig. 3). All trees except one of this selection were eliminated soon after planting and this one tree was kept for educational and photographic purposes. At 16 yr of age, it bears no marketable fruit.

At 16 years of age, the adjacent *C. aurantifolia* cv. Topara trees displayed good growth habits and productive capacity (Fig. 4). Stem pitting is rare and restricted to occasional deep isolated pits in wood one year or older, even though vein clearing can be seen on the leaves of all field trees. Since the mid-1990s, *C. aurantifolia* cv. Topara on UCLA rough lemon rootstock has been planted extensively along the Peruvian coast from latitude 3°S to latitude 20°S. Tree health and productivity remain uniform, suggesting that the isolate they contain protects against SP-CTV under varying climatic conditions. Also, the original 16 yr-old source tree is free of stem pitting symptoms and remains productive under high SP-CTV inoculum pressure. As of 2004, about 300 ha of orchards made with *C. aurantifolia* cv. Topara are profitable with no complaints from customers (Fig. 4). Many orchards are in areas where it would not be possible to grow the small fruited lime due to endemic severe SP-CTV strains. Significantly, the CTV strain in *C. aurantifolia* cv. Topara protects other susceptible citrus grapefruit and navel oranges from the severe Peruvian SP-CTV isolates. Because of these properties, *C. aurantifolia* cv. Topara trees continue to be a preferred source of budwood for commercial propagation.

Fig. 2. Clockwise from upper left. i) The superior budwood tree R1 selected from source orchard R and grown at the Topara nursery. ii) Twigs from Navelate showing little to no stem-pitting. iii) Twigs from Cara Cara navel trees protected with R1 and showing little or no stem pitting. iv) The fruit produced on source R1.

The extreme differences observed in the *C. aurantifolia* cv. XXX and *C. aurantifolia* cv. Topara lines are an indication of the diversity of CTV found in Peruvian coastal growing areas. Both lines have maintained their original characteristics over a long period suggesting these two isolates may maintain specific symptom characteristics during the lifetime of the host tree.

Protective character of Passiflora-derived CTV-isolates after 15 years. After 15 yr in the field, Duncan grapefruit trees protected with the isolate 37C remained free of pitting, continue to grow well and are highly productive with large fruits (Fig. 5). Isolates 37C and 37B protected other susceptible grapefruit cultivars (Star Ruby, Flame, Oroblanco and Marsh) from severe SP-CTV strains under Peruvian coastal conditions. Fig. 6 (top panel) shows uniformly sized Star Ruby grapefruits from trees protected with isolate with *C. aurantifolia* cv. Topara and 37C (rows 3 and 4 respectively). The control trees were not protected and show fruit of various sizes (rows 1 and 2). Fig 6 (bottom panel) shows severe stem pitting in branches from an unprotected Star Ruby grapefruit tree (compared to a similar unprotected Marsh grapefruit tree, Fig. 3). Isolate 37A protected Fukumoto, Navelina, Cara Cara, Navelate and Lane Late navels from SP-CTV under Peruvian coastal conditions.

The five Duncan grapefruit trees inoculated with isolate 40A developed stem-pitting after ten years in the field. By the fifteenth year all trees showed small fruit, severe stem pitting and related symptoms indicating breakdown of protection. This indicates that protection from isolate 40A is not persistent under the severe inoculum pressure in Peru.

Fig. 3. Clockwise from upper left. i) Vein corking in leaves of the *C. aurantifolia* cv. XXX trees. ii) A stunted and severely pitted non-protected Marsh grapefruit tree. iii) A severely pitted twig from the Marsh grapefruit iv) The small fruit and stunted growth of the *C. aurantifolia* cv. XXX trees.

The protective isolates from California have had no observed detrimental effect on other SP-CTV susceptible cultivars at the Topara nursery. However, based upon observations of uninoculated control trees, vector transmission may have moved protective isolates into these trees (data not presented).

CONCLUSIONS

Since the early 2000s the subsequent increase in the commercial production of several navel cultivars in coastal valleys of Peru has been significant. Also, highly productive Mexican lime orchards have been planted in areas where this was previously not possible.

After citrus infected with QD-CTV on sour orange rootstock was destroyed throughout Peru, a second disaster of severe stem pitting CTV destroyed the important navel orange industry in the coastal regions of the country. The magnitude of this disaster has been reviewed (6) and illustrated (8). When symptoms affect the productivity of the scion, changing rootstocks has little or no remedial effect and cross protection can be an effective way to revive the industry.

A concerted effort of judicious tree selection by the senior author over 20 yr yielded three sources of protective isolates. After 17 yr under severe inoculum pressure at the Topara nursery all three of these protective sources have withstood the test of time. In addition, they have opened up new industries not previously possible in Peru. New

Fig. 4. Clockwise from upper left: i) A grove of uniform and productive three year old lime trees. ii) The intact trunk of a 5-yr-old tree lime trees of *C. aurantifolia* cv. Topara. iii) A trunk with a peeled bark window showing no stem pitting. iv) Large fruit of high quality limes. v) Five-year-old grove of lime trees of *C. aurantifolia* cv. Topara.

navel selections as well as grapefruit and limes can now be grown in the coastal regions of Peru even in the presence of severe SP-CTV and its primary vector *T. citricida.* Also, production is excellent and the fruit size of these grapefruit and limes are large. Maintaining adequate fruit size is important since in most countries where grapefruit are grown in the presence of severe isolates of SP-CTV plus endemic *T. citricida*, the fruit size is materially reduced.

Protective isolates L and R have been genotyped using the multiple molecular markers method, and the major coat protein gene of each has been sequenced and the sequences compared to determine relatedness of the isolates (3). Biological indexing on sweet orange and grapefruit will be done using the '*in planta*' cultures (3).

We have shown that certain greenhouse produced isolates of SP-CTV derived via passage through *Passiflora* have successfully protected citrus in Peru under their severe inoculum pressure. This suggests an alternative method for developing protective isolates relatively rapidly rather than waiting for an industry to die and searching for surviving trees, which may take a long period of time.

It is important to realize that when *T. citricida* enters a country in the presence of severe SP-CTV, and causes the type of destruction seen in Peru (6), cross protection is an alternative solution to revive a citrus industry in decline. Because of the length of time and incredibly hard work and research required, it is important that citrus research stations worldwide invest in programs for cross protection to shorten the time required for development of protective strains of CTV. With *T. citricida* now present in

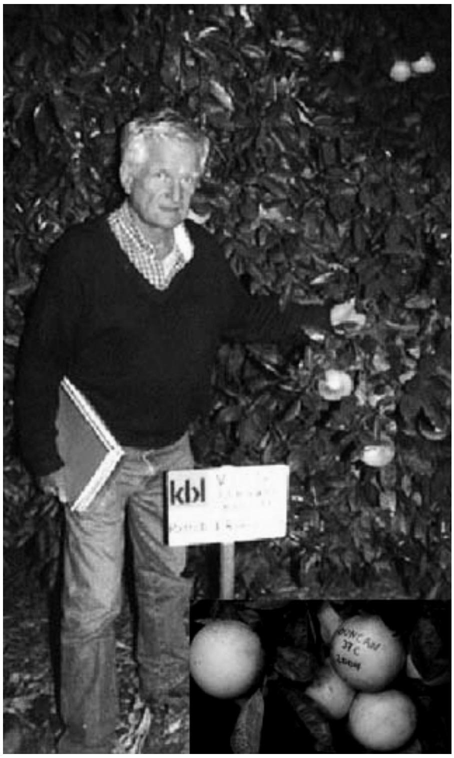

Fig. 5. Clockwise from upper left. i) The senior author standing next to a Marsh grapefruit tree protected with isolate 37C derived from passage of CTV through *Passiflora* by aphid vector. ii) Selection of large fruits produced on this tree.

Fig. 6. Top panel from left to right shows fruit from unprotected Star Ruby grapefruit trees (rows 1 and 2). Row 3 shows fruit from Star Ruby grapefruit trees protected with *C. aurantifolia* cv. Topara source, and row 4 shows fruits from trees protected with isolate 37C. Lower panel shows pitted stems from unprotected Star Ruby trees.

Europe, the United States (Florida), Mexico, the countries of the Caribbean, the research and work done in Peru and previously in Australia, Brazil, and South Africa could set an example and guidelines for perspective research. This applies as well to those countries of Africa, Asia, Indonesia and South America where *T. citricida* and severe CTV isolates are present and pose current and potential threats to their citrus industries.

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