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Research Article

The genetics of tolerance to tristeza disease in citrus rootstocks

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Abstract

Controlled pollinations between four elite citrus rootstocks, *Citrus limonia* - 'Limeira' rangpur lime (Cravo), *C. sunki* - 'Sunki' mandarin (Sunki), *C. aurantium* - 'São Paulo' sour orange (Azeda) and *Poncirus trifoliata* - 'Davis A' trifoliate orange (Trifoliata), resulted in 1614 nucelar and 1938 hybrid plants identified by the isozyme loci *Pgi-1, Pgm-1, Got-1, Got-2, Aps-1, Me-1, Prxa-1* and or by the morphological markers broadness of leaf petiole wing or trifoliolate leaves. Tolerance to the citrus tristeza virus (CTV) was evaluated under nursery and field conditions for several years by the reaction of Valencia orange infected with a severe strain of CTV and grafted onto the hybrids and nucellar clones. Genetic analyses indicated that tolerance was controlled by at least two loci designated here as *Az* and *t* interacting in dominant-recessive epistasis. Genotypes *Az*__ __ __ and __ __ *tt* were tolerant while *azaz T*__ was intolerant. The intolerant Azeda was *azaz TT*, the tolerant rootstocks Sunki and Cravo were *Azaz tt* and the Trifoliata was *Azaz TT*. The different degrees of intolerance seen in some hybrids may reflect the inability of segregating modifiers from parental clones to overcome the epistatic interaction that controls the major tolerance reaction.

Key words: *Citrus*, CTV, epistasis, rootstock, tristeza.

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Introduction

Compared to other plant diseases, citrus tristeza disease has received little attention in Brazil in recent years, despite its enormous historical importance and the economic threat that it still represents to other countries (Lee *et al.*, 1992; Yokomi *et al.,* 1994; Gottwald *et al.,* 1994, 1998; Rocha-Peña *et al.,* 1995; Hughes and Gottwald, 2001; Riac, 2001; Hughes *et al.*, 2002; Stover and Castle, 2002). To a large extent, this lack of interest reflects the success of agricultural research in this country in dealing with this disease since the late 1940s (Franco and Bacchi, 1944; Meneghini, 1946), but is also related to the characteristics and mode of transmission of the disease (Bitancourt and Rodrigues Filho, 1948; Bennett and Costa, 1949; Bitancourt, 1954). In addition, extensive tests with several hundred introduced rootstocks in diverse scion combinations of oranges, mandarins, limes and lemons have resulted in practical solutions and efficient measures to control this disease (Moreira *et al.,* 1949, 1954; Moreira and Salibe, 1969; Pompeu Junior, 1990). As a result, all of the sweet orange crop in Brazil, which accounts for 75% of the world's production, and 20% of the concentrated juice on the international market, derive from orchards established with rootstocks tolerant to the citrus tristeza virus (CTV) (Informativo Centro de Citricultura, 2002), a long RNA virus that damages plant phloem (Kitajima *et al.*, 1964). The characteristics and symptoms of tristeza disease, and its implications for the scion/rootstock relationship have been discussed in detail by Costa *et al.* (1949), Müller (1976), Adams (1991), Lee *et al.* (1994) and Bordignon *et al.* (2003).

Tolerance to CTV is seen in the ability of rootstock phloem to withstand the presence of the virus without adverse effects on normal cellular and physiological functions. Tolerance, as defined here, is distinct from the resistance seen in several *Citrus* and related species in which there is no multiplication and movement of the virus within the plant (Moreira *et al.,* 1949; Yoshida, 1993; Mestre *et al.,* 1997a,b). CTV is abundant in susceptible scions, such as sweet oranges, mandarins, limes and grapefruits (Moreira *et al.*, 1954). Since CTV occurs in all

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susceptible commercial scion sources in Brazil, and since the widespread distribution of the brown citrus aphid (*Toxoptera citricida*), the most efficient vector of CTV, means that no virus-free plants remain uninfected in any orchard, the use of tolerant rootstocks is essential, preferably together with tolerant scions.

For several decades, the rangpur lime (*Citrus limonia*), commonly known in Brazil as "limão Cravo" (referred to here simply as Cravo) has been the most widely used tolerant rootstock (Pompeu Junior, 1990), although there is concern about the need for rootstock diversification. Other species with tolerance to blight and trunk gummosis could also be useful in reducing the biological vulnerability of the crop to disease. During a breeding program at the Instituto Agronômico (IAC), thousands of controlled pollinations among elite rootstocks were done to obtain hybrids with desirable agricultural and industrial characteristics derived from different genitors. Tolerance to tristeza, the principal criterion for selection, was also evaluated in these hybrids. This tolerance is effective against all forms of CTV complexes (mild, regular, severe) responsible for symptoms such as quick-decline, leaf rolling, seedling yellows and stunting. Such CTV complexes have produced epidemics of tristeza in sweet oranges grafted onto sour oranges in major producing areas of the world. However, this tolerance is not effective against the Capão Bonito strains of CTV, to which the Rangpur lime is intolerant, or to the stem-pitting strains of xylem CTV complexes that affect scions, regardless of the rootstocks.

Based on the reaction of some hybrids imported from the USA, Costa *et al.* (1949) suggested that tolerance was a dominant characteristic, but did not examine this phenomenon any further. In this study, the reaction to CTV of 1614 nucellar plants and 1938 hybrids from four genitors was investigated in order to determine the genetic control of tolerance to the tristeza disease in citrus rootstocks.

Materials and Methods

Plant material

The genitors used in the crosses were from the Sylvio Moreira CAPTAC germplasm collection held at the IAC station in Cordeirópolis. These genitors (identifications used in the text are indicated in parenthesis) were: *Poncirus trifoliata* 'Davis A' - trifoliate orange, 848-Q.30 (Trifoliata or T), *Citrus limonia* - 'Limeira' rangpur lime, 863-Q.30 and Banco de Matrizes (Cravo or C), *Citrus sunki* - 'Sunki' mandarin, 200-Q.57, Coleção Clones Velhos and Banco de Matrizes (referred to as Sunki or S), *Citrus aurantium* - 'São Paulo' sour orange, 244-Coleção Clones Velhos and 285-Q.16 (Azeda or A).

Controlled crosses and hybrid production

Two series of controlled crosses were done in consecutive years (Series I and II) by emasculation, protection and pollination of more than 5,000 flowers. The mucilage, testa and integument were removed from the seeds of mature fruits and the seeds then germinated in moist Petri dishes. The embryos were subsequently removed from each seed and transplanted to seedling trays. The technique used in the crosses and in subsequent procedures have been described in detail elsewhere (Bordignon *et al.,* 1990; Bordignon, 1995). The results of the following crosses were studied: T x A, S x A, A x S, C x A, A x C, T x S, S x T, S x C and C x S.

Identification of hybrids and nucellar clones based on the isozyme profile, the presence of trifoliolate leaves and the broadness of the leaf petiole wing

After transplanting, the seedlings were subjected to isozyme analyses and/or were evaluated for leaf markers to identify hybrids and maternal nucellar clones.

Plants from the crosses A x C, C x A, A x S, S x A were analyzed mainly by the broadness of the leaf petiole wing (Ballvé *et al.*, 1997); doubtful cases were by isozyme analysis or were discarded. The dominant trifoliolate leaf marker (Toxopeus, 1962) was used to identify S x T hybrids.

Horizontal starch gel electrophoresis was used to identify the hybrids of T x A, T x S, C x S and S x C crosses (Ballvé *et al.,* 1991 1995). The PGI (phosphoglucoisomerase), PGM (phosphoglucomutase), GOT (glutamate oxaloacetate transaminase), PRX (peroxidase), ME (malic enzyme) and APS (acid phosphatase) enzymatic systems were screened to determine the genotype for the *Pgi-1, Pgm-1, Got-1, Got-2, Prxa-1, Me-1* and *Aps-1* loci.

Orange grafts and setting up of COS I and COS II plots

COS I and II, plots for observation and selection, were established using hybrid and nucellar clones from the crosses of series I and II, respectively. In COS I plot, the plants were transplanted to plastic bags in the nursery and bud grafted with Valencia sweet orange infected with a severe CTV strain. After eight months, the seedlings were pruned back and transplanted to the field in the spacing 8 x 4 meters. COS I consisted of 138 nucellar plants (48 Cravo, 48 Sunki, 9 Azeda, 33 Trifoliata) and 534 hybrids (65 T x A, 48 C x A, 20 T x S, 67 S x T, 207 S x C, 98 S x A and 29 C x S). For COS II, seedlings from the seedling trays were transplanted directly to the field and grafted with the same Valencia budwood as COS I. Because of the large number used, the seedlings were planted in double 1 x 0.5 m rows, 3 m apart. This plot consisted of 1410 nucellar clones (429 Cravo, 410 Sunki, 571 Azeda) and 1413 hybrids (845 S x A, 240 A x S, 139 C x A, 19 A x C, 147 S x C and 23 C x S).

Evaluations and observations in the nursery and in the COS I plot

Nursery plants from series I crosses were rated according to their general development and health conditions, leafiness, leaf rolling, presence of seedling yellows and stunting. These parameters were also recorded in 1996, 1997, 1998 and 1999 in the field. Scion diameter was evaluated in 1996 and 2000, rootstock diameter in 1996, 1998 and 2000, canopy diameter in 1998 and 2000, plant height in 1998 and 2000, vigor index [calculated from the original measurements, in centimeters, as: plant height + canopy diameter + (rootstock diameter x 10)/100] in 1998 and 2000, mean fruit weight (based on a maximum of 10 fruits/plant) in 1997, 1998 and 1999, and yield in 1997, 1998 and 1999. In 2000, all these parameters were consolidated into a single tristeza index that varied from 1 to 7 and reflected the intensity of the symptoms, with 7 being the most severe.

Evaluations and observations in the COS II plot

In COS II, the reaction of the hybrids and genitors to tristeza was initially evaluated six months after the Valencia clone had budded on the rootstocks. The plants were scored on three occasions for seedling yellows and stunting, whereas scion diameter was measured once. Based on these data, the hybrids were classified as tolerant or intolerant by comparison with the maternal nucellar progenies of the genitors.

Statistical analysis

Statistical comparisons were done with the chi-square (χ^2) test calculated using MINITAB software. Yates' correction for continuity (Little and Hills, 1978; Snedecor and Cochran, 1967) was applied because the segregations consisted of only two classes and one degree of freedom. A value of $p < 0.05$ indicated significance.

Results and Discussion

Tolerance to tristeza disease

The intensity of the tristeza symptoms in COS I varied from 1 to 7, with 7 corresponding to plants with the most extreme manifestation of intolerance, such as conspicuous seedling yellows, stunting, no yield or a yield limited to the production of a few small fruits. The intensity of the symptoms was not the same for all intolerant plants, as also reported by Costa *et al*. (1949) during early studies of the disease. For this reason, in the final classification, plants that scored higher than 2 were considered intolerant. This apparently rigid criterion was adopted because all 128 plants in the tolerant Cravo, Sunki and Trifoliata groups had scores of 1 or 2, and also because the Valencia scion is considered to be quite a tolerant clone. In the nursery, all 76 Azeda plants showed typical symptoms of seedling yellows and reduced growth, although the intensity was somewhat variable. The nine most vigorous plants of this group were transplanted to the field but, as expected, they soon stopped growing, became stunted, or died after producing a few small fruits; they all received a score of 7. The S x A, C x A, T x A, T x S and S x T hybrids yielded typically healthy, tolerant plants with scores of 1 or 2, as well as intolerant plants. The reactions of the Valencia orange grafted onto tolerant and intolerant rootstocks are shown in Figure 1. Although all plants from the nucellar Azeda rootstock invariably showed extreme symptoms of intolerance (score 7), hybrids of this stock also showed less severe symptoms (scores of 3 to 6). Plants with such scores were classified as intolerant because even with their less intense symptoms, their level of intolerance precluded any future attempt to select them. Additionally, the Valencia orange is more tolerant to tristeza than other well-established clones such as Pera. Most of the individuals in the field, and all of the Azeda nucellars and class 7 hybrids, were carefully checked for stem-pitting at the scion-stock junction, but none was found, despite the general occurrence of such at this site (Figueiredo *et al*., 1993).

The classification criterion for the COS II plot was less elaborate. The field was established with plants taken directly from the greenhouse trays and planted close together. This resulted in stressful conditions in which seedling yellows and stunting were more common (Figure 1). Comparison of the scion diameter of intolerant hybrids and Azeda with those of tolerant hybrids and of Sunki and Cravo indicated that there was reduced growth in intolerant plants compared to tolerant ones in both fields (Table 1). In COS II, plants with seedling yellows, small rolled leaves, stunting and or with diameters <2 cm were considered intolerant. All grafts onto Sunki and Cravo were healthy, the leaves were a normal dark green, and more than 95% of 839 plants had diameters >2 cm (mean = 3.4 cm), while all 571 plants grafted onto Azeda had seedling yellows and related symptoms, as well as a diameter <2 cm, (mean = 0.86 cm). Overall, the reaction to tristeza seen in both fields agreed fully with the expected tolerance of Cravo, Sunki and Trifoliata and with the intolerance of Azeda. Tolerant and intolerant individuals were observed among the hybrids, as discussed below.

The genetics of tolerance

Table 2 shows the number of tolerant and intolerant individuals among the parental plants and their hybrids in the COS I and COS II plots. In addition to the data in Table 2, any attempt to unravel the genetic control of tolerance must contemplate the following considerations:

a) The tolerance of Trifoliata, Sunki, Cravo, the Sunki x Cravo hybrids and their reciprocals is unquestionable, as is the absolute intolerance of Azeda.

b) There is genetic segregation between the Azeda hybrids and the tolerant Sunki, Cravo and Trifoliata (~1:1) and between Trifoliata x Sunki and their reciprocals (~3:1).

c) Selfing of Sunki and Cravo yielded only tolerant plants among the zygotic seedlings, as deduced from the observation that in commercial nurseries the frequency of zygotics is 5-15% (H.P. Medina Filho, R. Bordignon, un-

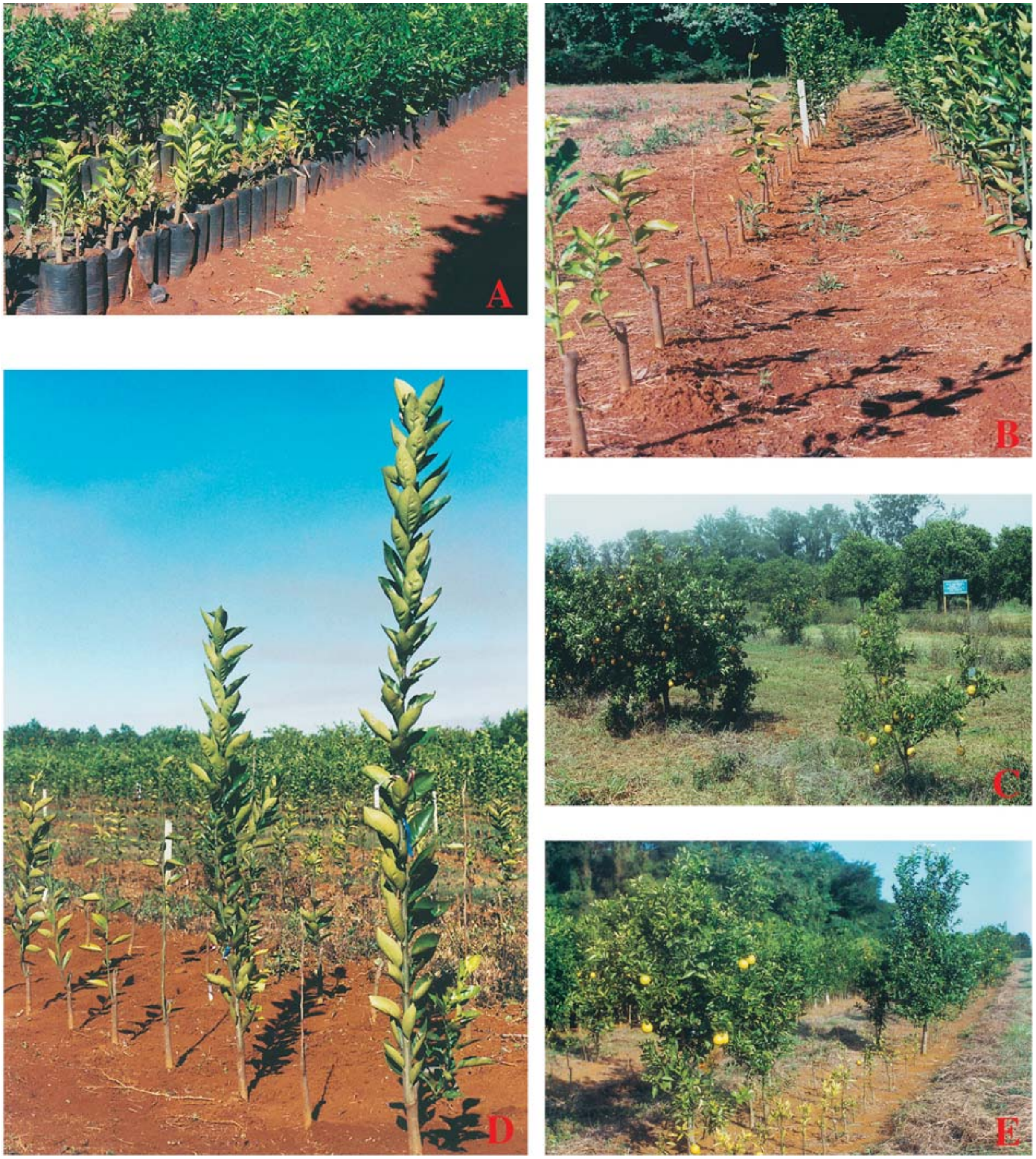

Figure 1 - Valencia orange scions grafted onto Azeda (intolerant) in the nursery before transplanting to the COS I plot (A) and in the plot COS II (B). The plants on the left show extreme symptoms of intolerance to tristeza (seedling yellows - score 7) when compared with tolerant plants (scores 1 and 2). When grafted onto segregating hybrids in COS I (C) and COS II (D and E), there was a conspicuous reduction in plant vigor in the intolerant hybrids.

published data) and the fact that no symptoms of tristeza were seen in grafted plants.

d) The selfing of Azeda yielded only intolerant plants, as shown by the fact that since the 1930s, when tristeza was introduced into Brazil and infected 10 million trees grafted onto Azeda, intensive efforts have been made to find tolerant individuals with the excellent qualities of Azeda as a rootstock. Although millions of trees have been investigated (many of which were certainly of zygotic origin), not a single tolerant individual has been found, neither in Brazil nor in any other citrus-growing region of the world. Invariably, all of the putative exceptions proved to be escapes,

Table 1 - Mean scion diameter (cm) for Valencia orange grafted onto Azeda (A), Trifoliata (T), Sunki (S), Cravo (C) and their hybrids segregating for tolerant (t) and intolerant (i) hybrids to tristeza, and the corresponding percentage reduction in this parameter in intolerant hybrids in COS I and II plots in 1998. The COS I and COS II plots contained 633 and 2823 plants, respectively. μ = average.

	COS I			COS II		
	i	t	% reduction	i	t	% reduction
A	2.7			0.83		
T		7.3				
S		9.2			3.5	
C		9.4			3.0	
TxA	4.3	7.4	42			
SxA	5.1	8.7	41	0.98	3.4	71
CxA	5.1	8.0	36	0.69	2.4	71
TxS	5.0	9.0	45			
SxT	4.4	8.5	48			
SxC		8.9			2.7	
CxS		8.5			2.3	
AxS				0.72	3.2	78
AxC				0.81	2.6	69
μ genitors	2.7	8.6	68	0.83	3.25	74
μ hybrids	4.8	8.4	43	0.8	2.8	71

Table 2 - Reaction to tristeza disease. Number (n) of tolerant (t) and intolerant (i) plants in the genitors Trifoliata (T), Sunki (S), Cravo (C), Azeda (A) and their hybrids scored in the plots COS I and COS II. The observed and expected ratios, the Chi-square (χ^2) values (corrected for continuity) and the corresponding probabilities (in percent) are also shown. The values for COS II plots are shown in bold italics.

	Scored plants N	Tolerant t	Intolerant i	Observed t:i	Expected t:i	χ^2	P %
T	33	33	0	t	t		
S	48	48	0	t	t		
	410	***410***	***0***	***t***	***t***		
C	47	47	0	t	t		
	429	***429***	***0***	***t***	***t***		
A	76	0	76	i	i		
	571	***0***	***571***	***i***	***i***		
SxA	103	60	43	1.39:1	1:1	2.81	7.9
	845	***523***	***322***	***1.62:1***	***1:1***	***47.81***	***0***
AxS	***240***	***118***	***122***	***1:1.03***	***1:1***	***0.067***	***100***
CxA	49	27	22	1.23:1	1:1	0.327	100
	139	***78***	***61***	***1.28:1***	***1:1***	***1.84***	***15.3***
AxC	***19***	***10***	***9***	***1.11:1***	***1:1***	***0.00***	***100***
TxA	53	21	32	1:1.52	1:1	1.89	15.3
TxS	24	16	8	2:1	3:1	0.50	100
SxT	60	44	16	2.75:1	3:1	0.02	100
SxC	207	207	0	t	t		
	147	***147***	***0***	***t***	***t***		
CxS	29	29	0	t	t		
	23	***23***	***0***	***t***	***t***		

hybrids or misnomers (Müller, 1976; Castle *et al*., 1989; Müller *et al*., 1990). A tolerant accession from China, known as Gou-Tou-Chen and that is under observation at the IAC station in Cordeirópolis, is also of hybrid origin (Castle *et al*., 1989; Müller *et al*., 1990) as confirmed by a close inspection of its fruits and isozyme profiles (H.P. Medina Filho, R. Bordignon, G.W. Müller, unpublished data).

Careful analysis of the genetic mechanism of tolerance indicated that no hypothesis based on quantitative inheritance or on the action of a single gene or two or more loci acting without epistasis could account for all of the data in Table II and for considerations a), b), c), and d) above. Among several known interacting systems (Sinnott and Dunn, 1939; Grant, 1975; Strickberger, 1976), a plausible alternative is the so-called dominant-recessive epistasis model that involves two loci, designated here as *Az* and *t*, that control the tolerance of the plant to CTV. Thus, the intolerant genotypes would be *azaz T_*, while the tolerant ones would be *Az_ _ _* and *_ _ tt*. Thus, the intolerant Azeda would be *azaz TT*, the tolerant Sunki and Cravo would be *Azaz tt* and Trifoliata would be *Azaz TT*.

Based on this reasoning, the selfing of Azeda would produce only intolerant zygotic progenies of the *azaz TT* genotype, while that of Sunki and Cravo would yield only tolerant plants (*Azaz tt* and *azaz tt*), and Trifoliata would yield tolerant (*AzAz TT*, *Azaz TT*) and intolerant (*azaz TT*) plants in a 3:1 ratio [see also points (c) and (d) above].

There is no information about the reaction of zygotic progenies of selfed Trifoliata to tristeza that would make it easy to check the heterozygous *Azaz TT* nature of this clone. However, this heterozygous nature is certain since crosses with Azeda (*azaz TT*) resulted in tolerant (*Azaz TT*) and intolerant (*azaz TT*) hybrids that were produced in an approximately 1:1 ratio. Additionally, the T x S hybrids and reciprocals that would be *AzAz Tt* and *Azaz Tt* tolerant and *azaz Tt* intolerant, respectively, in a theoretical ratio of 3:1, actually occurred in ratios of 2:1 and 2.8:1 (Table 2). Furthermore, other hybrids of *P. trifoliata* are referred to as tolerant or intolerant (Costa *et al*., 1949), which reinforces the conclusion that Trifoliata Davis A used here is heterozigous.

Crosses of Cravo or Sunki with Azeda (*Azaz tt* x *azaz TT*) would be expected to result in a 1:1 segregation of *Az az Tt* tolerant and *azaz* Tt intolerant progeny. Indeed, the segregations observed were close to this value (1.1-1.6:1; χ^2 not significant in 3 out of 6 crosses; Table 2). In the case of Cravo x Sunki (*Azaz tt* x *Azaz tt*), segregation would occur for *Azaz*, but not for *tt*, thus resulting in only tolerant hybrids, exactly as observed.

The present model of epistasis qualitatively explains the tristeza reaction seen in the four genitors, in their selfed zygotic progenies and in nine hybrid combinations studied in COS I and COS II plots. Follow-up investigations with selected F_2 progeny, additional crosses, and QTL analysis (Tanksley *et al*., 1982) of these populations would help to explain the genetic differences among individuals with different degrees of intolerance. The segregation of modifier loci could be one explanation since the major epistatic interaction conditioning the main tolerance reaction is not overcome.

Another aspect to be considered concerns segregations that partially deviate from the theoretically expected ratios of tolerant:intolerant individuals. A possible reason for this could be pre-zygotic selection and unequal viability of embryos, particularly if one considers the interspecific and the intergeneric nature of the crosses. Another possible source of distortion in the segregations could be the differential transmission of alleles as a result of meiotic irregularities, a common feature of interspecific hybrids. In this regard, it is possible that Cravo might not be a *bona fide* species, as judged by the fact that it is referred to as a lemon in Brazil, a lime in the USA, India and China, and a mandarin-like taxon (Hodgson, 1967), in addition to being classified as *C. reticulata* (a mandarin species) by Swingle and Reece (1967) and *C. limonia* by Tanaka (1954), the latter in allusion to its resemblance to lemons. In a recent study (Medina Filho *et al*., 2003), the segregation of isozyme alleles in the genitors of these hybrids showed marked distortion in the transmission of alleles of three Cravo isozyme loci. However, such observations does no alter the conclusions concerning the genetics of tolerance to tristeza addressed here.

Conclusions

The results of this investigation show that tolerance to tristeza disease, i.e., the ability of phloem tissue to tolerate CTV, is conditioned by at least two loci, *Az* and *t*, interacting in a dominant-recessive epistasis. Plants with the genotypes *Az _ _ _* and *_ _ tt* are tolerant and those with *azaz T _*, intolerant. The intolerant Azeda ('São Paulo' sour orange) is *azaz TT* while the tolerant Sunki mandarin and Cravo ('Limeira' rangpur lime) are *Azaz tt* and Trifoliata ('Davis A' trifoliate orange) is *Azaz TT*. Different levels of intolerance could be the result of segregation of modifier loci.

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