Distinction of populations infected with severe and common strains of the Citrus tristeza virus in Spain, by the ELISA-DASc (quantitative).

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DISTINCTION OF POPULATIONS INFECTED WITH SEVERE AND COMMON STRAINS OF THE CITRUS TRISTEZA VIRUS IN SPAIN, BY THE ELISA-DASc (QUANTITATIVE).

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ABSTRACT - ELISA-DASc (Enzyme Linked Immunosorbent Assay-Double Antibody Sandwich comparative) was assayed to compare different Citrus tristeza virus (CTV) isolates under quantitative conditions, using monoclonal antibody 3DF1 and a standard antigen. This method enabled us to discriminate between common and severe CTV strains inoculated on different Citrus hosts grown in a greenhouse. A positive correlation was found between strain severity and multiplication capacity or ability to yield high optical density values in ELISA-DASc. This correlation was not linked to the presence of the seedling yellows (SY) reaction.
Populations of Satsuma field trees infected with common CTV isolates, and a severe CTV strain, able to induce the SY reaction, could also be distinguished using ELISA-DASc. This method is simple and enables to perform massive analyses needed in CTV surveys or eradication programs. The method is also adequate to compare results obtained in different laboratories.

DISTINCTION DE POPULATIONS INFECTEES AVEC DES SOUCHES SEVERES ET COMMUNES DU VIRUS DE LA TRISTEZA DES AGRUMES, PAR ELISA-DASc (QUANTITATIVE)

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RESUME - La méthode ELISA-DASc (Enzyme-Linked Immunosorbent Assay Double Antibody Sandwich comparative) a été mise au point. Elle permet la comparaison de souches ou d'isolats du virus de la tristeza des agrumes (CTV), dans des conditions quantitatives en utilisant l'anticorps monoclonal 3DF1 et un antigène standard.
La méthode a permis la différenciation entre pathotypes communs et sévères du CTV, inoculés dans des différents hôtes cultivés en serre. Avec cette technique il est possible d'étudier une relation entre pathotypes sévères et pouvoir de multiplication ou capacité de produire des hautes densités optiques en ELISA-DASc, mais cette relation est indépendante de la réaction de seedling yellows (SY).
Avec la méthode ELISA-DASc, il est possible de distinguer des populations de Satsuma infectées en plein champ avec des isolats communs et des populations infectées avec un isolat sévère capable d'induire le syndrome de SY. L'utilisation de la méthode est simple et permet de réaliser les nombreuses analyses nécessaires dans des prospections ou des programmes d'éradication, permettant la comparaison des résultats entre laboratoires.

INTRODUCTION

The presence of numerous strains of the Citrus tristeza virus (CTV) is well known worldwide (GRAND and COSTA, 1951 ; GRAND and HIGGINS, 1957 ; MÜLLER and COSTA, 1972 ; BAR-JOSEPH and LOEBENSTEIN, 1973; Mc CLEAN, 1974 ; WALLACE and DRAKE, 1974 ; COHEN, 1976 ; BALARAMAN and RAMAKRISHNAN, 1977 and 1980 ; BURNS *et al.*, 1980 ; CALAVAN *et al.*, 1980 ; DODDS and BAR-JOSEPH, 1983 ; AUBERT and BOVE, 1984 ; DA GRAÇA, MARAIS and VON BROEMSEN, 1984 ; ROSNER and BAR-JOSEPH, 1984 ; BOVE *et al.*, 1988). Presence of different strains has also been studied in Spain (CUÑAT, GARRO and MOCHOLI, 1979 ; HERMOSO DE MENDOZA, BALLESTER and PINA, 1984 and 1988 ; BALLESTER-OLMOS, PINA and NAVARRO, 1988 ; BALLESTER-OLMOS *et al.*, 1988 ; MORENO and GUERRI, 1987) where most isolates are relatively mild by world standards.

Recently, a severe strain, able to induce the seedling yellows syndrome and to cause stem pits in wood of hosts known to be tolerant to common CTV strains, was introduced into Spain (BALLESTER-OLMOS, PINA and NAVARRO, 1988). Presence of this isolate is endangering the

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Spanish Citrus industry, based on the use of CTV tolerant rootstocks grafted with virus free varieties obtained through a comprehensive programme of Citrus improvement (NAVARRO, 1976). This introduced isolate not only will cause severe decline in older trees on sour orange, but will also cause crop loss in trees on tolerant rootstocks from its stem pitting effects ; for this reason, there is a governmental eradication plan (Ministry Order of 30th July, 1986) for the severe strain, which apparently is still established within the plant material illegally propagated. The original material consisted of extra early Satsuma mandarins [*Citrus unshiu* (Mak.) Marc.], probably of the Okitsu variety, from Japan (BALLESTER-OLMOS, PINA and NAVARRO, 1988). Budwood has been propagated on seedling rootstocks and also topworked on existing mature trees of different varieties.

Differenciation of severe from mild strains of CTV has been approached by several methods, in addition to the use of conventional Citrus indicator hosts under greenhouse conditions. CTV strains have not been differentiated by polyclonal antisera and serology trials using monoclonal antibodies (VELA *et al.*, 1986) have also not been successful. It may be possible to differentiate some strains (MORENO *et al.*, 1983 ; S.M. GARNSEY, personal communication, 1987), but no correlation between serotype, severe or aggressive pathotypes has been established. The presence of two or three different epytopes in the CTV capside (VELA *et al.*, 1988), still offers some possibility of finding such relationship.

Thus far, the most promising discrimination tests are based upon the analysis of double standard ribonucleic acids (ds-RNA) , according to the method of DODDS and BAR-JOSEPH (1983), and successfully tried by MORENO and GUERRI (1987) in Spain for CTV. Problems arise for application of the ds-RNA method to a large number of field samples, and for separation of conventional, but somewhat virulent strains from avirulent strains in Spain (BALLESTER-OLMOS *et al.*, 1988). The establishment of new viruliferous strains in populations of early Satsuma mandarin, prompted a new attempt of differentiating by serology, populations of trees infected with common or severe strains of CTV. To this purpose, a new variant of the ELISA-DAS method (BAR-JOSEPH *et al.*, 1979 ; CAMBRA, MORENO and NAVARRO, 1979) has been applied, allowing comparison of strains or isolates under quantitative conditions. The significance of setting up a serological method able to discern between isolates is crucial in Spain where there is underway a program for eradication of the severe strain.

MATERIAL AND METHODS

The ELISA-DASc method.

To ensure comparative quantification of CTV in plant extracts, the conventional ELISA-DAS method was modified (CAMBRA, 1983) utilizing it with monoclonal antibodies (VELA *et al.*, 1986) as follows : 1) DYNATECH, M129B plates were coated with 0.2 ml/well of solution containing 1 µg/ml 3DF1 monoclonal immunoglobulins (INGENASA) in carbonate buffer, pH 9.6, properly covered and incubated for 5 h at 35°C. 2) Plant extracts were prepared according to the method of CAMBRA (1983) and CAMBRA *et al.* 1988), using 4 young shoots (not suckers) per tree. Sample extracts were prepared by grinding the plant tissue in the ratio of 1 weight of tissue to 10 volumes extraction buffer (EB) : (physiological buffered saline, AFT, pH 7.2-7.4 + 1% PVP-10 or AFT + 0.2% DIECA or AFT + 0.2% merceptoethanol). 0.19 ml were dispensed per plate well. Ten healthy plant controls of the same species as the sample tested were placed on the plate (8 on the first column and 2 in another area of the microplate). In addition, 4 wells were used to dispense each dilution of standard dilution (SD) as positive CTV control. The SD was prepared starting from freeze dried Mexican lime material [*Citrus aurantifolia* (Christm.) Swing.], infected with the T-308 strain. Freeze dried antigen was maintained at -20°C. As needed, an extract was prepared at 1 weight in 60 volumes of extraction buffer (1W:60v), and filtered through cheescloth before dispensing in the plate. Four consecutive dilutions were prepared from the 1/60 stock dilution. 3) The rest of the operations, such as washings, conjugate addition and substrate preparation were accomplished as usual (CAMBRA, 1983), but using high quality conjugates of monoclonal origin (3DF1) at high dilution (usually 1/2000 in EB) of a stock solution stored in 50% glycerol at -20°C. 4) Plate reedings were made with a Titertek Multiskan (FLOW) automatic reader, at 405 nm. When the mean of positive controls (SD) reached a pre-established optical density, all wells were read. For comparison of Satsuma populations, the OD of the SD-1/60 was of 1.7-1.8. When CTV strains were compared, the readings were made when the SD-1/240 reached an OD value of 1.1-1.2. To better determine the most convenient time, serial readings were made every 30 min after substrate incubation at 35°C. The reader was zeroed on an empty plate from the same lot.

Differentiation of strains or isolates of CTV kept under greenhouse conditions.

Twenty two strains, thought to be common in Spain, from the collection of the Instituto Valenciano de Investigaciones Agrarias were comparatively tested by the ELISA-DAS technique (BALLESTER-OLMOS *et al.*, 1988), with the severe strain (coded T-388). All strains were inoculated to Mexican lime, sweet orange [*C. sinensis* (L.) Osbeck.] cv. Pineapple, citron (*C. medica* L.) and grapefruit (*C. paradisi* Macf.) cv. Duncan. One year after growing in a temperature controlled (18-26°C) greenhouse, 0.2 g of bark from the plant stem, was taken just below the first shoot. Samples from three plants of each species inoculated with the same strain or isolate were mixed and then analysed by the ELISA-DASc technique. Optical densities at 405 nm were measured and we calculated the mean of 4 replications per each strain and the mean of the 22 common strains.

Comparison of populations of early Satsuma trees infected with common CTV strains and with the severe strain.

During November and December, 270 samples from the early Satsuma cv. Okitsu were tested, using the ELISA-DASc technique, with the 3DF1 monoclonal antibody.

And after being tested for SY inoculation in the greenhouse, gave seedling yellows syndrome in specific indicator plants. For extract preparation, four young shoots from each tree were taken and each extract was dispensed into 4 wells. The same procedure was followed to test 148 samples of field trees of Satsuma infected with common strains present in Spain. For this purpose, areas of the Ribera Alta del Júcar (Valencia) were chosen for sampling. This area was known to be infected, presumably for years (MORENO *et al.*, 1983), and high rares of tristeza infection have been detected (CAMBRA *et al.*, 1988).

Virus-free Satsumas cv. Okitsu, obtained by shoot tip micrografting *in vitro* (NAVARRO, ROISTACHER, MURASHIGE, 1975) were used as negative controls.

Computerized distribution of optical density frequencies of OD was measured by grouping samples at 20 increasing intervals of OD from 0.00 to 0.5, up to 1.95 to 2.00.

Optical densities distribution in one single tree infected with the severe strain.

Twenty five shoots from 3 trees of the early Satsuma were tested for distribution of CTV by measuring optical densities obtained by ELISA-DASc assay. Four replications per sample were made. Trees infected with the severe strain were selected by biological tests.

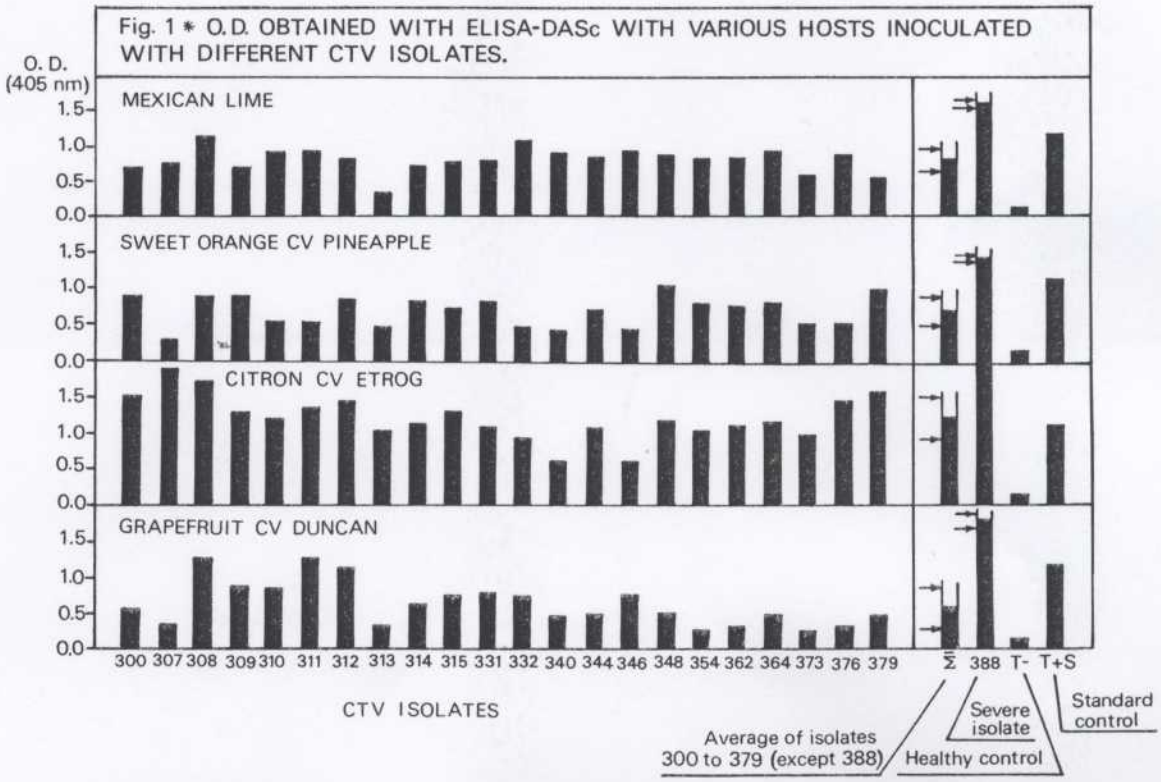
Parallel, infectivity ELISA-DASc and ds-RNA tests in trees infected with the severe strain.

Five budsticks, about 30 cm long, were chosen from each of the three trees previously used in the assays for OD distribution by ELISA-DASc. These samples showed the range of optical densities observed in a single tree in the previous experiment. One part of each budstick was kept for graft-inoculation on Mexican lime, sour orange (*C. aurantium* L.) and lemon [*C. limon* (L.) Burm. f.] cv. Lisbon, in the greenhouse, and the rest of the bark was used for ds-RNA analysis, following the method of DODDS and BAR-JOSEPH (1983), as modified by MORENO and GUERRI (1987).

RESULTS AND DISCUSSION

Mean OD values, obtained with 4 replications using the ELISA-DASc method, with different strains or isolates of CTV inoculated to different hosts in the greenhouse, are shown in Figure 1.

The recently introduced severe strain, coded T-388, gave the highest OD individual values ever recorded on any host assayed. The OD values of the severe strain were : 0.821 ; 0.734 ; 1.194, and were at least 0.780 OD units higher than the mean of all the other strains inoculated to Mexican lime, sweet orange, grapefruit and citron, respectively. The mean of the severe strain on citron surpassed the recording capacity of the automatic reader, and presumably, the difference with the mean OD in the rest of the strains would have exceeded 1.2 units.



Hosts providing the highest multiplication, for the common strains, were as follows, in decreasing order : citron, grapefruit, lime and sweet orange cv. Pineapple. Therefore, the severe strain seems to multiply more readily on Pineapple than the average of common strains assayed, since its mean differed significantly from them.

Strain T-308, known to be the most severe of conventional Spanish strains (BALLESTER-OLMOS *et al.*, 1988), provided the highest OD values from the common strains inoculated on lime, citron and grapefruit, but it consistently and clearly remained distant from the T-388 strain which is much more viruliferous (BALLESTER-OLMOS, PINA and NAVARRO, 1988).

The severe strain, therefore, could be clearly distinguished from the rest of strains assayed by the OD that it yielded in assays of experimental hosts grown under greenhouse. A close relationship between multiplication ability or OD produced in ELISA-DASc, using the monoclonal antibody 3DF1, and the virulence or aggressiveness of strain T-388 may be established, in these cases. Monoclonal antibody 3DF1 showed greatest affinity in previous assays with CTV strains (VELA *et al.*, 1986). Strain T-388 possesses, in addition, a ds-RNA profile different from the rest of the strains studied (MORENO and GUERRI, 1987) possibly because of its ability to cause the 'seedling yellows' syndrome (BALLESTER-OLMOS, PINA and NAVARRO, 1988).

Figure 2 shows the distribution of optical densities obtained by the ELISA-DASc technique in a population of 270 early Satsumas infected with the severe strain (SY). Mean optical density was 0.95. On figure 3 there is a similar distribution obtained with the ELISA-DASc technique under comparable distribution conditions to those in figure 2. Mean optical density of early Satsuma infected with common CTV strains was 0.51. Mean optical density of early Satsuma obtained from micrografting was 0.140± 0.025 in 250 tests conducted as control of all the experiments. In figure 4, both populations have been compared, one infected with the severe strain, and other with common CTV strains. The difference between both populations was clear-cut, and the OD means were significantly different.

Distribution of individual optical densities obtained from each of the three trees infected with the severe strain, was more similar to the distribution of the population infected with that strain than to that of trees infected with common strains. Individual means from each tree were : 0.94 , 0.89 and 0.98, respectively for trees A, B and C. Average of the three trees was 0.93 OD units at 405 nm, very similar to that reached by the population infected with the severe strain which was 0.95. Hence, OD distribution in a population reflects that obtained with their individuals. Means of optical densities of 25 shoots from one tree are also in agreement with the mean of a population of 270 trees in which 4 shoots per tree were taken.

Table 1 shows the results in OD units at 405 nm obtained by ELISA-DASc and the results of the infectivity tests and presence of typical 'seedling-yellows' ds-RNA profile, obtained from the five budsticks selected because of their distinct OD of the trees A, B and C, previously examined. In any of these trees the relation between SY reaction can be established on hosts grown under greenhouse, and presence of ds-RNA profile, typical of SY, according to MORE-

Fig. 2 * DISTRIBUTION OF O.D. (405 nm) OBTAINED WITH ELISA-DASc FROM EARLY SATSUMAS INFECTED WITH SEVERE STRAINS OF CTV-SY.

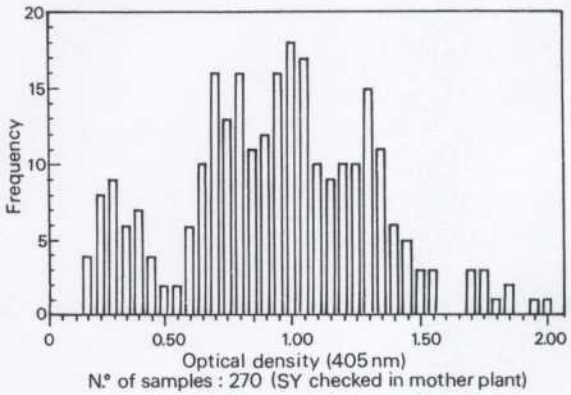


Fig. 3 * DISTRIBUTION OF O.D. (405 nm) OBTAINED WITH ELISA-DASc FROM EARLY SATSUMAS INFECTED WITH COMMON CTV STRAINS.

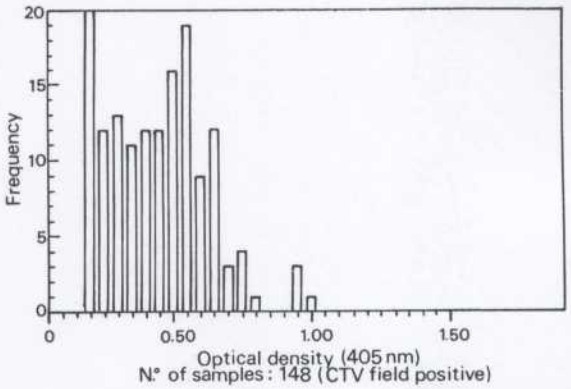


Fig. 4 * COMPARATIVE DISTRIBUTION OF O.D. FREQUENCIES (405 nm) OBTAINED WITH ELISA-DASc FROM EARLY SATSUMAS INFECTED WITH COMMON CTV AND SEVERE CTV-SY STRAINS.

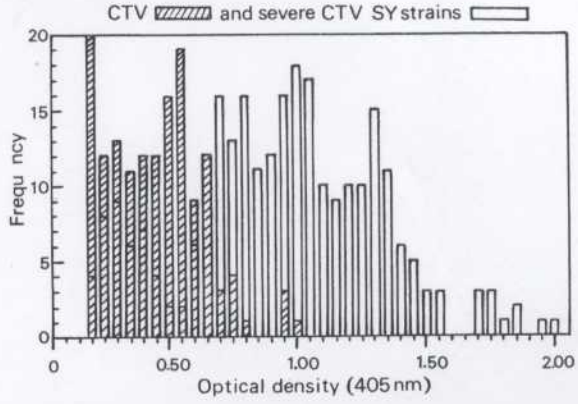


TABLE 1 - Optical density values (ELISA-DASc), symptom intensity and ds-RNA pattern obtained from different shoots of a single Satsuma (cv. Okitsu) tree infected with a severe CTV seedling yellows strain.

Tree	Budsticks	Optical density	Pittings on Mexican lime *	SY reaction *	ds-RNA pattern typical of SY
A	1	2,000	++++	++	yes
	2	1,341	++++	+++	yes
	3	0,857	++++	+++	yes
	4	0,674	++++	+++	yes
	5	0,483	++++	+++	yes
B	1	1,835	++++	+++	yes
	2	0,959	++++	+++	yes
	3	0,712	++++	+++	yes
	4	0,573	++++	+++	yes
	5	0,365	++++	+++	yes
C	1	2,000	++++	+++	yes
	2	1,733	++++	++	yes
	3	0,902	++++	++	yes
	4	0,730	++++	++	yes
	5	0,387	++++	+++	yes

* - Pitting severity and seedling yellow (SY) symptoms :
+ mild ++ moderate +++ severe ++++ very severe

NO and GUERRI (1987). This relation was, in addition, not dependent upon the specific optical density of each budstick. Therefore, the low optical density of a single sample was useless in trying to exclude the future production of SY syndrome by ELISA-DASc. However, identical profile of ds-RNA was obtained in the five budsticks of one tree which provided very different OD's.

CONCLUSION

There appears to be certain relationship between aggressive pathotypes and multiplication ability or capacity to provide high optical densities with the ELISA-DASc for strain T-388.

Thus, distinction of the severe strain T-388 by serology, using the ELISA-DASc technique appears to be feasible. The other strains assayed, common in Spain, when multiplied on different hosts grown under greenhouse, show consistently a lower titer when assayed by ELISA. The mean of optical densities of the common strains differed very significantly from the mean of the severe strain in all the hosts assayed.

Citron seemed to be the best host tested for the severe strain. This implies that citron allowed more successful virus multiplication, or at least, of its protein sub-units. Hence, it would seem appropriate to recommend it as a host for multiplication of this strain for purification or any other experimental work. One problem is that growth is strongly suppressed by the inoculation with that strain. Sweet orange, which does not undergoes such depression, is also a highly advisable host.

Populations of early Satsuma mandarin infected with the severe strain can be clearly distinguished from those infected with other strains or mixed with them, commonly occurring in Spain before the introduction of the severe strain. However, the distinction of severe strain in individual trees appears to be more troublesome using this method. The number of samples to be collected from each tree to ensure a conclusive assay would be large, and hence, not feasible as to be done routine use at large scale.

The enzyme linked immunosorbent assay, ELISA-DASc under the conditions of Spain, could, however, be implemented for the distinction between populations of early Satsuma trees infected with the severe strain and populations of trees comtaminated only with common strains. The conclusion could be extended to other Citrus growing countries in which, if there are no severe strains, the distinction between recently introduced strains on a certain host, and those currently occurring, would be feasible.

The implementation of the ELISA-DASc is simple and allow the analysis of numerous samples that require comprehensive surveys in an eradication programme. The only delicate point is extract preparation for analysis, which requires greater care than the conventional method, but much less than the necessary to perform the ds-RNA analysis.

The ELISA-DASc method provides only partial information, and hence, for research programmes or for final cataloguing of one strain there will be necessary to turn to infectivity tests, to the analysis of ds-RNA, and to other tests available. Nevertheless, as long as the severe strain continues to remain established in early Satsuma mandarins, the ELISA-DASc test may be highly valuable for preliminary analyses aiming at eradication of severe strains in Spain and in other countries with similar conditions within the Mediterranean basin.

There exists a positive correlation between SY reaction on hosts grown under greenhouse, upon inoculation with the severe strain of early Satsuma, and the presence of a

ds-RNA SY typical profile. This relation is not dependent on the OD found by the use of ELISA-DASc. It therefore implies that SY is probably a component of the viral genetic information, as it was reported by a number of authors. Any budstick from a tree infected with the severe strain, will be carrier of the ability of triggering SY symptoms.

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ERKENNUNG MIT ELISA-DASc (QUANTITATIV) VON POPULATIONEN, DIE MIT VIRULENTEN UND GEMEINEN STÄMMEN DES TRISTEZA-VIRUS DER ZITRUSFRÜCHTE INFIZIERT SIND.

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KURZFASSUNG - Die ELISA-DASc-Methode (Enzyme-Linked Immunosorbent Assay Double Antibody Sandwich comparative) wurde entwickelt ; sie ermöglicht eine Vergleichsstudie von Stämmen oder Isolaten des Tristeza-Virus der Zitrusfrüchte (CTV) unter quantitativen Voraussetzungen und unter Verwendung des monoklonalen Antikörpers 3DF1 und eines Standardantigens.
Die Methode ermöglichte eine Differenzierung zwischen gemeinen und virulenten CTV-Pathotypen, mit denen verschiedene Gewächshauswirte geimpft worden waren. Mit diesem Verfahren können Wechselwirkungen zwischen virulenten Pathotypen und dem Vermehrungspotential bzw. der Produktionskapazität bei hohen optischen Dichten mit ELISA-DASc analysiert werden, aber diese Wechselwirkungen sind unabhängig von der SY-Reaktion («seedling yellows»).
Die ELISA-DASc-Methode erlaubt eine Differenzierung zwischen Satsuma-Beständen, die im Freiland mit gemeinen Isolaten infiziert sind, und Populationen, die mit einem virulenten Isolat befallen sind, das das SY-Syndrom induzieren kann. Der Einsatz der Methode ist einfach und ermöglicht zahlreiche Analysen, die für Prospektionskampagnen und Ausrottungsprogramme notwendig sind, wobei Laborergebnisse miteinander verglichen werden können.

DISTINCION DE POBLACIONES INFECTADAS CON RAZAS SEVERAS Y COMUNES DEL VIRUS DE LA TRISTEZA DE LOS CITRICOS EN ESPANA, MEDIANTE ELISA-DASc (CUANTITATIVA).

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RESUMEN - Se ha puesto a punto el método ELISA-DASc (Enzyme-Linked Immunosorbent Assay Double Antibody Sandwich Comparative) que permite la comparación de razas o aislados del virus de la tristeza de los cítricos (CTV), en condiciones cuantitativas, utilizando el anticuerpo monoclonal 3DF1 y un antígeno standard.
El método ha permitido la diferenciación entre patotipos comunes y severos de CTV, inoculados en diferentes huéspedes cultivados en invernadero. Se ha podido establecer relación entre patotipos severos y poder de multiplicación o capacidad de proporcionar altas densidades ópticas en ELISA-DASc. Esta relación es, sin embargo, independiente de la reacción de seedling yellows (SY).
Con el método ELISA-DASc, se han podido distinguir poblaciones de Satsuma infectados en campo con aislados comunes, de las infectadas con un aislado severo capaz de inducir el síndrome de SY. La utilización del método es simple y permite realizar los numerosos análisis que son necesarios en prospecciones y programas de erradicacion. El método es también adecuado para comparar los resultados obtenidos en diferentes laboratorios.