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Full Length Research Paper

Discrimination of citrus tristeza virus (CTV) strains using Mexican lime/citrange Troyer combinations (*Citrus poncirus*/*Citrus trifoliata* x *Poncirus sinensis*)

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Two strains of citrus tristeza virus (CTV) were studied for six years in Yaounde in the forest zone of Cameroon. These strains, S_NCL2 and S_NCL4, were characterized on Lisbon lemon in Nyombe in the littoral zone of Cameroon. They were inoculated onto combinations of Mexican lime/citrange Troyer. The virulent strain S_NCL2 induced a lower lateral growth of the trunk of stocks and scions of six years old combinations. These observations were made three years after inoculation. Tristeza symptoms could not allow for differentiation between the two strains of CTV.

Key words: Tristeza, viral strains, discrimination.

INTRODUCTION

Tristeza, caused by the citrus tristeza virus (CTV), is one of the most important factors limiting citrus production worldwide by infection of all citrus species, hybrids, and relatives (Roberts et al., 2001; Brlansky et al., 2005). CTV, a member of the closterovirus group, is the largest known plant virus, having the shape of a long flexuous rod about 11 x 2,000 nm in size. The genetic material of the virus consists of a single strand of ribonucleic acid (RNA), which is enclosed by a protein coat. Recent molecular studies have allowed complete sequencing of several isolated and have revealed that there are exten-sive differences among various isolates (Roberts et al., 2001). Stephen and Brlansky (2005) report that, CTV strains or isolates may vary from mild to severe, causing little damage to severe decline, especially on trees gra-fted on sour orange rootstock. In cases of infection with mild isolates in trees grown on susceptible rootstocks, trees may be reduced in size, vigour, and fruit yields. Trees with a severe strain may quickly decline and die, with the first symptoms being leaf wilt and ultimate tree death in several weeks.

Studies and observations made in Cameroon reveals the presence of the stem pitting effect of tristeza (Rey, 1982; Aubert, 1986). The damages and losses caused by this effect exceed those caused by the declining of varieties grafted on sensitive stocks (Salibe, 1977) . In Cameroon, the disease and its most important vector, the brown citrus aphid *Toxoptera citricidus* kirk, are found in all the wetland with north limit at the buttresses of Adamaoua massive.

Several techniques allowing the characterization of viral strains are either under investigation, or no transposable of an ecosystem orchard to another. The characterization associated with the appreciation of the varietal behaviour in natural conditions permit the intervention against tristeza in zone where the disease is endemic. This study is aimed at discriminating between two tristeza virus strains. These strains were characterized in littoral zone on Lisbon lemon tree.

MATERIALS AND METHODS

Plant material

The plants used for the viral strains discrimination were grown under isolation cage. These plants were obtained by the grafting of Mexican lime on citrange Troyer. The citrange Troyer was used to minimize the risks of destruction of plants by *Phytophthora gummosis*. Sowings of citrange Troyer were carried out at a distance of 20 x 30 cm. The scions were taken in Garoua (north Cameroon) on the same disease-free tree. The absence of infection of this tree by the CTV was confirmed by enzyme-linked immuno-

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Table 1. Tristeza symptoms tolerance index.

Reaction level	Tolerance index	Vein clearing on foliar veins	Stem pitting on wood-and-bast base
0 absent	4 (++++)	No symptom	No symptom
1 weak	3 (+++)	Scare discoloured dashes on foliar veins	Few dense little cortical growths
2 moderate	2 (++)	Discoloured dashes of average size but not very dense	Small and very dense cortical growths or growths of average size but few dense
3 severe	1 (+)	Discoloured and dense dashes of average size but without coalescence	Big and dense cortical growths but without invaginations of Peel back
4 very severe	0 (-)	Enlightenment of all vein length	Dense cortical growths of big size and presence of hollow on Peel back

sorbent assay (ELISA), a rapid laboratory test that detects CTV in plant tissue by means of antibodies reaction with the virus. The plants obtained were regularly pruned below the cage height.

Strains characterization

The two viral strains used were characterized at Nyombe (littoral zone of Cameroon) on Lisbon lemon tree. The S_NCL2 viral strain provokes severe stem-pitting symptoms, whereas the S_NCL4 strain provokes moderate symptoms.

Plants inoculation

Inoculation was carried out on three years old healthy plants, by double grafting of the shield of Mexican lime on citrange Troyer. The experiment was carried out according to the randomised complete design with eight repetitions per strain and controls. A coloured plastic fabric marked the various treatments. The symptomatic observations and measurements were made on these plants three years after inoculation.

Foliar and cortical symptoms notation technique

Tristeza symptoms intensity is evaluated on the basis of the size and density of cortical or foliar anomalies (stem pitting or vein clearing, Table 1). Stem pitting indicates projections of bark in wood, with formation of invaginations under depressed on the surface of sapwood (Salibe, 1977). Vein clearing indicates observable clear transparent dashes on the segments of veins of the leaves that have just reached their physiological maturity. These segments of vein are abnormally gorged with water (Aubert, 1982).

The notation scale includes five levels of reaction varying between 0 (absence of reaction) to 4 (very severe reaction). The evaluation of symptoms intensity at the canopy level takes account of the growth stage of this one. According to the tree orientation compared to sun orientation, the canopy is divided into eight sub sectors (Aubert, 1985). A piece of branch of 6 to 8 months old and 4 cm length is taken at the level of each sub sector for the evaluation of stem-pitting reaction level after having removed the bark. Eight branches are thus observed per plants. In the same way, four new mature leaves were observed by sub-sector of the canopy. Thirty two leaves were thus observed per plant. The leaves were checked for transparency under the sun for evaluation of the vein clearing reaction.

The percentages of organs per type and level of reaction were calculated, which allow for calculating an index of tolerance, a weighted average. This index allow to establish a scale of tolerance defined by "+" and "±". A sign "+" correspond to a point of index of tolerance, a sign "±" correspond to the fractions of index of tolerance varying from 0.25 to 0.75. The other fractions are round by defect or excess.

ELISA

ELISA serologic test of CTV detection was carried out by utilisation of bark rough extracts to confirm the infection and to ensure themselves of the absence of infection of healthy plants. The standard technique of double sandwich (DAS-ELISA) was used in accordance with the protocol recommended by the kit diagnosis marketed by SANOFI Animal Health and INRA Knowledge.

RESULTS

Pathogenic reactions caused by various strains on Mexican lime/citrange troyer combinations

Cortical and foliar traditional anomalies observed on S_NCL2 and S_NCL4 strains inoculated plants did not allow the differentiation of viral strains by the means of symptoms notation technique. The index of sensibility for the vein clearing symptom was 1.62 and 1.87 for S_NCL2 and S_NCL4, respectively, whereas for stem-pitting symptom, this index was 1.68 and 1.62, respectively, for S_NCL2 and S_NCL4 viral strains (Table 2).

The delay under which the vein clearing on leaves of flushes in emission appears was 21 days for the two strains. Some more or less atypical reactions were observed compared to the healthy plants. Beyond the date of appearance of the first discoloured dashes on leaves, the young branches twisted, the internodes became shorter, the liberated leaves were reduced and presented a yellowing more or less characteristic of a zinc deficiency (Anonyme, 1975). All these phenomena (more severe for the strain S_NCL2) resulted in a much earlier growth stop than healthy controls. The liberated leaves were much more stunted compared to those liberated from healthy plants.

Influence of S_NCL4 and S_NCL4 strains on the growth of Mexican lime/citrange Troyer combinations

The strain S_NCL2 had a significant negative influence

Table 2. Symptomatologic tolerance index and scale on Mexican lime/citrange Troyer.

Strains	Vein clearing on leaves		Stem pitting on branch	
	Tolerance index	Tolerance scale	Tolerance index	Tolerance scale
S_NCL2	1.62	++±	1.68	++±
S_NCL4	1.87	++	1.62	++±
control	4	++++	4	++++

Table 3. Mean diameter of scions and stocks infected or uninfected by S_NCL2 and S_NCL4 viral strains.

Strains	Mean diameter (cm)			
	scion	C.V. (%)	stock	C.V. (%)
S_NCL2	2.18b[X]	23.3	1.89b	24.3
S_NCL4	2.70a	10.7	2.46a	11.3
Control	2.88a	7.9	2.56a	7.8

C.V: coefficient of variation
X: Means sharing similar letters within the same column do not differ from each other at P = 0.05

Table 4. Mean compatibility index (MCI) and mean size of young growths (MSG21) at 21 days.

strains	MCI	C.V. (%)	MSG 21	C.V. (%)
S_NCL2	0.87b[X]	4.5	17.62a	20
S_NCL4	0.89ab	3.2	26.62a	25
Control	0.91a	2.1	26.62a	20

C.V.: coefficient of variation.
MCI: fraction of stock diameter over scion diameter.
X: Means sharing similar letters within the same column do not differ from each other at P = 0.05.

($P \leq 0.05$) on the diametrical growth of the two parts of combination, like on their compatibility. This resulted in particular in circumferences of the stock and scion lower than those of the healthy controls and plants inoculated by the strain S_NCL4 (Table 3). The grafting compatibility index is also significantly different between the healthy plants and the plants inoculated by the strain S_NCL2 (Table 4).

The side growths of plants were not significantly different between the plants inoculated by the strain S_NCL4 and healthy controls (Tables 3 and 4). The average diameter of the stocks and scions of the combinations showed a greater variability for S_NCL2 strain compared to S_NCL4 strain and controls (Table 3). Concerning the young growths size 21 days after their emission at the beginning of the rains, no significant difference between the three treatments was observed (Table 4).

DISCUSSION

The tristeza virus is associated with phloem cells (Aubert, 1982). At the level of this tissue complex, CTV cause anatomical, physiological and biochemical disturbances whose intensity varies according to strain virulence and viral titre. This explains the differences observed on the side growth of plants caused by the disturbance of the functioning of sapwood that controls the side growth. The greatest variability of side measurements observed on the plants infected by the S_NCL2 strain should result from the differential migration of infecting viral complex isolates and which appeared at the level of grafting shields. On this assumption, it is question of determining if a selective pruning based on a sectoral discrimination of the strains infecting a crown would not allow the reduction of the tristeza effects. Such strains discrimination could be based on immunological and molecular techniques or on fine evaluation and pathogenic reactions (Ndongo-Bekolo, 1989).

As for the viral titre, it is normally low in season of vegetative rest (Bar-Joseph et al., 1988). This titre increases gradually at the favour of first flushes phloem until a certain threshold that corresponds to first leaves symptoms expression. Beyond this threshold, the physiological disturbances resulting in morphological changes become so significant and induce reduction in early growth compared to the healthy controls. These disturbances would also affect subsequent flushes emission and growth that depend on conducting channels production by first growths for sap transport. Time is thus necessary to be able to observe the reliable differences on the apical growth between healthy and infected plants. Roberts et al. (2001) report that Mexican lime has been the standard indicator (bioassay plant) used to confirm the presence of CTV. However, it is not useful for differentiation of seedling yellows, decline on sour orange, or stem-pitting components. Indeed, the great sensitivity of the Mexican lime masks the differences which make the strains discrimination at the symptomatologic level possible. All these considerations make Mexican lime use not reliable technique for strains discrimination. A good discrimination technique must be fast and sensitive (Cambra et al., 1979). In addition, the specificity of host-parasite relation always does not allow results extrapolation.

Nevertheless, the results of this work show that Mexican lime can be an excellent indicator of strains virulence in zone where the disease is endemic. In addition, these results also show that there is a potential of mild strains in the viral complex present in Cameroon. It is possible to combine the advantages offered by the mild strain cross protection techniques with biological control strategies against plant aphid's vectors. Such a cocktail would make limitation of the harmful effects of the surinoculations of virulent strains by plant aphids possible. Caver et al. (1993) report that *Aphis colemani* Viereck is an effective agent of biological control of *T. citricidus* Kirk, the efficient

vector of CTV.

Conclusion

The Mexican lime/citrange Troyer combination made the discrimination of two tristeza virus strains different by their virulence possible. These strains were characterized on Lisbon lemon tree. The parameter used for this discrimination was the side growth of the trunks of scion and stock combinations. These measurements were carried out three years after inoculation of three years old plants. In practice, it is not convenient to wait six years to be able to discriminate the virus strains. But the results obtained show that the port of the Mexican lime can be a good indicator of tristeza virus strains virulence in natural conditions. The pathogenic reactions and the size of the young growths at 21 days did not allow discrimination of the two CTV strains.

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