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Assessment of the sanitary status of citrus germplasm

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SUMMARY - This paper is focused on the assessment of the sanitary status of citrus germplasm. In addition to the common laboratory methods, an overview is given of the use of biological indicators, which are still compulsory in the sanitary assessment of selected germplasm and field observations, collection and storage of plant material for the success of the sanitary assays.
The detection facilities and the laboratory assays for the most important citrus graft-transmissible diseases, such as serological and molecular techniques, including ELISA, DTBIA, sPAGE, Dot Blot and PCR are reviewed as well.

Key words: Dot blot, DTBIA, ELISA, PCR, graft-transmissible pathogen, indexing, electrophoresis

RESUME *Le travail est axé sur l'évaluation de l'état sanitaire des ressources phylogénétiques; en plus des méthodes courantes de laboratoire, un aperçu est donné sur l'utilisation des indicateurs biologiques, qui restent d'une importance primordiale dans l'évaluation de l'état sanitaire des ressources génétiques sélectionnées, et les observations au champ, collecte et conservation du matériel végétal pour la réussite des tests sanitaires.*
Les structures utilisées pour la détection et les tests de laboratoires des principales maladies transmissibles par greffage, entre autres sérologiques et moléculaires, incluant l'ELISA, le DTBIA, le sPAGE, le Dot Blot et la PCR sont aussi passés en revue.

Mots clés *Dot Blot, DTBIA, ELISA, PCR, pathogènes transmissibles par greffage, indexage, électrophorèse*

Introduction

The status of germplasm collections is lamentably poor in many countries because of diseases, pests and technical and financial management shortcomings. Since most of the pests are quarantine pests, the exchange of material is seriously hampered.

The detection of citrus virus and virus-like diseases has significantly developed over the past half century. At present, in addition to biological indicators, which are still compulsory in the sanitary assessment of selected

germplasm, many laboratory methods ranging from culturing of the causal agent (i.e. *Spiroplasma citri*) to molecular biology have been devised along with field techniques, such as water uptake or mineral content of trees (i.e. blight disease) which can detect physiological changes.

Serological and molecular techniques are a valuable adjunct to field observation. They are user-friendly, cost-effective and time-saving, while allowing mass detection of the pathogen. Moreover, their use is also desirable for a preliminary screening within the framework of sanitary assays to be carried out on the selected germplasm.

Any accurate assessment cannot do without biological indexing in citrus, as some diseases are of unknown aetiology and serological and molecular techniques are not 100% reliable.

Hence, positive and negative controls must be included in each test so as to provide indication of possible environmental, pest- or contamination-related damage and help with pathogens or diseases not yet characterized.

Collection of citrus plant material for sanitary assays

The collection of samples and their storage are of utmost importance to successful detection. The type of explant, pathogen, sanitary assay and time of the year when the pathogen is more concentrated in plant tissues or organs have to be taken into account.

Care must be taken to use disinfected clippers when moving from a tree to the next and keep samples moistened and fresh using plastic bags or Petri's dishes and an ice chest, after labelling. Plant material is preferably used fresh but it can also be maintained for some time, according to the type of explant and pathogen.

The detection of viroids and phloem-restricted prokaryotes requires samples which have been collected late in the summer when temperatures have remained high (more than 30°C) for an extended period of time, whereas samples for virus and virus-like disease detection are best collected in the spring.

Preliminary screening

Field observations

Citrus graft-transmissible pathogens may induce symptoms in field trees which can be diagnostic for a specific disease. Generally speaking, any consistent, measurable or striking change in the morphology or chemistry of

the plant brought about by the direct or indirect presence of a pathogen can be considered as an index. However, clear diagnostic symptoms are not always apparent as they may be induced by other pests or environmental conditions.

It is also important to highlight the latency of those pathogens which do not cause symptoms in a number of hosts and may give little or no diagnostic evidence of their presence in the field (i.e. certain CTV isolates in mandarins; cachexia viroid in grapefruit.

Visual inspection in the field has then to be complemented by laboratory and greenhouse diagnostic methods.

Enzyme-linked Immuno Sorbent Assay (ELISA) and Direct Tissue Blot Immuno Assay (DTBIA)

In recent years, serological detection has been developed for several citrus pathogens which play a key role in diagnosis. ELISA test, the most commonly used being Double Antibody Sandwich (DAS) and Triple Antibody Sandwich (TAS), has been standardised and validated for sensitivity and specificity using commercial antibodies (polyclonal and/or monoclonal) which are available for the following pathogens: citrus tristeza virus (Bar Joseph *et al.,* 1979), citrus infectious variegation virus (Davino and Garnsey, 1984), citrus psorosis virus (D'Onghia *et al.,* 1998; Potere *et al.,* 1999; Alioto *et al.,* 1999; Djelouah *et al.,* 2000), citrus vein enation virus (Clark and Da Graça, 2000), *S. citri* (Saillard *et al.,* 1980), greening liberobacters (Garnier *et al.,* 1987), citrus variegated chlorosis and citrus canker (Lee *et al.,* 1992; Garnier *et al.,* 1993).

Some other pathogens may be ELISA-detected, such as satsuma dwarf virus (Kuhara *et al.,* 1982; Iwanami and Koizumi, 1992), citrus tatterleaf virus (Su and Tsai , 1990) and the agent of Witches' broom (Bové, 1995).

DTBIA a technique which requires very little sample manipulation, has also been successfully implemented for a rapid detection of CTV and CPsV using commercial antibodies. It has proved more sensitive and cheaper than ELISA (Garnsey *et al.,* 1993; Cambra *et al.,* 2000; D'Onghia *et al.,* 2001; Djelouah *et al.,* 2001).

Dot blot hybridization

A preliminary detection of the main citrus viroids, citrus exocortis (CEVd) and citrus cachexia viroid (CCaVd) can be successfully carried out by molecular hybridization, using digoxigenin-labelled RNA probes. The

hybridization of total RNA extracts from a small amount of citrus leaves ensures high sensitivity for the detection of these viroids in symptomless trees (Palacio *et al.,* 2000; Minafra *et al.,* 2001).

Completion of the sanitary assessment

In order to complete the assessment of the sanitary status of citrus germplasm stocks, which were found to be negative during the preliminary sanitary assays, biological indexing and electrophoresis procedures should be performed.

Graft transmission

Except for viroids which are detected by electrophoresis, graft transmissions using woody indicators remain compulsory in the production of healthy citrus germplasm whenever absolute certainty of sgraft-transmissible diseases freedom is essential.

The biotest is valuable especially for the detection of diseases induced by pathogen which is unknown or not detectable by other means.

Among several types of grafts, bud and bark graftings are the preferred ones for the inoculation of most virus and virus-like diseases (Roistacher, 1991).

Indicator plants may show symptoms which are specific for certain pathogens (i.e. vein clearing in a CTV-infected Mexican lime) or may only suggest a general infection (i.e. oak leaf pattern which may highlight the presence of concave gums or cristacortis or impietratura).

The time of onset of the first symptoms in indicator plants under optimum growth and temperature conditions varies widely with the citrus diseases indexed. When plants start to flush, observations are daily made for symptom expression, because they may disappear after a certain period (Roistacher, 1991) .The recommended indicator plants and the pathogens which can be detected are summarized in Tab. 1.

Tab. 1: Recommended indicator plants and detectable diseases

Citrus disease	Agent	Woody indicators (22-25°C)
Viroid		
Cachexia	CCaVd	Parson's special mandarin *
Exocortis	CEVd	Etrog citron *
Virus		
Leaf rugose	CiLRV	Mexican lime, Eureka lemon
Tristeza	CTV	Mexican lime
Vein enation-Woody gall	CVEV	Mexican lime, Sour orange*
Inf. variegation- crinkly leaf	CVV	Eureka lemon, Etrog citron
Psorosis	CPsV	Madame vinous sweet orange, Dweet tangor
Satsuma dwarf	SDV	Dweet tangor, Etrog citron
Tatterleaf	CTLV	Troyer citrange, Citrus excelsa
Mosaic	CiMV	Dweet tangor
Virus-like		
Concave gum, Cristacortis, Impietratura		Dweet tangor, Navelina orange, Grapefruit
Kumquat disease		Troyer citrange
Turkish citrus chlorotic dwarf		Mexican lime
Phloem-restricted prokaryotes		
Stubborn	*S. citri*	Madame vinous sweet orange*
Greening	*Liberobacter* sp	M. vinous orange*, Mexican lime*, Ponkan*
Witches' broom	WBDL	Troyer citrange*, Sweet lime*

*** T = 35°C**

Mechanical transmission

The use of herbaceous hosts is also desirable for pathogens which are mechanical transmissible as well as for the isolation and characterisation of new agents.

This method is used in citrus diagnosis concurrently with graft transmission with infectious sap which can be more effective

Exocortis, cachexia and other citrus viroids are primarily mechanical transmissible through cutting tools. Other pathogens may also be transmissible by this mode using sap inoculations into herbaceous hosts: satsuma dwarf, tatterleaf, psorosis, infectious variegation, leaf rugose, citrus mosaic viruses (Roistacher, 1991)

Tab. 2 : The main herbaceous hosts used for mechanical transmission.

Species	Disease	Pathogen
Chenopodium spp.	Psorosis, Tatterleaf, Leaf rugose	CPsV, CTLV, CiLRV
*Catharanthus roseus**	Greening, Stubborn, W. broom	*Liberobacter sp*, Spiroplasma citri,
Crotalaria spectabilis	Infectious variegation, Leaf rugose, Psorosis	CVV, CiLRV, CPsV
Chrysanthemum spp	Exocortis	CEVd
Cucumis sativus	Leaf rugose, Infectious variegation, Cachexia	CiLRV, CVV, CCaVd
Gomphrena globosa	Psorosis	CPsV
Gynura aurantiaca	Exocortis	CEVd
Petunia hybrida	Satsuma dwarf, Exocortis	SDV, CEVd
Phaseolus vulgaris	Leaf rugose	CiLRV
Sesamum indicum	Satsuma dwarf	SDV
Vigna spp	Tatterleaf, Infectious variegation, Leaf rugose	CTLV, CVV, CiLRV

* ***Transmission by dodder***

Electrophoresis

The electrophoretic analysis of the viroid RNAs present in the various exocortis isolates has demonstrated that, besides CEVd viroid RNA, other viroid RNAs are present.

Electrophoresis used to detect citrus viroids such as cachexia, exocortis and many of the more recently discovered citrus viroids is becoming the primary index for viroids difficult to detect by other methods. Where the laboratory is available and experience proven, sequential polyacrylamide gel electrophoresis (sPAGE) technique for detection of citrus viroids is preferred over the long-term biological index.

Semancik and Duran Vila (1991) outlined a concept for the grouping of citrus viroids based on molecular weights and catalogued five viroid groups as CEVd, CV I, CV II, CV III, CV IV.

Optimum resolution of viroid RNA is obtained by a sequential gel electrophoresis procedure, involving migration of the sample onto a standard gel (5%PAGE), followed by excision of a piece of the gel which is placed on a denaturing gel containing urea.

Electrophoresis is a very costly and delicate method, for which skilful personnel is required and therefore, indexing (graft- transmission) is still used in the assessment of citrus sanitary status.

Detection facilities

Laboratories equipped for serological, electrophoretical and molecular detection are necessary beside a facility for biological indexing.

Laboratories

Laboratories well equipped for serological (ELISA, DTBIA), molecular (Dot blot hybridization) and electrophoretical (sPAGE) detection are necessary. The preliminary assessment is carried out for some pathogens using diagnostic reagents available on the market.

Greenhouse and screenhouse

A heated greenhouse for the production of woody indicators and a controlled-climate greenhouse for biological assays make it possible to continuously test for different pathogens using three ranges of temperatures:22-24°C for virus and virus-like diseases; 32-35°C for viroids and phloem-restricted prokaryotes; 27-30°C to grow indicator plants. In addition, the soil mixture is considered of utmost importance, with its balanced supply of micro and macro nutrients for the production and care of indicators plants.

A collection of infected source plants, containing mild and severe citrus graft-transmissible diseases, should be maintained under an insect-proof screenhouse, which represents a *virus bank,* periodically indexed for the presence of pathogens.

The most important requirement to fulfil in all these facilities is to grow plants under isolated conditions (conservation of positive controls; indexed plants), to prevent outdoor contamination or disease spreading in the area (i.e. double net insectproof at the window openings of the greenhouse and in the screenhouse; double doors; sterile soil mixtures etc...).

Conclusion

The best method for the detection of citrus graft-transmissible pathogens must meet some requirements as being rapid, not expensive, reliable and

environmentally friendly, diagnostic methods for citrus plant viruses are being continuously improved; in recent years, research on nucleic acid has considerably advanced leading to the development of new methods for detecting genomic components of citrus viruses. However, although these new methods have been improved to detect the pathogens at levels below economic thresholds, they are not yet accessible to non-specialised laboratories.

Serological tests remain very useful as detection methods, due to their sensitivity, easy adaptability, relatively low cost and excellent large-scale use. Nonetheless, the time of the year, the sampling method, the antisera quality and the conditions under which the test is carried out have to be taken into account.

Despite the disadvantages of being laborious, time-consuming, and skill-demanding, woody indexing still remains necessary for the assessment of citrus sanitary status. In the future, even if new technologies may improve diagnostic reagents and methods, biological indexing will always represent the basis for the detection of most citrus graft-transmissible diseases.

It should be noticed that, in the last decade, more causal agents of these diseases have been discovered. Moreover, it has been demonstrated that some diseases which seemed to be caused by a single pathogen might involve several agents.

On the other hand, many strains require the use of different techniques as it occurs with severe and mild strains of CTV for which biological indexing and ELISA have to be applied, thus combining the use of indicator plants and electrophoretic analysis for viroid RNAs diagnosis.

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