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Technical procedures for the monitoring of *Citrus tristeza virus* (CTV)

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Abstract. Several Mediterranean countries are now strengthening phytosanitary measures to contrast the spread of Citrus tristeza virus (CTV) in accordance to the European Community (EC) regulations for the mandatory control of CTV. In this paper, an account is given on technical methods and procedures for planning, sampling, testing and plant elimination, which should be included in a regulation for an accurate control of CTV. An advanced organization of the virus monitoring and eradication programme is presented in order to provide Phytosanitary Services with the most advanced methods and efficient procedures.

Keywords. Citrus tristeza virus – Mediterranean – Monitoring – Sampling – Vector.

Amélioration des procédures techniques pour le suivi du virus de la tristeza des agrumes (CTV)

Résumé. *Plusieurs pays méditerranéens sont en train de renforcer leurs mesures phytosanitaires afin de bloquer la diffusion du virus de la tristeza des agrumes (CTV), ceci conformément à la réglementation de la Communauté Européenne (CE) sur le contrôle obligatoire du CTV. Dans cet article, un compte rendu est donné sur les méthodes techniques et les procédures à mettre en œuvre pour la planification, l›échantillonnage, les analyses et l›élimination des plantes, qui doivent êtres inclus dans une règlementation, ceci afin de contrôler efficacement le CTV. Une organisation plus accru du suivi du virus et du programme d›éradication est présentée, afin de mettre a disposition des services phytosanitaires, des méthodes plus récentes ainsi que des procédures plus efficaces.*

Mots-clés. *Virus de la tristeza des agrumes – Méditerranée – Suivi – Échantillonnage – Vecteur*

I – Introduction

Control of CTV is extremely difficult once the virus gets established in a region where its natural aphid vectors are also present. The ability to control the disease damages depends to a large extent on CTV incidence and on the virus strains and citrus varieties predominant in each region.

Several approaches have been used to control the losses caused by CTV, particularly, in areas where CTV is rare and its natural spread is limited. Beside the strict quarantine measures and the establishment of certification programs, elimination of infected trees is considered as the best means to avoid or delay an epidemic, if CTV has a low incidence and the infected trees are grouped in a limited number of foci.

Accurate information on the distribution and incidence of CTV-infected trees are of utmost importance for decisions to implement a CTV eradication or suppression program; if a larger number of infected citrus trees in the open field are destroyed before the onset of new infections, the incidence can be maintained at reasonable levels.

Accordingly, there is an urgent need for enforcing the national CTV control strategy in the Mediterranean countries, this strongly suggesting the need for continuously virus and vector monitoring. In this context, several procedures were employed over the world, but most of them were not extensive and were designed only for quantitative data, such as the number of infections per plant.

Recently, hierarchical sampling procedures were set up (Gottwald and Hughes, 2000), which increased accuracy and precision of estimates of CTV incidence without appreciable increase in the number of laboratory assays required. These slightly modified methods have been successfully implemented in Apulia region.

II – Legislation

A Ministerial Decree or a specific regulation for the mandatory control of CTV and its vectors, is the first step that a country has to take toward the establishment of a virus and vector monitoring programme. Moreover, laws and regulations are necessary for the certification of citrus nursery production, which represents one of the basic actions to control citrus quarantine pests.

In the Mediterranean basin, especially at EU level, several regulations (EU Directives 93/48/EEC, 93/64/EEC, 93/79/EEC) have been issued to control the entrance and dissemination of citrus pests or plant products and to set forth the phytosanitary requirements for the free movement of citrus propagating material throughout the Community.

III – Identification of the areas to be monitored

At the beginning of each monitoring campaign, the Plant Protection Service (PPS), in cooperation with all the stakeholders, draws up an action plan based on data collected in the previous years and on notifications and reports.

Monitoring is carried out in the areas listed below and according to the following priorities:

a) in groves where citrus trees are located in the range of at least 1 km from citrus nurseries, to be sure that nurseries are established in virus-free areas;
b) in groves where citrus trees are located in the range of 1 km from certification facilities (conservation, pre-multiplication blocks, mother blocks);
c) in groves where CTV-infected plants have been found and in contaminated areas and buffer zones for three years after the virus detection;
d) in citrus nurseries;
e) on extra-regional material, introduced also for scientific purposes;
f) in ornamental gardens, botanical gardens, variety collections, public and private parks;
g) in commercial citrus groves.

Whenever suspected cases are reported, the PPS shall provide for completion of visual inspections and tests, even if the areas concerned are not included in the annual monitoring program.

At the beginning of each monitoring campaign, the team coordinated by the designated phytosanitary inspector shall delimit the intervention areas based on the above criteria. These areas can be easily identified using aerial photographs, that is to say orthophotographs (with cadastral grid) which allow to visualise the citrus groves and the relative cadastral coordinates.

The annual planning takes into account the possible outbreaks which have previously been identified and the related contaminated areas. It is therefore possible to identify groves which shall be inspected, subdivided according to:

- locality;
- map sheet;
- land parcel (using the cadastral data).

IV – Sampling

1. Survey notification

After setting the survey time schedule, the PPS shall send an official notice to the interested person at least 10 days before the scheduled inspection date.The notice shall indicate the survey date and time and provide the interested person with:

- the right to be assisted by a trustworthy technical expert in all inspection steps and during the collection of samples which will be tested;
- the opportunity, when necessary, to delegate in written form a third party to be present during the inspection.

If on the scheduled monitoring day, the interested person or his/her delegate are not present and no major obstacle prevents the access to the field, sampling will be anyway carried out.

2. Sampling method

In order to optimise preventive actions while reducing monitoring time and costs, surveys must be organised per limited areas and exploiting at best the available working hours.

On the day scheduled, the monitoring team, in the presence of the owner or of a delegate, shall start surveying the citrus grove and collecting samples.

Two hierarchical sampling methods can be adopted according to whether one surveys CTV-free areas, contaminated areas, buffer zones or areas in the range of 1 km from nurseries (when commercial groves and nurseries are investigated). In all the other instances, not included in the above list (mother plants, budwood and seed sources, public and private gardens, scientific collections) samples shall be collected individually.

Besides the samples collected at random, some “out-of-scheme” samples are included, that is to say plants which display suspected tristeza symptoms.

A team unit is composed by three people:

- the first one shall carry out visual inspections, map out, draw up the scheme and “label” the plants to be sampled with an identification code and number;
- the second one shall collect the samples;
- the third one shall store the samples in polyethylene bags reporting the identification code or the membrane immunoprinting code.

Once the samples have been prepared, they are stored at the temperature of about 4°C. Alternatively to bag-storage, the sample can directly be immunoprinted onto a nitrocellulose membrane for further testing. The membrane is properly numbered and stored at room temperature until testing.

3. Sample collection

A. CTV-free area

Commercial groves. As set out and described in Fig. 1, an area of two hectares, representative of 10 hectares, shall be identified trying to include, where possible, a whole land parcel, homogeneous for the species and age. Hundred single samples (4 twigs per plant) shall be collected along the edge of the delimited plot.

Nurseries. 10% of individual samples are collected randomly from homogeneous lots as regards to the variety and age of the plants intended for sale.

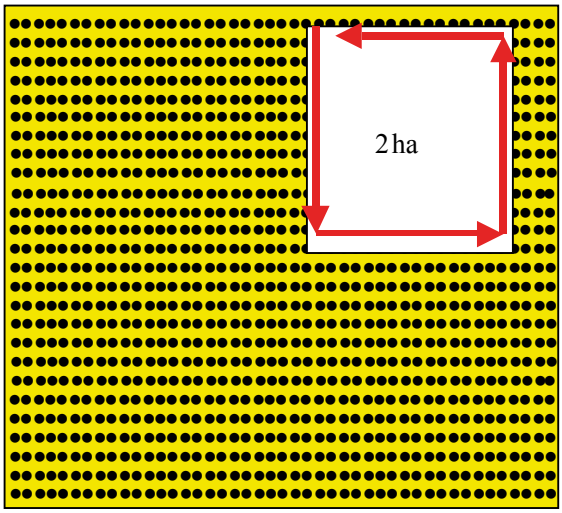

Figure 1. Sampling method adopted in CTV free area.

B. Contaminated areas, buffer zones and areas in the range of 1 km from nurseries and certification facilities

Commercial groves. Samples are collected per homogeneous citrus groves and managed by the same tenant, subdividing the plot according to the species/variety and age (where possible).

To collect the samples, the hierarchical sampling scheme is adopted (Gottwald and Hughes, 2000), samples are collected from 25% of the plants in the grove.

In the sampling method, a citrus plot is subdivided into squares made up of 4 trees each. To this end one has to start from the inter-row, counting 6 pairs of trees and sampling the seventh and eighth pair. In case of an irregular row spacing, it is also possible to go along each single row, sampling 4 trees and leaving aside 12 (Fig. 2). From each of the 4 trees in the square, four young twigs are collected from the different plant sides (since CTV is irregularly distributed in the plant) and they are individually tested through Direct Tissue Blot Immuno Assay (DTBIA).

The square plant samples are numbered clockwise, labelling the first plant of each group; the map shall also report the plants which are not sampled as well as the possible missing plants or plants belonging to other species. The single samples from the four plants, properly labelled, are placed into a bag, marked by a clear field code and the sample progressive number.

Nurseries. At the beginning, 25% of the plants in the lots intended for sale are sampled. They must be homogeneous as regards the variety and age. If no sample proves to be positive, the sampling shall be extended to the remaining 75% plants. Twenty five percent of the lot plants is represented by a number of samples (5-10) along the lot row which are collected from each of 15-30 plants; obviously, this scheme varies according to the lot age and its lay-out which may be regular or not.

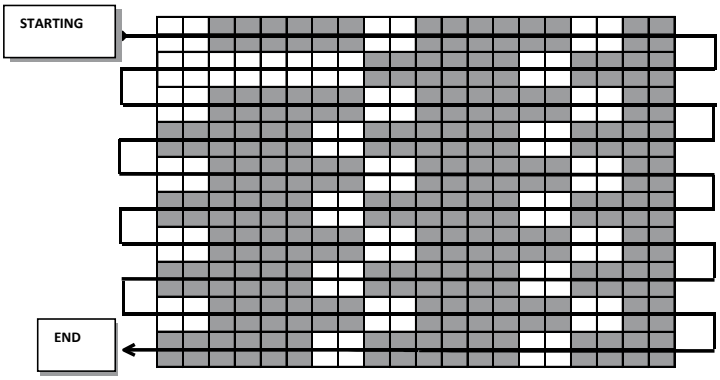

Figure 2. Hierarchical sampling scheme (Gottwald and Hughes, 2000),

4. Sampling report

During the survey the phytosanitary inspector must draw up the sampling report, which shall be signed by the parties and shall include the following:

- the identification data of the holding and of the grove, nurseries etc. (tenant, surface area, map sheet, land parcel);
- the plot data (total number of plants, species, rootstock, age and origin of the material, etc.)
- the persons present and their role;
- the number of plants present and the number of samples collected;
- the name of the accredited laboratory which will run the tests;
- the possible remarks.

A copy of an orthophotograph of the citrus grove shall be enclosed, if available, indicating the plots sampled. After the sampling, the bags with the samples shall be taken by the technical expert of the reference laboratory.

V – Laboratory tests

1. Sample analysis

The collected samples shall be analysed by the DTBIA method (Garnsey *et al.*, 1993) at the PPS laboratory or at an accredited laboratory designated by the competent authority. The analysis results shall be notified by the laboratory to the PPS no later than 10 days from the survey and, subsequently, to the citrus plot owner.

2. Confirmation tests

Confirmation tests are carried out by other laboratories, accredited for diagnostic analyses by biological, molecular or electronmicroscopy methods, when:

- the DTBIA result is considered doubtful by the laboratory which, therefore, asks the PPS to conduct a confirmation test;
- the infection is detected for the first time in a given area, considered free till that time;
- whenever deemed necessary.

In these cases the PPS shall promptly contact the plot tenant to inform him/her that a second survey shall take place to collect all samples requested for the confirmation tests.

The official laboratory technical expert and the phytosanitary inspector shall collect the samples in a sufficient amount to perform the confirmation tests. A report shall be drawn up during this survey as well. The confirmation test results shall be communicated to the PPS no later than 10 days from the sampling.

VI – Eradication of outbreaks

In order to prepare the mandatory eradication provisions, the PPS shall delimit the contaminated area and the buffer zone on map sheets. These sheets and the relevant data on the infected plot (locality, surface area, land parcel, map sheet, number of infected plants) and on the tenant (name, address and phone number) will be a basic part of the provision which designates the contaminated area and the buffer zone.

The contaminated area is delimited as follows:

- when the **infection percentage in the citrus grove is lower than 30%,** the distance of 500 metres shall be measured from the farthermost infected plants in the focus;
- when the **infection percentage is higher than 30%,** the distance of 500 metres shall be measured from the vertices of the infected plot and form the half of each plot side. The contaminated area shall be delimited on the map by joining with straight lines the farthest point of the circle with a radius of 500 metres.

The buffer zone shall be delimited extending by 500 mt all the sides of the contaminated area. If a plot or a nursery falls also partially within the buffer zone, it will be totally included.

The contaminated area and the buffer zone shall be included for three years in the monitoring programme for the area to be designated as decontaminated.

Based on the technical procedures, administrative provisions are then applied for:

- the mandatory eradication of virus-infected plants;
- the designation of virus contamination;
- the establishment of the buffer zone.

The PPS shall later notify the citrus grove owner of the mandatory eradication and shall send a phytosanitary inspector who will be present during the operations of uprooting and destruction of the infected plants and will draw up a report.

VII – Extension and training

Extension is at the core of a successful monitoring programme. Extension activities shall be intensively carried out at any time during monitoring, be CTV present or not. Only extension can gradually raise the stakeholders' awareness (farmers, professional associations, public bodies etc.) about the risks related to the spread of this disease in the territory. Indeed this is a true natural disaster which can sweep away the citrus industry in a country, with severe socio-economic repercussions and in many instances, also, endanger the cultural or landscape resources featuring a territory. This information campaign shall be conducted also mobilising the technical experts involved in the virus study and control and through proper media communication (brochures, posters, videos, TV ads, courses, workshops, web sites etc.). Training of people in charge of monitoring is fundamental for the success of all operations which are to be carried out in compliance with the laws in force in a country, timely and professionally.

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