Arab Organization For Agricultural Development

# Short Course On

# DIAGNOSIS OF PLANT VIRAL DISEASES

**MAI-Bari,Italy**

**8-13-September 2003**

# Course Programme

# P O R O G R A M M E

| Days | Time Table | Activity | Lecturers |
|---|---|---|---|
| 7th | | Arrival at Bari airport<br>Transfer to the Institute | |
| 8th | 09:00-13:00 | Main plant virological problems and their detection<br>Organization of a Centre for virological detection | A. Myrta |
| | 15:00-17:00 | Visit to the detection facilities of IAMB | |
| 9th | 09:00-13:00<br>13:00-17:00 | Virus identification by: Serological assays | K. Djelouah |
| 10th | 09:00-13:00 | Virus identification by: Serological and molecular assays | K. Djelouah<br>M. Rwahnih |
| 11th | 09:00-13:00<br>13:00-17:00 | Virus identification by: Molecular assays | M. Rwahnih |
| 12th | 09:00-13:00 | Virus identification by: Biological assays | A.M. D'Onghia |
| | 14:00 | Closing ceremony | |
| 13th | 09:00-17:00 | Technical visit: certification facilities and experimental fields | M. Digiaro<br>A. Myrta |
| 14th | | Departure from the Institute | |

# Foreword

# Foreword

In the framework of cooperation between the Arab Organization for Agricultural Development (AOAD) and the Mediterranean Agronomic Institute of BARI (CEUIAM), AOAD organized a regional short-course on the "**Diagnosis of Plant Virus Diseases**", which was jointly held at BARI headquarters in Italy during the period from 13 to 18 September 2003.

Participants from nine Arab countries attended the course which was oriented capacity building for Arab Cadres working in the field of diagnosis of plant viral diseases.

The course programme included some principal theoretical and practical sessions on virus identification by biological assays, serological assays, identification of viruses by molecular and direct tissue blotimmuno assay, and detection of the virological problems. The programme also included technical visits to the certification facilities and the experimental fields of Bari Institute, to let the participants be fully acquainted with the different virus diagnostic techniques.

In conclusion it gives us pleasure to extend our sincere thanks to his Excellency Dr. Cosiom Lagariznola, Director of BARI Institute, and their staff for their cooperation in organizing this training course. Thanks are also extended to the participants for attending the course and their will to convey what they have studied to their subordinates.

**Dr. Salem Al-Lozi**

**Director General**

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## التي إنعقدت بالتعاون مع معهد باري/سيهام
## إيطاليا 8 – 13/ سبتمبر (أيلول)2003

**خلفيـــــة :**

تم عقد هذه الدورة بالتعاون مع معهد باري بإيطاليا ، وذلك في إطار إتفاقية التعاون التي وقعتها المنظمة مع المعهد في يونيو (حزيران)2001. وقد تم إدراج هذه الدورة ضمن أنشطة خطة عمل المنظمة للعام 2003 – ضمن مكونات برنامج التعاون مع المؤسسات الدولية والإقليمية.

وقد أجرت المنظمة إتصالاتها منذ أول العام 2003 بمعهد بارى/ بإيطاليا التابع لمركز سيهام الدولى، حيث رحب المعهد بالتعاون مع المنظمة لعقد تلك الدورة خلال الفترة 8–13/سبتمبر(أيلول)2003.

وقد أتخذت كافة الترتيبات وتم عقد الدورة في الموعد المحدد لها ، وقد كانت ناجحة بكل المقاييس ومهدت لمزيد من التعاون مع مركز سيهام في المجالات التي تتسق مع مجالات عمل المؤسستين.

- **مبررات عقد الدورة :**
  - النقص في الكوادر المدربة على التقانات الحديثة المستخدمة عالمياً في مجال تشخيص الأمراض النباتية الفيروسية في المنطقة العربية.
  - ضعف إنتاجية المحصولات الزراعية من جراء تأثرها بالإصابات الفيروسية.

- **أهداف الدورة :**
  - تأمين كوادر عربية عالية التدريب في مجال إستخدام الأساليب التقنية الحديثة لتشخيص الأمراض النباتية الفيروسية.
  - زيادة الإنتاج وتحسين نوعيته من خلال السيطرة على الإصابات الفيروسية التي تتعرض لها المحاصيل الزراعية في المنطقة العربية.

- **الفئات المستفيدة من الدورة :**

إستهدفت هذه الدورة حملة البكالوريوس من العاملين بإدارات وقاية المزروعات بوزارات الزراعة العربية ، ممن لا تقل مدة خدمتهم في المجال عن ثلاث سنوات ، وبما أن الدورة قد نفذت على المستوى الإقليمي فقد شاركت في أعمالها ثمان دول عربية ، من بين الدول ذات الثقل في الإنتاج الزراعي في المنطقة العربية .

- **موعد ومدة ومكان إنعقاد الدورة :**

تم عقد هذه الدورة بمدينة بارى/بمقر المعهد في فالينزانو بإيطاليا خلال الفترة 8 – 13 سبتمبر (أيلول)2003.

- **النتائج المتوقعة من الدورة :**

- تأمين كوادر مدربة للعمل كمدربين لمن يليهم في مجال تشخيص الأمراض الفيروسية التي تصيب المحاصيل الزراعية.
- إنخفاض الإصابات الفيروسية في المزروعات وزيادة الغلة المحصولية.

- **موضوعات التدريب : تشمل :**

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1. الأمراض الفيروسية الرئيسية التي تصيب الأشجار المثمرة والمحاصيل الحقلية في منطقة البحر الأبيض المتوسط.
2. إختبارات الكشف عن الأمراض الفيروسية التي تصيب النبات:
   - ❖ طرق الكشف البيولوجي.
   - ❖ طريقة التطعيم.
   - ❖ الكشف السيرولوجي.
   - ❖ طريقة الإنتشار المناعي في الأجار.
   - ❖ طريقة الكشف بإستخدام الإليزا.
   - ❖ طريقة الكشف المناعي من خلال الإنتشار الخلوى.
   - ❖ إستخدام الإختبارات الجزيئية الحيوية.
   - ❖ إختبار التفاعل على سلسلة البلمره.
   - ❖ طريقة التهجين الجزيئي.
3. طرق وأساليب التحكم في تفشي الأمراض الفيروسية.
4. إجراءات الحجر وإصدار الشهادات الصحية.
5. إستخدام الهندسة الوراثية في إستنباط إصناف نباتية مقاومة للأمراض الفيروسية
6. إستخدام الهندسة الوراثية في إستنباط إصناف نباتية مقاومة للأمراض الفيروسية.

- **المقترحات التي طرحها المشاركون في الدورة :**

لتقييم أعمال الدورة والتثبت من جدواها صممت المنظمة إستمارة إستبيان تم توزيعها على المشاركين لتعبئتها وإعادتها بدون أسماء ، ليتمكنوا من تسجيل ملاحظاتهم ومقترحاتهم بحرية تامة، وفيما يلي أهم ما ورد بتلك الإستمارات من ملاحظات :

1. يقترح المشاركون مد فترة مثل تلك الدورات لأسبوعين على الأقل لتمكينهم من إتقان إستخدام التقانات التشخيصية الحديثة التي يتيحها برنامج الدورة.
2. أمن المشاركون جميعهم على أهمية هذه الدورة بما أتاحته لهم من الإلمام بالجوانب التطبيقية للتقانات الحديثة المستخدمة في مجال تشخيص الأمراض النباتية الفيروسية ولمساعدتهم في التعرف على زملائهم المختصين في هذا المجال في الدول العربية الأخرى ، مما سيتيح لهم مزيداً من الترابط وتبادل الخبره ونتائج البحوث.
3. يقترح المشاركون أن يتم تكرار عقد هذه الدورة كل ثلاث سنوات لذات المجموعة ــ من أجل متابعة التطورات في المجال.
4. أمن المشاركون على ضرورة قيام المنظمة وبالتعاون مع وزارات الزراعة العربية بإقامة شبكة للبحوث في مجال الفيروسات التي تصيب المزروعات.

- **المشاركون في الدورة :**

شارك في أعمال هذه الدورة متدربون من ثمان دول عربية ، هي الدول التي تمكنت من الحضور من بين (11) دولة وجهت لها الدعوة ، وبيانهم كما يلي :

| الأســـــــم | البلد |
|---|---|
| د. عدنان عثمان | الجمهورية العربية السورية |
| ناصر عبد العزيز التميمي | المملكة الأردنية الهاشمية |
| عبد الله سالم أحمد جنعان | دولة الإمارات العربية المتحدة |
| محمد خليفه لقرن | الجمهورية التونسية |
| يوسف بن محمد بن مراد الرئيسي | سلطنة عمان |
| معاوية العيدروس | جمهورية السودان |
| د. إيليا شويري | الجمهورية اللبنانية |
| بورزيق ميمون | المملكة المغربية |

- **تقييم أعمال الدورة :**

تم تقييم أعمال هذه الدورة من خلال إستمارات إستبيان ، تم توزيعها على المتدربين للأخذ ملاحظاتهم على محتوى البرنامج ومستوى تنفيذه ، وقد كانت النتائج كما يلى :

| البيـــــــان | النسبـة المئويـة للإجابـات | | |
|---|---|---|---|
| | ممتـاز | جيـد | مقبـول |
| 1. مدى شمولية تغطية برنامج الدورة للموضوعات الهامة في مجال تشخيص الأمراض الفيروسية | 87.5 | 12.5 | 0 |
| 2. مدى توازن الجانبين النظري والعملي في برنامج الدورة | 62.5 | 37.5 | 0 |
| 3. إلى أي مدى كان الجانب التطبيقي (العلمي) مفيداً | 88.0 | 12.0 | 0 |
| 4. إلى أي مدى أضاف برنامج هذه الدورة معلومات ومهارات جديدة لك | 87.5 | 12.5 | 0 |
| 5. مدى الاستفادة من تجارب الدول المشاركة في الدورة ، من خلال ما طرحه ممثليها من المشاكل والصعوبات التي تواجههم | 60.0 | 34.0 | 6.0 |
| 6. مدى الاستفادة فنياً واجتماعيا من الدورة | 63.0 | 25.0 | 12 |
| 7. مستوى تنفيذ برنامج الدورة من قبل المحاضرين ومساعديهم | 87.5 | 12.5 | |

# Serological Detection of Viruses Included in Certification Protocols for Stone Fruits

# Serological detection of viruses included in certification protocols for stone fruits

**DONATO BOSCIA**
CENTRO DI STUDIO DEL CNR SUI VIRUS
LE VIROSI DELLE COLTURE MEDITERRANEE
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**Summary:**

Stone fruits are affected by many viruses which are routinely detected by serological methods. The paper focuses specially on ELISA and other serological techniques reviewing reports for Trichoviruses, Potyviruses, Ilarviruses and Nepoviruses reported on stone fruits. Diagnostic problems that disturb the reliability of ELISA results are also discussed.

***Key words:*** stone fruits, serology, ELISA, plant viruses, certification.

***Resume***: Les essences à noyaux sont affectées par bon nombre de virus qui sont détecés par des méthodes sérologiques de routine. Dans le présent travail, l'accent est mis sur l'ELISA et d'autres techniques sérologiques, en passant en revue les Trichovirus, les Potyvirus, les Ilarvirus et les Nepovirus signalés sur les essences a noyaux. On discute également les problémes qui inerférent avec la fiabilité des résultants de l'ELISA.

**Mots-cles:** essences a noyaux, serologie, ELISA, virus des vegetaux, certification.

**Introduction:**

Serology is a traditional technique for virus detection, based on the use of antibodies, proteins of the immunoglobulin type, raised in animals and capable of specific binding to antigens. Early researches carried out with antibodies have been of great importance for the identification and classification of viruses, but, especially in the case of woody plants, they were not sensitive enough for routine diagnosis. A tremendous improvement in sensitivity was achieved with the development of immunoenzymatic techniques, i.e. ELISA (enzyme linked immunosorbent assay), based on the use of antibodies conjugated to an enzyme, able to reveal minimal concentrations of antigen.

The potential application of the serological diagnosis is limited to viruses for which antibodies are available; consequently only known viruses, but not diseases of unknown origin, can be detected. Moreover, since the antigenic properties reside in the coat proteins, viroids cannot be detected by this means.

**1. ELISA:**

ELISA is a diagnostic technique largely utilized for the identification of plant viruses. The presence of the antigen in infected sap is indirectly detected through a colorimetric reaction, that develops because of the reaction of an enzyme (alkaline phosphatase, horseradish peroxidase) conjugated to antibodies in the presence of an appropriate substrate (p.nitrophenylphosphate, tetrametylbenzidine). Among several variants of ELISA (Fig.1), DAS (Double Antibody Sandwich) ELISA is mostly used in routine diagnosis. Schematically, antigens are first trapped by antibodies coating the internal surface of polystyrene wells and then covered by the same enzyme-conjugated antibodies. Finally the addition of the substrate induces a colorimetric reaction, if the complex antigenenzyme antibody-conjugated is present.

The success of ELISA , that has been rapidly adopted in most diagnostic laboratories, is due to the numerous advantages that this technique offers in comparison with others (Clark and Bar Joseph, 1984).

- Sensitivity for detecting very small amounts of virus, i.e., antigen concentrations as low as 1-10 ng/ml.

- speed of reaction - results are usually available within 6-24 hr.
- scale of operation - several hundred samples can be readily handled, either individually or in groups.
- use with plant extracts and purified virus preparations.
- specificity, for differentiating serotypes.
- suitability for both intact and fragmented virons of different size or morphology.
- possibility of obtaining quantitative measurements.
- possibility of automation and of standardizing tests by the production and use of home made or commercial kits.
- low cost and relatively long shelf life of reagents.
- basic requirement for simple equipment.
- economical and efficient use of antibodies and antisera.

ELISA has been applied to viruses of stone fruit trees since its first introduction into plant virology in 1976. The first approach was with arabis mosaic virus (ArMV) and plum pox virus (PPV), representatives of isometric and filamentous viruses, respectively (Voller et al., 1976; Clark et al., 1976). The technique was applied later to the majority of the viruses for which antisera were already available.

Up to now, 22 viruses are reported as actual or putative agents of diseases affecting stone fruits. Considering that some have been reported only in North America, still 14-16 viruses remain (Table 1) for which tests have to be done for the production of certified plant propagating material in the EPPO countries: Trichovirus (ACLSV); Potyvirus (PPV); Ilarviruses (ApMV, PDV and PNRSV); Nepoviruses (ArMV, CLRV, CRLV, MLRSV, RRSV, SLRSV, TBRV and TomRSV); Foveavirus (CGRMV) Closterovirus (LCV) and possibly, Tombusvirus (PeAMV).

The above listed viruses, belonging to different genera or families, possess different antigenic properties and, consequently, induce the production of antisera and serological reagents characterized by different levels of sensitivity that, in some cases, are of low diagnostic power. This aspect, together with other important parameters, as the choice of the sample and the season of sampling, is of major importance in order to consider the ELISA

results reliable enough for satisfactory evaluation of the sanitary status of the plants.

**Monoclonal antibodies:**

For the absolute majority of viruses that have been isolated, purified and characterized as causal agents of a disease, serological diagnosis has been performed using polyclonal reagents and more recently, monoclonal antibodies as well. Monoclonal antibodies specific for the majority of the viruses infecting stone fruits have been already produced (Halk et al., b; 1984; Boari et al., 1998b; Myrta et al., 1998). Particularly important for the possibility to specifically identify the different virus strains are the PPV strain-specific monoclonal antibodies (Cambra et, at., 1994). Beside the well known advantages of monoclonal antibodies (specificity, unlimited production, reproducibility of results, easier immunization, possible utilization of mix infected virus sources) these reagents should not be always preferred to polyclonal antisera in routine detection work, not only because they are more expensive, but mainly for the extreme specificity that, in some cases cause false negatives, highly undesirable in sanitary controls of plants to be certified. A good example is the case of PDV and PNRSV that, being characterized by a high level of serological variability, can not be safely detected by single monoclonal antibodies, unless appropriated cocktails are used (Boari et al., 1998b).

**Artificial polyvalent antisera:**

To overcome strain specificity, also poyclonal antisera to several different strains can be mixed (Koenig et at., 1979; Uyemoto, 1980).

Several viruses can be detected simultaneously by using mixed or artificial polyvalent antisera (Cambra et al., 1983). The sensitivity of the detection using polyvalent antisera is not compromised if similar conditions are required for the reliable detection of the different viruses (James, 1997).

**2. Other serological techniques alternative to ELISA:**

**Dot Immunobinding Assay (DIBA):**

DIBA has the same sensitivity of ELISA, needs little equipment. It is based on the use of membranes (nitrocellulose or other) in substitution of the plates used for ELISA, thus eliminating the need for ELISA plate reader (Makkouk et al., 1993; Poggi Pollini et al., 1993).

**Tissue blot immunoassay:**

While it may not always reach the same sensitivity as ELISA or BIBA, tissue printing is remarkably rapid (sample grinding and preparation is virtually eliminated) and, as DIBA, it can be performed with very little equipment. In addition, tissue printing can provide data on virus localization within plant organs (Makkouk et. Al., 1993; Knapp et al., 1995).

**III. Stone fruit viruses:**

***1. Trichoviruses:***

***Apple chlorotic leafspot virus (ACLSV):***

The virus has a medium immunogenic power and is detectable by serological tests, mainly ELISA or immono tissue printing (Knapp et al., 1995). Immuno electronmicroscopy can also be used for detection of ACLSV (Kerlan et al., 1981; Kalashjan and Lipartia, 1986).

In detecting ACLSV by ELISA with the diversity of its isolates, difficulties were encountered stemming from virus lability and low concentrations. So flegg and Clark (1979) modified the DAS technique by incubating the enzyme conjugate immediately with the sap instead of adding it subsequently, whereas Detienne et al., 1980 failed to detect the virus in apricot, even by using the same modified procedure. A positive reaction was obtained, however, with the DAS procedure if, during the extraction, stabilizing agents were used and nicotine was added to the plant extract to neutralize the tannins. The modified version of the ELISA technique for detecting ACLSV, (Flegg and Clark, 1979), was used successfully by other researchers (Fuchs et at. 1979; Fuchs, 1980, 1982,1983).

According to the work of Fuchs (1980, 1982), the concentration of virus in apple trees increases at the beginning of March reaching its maximum in May and June. The ELISA, however, could be carried out even in September with sap from forced buds. Detienne et al., (1980) could detect ACLSV under field conditions even in September, by using special buffers.

There are considerable differences in the virus concentration between tissues of different plant parts. Fuchs (1980, 1982) recommended to test flower petals for the detection of ACLSV in ELISA, whereas Llacer et al. (1985) suggested fruit tissue. Barba and Clark (1986) reported an erratic distribution of ACLSV, with leaves at the base of the branches containing more virus than apical ones. Bark stripped from one or two year-old wood was the most reliable tissue for assay, particularly later in the season.

Although ACLSV has a high degree of symptomatological variability, its antigenic properties are very stable and polyclonal reagents to detect all known strains of the virus. The difference in antigenic properties of the various strains do not have any impact on the polyvalence of polyclonal reagents, therefore, the search for specificity in serological detection called for the production and use monoclonal antibodies (Poul and Dunez, 1990; Malinowski et al., 1997).

# Biological diagnosis of virus and virus-like diseases
# A special reference to stone fruit certification

# Biological diagnosis of virus and virus-like diseases
## A special reference to stone fruit certification

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**Summary:**

The biological diagnosis of virus and virus-like diseases of stone fruit trees is briefly discussed. Mechanical transmission and graft inoculation are separately exposed with a special reference to certification programs. Indexing facilities and the activities conducted in the framework of certification are also described. A list of virus and virus-like diseases of stone fruits and their main indicators are given. Finally, it is underlined that biological diagnosis in general and woody indexing in particular still remain a compulsory approach, at least for sanitary selection and certification programs.

**Key words**: stone fruits, plant viruses, virus-like diseases, biological diagnosis, plant certification

**Resume:**

Dans ce travail, on parcourt briévement le diagnostic biologique des maladies a virus et de type viral des essences a noyau. La transmission mécanique et l'incoculation par greffage sont discutées séparément, mais en se référant toujours aux programmes de certification. Les structures d'indexage et les activités conduites dans le cadre de la certification sont également décrites. En outré, une liste des maladies a virus et de type viral des essences a noyau et de leurs indicateurs principaux est presentée, Enfin, on meten evidence qui le diagnostic biologique en général en indexage sur les indicatevis ligneox, en particulier, demeurent encore une pratique indispensable, au moins pour la selection sanitaire et les programmes de certification.

**Mots-cles**: *essences a noyaux, virus des vegetaux, maladies de type viral, diagnostic biologique, certification des plantes.*

## 1. Introduction:

Diseases caused by virus or virus-like agents induce heavy losses worldwide every year in fruit trees (Németh, 1986; Roistacher, 1992; Martelli, 1993). Only in fruit trees more than 150 diseases caused by viruses, viroids, phytoplasmas and unknown graft-transmissible, agents are reported. In order to control these disease, the first and essential step is to identify the causal agent and to determine its properties. The recent advances of serological and molecular (probe hybridization and PCR-based) techniques (Dunez *et.al.*, 1994; Candresse, 1995) for the rapid identification of virus infections tend to replace biological tests. Nevertheless, the inoculation of plant viruses to different herbaceous or woody plants remains a simple and most useful tool and it is still essential for studying new agents or diseases and producing virus-free propagating material. Regarding diagnosis, as long as the causal agent is not known, the presence of the disease can be demonstrated only by biological transmission to susceptible indicators.

## II -Diagnosis problems for virus and virus-like agents in fruit trees

Some general considerations are needed when the samples are to be checked for the presence of virus or virus-like agents in fruit trees: (i) the choice of plant material to be sampled is of great importance for successful diagnosis; (ii) the uneven distribution of infectious agents within the plant (e.i. plum pox virus, phytoplasmas) can make appropriate sampling difficult; (iii) environmental factors influence the agent concentration in a tree impiring the possibility of detection in different seasons (virus concentration is normally higher in the spring, whereas phytoplasmas and viroids later in the season).

The large number of diseases reported for fruit trees is also due to the differential expression of the same pathogen under different climatic conditions, species and varieties. Among the ilarviruses, *Prunus* necrotic ringspot virus (PNRSV) and prune dwarf virus (PDV) cause in the stone fruit trees 14 and 10 diseases respectively (Dunez, 1988). Different diseases caused by apple chlorotic leaf spot virus (ACLSV) were also reported from Desvignes and Boyé (1988). Moreover often diseased plants in the field can be infected with more

than one graft transmissible agent making the diagnostic procedure more complex.

When appraising the relative merits of different diagnostic methods, the following factors should be considered: (a) sensitivity, how small an amount of virus can be measured or detected; (b) accuracy and reproducibility; (c) number of samples that can be processed in a given time by one operator; (d) cost and sophistication of the apparatus and materials needed; (e) the level of training required for operators; (f) adaptibility to field conditions.

The last points may prove to be particularly important in many Mediterranean countries and, as a result, the development of proper biological testing may be especially useful for these countries.

**III -Biological indexing**

Indexing may be defined as any test that will reproducibly assess the presence or absence of a transmissible pathogen, or identify a disease on the basis of the reactions induced on specific host plants that are artificially inoculated.

The recognition that symptoms caused by virus and virus-like diseases could be reproduced from one plant to another by the transfer of a "contagium vivum fluidum" was significant for the birth of plant virology as a science. It was the end of the 19th century in the USA (Smith, 1888), when the infectious nature of peach yellows was demonstrated by graft transmission from diseased to healthy trees.

Until the late 1950s, virus diagnosis was dominated by field symptomatology and biotests. Early virologists found that not only symptoms could be transferred from one plant to a similar one, but also that other genera of plants could be infected and, moreover, that the symptoms produced on these plants were characteristic for specific viruses. Although biotests lack the clinical objectivity of other recently developed serological and molecular techniques, they still play a major role in routine diagnosis.

## 1. Mechanical inoculation to herbaceous plants

In the detection of fruit tree viruses, the mechanical transmission to herbaceous indicators is an important diagnostic procedure that provides a good hint for further determination of the agent. The first mechanical transmission of a fruit tree virus was carried out successfully only in the late '40s, when cherry ringspot virus was transmitted to cucumber (Moore *et. al.*, 1948). The use of some families of herbaceous plants has greatly improved the diagnostics of stone fruit viruses, e.g. *Cucurbitaceae* for ilarviruses, *Chenopodiaceae* and *Solanaceae* for nepoviruses and filamentous viruses. Beside virus diagnosis, herbaceous hosts are sometimes used for strain identification e.g. Sutic *et al.* (1971) reported three PPV strains on the basis of symptoms obtained on *Chenopodium foetidum* Schrad.

Very valuable properties of herbaceous indicators are: (i) grow easily and fast under controlled environmental conditions; (ii) show symptoms rapidly and clearly; (iii) have low tannin and inhibitor content. The herbaceous plants are less costly and time-requiring for reaction than woody indicators, symptoms can be reproduced under controlled conditions all the year round and they also allow the detection of latent viruses which cannot be recognized otherwise. However, herbaceous indicators are not always specific and reliable like woody plants, and only about one-third of the known fruit tree viruses can be transmitted mechanically so far. The fact that a virus is not mechanically transmissible is due probably not to the virus itself, but to the source and acceptor plant (Németh,1986).

However, the use of herbaceous hosts, for certification purposes, is omplementary to woody indexing and serological techniques. The minimal host range of herbaceous host to be used in the sanitary controls for the certification of stone fruits should include at least: *Cucumis sativus* L., *Chenopodium quinoa* Wild. nd *Nicotiana occidentalis* Weeler.

### 1.1. *Inoculation procedure*

The procedure for mechanical inoculation can be applied with a reasonable degree of success if some basic requirements in the management

of herbaceous hosts and handling of the inoculum are fulfilled. In principle, parts of any living organ of an infected plant can serve as a source of inoculum for sap transmission. In practice, however, the chances for successful transmission are best if young, tender tissues from developing leaves, flowers or root tips are used. The collected samples should avoid exposure to direct sun and be kept in a refrigerator box during transportation. In the laboratory the samples are processed immediately or placed in a refrigerator at 4°C until used. Prolonged storage in a freezer at -20°C is possible but not devoid of risk, for the particles of certain viruses may disassemble during thawing, thus decreasing the infective power of the extract. For numerous viruses the main extraction medium is phosphate buffer, *0.1* M, pH 7 containing 2.5 % nicotine as antioxidant.

**1.2. *-Symptom expression***

Inoculated plants are grown at a temperature ranging preferably from 18°C to 26°C and are checked for symptom appearance. (Fig. 1-3).

**2. Graft inoculation to woody indicators**

As there are still many viruses that can not be transmitted to herbaceous plants and, with phytoplasma and xylem-limited bacteria mechanical transmission has failed so far, the main and most reliable diagnostic method is still biological testing by woody indicators.

An ideal woody indicator plant should: (i) be free of viruses, resistant to other pathogens and pests; (ii) be easily grown; (iii) react rapidly and specifically to a given virus; (iv) possibly be polyvalent, i.e. suitable for detecting more than one virus; (v) exhibit identical symptoms under different conditions; (vi) by its use a transmission ratio of at least 80% should be attained; (vii) be ready for use during the whole year. Since only a few woody indicators have so many attributes, research continually aims at finding new, polyvalent indicators, surpassing in quality the earlier used plants.

The indicator plants may be either cultivated varieties susceptible to individual viruses or wild-growing species grafted to virus-free rootstocks. In the

latter case the selection of suitable rootstocks is very important. So the sweet cherry *(Prunus avium* L.) cv. Bing, used as indicator, produces more enations on *P. mahaleb* rootstock than on *P. avium, P. serrulata* cv. Kwanzan takes better on P. *avium* than on *P. mahaleb* L. (Németh, 1986). In many cases the rootstock itself can be used as an indicator plant ( *P. persica* (L.) Batsch cv. GF 305, P. *tomentosa* Thunb., *P. mahaleb)* or the indicator can be self-rooted *in vitro* (Shirofugen, GF 305, etc).

### 2.1. *Field indexing*

This method was the first used among biological tests. It consists in grafting the plant material to be indexed onto indicator directly in the field in different seasonal periods. This method requires long-term observations on trees ranging generally from two to five years; it is quite indispensable for the reliable detection of virus-like diseases of stone fruits. A longer period is necessary to observe symptoms on fruits.

In different indexing centres field indexing is anticipated in greenhouse for 4-5 months at 22-24 °C. Indexing under greenhouse conditions is checked weekly for symptom expression. The preliminary observations under protected conditions are mainly focused on cherry green ring mottle on *P. serrulata* cv. Shirofugen; ilarviruses, nepoviruses, PPV and ACLSV on GF 305; cherry twisted leaf, cherry mottle leaf on P. *avium* cv. Bing and apricot pit pox on P. *armeniaca* cv. Tilton. Several indicator plants need to be transferred in the field to be observed for two more seasons (i.e. *P. avium* cvs. Bing or Sam for cherry mottle leaf, cherry rusty mottle, cherry necrotic rusty mottle, stem pitting; P. *armeniaca* cv. Luizet for apricot leaf roll, etc).

### 2.2. *Glasshouse indexing*

Although woody indexing of fruit tree virus and virus-like diseases is a reliable diagnostic method, field indexing proved to be very costly in terms of time, space and labour. So, efforts have been made to transfer indexing from the field to the glasshouse, to the use of new indicators and to improve the detection of several agents (Fridlund,1970; Devignes, 1976; Boy and

Desvignes, 1986; Cornaggia and Desvignes, 1986; Gilles and Bormans, 1986). The change from field to greenhouse indexing became possible because the appropriate management of environment and hosts yielded more rapid and accurate results The average time for symptom development can be reduced to a few months in greenhouse, from one to five years in the field (Fridlund, 1980a; 1980b).

In order to improve greenhouse indexing, investigations were conducted for many years under controlled temperatures for many virus disease isolates on different woody indicators, already used in field indexing. Clear-cut symptoms of most host virus combinations occurred at 18 or 22°C. However, the optimum temperature for maximum symptom intensity of a particular disease was shown to vary substantially among different indicators (Fridlund, 1970).

More recent studies confirm that greenhouse indexing can substitute for field indexing for many virus diseases, but till a complete correlation between greenhouse results and field observations of symptom production does not occur, the field indexing remains useful (Fridlund, 1980).

Greenhouse indexing had a sudden boost with the use of the polyvalent GF 305 peach indicator for the indexing of graft-transmissible agents of stone fruit trees (Bernhard and Marenaud, 1962; Bernhard *et al.*, 1969). The indexing with GF 305 takes generally from 3 weeks to some months. By this method ilarviruses, nepoviruses, PPV, ACLSV, as well as some phytoplasmas and virus-like diseases are detected. The time needed for symptom expression is related to how indicator is used (selfrooted or grafted on rootstock). When simple indexing is combined with the cross-protection technique peach latent mosaic can also be detected (Desvignes, 1976; Boye and Desvignes, 1996).

**IV -Indexing facilities for a certification program**

The use of differential woody indicators is a compulsory step in any certification program because there are virus-like and latent diseases for which it represents the only possible way known to date for ascertaining the occurrence of infections.

Indexing programmes, regardless of whether they are carried out on a small or large scale, i.e. for experimental or commercial purposes, require adequate facilities in terms of: (i) greenhouse for biological tests; (ii) indicator mother plots and fields for bioassays; (iii) grafting area; (iv) sheds and rooting benches; (v) forcing chambers; (vi) cold chambers; (vii) soil mix box; (viii) storehouse; (ix) screenhouse and (x) diagnostic laboratory.

Availability of nursery land is of utmost importance for growing mother tree indicators and indicators that have been graft-inoculated.

## 1. Correct indexing

### *1.1.Establishment and care of mother indicator plants*

Mother indicator plantings constitute the source of wood or seeds for indexing. Errors in the establishment and care of these plantings may compromise indexing programmes.

Mother plant plots for stone fruit indicators should meet as many of the following criteria as possible: (i) be located on grounds reasonably close to the research unit in charge of indexing; (ii) be established on good quality, well-drained and clean soil, preferably with no fruit tree history or at least free of them for at least 5 years; (iii) be at least 100 m far from any fruit orchards in case of mother plants for bud production and 300 m when plants are for seed production *(P. mahaleb,* GF 305, etc.); (iv) stay in place for no more than 10 years if destined for bud or wood production and 12 years for seed production; (v) not to be allowed to bloom if destined to bud production; (vi) be protected from possible contamination from adjacent plots through irrigation, water, flooding and cultivation; (vii) be large enough to accommodate other optional indicators in addition to those used routinely.

The soil must be free from nematodes, in particular the virus vector species. Spraying schedules for the chemical control of possible airborne vectors should be devised according to necessity and local conditions.

### 1.2. *Growth care of grafted indicators*

Regardless of whether grown under greenhouse, screenhouse or open field, indicators should be forced to grow vigorously and be protected from diseases and pests which may obscure symptoms and even endanger their survival. For field grown indicators, cultural practices are the same as those routinely used in nurseries.

Symptoms are usually read four times a year in the open field conditions: two between March and May, once during summer and once in autumn. Records of indicator reactions should be kept for each candidate accession or selection subjected to indexing. The time and appearance of symptoms, their type and severity are noted and compared with the responses of posikue controls. Each plot should also contain negative controls, ie. healthy, non-inculated indicator plants. The positive and negative controls should be distributed in 2-3 replications over the indexing plot.

## 2. Successful grafting in indexing:

The types of grafting used in the indexing are those used in the normal horticultural practice. The choice depends on : (i) the growing stage of scion and rootstock; (ii) the period in which the grafting is made; (iii) the quantity of available plant material. The types of grafting commonly used in indexing works are thoroughly illustrated in bibliography (Németh, 1986; Desvignes, 1990; Roistacher, 1992; Martelli, 1993).

## V -Stone fruit indicators

A full list of viruses and viroids mentioned in the text is given in Table 1, whereas the main indicator plants for stone fruit indexing are listed in Table 2. All indicators are liable to be infected by the whole range of graft-transmissible disease agents, but the symptoms they express may be specific enough for a reliable diagnosis of given diseases.

**1. Diseases and agents identified**

A comprehensive description of the subject is reported in several papers (Németh, 1986; Desvignes, 1990; Boyé and Desvignes, 1996; Barba *et al.*, 1998), which the readers are referred to:

(i) Ilarviruses (PDV, PNRSV, ApMV) generally induce, on *P. persica* GF 305 or Elberta in greenhouse conditions, delayed sprouting, reduced growth, and chlorotic line pattern mainly for ApMV (Fig. 4); on *P. serrulata* cv. Shirofugen infected inoculated buds cause local necrosis when infected with PDV; on P. *avium* cv. Bing, in open field conditions chlorotic rings and spots (Fig. 5).

(ii) Nepoviruses (ArMV, TomRSV, TBRV, SLRV, RRSV, CLRV, CRLV, MLRSV) generally induce, in *P. persica* GF 305 or Elberta in greenhouse conditions, reduced growth, stunting, rosetting (Fig. 6). Sometimes symptoms by TomRSV develop only in the second season.

(iii) ACLSV: induces on GF 305 dark-green small spots, slight deformation of the leaf surface and, for some strains, severe spotting with leaf deformation.

(iv) PPV: generally induce on *P. persica* GF 305 or Elberta in greenhouse conditions, vein clearing and distortion of young leaves (Fig. 7). In field conditions P. *tomentosa* shows distortion and epinasty of first leaves; later chlorotic spots which become necrotic by mid-summer.

(v) Viroids: peach latent mosaic viroid (PLMVd) is detected successfully on GF 305 by cross-protection with a severe and stable strain of it (Fig. B), whereas hop stunt viroid (HSVd) indexing on GF 305 is not very reliable, and needs more studies.

(vi) Phytoplasmas: apricot chlorotic leaf roll in field conditions induce leaf roll on P. *armeniaca* cvs. Luizet or Priana, whereas, parts of leaves become chlorotic, red and finally drop on GF 305 in greenhouse conditions after 3-4

months. The most severily infected plants wilt rapidly. Other phytoplasmas (cherry X disease, peach yellows, peach rosette, etc) can be also detected on GF 305.

(vii)Virus-like diseases: There are several graft-transmissible diseases of stone fruits whose causal agent is still obscure. For these diseases, woody indexing, represents the only diagnostic tool avaiiable today. A list of these with their respective possible indicators is presented in Table 2.

In Tab. 3 a minimum of indexing protocols to be used in the framework of a certification program for stone fruits is given, in order to obtain "virus-free" plant propagating material.

**VI -Conclusions**

Despite its disadvantages of being laborious, time-consuming and skill-demanding, woody indexing still remains a compulsory approach, at least for the sanitary selection and certification of propagated material. In the next future, together with the advent of new technologies for the improvement of diagnostic methods for these agents, the biological indexing will continue to be useful and its improvement important.

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**Tab. 1. Viruses and viroids mentioned in the text, their acronym and taxonomic group**

| English name | Acronym | Taxonomic group |
|---|---|---|
| ***virus*** | | |
| Apple chlorotic leaf spot | ACLSV | *Trichovirus* |
| Apple mosaic | ApMV | *Ilarvirus* |
| Arabis mosaic | ArMV | *Nepovirus* |
| Cherry green ring mottle | CGRMV | *Fovea virus* |
| Cherry leaf roll | CLRV | *Nepovirus* |
| Cherry rasp leaf | CRLV | *Nepovirus* |
| Myrobolan latent ringspot | MLRSV | *Nepovirus* |
| Plum pox | PPV | *Potyvirus* |
| Prune dwarf | PDV | *Ilarvirus* |
| Prunus necrotic ringspot | PNRSV | *Ilarvirus* |
| Raspberry ringspot | RRSV | *Nepovirus* |
| Strawberry latent ringspot | SLRV | *Nepovirus* |
| Tomato black ring | TBRV | *Nepovirus* |
| Tomato ring spot | TomRSV | *Nepovirus* |
| ***Viroid*** | | |
| Hop stunt | HSVd | |
| Peach latent mosaic | PLMVd | |

**Tab. 2. Main indicators for the detection of virus and virus-like diseases of the stone fruits**

| Indicators | Virus and virus-like diseases detected |
|---|---|
| *Prunus persica* cv. GF305 or Elberta | ACLSV, ApMV, PPV, PDV, PNRSV, CLRV, CRLV, ArMV, RRSV, SLRV, TBRV, TomRSV, MLRSV, PLMVd, |
| *P. persica* cv. Springtime | peach asteroid spot; |
| *P. armeniaca* cv. Luizet or Priana | apricot chlorotic leafroll; |
| *P. armeniaca* cv. Luizet or Tilton | apricot ring pox; |
| *P. avium* cv. Sam | LCV, cherry necrotic rusty mottle, cherry European rusty mottle, |
| *P. avium* cv. Bing | cherry rusty mottle, cherry mottle leaf, cherry twisted leaf, cherry European rasp leaf, nepoviruses; |
| *P. avium* cv. Canindex | LCV |
| *P. serrupata* cv. Shirofugen or Kwanzan | CGRMV, PNRSV, PDV; |
| *P. tomentosa* IR 473/1 or IR 474/1 | PNRSV, PDV, ACLSV, TomRSV |

**Tab. 3. Indicated protocols to assess the sanitary status of stone fruits for the production of "virus free" propagating material**

| Agent | Species | Woody indicator | Herbaceous indicator (*) | Other tests |
|---|---|---|---|---|
| ACLSV | (1,2,3,4,5) | GF305 | (*) | ELISA |
| ApMV | (1,2,3,4,5) | GF305 | (*) | ELISA |
| ArMV | (3) | GF305 | (*) | ELISA |
| CGRMV | (3,4) | Kwanzan or Shirofugen | | |
| CLRV | (3) | GF305 | (*) | ELISA |
| CRLV | (3) | GF305 | - | ELISA |
| MLRSV | (5) | GF305 | (*) | ELISA |
| PDV | (1,2,3,4,5) | GF305 | (*) | ELISA |
| PNRSV | (1,2,3,4,5) | GF305 | (*) | ELISA |
| PPV | (1,2,3,4,5) | GF305 | (*) | ELISA |
| RRSV | (3) | GF305 | (*) | ELISA |
| SLRV | (3,4) | GF305 | (*) | ELISA |
| TBRV | (3,4) | GF305 | (*) | ELISA |
| TomRSV | (3,4,5) | GF305 | (*) | ELISA |
| LCV | (3) | Sam | - | ELISA |
| **Viroid** | | | | |
| PLMVd | (4) | GF305 | - | - |
| Phytoplasma | | | | |
| Apricot chlorotic lesproll | (2,5) | Luizet or Priana | - | - |
| Apricot ring pox | (2) | | - | - |
| Cherry mottle leaf | (3) | Bing | - | - |
| Cherry twisted leaf | (3) | Bing | - | - |
| Necrotic rusty mottle | (3) | Sam | - | - |
| Peach asteroid spot | (2,4) | Springtime | - | - |
| Rusty tmottle (European) | (3) | Sam or Bing | - | - |

(*)The herbaceous indicators for mechanically transmissible viruses are: *Chenopodium quinoa, Cucumis sativus* cv Marketer and *Nicotiana occidentalis.*

(1) almond; (2) apricot; (3) cherry; (4) peach; (5) plum.

**Poty Virus**
***Plum pox Virus (PPV)***

# Poty Virus
## *Plum pox Virus (PPV)*

*Biagio DI Terlizzi*
*Arben MYRTA*

PPV infects many cultivated and wild *Prunus* species and is the causal agent of Sharka, the most serious virus disease of Prunoidae. The main problems are caused to plum, apricot and peach, however, recently it was also reported from sweet and sour cherry (Crescenzi *et a/.,* 1994; Kalashjan *et a/.,* 1994). Actually four strains of PPV are known, with differences in the biological, serological, molecular and epidemiological properties: Marcus (PPV-M), Dideron (PPV-D), El Amar (PPV-EA) and Cherry (PPV-C) (Pasquini and Barba, 1997; Candresse *et a/.,* 1998; Boscia *et a/.,* 1998a). PPV-M, already endemic in South-Eastern Europe, spreads rapidly, particularly in peach orchards. The isolates belonging to this strain lack the site Rsa I, which is present in PPV-D (Candresse *et a/.,* 1994). Candresse *et a/.* (1995) have designed serotype-specific primers for M and D strain. PPV-M is also differentiated from PPV-D by coat protein subunits mobility (38 KDa) in SDS PAGE (Adamolle, 1993; Pasquini and Barba, 1994). PPV-D is common in apricot and plum orchards, whereas is reported rarely on peach. Candresse *et a/.* (1994, 1995) identified the strain by enzymatic digestion and serotype-specific primers.

PPV-EA strain is an Egyptian isolate from apricot. This isolate shows high heterology level of the nucleotide and amino acid sequence by comparison with other isolates. Hammond *et a/.* (1998) designed primers based on Nib (replicase) sequence able to differentiate PPV-M from PPV-EA. The PPV-C isolates are identified by RFLP of PCR products (Nemchinov and Hadidi, 1996; Hammond *et a/.,* 1998), RNA probes specific to PPV-C (Nemchinov *et a/.,* 1996), and the use of strain-specific primers (Nemchinov and Hadidi, 1997).

PPV has a good immunogenic power. ELISA (using polyclonal or, more recently, specific monoclonal antibodies) was used for this virus since 1977 (Dunez, 1977) and is today the most common technique for diagnosis (Polak,

1988; Pasquini *et a/.,* 1995). When testing for PPV Nyujto *et a/.* (1986) could obtain amplified sensitivity of ELISA using avidin-biotin reagents with slight modifications of the procedure described by Guesdon *et a/.* (1979). The method was used for testing apricot, peach and plum seeds for the presence of PPV (Nemeth and Kolber, 1983).

Beside polyclonal antibodies, widely utilised for routine detection of PPV, monoclonal antibodies can now be routinely used not only for the virus detection, but for easy and quick identification of its strains. In fact, an antibody (MAb5B) was identified as specific for one antigenic determinant present in all the known isolates of PPV (Cambra *et a/.,* 1994). To determine the PPV strain, four Mabs are now available, specific for four strain-specific antigenic determinants: MAb4DG5 for PPV-Dideron (Cambra *et a/.,* 1994), MAbAL for PPV-Marcus (Boscia *et a/.,* 1997), MAbAC for PPV-Cherry (Boscia *et a/.,* 1998b), and MAbEA24 for PPV-El Amar (Myrta *et a/.,* 1998).

Virus concentration in the leaves is highest in the early season (May-June). Later, the concentration is always higher in the lower leaves of the shoots than in the top ones (Nemeth *et al.,* 1980). PPV can be detected in leaves, flowers, fruits, bark and roots (Clark *et al.,* 1976; Adams, 1978; Dosba *et al.,* 1986; Llacer *et a/.,* 1986; Adams *et al.,* 1998) and the assessment is largely influenced by the host species and the buffers used (Dunez, 1977). Dosba *et al.* (1986) found the detectability on peach trees to be the most reliable in the bark of one or two-year-old twigs and in young shoot leaves, and on apricot trees in flowers, very young shoots and late summer leaves.

In PPV testing it has to be remembered that the virus is not evenly distributed in the tree and so infected and virus-free areas can be found not only within the same leaf but also within the tree because only some branches may be infected (Dunez, 1977; Casper, 1979). Hamdorf (1983) could detect the virus from leaves only after the appearance of symptoms in 9 to 12-year-old, originally virus-free mother trees which had been previously infected by aphid vectors. A very accurate analysis of an adult tree infected by PPV for many years demonstrate the uneven distribution of this virus: over 55% out of 700 samples taken from that tree resulted ELISA negative (but only 15% when the same samples were tested by molecular hybridisation) indicating the absence

or very weak concentration of the virus in many parts of the plant (Varveri *et al.,* 1988). The absence of precise rules suggests a scheme consisting in dividing the tree in sectors and collect three samples from each quadrant: this is obviously a rather cumbersome procedure as it means taking and analysing different samples per tree.

These observations indicate that even a very sensitive method like ELISA does not guarantee freedom from PPV, even when the test is negative.

Immuno tissue printing was also used mainly for resistance studies (Dicenta and Audergon, 1994; Knapp *et al.,* 1995). Immunoelectron microscopy can also be utilised (Noel *et al.,* 1978; Kerlan *et al.,* 1981; Himler *et al.,* 1988).

**Ilarviruses**

Ilarviruses can be detected by ELISA. However, it has been noted (Boari *et al.,* 1998b; Fulton, 1968) that there is a great serological variability, especially with PNRSV and PDV.

*Apple mosaic virus (ApMV)* ApMV is routinely detected by ELISA (Clark *et al.,* 1976; Voller *et al.,* 1976; Thresh *et al.,* 1977; Barbara *et al.,* 1979; Korpraditskul *et al.,* 1979; Hardcastle and Gotlieb, 1980; Torrance and Dolby, 1984).

ELISA detection can be done throughout the growing season in individual samples of young leaves or twigs with newly formed buds, and less readily in mature leaves after June (Torrance and Dolby, 1984), however it was found to be easiest from mid-April to mid-June (Fuchs, 1980).

There are considerable differences in virus concentration between tissues of several plant pots. As for ACLSV, fuchs (1980, 1982) recommended the flower petals for direct ELISA of ApmV.

Studies made with monoclonal antibodies in two different Italian laboratories (Pasquini and Barba, 1991; Boari *et al.,* 1998b) indicated that the serological properties of the ApMV population are very stable and, consequently, single MAbs are suitable for its routine detection.

***Prune dwarf virus (PDV)***

PDV is also detectable by serological tests, and, particularly, by ELISA (Casper, 1977; Torrance and Dolby, 1984; Mink and Aichele, 1984a). ELISA detection of PDV has been reported in germinating *Prunus avium* seeds (Casper, 1977). The method is used in routine testing of seedlings for PDV and PNRSV in the seed of *P. avium, P. mahaleb, P. cerasifera, P. persica* (Mink and Aichele, 1984a).

Protein A ELISA (PAS ELISA) was developed by Cooper *et al.* (1986) for CLRV and PDV diagnosis in cherry seeds. Such tests can provide a quick assessment of seed lots requiring the issue of phytosanitary certificates. DASI-ELISA with monoclonal antibodies was applied to study seed transmission of PDV in *Prunus mahaleb* (Boari *et al.,* 1998a).

In plum and sweet cherry, PDV can be detected during the whole vegetation period in young leaves or in newly formed buds (Torrance and Dolby, 1984).

PDV isolates are characterised by a great serological variability. Boari *et al.* (1998b) identified 36 different serogroups in 128 isolates originated by different *Prunus* species, consequently, if monoclonal antibodies are used for PDV detection, it is advisable to prepare appropriate cocktails to reduce risks of false negatives.

***Prunus necrotic ringspot virus (PNRSV)***

ELISA is widely used for the detection of PNRSV in tissues collected early in the vegetation period (Thresh *et al.,* 1977; Barbara *et al.,* 1978, 1979; Barbara, 1980; Thomas, 1980; Mink and Aichele, 1984 a, b; Torrance and Dolby, 1984). The method is also used in routine testing of seedlings in the seed of *P. avium, P. mahaleb, P. cerasifera, P. persica* (Mink and Aichele, 1984a). A specific study carried out by Torrance and Dolby (1984) ascertained that PNRSV can be detected in plum during the whole vegetation period in young leaves or in newly formed buds. The sensitivity of DAS-ELISA was ten-fold increased when an amplification of the enzyme reaction was applied (Varveri, 1994).

Immuno tissue printing has been also proved to be effective for this virus (Knapp *et al.,* 1995).

Different serological strains of PNRSV have been reported (Casper, 1973; Mink *et al.,* 1987); they became very important when monoclonal antibodies are used, because of the high specificity that, often, may lead to missing detection of isolates belonging to different serological strains. Boari *et al.* (1998b) identified 17 serological variants among 38 isolates tested. Consequently, as for PDV, when using monoclonal antibodies the adoption of cocktails is strongly recommended.

In the past a strong specificity of polyclonal reagents was also reported (Johnstone *et al.,* 1995) but it was probably originated the erroneous identification of the isolate HP-1, initially classified as PNRSV, but later identified as ApMV.

**Nepoviruses**

Nepoviruses, in general, are good immunogenics and can be readily detected by ELISA Clark *et al.,* 1976; Korpraditskul *et al.,* 1979;Voller *et al.,* 1976; György, 1979; Thomas, 1980, Dunez, 1977, Gonsalves, 1979; Lister *et al.,* 1980; Parish and Converse, 1981; Bitterlin *et al.,* 1984; Hoy and Mircetich, 1984; Hoy *et al.,* 1984; Powell, 1984; Powell *et al.,* 1984; Bitterlin and Gonsalves, 1986).

Protein A ELISA (PAS ELISA) was developed by Cooper *et al.* (1986) for CLRV diagnosis in cherry seeds. This test provides a quick assessment of seed lots requiring the issue of phytosanitary certificates.

Different serological strains of TBRV have been reported (Kerlan *et al.,* 1982). Antigenic differences may lead to a strong specificity of polyclonal reagents.

Many authors reported the detection of Tomato ringspot virus by ELISA. However the virus is irregularly distributed in some hosts. Lister *et al.* (1980) reported that ToRSV could be detected by ELISA in apple trees most easily from leaf- and bark extracts of root suckers and in peach trees from root extracts. Bitterlin *et al.* (1984) showed, that in apple trees this virus can be

detected most consistently in leaves, slightly less in the bark and only erratically in the roots. Towards the end of the growing season the reliability of detection decreases. Bitterlin and Gonsalves (1986) found the distribution of TomRSV in peach trees to be irregular but mostly concentrated at and below the soil line.

**Closteroviruses**

Initially defined by transmission on sensitive cherry varieties (Van or Sam), indexing of the closterovirus identified in the trees affected by "little cherry" can be done by serological or molecular techniques (Eastwell *et al.,1996).* However, for the time being, the association of this closterovirus to the "little cherry" disease is basically related to the strict correlation between the presence of the virus and the presence of symptoms.

**Foveaviruses**

A RT -PCR system for the detection of Cherry green ring mottle virus (CGRMV) has recently been developed (Zhang *et al.,* 1998). No serological assays are available.

**Tombusviruses**

Petunia asteroid mosaic virus (PeAMV) is unevenly distributed within diseased trees. The virus seems to be mainly restricted to the symptom-bearing tissue in the different plant parts tested (leaves, fruits, young twig-tips, bark).

Trees showing few or no symptoms of the disease react rarely positive in ELISA test. Therefore, reliable indexing for latent infections with PeAMV by means of serology is not possible at the moment (Diekmann and Putter, 1996).

**Diagnostic problems**

***1. Sampling***

The nature of the sample has a great influence on the results. The use of old leaves, often rich in tannins and oxydating substances is not advisable. When imported, the material is often in the form of dormant material (Babovic and Bulajic, 1995). Sanitary control may therefore be carried out in three ways:

i) sampling of buds and grafting on rootstocks to carry out a further test; ii) forcing the budsticks to sprout and assay fresh tender tissues; iii) direct analysis of the bark: although virus concentrations in dormant material is generally low, this can be done with a certain amount of success.

***2. Uneven distribution of the pathogen in the infected plant***

This phenomenon is very frequent with many viruses infecting stone fruits: not only if the virus is localized around a point of infection, but also classical systemic infection situations when the virus is unevenly distributed and/or is in a variable concentration in the plant. This is particularly frequent in woody plants, very true for PPV but applies also to other viruses.

***3. Grouping of samples***

ELISA is well suited for testing routinely large numbers of samples in numerous host-virus combinations and for different samples to be mixed and tested together (group testing). According to Torrance and Dolby (1984) the sampling date influences also the efficiency of group-testing of leaves. PDV was detected in 1/40 (infected/total leaves) cherry leaves in April and May and in 1/40 plum leaves until July, whereas PNRSV was detected in 1/20 cherry leaves until July and 1/20 apple or plum leaves until May. ApMV was detected in 1/20 apple or plum leaves until July.

**4. The variability of pathogens**

The direct DAS ELISA is highly strain specific; the selectivity may be so high that conjugates prepared for a given strain are unable to detect other, serologically related strains. So not only distant serological relationships, but in some viruses also intermediary ones remain undetected (Koenig, 1978; Lister *et al.*, 1980). The high degree of selectivity may be an advantage in epidemiological studies where different virus strains need to be distinguished; it is a major drawback, however, in diagnostic work and extensive routine testing where all the strains of a virus, both known and unknown, have to be detected.

As reported by van Regenmortel and Burckard (1980) and Lommel *et al.*

(1982), strain specificity does not present a problem with the indirect ELISA (T AS) systems in which, instead of antiviral antibodies, anti-Ig antibodies are labelled by the enzyme.

Although ACLSV has a high degree of variability at the symptom level, its antigenic properties are very stable and polyclonal reagents allow to detect all known strain of the virus.

Cherez and Lister (1973b) reported on the existence of different serotypes of ACLSV, as well as Barba and Clark (1986). However, an investigation carried out by Poul and Dunez (1989) with 8 different Mabs on 29 virus isolates demonstrated that the antigenic properties are very stable. These latter results were confirmed by Candresse *et al.* (1995) by the comparative analysis of coat protein genes of several isolates of the virus.

**5. Serological cross-reactions:**

Serological cross-reactions are reported for isometric and filamentous viruses of stone fruits. The cross-reaction may occur with viruses members of the same taxon or different ones. James *et al.* (1994; 1996) reported the reaction of a PPV polyclonal antibody with Prunus virus isolates, whereas Jordan and Hammond (1991) described several potyvirus-specific Mabs which reacted with PPV.

Serological relationships between ApMV and PNRSV have been reported (Fulton, 1968; Barbara, 1988a,b; Halk *et al.,* 1984). However, recent investigations carried out with monoclonal antibodies were incapable to confirm this finding, at least with Italian isolates (Pasquini and Barba, 1991; Boari *et al.,* 1998b).

A combination of diagnostic procedures may be necessary to confirm virus identity, especially when the disease is of quarantine and economic importance.

## Conclusions

Serological tests remain still very useful as detection methods in general, and for certification purposes in particular, due to their sensitivity, adaptability, low cost and large-scale use. However, it is necessary to avoid false negative reactions due to the wrong choice of the period and method of sampling, quality of antisera and correct conduction of ELISA. Serological methods may not satisfy all requirements for sanitary assessment of the plants (biological assay still remain priority), however they have an important role in a range of detection and analytical techniques, for their adaptability to perform quick large scale assessment, while molecular tools as PCR, for their tremendous sensitivity should now be taken in considerations in the steps of certification programmes where a minimum number of trees are conserved.

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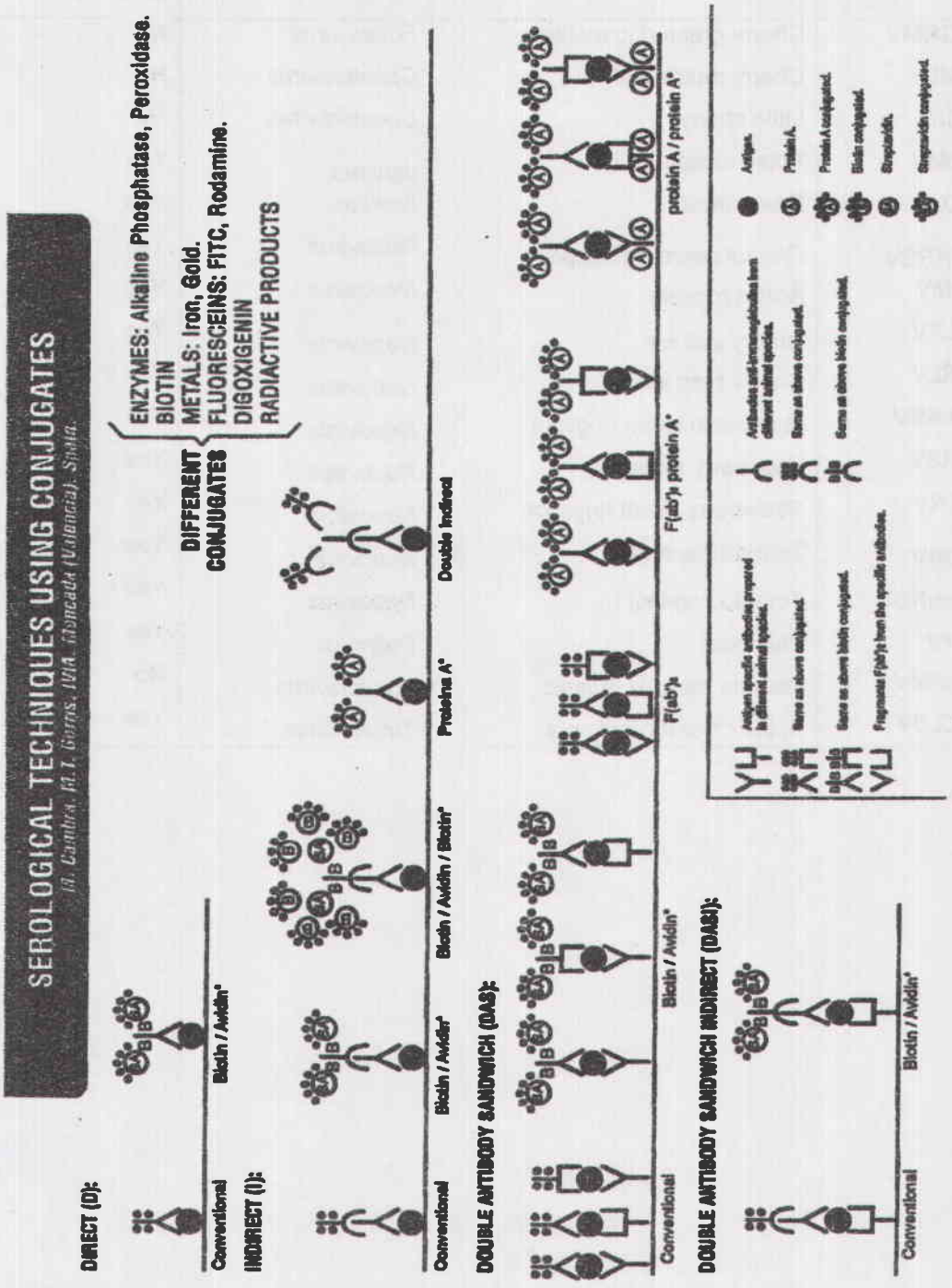
**Table1. Viruses indicated in the certification protocols for EPPO countries to assess the sanitary status of stone fruits (Anonymous, 1992)**

| Acronym | Virus | Taxonomic group | Availability of Elisa Kit |
|---|---|---|---|
| CGRMV | Cherry green ring mottle | *Fovea virus* | No |
| CMLV | Cherry mottle leaf | *Cposterovirus* | No |
| LChV | Little cherry | *Cposterovirus* | No |
| ApMV | Apple mosaic | *Ilarvirus* | Yes |
| PDV | Prune dwarf | *Ilarvirus* | Yes |
| PNRSV | *Prunus* necrotic ringspot | *Nepovirus* | Yes |
| ArMV | Arabis mosaic | *Nepovirus* | Yes |
| CLRV | Cherry leaf roll | *Nepovirus* | Yes |
| CRLV | Cherry rasp leaf | *Nepovirus* | Yes |
| MLRSV | Myrabolan latent ringspot | *Nepovirus* | Yes |
| RRSV | Raspberry rings pot | *Nepovirus* | Yes |
| SLRV | Strawberry latent ringspot | *Nepovirus* | Yes |
| TBRV | Tomato black ring | *Nepovirus* | Yes |
| TomRSV | Tomato ringspot | *Nepovirus* | Yes |
| PPV | Plum pox | *Potyvirus* | Yes |
| PeAMV | Petunia asteroid mosaic | *Tombusvirus* | No |
| ACLSV | Apple chlorotic leaf spot | *Ttrichovirus* | Yes |

Fig. 1. Variants of ELISA mainly used for the diagnosis of stone fruit viruses (Cambra and Gorris, 1996).



# *Serological Techniques for Plant Viruses*

**K. DJELOUAH**

**Istituto Agronomico Mediterraneo -Bari**

## *General Principles*

### *Fundamental basis of Serology*

*Capacity of animals to be immunised against a wide variety of substances (antigens) unrelated to pathogens*

## General Principles

*Fulfilling its role of defending the organism against infection, the immune system is able to recognize and destroy a wide variety of pathogens Avoid recognizing and reacting against itself (self tolerance)*

*Since both pathogens and host are composed of proteins, carbohydrates, lipids and nucleic acids, immune recognition must be finely tulned, otherwise*

1- *fatal infection*

2- *self destruction can occur*

## General Principles

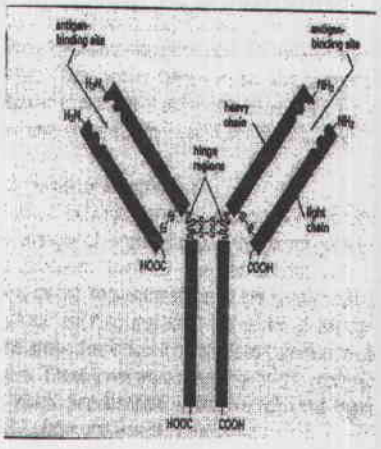
*In higher vertebrates there is a continuous random production of thousands of different receptors. Each antigen-binding receptor is carried out by a cell and each cell carries only one kind of receptor. In a human being there are 2x1012 of these cells (lymphocytes) able to bind more or less efficiently, to any possible antigen, population of lymphocytes are T cells and B cells.*

*B cells have on their surface specific receptors identified as immunoglobulin Ig.*

## General Principles

*Igs are formed by the covalent linking of larger heavy polypeptide chains with smaller light chains. The basic unit of Ig is a Y -shaped molecule*

*Antigen recognition is achieved by two identical antigen binding sites, (Paratopes) situated at the ends of the arms of the Y. Each of these arms is known as Fab = antibody fragment; both Hland L chains contribute to the binding site*




## General Principles

*Antigen: Any substance capable of inducing an immune response when it is introduced in appropriate animal and consequently reacting specifically with the induced antibodies or with the receptor of lymphoid cell*

*Antiserum: The component of blood remaining liquid after clotting (serum) which contains a population of antibodies reactive with a specified antigen, polyspecific, reacting with all the antigenic determinants of the antigen (s)*

# General Principles

***Antibodies:*** *Proteins of the immunoglobulin type, capable of specific bindings to antigens, found in serum and produced by lymphoid cells, predominantly plasma cells, in response to stimulation by an antigen. Antibodies can be obtained from polyclonal antisera or from eggs.*

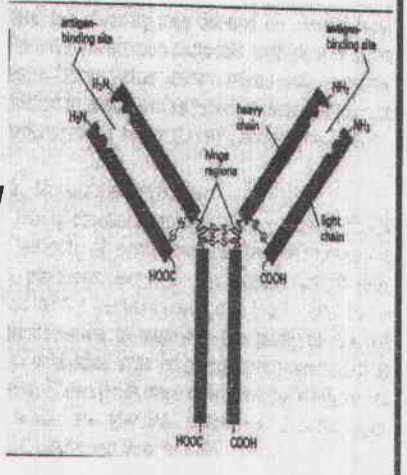
***Monoclonal Antibody:*** *An antibody secreted by a single clone of cells. All molecules of this antibody are identical in structure and therefore the section can be stored frozen and is immortal so exactly the same antibody is in principle always available in unlimited supply, they are monspecific.*

# General Principles

*The Igs of higher vertebrates have been placed in five different classes (IgA, IgD, IgE, IgG, IgM); based on their biochemical and biophysical properties*

*The most common Igs in serum are IgG, then Ig./M*

*In most mammalians IgG can be found in a number of forms called subclasses, related to the heavy chain structure with exception to the rabbit which appears to possess only one form of IgG*



## Immunoassays in plant virology

*These assays are possible because plant viruses (like animal viruses) possess specific antigenic determinants, primarily protein and rarely lipids)*

*Early immunoassays lacked sensitivity for antigen or antibody detection, new methods are very sensitive (ELISA, ISEM, DTBIA)*

*ISEM was first reported by Derrick (1973), later Clark and Adams (1977) described an adaptation of the medical immunodiagnostic procedure (ELISA) for the quantitative detection of plant viruses*

## Main goal of the immunoassays

1. *Selection of healthy plant propagating material*
2. *Identification and classification of viruses*
3. *Localisation of a virus in host tissues or cells*
4. *Quantification of the virus (purification)*

# *Types of serological tests*

**1. *Aggregation tests***

*Simple tests: Antibodies and antigens form insoluble aggregates that can be seen directly or under low magnification.*

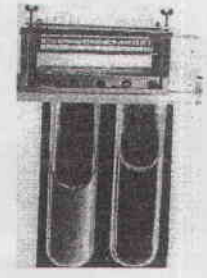
* *Amplified tests: One of the reactants, generally Ab is bound to larger particles (latex, red blood cells) with the aim to amplify the reaction*

# *Types of serological tests*

## 2. *Immunosorbent tests*

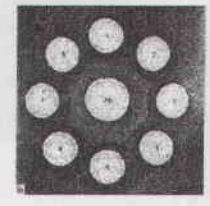
*The absorption of one reactant (mostly Ab) to a fixed solid phase i.e. the surface of plastic tubes, wells, spheres or support films for electron microscopy*

## *Types of serological tests*



### *Aggregation tests*

1. ***Simple tests***
   - * *Slide precipitin test*
   - * *Immunodiffusion*
   - * *Radial diffusion*



2. ***Amplified tests***
   - * *Chloroplast agglutination assay .*
   - * *Latex test*

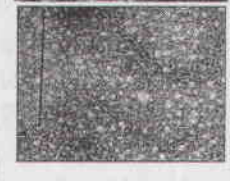
## *Types of serological tests*

### ***Immunosorbent assay***

1. ***Simple tests***
   - *ImmunoSorbent Electron I1icroscopy (ISEM")*
   - *ImmnoElectronMicroscopy (IE/M)*
   - *Enzyme Linked ImmunoSorbent Assay (ELISA)*

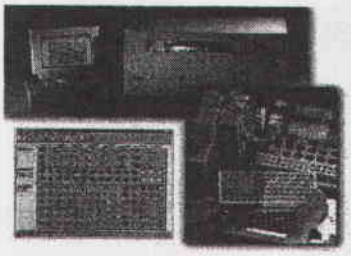
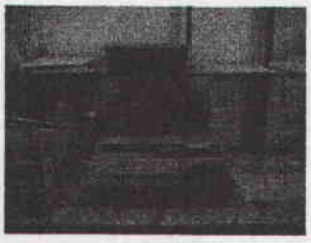
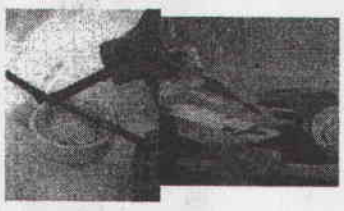
*(Abs acts as link between Ag and enzyme),*

- *Direct Tissue Blot Immunoassay (DTBIA)*



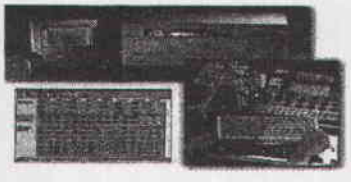
2. ***Amplified tests***
   - *(increase the sensitivity)*
   - *Adding antispecies Ab or other trapping systems such as Avidin -Biotin*

## *Advantages of the serodiagnosis*

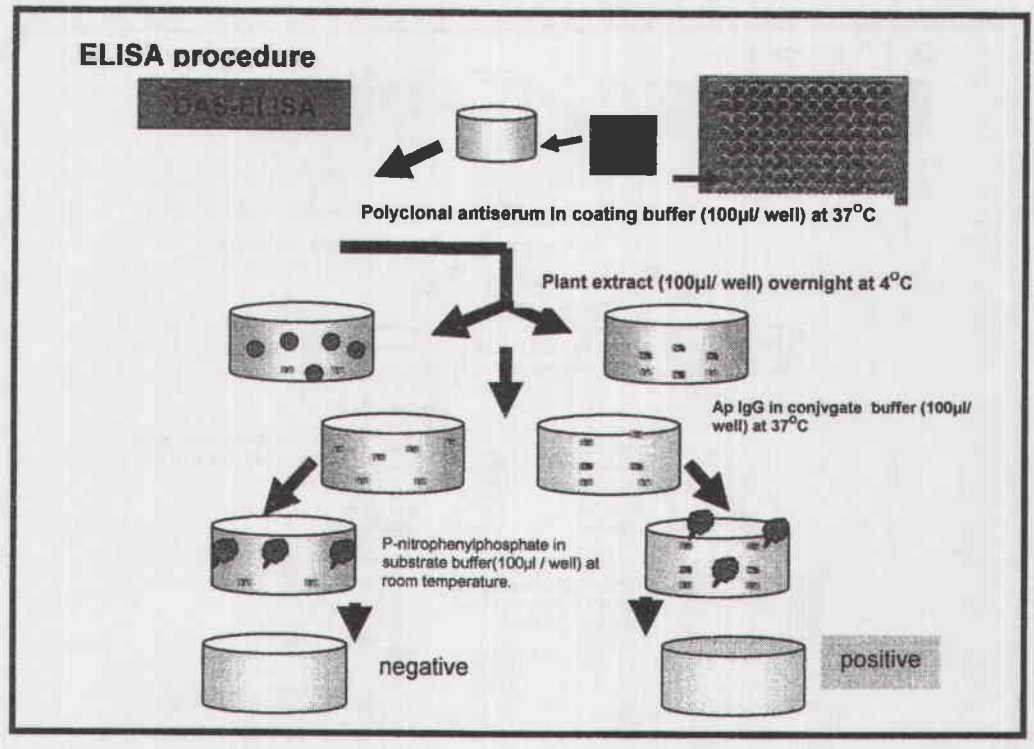





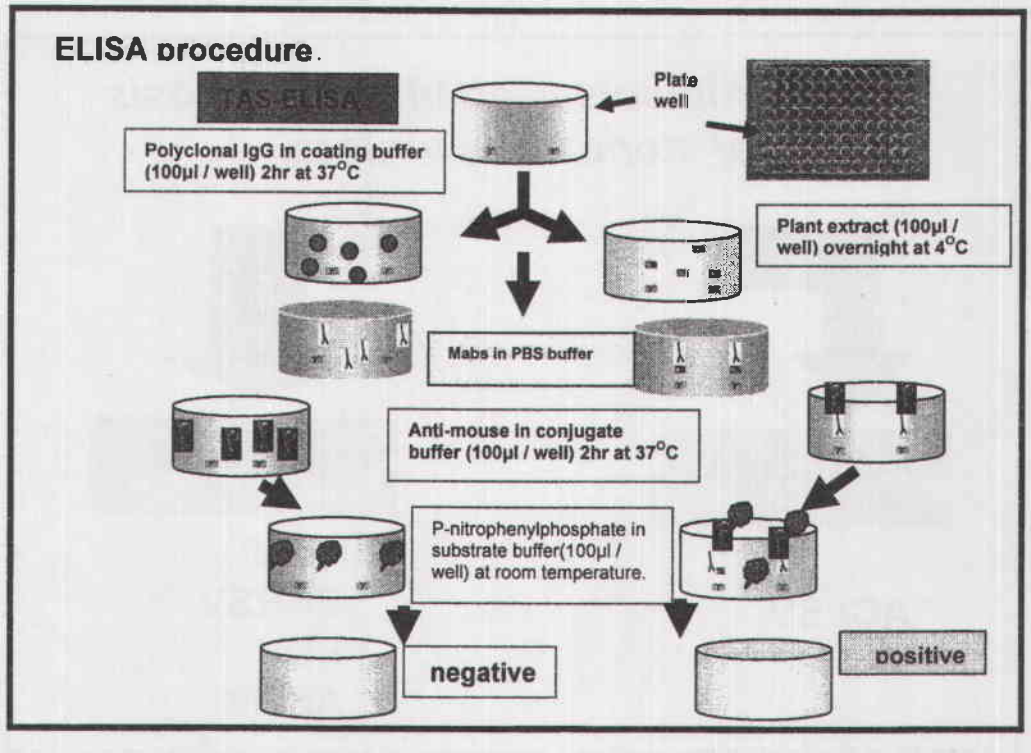
Compact use of space
Mass diagnosis
Short time
No risk of contamination and safety
Modest level of technology required
Reliability/ Low costs

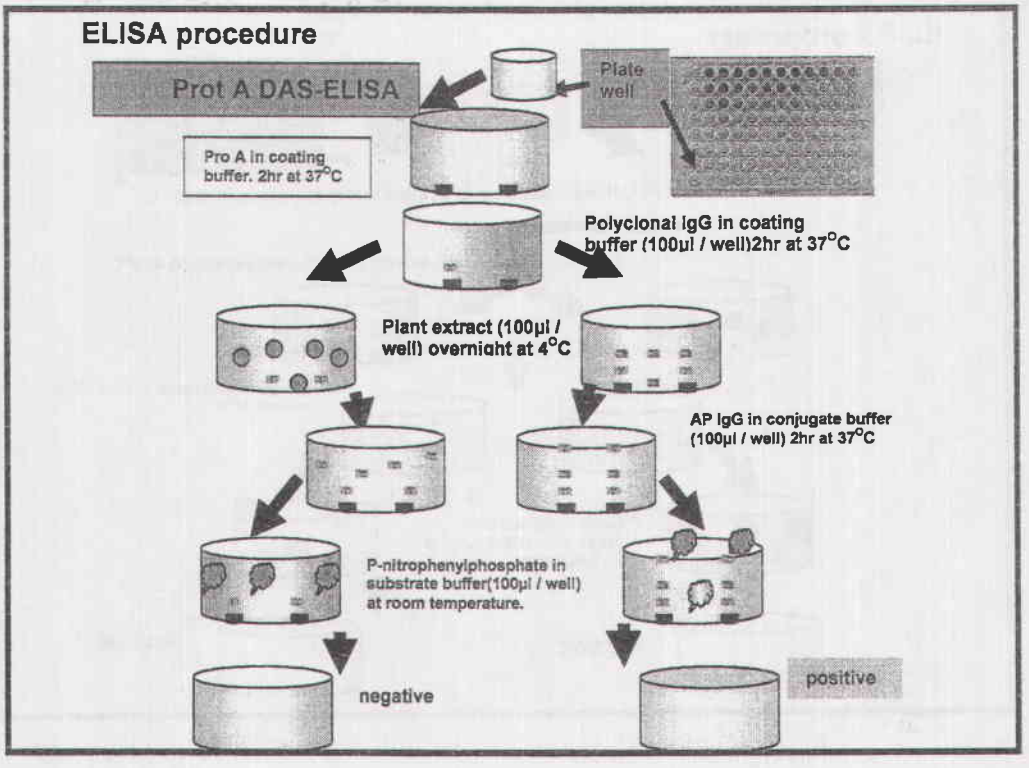
## *Limitations of the serodiagnosis*



* *Detection of unstable viruses like: cucmoviruses and ilarviruse;*
* *Viruses with poor immunogenic properties;*
* *Viruses requiring laborious purification procedures with host contaminants still present (phloem limited viruses);*
* *.Detection of non encapsidated double-stranded (ds) or single stranded (SS) viral RNA/ Detection of viroids*
* *.Detection of defective virus particle (e.g. tobraviruses),interfering R..IVAs (DI-R,NAs) or satellite RNAs;*
* *Differentiation of very closely related virus strain*


ELISA procedure
DAS-ELISA
Polyclonal antiserum in coating buffer (100µl/ well) at 37°C
Plant extract (100µl/ well) overnight at 4°C
Ap IgG in conjvgate buffer (100µl/ well) at 37°C
P-nitrophenylphosphate in substrate buffer(100µl / well) at room temperature.
negative
positive


ELISA procedure.
TAS-ELISA
Plate well
Polyclonal IgG in coating buffer (100µl / well) 2hr at 37°C
Plant extract (100µl / well) overnight at 4°C
Mabs in PBS buffer
Anti-mouse in conjugate buffer (100µl / well) 2hr at 37°C
P-nitrophenylphosphate in substrate buffer(100µl / well) at room temperature.
negative
positive



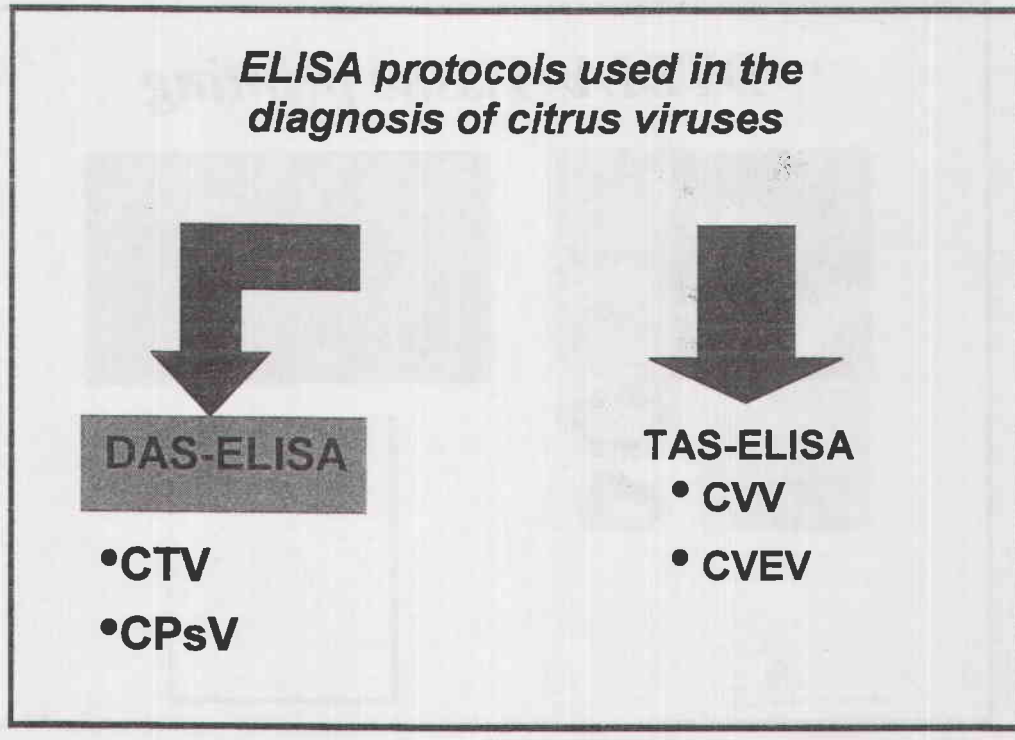

## *ELISA protocols used in the diagnosis of stone fruits viruses*

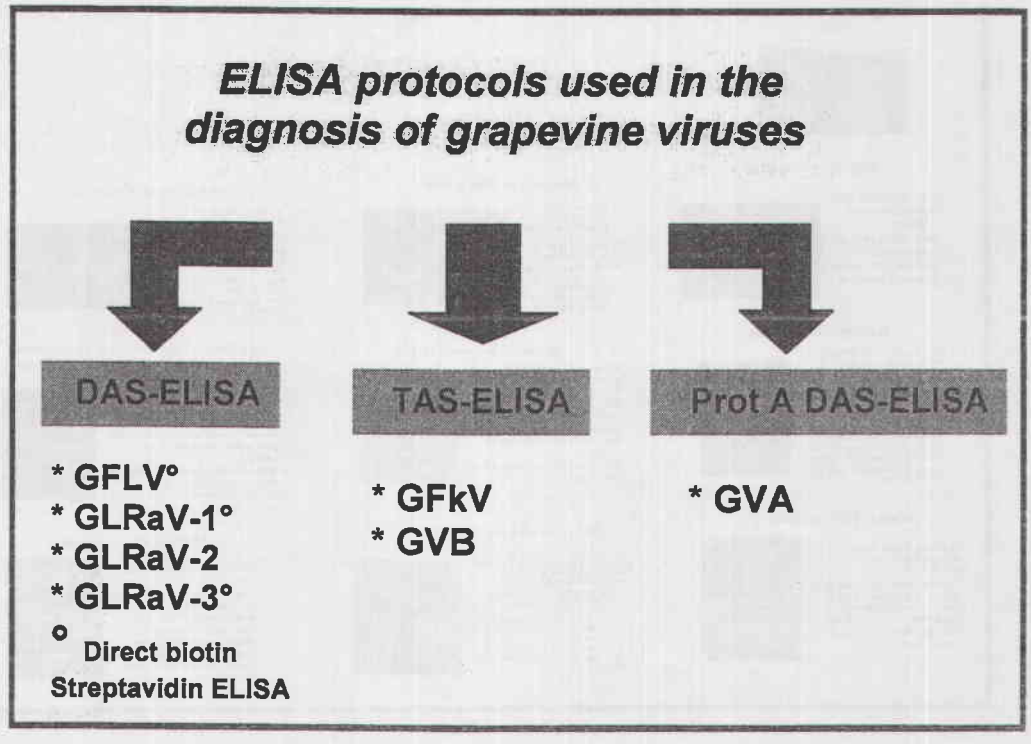
**DAS-ELISA**

- **ACLSV**

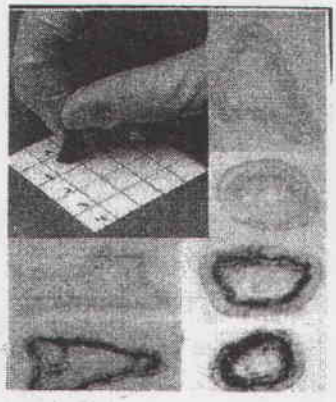
**TAS-ELISA**

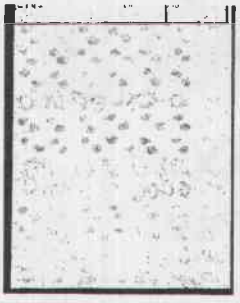
- **PPV**
- **PNRSV**
- **PDV**
- **ApMV**

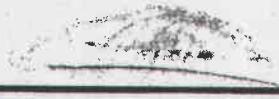

ELISA protocols used in the diagnosis of citrus viruses
DAS-ELISA
•CTV
•CPsV
TAS-ELISA
• CVV
• CVEV


ELISA protocols used in the diagnosis of grapevine viruses
DAS-ELISA
* GFLV°
* GLRaV-1°
* GLRaV-2
* GLRaV-3°
° Direct biotin Streptavidin ELISA
TAS-ELISA
* GFkV
* GVB
Prot A DAS-ELISA
* GVA

# DTBIA/ Tissue printing







Direct tissue blot immunoassay
DTBIA

## DTBIA/ CTV-CPsV

### Collecting samples

- In the blooming time closed flowers are collected
- They can be used as fresh material or stored at -20° in petri disks well wrapped

### Membrane preparation

- Diverse type of nitrocellulose membranes can be used, depending (0,20-0,45µm)
- The membrane is prepared by delimiting distinct squares using a pencil

### Membrane printing

- A fresh clean cut is made with a sharp razor blade across each sample and the cut surface is gently pressed on the trasabled nitrocellulose membrane
- The membrane is allowed to dry at least 30 min.

### Membrane blocking

- 1% bovine serum albumin (BSA) solution in distilled water is prepared
- The printed membrane is placed in appropriate container
- Cover with the albumin solution using a pipette and incubated for 2h at room temperature or overnight at 4°, blocking the aspecific sites
- Slight agitation is beneficial over this step.

### Membrane washing

- Washing buffer is prepared using a PBS Tween buffer as for ELISA
- The membrane is rinsed in the container with 10 ml or more of washing buffer for 3 minutes in orbital shaker
- The operation is repeated three times

### Addition of monoclonal antibodies conjugated with alkaline phosphatase

- Solution containing specific monoclonal antibodies linked with alkaline phosphatase diluted in conjugate buffer is prepared and used to cover the printed membrane in the container
- After the incubation for 2-3h at room temperature the conjugate solution is discarded

### Membrane washing

- Washing buffer is prepared using a PBS Tween buffer as for ELISA
- The membrane is rinsed in the container with 10 ml or more of washing buffer for 3 minutes in orbital shaker
- The operation is repeated three times

### Membrane development

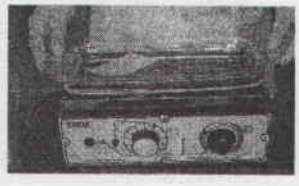
- The substrate buffer is prepared by solving 1 tablet (BCIP-NBT Sigma Fast) in 10ml distillated water
- The membrane is covered with this solution and incubated until the appearance of purple-violet colour in the positive control (3 to 10 min.)
- The reaction is stopped by washing the membrane with tap water

### Membrane reading

- The membrane is dried on filter paper
- The printing are observed by using a low power magnification (X10-X20)
- The presence of purple-violet precipitates in the vascular area of plant print reveals the presence of the virus

## DTBIA Advantage

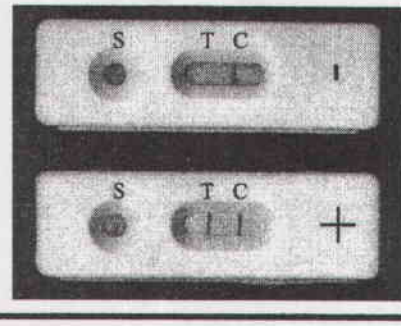
* *Simple and faster than ELISA test;*
* *Tissue imprints can be performed in field condition;*
* *The observation of tissue structure sometimes is veryhelpful (localization);*
* *Inexpensive (no buffers nor equipment is required);*
* *Few amount of plant material is needed;*
* *Reduced risk of contamination;*
* *The imprints can be easily stored and mailed;*
* *Can be safely used with quarantine plan pathogens.*

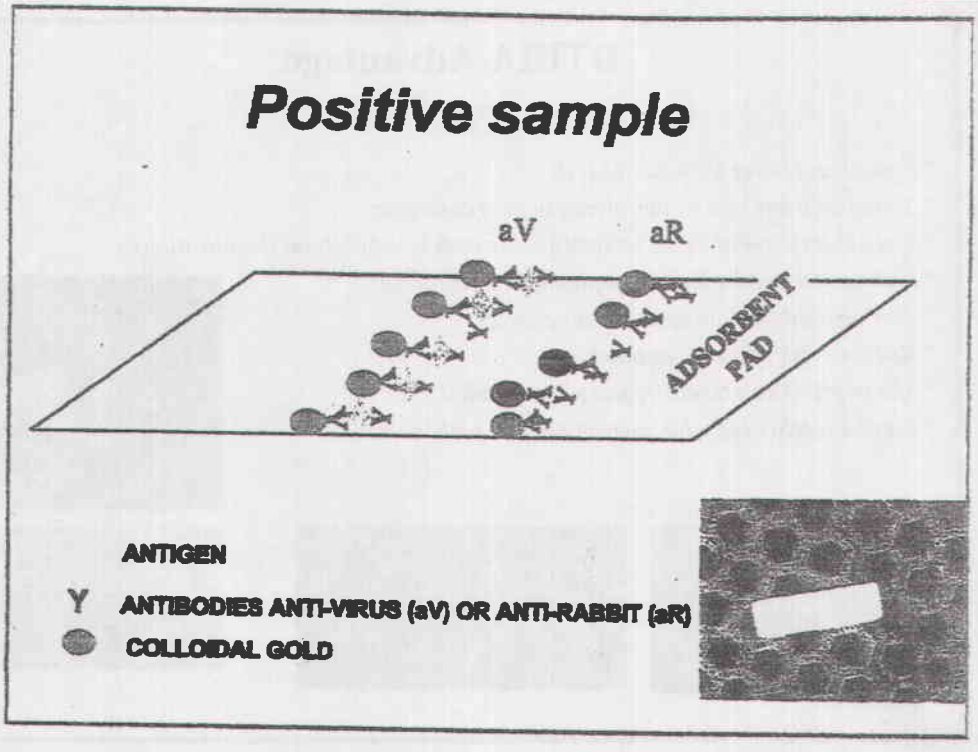


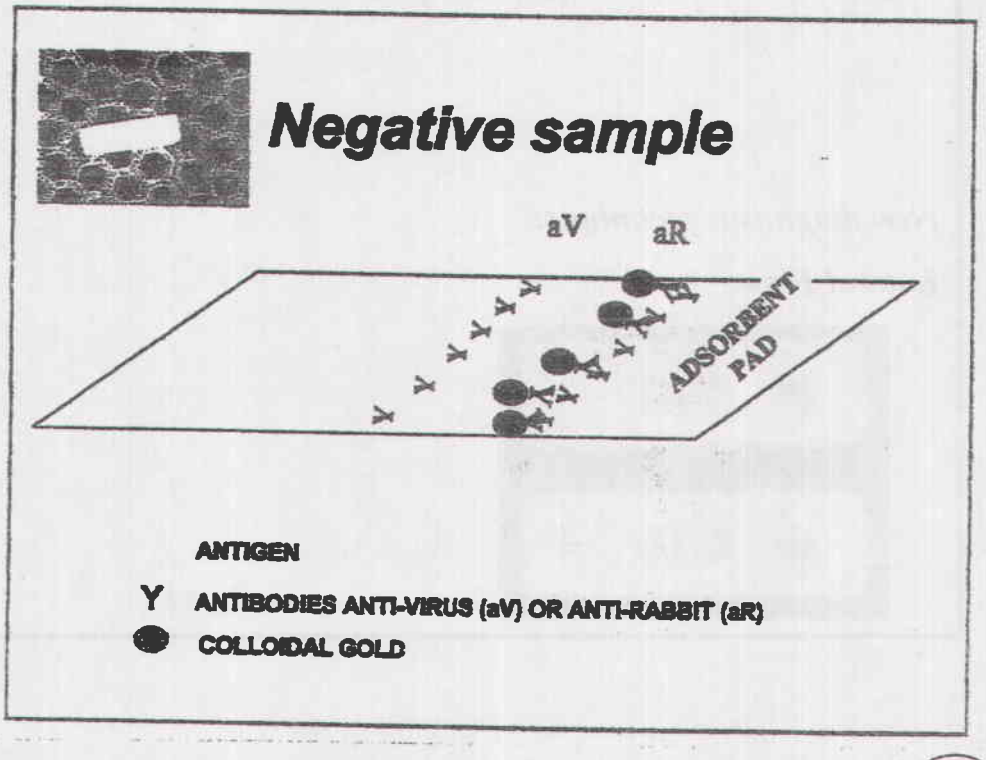
## Advanced serological for plant viruses detection

***New diagnostic technique:***

***Lateral Flow***



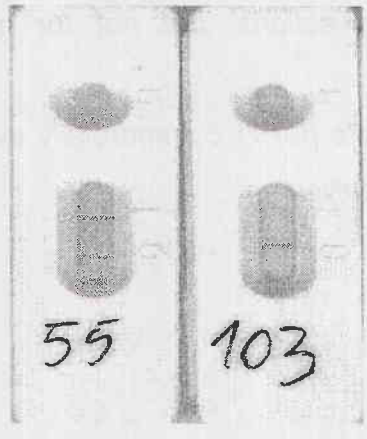

Positive sample
aV
aR
ADSORBENT PAD
ANTIGEN
ANTIBODIES ANTI-VIRUS (aV) OR ANTI-RABBIT (aR)
COLLOIDAL GOLD

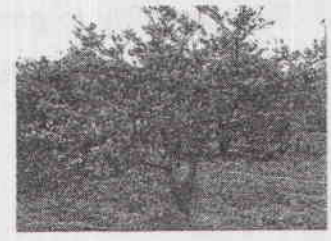

Negative sample
aV
aR
ADSORBENT PAD
ANTIGEN
ANTIBODIES ANTI-VIRUS (aV) OR ANTI-RABBIT (aR)
COLLOIDAL GOLD

## ELISA and Lateral Flow

| | ELISA | LAT FLOW |
|---|---|---|
| Results | 1-2 days | 10 minutes |
| Place | Laboratory | Field |
| Professional skill | Medium | Limited |
| Lab. Tools | Needed | Not needed |
| N°samples | Hundreds | Few |
| Cost | Low | High? |
| Reliability | Good | ? |

***Evaluation of the possibility to apply LF to the CTV detection***



## *Main advantages*

* *Sensitivity 90% = reliable*
* *Help when immediate actions are needed*
* *Help in training*
* *Immediate answer*
* *No need to label plants and move samples*

## *Limits*

* *5% false positives: need of confirmation.*
* *Valid for taking safeguarding actions, but not for mandatory disruption of trees*
* *The possibility to produce LF kits (CTV) commercially available is now object of evaluation (CNR Italy)*

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Djelouah K., Potere O., Boscia D., D'Onghia A.M., Savino V., 2000. Production of monoclonal antibodies to Citrus Psorosis associated virus. Proceedings 14 th Conference of International Organization of Citrus Virologists, Campinas 1998, 152-158.

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# Short Comuniation: Detection of Citrus Psoriosis Boldtitalics by Direct Tissue Blot Immunoassay

## SHORT COMUNIATION

# DETECTION OF *CITRUSPSOROSIS BOLDTITALICS BY* DIRECT TISSUE BLOT IMMUNOASSAY

**A.M. D'Onghia, K. Djelouah", D. Frasheril and O. Potere[2]**

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**Summary**:

Detection *of citruspsoris virus* CPsV) by direct tissue blot immunoassay (DTBIA) was attempted using different tissues from CPsV-infected sources. Freshly cut surfaces for different plant organs were gently pressed on nitrocellulose membranes, exposed to a CPsV monoclonal antibody (MAb Ps29) conjugated with alkaline pnosphatase, and stained with BCIP-NBT Sigma fast. For comparison some explants were tested also by DAS-ELISA using the same MAb and a polyclonal antiserum for plate coating Purple. Staining positive reaction) was readily observed when flower explants were used, especially the ovary. DTBIA from ovaries correlated with DAS-ELISA. proving reliable and sensitive for the rapid detection of CPsV infections.

***key words:*** Citrus psorosis virus diagnosis, immunoprinting.

Psorosis, a severe disease of cilios with a worldwide distribution (Roistacher, 1993), is caused by *Citrus psorosis virus (*CPsV), genus *Ophiovirus (*Milne *et al.,* 2000). For many years, laborious and costly indexing on cirtus ir.dicators was the only diagnostic method available Roistacher, 1993) but laboratory procedures, suchas ELISA D'Onghia et al 1998, 2001, Pofere et al 1999 Aloto *et al.,* 1999, 2000: Djelouah *et al, (*2000) and RT -PCR : Garcia *et*

*al.*, 1997: Legarreta *et al.*, 2000; D'Onghia *et al.*, 2001), are now being utilised.

Direct tissue blot immunoassay (DTBIA), a technique, that requires very little sample manipulation, has been used for the rapid delection of *Citrus trlsteza virus (*CTV), providing to be more sensitive and cheaper than ELISA Garnsey *et al.*, 1993) and comparing well with the more recently described in *situ* immunoassay (ISLA) Youjian *et al.*, 2000). *Thus,* as reported in the present paper, DTBIA was tested for the rapid detection or CPsV.

Preliminary assays were done on roots, stems, leaves, fruit pedicels and flowers from five citrus genotypes (Table 1) from a field-grown collection of the Mediterranean Agronomic Institute (IAMB) and the university or Bari (UBA), all of which were known to be infected with CPsV, as determined by indexing and serology (Potere *et al.*, 1999; Djelouah *et al.*, 20001. Explanls from a CPsV-free Navelina' orange, were used as controls.

The high background in the blots from roots and the uncertain reactions given by stems and fruit pedicels, suggested to concentrate on leaves and flowers which appeared to be more promising sources *of* antigen. tests were done twice at blooming time using no less than 50 samples of different types *of* leaves (young exptanaing, fully expanded, and mature) and flowers, from each of the CPsV- infected sources. Open and closed flowers were collected dissected into style, stigma, and ovary, which were blotted immediately or after storage at -20 and 70°C :or 20,60, and 120 days.

**Table 1: Citrus psorosis virus-infected sources used in the tests**

| Accession | Species/variety | Origin |
|---|---|---|
| IAMB-UBA - 191X | Navelina | Italy |
| IAMB-UBA-269X | Bonanza orange | Italy |
| IAMB-UBA-654X | Grapefruit | Italy |
| IAMB-UBA-655X | Lemon | Italy |
| P216 | Dweet tangor | USA |

DTBIA was as described by Garnsey *et al.* 1993) with minor modification. A fresh cut was made with a shap razor blade across each sample and ,the cut surface was gently pressed on a Bio-Rad transblot nitrocellulose membrane. Leaf blades were rolled up before curling and blotting. Gloves or tweezers were used when handling the membranes and in the blotting process.

Blotted membranes were allowed to dry for 20-30 min at room temperature and, betore testing, were marked with a pencil. so as to record the position or individual samples. Membranes were then placed in a 1% solution of BSA in distilled water and incubated for 2 h at room temperature, or overnight al 40°C in a plastic Container on a shaker stirrer to block protein binding sites. after washing with PBS containing 0.05% Tween 20 blotted membranes were exposed for 3h to an alkaline phosphate conjugated monoclonal antibody 1:250 dilution in conjugate buffer (Porere *et al.,* 1999).

Membranes were stained by dissolving one tablet of BCIP-NBT Sigma fast in l0 ml distilled water and incubated until a purple-violet colour appeared in the positive control. The reaction was stopped by washing with tap water. after drying at room temperature. the membranes were Observed with a low power magnification lens.

Leaves and whole flowers assayed by DTBIA were also tested by DAS-ELISA as described (Potere *et al,1*999) using a polyclonal antiserumfor plate coating and the alkaline pnosphalase-conjugatecd Mab Ps29 at l :500 dilution in conjugate burrer as second antibody Explants from the tree inrected with the CPsV isolate used for raising Mab Ps29 served as positive controls. Colour development on botled membranes usually appeared within 10 min from the addition or subsrrate and the reaction was stopped) 5-10 min later.

Purple-stained areas were observed in imprints or all CPsV, infected flower explants (ovary, style and stigma) (Fig.1). The localization and distribution of the stain was intense and homogeneous in blots from ovaries (Figs 1 and 2) and less clear-cur in those from stiles and stigmas (Fig 1). Little or no reaction was observed in blots from leaves (Fig. 2). Control blots remained virtually unstained (Figs 1 and 2 .The totality of overies from closed flowers were DTBIA positive, whereas positive reactions from open flowers

were fewer *(ca* 80% Interestingly, no apparent differences were found between fresh and frozen ovary explants from closed flowers. regardless of the duration of the storage in the cold.

DAS-ELISA confirmed the results of immunopriting. Strong posirive reactions were obtained, especial from ovaries which showed OD 405 reading from 35 to 65 higher than readings from leaves.

Based on the above the conclusion can be drawn than DTBIA correlates with ELISA and is a sensitive and simple procedure for CPsV detection. Ovaries a better organs than leaves for DTBIA in line with previous observations that reported lower concentration and irregular disrribution of CPsV in infected leaves compared with flowers (Dielouah *et al,* 2000 D;Onghia et al, 2001) interestingly in our experiments blots from the a single ovary consistently detect CPsV, whereas with CTV, multiple prints from t same the sample were needed (Garnsey *et al,* 1993).

High sensitivity, short assay time and limited cost are the main advantages of DTBIA, which also represents very convenient and safe system for shipping blotted membranes from one place to another. The short slowing period *of* most citrus species may not represent limit to the use *or* DTBIA for large-scale routine testing since storing flowers at -20 - 70C for 1 to four months, apparently does not affect the results of the test.

# Serological Diagnosis of Citrus Psorosis Virus and Citrus Tristeza Virus Using Flower Parts

AOAD

# Serological Diagnosis of Citrus Psorosis virus and *Citrus tristeza virus* Using Flower Parts

K. Djelouah, D. Frasheri, and A. M. D'Onghia
Serological Diagnosis of *Citrus psorosis virus*

**Abstract**:

Citrus p*sorosis virus (CPsv)* and *Citrus tristeza virus* CTV) isolates from citrus species of different *origin were* tested at flowering time by DTBIA and *ELISA,* using ovaries, or .petioles and leaves. Reagents were commercial antibodies to CPsV and commercial antibodies and kits for CTV. Compared to petioles or leaves, the Use of flower parts (ovary and *pistil)* gave better reactions in DTBIA and ELISA. Ovary prints from CPsV- and CTV- infected plants stained *uniformly* and intensely, whereas no reaction was observed prints from comparable healthy tissue. Ovaries could be processed fresh or after up to 1.yr storage at -20°C. Printed membranes could be stained Immediately or processed after 1 yr using the same types of buffer with out apparent loss of sensitivity.

Tristeza and psorosis are severe diseases of citrus with a worldwide distribution (11, 12)- As alternatives or additional to biological indexing, simple and rapid procedures such as ELISA and DTBIA. are now being utilized (1, 2, 3, 5, 6, 7, 8, 9, 13) to detect the causal viruses, and commercial kits make it Possible to test a large number of samples with high sensitivity and specificity.

The disease agents, *Citrus tristeza virus (CTV;* and *Citrus psorosis virus* CPsV have differing distributions in the host plant, so different tissues are used for diagnostic assays, i.e. young shoots and leaf petioles for CTV (3) and mature leaves for CPsV (5). Sampling should be done in autumn and Spring when the temperature is *18-24C* and the virus concentration is highest.

Recently; flower parts have been successfully used for detection of both viruses using ELISA and DTBIA (7, and unpublished information). At

flowering time, closed flowers and mature leaves were sampled from two separate collections of cit citrus genotypes of different origin, mainly Mediterranean, infected by C1'V and CPs V respectively: The citrus types covered sweet oranges, mandarins, grapefruits, lemons, *Pummelo,* kumquat and *Citrus excelsa.* These collections were maintained in insect-proof screen houses and have been biologically and serologically characterized (4, 5, 13, and unpublished information).

Samples of 50 flowers and leaves were collected from each source, and subsamples were stored at 4°C, -20°C or -70°C. These were tested, respectively; at daily, weekly and monthly intervals.

For CTV, leaf petioles as well as flower parts (pistils and ovaries) were tested by ELISA and DTBIA. With CPsV, ELISA Was used on pistils and mature leaves while DTBIA Was only applied to the ovaries; this Was because DTBIA proved not to give a consistent signal with CPsV . infected leaves (7) although with later and very recently reported experiments (9) it has been found useful also for young shoots and leaves- Similar tissues from the corresponding healthy species were used as controls.

ELISA assays were done with commercial monoclonal antibodies - specific to CTV (Domaines Royales, UCP, Morocco) and to CPsV (Agritest-Italy; 10). EXtracts were prepared by grinding different types of tissue (0.5 g of mature leaves or petioles and one pistil) in the extraction buffer (1:10 dilution). Each sample was assayed in two wells. Optical densities were measured at 405 nmin a tikertck Multiscan photometer. The results were based on the mean absorbance values of the two sample wells, which were considered positive when three or more times higher than the healthy controls.

DTBIA was carried out using the Plant Print Diagnostics kit (Spain) *for* CTV; for CPs V the conjugated monoclonal antibodies of agritest (Italy) were used, with 0.45 nm nitrocellulose membranes (BioRad). The blotted membranes were allowed to dry at room temperature before being analyzed. About 20 additional blotted membranes were also stored dry and tested monthly. After blocking with BSA, the alkaline phosphatase-conjugated antibodies were added. The membranes were then rinsed in washing buffer and

developed using the substrate. The color reaction was stopped by washing with tap water and, after drying at room temperature, the membranes were examined with a 10 or 20x lens.

All samples from CTV- and CPsV infected sources were positive using both serological techniques (data not shown). In ELISA, higher values were obtained, with CTV; from pistils than from petioles, and with CPs V, from pistils than from mature leaves.

With DTBIA, purple-stained areas appeared in the ovary prints of all CTV- and CPSV infected samples, whereas control blots were virtually unstained, confirming the ELISA results. With CTV-, the localization and distribution of the stain was intense and homogenous in blots from ovaries (Fig. 1), whereas petiole imprints were less colored (not shown).

No differences were noted in the use of fresh or frozen flower parts tor ELISA or DTBIA; however, leaves could not be frozen, and could be stored at 4°C for only a week. In contrast, blotted membranes could be kept *for* at least 1 yr before processing, as previously described (3, 8).

Our results indicate that CTVand CPsV infected flowers are better antigen sources than other tissues (leaf petioles and leaf blades) for both ELISA and DBTIA. Flowers can easily be collected in the field without damaging the trees, and can be stored for long periods without deterioration; moreover each *virus* can be consistently detected in a single ovary.

*W-e* conclude that CTV and CPSV can be rapidly and reliably detected by DTBIA using ovaries. For large scale surveys in the field, a few flowers can be collected from each tree for fresh printing and storage at *-20°C.* However, in most cases, only one flower is sufficient for successful blotting. Blotted membranes or frozen pieces can be readily processed throughout the year.

Although flowers gave best results, these are only available for brief periods. At other times CTV can be detected by DBTIA using leaf petioles, stem sections or fruits, whereas ELISA using mature leaves is needed for CPsV.

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## APPENDIX

1. Microtiter plates: Falcon 3911 MicroTest III Flexible Assay Plate (U-bottom) Becton Dickinson Labware. Becton Dickinson and Co. 1950 Williams Drive, Oxnard, CA 93030.

2. Buffers

A) PBS (pH 7.4)

8.0 g NaCl
0.2 g $KH_2PO_4$
2.9 g $Na_2HPO_4 \cdot 12H_2O$ (1.15 g anhydrous) — make up 1 liter
0.2 g KCl
0.2 g $NaN_3$

B) PBST = PBS + 0.5 ml Tween20 per liter

C) Coating Buffer (pH 9.6)

1.59 g $Na_2CO_3$
2.93 g $NaHCO_3$ — in 1 liter $H_2O$
0.2 g $NaN_3$

D) Sample extraction buffer

a) PBS extr. buffer: (pH 7.4)
PBST + 2% PVP-25

b) Tris extr. buffer (pH 8.2)
60.5 g TRIS
8.0 g NaCl
20.0 g PVP-24
10.0 g PEG 6000 — make up to 1 liter
0.2 g $NaN_3$
0.5 ml Tween 20

E) Enzyme Conjugate Buffer (pH 7.4)

PBST + 2% PVP-25 + 0.2 % BSA (Bovine Serum Albumin, Sigma A-4503)

F) Substrate buffer

97 ml Diethanolamine
800 ml $H_2O$
0.2 g $NaN_3$ — make up to 1 liter
Add HCl to give pH 9.8

3. Substrate: Sigma 104 Phosphatase substrate.
4. Enzyme: Sigma Alkaline Phosphatase Type VII-S (P-5521)

**The kit contains:**

Plastic bag 1: Silicate gel and test disposal
Plastic bag 2: Cheesecloth for sample preparation
Extraction buffer (as for ELISA extraction buffer, plus 9,5% Triton X 100)

**Protocol:**

Pour the extraction buffer on the cheesecloth.
Put some leaves fragments (around 0,25g) in the plastic bag and grind. The solution should become green, otherwise grinding of vegetal tissue is insufficient.
Open Plastic Bag 1. Immediately put a few drops (around 150 µl) of the vegetal extract into the small circular test window.
Wait for a few minutes and evaluate the result as follows:
If you only have a single line next to the "C", the test worked and the sample is negative;
If you have two lines, one next to the "C" and the other next to the 'T' , the test worked and the sample Is positive;
If you do not have any line after 15 minutes, the test did not work and it should be repeated.

**Important**:

Do not open plastic bag 1 (containing silicate gel and test) before the plant extract is ready: air humidity can damage the test.
Do not use the same kit more than ones: each sample should be run just on a single test.

# Molecular Techniques for Detection of Plant Viruses and Viroids

AOAD

# Molecular Techniques for Detection of Plant Viruses and Viroids

ALRWAHNIH Maher
MAI-Bari
2003

## Principle of Molecular techniques

Molecular techniques are based on recognizing of the nucleic acid (DNA or RNA) in the infected plants.

- Molecular hybridization
- Polymerase chain reaction (PCR)
- dsRNA

## Molecular hybridization

Hybridization: two complementary sequences will form hydrogen bonds between their complementary bases (G to C and A to T or U) and form a stable double- stranded anti-parallel "hybrid" helical molecule.

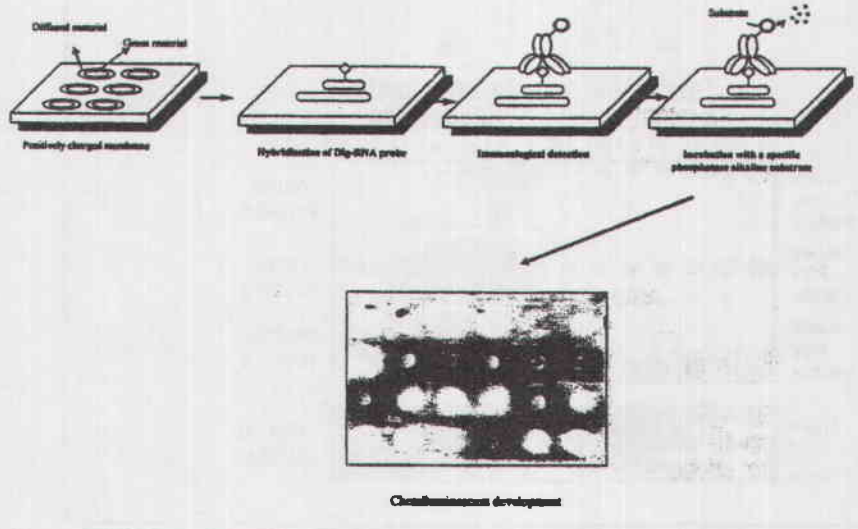
## Molecular hybridization

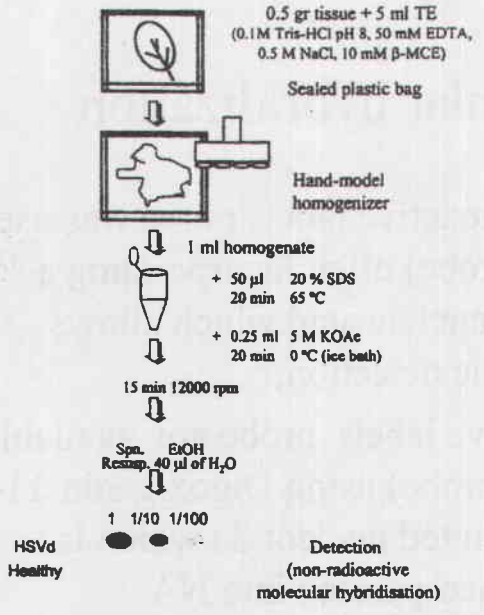
- Nucleic acid (NA) hybridization on membrane is a simple sensitive and specific means of detection nucleic acid of interest;
- Denatured target NA fixed on positively charged membrane and anneals to it labelled specific single-stranded probe nucleic acid.

# Molecular hybridization

- Previously radioactive labels probe was used (hot labelled probe) often incorporating a-$^{32}$P dCTP into the nucleic acid which allows autoradiographic detection;
- Non–radioactive labels probe are available (cold labelled probe) using Digoxigenin-11-dUTP as substituted nucleotide which is enzymitcally incorporated into NA

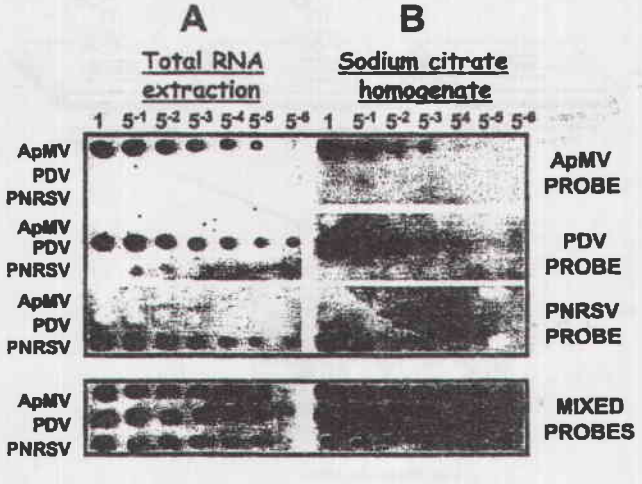
## SCHEME OF MOLECULAR HYBRIDISATION



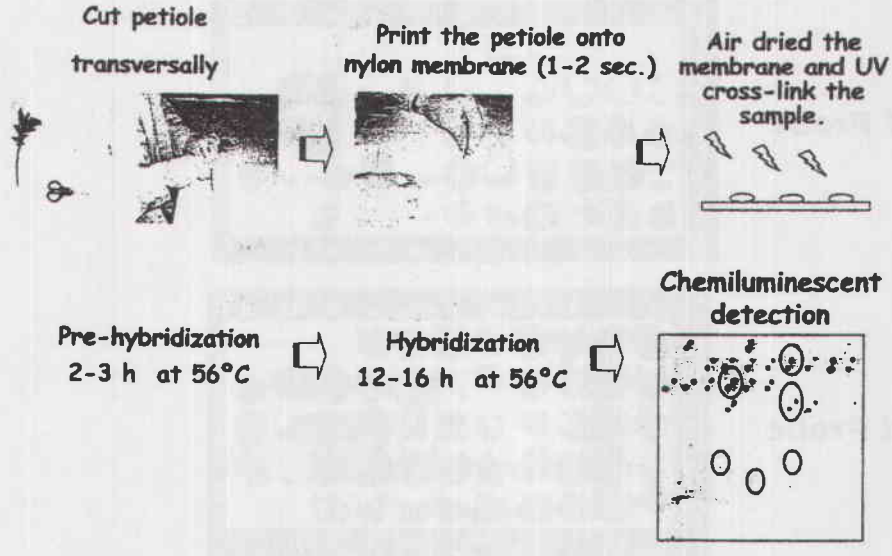



*Pallás lab, 1997*

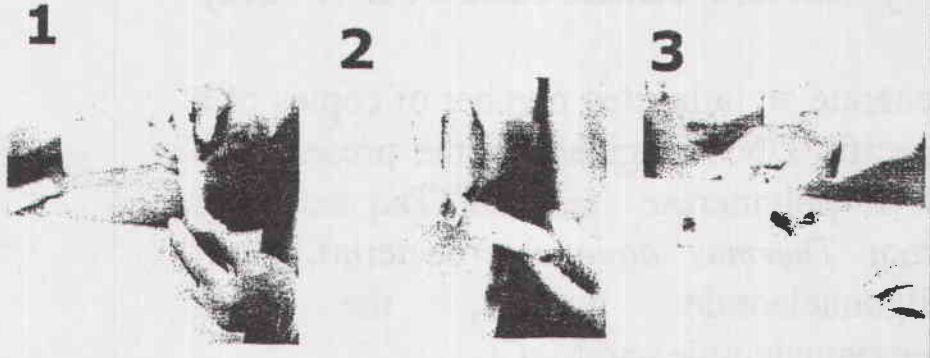
## Simultaneous Detection of Three Ilarviruses by Molecular Hybridization (Saade et al ., 2000)



## Tissue Printing Methodology

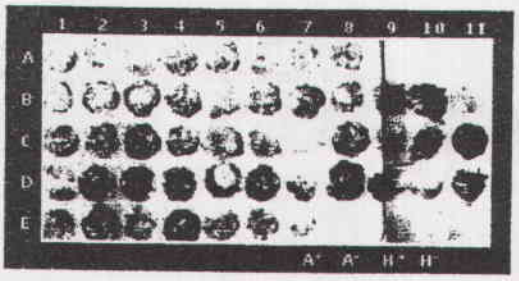



## Tissue printing on fruits

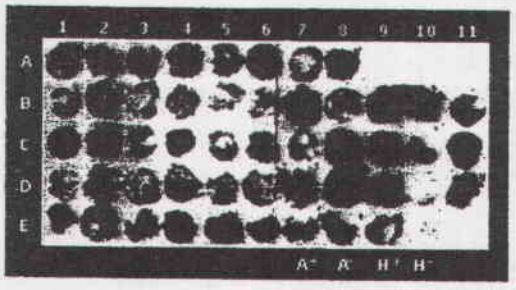


## Apricot Results

**ACLSV Probe**




**HSVd Probe**




## Polymerase chain reaction (PCR)

generate an unlimited number of copies of a specific DNA fragment in the presence of DNA polymerase enzyme (Taq extracted from *Thermus aquaticus* bacteria), short oligonucleotide primers, the four deoxynucleotide and $MgCl_2$

## The Main Features of PCR Technique

- The ability to detect plant pathogens present at levels below the detection limits of previous methods;
- The identification and characterization of unknown viruses;
- The establishment of phylogenetic relationship.

## ADVANTAGES PCR

- Sensibility (600 times > probe)
- Specificity (at strain level)
- Quick
- Degenerate primers
- Multiplex primers
- Sequencing

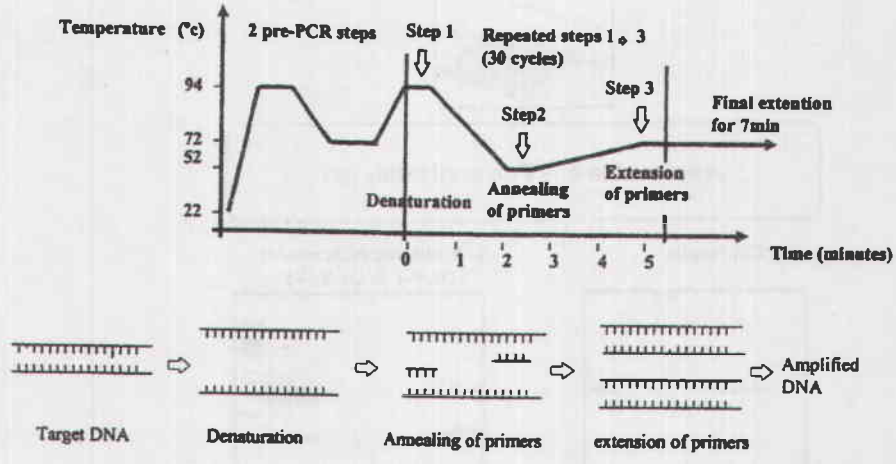
## DISADVANTAGES of PCR

- **Costly**
- **expertised technicians**
- **Low number of samples**
- **Purification of viral template**
- **Effect of plant inhibitors**
- **Electrophoresis**
- **Steps of RT-PCR for RNA virus**

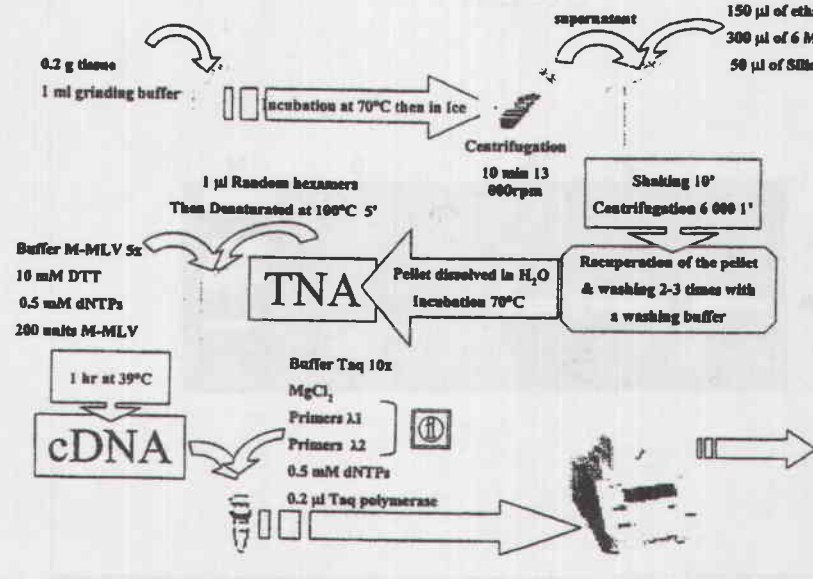
## Types of PCR

- Reverse transcriptase PCR (RT-PCR)
- Immunocapture PCR (IC-PCR)
- Nested PCR
- Co- operational amplication Co-PCR
- Real time PCR

## Scheme of the PCR protocol



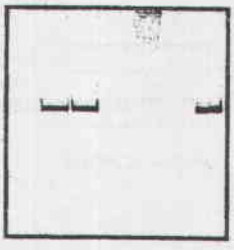

## RT-PCR (Silica extraction)



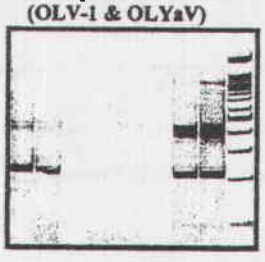
# RT-PCR (Silica extraction)


Analysed in 6% Polyacrilamide gel

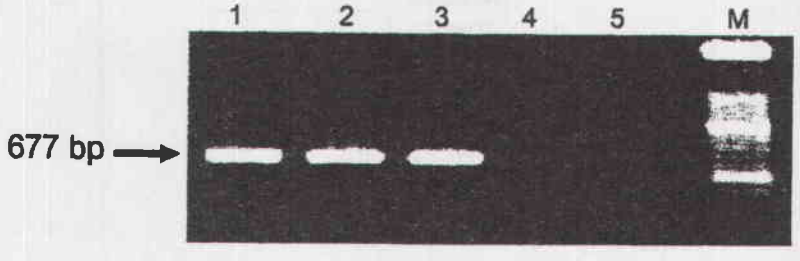
A- PCR results



B- Multiplex PCR results (OLV-1 & OLYaV)



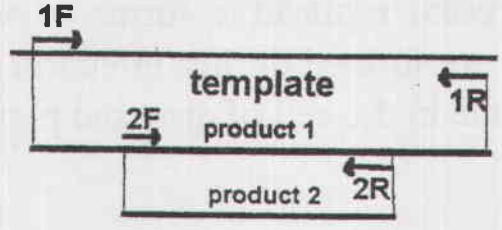
## RT-PCR for Apple chlorotic leaf spot virus (ACLSV)



# Nested PCR

**nested primer PCR:** PCR amplification is performed with one set of primers, then some product is taken - with or without removal of reagents - for re-amplification with an internally-situated, "nested" set of primers. This process adds another level of specificity, meaning that all products non-specifically amplified in the first round will not be amplified in the second.

**NESTED PRIMER PCR:**




# ALWAYS REMEMBER

- **WORK CLEAN**
- **TITRATE MAGNESIUM**
- **DON'T USE TOO MUCH TEMPLATE DNA**
- **DON'T USE PCR PRODUCTS IN PCR PREPARATION AREAS**
- ***ALWAYS, ALWAYS* INCLUDE WATER AND VERY DILUTE POSITIVE CONTROLS IN EVERY EXPERIMENT**
- **WEAR GLOVES**
- **USE PLUGGED TIPS**

## dsRNAs

- Double-stranded RNAs (dsRNAs) are paired molecular of viral genomic or sub genomic RNAs made up of a positive sense RNA strand;
- dsRNAs represent replicative forms of viral RNAs which are formed during infection and may accumulate in the cell of diseased plants;

## dsRNAs

- dsRNAs are stable molecule complexes with a size double that of genomic and sub genomic single-stranded RNAs;
- By estimating the relative size of dsRNAs, it is possible to identify the taxonomic group to which the eliciting virus is belong.
- Use for the synthesis of cDNA in order to run PCR.

# dsRNAs
## (CF-11 cellulose extraction)

1. Selection and processing of plant tissue

(5 –10g )

2. Extraction of TNA


Phenol:Cloroform extraction

3. Purification of dsRNA

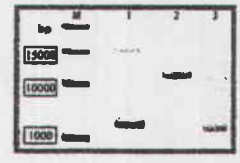
A) CF11 purification


5 washing by 1xSTE & ETOH

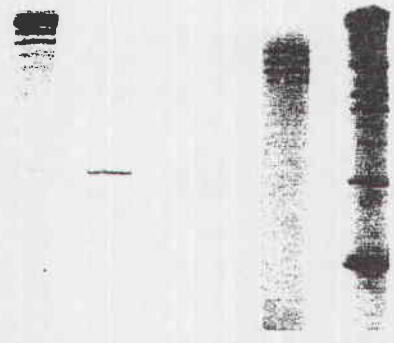
Concentration of dsRNA

B) Enzymatic digestion


4. dsRNA analysis in 6% polyacrylamide gel




# dsRNA for ApLV



Lane 1 ApLV - infected GF305, Lane 2 ACLSV-infected C. quinoa, Lane 3 healthy GF305, Lane 4 GVA-infected N. benthamiana Lane 5 GLRaV-2-infected N. benthamiana. The arrow indicates the presumed ApLV full-genome dsRNA.

# Routine Detection of Citrus Tristeza Virus by Direct Immunoprinting - ELISA Method Using Specific Monoclonal and Recombinant Antibodies

# Routine Detection of Citrus Tristeza Virus by Direct Imununoprinting-ELISA Method Using Specific Monoclonal and Recombinant Antibodies

M. Cambra, M. T. Gorris, M. P. Roman, E. Terrada, S. M. Garnsey, E. Camarasa, A.. Olmos, ann M. Colomer

**ABSTRACT**:

Extract prepartion is the most limiting factor for large-scale plant virus testing. Direct tisue print of fresh cross sections of tender shoots or leaf petioles on cellulose membranes, allows the collection of samples and testing of a large number of plants (1,250 plants per team of two workers per day). The printed membranes can be analyzed in the field, mailed, or kept for several months before testing. The analysis is performed by a simple and fast (3 h) direct ELISA protocal using a mixture of citrus tristeza virus (CTV)-specific, alkaline phosphatase conjugated monoclonal antibodies 3DFI and 3CA5 or using a mixture of 3DFI adn 3CA5 scFv-AP/S recombinant antibodies expressed in *E. coli* as a fusion protein with the alkaline phosphatase enzyme. The sensitivity of immunoprinting-ELISA method was the same as immunocapture-PCR, but it was more reliable. A kit has been designed and evaluated under nursery conditions. This kit has been succefully used by nurserymen to test more than 600.000 plants over the last five years.

***Index words***: citrus tristeza virus, detection kit, immunoprinting-ELISA, tissue print-ELISA, monoclonal antibodies, recombinant antibodies, IC-PCR.

Testing for citrus tristeza virus (CTV) has been performed for many years by grafting on the biological indicator, Mexican lime (16). The application of DAS-Elisa (1, 6) revolutionized the diagnosis by allowing the process of large number of samples in a short period. The Elisa was used for large-scale surveys, CTV control in citrus nurseries, epidemiological, and other studies (4, 8). The ELISA techniques has provided diagnosis with a high level of sensitivity

and low cost. In addition, the production of monoclonal antibodies (MCAs) specific to CTV in 1982 (14,15) and its commercialization by Ingenasa (Madrid) made it possible to test a large number of samples for CTV with a high sensitivity and specificity. Among the available MCAs (12), the mixture of the well-characterised 3DF1 and 3CA5 is able to recognize all CTV isolates tested from different collections (5). This mixture has been extensively used in routine tests and in several ELISA variants (4). The change in CTV diagnosis due to ELISA with MCAs is illustrated by the fact that more than 2 million samples have been tested using these MCAs.

The most important limitation for detection of CTV by conventional ELISA, however, is the necessity to prepare plant extracts which is a laborious and time-consuming process especially in woody plants and also enhances the risk of contamination.

Furthermore, conventional ELISA requires skilled specialistsa and expensive equipment for reading the plates. A laboratory is necessary for sample extract preparation and testing , and additionally, samples collected fro Elisa can only be stored for a maximum of 1 week at 4 degrees C, before the exract preparation.

The use of membranes to capture and immobilize targets constitutes a good alternative to extract preparation. The development of direct tissue blot immunoassay, immunoprinting-Elisa or tissue print-Elisa in plant pathology (11) and its application to CTV (2,3,9) again revolutionized and simplifoied the detection of the virus. Immunoprinting-Elisa allows the sensitive analysis of thousands of samples in an easy way, without the need of extract preparation, by directly printing sections of plant material on nitro-cellulose membranes.

A complete kit, with improved protocol for CTV detection has been produced by Plant Print Diagnostics (Valencia) in agreement with Instituto Valenciano de Investigaciones Agrarias (IVIA). With this kit more than 600,000 plants have been analysed since 1994 by nurserymen to test their multiplication blocks, apart from the official control. In this paper, we have evaluated this methodology for its sensitivity, simplicity, cost, as wellas the possibility of its use by non-specialized workers, and direct field application under nursery

conditions. We also have compared the routine u se of conventional MCAs (3DF1 + 3CA5) conjugated to alkaline phosphatase against the use of CTV specific recombinant single chains (scFv-3DF1 and scFv-3CA5) genertically fused to alkaline phosphatase (scFv-Ap/s) for the direct detection of CTV by tissue print-Elisa.

**Material and Methods:**

**Direct immunoprinting-ELISA protocol**. The improved protocol for direct immunoprinting-ELISA was performed in three steps: a) Sample imprinting on nitrocellulose membranes; b) Blocking and addition of CTV-specific antibodies alkalin phosphastase conjugate and; c) Substrate addition and reading. The protocol is performed as follows:

1. **Preparation of plan samples (membrane printing)**: Make clean cuts on tender shoots, leaf petioles or fruit peduncles. Press carefully the freshly made sections against the nitrocellulose membrane 0.45 mm (Millipore). Let the trace or the print dry for a few minutes. Printed membranes can be kept for several years in a dry place. For adult plants select five tender shoots (from last flush) or 10 leaves from around the canopy (preferably from the top area) for sample imprint. For seedlings select two shoots, or four leaves and analyze. Perform two printings per shoot or one per leaf.
2. **Membrane blocking**: Prepare 1% solution of bovine serum albumin (BSA) in distilled water. Place the membranes (about 7x13cm) in an appropriate container (tray, hermetic container, plastic bag). Pour the BSA solution over the membrane covering them, and incubate for 1 h at room temperature, or overnight at 4 degrees C. A slight agitation is recommended during this step. Discard the albumin solution and keep the membranes in the same container.
3. **Addition of monoclonal antibodies/alkaline phosphatase (AP) linked or recombinant antibodies AP/S fused.** Prepare a solution of CTV specific 3DF1 + 3CA5 MCAs linked to AP (about 0.1 ug/ml each MCA in PBS) or of 3DF1 scFv-AP/S + 3CA5 scFvAP/S fusion

proteins expressed in *E coli* 1/4 diluted in PBS. Pour the solution on the membranes, covering them and incubate for 2 to 3 h at room temperature, then discard the conjugate solution.

4. **Washing of membranes**: Prepare 1l washing buffer (PBS + 0.05% Tween 20) for 10 to 15 membranes each of 7 x 13 cm. Rinse the membranes and the container with 100ml of washing buffer. Wash by shaking (manually or mechanically) with 400 ml buffer for 5 min. Discard the washing buffer and repeat the process with the remaining buffer.
5. **Membrane development**: Prepare substrate buffer by dissolving 10 BCIP-NBT, Sigma Fast tablets in 100 ml (for 10 to 15 membranes) distilled water. Pour over the membranes and let incubate until appearance of purple violet color in positive controls (3 to 7 min). Stop the reaction by washing the membranes on absorbent paper and let them dry.
6. **Membranes reading**: Observe the printings by using a low power magnification (x10 to x20). Presence of purple-violet precipitates in the vascular region of plant material reveals the presence of CTV.

**Evaluation in citrus nurseries**: A complete kit (Plant Print Diagnostics) based on the above described protocol including all reagents and pre-printed controls, was evaluated, in two Spanish nurseries. In Viveros Valencia (Peniscola, Spain), the method was tested on samples from increase blocks in open field. In Viveros Alcanar (Alcanar, Spain) the samples were collected from an insect-proof screen-house tunnel. Two-person teams performed the routine analysis of nursery plants. The total number of samples collected daily and printed on membranes was evaluated and compared with the number of extracts prepared from the same samples and analyzed by DAS-ELISA and immunocapture-PCR (IC-PCR). The economic cost of the analysis per nursery plant was also calculated from all assayed techniques.

**Comparison with other detection techniques**. Samples (five young shoots/tree) from 65 sweet orange trees cv. Washington Navel were analyzed

in October by immunoprinting-ELISA. An extract of the same plant material was also analyzed by IC-PCR (7). All these trees have been assayed by DAS-ELISA (15) a year before and proved to be CTV-free. The trees that gave differential diagnostic by both techniques were analyzed again 1 month later (November) by immunoprinting-ELISA and IC-PCR. Samples were collected again from trees that gave different test results and analyzed by immunoprinting -ELISA and nested-PCR in single closed tube (13) and grafted on Mexican lime seedlings. In addition 200 seedlings (about 25cm high) of Mexican lime, Alemow, sour orange and sweet orange (total of 800 plants) were cultivated in field from May to July at Moncada in an Clementine plot with 85% CTV infection. The seedlings were individually analyzed by DAS-ELISA biotin/streptavidin system (Ingenasa), tissue print-ELISA and IC-PCR after 6 months of growth in an insect proof screenhouse.

Production and use of CTV-specific recombinant single chain Fv fragments (scFvs) fused with alkaline phosphatase. The variable domains of 3CA5 and 3DF1 antibody genes were amplified from mRNA isolated from MCAs-production hybridoma cells kept at Ingenasa (Madrid) and cloned into pDAP2/S (10) vector. The expression of this construct in *E. coli* produced a single chain Fv fragment, in which the two variable domains are connected by a generically encoded linker, fused to alkaline phosphatase protein. A mixture of both recombinant conjugates 3DF1 scFv-AP/S and 3CA5 scFv-AP/S were assayed by tissue print-ELISA. Parallel assays were performed with conventional MCAs conjugated with alkaline phosphatase.

**Results:**

The collection of nursery plant samples and their direct printing in screen house or field allowed the analysis of 1,250 plants/day by a two-person team. About the same number of plants were analyzed daily collecting leaf samples in the field, printing and subsequent analysis in the laboratory conditions. (Fig. 1 to 5).

Leaf petioles were the most convenient analysis for tissue print-ELISA in both nurseries. This material remains succulent over the growth of the plants in the nursery and can be easily collected without damaging the plants. In addition prints from leaf petioles occupy less space on a membrane than sections of the stems, allowing a higher number of tests per membrane. Table 1 summarizes the evaluation of the routine analysis of nursery plants performed by a two-worker team by different techniques.

The results of the analysis by DAS-ELISA, tissue print-ELISA and IC-PCR of citrus seedlings exposed to natural CTV infection in Moncada, were coincident. Only five plants out of 800 ware detected as CTV infected by all the assayed techniques. Nevertheless, discrepancies were observed in the analysis of mature sweet orange trees. The comparative analysis by tissue print-ELISA and IC-PCR of 65 recently CTV-infected Washington Navel trees resulted in 37 positive and 16 negative trees by both techniques assayed, five trees tested positive by tissue print-ELISA but negative by IC-PCR, and 7 trees were positive only by IC-PCR. Coincident results were obtained in 81.5% of the analyzed trees but a different diagnosis was rendered in 12 trees These 12 questionable trees were analyzed again 2 months later. The five trees that were positive by tissue print-ELISA were confirmed as infected by both techniques. Of the seven trees which were positive by IC-PCR only four were positive by IC-PCR and negative by tissue print-ELISA, and the remaining three were CTV negative by both techniques. The four trees CTV positive only by PCR were sampled again and analyzed by nested-PCR in a single closed tube and indexedon Mexican lime indicator plant, and all were found negative by both methods.

Printed sections of leaves and stems from CTV infected sweet orange and clementine trees were analyzed 1 and 2 years after preparation. No differences were observed in this comparison with other samples fresh-printed and subsequently analyzed on the same membrane.

The recombinant antibodies were used to detect CTV coat protein by tissue print-ELISA and the results compared with the commercial detection kit (Plant Print Diagnostics) based on the same technique but using the conventional MCAs 3DF1 and 3CA5. The developed prints show a similar intensity and number of stained areas in the vascular regions of sections of sweet orange shoots when using conventional MCAs or recombinant antibodies (data not shown).

**DISCUSSION**

Direct immunoprinting-ELISA or direct tissue print-ELISA performed with universal CTV-specific MCAs is a reliable, sensitive and economic procedure for routine detection of CTV in citrus plants. The sensitivity of the test, using the proposed protocol and rested reagents, is the same as conventional DAS-ELISA or IC-PCR. The advantages of the evaluated tissue print-ELISA kit were their increased reliability compared to IC-PCR, simplicity (that allows its use for non-specialized workers), efficiency (that allows to collect and process up to 1,250 nursery plants per day) and the low cost ($US 0.26/plant including sampling). In addition, the immunoprinting-ELISA method allows CTV detection in the field or greenhouse in only 3 h after imprint preparation.

The use of immobilized targets on membranes by tissue print or squash, constitute a good alternative to extract preparation. The printed membranes can be stored at room temperature or at 4°C for long periods. Tests performed 2 yr after imprint of samples on a membrane were still producing reliable results. This fact represents a great advantage allowing the storage at room temperature and the submission of pre-printed membranes by conventional courier, if necessary.

The use of tissue print-ELISA kits is very convenient for large surveys in field or nursery plants, and especially more convenient to work in remote sites.

The use of scFv-AP/S fusion proteins which are CTV specific have also proven to be excellent conjugates in immunoprinting-ELISA. Recombinant conjugates efficiently reacts against CTV and may substitute conventional antibodies linked with alkaline phosphatase by glutaraldehyde in a near future because of its easier production and lower cost.

**Table 1:**

**evaluation of three detection methods for the routine analysis of citrus nursery plants by a two-worker team**

| Category | CTV Diagnostic methods | | |
|---|---|---|---|
| | DAS-ELISA (extracts) | Immunoprinting-ELISA (tissue prints) | Immunocapture-PCR (extracts) |
| Sensitivity | +++ | +++ | +++ |
| Reliability | +++ | +++ | ++ |
| Simplicity | ++ | +++ | + |
| Capacity for routine processing | + | +++ | + |
| Number of analyzed plants/day | 600 | 1,250 | 150 |
| Sampling cost per plant | 0.09 | 0.09 | 0.09 |
| Extract preparation cost per plant | 0.10 | 0.00 | 0.22 |
| Reagents, buffers and material cost per plan | 0.50 | 0.17 | 1.20 |
| Total cost of the analysis per plant | 0.69 | 0.26 | 1.51 |
| Laboratory equipment necessary (cost) | 15,625 | 1,250 | 12,500 |

'Cost in US$

The number of + symbols indicate how methods rate from accpetable (+) to optimum (+++).

Our experiments in the field clearly showed a higher reliability of the tests performed by immunoprinting-ELISA than by IC-PCR in spite of IC-PCR great theoretical sensitivity. Tissue print-ELISA method can be easily adopted and adapted to particular conditions in private nurseries. The availability of the evaluated kit makes possible the official Spanish policy to produce only pathogen-free citrus plants. Zero tolerance for CTV in nurseries, in spite of the fact that plants will be infected by viruliferous aphids in the field, ensures that at least sometime exists for the sate use of susceptible rootstocks such as Alemow and that no CTV isolate will be spread from the nurseries (including severe CTV isolates that could be introduced into nursery plantings.)

The high number of tests performed by private nurserymen, in addition to the official control, also demonstrate the suitability of the method.

## ACKNOWLEDGMENTS

This work was supported by grants form INIA (SC98-060), European Union (AIR-CT94-1046 and SMTA-CT98-2252) and by IVIA-Plant Print Diagnostics S.L. (No. 7809) and IVIA-AVASA (No. 7602) agreements. The authors are grateful to Jose Antonio Ximenco and Rafael Alcaide, technical directors of Viveros Alcanar and Viveros Valencia, respectively, for their evaluation of the kit in nursery conditions, and to Maribel Soler and Ana Sancho (from Viveros Alcanar) and to Maria Jose Colom and Cristina Genoves (from Viveros Valencia) for excellent technical assistance in collecting, printing and analyzing samples.

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# Direct Tissue Blot Immunoassay (DTBIA) for Detection of Citrus Tristeza Virus (CTV)

# Direct Tissue Blot Immunoassay (DTBIA) for Detection of Citrus Tristeza Virus (CTV)[1]

S. M. Garnsey, T.A. Permar, M. Cambra, and C.T. Henderson

## ABSTRACT:

A direct tissue blot immunoassay (DTBIA) procedure was tested for detection of citrus tristeza virus (CTV). Freshly cut stem, petiole or fruit pedicel tissue was carefully pressed to nitrocellulose membranes. The membranes were blocked by incubation in dilute bovine serum albumin and then incubated with unlabeled or biotinylated monoclonal or polyclonal antibodies. Antigen-bound biotinylaed antibodies were detected by exposure to a streptavidin-alkaline phosphatase conjugate (APC) and antigen-bound unlabeled antibodies were detected by a goat anti-mouse or goat, anti-rabbit IgG-APC. The substrate was NBT-BCIP. Localized areas of the tissue imprints of CTV-infected plants stained intensely and were easily recognized under 10X magnification. Location of CTV in phloem tissues was determined easily without sectiononig or other cytological techniques. No comparable staining was observed in imprints of healthy tissue. Assays of 858 healthy and CTV-infected trees in Florida and 560 trees in Spain by ELISA and by DTBIA indicated similar rates of CTV infection. Strain differentiation was accomplished by making duplicate impressions on different test sheets and processing one with the strain-selective monoclonal CTV-MCA13 and the other with polyclonal antibodies, or a mixture of monoclonal antibodies which react to all isolates. DTBIA is rapid, requires little sample preparation, and tissue blots could be stored at room temperature at least 30 days prior to assay. Blotted membranes can can be sent safely to another location for testing. DTBIA has been adapted for commercial diagnostic purposes.

[1]Mention of a trademark, warranty, proprietary product, or vendor does not constitute a guarantee by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products or vendors that may also be suitable.

***Index words:*** *CTV-MCA13, 3DF1, and 3CA5 monoclonal antibodies, biotinylated antibody, streptavidin, ELISA, immunoblotting.*

The use of enzyme-labeled antibodies in serological assays has provided diagnostic probes with a high level of sensitivity, stability, low cost, and safety (7, 12). ELISA is the most commonly used diagnostic procedure for plant viruses which combines use of an enzyme-labeled antibody and binding of the antigen or antibody to a solid phase (the ELISA plate). A number of variations of ELISA have been developed for CTV, and sensitivity has been enhanced through use of secondary antibodies and biotin-streptavidin linkages (8). Immunoblot procedures are a form of ELISA where one of the reactants (usually the antigen) is bound to a membrane, such as nitrocellulose, which has protein building properties, and is detected directly or indirectly with a labeled probe. An immunoblotting procedure for CTV was recently described by Rocha-Pena et al. (20, 21). Immunoblotting procedures are rapid, require only minimal equipment, and can have good sensitivity, but background color and lack of quantitative measurements of results can be a problem in some applications (12).

Lin et al. (15) recently described a variation of the immunoblot technique where the tissue sample is blotted directly to the membrane. They obtained good results with several virus and mycoplasmalike pathogens, including two which are phloem-limited. Application of this technique to tomato spotted wilt virus has also been reported (14). The direct tissue blotting assay (DTBIA), also described as an immuno-printing ELISA, requires no sample preparation or extraction and provides information on distribution and localization of the pathogen in host tissues. We felt that DTBIA should also work well with CTV because it is phloem-limited, the tissue area to observe for a virus-specific reaction is well defined and previous cytological studies have indicated that large amounts of virus are present in some cells of the phloem of CTV -infected plants (3, 9).

Polyclonal antisera have been prepared to several CTV isolates and work well for general detection of C~TV (1, 2, 8). Monoclonal antibodies have also been developed. Some are specific to well conserved epitopes and react to

most isolates (22, 23). A strain~selective monoclonal, CTV-MCA13, has also been described (18). The large variety of serological detection methods which have been developed for CTV since the advent of high quality, virus-specific antibodies was recently reviewed (19).

This paper reports development and evaluation of DTBIA for CTV, which is sensitive, reliable, requires minimal equipment and sample preparation, and is adaptable for large scale testing. An abstract has been previously published (17).

## METHODS AND MATERIALS:

**Tissue blotting technique**. Tissue blots were prepared essentially as described by Lin et al. (15). Blots were made from stem pieces, leaf petioles, fruit pedicel, vascular cores, bark cut from larger stems, and roots. Vascular cores of fruit and bark samples were trimmed to an appropriate size for blotting. A smooth fresh cut was made with a razor blade and the cut surface was pressed gently and evenly to the membrane. In some cases, especially with succulent tissue, two blots were made sequentially from the same cut. Both ends of stem pieces were frequently blotted to increase testing of each sample. To compare different antibodies, or different treatments, blots were made from the same tissue piece on separate membranes. A fresh cut was made between each blot and only a thin slice of tissue was removed so that the blots would be as comparable as possible. Disposable gloves or tweezers were used when handling the membranes and in the process of blotting

Blotted membranes were allowed to dry for 10-30 minutes. In most cases blots were processed within several hours, but in some cases blotted membranes were stored for longer periods, and a comparison was made of temperature, duration and desiccation effects on DTBIA.

**Membranes and membrane processing**. Bio-Rad Trans-Blot nitrocellulose membranes (Bio-Rad Laboratories, Hercules, CA 94547) were used for most studies. The 15-cm sq membranes were cut to an appropriate size for the number of samples to be blotted. The membranes were usually pre-marked with

an indexed grid of suitable size so the position of individual samples on a membranes could be recorded. Other membranes tested included Bio Blot nitrocellulose (Costar, Cambridge, MA 02140), Millipore 0.45 um filter membranes (Millipore Corp., Bedford, MA 01730), Photo gene nylon membrane (GIBCO BRL, Gaithersburg, MD 20877) and ZetaProbe membranes (Bio-Rad Laboratories, Hercules, CA 94547).

*Blocking.* After the membrane was imprinted with the tissue samples and dried, it was usually placed in a solution 1 % BSA in PBS and incubated for 1 hr at 25 C, or overnight at 4-6 C to block any remaining protein binding sites. Other blocking agents were used in specific tests as described below.

*Incubation.* Membranes were incubated in plastic dishes on a bench top shaker, in resealable plastic bags attached to a slowly rotating wheel, or in a Robbins Model 310 Hybridization Incubator (Robbins Scientific Corp, Sunnyvale, CA 94086). Incubation times were normally 1 to 2 hr at room temperatures for the virus specific antibody or secondary antibodies, and 1 hr for streptavidin conjugates.

*Washing.* Membranes were washed three times between steps in PBS-Tween (7) for 5 min under gentle agitation.

**Immunological methods and antibody sources**. *Immunoblots.* Four basic procedures were used and are diagrammed in Fig.1 The first was a direct method where the blotted membranes were exposed to CTV-specific antibodies conjugated to alkaline phosphatase (1, 7). The second procedure was an indirect method where the blotted membrane was exposed first to unlabeled CTV-specific antibodies and then used to commercially prepared alkaline phosphatase-labeled secondary antibodies (goat anti-rabbit for polyclonals and goat anti-mouse for monoclonals). In the third method the blotted membranes were incubated with biotiny-lated CTV-specific antibodies (13) and then with a commercially prepared streptavidin-alkaline phosphatase conjugate. In the fourth variation, the blotted membranes were incubated sequentially with unlabeled CTV-specific antibodies, a commercially prepared biotinylated secondary antibody, and a commercially prepared streptavidin-alkaline

phosphatase conjugate. The source of commercial alkaline phopshatase and biotinylated antibodies was Boehringer Mannheim Biochemicals, Indianapolis, IN 46250.

The CTV polyclonal antibody (PAB) 1052 to the Florida isolate T36 (18) was used for most tests. Several other polyclonals were used in limited tests. The 873, 894 and 879 PABs are to the Florida CTV isolate T4 as described previously (2). The 1051 and 1053 PABs are to the Florida CTV isolates T30 and T26, respectively, and have also been described (20). The 908 PAB was prepared to whole unfixed virus of the Florida CTV isolate T3 and has been used successfully for ELISA (Garnsey, unpublished).

Several different monoclonal antibodies (MABs) were used. The 3DFI and 3CA5 MABs (23) are reactive to most isolates of CTV, and are specific to two separate and widely conserved epitopes on the CTV coat protein (11). A mixture of 3DFI and 3CA5 was used in some cases to ensure detection of all isolates (5). TheCTV-MCA 13 is a MAB which reacts with severe sources of CTV, but does not react to mild isolates from Florida and some other countries (18). The 3E10 MAll is a broadly reactive MAB from Taiwan (22).

In most cases, purified IgG was used as a source of polyclonal antibody. Ascites and purified IgG were used as sources for MABs. Dilutions were made in PBS or in PBS which contained 1% BSA (8). Concentrations of IgG varied with the different sources and applications but, in general, dilutions for unlabeled CTV-specific antibodies ranged from 1/5,000 to 1/50,000 when made from ascites or from 1 mg/ml stock solutions of purified IgG. Commercially labeled secondary antibodies and streptavidin conjugates wre used at the manufacturere's recommended dilution.

**ELISA**. Double antibody sandwich (DAS) and double antibody sandwich indirect (DAS-I) procedures (4,8) were used in different studies. The 1052 PAB was used for coating and conjugate in DAS and as the coating antibody for DAS-I. Several monoclonals, including 3DF1, 3CA5, a mixture of 3DFI and 3CA5, and CTV-MCA 13 were used as intermediate antibodies. The labeled secondary antibody was as described above.

*Substrates.* In most tests, the substrate was a freshly prepared mixture of

NBT (nitroblue tetrazolium) and BCIP (5-bromo-4-chloro3-indoly phosphate) (12). Stock solutions were made in N, N' dimethylformamide (DMF) at 75 and 50 mg/ml respectively. The substrate mixture was 0.33 mg/ml NBT and 0.175mg/mlBCIP in substrate buffer (0.1 M Tris-HCl, 0.1 M NaCl, 5 mM MgCl, pH 9.5). In some tests substrate was prepared from Sigma Fast BCIP/NBT tablets (Sigma Chemical Co., St. Louis, MO) or from Vector Stain (Vector Laboratories, Inc. Burlingame, CA 94010). Incubation time in the substrate solution varied 5 to 20 min. The reaction was stopped by washing the membranes in distilled water or in 0.001 MEDTA prepared in 0.01 M Tris-HCl, pH 7.5.

*Observation of blots.* The processed membranes were placed in water in a petri dish or in a p]astic bag with a small quantity of water and examined under a dissecting microscope at a 10 to 25X magnification. Dried membranes were stored in envelopes in the dark for future reference.

**Virus isolates and tissue sources**. A large number of CTV isolates were tested. The Florida isolate T36 (18,20) was used in many routine tests to define optimum parameters fro testing differential reaction of CTV-MCA13 in DTBIA. Several different Florida mild isolates were also tested , including T30, T55-1 (T55a) and T69. These isolates cause very mild symptoms on Mexican lime and do not cause decline in trees grafted on sour orange or stem pitting in grapefruit or sweet orange. Plants infected with citrus tater leaf virus and citrus exocortis viroid as described previously (18) were also included for testing. The CTV isolates from field trees in Florida were not characterized.

Twenty-three Spanish isolates of CTV from the collection at I.V.I.A. at Moncada (16, 23) and 74 different CTV isolates from the exotic CTV isolate collection at Beltsville, MD (10) were tested. The latter came from nine countries plus California and Hawaii and represented a wide range of strain severity. DTBIA tests of exotic isolates were made at the USDA quarantine facility at Beltsville.

Tissues were collected from glass-house and field grown plants. Madam Vinous sweet orange and Mexican lime were the glasshouse sources most commonly tested, but blots were made from other varieties as well.

Hamlin and Valencia sweet oranges were the field sources most commonly tested in Florida. Varieties tested in Spain included Washington Navel, Clementines and Nova. Where possible, tissue sources were stem or petiole tissue from a new or recent flush of growth. In field tests in Spain, blots were made of a composite sample which consisted of three twigs from each of five trees (6). A cut was made across a bundle of 15 twigs and the ends were blotted simultaneously to the membrane. Tissues were stored at 4-6 C if blots could not be done at the time of collection.

## RESULTS

Initial tests were made by making blots of CTV-infected tissue and healthy citrus stem tissue on nitrocellulose membranes with procedures similar to those described by Line et al. (15). These blots were tested by the MAB-indirect and MAB-BIO/SA methods (Fig. 1) with 3DF1MAB as the CTV-specific antibody. Under 10X magnification, the outline of the stem imprint was clearly visible and intense areas of deep purple staining were present in the imprint area which corresponded to the phloerm of CTV-infected stems (Fig. 2C-D). These intensely stained areas were not present in blots of comparable healthy tissue (Fig. 2B). When appropriate antibody concentrations and incubation times were used, the uninfected tissue imprint was pink, and the remaining membrane was white or a faint pink. The pink background was easily distinguished from the intensely stained areas in the phloem of CTV-infected tissue. Best results were obtained when the tissue was pressed to the membrane just firmly enough to leave a faint green image of the tissue without a strong imprint in the membrane. Nonspecific background increased when imprints were made too forcefully onto the paper.

Generally, a number of intensely stained areas were present and these sometimes coalesced to form a ring of staining corresponding to the phloem region. In most cases, positive blots were instantly and easily identified even when only one or two small areas of intense staining were present. As with ELISA, inclusion of known healthy and infected controls with each sheet was essential to confirm that the reactant concentrations and test procedure were

appropriate and to determine the normal background color to be expected. A set of standard controls for a series of blots was generated by blotting a single membrane repeatedly with CTV-infected and healthy tissues freshly cut for each impression. Portions of this membrane with paired CTV-infected and healthy tissue imprints were included with a series o ftest sheets as a reference standard.

*Comparison of procedures.* The MAB-indirect, MAB-BIO/SA, and the MAB-BIO-I/SA methods were similar and gave better signal to background ration and a more sensitive assay than the direct method. The MAB-BIO/SA requires an additional step, but has the advantage that no preparation or labeling of the CTV-specific antibody is required. It has been used extensively for commercial applications during the past year with excellent results. The considerations which affect choice of method for DTBIA are essentially similar to those indicated fro ELISA (8).

*Membranes.* All sources of nitrocellulose membranes tested gave acceptable results. Bio Blot nitrocellulose tore less than the other membranes tested. Differences were noted between different lots of membrane from the same source. Photogene nylon and Zeta Probe membranes also worked. The Zeta Probe, generally used for binding nucleic acids, showed a marked overall color development, but the CTV-specific stained areas could be clearly differentiated. Nitrocellulose membranes were white immediately after incubation in substrate, but frequently developed a general pink cast with times, especially if exposed to light. This color development varied from test to test and did not interfere with readings. Membranes stored in the dark could be read for up to 12 months.

Blocking agents. Blocking with 0.5 or 1% BSA gave satisfactory results and was used routinely. Tests with Blotto (5% non-fat dry milk with 0.02% NaN3 in PBS), Blotto plus 0.2% Tween, and 1% milk did not show marked differences in a MAB BIO-I/SA, and in fact, the control without blocking ingredients produced a usable blot. Ovalbumin was unsuitable as a blocking agent.

*Incubation schedules.* A typical incubation schedule for DTBIA is indicated in Fig. 3. Considerable flexibility was found in incubation times and conditions as previously indicated (15).

The blocking steps or one of the antibody incubations can be done overnight at 4-6 C rather than at room temperature. Two-hour incubations were used intially for the various antibody incubation steps, but later, shorter periods were used and background color decreased. Incubations were done in glass cylinders of a hybridization oven, in flat plastic containers placed on a bench top shaker, and in sealed plastic bags attached to a slowly rotating wheel oriented at a 45-degree angle.

Results were comparable, but changing solutions was easier with the bag or dish system, and the bag system required the least antibody solution.

Incubation time in the substrate was critical. Over incubation increased background color and did not increase the specific signal. Color development usually began within 5 min after addition of the substrate and the reaction was stopped 5-10 min later, or as soon as any color appeared in the membrane away from the imprint areas. The most convenient procedure was to observe the imprint of a known positive control and to stop the reaction when the desired reaction appeared. A strong background color soon after addition of the substrate indicated that concentration of the antibodies or enzyme conjugate was too high. As a general rule we found that a concentration approximately one-half that used for ELISA was optimum. Initial tests with several 10-fold dilutions around the anticipated optimum should be made and the greatest dilution which permits full color development should be selected.

**Comparison of different polyclonal and monoclonal antibody sources:**

Several different polyclonal antisera and monoclonal antibodies were tested. Results of a comparative test of seven PABs in a PAB BIO-I/SA protocol are shown in Table 1. Antisera to five different isolates worked, and antisera to fixed whole virus, unfixed whole virus and to SDS-degraded coat protein (2) of a single isolate also worked. A nonspecific background reaction was observed with PAB 894 as observed previously in ELISA (2). It did not prevent detection

of the CTV-specific reaction. Correspondingly, four different MABs (3DF1, 3CA5, CTV-MCA13, and 3E10) also all worked well in a MAB BIO-I/SA protocol.

The specificity of CTV-MCA13 for certain CTV isolates observed in ELISA (17) was also true for DTBIA. Isolates inducing decline and stunting in Florida which reacted to CTV-MCAI3 in ELISA also gave a strong reaction in DTBIA. Isolates which did not cause decline and stunting did not react in ELISA or DTBIA using CTV-MCA13, but did react strongly to 3DF1 MAB and the 1052 PAB. Differentiation of isolates could be done by blotting each sample to two separate membranes and processing these with CTV-MCA13 and with a broadly reactive antibody (Fig. 2). Results for a comparative assay of 13 different isolates of CTV by ELISA and DTBIA using the broadly reactive 3DF1MAB and the severe-strain-selective CTV-MCA13 MAB are shown in Table 2.

Isolate and host effects. DTNIA detected the wide vanity of CTV isolates tested in Florida and Spain, and deteced all 74 sources tested from the international CTV collection at Bellsville. Direct tissue blots were done successfully with numerous citrus hosts including Hamlin, Valencia, and navel sweet oranges, Marsh and Red Blush grapefruit, Mexican lime, alemow, Citrus hystrix, pummelo, and rough lemon. There was no evidence for host associated nonspecific reactions with any of the varieties tested. As expected, negative tests were obtained with hosts that are immune to CTV such as trifoliate orange or Carrizo citrange. Blots of tissue infected with tatter leaf virus or citrus exocortis viroid were negative.

Tissue source. CTV infection was detected by DTBIA from different infected tissues, including stems and leaf petioles of different ages, fruit pedicel, the vascular core of mature fruit, bark patches cut from the trunk of large trees, and roots. In general, the best reactions were obtained from young flush tissue or from twigs directly below a young flush with good cambial activity. Good reactions were also obtained with bark from older limbs and main stem (trunk ) the stained areas in the trunk bark were often scattered and small, but were very distinct. Stem pieces 3-7 mm in diameter and leaf petioles were the easiest to blot and were used in most tests.

To test location effects within a plant, a chronically infected 2-yr-old navel orange was sampled at multiple sites. Stem pieces from at least four distinct grwth flushes were tested. All 17 sites tested were positive. The strongest

reactions were obtained in new flush tissue. The oldest stem pieces gave weaker but clearly positive reactions. In several experiments large numbers of twigs or leaves were taken from a single infected tree and all tested positive. In tests to compare membranes and other variables, a large number of blots from a single stem were made. A thin slice was removed between blots so that, in effect, multiple sites were tested along the stem. All 48 blots made from individual stems infected with each of four different isolates were positive.

Storage of blotted membranes prior to assay. To test storage effects on the blot assay, blots were made of healthy and T36-infected sweet orange. Each sample set consisted of two blots each of healthy tissue and three sources of T36-infected tissue which varied in reaction intensity. These were stored at 4 and 30°C at room humidity and over a desiccant. Assays were completed at 1, 15,7 and 30 days after the initial blots were made. The assay system was Biotins/SA with MCA13. Membranes stored at 30°C gave a stronger reaction than those stored at 4°C. Membranes stored under normal room humidity were also slightly better than those stored over a desiccant. There were no obvious differences between the 1- day and the 15- or 30-day storage periods for the same treatment combination. Other tests have given good results.

TABLE 1
REACTION OF DIFFERENT POLYCLONAL ANTIBODIES (PAB) TO CITRUS TRISTEZA VIRUS (CTV) DIRECT TISSUE BLOT IMMUNOASSAYS (DTBIA)

| | | | Reaction in DTBIA* | | | | |
|---|---|---|---|---|---|---|---|
| Antibody | Isolate | Inject antigen* | Healthy | T – 30 | T-55-1 | T-G8 | BKGD* |
| 873 | T4 | Whole F | 0/2 | 2/2 | 2/2 | 2/2 | Low |
| 879 | T4 | Whole UF | 0/2 | 2/2 | 2/2 | 2/2 | Low |
| 894 | T4 | Coat P | 0/2 | 2/2 | 2/2 | 2/2 | Mod. |
| 908 | T3 | Whole UF | 0/2 | 2/2 | 2/2 | 2/2 | Low |
| 1051 | T30 | Whole UF | 0/2 | 2/2 | 2/2 | 2/2 | Low |
| 1052 | T36 | Whole UF | 0/2 | 2/2 | 2/2 | 2/2 | Low |
| 1053 | T26 | Whole UF | 0/2 | 2/2 | 2/2 | 2/2 | low |

- Numper of imprints positive over number tested. Stem imprints were made on nitrocellulose membranes, and processed with PAB-B10-1/SA procedure (Fig. 1). Concentration of PAB was 1 ug/ml, the biotinylated goat anti-rabbit was used at 1/5000 and the streptavidin-alkaline phosphatase conjugate was used at 1/4000.
- Whole F = formalin-fixed purified virus, whole UF = untreated whole virus, and Coat P. = denatured coat protein from purified virus.
- BKGD = Back ground color reaction in tissue.

**TABLE 2**
COMPARISON OF ELISA ANE DIRECT TISSUE BLOT IMMUNOASSAY (DTBIA) FOR DIFFERENTIAL DETECTION OF MILD AND SEVERE ISOLATES OF CITRUS TRISTEZA VIRUS IN FLORIAD

| | 3DFI Antibody | | CTV- MCA13 Antibody | | |
|---|---|---|---|---|---|
| Isolate | ELISA* | DTBIA* | ELESA | DTBIA | Bioassay* |
| T-30 | + | + | - | - | M |
| T-36 | + | + | + | + | S |
| T-55-1 | + | + | - | - | M |
| T-66 | + | + | + | + | S |
| FS-506 | + | + | + | + | S |
| FS-537 | + | + | - | - | M |
| FS-539 | + | + | + | + | S |
| FS-542 | + | + | - | - | M |
| FS-546 | + | + | + | + | S |
| FS-549 | + | + | + | + | ND |
| FS-550 | + | + | + | + | S |
| FS-556 | + | + | - | - | M |
| FS-557 | + | + | - | - | M |
| Healthy | - | - | - | - | 0 |

- ELISA was done by DAS- I method with PAB 1052 USED AS COATING ANTIBODY.
- DTBIA was done by B10-SA procedure in Fig. 1.
- M = no symptoms in infected sweet orange grafted on sour orange; S= stunting and/or decline effects in infected sweet orange grafled on sour orange; ND = not determined and 0 = no reaction.

Comparison of DTBIA and ELISA for field assays. In a large scale comparison of ELISA and DTBIA, shoots of new flush growth were collected from 858 vigorous 3-year-old Hamlin and Valencia orange trees in field planting near Clewiston, FL. These trees were part of an epidemiology experiment to study natural spread of CTV into a virus-free planting. The two previous annual surveys indicated a low, but increasing incidence of CTV. Comparative assays were made from each shoot collected. An 8-10 cm stem section was selected and each end was freshly cut and blotted to nitrocellulose. An extract from a 0.5 g sample of diced bark from the remaining stem piece was prepared and tested by DAS ELISA (1) . Identical results were obtained with 852 trees by each method, 51 trees were infected, and 801 were virus-free. A discrepancy occurred with six trees showed that four of the six trees had originally been misdiagnosed by ELISA and two had been misdiagnosed by DTBIA. In Spain,

560 trees were tested as five tree composities and the composite samples with infected trees were identified equally well by DTBIA and ELISA.

Comparision of sensitivity of DTBIA, ELISA, and immunoblotting. A limited test was made of tissue of different ages from sweet orange infected with mild and severe isolates of CTV. Blots were made from the diffeent sources and extracts were made and tested by DAS-1 ELISA and by immunoblotting at 1/50 and 1/500 dilutions. MAB and secondary antibody concentrations were the same for DAS-1 and immunoblotting. Immunoblotting failed to detect infection at a 1/500 dilution of some extracts which were detected by ELISA. Even weak sources whose extract were positive by ELISA only at a 1/50 dilution were detected by DTBIA.

**Discussion:**

DTBIA is a reliable and sensitive procedure for detection of CTV. Sensitivity, assay times, and cost compare favorably with other previously described procedures for serological detection of CTV. The assay makes efficient use of virus specific antibodies, and by using an indirect or the BIO-1/SA method. The assay can be done without any labeling or conjugation of antibodies. DTBIA has several advantages over conventional immunoblot procedures. It requires no preparation or extraction of the sample, eliminating the need for homogenizers, or for tubes and containers to store extracts prior to testing. It provides precise delivery of the sample to the membrane without need for manifolds or other loading devices. It can be easily tailored to varying numbers of samples by cutting the membrane to an appropriate size.

DTBIA provides direct information about distribution of the virus within the host. Even samples which give weak positive reactions by ELISA or by regular immunoblots usually give clear results with DTBIA, since only one infected cell group is needed to give a clear signal.

In general, procedures where the antigen is trapped to the solid phase are less sensitive for detection of viruses in plant extracts than procedures where the antigen is trapped by an antibody bound to the solid phase. In both ELISA and conventional immunoblots there is competitive binding of host

proteins and antigens in the extract to the solid phase and when the virus titer is low there may be insufficient binding of the pathogen-specific antigen. In DTBIA there is direct binding of the virus from infected cells on the cut surface of the tissue without dilution by proteins from no infected cells in other locations. Thus, strong signals are formed in localized areas which are easily detected. If the sample is ground and the extract is tested by ELISA or immunoblotting, the advantage of localization is lost and a weak signal is obtained.

DTBIA provides a very convenient method to ship a sample for testing from one location to another. No live tissue is present and possible introduction of other pests or pathogensis eliminated. The sample is stable on the membrane. Refrigeration or protection of the sample is not required, and shipping costs are minimized. DTBIA is extremely convenient for field survey work in remote sites. All an investigator needs to carry are several sheets of nitrocellulose membrane, a few razor blades and disposable gloves.

Because of the intense reaction in localized areas where CTV is concentrated in the phloem of infected plants, cross reaction to host antigens by antibodies to host proteins in the serum is less of a problem than for ELISA or conventional immunoblot assays. The reaction to host proteins is more uniform and the background does not interfere with observation of the intense CTV-specific reaction sites in the blot. Several of the polyclonal antisera used successfully for DTBIA in this test give high background readings in ELISA.

The major disadvantage of DTBIA is that it is not convenient to precisely quantitate results. In many applications this is not important, but for those situations where quantitiation is needed, ELISA is a preferable assay. DTBIA is also less convenient than ELISA or conventional immunoblot assays when multiple tests of a single sample by different antibodies are needed. For example , panel assays against several different monoclonals are easy to perform from a single extract in ELISA, but require preparation of separate sheets for each MAB in DTBIA.

Since only the plane of the cut surface is probed, DTBIA would be less likely to detect a poorly distributed pathogen than a procedure where a larger amount of tissue is tested. In our experiences, this was not a problem with CTV

and can be overcome by making multiple blots of the same sample.

We found that it takes more time to precisely log in sample information and to record results with DTBIA that it did with a computer assisted ELISA system. Nitrocellulose membranes are also more fragile to handle than ELISA plates. Use of commercial kit with remarked membranes and data sheets for sampling (Nokomis Corp., Altamonte Springs, FL) reduced blotting time and provided protection to the membranes.

In common with other assays, some experience is helpful to accurately read blots, especially where the reaction is weak. It is essential that appropriate healthy and infected controls be included in each membrane for reference. Some preliminary testing with known healthy and CTV - infected tissue should be done to define optimum dilutions and incubation periods for the antibodies and reagents to be used.

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# Simultaneous and Co-Operational Amplification (CO-PCR): A New Concept for Detection of Plant Viruses.

# Simultaneous and co-operational amplification (Co-PCR): a new concept for detection of plant viruses

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## Abstract

A new and highly sensitive method for the amplification of viral RNA targets from plant material has been developed and patented. This technique called Co-operational amplification (Co-PCR) can be carried out easily in a simple tetra primer reaction based. On the simultaneous action of four primers. The reaction process consists of the simultaneous reverse transcription of two different fragments from the same target, one containing the other; the production of four amplicons by the combination of the two pair of primers, one pair external to other; and the co-operational action *of* amplicons for the production *of* the largest fragment. The technique was used successfully, both in metal block and capillary air thermal cyclers for the detection of plant RNA viruses *(Cherry leaf roll Virus,* Strawberry latent ringspot virus, *Cucumber mosaic Virus, Plum pox virus* and *Citrus tristeza virus).* The sensitivity observed is at least 100 times higher than that achieved with RT-PCR and similar to nested RT-PCR. Colorimetric detection was coupled with this methodology facilitating its introduction for routine indexing programs and for phytosanitary selection of virus-free plant material. 

***Key words***: RT-PCR; Nested RT-PCR; Co-RT-PCR; RNA and DNA targets

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## 1. Introduction

There is a need for sensitive, specific and reliable methods to detect and characterize plant RNA viruses because of their agronomic and economic impact. These techniques are particularly relevant in indexing, phytosanitary and virus eradication programmes where easy application is required.

Routine detection and characterization of viral targets is mainly performed by serological methods (ELISA) based on polyclonal or specific monoclonal antibodies. When the amount of target is limited and minute quantities of RNA are present more sensitive techniques are necessary. The detection of viral targets in plant tissue prints or in single aphid vectors squashed on paper 3MM (Olmos et al., 1996) saving time and nucleic acids extraction is possible. Immunocapture (IC)- RTPCR-based technology (Wetzel et al., 1992; Nolasco et al., 1993) greatly improves the sensitivity which is enhanced dramatically when coupled with nested amplification (Olmos et al.. 1997). However, nested RT-PCR continues to require two rounds of amplification. The general use in nested RT-PCR of two rounds of amplification in different tubes results in a high degree of contamination risk (Roberts, 1996). A few interesting alternatives with single closed tubes have been developed in order to avoid this problem (Yourno, 1992). The development of nested-PCR in a single closed tube using a single compartmentalised Eppendorf tube is another interesting alternative (Olmos et al., 1999) but this method requires the use of 0.5 ml Eppendorf tubes and cannot be used in rapid reaction capillary tubes. In this article we describe a new time-saving, economic method, called Co-operational PCR (Co-PCR) which is at least as sensitive as the nested technique, using one-reaction, one tube and one-manipulation of the cocktail, based on the action of different primers and amplicons (Fig. 1). This co-operational amplification usually produces the largest amplicon, in comparison with nested-PCR, that requires two sequential reactions, and obtains the lowest fragment. The method has been patented as Co-PCR (Spanish patent 31 October 2000; P20002613). We have applied this system successfully to both metal block and capillary air thermal cyclers for the detection of some plant RNA viruses from different genera, and to a bacterium showing the ability of this

new method for the amplification of DNA targets. Hybridisation and colorimetric detection using specific internal probes to amplicons were employed to facilitate routine applications.

## 2. Material and methods

### 2.1. *Plant material, virus isolates preparation of plant samples and RNA purification*

The virus sources included *Chenopodium quinoa, Olea europaea, Nicotiana tabacum* (Xanthi), *Nicotiana glutinosa, Prunus persicae* (GF305 / peach seedling) and *Citrus sinensis. Cherry leaf roll virus* (CLRV) isolates PV 200 and PV 278 in C. *quinoa;* Strawberry latent ringspot virus (SLRSV) isolate PV 0247 in C. *quinoa; Cucumber mosaic virus* (CMV) isolate M13-96 in *N. tabacum* and isolate P16-97 in *N. glutinosa; Plum pox virus* (PPV) isolates RB3.30 (D type) and Ms89 (M type) in *P. persicae;* and *Citrus tristez virus* isolates T-300, T-302, T-304, T-308, T-318, T-388 and T-407 from IVIA Collection in Cinensis, were used to prepare positive controls by mixing extracts from healthy plants of the respective infected plant virus sources.

Samples were prepared by grinding plant material 1/20 (w/v) in PBS buffer, pH 7.2, supplemented with 2% (w/v) polyvinylpyrrolidone (PVP-10) and 0.2% (w/v) sodium diethyl dithiocarbamate. About 10-fold serial dilutions of extracts from infected plants were prepared in extracts from healthy for sensitivity analysis. In addition 30 selected samples from *P. persicae, P. salicina* and *P. domestica* healthy and PPV infected, 32 *europaea* samples from asymptomatic nursery plants and 31 samples from citrus trees, were analysed by R T-PCR, nested RT -PCR and Co-PCR for analysis of PPV, viruses that infect olive trees (CMV, CLRV and SLRSV) (Bertolini et al., 2001) and CTV, respectively. Viral RNA isolation procedure from plant tissue samples was performed using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's method.

## 2.2 Primers and probes design

Primers and probes were designed according Bertolini et al. (2001). Briefly, sequenced regions of each virus were recovered using the Nucleotide Sequence Search program located in the Entrez Browser program provided by the National Centre for Biotechnology Information (NCBI) (http:// www3.ncbi.nlm.nih.gov/Entrez) (Bethesda, MD, USA). Conserved regions for each virus were studied using the similarity search tool Advanced BLAST 2.0, with the BLASTN program designed to support analysis of nucleotides (http:// www3.ncbi.nem.nih.gov / blast/beast.cgi?Jform = 1) (Altschul, et al., 1997). The alignment view was performed as master-slave with identities, to analyse significant nucleotide homologies in the molecular data retrieved from NCBI's integrated databases, GenBank, EMBL and DDBJ. Specific nucleotide regions were selected. Specific primers with similar annealing temperature based on the OLIGO program were subsequently designed for the viruses (http://www.lifescience-software.com / oligo..htm) (LRS, Long Lake, MN, USA). Following this methodology, external and internal primers to obtain amplification products for CLRV, SLRSV and CTV, were designed. A modification and reformulation of CMY primers previously described (Rizos et al., 1992) was necessary for their use as external primers. A new pair was designed for internal use. For PPV detection, PI and P2 universal PPV primers (Wetzel et al., 1991) were used as internal primers, and P10 and P20 were designed as external primers. Internal probes to amplicons were designed following this methodology. Table 1 shows nucleotide sequences of designed primers and probes.

## Table 1. Primers and probes

| Virus | Oligonucleotide | | Amplicon lengh (bp) | Sequence ( 5´- 3´) |
|---|---|---|---|---|
| CLRV | External primers | CLRV 1 | 283 | CATTTCCATGCGACCGGTCTT |
| | | CLRV 2 | | AGTCCGACACTCATACAATAAGC |
| | Internal primers | CLRV 3 | 171 | GTTAACGAATATCTACTGC |
| | | CLRV 4 | | CAAATATTGCTAAACAACC |
| | Probe (3DIG) | | | AAGCCCAAGAATTTAGGGGGTTATGTGGGTAGATAGCGTT |
| SLRSV | External primers | CLRSV 1 | 181 | GTTACTTTTACCTCCTCATTGTCCATGTGTGTTG |
| | | CLRSV 1 | | GACTATCGTACGGTCTACAAGCGTGTGGCGTC |
| | Internal primers | CLRSV 1 | 109 | TGGACCTTTATTGGTTGGAT |
| | | CLRSV 1 | | ATCTGCCACTGATTCTCAC |
| | Probe (3DIG) | | | AGTAAGCAGCCGCTAGCGTTCTGGAWTTCCAGGCAYAGTG |
| PPV | External primers | P10 | 359 | AAAGCATACATGCCAAGGTA |
| | | P20 | | GAGAAAAGGATGCTAACAGGA |
| | Internal primers | P1 | 243 | ACCGAGACCACTACACTCCC |
| | | P2 | | TCGTTTATTTGGGCTTGGATGGAA |
| | Probe (3DIG) | | | CATCTGATTGAAGTGGAC |
| CTV | External primers | PEX 1 | 241 | TAAACAACACACACTCTAAGG |
| | | PEX 2 | | TATCACTAGACAATAACCGGATGGGTA |
| | Internal primers | PIN 1 | 132 | GGTTCACGCATACGTTAAGCCTCACTT |
| | | PIN 2 | | AGTCTTTAAAATGATCGAGGGGAAAATTAACC |
| | Probe (3DIG) | | | CTTTCTCATGGATGCTTCTC |
| CMV | External primers | CMV 1 | 885 | GCCGTAAGCTGGATGGAC |
| | | CMV 2 | | ACTATTAACCACCCAACCT |
| | Internal primers | CMV 3 | 172 | TTTGAATGCGCGAAACAAG |
| | | CMV 4 | | AATCCTTTGCCGAAATTTGATTCTACCGTGTGGGT |
| | Probe (3DIG) | | | |

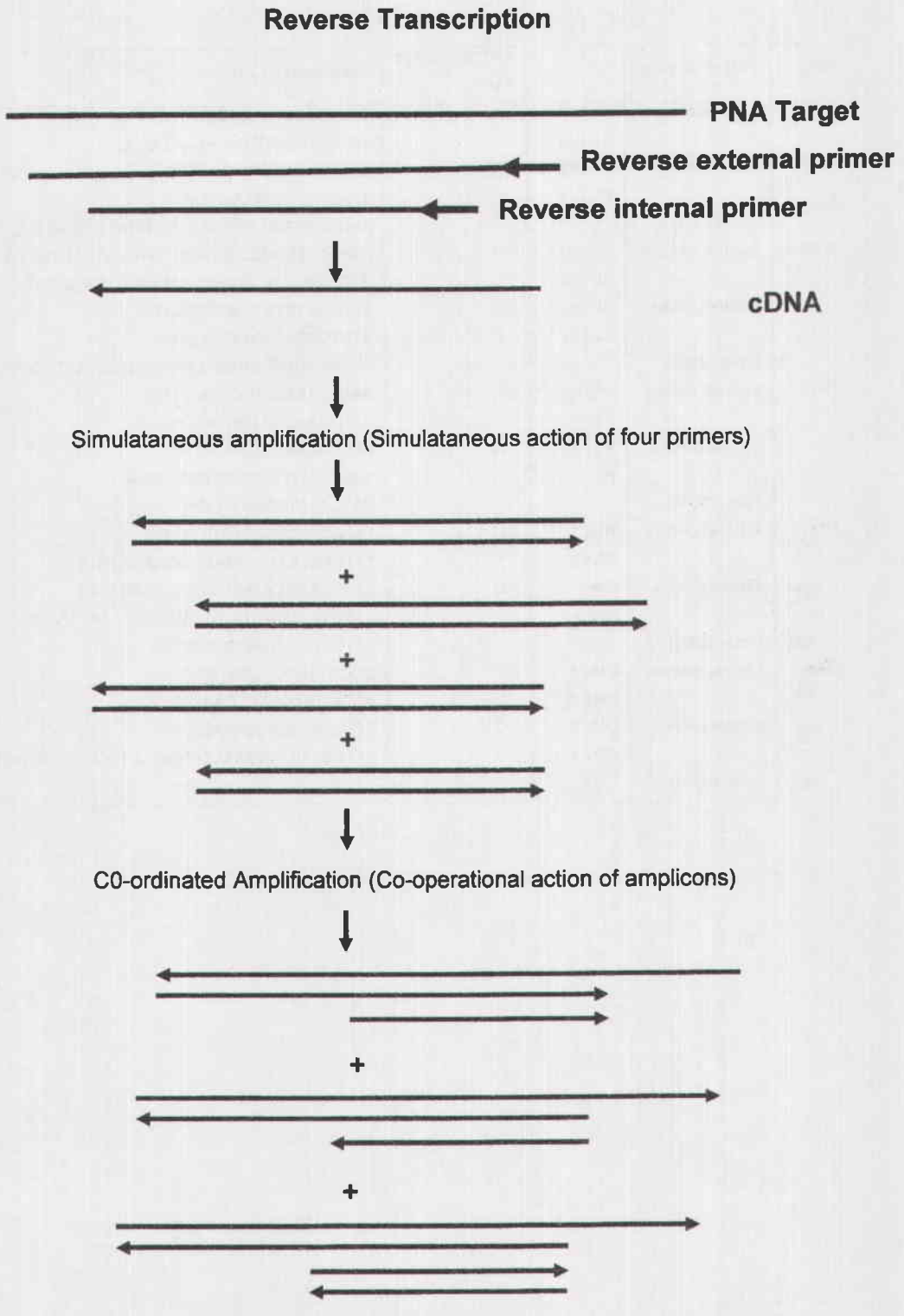


Fig 1. Scheme of the Co-PCR procedure

### 2.3. *Amplification methods*

#### 2.3.1. *RT-PCR*

The RT-PCR one step protocol (Wetzel et al., 1992) with minor modifications was used for amplification purposes. Briefly, 20 ul of RT -PCR mix consisting of 10 mM Tris-HCl pH 8.9, 50 mM KC1, 0.3% Triton X-IOO (w/v), 1 l-IM of each primer (CLRVI, CLRV2 for CLRV detection; SLRSV1, SLRSV2 for SLRSV detection; CMVI, CMV2 for CMV detection; P1, P2 for PPV detection; PIN1, PIN2 for CTV detection), 250 uM dNTPs, 0.25 units of AMV-RT (Promega), 0.5 units of Taq DNA polymerase (Promega)] were added directly to tubes containing 5 ul of RNA sample. Two types of thermal cyclers were employed, the metal block thermal cycler PHC3 (Techne) and the capillary air thermal cycler RapidCycler (Idaho Technologies). The cDNA synthesis and amplification were carried out at 42 °C for 45 min followed by a denaturation phase at 92 °C for 2 min and 40 cycles of amplification [92 °C for 30 s, 50 °C (CLRV, SLRSV, CMV) or 60 °C (CTV, PPV) for 30 s and 72 °C for 1 min]. The primer sequences are shown in Table 1. PCR products (10 ul) were analysed by electrophoresis in 2% agarose gels and stained by ethidium bromide.

### 2.4. *Nested RT-PCR in a single closed tube*

The method and device based on the use of a compartmentalised Eppendorf tube (Spanish patent P9801642, Spain) was used (Olmos et al.,1999). The cocktail for reverse transcription and external amplification was a mixture of 30 ul containing 50 mM KC1, 10 mM Tris-HCl (pH 9.0 at 25 °C), 0.3% Triton X-100 (w/v), 3 mM $MgCl_2$, 250 uM dNTPs, 0.1 uM (CLRV, SLRV, CMV, PPV) or 0.5 uM (CTV) of external primers, DMSO 5%,1.2 units of AMV-RT (Promega), 0.6 units of Taq DNA Polymerase (Promega) and 5 ul of RNA sample. The cocktail for the second (internal) amplification was a mixture of 10 ul containing 50 mM KC1, 10 mM Tris-HCl (pH 9.0 at 25 °C), 8 ilM of internal primers. RT-PCR was carried out in a thermal cycler PHC3 (Techne) at 42 °C for 30 min followed by a denaturation at 94 °C for 2 min and 23 cycles of amplification [92 °C for 30 s, 50 °C (CLRV, SLRSV, CMV, PPV) or 45 °C (CTV)

for 30 s and 72 °C for 1 min 20s]. After RT -PCR, tubes were vortexed and centrifuged (6000 x *g* for 2 s). Nested PCR began with a denaturation phase of 2 min at 94 °C, followed by 40 cycles of amplification, with a temperature profile of 30 s at 92 °C, 30 s at 50 °C (CLRV, SLRSV, CMV) or 60 °C (PPV, CTV) and 1 min at 72 °C. The primer sequences are described in Table 1 PCR products (10 ul) were finally analysed by electrophoresis in 2% agarose gels and stained by ethidium bromide.

**2.5 Co-operational RT-PCR (Co-PCR):**

The Co-PCR one step cocktails consisted of 20 ul of Co-PCR mix [10 M Tris-HCl pH 8.9, 50 mM KC1, 0.3% Triton X-I00 (w/v) , 0.1ul of external primers, 0.05 uM of internal primers, 400 uM dNTPs, 5% DMSO, 0.25 units of AMV-RT (Promega) and 0.5 units of Taq DNA polymerase (Promega)] that was directly added to tubes containing 5 ul of RNA sample. Two types of thermal cyclers were employed, the metal block thermal cycler PHC3 (Techne) and the capillary air thermal cycler RapidCycler (Idaho Technologies). The cDNA synthesis and amplification *was* performed at 42 °C for 45 min followed by a denaturation phase at 92 °C for 2 min and 60 cycles of amplification [92 °C for 10 s, 50 °C for 10 sand 72 "C for 15 s]. The sequences of primers are described in Table 1, PCR products (10 ul) subsequent colorimetric detection were finally analysed by electrophoresis in 2° agarose gels and stained by ethidium bromide, and 1 ul was dispensed onto nylon membranes for subsequent colorimetric detection.

**2.6. Colorimetric detection by dot blottinghy bridisation of amplicons**

About 1ul of the amplicons was dispensed onto a nylon membrane positively charged (Roche) dried at room temperature and crosslinked by UV in a transilluminator for 3 min 30 s. Membranes were submitted to a prehybridisation phase in a hybridiser Roller-Blot HB-3D (Techne).

Prehybridisation was performed at 60 °C (CLRV, SLRSV and PPV) or 50 °C (CTV and CMV), during 1 h in a buffer containing 5 x SSC, 0.1 % (w/v) *N* -lauroyl-sarcosine, 0.02% (w/v) SDS and 1% Blocking Reagent (Roche). After this step, the solution was discarded and the hybridization was performed

mixing 10 pmol/ml of 3'DIG labelled specific-probe for each virus (Table 1) with prehybridisation buffer. Conditions were fixed at 60 °C (CLRV, SLRSV and PPV) and 50 °C (CTV and CMV), for 2 h. The membranes were washed twice for 15 min with 2 x SSC supplemented with 0,1% SDS and twice for 15 min with 0.5 x SSC supplemented with 0.1% SDS.

Colorimetric detection with NBT and BCIP was subsequently performed. Membranes were equili brated for 2 min with 100 mM maleic acid, 150 mM NaCl, 0.3% (v/v) Tween 20, pH 7.5 (Roche) and blocked for 30 min with blocking buffer [100 mM maleic acid, 150 mM NaCl pH 7.5 and 1% (wiv) blocking reagent (Roche)]. Membranes were incubated at room temperature with anti-digoxigenin-alkaline phosphatase antibodies in a concentration of 150 mU/ml diluted in blocking buffer for 30 min. Membranes were washed twice 15 min with 100 mM maleic acid, 150 mM NaCl, 0.30% (v / v) Tween 20, pH 7.5 and equilibrated for 2 min with 100 mM Tris-HCl, 100 mM NaCl, pH 9,5. Substrate consisting of 315 ug/ml NBT and 175 ug/ ml BCIP in 100 mM Tris-HCl, 100 mM NaCl, pH 9.5 was used for detection. The reaction was stopped with sterile water or 10 mM Tris- HCl, 1 mM EDTA, pH 8.0,

**2.7. Southern blot hybridization:**

To confirm the specificity of the amplicons obtained by Co-PCR a Southern transfer and hybridisation was undertaken. After electrophoresis DNA was blotted overnight from the gel by capillary transfer to a positively charged (Roche) membrane, using 20 x SSC (Sambrook et al., 1989). DNA was crosslinked by UV in a transilluminator for 3 min 30 s and hybridisation and colorimetric detection were performed as indicated above (see colorimetric detection by dot blottinghybridisation of amplicons).

## 3. Results

### 3.1. Co-PCR optimization for detection of RNA viruses:

The designed primers were able to amplify all isolates tested for each virus assayed (CLRV, SLRSV, CMV, CTV and PPV). Several parameters were assayed in order to optimise Co-PCR amplification. Annealing temperatures

(45, 50. 55, 60 and 65. °C), denaturation, annealing and elongation times (5, 10, 15, 30 s and 1 min), number of cycles (30, 40, 50, 60, 70), primers concentration (0.01, 0.02, 0.03, 0.05, 0.1, 0.2, 0.5 and 1) uM) and combinations to obtain the best. ratio between external and internal primers. The best results were obtained at 50 °C annealing temperature, 10 s for denaturation and annealing steps, 15 s for elongation step, 60 cycles, 0.1) uM of external primers and 0.05 ) uM of internal. Primers were used. Amplified fragments of the expected size were observed in positive controls and South ern blot analysis demonstrated the specificity of amplicons. In the case of CLRV, SLRSV, PPV and CTV, onJy the largest amplicon was obtained, and in the case of CMV four specific fragments corresponding in size to the combinations of the four primers were obtained.

### 3.2. Optimisa!ion of dot blotfing-hybridisation

The designed probes, internal to amplicons were able to hybridise with all isolates tested for each virus, facilitating the interpretation of results. Some conditions such as hybridisation tempera tures (45, 50, 55, 60 and 70 °C), probe concentrations (1, 5, 7 and 10 pmol/ml) and time of hybridisation (1, 2, 3, 4 and 6 h) were assayed. Colour precipitated in spots corresponding to positive controls and infected samples but not in healthy or negative controls.

### 3.3. Comparison of RT-PCR, nested RT-PCR and Co-PCR

Comparison of the three techniques demonstrated that Co-PCR and nested RT-PCR in a single closed tube were at least 100 times more sensitive than the conventional RT -PCR, for each virus tested. Fig. 2 shows the results of the comparison of sensitivity using CLRV and SLRSV as models. In the case of CLRV, the amplification products obtained by RT-PCR were detected as a clear band upon agarose gel electrophoresis up to the $1:10^3$ (corresponding to a J 03 dilution factor of 1:20 infected plant material weight to grinding buffer volume). The nested RT-PCR amplification products were detected up to the $1:1^5$ (corresponding to a $10^5$ dilution factor of 1:20 infected plant material weight to grinding buffer volume).

Similarly when Co-PCR was employed the amplification products were detected up to the $1:10^6$ (corresponding to a $10^6$ dilution factor of 1:20 infected plant material weight to grinding buffer volume). In the case of SLRSV, a clear band of amplicons by RT-PCR was observed up to the 1:102. The nested RT-PCR amplification products were detected up to the $1:10^4$. Co-PCR detected products of amplification up to the $1:10^4$.

### 3.4. Application of Co-PCR coupled u'ith colorimetric detection to plan! material

Coincidental results of the 30 selected samples from *P.persicae*, *P. salicina* and *P.domestica* healthy and PPV infected, were observed by RT PCR, nested RT-PCR and Co-PCR. The analysis of these 30 samples by Co-PCR is shown in Fig. 3.

The analysis of the 32 *O.europaea* samples from asymptomatic nursery plants revealed that only four samples (two infected by CLRV and other two infected by SLRSY) gave positive results by nested RT-PCR and Co-PCR. RT-PCR was not effective for the detection of these viruses. In the case of the selected 31 samples from *C.sinensis*, similar results were obtained by RT-PCR, nested RT-PCR and Co-PCR.

## 4. Discussion

According to our hypothesis, shown in Fig. 1, Co-PCR is a new method for amplification, and this tetra primer-reaction allows the simultaneous action of four primers in a single reaction, in contrast to nested RT-PCR that requires two sequential reactions. The reaction begins with the reverse transcription of the RNA molecule target, producing simultaneously two different cDNA fragments, the largest containing the shortest, because the simultaneous action of the two reverse primers (external and internal primers). During the initial cycles of this amplification, four specific amplified products are generated by the co-ordinated action of the four primers. The largest amplicon obtained by the action of the external primers, the shortest amplicon obtained by the action of the internal primers, and two medium amplicons in length obtained by the combination

between one external and one internal primer. In the subsequent cycles the shortest and the medium in length amplicons are used as additional primers to yield the largest amplicon which is added to that created by the action of the external primers.

As expected, when the primers could be de signed in optimal regions (CLRV, SLRSV, PPV, CTV) Co-PCR clearly favoured the creation of the largest amplicon in contrast to nested RT. PCR that amplifies the shortest amplicon. Nevertheless, when amplifying CMV targets the four bands corresponding to the specific bands generated by the combination of the four primers were observed. Specificity of CMV amplified products were confirmed by Southern blot hybridisation.

**PPY UNIVERSAL PROBE**

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| + | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 |
| 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 |
| 24 | 25 | 26 | 27 | 28 | 29 | 30 | - |

Fig 3. Diagnosis of PPV in thirty selected samples (1-30) trom different *Prunus* species by Co-PCR and colorimetric detection using a PPV-universal digoxigenin labelled probe. +, positive control: -, cocktail control: 3-7,10, II, 15, 16, 18, 20, 24, 25, 27, 28 and 30 samples with positive reaction.

The sensitivity was enhanced greatly in all cases, in addition, Co-PCR was successfully coupled to a colorimetric detection, facilitating the interpretation of results and allowing the detection of isolates as previously demonstrated by Bertolini et al,(2001). Comparison of the sensitivities achieved by RT-PCR, nested RT-PCR in a single closed tube, and Co-PCR, showed that traditional RT-PCR was at least 100 times less sensitive than other methods evaluated (Olmos et al 1999). Although nested RT-PCR in a single closed tube and Co-PCR resulted in similar sensitivities, the simpler Co PCR procedure based on one step, one reaction and one manipulation protocol demonstrated its relative simplicity and suitability. An important advantage of the developed methodology is the possibility, of using Co-PCR in capillary air thermal cyclers,

thus, reducing time without any yield losses. It implies that Co-PCR overcame for the first time the limits of sensitivity described for this type of thermal cycler where the possibility of performing nested RT-PCR still does not exist (Sambade et al" 2000).

The successful. application of Co-PCR to different plant materials validates its use for plant pathogen diagnosis, Coincidental results were observed by different amplification procedures when were used to test CTV and PPV infection in the selected woody plants such as *Citrus* and different *Prunus* species, respectively, However, the analysis of olive tree samples from different cultivars gave higher number of positive results by nested-PCR and Co-PCR than by simple RT PCR, probably due to the low viral titre in asymptomatic olive trees (Bertolini et al., 2001). The method seems to be very sensitive to PCR inhibitors, When the method was used without prior purification (IC), amplification failed frequently. The low amount of reagents (ten times less than in conventional PCR) probably increases susceptibility to inhibitors. In conclusion, this methodology was applied to both metal block and capillary air thermal cyclers, for the detection of different RNA viruses. This method is specific, rapid, easier, and more economical than other methods. It opens new possibilities for the detection and characterisation of RNA viruses. This methodology facilitates the preparation of PCR kits based on the simple cocktail used. In addition the method has been successfully assayed for the detection of the bacterium *Ralstonia solanacearum* (data not shown) showing the versatility of the method that also can be easily adapted for DNA amplification. This method opens new possibilities for sensitive molecular detection and characterization of RNA targets.

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# Dot - blot Hybridization for PLMVd

## Dot-blot hybridization for PLMVd

### Total RNA extraction

Total RNA extraction was done by non-organic method, described by Astruc *et al.* (1996) with some slight modifications.

Half gram of leaf tissue was homogenized in a sterile plastic bag in the presence of 5 ml of extraction buffer [100 mM Tris-HCl pH 8.0, 50 mM EDTA pH 7.0 500 mM NaCl, 10 mM 2-mercaptoethanol (1:1000)]. 1 ml of the extract was incubated at 65°C for 30 min in the presence of 50 ~l of SDS (20%). Then 250ul1 of 5 M KOH solution was added to each eppendorf and mixed. Aliquots were incubated in ice bath for 20 min and centrifuged at 13,000 rpm for 15 min. Supernatant was precipitated overnight at -20°C in the presence of 2.5 volumes of absolute ethanol and 0.1 volume of 3M sodium acetate (pH 5.5) solution.

### Ethanol precipitation

Samples taken from -20 °C were centrifuged at 13,000 rpm for 15 min and supernatants discarded. Pellets were washed with 1 ml of 70% cold ethanol and centrifuged at 13,000 rpm for 5 min. Ethanol was discarded and the pellets were dried under vacuum. Dried pellets were resuspended with 50ul RNase-free water and vortexed. Fifteen ul of resuspended pellets were used for the dot-blot analysis.

Molecular hybridization was carried out as described by manufacturers' instructions (DIG Chemiluminescent detection Kit -Roche).

### Sample denaturation

Fifteen ul of freshly prepared denaturation buffer (100 mM NaOH, 5mM EDTA) were added to each eppendorf tube containing 15ul of TNAs and

incubated at RT for 5 min., then applied onto a positively charged nylon membrane (Hybond N+) using a Dot blot apparatus (ABN vacuum systems micro sample blotting apparatus).

Then the membrane was exposed to UV for 2-3 min for fixing nucleic acids (DNA control must be loaded and used as homologous control for the probe).

**Prehybridization and hybridization**

The membrane was soaked in 2X SSC solution just before use, and then rolled and placed in an incubation cylinder containing 20 ml of prehybridization solution (Formamide 50 %, 5x SSC blocking reagent 2 %, sodium lauryl sarcosine Na LS 0,1 % and SDS 0,02 %). The prehybridization step was done for 2h at 55°C. The solution is then changed and the Riboprobe was added at a concentration of 50-100 ng/ml. The tube is incubated overnight in the hybridization oven at 55°C to perform the hybridization. Buffers and solutions used in molecular hybridization tests are shown in Annex 1.

**Chemiluminescent detection**

The next day, the membrane was washed 2 times in 50ml washing solution 2X SSG + 01 % SDS at RT for 5 min each time, then the membrane was washed 2 times in 50 ml washing solution 0.1X SSC + 0.1%SDS at 68°C in hybridization apparatus, for 15 min each washing. The membrane was then washed with 2X SSC + RNase for 30 minutes at RT with shaking. Another washing was done in SSC 2X on shaker for 5 min at RT. The membrane was placed in washing buffer 1 (1X + 0.3% Tween 20) on the shaker for 5 min at RT.

The blocking step was then performed. The membrane was placed between two plastic sheets and Buffer 2 (Blocking stock solution diluted 1: 10 in Buffer1 1 X) (0.15ml/1 cm2 of membrane) was added. The sachets were then left shaking for 1 h at RT. The buffers 2 were removed and anti-digoxigenin-AP diluted in buffer 2 (at the dilution indicated by the manufacturer instructions) added inside the plastic sachet and left on shaker for 30 min at RT.

The unbound conjugated antibodies were removed by washing twice the membrane in washing buffer for 15 min each washing at RT. The membrane

was stabilized for 1-2 min in Buffer 3, then exposed to the substrate (diluted 1:100 in buffer 3) for 5 min at RT.

The membrane was dried on tissue paper, and then covered with plastic film and put in a dark folder at 37°C in order to activate the enzyme for 15min. The membrane was then exposed to X ray film in dark room for 30 min. The composition of buffers and solutions used are shown in annex 2.

**Tissue-printing hybridization**

In spite of this procedure, any extraction procedure is a tedious step being then very convenient that for routine analysis sample manipulation could be reduced to a minimum Pallas *et al.* (2003). The imprint of the nylon membranes is prepared by using transversal section of petioles and/or stems (by duplicates), no further treatment is needed after sample application and the procedure is the same as protocol of dot blot hybridisation.

**Reverse transcription-polymerase chain reaction (RT -PCR) for ACLSV**

**Synthesis of the complementary DNA (cDNA)**

TNA was used as template to generate the single stranded cDNA. To achive this aim, 7 ul of TNAs in the presence of 22 ul sterile water and 1 ~l of random hexanucleotide primers (1 ug/ul), were denatured at 95°C for 5 min and placed on ice for 3 min. Then 1 0 ul of 5X reverse transcriptase (RT) buffer (Promega), 1 ul dNTPs (10 mM), 1 ul M-ML V (200 Units / ul), 2 ul DOT 0.1 M and 5 ul H2O were added to the mixture which was incubated at 42°C for 1 hour.

**Primers used**

ACLSV primers described by Menzel *et al.* (2002)

downstream primer

ACLSV-as (5'AAGTCTACAGGCTATTTATTATAAG TCTAA3')

upstream primer

ACLSV-s (5'TTCATGGAAAGACGGGGCAA3')

Intended to amplify a genome fragment of 677 bp.

**PCR**

The amplification of the nucleic acids was carried out as described by Menzel *at al.* (2002). Briefly, 5 ul of the cDNA were mixed with 45 ul of the amplification mixture [5 ~l of the reaction buffer 10X (Promega), 2 ul of $MgCl_2$ (25 mM), 1 ul dNTPs (10mM), 1 ul of the primer ACLSVs (1 0 uM), 1 ul of the primer ACLSVas (10 uM), 0.25 ul of Taq DNA polymerase (promega) and 34. 75 ul of sterile water]. Initial denaturation was at 94°C for 90 sec and cycling was as follows: denaturation for 30 sec at 94 °C, annealing for 30 sec at 62°C, extension for 60sec at 72°C for 30 cycles, and final extension for 5 min at 72°C.

**Analysis of PCR products**

PCR products were analyzed by electrophoresis in 5% polyacrylamide vertical slab gel. Polyacrylamide gel was prepared and poured in gel plates and let to polymerize for 15 min (Annex 6). Electrophoresis was carried out in 1 x TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDT A, Ph 8.0). Aliquots of 1 0 ul of the PCR products were mixes with 3 ul of loading dye. Marker XIV (Roche) was used as DNA marker. The gel was run at 100 V for 1 h. The products were visualized by silver staining.

**Silver staining**

The gel was incubated with 100 ml of 10% acetic solution for 20 min and washed 3 times with distilled water for 30 sec each. The gel was incubated with 100 ml of 1 % nitric acid solution for 3 min, rinsed 3 times with distilled water, then incubated with AgNO3 solution (Annex 7) for 20 min. After washing with distilled water, a developing solution (Annex 7) was added to the gel until clear bands appeared. Staining was stopped by a solution of 10 % acetic acid.

**Annex 1**

**Buffers and solutions used in molecular hybridization tests:**

| | |
|---|---|
| *Extraction Buffer* (1X) pH 7.0 | |
| Tris-HCl pH 8.0 | 100 Mm |
| EDTA | 50mM |
| NaCl | 500mM |
| 2- Mercaptoethanol | 1:1000 |
| *Denaturation Buffer* | |
| NaOH | 100mM |
| EDTA | 5mM |
| *SSC Solution* (20X) pH 7.0 | |
| NaCl | 3M |
| Na-Citrare | 0.3M |
| *Prehybridisaton Solution* | |
| Formamide | 50% |
| SSC | 5X |
| *Blocking Solution* | 2% |
| NaLS | 0.1% |
| SDS | 0.02% |

Blocking Stock Solution

Blocking reagent 10% (w/v) in Buffer 1, autoclaved and stored 4 °C

*Washing Buffer* (for 1ml)

| | |
|---|---|
| SSC | 2X |
| SDS | 0.1% |

## Annex 2

## Buffers used in chemiluminescent detection

*Buffer* 1 pH 7.5

| | |
|---|---|
| Maleic acid | 0.1M |
| | 0.15M (adjust with solid or concentrated NaOH autoclaved) |
| NaCl | |

*Washing Buffer*

| | |
|---|---|
| Buffer 1 | 1x |
| Tween-20 | 0.3% (w/v) |

*Buffer 2* 1%

(1% final concentration of blocking reagent, blocking stock solution is diluted 1:10 in buffer 1)

*Buffer 3*

| | |
|---|---|
| Tris-HCl | 0.1M |
| NaCl | 0.1M |
| $MgCl_2$ | 50mM pH 9.5 |

*Substrate Solution* CSPD diluted in 1:100 in buffer 3 (1x)

**Buffers and solutions used in TNAs extraction**

*Grinding Buffer*
4.0 M guanidine thiocyanate
0.2 M NaOAc Ph 5.2
25 mM EDTA
1.0 M KOAc
2.5% wt/vol PVP-40
2% Na2S2O5 (added just before use)

*Nal*
Dissolve 0.75g Na2SO3 in 40ml water
Add 36g Nal (Sigma SS379) MW 149.9
Stir until completely dissolved
Store in dark bottle at 4°C

*Preparation of Silica particle suspension*
In measuring cylinder, add 60g silica particules (Sigma SS631) to 500 ml H20, mix
Settle for 24 hours at 24 °C
Discard the upper 470 ml supernatant
Add H20 to 500 ml and mix well
Sette for 5 hours
Discard 440 ml supernatant
Adjust the remaining 60 ml slurry to a pH of 2.0 with HCl
Autoclave and store in dark bottle at room temperature.

*Washing solution* 10.0 mM Tris-HCl. PH 2.0 with HCl
0.5 mM EDTA
50.0 Mm NaCl
50% ethanol
store at 4°C

**Acrylamide gel for PCR products (5%)**

*Reagent*

| | |
|---|---|
| 40% Acrylamid/Bis | 1.3ml |
| 10X TBE Buffer | 1 ml |
| Distilled water | 7.7 ml |
| 10% APS* | 150 ul |

* APS: Ammonium persulfate

## Staining and developing solution for acrylamide gel

| | |
|---|---|
| *Silver nitrate solution* | |
| AgNO3 | 100mg |
| 37% formaldehyde solution | 150 µl |
| | |
| *Developing solution* (100 ml) | |
| Sodium carboanate (Na 2CO3) | 0.3g |
| 37% formaldehyde solution | 150 µl |
| Sodium thyosulfate | 6µl |

# Molecular diagnostic techniques and their potential role in stone fruit certification schemes

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**Summary**:

Stone fruit trees are affected by a large number of viruses that cause important economic losses. At the moment, unlike bacterial and fungal diseases, no chemical exist to be applied directly to control viral or viroid diseases. So, the early detection by means of sensitive diagnostic methods is the main way to control them. The extraordinary progress made in the nucleic acid research recently and the application of recombinant DNA technology in plant virology have permitted the use of diagnostic methods based on the genomic component of viruses and viroids. Among them, molecular hybridization and polymerase chain reaction (PCR) have received great interest lately and have been incorporated in the diagnostic field of plant virology. This review focuses on the molecular basis of these two techniques, their application to the diagnosis of stone fruit viruses and viroids and their potential incorporation into the certification schemes.

**Key words** -stone fruit viruses, viroids, non radioactive molecular hybridisation, PCR, diagnostic methods

***RESUME:***

Les essences à noyaux sont affectées par bon nombre de virus qui occasionnent des pertes économiques importantes. Actuellement, a l'opposé des maladies bactériennes et cryptogamiques, il n'existe aucun produit chimique qui puisse étre utilisé dans la putte directe contre les viroses ou les maladies a viroides. Par conséquent, le dépistage précoce a travers des méthodes de diagnostic sensibles represente la voie principale pour les combattre. Ces dernieres annees, les

progres extraordinaires faits dans l'étude des acides nucléiques et l'application de la technologie du DNA recombinant ont permis d'utiliser des méthodes de détection basées sur la composante génomique des virus et des viroides. Parmi ces techniques, l'hybridation moleculaire et l'amplification de sequence (PCR) ont suscité un grand intérét et elles ont été adopteés pour le diagnostic en virologie végétale. Le présent travail illustre les bases moléculaires de ces deux techniques, leur application au diagnostic des virus et des viroi des essences a noyaux et leurpossible introduction dans des programmes de certification.

***Mots-clés*** -virus des essences ànoyaux, viroiides, hybridation moléculaire non radioactive, PCR, méthodes de diagnostic.

## I. Introduction

Stone fruit trees are affected by a large number of viruses that exhibit very different biological properties as well as structural characteristics and genome expression strategies. They belong to different genera such as ilarvirus (ApMV, PDV, PNRSV), nepovirus (ArMV, RRSV, SLRV, TBRV, MLRSV, CLRV), trichovirus (ACLSV), tombusvirus (PeAMV), and potyvirus (PPV). Economic losses caused by these viruses vary from slight (as in the case of ACLSV) to extremely severe (PPV) (Uyemoto and Scott, 1992). Two different viroids are known to infect stone fruit trees, hop stunt viroid (HSVd), originally detected as causing a stunt disease of hops, and found in plum, peach, apricot, and almond (Shikata, 1990, Astruc *et al.,* 1996). HSVd cause of dapple fruit disease in plums and peaches (Shikata, 1990). Another viroid, peach latent mosaic (PLMVd) (Hernandez and Flores, 1992) is the causal agent of the peach latent mosaic disease (Desvignes, 1980). Both PLMVd and HSVd occur in high incidence in several Mediterranean areas in varieties of peach and apricot, respectively (Flores *et al.,* 1992; Cafiizares *et al.,* 1997; Badenes and Llacer, 1997; Loretti *et al.,* 1998). In addition, PLMVd has occasionally been detected in cherry, plum, and apricot germplasm from countries in Europe or Asia (Hadidi *et al.,* 1997).

At the moment, unlike for bacterial and fungal diseases no chemicals exist that could be used as a direct field control of viral or viroid diseases, and therefore the early detection by means of sensitive diagnostic methods is the main way to control them (Mathews, 1991; Hull, 1993). Plant virus composition relies on both traditionally called informative molecules (nucleic acids) and functional molecules (proteins). Methods for plant virus diagnosis have evolved in a parallel way to the progress in the knowledge of these components. Until very recently only methods based on the protein component of the viral particle were routinely used in plant virus detection. Among them, the serological ones (ELISA) were used due to their easy use, sensitivity and automation. However, one disadvantage of serology lie in the fact that only 2-5% of the genetic information of viral genome occur as antigenic determinants on the surface of the coat protein (Hull, 1986). However, serological techniques can not be applied to viroid diagnosis because viroids lack of specific-encoded proteins. Therefore, viroid detection must rely on bioassays or by direct detection of the genomic viroid RNA. Equally, bioassays are not appropriate for screening large populations. Likewise, gel electrophoresis techniques, used on the basis of the distinct mobility of small circular viroid RNAs, would not be suitable for large sample numbers. The extraordinary progress made on the nucleic acid research, during the last years, and the application of recombinant DNA technology to plant virology have permitted using diagnostic methods based on the nucleotide sequences of the genome component of viruses and viroids. Among them, molecular hybridisation and polymerase chain reaction (PCR) were recently incorporated into the diagnostic field of plant virology. This is a review of two molecular two techniques, their application to the diagnosis of stone fruit viruses and viroids, and their potential incorporation into the certification schemes. For additional information, excellent reviews on molecular hybridization (Hull, 1993; Miller and Martin, 1988) and PCR technologies, have been published (Henson and French, 1993).

## II -Molecular hybridisation techniques

### 1. Basic methodology:

Molecular hybridisation as a diagnostic tool in plant virology was first used to detect viroids (Owens and Diener, 1981) and later, applied to plant viruses (Maule *et al.,* 1983; Garger *et al.,* 1983). Molecular hybridisation, based on specific interaction between complementary purine and pyrimidine bases forming A-T and G=T base pairs, result in a stable hybrid formed by part (or the totality) of the nucleic acid sequence of the pathogen to be detected (target molecule) and the labelled complementary sequence (probe). The stability of the hybrid depends on the number of hydrogen bonds formed and on both electrostatic and hydrophobic forces. Electrostatic forces rely on the phosphate molecules of the nucleic acid backbone whereas hydrophobic interactions are maintained between the staggered bases.

The most common method for molecular hybridisation, the dot-blot hybridisation technique, involve the direct application of a nucleic acid solution to a solid support, such as nitrocellulose or nylon membranes, and subsequent detection with appropriate specific probes.

Several aspects affecting the different steps of the molecular hybridisation technique (which include the synthesis of the labelled probe, sample preparation, hybridisation and detection) will be discussed below. Detailed protocols for all these steps can be found in previous reviews (Hull, 1993; Pallàs *et al.,* 1997).

### 2. Synthesis of the Probe

Use of non-radioactive precursors to label nucleic acids, made the molecular hybridisation technique more accessible, and currently being used in an increasing number of virus-host combinations. Among non-radioactive precursors, those derived from biotin and digoxigenin molecules are most widely used. The biotinyl labelled nucleic acids are recognised with great efficiency by avidin or its microbial analogue, streptavidin, taking advantage of the exceptionally high affinity of the avidin-biotin complex. The main disadvantage of this system occur when sap extracts were used, where the

endogenous biotin may cause false positives or, alternatively, the presence of glycoproteins that bind avidin or biotin-binding proteins give rise to unworkable high background. Another widely used molecule to non-radioactively labelled nucleic acids is the hapten digoxigenin which is bound via a spacer arm (eleven carbon residues) to uridin-nucleotides and incorporated enzymatically into nucleic acids by standard methods.

Viroids and most of the plant viruses, including the totality of viruses affecting stone fruit trees have RNA genomes. RNA-RNA hybrids were more stable than RNA-DNA hybrids; therefore more stringent hybridisation conditions can be selected in the case of RNA-RNA hybrids that will help to increase specificity and lower nonspecific background. Hence, RNA probes were preferred over DNA ones to detect stone fruit viruses. Nonradioactive RNA probes (riboprobes) were synthesised by incorporating the digoxigenin hapten into a cRNA by means of an in vitro transcription reaction from cloned viral cDNA (Fig. 1A). To check the success and/or the yield of the riboprobe the electrophoretic mobility in TBEàagarose gels of the transcription products obtained in the presence and absence of the precursor DIG-UTP must be compared. If the digoxigenin was incorporated into the cRNA, the electrophoretic mobility of the transcript will be slower than that of unlabelled transcript (Fig. 1 B). Alternatively, transcription products may be serially diluted and spotted on nylon membranes which developed as described below (Fig. 4). Nonradioactive riboprobes for detecting viruses and viroids affecting stone fruit trees have been obtained for CLRV (Màs *et al.*, 1993; Màs and Pallàs, 1995), PNRSV (Heuss-LaRosa *et al.*, 1995; Sànchez-Navarro *et al.*, 1998), PPV (Nemichov *et al.*, 1996), CLSV (Kummert *et al.*, 1995), PLMVd (Ambros *et al.*, 1995; Badenes and Llàcer, 1998; Hadidi *et al.*, 1997), and HSVd (Romero-Durbàn *et al.*, 1995; Astruc *et al.*, 1996).

### 3. Sample preparation

There are no universal sample processing conditions for nonradioactive molecular hybridisation analysis. Choise of process protocol will depend on the virus being detected, the host, and the method used for detecting the

digoxigenin-labelled nucleic acids. For instance, when clarified sap extracts were used, the natural green-brownish colour of leaves on the membranes interferes directly with the colorimetric detection, probably due to the reduction of the nitroblue tetrazolium by components of the plant sap, while the light emission may not be altered by the presence of these components (Màs *et al.,* 1993; Pallàs *et al.,* 1997). An extraction buffer used for most of the stone fruit virus assays was applied for PPV (Varveri *et al.,* 1987) and consisted of 50 mM sodium citrate pH 8.3, containing 20 mM diethyldithiocarbamate (DIECA) and 2% (w/v) polyvinilpyrrolidone (PVP). Samples were homogenised, clarified by centrifugation at 5000 g for 5 min at 4°C, and denatured by heating at 60°C for 15 min in the presence of formaldehyde. This last step was optional for viruses since it increased only slightly the sensitivity limit. However, it was necessary for viroids due to their high degree of self complementarity (McQuarie *et al.,1984;* Flores, 1986; Astruc *et al.,* 1996). Most methods used for (viroid) RNA extraction require use of phenol or other toxic organic solvents, making them undesirable for diagnostic laboratories that process large number of samples. Recently, an extraction method that avoids the use of phenolics, previously described for obtaining plant genomic DNA (Dellaporta *et al.,* 1983), to enrich partially purified extracts in viroid-like RNAs (Pallàs *et al.,* 1987) or in the purification of double stranded viral RNAs (De Paulo and Powell, 1995) was adapted for viroid detection (Astruc *et al.,* 1996; Canizares *et al.,* 1998). For outline of the method, see Fig. 2.

For routine analysis in scheme certification programs, sample manipulation must be reduced to a minimum. This was achieved by using the tissue-imprinting technique that avoids sample extraction and only require the direct transfer of the plant material (stem, cutting, leaf) onto a nylon or nitrocellulose membrane. This technique was first described to detect proteins by immunocytolocalization (Cassab and Varner, 1987), later, applied to RNA detection (McClure and Guilfoyle, 1989), and then, adapted for detection and localisation of plant viruses (Mansky *et al.,* 1990; Chia *et al.,* 1992). Regarding viruses and viroids of stone fruit trees, the nonisotopic tissue-imprinting hybridisation has been applied to CLRV (Màs and Pallàs, 1995, see Fig. 3),

PNRSV (Sànchez-Navarro and Pallàs, unpublished results), and HSVd (Romero-Durbàn *et al.*, 1995; Astruc *et al.*, 1996). Immuno-tissue imprinting was used to detect ACLSV and PPV (Knapp *et al.*, 1995). Alternatively, samples may be applied to membranes by using sap impregnated cotton buds and uniformly pressing them until lateral diffusion occur (Fig. 4 and Sànchez-Navaro *et al.*, 1996).

**4. Hybridisation and nucleic acid detection**

Samples (nucleic acids) must be fixed onto a membrane by baking 2 h at 80°C, or at 120°C for 30 min, or by uv cross-linking (in the last two cases only Nylon membranes positively charged can be used). The last method result in a 5- to 10 fold increase in sensitivity over the baking methods.

The hybridisation process depend on several factors such as the complexity (length and composition of the nucleic acid), concentration of the probe, the temperature, salt concentration, base mismatches and hybridisation accelerators. The temperature at which half of the strands separate is the melting temperature (Tm). The stringency of the hybridisation conditions and the stability of the formed hybrid complexes determine the specificity of hybrid formation. In general, high temperatures and low salt increases stringency. The presence of formamide in the hybridisation solution, also increases stringency, favours correct base pairing and reduces background noise. For plant RNA virus detection, hybridisations were often carried out at 65-68°C. For viroids, good signal to background ratio was achieved at 70-72°C in 50% formamide.

Hybridised filters were either processed immediately or stored dry. The labelled hybrids were detected by an ELISA reaction using conjugates composed of high affinity DIG-specific antibodies coupled to alkaline phosphatase (AP). After three washing steps, a reaction was obtained by subsequent addition of AP substrates, either the colour substrates BCIP and NBT or with the chemiluminescent substrate CSPD®.

## III -Polymerase chain reaction techniques

### 1. Basic methodology

The polymerase chain reaction (PCR) was rapid, versatile, specific, and sensitive. PCR has been broadly used in plant pathology for the detection and diagnosis of pathogens as viroids, viruses, bacteria, phytoplasma, fungi, and nematodes (reviewed by Henson and French, 1993).

Principles, applications, and protocols for PCR have been detailed in numerous reviews. For this reason, this section will summarise the general guidelines, useful in the potential application of the technique in certification programs of stone fruit trees. The PCR method utilises an enzymatic and an exponential amplification of specific DNA sequences (Fig. 5). This goal may be achieved through multiple cycles of three steps performed at different temperatures to: (i) denaturate the DNA, (ii) anneal two oligonucleotide primers to the denatured DNA strands, and (iii) primer extension by thermostable DNA polymerases to synthesise the target sequence whose ends were defined by the primers. The presence of amplified DNAs was determined by gel electrophoresis analysis. In the case of RNA pathogens viroids and most plant viruses, a previous reverse transcription step (RT) must be included to copy the target RNA into cDNA prior to being amplified.

### 2. Considerations, advantages and disadvantages of PCR

PCR, a very specific and sensitive molecular technique suitable for diagnosis, is dependant upon the design of specific primers to initiate DNA synthesis. Primer sequences were obtained from the pathogen genome. Oligonucleotide primers must be 18-25 (annealing) nucleotide residues in length, with a 50 % G+C content, no annealing 3' ends, no secondary structures, and high G+C content at the 3' ends. Primers may be targeted either to conserved regions (to amplify sequences from groups of pathogens) or to variable regions (to discriminate between strains). The annealing temperature of primers will affect specificity of PCR and successful reaction depend on primer length, its G+C content, and primers around 20 nucleotides require increases of

up to 2°C for every A or T and 4°C for G or C. With stone fruit viruses, RT-PCR was applied in detecting of PPV, CLRV, ACLSV, PDV, ApMV, PNRSV, and TomRV (Wetzel *et al.,* 1992; Borja and Ponz, 1992; Rowhani *et al.,* 1995; Griesbach, 1995; Nemichov *et al.,* 1995; Parakh *et al.,* 1995; Rosner *et al.,* 1997; MacKeinze *et al.,* 1997; Sànchez-Navarro *et al.,* 1998)

Other factors affecting specificity were the incubation times for the different steps and concentrations of salts (KCl and $MgCl_2$), primers and enzyme. Size of the amplified product was also important; e.g. average of 200-500 residues (no longer than 1000) to maximise efficiency. Recently, Rosner *et al.,* (1997), used two different pairs of primers yielding a short (200 base pairs, bp) or long product (785 bp) and demonstrated that PNRSV was more reliably detected in situations of low virus titer by amplification with the short primers and at higher virus titer with the long primers. Since several factors affected the PCR reaction process, a series of detailed experiments to optimise the assays must be performed. The influence of different sample treatments and concentrations should also be tested in a later stage at the design of the diagnosis approach.

Theoretically, the sensitivity of the technique allows amplification of a single nucleic acid molecule from a complex mixture. Because of this extreme property, every caution must be taken in order to minimise contamination risks and 'one-use' aliquots should be prepared from 'mother' stocks of primers, reagents, and enzymes. Similarly, positive and negative controls must be included with every group of PCR assays performed.

Initially, reasonable pure nucleic acid preparations may be used to check primers and optimise PCR parameters and buffer composition. However, the procedures for obtaining those pure preparations are laborious, time-consuming and may require specialised equipment. Less-purified preparations, like those obtained with the non-phenolic extraction method described above (Fig. 2), may be used to determine their suitability for routine use. An important point to consider will be the design of experiments to detect possible inhibitors of the PCR reaction (i.e., serial dilutions of samples added to positive control

mixtures). Stone fruit trees are rich in polysaccharides, known to inhibit polymerase activity. Removal of such inhibitors from test samples may be achieved by using cation-exchange resins or polyvinyl pyrrolidone (PVP), which binds polyphenolic compounds. Gene Releaser$T_M$ polymeric matrix was used to eliminate inhibitors in assays of viroids (e.g. Hadidi and Yang, 1990; Levy *et al.*, 1994). A method for the extraction of high-quality RNA from woody plants that employs commercially available spin-column matrices and mitigates the inhibitory effects of plant polysaccharides and polyphenolic compounds were recently described (MacKeinze *et al.*, 1997).

Although unquestionable in advantages, PCR is very expensive requiring costly accessories such as thermocycler, and cost of enzymes and other molecular biology grade reagents. In addition, PCR-based techniques are prone to render false positives due to the extreme sensitivity and with the ease of contamination by aerosols, hair, skin, gloves, contaminated reagents, commercial preparations of Taq DNA polymerase, or even autoclaved material containing target sequences (Dwyer *et al.*, 1992; Henson and French, 1993). Therefore, it might not be a 'first choice' for large scale indexing. However, the sensitivity and rapid response make PCR a convenient approach for testing 'mother' plants.

**3. PCR for diagnosis of viroids**

RT-PCR was successfully applied in the detection of viroids from pome (Hadidi and Yang, 1990) and stone fruits (Shamloud *et al.*, 1995; Hadidi *et al.*, 1992; 1997), citrus (Yang *et al.*, 1992), grapevine (Rezaian *et al.*, 1992; Staub *et al.*, 1995; Wan Chow Wah and Symons, 1997), and avocado (Schell *et al.*, 1997). In most cases, the nature of the tissue to be analysed and/or the low titer of the viroid made necessary the use of laborious and time-consuming sample extraction protocols. Good PCR reactions were obtained with standard viroid extraction methods (Fig. 58). Recently, efforts were made conducted to simplify these extraction methods, either with the use of commercial products (Levy *et al.*, 1994), or alternative protocols not requiring organic solvents (Wan Chow Wah and Symons, 1997). Our work showed that a simple non-phenolic

extraction protocol (Fig. 2) (Astruc *et al.*, 1996) may be used, coupled with RT - PCR to detect HSVd in apricot (Khalid *et al.*, unpublished). Presumably, this procedure will be useful with other stone fruit species.

In primers designed for viroid detection, it should be noted that intra-molecular base pairing of viroid molecules (due to the high degree of self-complementarity of viroid RNAs) compete for primer annealing. To partially overcome this problem, longer oligonucleotides with higher annealing temperatures were recommended. In our laboratory, viroid PCRs were usually done at annealing temperatures no lower than 60°C. In order to maximise the detection of all sequence variants, primers should be located in the well known conserved sequences which includes the central conserved region in HSVd and the hammerhead region in PLMVd (Astruc *et al.*, 1996; C. Hernàndez and R. Flores, personal communication). It was not necessary to amplify complete viroid sequence and equally good results were obtained with shorter amplified products. It was demonstrated that different primer combinations resulted in different yields and sensitivity of the PCR reaction (Wan Chow Wah and Symons, 1997).

**IV- Combined Techniques:**

Serological and molecular techniques differ not only in the viral component to be detected but in their specificity, sensitivity, and facility of automation. Recently, the specificity and facility of automation for serological methods were combined with the sensitivity of the PCR technique in a single assay in which viral particles were initially antibody-captured and then amplified by PCR (Jansen *et al.*, 1990; Wetzel *et al.*, 1992; Nolasco *et al.*, 1993). This attractive technique, called immunocapture-PCR (IC-PCR) was 250 times more sensitive than direct PCR. ICPCR avoids using the level of purification of the extract usually required in order to eliminate the interfering compounds that affect the PCR-based methods. More recently, it was shown that the immunocapture step may be substituted by direct virus immobilisation, not requiring antiserum (direct binding-PCR; DB-PCR; Rowhani *et al.*, 1995), and the detection levels achieved by the DB-PCR were generally lower than those

of IC-PCR.

Another attractive PCR-based alternative was the one described by Olmos *et al.* (1996) in which the simplicity of the tissue-imprinting technique (see above) was combined with the specificity and sensitivity of the IC-PCR. This technique, called print-capture PCR (PC-PCR) avoids the need for grinding the samples without lost of sensitivity. This technique was applied for detecting PPV and ACLSV (Olmos *et al.*, 1996).

**V-Concluding remarks and future prospects.**

Diagnostic methods for plant viruses are being continuously improved. In recent years, considerable progress on nucleic acid research had advanced newer methodologies in detecting the genomic components of plant viruses. Although molecular hybridisation and PCR have gained new levels of sensitivity compared to serological ones, an acceptable level of automation was lacking. For a stone fruit certification programme, a compromise between simplicity of automation and sensitivity must be chosen. As a general rule, certified or certifiable material may be assayed by serological or nonradioactive molecular hybridisation methods, whereas more sensitive techniques, but also less affordable, such as those derived from the PCR approach could be used for primary sources or pre-basic material as well as for imported, dormant budwood during postentry quarantine or sanitation purposes.

The simplicity and sensitivity of new molecular methods have been sufficiently improved to detect most of plant viruses at levels below economic thresholds. The goal for the coming years will rely on making these methods more accessible to non specialised laboratories

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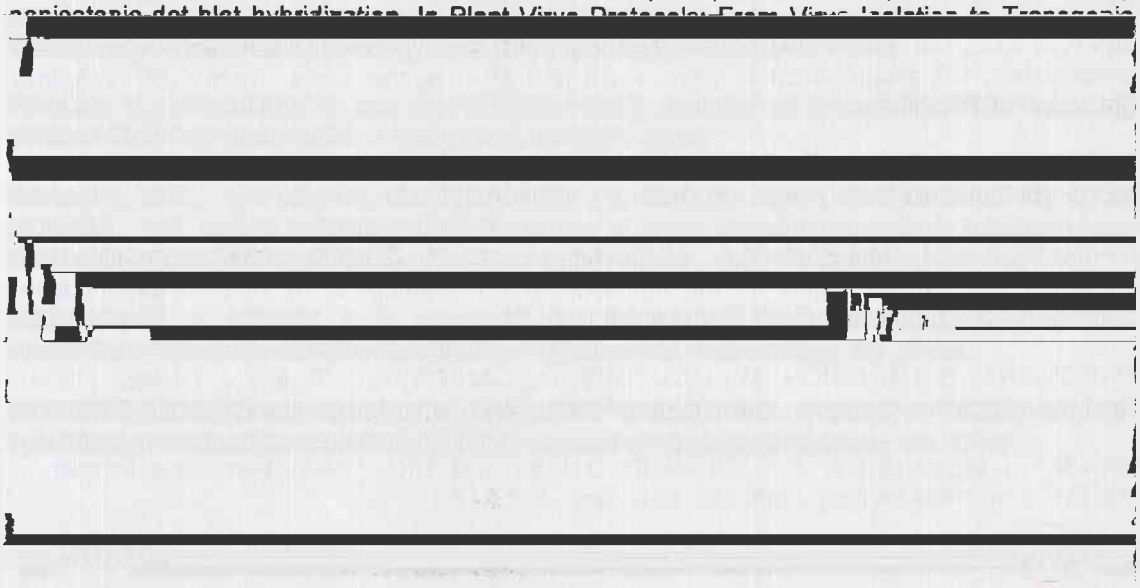

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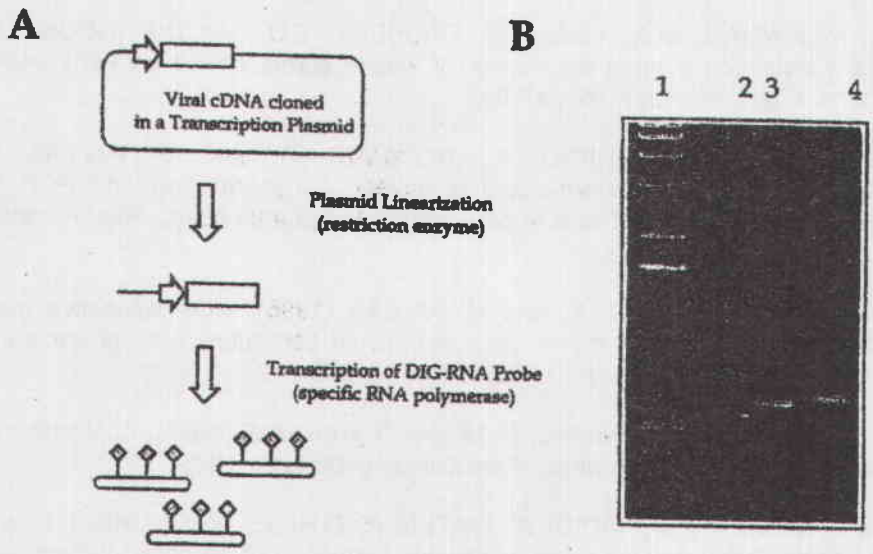


**Fig. 1. (A) Schematic diagram of the procedure used for the synthesis of a viral-specific digoxigenin-labelled RNA probe.**

The cloned viral cDNA sequence is represented by an open box under the control of an RNA polymerase promoter (arrow) specific of T3, T7 or SP6 phage polymerase. Plasmid was linearised downstream of the viral sequence and then in vitro transcribed to produce the digoxigenin-labelled RNA (the digoxigenin hapten is represented by diamonds). **(B) Agarose gel electrophoresis for the analysis of digoxigenin-labelled RNA probes.** Lane 1, DNA molecular weight marker; lane 2, linearised plasmid DNA; lane 3, RNA synthesised from the plasmid DNA shown in (2) in the presence of non-labelled UTP; lane 4, RNA synthesised from the plasmid DNA shown in (2) in the presence of DIG-UTP (note the slight slower mobility due to the presence of the digoxigenin hapten).

# Appendix

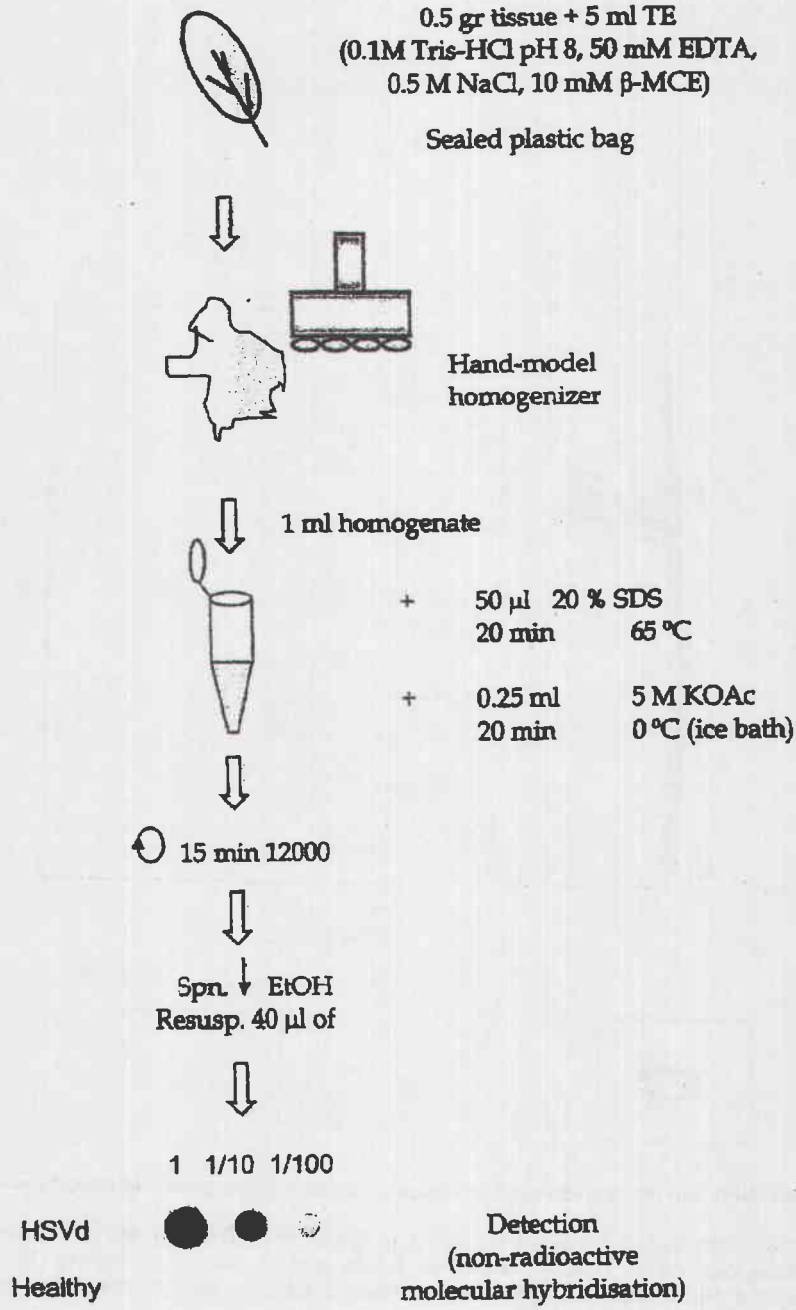


**Fig. 2. Schematic diagram of the non-organic sample processing procedure used for viroid detection.**

The procedure has been adapted for processing small volumes and managing a large number of samples. Aliquots of the samples resuspended in $H_2O$ were finally dotted onto Nylon membranes, fixed by uv cross-linking, and hybridised and developed as outlined in Fig. 4.

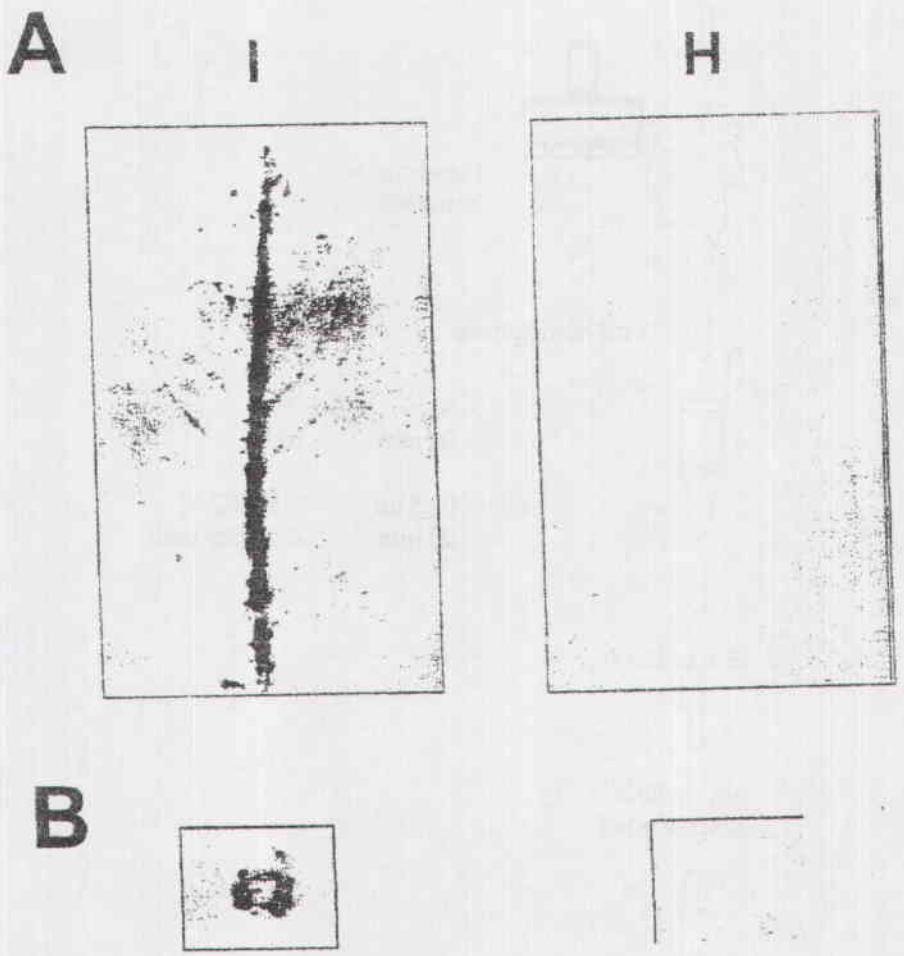


**Fig. 3. Viral RNA detection by hybridisation of tissue prints to a digoxigenin-labelled specific RNA probe.**

Leaves from either cherry leaf roll nepovirus-infected (I) or healthy (H) walnut (var. MBT-231) were tissue-printed directly onto Nylon membranes. Leaves were either directly printed onto the membrane (A), or tightly rolled, cross-sectionally cut with a razor blade, and the section printed onto the membrane (B). Membranes were analysed and developed as outlined in Fig. 4

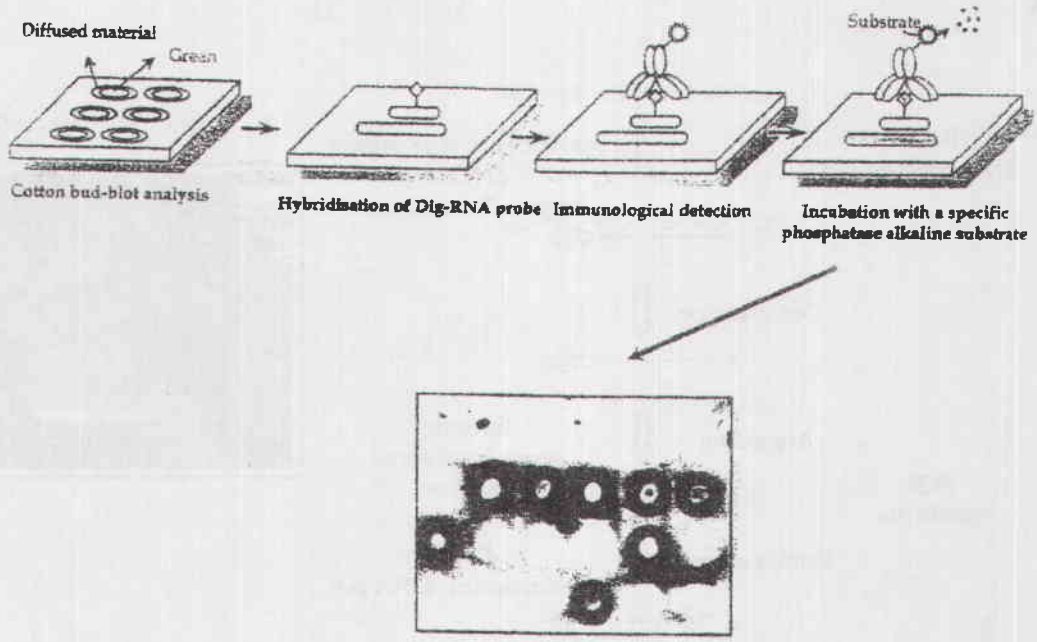


**Fig. 4. Schematic diagram of the procedure used for the analysis of samples by nonradioactive molecular hibridisation.**

The samples on the membranes were hybridised to a specific digoxigenin-labelled RNA probe, overnight at 50-68 °C in the presence of 50% formamide; the hybridised probe was detected by immunological binding to an anti-DIG antibody conjugated to either alkaline phosphatase (in this example) or horseradish peroxidase; finally, the reaction was developped with a substrate specific for the conjugated enzyme, either chemiluminiscent (in this case, CSPD®) or colorigenic. In this example, samples were applied onto the membrane by the 'cotton-bud' technique (see text).

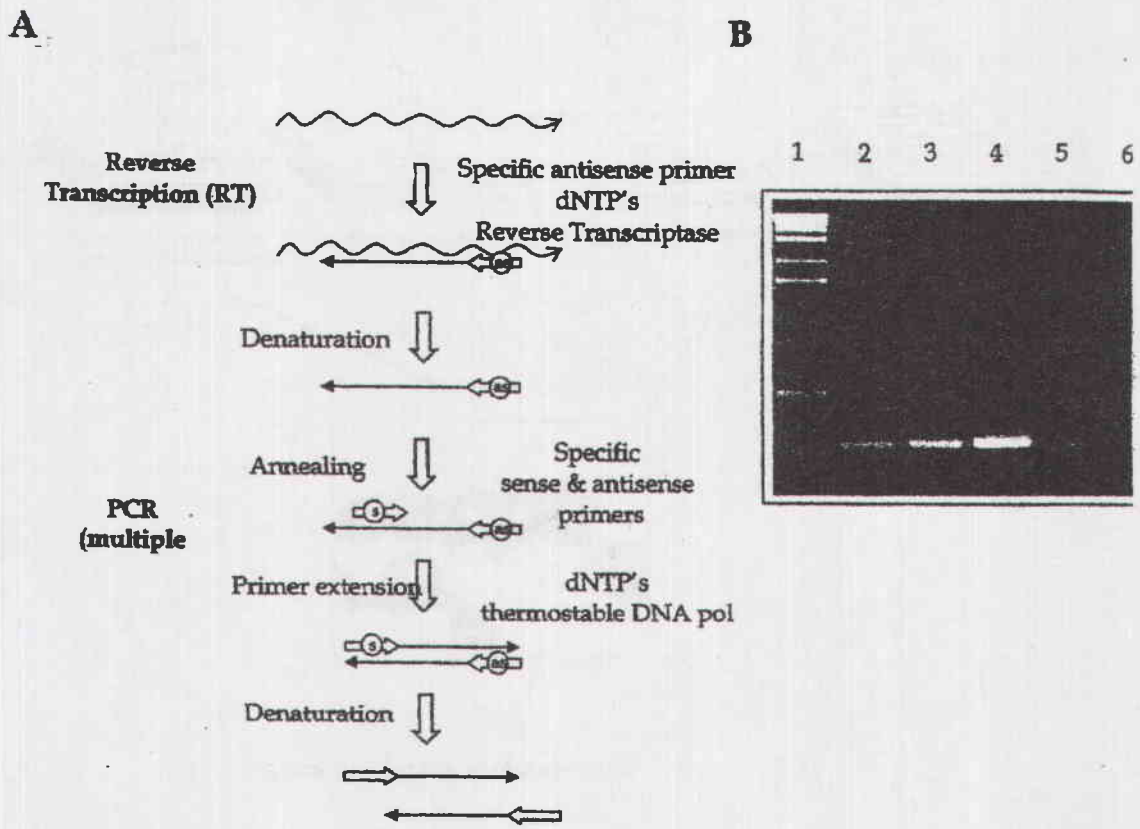


**Fig 5. (A) Schematic diagram of the RT-PCR procedure used for the detection of plant virus and viroids.**

The pathogen RNA (wavy line) was first reverse transcribed to cDNA using a **specific antisense (as)** oligonucleotide primer, and then exponentially amplified through multiple cycles of PCR using specific sense (s) and antisense (as) oligonucleotides. **(B) Agarose gel electrophoresis of the RT-PCR products obtained in the analysis of field samples for the presence of HSVd.** Lane 1, DNA molecular weight marker; lane 2, control sample from HSVd-infected peach GF-305; lanes 3 to 6, samples from different apricot trees (samples 3 to 5 were found infected with HSVd while sample 6 was viroid-free).

## Names of Participants

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| Names | Country |
|---|---|
| 1- Nasir Abdel Aziz El Tamimi | Jordan |
| 2- Abdulla Salim Ahmed Janaan | U.A. Emirates |
| 3- Mohamed Khalifa Lagran | Tunisia |
| 4- Moawia El Aiderous | Sudan |
| 5- Dr. Adnan Osman | Syria |
| 6- Yousuf bin Mohamed Al-Raeesy | Sultanate of Oman |
| 7- Dr. Elia Choueiri | Lebanon |
| 8- Bourziq Mimoun | Morocoo |