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A guideline for the detection of selected plant viruses in forest tree seeds

Ein Leitfaden zum Nachweis ausgewählter Pflanzenviren in Forstsaatgut

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Summary

We report about the transmission of plant viruses in forest tree seeds and give a guideline for the detection of viruses in deciduous forest tree seeds in general and then focuse on the detection of widespread infections caused by seedtransmissible pathogen cherry leaf roll virus (CLRV).
Serological methods, dot blot hybridization and as well as a modified immunocapture RT-PCR (IC-RT-PCR) are demonstrated to detect the most described and observed seedtransmissible viruses as there are nepoviruses, ilar- cucumo- and tobamoviruses. Applied methods are valued and discussed.
The application of a conserved primer pair is shown to be suitable for the detection of e.g. CLRV which was easily detectable all over the year in collected seeds collected from a CLRV-infected birch-tree.

Key words: plantviruses, forest tree seeds, diagnosis, test procedures, cherry leaf roll virus

Zusammenfassung

Wir berichten über die Übertragung von Pflanzenviren mit Forstsaatgut und geben einen Leitfaden zum Nachweis der Viren in Samen von Laubgehölzen im allgemeinen und fokussieren dann auf die Diagnose weitverbreiteter Infektionen, die durch das samenübertragbare Kirschenblattrollvirus (cherry leaf roll virus, CLRV) verursacht werden.
Serologische Methoden, dot-blot Hybridisierung und eine modifizierte immuno-capture RT-PCR (IC-RT-PCR) werden vorgestellt, um die am häufigsten beobachteten und beschriebenen samenübertragbaren Viren, die Nepo-, Ilar-, Cucumo- und Tobamoviren nachzuweisen. Die vorgestellten Methoden werden diskutiert und bewertet.
Die Anwendung eines hochkonservierten Primerpaars wird als geeignete Methode beispielsweise zum Nachweis des CLRV gezeigt. Damit lässt sich der Erreger während des ganzen Jahres leicht in Samen einer CLRV-infizierten Birke nachweisen.

Stichwörter: Pflanzenviren, Forstsaatgut, Diagnose, Testverfahren, cherry leaf roll virus

Introduction

Investigations on spread of viruses in selected German forest ecosystems and nurseries of North and West Germany confirm former studies from different countries, as NIENHAUS and CASTELLO (1989) reviewed, that many diverse deciduous trees of any age are virusinfected (FÜHRLING and BÜTTNER, 1997).
From the economic point of view one should be aware that virus diseased plants may increase production costs because of the possibly decreased growth of infected stock plants and that they may damage subsequent field performance. Virus infection alters plants predisposition and these trees become more susceptible to abiotic and biotic stress impact and may even lead to completely degenerating, dieing trees.
Referring to the International Committee on Taxonomy of Viruses (ICTV) there are 32 groups of well-characterized viruses but only about twelve of these have been commonly detected in trees and shrubs (COOPER, 1993) and there are only a few virus groups with a marked preference for woody perennial hosts use pollen and/or seed to an important extent for their dissemination as there are nepoviruses, ilarviruses and cucumoviruses. Seed transmission may be of very considerable economic importance, because viruses may persist in seed for long periods so that commercial distribution of a seedborne virus over long distances may readily occur (BÜTTNER and BANDTE, 2000).

Nepoviruses being transmitted by seed and pollen and by other causes such as nematodes and mechanical means have been frequently detected in trees and shrubs: cherry leaf roll virus (CLRV) is the most common and the most detected virus in many species as there are e.g. *Betula* sp., *Cornus* sp., *Fagus sylvatica L.*, *Fraxinus excelsior* L., *Juglans regia* L., *Rhamnus catharticus* L. and *Sambucus nigra L.*. The virus causes chlorotic ring spots and lines, in many cases small leaves and may finally lead to degenerating twigs or trees (BANDTE and BÜTTNER, 2001). Furthermore arabis mosaic virus (AMV) occures in ash.
Ilarviruses tend to be host specific and be transferred via pollen. They are widespread in fruit trees and cause serious losses. Elm mottle mosaic virus (ElMMV) in *Syringa vulgaris* L. and in *Ulmus glabra* L. has been recorded as well as apple mosaic virus (ApMV) in *Rosa* spp. and *Corylus avelana* L. and prunus necrotic ringspot virus (PNRV) in *Aesculus hippocastanum* L.
Cucumoviruses are transmitted in seeds and are very common to be found in cultivated plants. The main member cucumber mosaic virus was detected in *Lonicera periclymenum* L. and *Ligustrum* spp..
Tobamoviruses are known to be transmitted by contaminating the seed coat and infect the seedling when germinating. Tobamoviruses have been detected in *Quercus robur* L. and *Acer* spp. (FÜHRLING and BÜTTNER, 1998).
Most of the virus infected plants cause a serious source for the widespread of the pathogens, because they are easiliy transmitted by seed and pollen and/or by soil, water or through vectors.

Two general types of seed transmission can be distinguished. Seed transmission may be the result of contamination of the seed coat with virus particles, resulting in subsequent infection of the germinating seedling by mechanical means. The external virus can be inactivated by certain treatment eliminating almost all seed-borne infection.

In the more common type of seed transmission the virus is found within the tissues of the embryo which may become infected through the ovary or via the pollen.
Most seed-transmitted viruses are also transmitted through pollen from infected plants, and all known pollen transmitted viruses are also seed transmissible. The mechanisms transmitting pollen are either within the sperm cell nucleus or cytoplasma or second on the exine of the pollen grains. The germ tubes growing from such pollen may then pick up virus particles and carry active to the ovule.

In case that cure of virus infections is not possible, prevention is an important tool to control plants. Except for eliminating affected and unthrifty trees, it is usually impractical to prevent the spread of viruses between trees in the field after planting into the field. Attention is inevitably focused on the nursery production of planting stock where critical examination and assessment of vigour can be practised routinely.
Prevention tools are possible when the pathogen is detected and its general properties including its mechanisms of natural dispersal are known. Therefore diagnostic trails have to be established (BÜTTNER et al., 1996).
In this paper we will give a guideline and a laboratory manual for diagnostic tools to detect the most common seed- and pollen transmissible viruses affecting woody perennials.

Material and Methods

In regard to the most common virus in forest trees we focuse on the nepovirus CLRV which is a seed- and pollen transmissble pathogen to demonstrate a modified PCR-technique to detect the pathogen in leaves and seeds. Furthermore the hybridization technique is applied to detect previously unknown tobamoviruses of diseased oak- and acer trees. The ilarviruses and the cucumoviruses were identified by the serological test ELISA.
When testing seeds, there are two trails to prepare the samples, first the direct preparation of the seeds when possible. And second using leaf tissue after the seeds have germinated. In all cases of the procedures the seed coat has to be removed before starting the sample preparation.
The electronmicroscopy has to be regarded as not reliable and a less suitable tool to detect viruses directly from seeds, because of the irregular distribution of the pathogens in seed lots and the low concentration of the pathogens. In some cases the ELISA overcomes this problem, but still phenolic compounds may disturb clear results. Only the methods based on nucleic acid detection such as hybridization or amplification of characteristic fragments have shown progresses to reach a higher sensitive testing and reliable results.

Double antibody sandwich ELISA (Enzyme linked immunosorbent assay)
(CLARK AND ADAMS, 1977, VAN REGENMORTEL, 1982)

If bacterial contamination is likely to occur, 0.02 % sodium azide may be added to all buffers used in the assay.

10 x PBS (phoshate buffered solution, pH 7.4): NaCl (80 g), KH_2PO_4 (2 g), Na_2HPO_4 x 2 H_2O (14,4 g), KCl (2 g) add aqua dest. 1000 ml

Preparation of the antibody

the globulins should be prepared by precipitation from antiserum with an equal volume of 4 M ammonium sulfate

↓

stir solution slowly for an hour at room temperature

↓

centrifuge the solution (8000 rpm for 10 min)

↓

resolve the pellet in 1ml phosphate-buffered saline pH 7.4 (PBS) (half concentration)

↓

remove ammonium sulfate by dialysis using PBS (half concentration)

ELISA-Testing

Step 1: **coating** (specific antibodies are adsorbed to the plate)

antibodies are diluted in coating buffer (usually 1:1000, but has to be tested before in dependance on the quality of the anitbodies)

↓

use 200 µl / well for coating (coating by passive adsorption)

↓

incubate at 37 °C for 4 hrs

↓

wash 3 times with PBS-T

(PBS containing Tween 20 (0,05 %)) Use 3 minutes intervals between washes

Step 2: sample preparation (virus is added)

viral antigens in seeds or leaf material are extracted (1:10 - 1:30 in PBS-T)

↓

transfer 200 µl of the sample into the wells

↓

incubate overnight at 4 °C

↓

wash 3 times with PBS-T, use 3 minutes intervals between washes

to reduce nonspecific reactions and increase the sensivity of the virus detection polyvinyl pyrrolidone (1-2 %), urea (1 M) or reducing agents (Guggerli, 1979) may be added to the buffer

Step 2 a: blocking (gives in some cases better results)

block with 200 µl 1 % Bovine serum albumin (BSA) per well

↓

incubate for 2 hrs at room temperature

↓

wash 3 times with PBS-T

↓

use 3 minutes intervals between washes

Step 3: conjugate (enzyme labeled specific antibodies are added)

antivirus enzyme conjugate is diluted in PBS-T (usually 1:500-1:1000, but has to be determined empirically)

↓

transfer 200 µl per well

↓

incubate at 37 °C for 4 hrs

↓

wash 3 times with PBS-T, use 3 minutes intervals between washes

the enzyme conjugate most commonly used is prepared with alkaline phosphatase (Boehringer, Mannheim or Sigma, St. Louis, Missouri) by coupling the globulins with enzyme at 1 ml:0,1 ml (v:v, globulin:enzyme) using 0,06 % glutaraldehyde. The conjugate should be stored at 4 °C in the presence of 1 % bovine serum albumin.

Step 4: substrate (enzyme substrate is added)

dilute (just before use !) the substrate *p*-nitrophenyl phosphate (1 mg/ml) in (0,1 M diethanolamine buffer pH 9.8)

↓

transfer 250 µl into each well

↓

results are scored visually by appearance of a yellow colour, or adsorbances at 405 nm are read by spectrophotometer or ELISA-reader

↓

reaction is stopped by the addition of 50 µl of 3 M NaOH to each well

Results are considered positive if the adsorbance is twice that found with healthy controls, or alternatively, if it is two standard deviation units higher than the mean of a negative control curve.

Immunocapture-reverse transcriptase polymerase chain reaction (IC-RT-PCR) (BÜTTNER et al., 1996; WERNER et al., 1997)

sample preparation

a)

grind plant tissue under liquid nitrogen

↓

homogenize the powder in buffer; 1:10, w.v

(PBS-Tween, 2% (w/v) Polyvinylpyrrolidone 40 (PVP 40))

b)

homogenize plant tissue with sterile seasand in buffer; 1:10, w.v
(PBS-Tween, 2% (w/v) Polyvinylpyrrolidone 40 (PVP 40))

Coating and reverse transcription

coat sterile reaction tubes (0,5 ml, Eppendorf Safelock) with 50 µl purified CLRV specific antibodies (40 µg/ml in 50 mM carbonate buffer, pH 9.6)

↓

incubate for 3 hrs at 37 °C

↓

wash the tubes 3 times for 3 minutes with 150 µl PBS-Tween
(20 mM phosphate buffer, pH 7.4, 135 mM NaCl, 0.05% (v/v) Tween 20)

↓

incubate the coated tubes with 50 µl of the plant extracts (see sample preparation) over night at 4 °C

↓

wash 3 times for 3 minutes with 150 µl PBS-Tween

↓

centrifuge the tubes briefly and remove remaining washing buffer

↓

add buffer to a total volume of 20 µl; 50 mM Tris-HCl, pH 8.3, containing 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 1 mM dNTP's, 20 U ribonuclease inhibitor (Promega, Madison), 200 U M-MLV reverse transcriptase (GIBCO BRL, Inchinnan), 100 pmol CLRV specific first strand primer (5'-GTC GGA AAG ATT ACG TAA AAG G-3')

↓

incubate the captured virions for 1 h at 37 °C,

↓

heat denaturation at 95 °C for 3 min

↓

amplify aliquots of 2 µl of the reverse transcription reaction in a subsequent PCR

Polymerase chain reaction

mix:	2	µl	aliquot of the reverse transcription reaction
	20	pmol	first strand primer
	20	pmol	second strand primer
	2,5	U	Taq-DNA-Polymerase

↓

add 100 µl buffer (10 mM Tris-HCl, pH 8.3, containing 1,5 mM $MgCl_2$, 50 mM KCl)

↓

amplification of the viral cDNA in a thermocycler

35 cylcles:	primer annealing at 51 °C for 60 s
	chain elongation at 72 °C for 60 s
	denaturation at 95 °C for 30 s

Gelelectrophoesis of the amplification products

melt 2 g agarose in 100 ml 1 x TBE-buffer
(10 x TBE: 0,9 M Tris, 0,9 M boric acid, 50 mM EDTA)
↓
cool down to approximately 60 °C
↓
add ethidium bromide (7 µl of a 10 mg/ml stock solution)
↓
transfer agarose into a electrophorese chamber (Biometra midi-gel)
↓
prepare samples (aliquotes of the PCR products); add 1:1 loading buffer (gylcerol (87 %) 8 ml, 10 x TBE 4 ml, aqu. dest. 28 ml, 0,01 % bromephenolblue, 0,01 % xylencyanol)
↓
carry out electrophoretic separation at 70 mA
↓
visualize nucleic acid fragments under UV-light

Dot blot hybridization (MILTENBURG ET AL., 1995)
It is necessary to use sterile Eppendorf tubes and DEPC-treated aqua dest. to produce the mentioned buffers.

RNA-extraction from plant tissue

grind 200 mg plant tissue in a mortar with liquid nitrogen to a fine homogenous powder
↓
transfer to a sterile eppendorf tube
↓
add 800 µl denaturation buffer (4 M guanidinium isothiocyanate, 0,2 M mercaptoethanol) and vortex the mixture
↓
centrifuge at 12.000 rpm for 5 min
↓
suck off 500 µl supernatant and transfer to a fresh tube
↓
for the chloroform/phenol extraction add 250 µl phenol and 250 µl chloroform/isoamylalcohol (v:v, 24:1) and vortex the mixture
↓
centrifuge at 12.000 rpm for 3 min
↓
repeat the chloroform/phenol extraction two more times
↓
transfer than the supernatant to a fresh tube
↓
add 500 µl chloroform
↓

centrifuge at 12.000 rpm for 3 min

↓

suck of the aqueous phase and transfer to a fresh tube

↓

ethanol precipitation:

a) when using leaf tissue of soft wood
add 50 µl 4 M LiCl and 1,3 ml ethanol (96%) and vortex

b) when using seeds, leaf tissue of *rosaceae* or hardwood
add 60 µl 6 M NaCl and 1,3 ml ethanol (99,6%) and vortex

↓

store at: a) for 1 h at -80 °C or overnight at -20 °C
b) for 1 min on ice

↓

centrifuge at 10.000 rpm for 30 min

↓

remove supernatant and discard

↓

add 1 ml ethanol (70 %)

↓

continue sample preparation or store at -80°C when needed

sample preparation

centrifuge the tube at 15.000 rpm for 20 min

↓

remove ethanol and discard

↓

add 500 µl ethanol (70 %) and vortex

↓

centrifuge at 15.000 rpm for 10 min

↓

remove ethanol and discard

↓

add 500 µl ethanol (70 %) and vortex

↓

centrifuge at 15.000 rpm for 10 min

↓

remove ethanol and discard

↓

dry pellet (dry by air or by speed vac 2 x 2 min)

blotting procedure

resolve pellet in 200 µl formaldehyde
(6 M in 10 x SSC (1,5 M NaCl, 0,15 M trisodiumcitrate))

↓

incubate 15 min at 65 °C

↓

chill on ice

↓

wet membrane (positively charged Nylon membrane) in sterile water and incubate for 15 min in 20 x SSC

↓

incubate blotting paper (whatman 3mm) for 15 min in 20 x SSC

↓

wash sample wells of the blotting apparature with 400 µl 20 x SSC

↓

load RNA samples into the sample wells (concentration has to be determined by experiments, use two concentration steps)

↓

incubate for 30 min at room temperature

↓

suck RNA samples through the membran with vacuum

↓

wash with 500 µl 20 x SSC

↓

incubate the membran for 1 h at 80°C
or UV-crosslinker the membrane (2000 µJoule x 100)

hybridization

transfer the membrane (printside up) into a hybridization flask

↓

add 10 ml hybridization buffer (5 x SSC, 0,1% N-Lauroylsarcosin, 0,02 % SDS, 50 % formamide, 2 % blocking reagent (Boehringer, Mannheim) and 125 µg/ml herring sperm DNA)

↓

incubate for 1 h at 68 °C with continuous agitation

↓

add 50 µl of the hybridization probe (BÜTTNER and FÜHRLING, 1997)

↓

incubate for overnight at 68 °C

↓

remove hybridization buffer (can be reused for several times)

↓

wash the membrane in 30 ml of wash solution I (2 x SSC, 0,1 % SDS) at 65 °C, three times for 15 min each

↓

wash the membran in wash solution II (0,1 x SSC, 0,1 % SDS) at room temperture twice for 5 min each

detection

equilibrate the membrane in buffer I
(buffer I: 0,1 M maleic acid, 0, 15 M NaCl; pH 7.5, add 0,3 % Tween 20)
↓
block the membrane by gently agitating the buffer II for 60 min
(buffer II: 1 % blocking reagent disolved in buffer 1)
↓
dilute Anti-DIG-alkaline phosphatase 1:10000 (75 mU/ml) in buffer II. Mix gently by inversion (the solution is stable for several days at 4 °C)
↓
incubate the membrane in the antibody solution for 30 min (agitate the tray gently, ensure that the membrane is always covered)
↓
discard antibody solution and wash the membrane twice for 15 min in buffer I
↓
equilibrate the membrane in buffer III for 2 min
(buffer III: 0,1 M Tris HCl, pH 9.5; add 0,1 M NaCl, 0,05 M $MgCl_2$)
↓
dilute CSPD in buffer III (1:100)
↓
place wet membrane between two sheets of plastic page protectors. Lift the top sheet and add diluted CSPD (0,5 ml/100cm2), distribute the drops over the surface by rocking the membrane; wipe the top sheet to remove any bubbles to create a closed liquid seal
↓
incubate the membrane for 5 min at room temperature
↓
seal semi-dry membrane in a plastic bag
↓
incubate for 15 min at 37 °C
↓
expose the membrane to Hyperfilm-ECL (Amersham); start with 15 min
↓
develop the film and expose the membrane again as long as needed

Results and Discussion

Viruses of woody forest plants and of their seeds are known to be difficult to detect due to phenolic compounds in plant extracts and an often irregular distribution or the low concentration of the pathogens within the plants and seeds when using serological methods as a tool (BÜTTNER and FÜHRLING, 1996). But still these methods have to be regarded as important diagnostic tools when considering the restricted capacity of the test.

The objectivity of electron microscopy has increased by the use of immunological reagents, but we cannot recommend it as a reliable test searching for viruses in seeds of

woody plants. When using antiserum coated electron microscope grids, virions can be selectively adsorbed from plant extracts by additional precision when decorating the particles with specific antibodies applied to the grids.These methods have been used to locate cherry leaf roll virus on and in pollen from birch or walnut (MASSALSKI and COOPER, 1984; MASSALKI et al., 1988), but is not suitable for routine testing.

The ELISA is a most sensitive immunological system. COOPER et al. (1986) detected ilarviruses in cherry *(Prunus avium)* seed and poplar mosaic virus in large-scale surveys of poplar germplasm. There are no reports on detecting cucumoviruses in forest tree seeds by ELISA, but studies on seeds from herbaceous hosts confirm that the ELISA is a reliable test.

Methods which detect nucleic acid are often essential when the pathogen being sought lack protein. A new perspective for the diagnosis gives the combination of grafting plants to transmit the pathogen to host plants and a modified technique of the polymerase chain reaction (PCR) as well as the hybridization technique to detect the assumed pathogen (BÜTTNER et al., 1996). The PCR and hybridization technique are sensitive methods to detect viruses of smallest amount. WERNER et al. (1997) evaluated a method for detecting cherry leaf roll virus (CLRV) in seeds of birch and concluded that PCR followed by immunocapture-reverse transcriptase is the most sensitive way to detect the viral RNAs without a radioactive detection, which makes the system cheaper and more reliable for routine use.

CLRV was detected in single birch seeds but not in all seeds from an infected tree, even if they derived from the same female inflorescence. This finding confirms previous observations on the vertical spread of CLRV in cherry trees (LÖW, 1995) and birch, where healthy progeny could be issued from infected trees (COOPER, 1993). We have shown 200 birch seeds from an infected tree to estimate the vertical transmission rate and yielded 16 seedlings. All seedlings were tested for CLRV-infection by IC-RT-PCR. Five of them were strongly infected, one showed a weak signal, and the other 10 seedlings seem to be uninfected (data not shown). A serial dilution of plant sap from leaf buds of a CLRV infected tree was carried out to determine the dilution limit of plant sap for detection of CLRV by IC-RT-PCR. In comparison to ELISA techniques RT-PCR approaches represent a remarkable improvement in sensitivity (WETZEL et al., 1992, NOLASCO et al., 1993). Our results confirm that the RT-PCR assay is 1000-10.000 times more sensitive than the ELISA. The IC-RT-PCR enables the detection of CLRV in samples of very small amount such as in seeds (WERNER et al., 1996). In plant sap dilutions and seeds, stored at 4 °C, CLRV could be detected by IC-RT-PCR within 3-4 weeks.

As shown by COOPER (1993) seed transmission rate of CLRV is very variable and depends on whether male or female gametophytes derive from a CLRV infected tree. The rate of vertical transmission of viruses also depends on host factors, viral strains and environmental conditions (MAULE and WANG, 1996), but the high sensitivity of the IC-RT-PCR provides good means to detect CLRV infections even at low transmission rates in spot-checks of seed material.

The hybridization technique can be applied to detect particular plant RNA viruses or viroids in case that a specific RNA-probe is available. This probe has to be produced by molecularbiological methods. In comparison to the described IC-RT-PCR it is not nec-

essary to have informations concerning the genom sequence to construct a probe. Whereas the selected oligonucleotide primers used in the PCR are synthesizied based on the sequence of conserved genom regions. Variing the hybridization conditions (especially temperature) a sequence homology of 60-70% in between the specific RNA probe and the sample RNA is sufficient for the detection. If the reagents for both methodes, the IC-RT PCR and the hybridization, are available the hybridization should be applied if the identification of virus strains is not object of the investigation. The dot blot hybridization is particularly qualified for routine diagnostic testing of a large scale samples. The PCR is more specific than the hybridization and enables the differentiation of virus strains. This specifity excludes the application of the method for general virus diagnosis purpose. Furthermore technical equipement and chemicals which are more expensive.

Concluding remarks

By the worldwide shift from extensive to intensive forest management, systems of high quality forest stands have been established. To preserve their value, more resources must be committed to the protection of forest stands. Forest tree pathology plays a major part in conserving the economic value of a forest stand (HUBBES, 1993). Therefore potential and existing disease threats must be identified and prophylactic methods including disinfecting trails for disease control have to be developed (BÜTTNER and Bandte, 2001). In contrast to other pathogens viruses can not be controlled by curative treatments. Therefore prophylactic measures and the planting of virus-free seedlings are the basic requirement to ensure a long-term economic forest stand. For instance, CLRV is widely distributed, and early detection is an important part of the strategy for prevention of the spread of the disease. In regard to CLRV, new stands should be established with CLRV-free seedlings. As this pathogen is seed-transmissible to a high degree, seed lots as well as growing seedlings have to be tested for CLRV infection before planting into their final stand. A new infection in a stand may be introduced by contaminated soil or water, but these transmission modes are epidemiologically of minor importance. Molecular biological techniques may offer the basis for the establishment of a test suitable for routine diagnosis (BÜTTNER et al., 1996). The required method demands high reliability and sensitivity and has to be suitable for seeds as well as leaves and buds in regard to practical approach.

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