Digoxigenin-Labeled Riboprobes Applied to Phytosanitary Certification of Tomato in Italy

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ABSTRACT

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A dot-blot hybridization system using digoxigenin-labeled riboprobes and chemiluminescent detection was developed for the diagnosis of infections by cucumber mosaic cucumovirus (CMV), tomato spotted wilt tospovirus (TSWV), potato Y potyvirus (PVY), tomato yellow leaf curl geminivirus (TYLCV), and, occasionally, alfalfa mosaic alfamovirus (AMV) and tomato mosaic tobamovirus (ToMV) in tomato seedlings. This system was successfully applied to sanitary certification purposes. Tomato samples were collected using a systematic sampling method. The optimal ratio of tissue sample to extraction solution was 1.2 g to 6 ml. Hybridization reactions were done using a riboprobe mixture. This procedure saved time and cut costs without reducing sensitivity. TSWV was detected up to a ratio of 21 µg of infected tissue per spot; whereas the other viruses were detectable at a ratio of 17 µg of infected tissue per spot. The method allowed the analysis of 400 to 500 samples (representative of approximately 1.15 million tomato seedlings) per day and fulfilled the requirements for virus detection in routine diagnosis.

Additional keyword: viral infection

In the last few years, tomato (*Lycopersicon esculentum* Mill.) crops of southern Italy have been frequently infected with viruses causing heavy losses and a dramatic reduction of cropping areas (5). Serious outbreaks of cucumber mosaic cucumovirus (CMV), tomato spotted wilt tospovirus (TSWV), potato Y potyvirus (PVY), tomato yellow leaf curl geminivirus (TYLCV), and, occasionally, alfalfa mosaic alfamovirus (AMV) and tomato mosaic tobamovirus (ToMV) were recorded (4,6; D. Gallitelli, *unpublished*). These viruses occurred in single or, more frequently, multiple infections (3,4; D. Gallitelli, *unpublished*).

One potential source of primary inoculum could be infected tomato seedlings available on the market. To address this concern, a voluntary phytosanitary certification program was established in Apulia (southern Italy) in 1994. To assess the phytosanitary status of seedling lots in the program, a simple and sensitive procedure was developed, which consists of a dot-blot hybridization assay of tissue extracts with a mixture of six chemiluminescent riboprobes transcribed from cDNA clones of the above viruses.

This paper describes the experimental protocol that was developed to test 14 million and 31 million tomato seedlings in 1994 and 1995, respectively.

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MATERIALS AND METHODS

Virus sources. CMV, PVY, AMV, ToMV, and TSWV were collected from Apulian tomato plants, and a Sicilian isolate of TYLCV was provided by M. Davino (Istituto di Patologia Vegetale, Catania, Italy). All viruses were maintained and propagated in tomato seedlings cv. Rutgers grown in a temperature-controlled glasshouse at 22 to 24°C and were used as positive controls. Healthy seedlings served as negative controls.

Field sampling and sample preparation. Tomato samples assayed in the voluntary certification program were collected from four- to six-true-leaf-stage seedlings in nursery lots. The lot of tomato seedlings was the actual group of seedlings of the same age growing in the same greenhouse. On average, each lot consisted of 100,000 seedlings. For systematic and reproducible sampling, 24 subsamples of leaf tissue were collected along a W-shaped sampling pattern (1) for each 2 m^2 of polystyrene tray in which seedlings (2,300 plants) were grown. The 24 subsamples constituted a sample unit. The formula of Clayton and Slack (2) was used to calculate the probability of erroneous acceptance (PEA) of infected seedling lots.

Sample units (1.2 g) were collected with a simple homemade instrument (Fig. 1) that composited directly into a plastic bag (Fig. 2). Samples were then placed in a portable icebox. Tissues were ground in the plastic bag with a roll press in the presence of 3 to 6 vol of 50 mM NaOH and 2.5 mM EDTA, pH 8.0. The extract was incubated at room temperature for 5 min, then 5 µl was spotted onto a nylon membrane (Hybond N^+; Amersham, UK). For routine assays, up to 200 sample units were spotted on a 20 × 10 cm membrane.

Tissue and buffer optimization. The optimal ratio between tissue weight and volume of extraction solution was also assessed. The weight of 100 sample units was measured, and the average was determined to be 1.2 g. This was selected as the weight of the experimental sample unit used for standardizing the method, i.e., 50 mg of artificially infected tomato tissue was mixed with 1.15 g of healthy tissue, a ratio that occurs when only 1 plant out of 24 is infected.

cDNA clones and labeled RNA probes generation. Six recombinant plasmids were used for digoxigenin-labeled RNA (DIG-cRNA) probe synthesis. The plasmids PL-1037, PL-1055, and PL-1063 were supplied by DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen, GmbH, Braunschweig, Germany). PL-1055 and PL-1063 contained specific PVY and TSWV sequences, respectively, cloned in the transcription vector pT7/T3. The plasmid PL-1037 contained a cDNA sequence to TMV RNA cloned in pUC9. After digestion with *Eco*RI and *Hin*dIII, the resulting fragment (1 kb) was cloned in the *Sma*I site of pGEM-4Z vector.

The plasmid containing the complete cDNA sequence of AMV-S RNA3 cloned between the *Eco*RI/*Sal*I sites of pGEM1 vector was provided by L. Pinck (IBMP, Strasbourg, France). To remove the 3′ poly (A) tail, a 200-bp fragment was excised after digestion with *Dra*III and *Pst*I, and the resulting linearized plasmid (pAlMV) was incubated with T4 DNA polymerase, religated using T4 DNA ligase, and used to transform *Escherichia coli* strain DH5α competent cells (9). The plasmid pTom4 (from G. P. Accotto, Istituto di Fitovirologia Applicata del CNR, Torino, Italy) contained a 2,770-bp DNA sequence corre-

sponding to the complete genome of a Sardinian isolate of TYLCV, cloned in pUC118. The viral insert was recloned in the *Sma*I site of the vector pGEM-4Z and was labeled pGETY.

The plasmid pCMVS3 (supplied by F. Cellini, Metapontum Agrobios, Metaponto, Italy) contained a cDNA sequence corresponding to 1.6 kb of RNA 3 of CMV-S, cloned in the *Kpn*I site of the pGEM-3Zf+ vector.

One microgram of the above plasmids was linearized by digestion with the appropriate restriction enzymes and in vitro–transcribed using T3, T7, or SP6 RNA polymerases to give minus-sense riboprobes. Generation of DIG-RNA probes was performed using a DIG RNA Labeling kit (Boehringer Mannheim, GmbH), following the manufacturer's instructions. The yield of each transcription was evaluated by agarose gel electrophoresis and compared to the controls of the kit. Generation of radioactive riboprobes labeled with ^{32}P-UTP (800 Ci/mM, Amersham International, UK) was performed with the SP6/T7 Transcription kit (Boehringer Mannheim, GmbH), following the manufacturer's instructions.

Nonradioactive dot-blot hybridization assay. Spotted nylon membranes were prehybridized for 1 h at 55°C in a hybridization mix (150 µl/cm^2) containing 5× SSC (20× SSC = 3 M sodium chloride and 300 mM sodium citrate), 50% deionized formamide, 0.02% sodium dodecyl sulfate (SDS), 0.1% sodium lauryl sarcosine, and 2% blocking reagent (Boehringer Mannheim, GmbH) and hybridized overnight with a DIG-labeled mixture of the six riboprobes containing approximately 100 ng of each probe per ml.

Filters were washed three times for 30 min at 65°C in 0.1× SSC and 0.01% SDS, then incubated for 30 min at room temperature in 2× SSC containing 1 µg of RNase A per ml (8) to remove aspecifically bound riboprobe, washed again in 2× SSC for 5 min, and used directly for chemiluminescent detection with the DIG luminescent detection kit. Some modifications were introduced in the manufacturer's protocol: (i) the blocking step was prolonged up to 60 min; (ii) anti-DIG IgG alkaline phosphatase conjugate (anti-DIG-AP, Fab fragments) was diluted to 1:5,000 in the blocking solution, and membranes were incubated for 60 min; and (iii) blocking and antibody reaction steps were performed in plastic bags using 250 µl of solution per cm^2 of membrane. Damp membranes, sealed in transparent polyester sheets, were exposed to X-ray film for 1 to 2 h or sometimes overnight.

Hybridization assays with radioactively labeled riboprobes were made as described by Crescenzi et al. (3). Samples were spotted onto Hybond N$^+$ membrane using 1× BLOTTO as a blocking agent (9).

RESULTS

PEA. About 43 sample units representing at least 1,043 plants were collected from each lot of seedlings. This figure gives PEA values of 35 and 0.03%, assuming 0.1 and 1% frequency of infection, respectively.

Optimization of hybridization tests. Using homologous probes in separate reactions, all six viruses were easily detected up to a volume ratio of 1.2 g of tissue per 15 ml of extraction solution, corresponding to 400 µg of plant tissue per spot. However, some viruses (PVY, ToMV, TSWV, and TYLCV) could be detected at a ratio of 1.2 g per 24 ml (i.e., 250 ng of plant tissue per spot). Since only one out of 24 plants was infected, the limit of sensitivity using 15 ml of extraction solution was 17 µg of infected tissue per spot. To apply more plant tissue per spot without affecting sensitivity caused by the interference of plant constituents with riboprobes, the ratio of 1.2 g of plant tissue per 6 ml of extraction solution was applied routinely.

Standard transcription assays showed a similar efficiency of the six templates. The reaction yielded more than 10 µg of DIG-RNA from 1 µg of linearized plasmid. This allowed hybridization of about three nylon membranes, each containing 200 samples.

The chemiluminescent detection assay described in the manufacturer's protocol was clearly improved. The prolonged blocking step and the RNase treatment of the hybridized membrane significantly reduced the background signal. This allowed a prolonged exposure time (from 5 h to overnight), although 90% of the positive signals were already visible after 1 h of exposure. Moreover, the detection sensitivity was increased by use of a higher concentration of anti-DIG-AP Fab fragments without any appreciable increase of background. None of the riboprobes hybridized with healthy tomato tissue (Fig. 3A, lane H).

Simultaneous use of six riboprobes. All the probes gave clear-cut positive signals at a tissue–volume ratio of 1.2 g per 6 to 9 ml (Fig. 3B). The limit of detection was represented by TSWV, with a signal clearly visible up to a ratio of 1.2 g per 12 ml (i.e., 21 µg of infected tissue per spot), while the other five viruses were still detectable at a ratio of 1.2 g per 15 ml (i.e., 17 µg of infected tissue per spot). Heterologous hybridizations were not observed (not shown).

Comparable results were obtained with radiolabeled riboprobes (Fig. 4A and B), although the interference that reduced the limit of TSWV detection in the simultaneous hybridization with DIG-labeled riboprobes was not observed (Fig. 4B).

During the phytosanitary certification campaign, four operators were able to collect and analyze 400 to 500 sample units

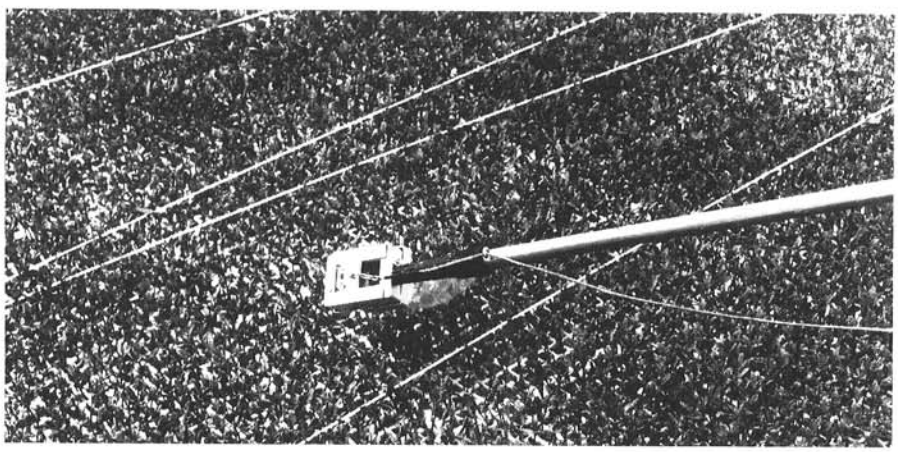

Fig. 1. Systematic sampling along arms of a W-shaped frame layered on tomato seedlings. Sampling sites were marked with red tape. Leaf tissues were cut with a coil spring–driven razor blade mounted on an aluminum box connected with a telescopic tube.

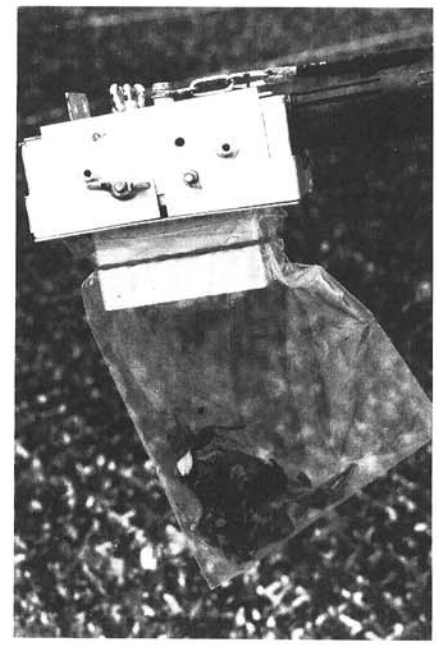

Fig. 2. Tomato leaf subsamples collected directly into a plastic bag ready for further processing.

per day and to give the results to the Regional Extension Service for Plant Protection (RESPP) and to nurserymen within 3 days from sample collection. During 2 years of testing, about 20,000 sample units, representative of approximately 45 million seedlings, were analyzed and only eight were found to be infected by ToMV. The overall cost per sample unit was approximately 2.3 US$, i.e., approximately 0.1 US cent per plant certified.

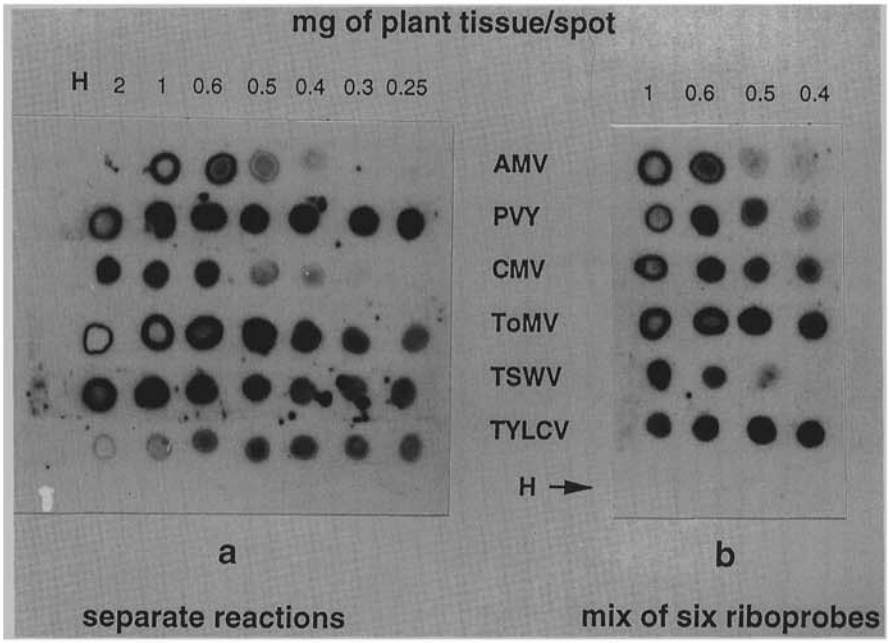

Fig. 3. (A) Chemiluminescent detection of six dot-blot hybridization assays of alfalfa mosaic alfamovirus (AMV), potato Y potyvirus (PVY), cucumber mosaic cucumovirus (CMV), tomato mosaic tobamovirus (ToMV), tomato spotted wilt tospovirus (TSWV), and tomato yellow leaf curl geminivirus (TYLCV) infected tomato plants. Healthy sap control is present on each row (lane H). Each blot was hybridized with the homologous DIG-labeled riboprobe. **(B)** Chemiluminescent detection of a dot-blot hybridization assay of AMV, PVY, CMV, ToMV, TSWV, and TYLCV infected tomato plants and healthy sap control (H). The blot was hybridized with a mixture of the six DIG-labeled riboprobes.

DISCUSSION

In surveying for tomato viruses to assess the sanitary status of seedling lots, we faced three problems: (i) the choice of a suitable sampling pattern, (ii) the number of samples to collect, and (iii) how to save time and lower costs.

To limit errors due to biases introduced by the operator, we opted for a systematic sampling design. In principle, unrestricted random sampling, where each individual plant has the same chance of being chosen, should be preferred, but as pointed out by Barnett (1), systematic sampling also has a form of randomness because the first sample is selected in a random manner and only the following samples are taken at a fixed distance. Barnett (1) proposed sampling patterns that would be appropriate for different survey situations. The W-shaped pattern would be recommended for large fields because most of the field would be covered and because, unlike other patterns, it does not amplify edge effects. Such effects could be relevant in greenhouses where seedlings can be subjected to different environmental conditions (light intensity, air flow, humidity) and degrees of protection from incoming viruses according to the position (central, side, along passages) they occupy.

The question of sample size is a general problem focusing on the probability of

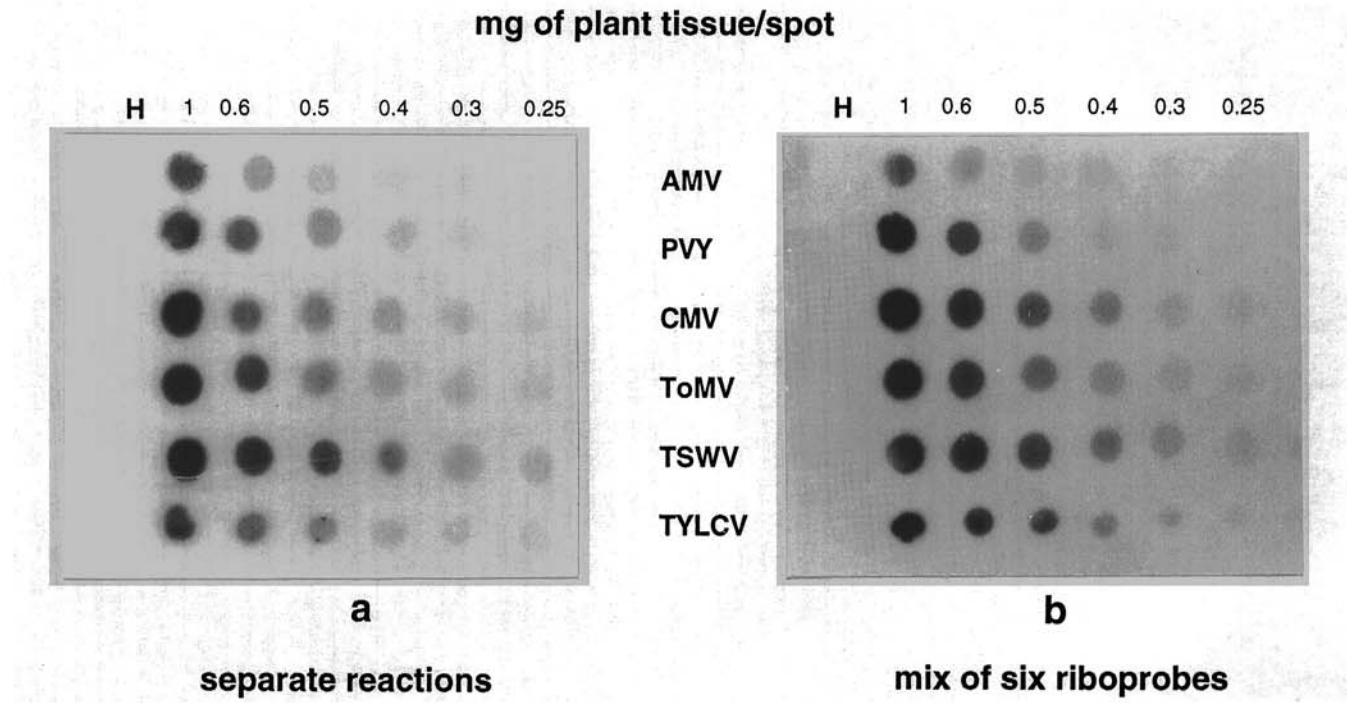

Fig. 4. (A) Radioactive detection of six dot-blot hybridization assays of alfalfa mosaic alfamovirus (AMV), potato Y potyvirus (PVY), cucumber mosaic cucumovirus (CMV), tomato mosaic tobamovirus (ToMV), tomato spotted wilt tospovirus (TSWV), and tomato yellow leaf curl geminivirus (TYLCV) infected tomato plants. Healthy sap control is present on each row (lane H). Each blot was hybridized with the homologous ^{32}P-labeled riboprobe. **(B)** Radioactive detection of a dot-blot hybridization assay of AMV, PVY, CMV, ToMV, TSWV, and TYLCV infected tomato plants and healthy sap control (H). The blot was hybridized with a mixture of the six ^{32}P-labeled riboprobes.

erroneously accepting (PEA) seedling lots where a virus is present. However, sampling should adequately characterize a population at reasonable cost and labor. Clayton and Slack (2) proposed a formula to estimate PEA that can be used in this circumstance for hypothesizing the frequency of infection. With 0.1% frequency of infection, our sample size gave a PEA of 35%, which can be considered too high. However, this and other PEA values that could be calculated assuming different frequencies of infection may be not realistic because, although in principle each sample unit should represent 24 single plants, very likely it represented more than that. Seedlings grow very close to each other, and the tool we used cannot collect tissue from single plants. Therefore, the PEA values could be much lower, although this cannot be estimated exactly. Additionally, sampling was repeated each 2 m^2 in the same greenhouse, which, on the whole, represents a quite uniform sampling area. These considerations should alleviate any concern about the validity of the sample size we used.

Separately analyzing thousands of samples representing millions of seedlings requires much time and labor and is costly. In this particular instance, six different hybridization reactions had to be carried out with six replica filters prepared for each sample. However, since the standards fixed from Apulian RESPP for rejection of lots of tomato seedlings were 10% of the sample units tested regardless of the virus detected, there was no need to identify which of the six viruses was present. Therefore, to save time and reduce costs and labor, we tried the simultaneous use of the six riboprobes in the hybridization reaction. With the exception of TSWV, the six probes did not interfere with one another and did not cross-hybridize with heterologous target nucleic acid or give nonspecific reactions with healthy sap. We have shown that the assessment of the optimal ratio of tissue weight to extraction solution volume ensured a limited interference of plant sap components, which is critical for chemiluminescent detection assays. Compared to the radioactively labeled riboprobes and to the classical dot-blot analysis (7), the multiple detection method described here was not more time-consuming or elaborate, retained the same characteristics of sensitivity and reliability, and did not need special equipment. Finally, the possibility of detecting up to six viruses in a one-step assay allowed us to carry out the complete protocol in 3 days, from sample collection to producing results.

Phytosanitary certification requires the timely and quick processing of a large number of samples, which, in turn, requires the use of a rapid and sensitive detection method. We think that the multiple chemiluminescent dot-blot procedure devised has fulfilled these requirements. In addition, this method is simple and can readily be used in laboratories not equipped for handling radioactive material.

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