European Journal of Plant Pathology **110:** 275–283, 2004.

Development of a non-radioactive dot-blot hybridisation assay for the detection of Pelargonium flower break virus and Pelargonium line pattern virus

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Accepted 7 November 2003

Key words: chemiluminescent detection, PFBV, PLPV, RNA probes, viral diagnosis

Abstract

The ornamental geranium, *Pelargonium* × *hortorum* Bailey, is a traditional ornamental plant widely cultivated in Europe and Northern America. Vegetative propagation facilitates rapid spread of viral infections which have detrimental effects on the production and the quality of the crop. A non-radioactive nucleic acid hybridisation method was developed for detection of Pelargonium flower break virus (PFBV) and Pelargonium line pattern virus (PLPV) in infected host plants. This method was significantly more sensitive than the conventional ELISA test when using either purified viral preparations or crude plant extracts. The distribution of the viruses was studied by means of the non-isotopic hybridisation technique. The results indicated that the petioles and the apical blade regions of fully expanded leaves were the best source of test material. The hybridisation procedure enables the detection of PFBV and PLPV in a single assay, and its simplicity allows its application to routine large-scale indexing.

Introduction

The geranium (*Pelargonium* spp.) is one of the most popular bedding plants for both indoor and outdoor use. However, its production is often affected by a number of factors, in particular, disease provoked by viral pathogens. These infectious agents usually do not kill the plants but reduce growth and quality by inducing inflorescence distortions, white flower streaking, chlorotic/necrotic spotting of leaves and stunting (Stone, 1980; Welvaert and Samyn, 1985). In the last few years, a progressive increase in geranium viral infections has been observed in western Europe, which may have been facilitated by vegetative propagation and the frequent absence of obvious symptoms in the affected plants. This situation emphasises the need to produce and maintain mother-stocks free of viral pathogens. The availability of reliable and sensitive methods for virus detection is essential for effective control, which is not easily achieved in the case of geranium since many failures in the detection procedures have been encountered with this crop (Welvaert, 1974; Peña Iglesias et al., 1974; Abo El-Nil et al., 1976; Stone, 1980; Albouy and Poutier, 1980; Stone et al., 1981; Paludan and Begtrup, 1987).

Although at least 18 viruses have been isolated from naturally-infected Pelargonium, the most frequent viral infections in geranium are caused by Pelargonium flower break virus (PFBV) and Pelargonium line pattern virus (PLPV) (Bouwen and Maat, 1992; Krczal et al., 1995; Frank and Loebenstein, 1994; Blystad et al., 1995; Borja et al., unpublished data). PFBV is a member of the genus *Carmovirus* within the family *Tombusviridae*, whereas the taxonomic position of PLPV is unclear (Lommel et al., 2000). Both viruses have 30 nm isometric virions, contain a monopartite genome of positive sense RNA of ~4 kb, and produce 3′ coterminal subgenomic RNAs to express internal open reading frames (Morris and Carrington, 1988; Lommel et al., 2000).

Various methods have been reported for detection of PFBV and/or PLPV, including bioassays on indicator plants, electron microscopy, serological techniques and RT–PCR (Stone, 1980; Bouwen and Maat, 1992; Blystad et al., 1995; Frank et al., 1997). PFBV and PLPV are usually detected by ELISA in propagation nurseries but the results are often erratic because of the uneven distribution of the viruses in the plant, the low viral titers and/or the presence of inhibitory compounds in geranium leaves (Stone, 1980; Stone et al., 1981; Bouwen and Maat, 1992). Nucleic acid hybridisation, a procedure which has been applied successfully to different virus–host combinations, might be a good alternative to ELISA (Maule et al., 1983; Varveri et al., 1988; Roy et al., 1988). In fact, detection of PFBV in geranium by molecular hybridisation with ^{32}P-labelled probes has been reported (Frank et al., 1996). However, the use of radioactive isotopes is inappropriate for routine diagnosis, a problem which may be circumvented by using non-radioactive precursors to synthesise the probes (Más et al., 1993; Harper and Creamer, 1995; Wesley et al., 1996; Saldarelli et al., 1996; Hu and Wong, 1998; Sánchez-Navarro et al., 1996; 1999).

In this work a non-isotopic molecular hybridisation method has been developed for the detection of PFBV and PLPV in infected geranium plants. The sensitivity and the viability of the method have been compared to those of ELISA. Moreover, the distribution of both viruses in infected plants has been studied in order to select the most suitable tissue for the detection assays.

Materials and methods

Virus sources and viral purification

Geraniums (*Pelargonium* × *hortorum* Bailey) plants naturally infected with different isolates of PFBV and/or PLPV were collected and maintained by the breeding company Fundación Promiva (Madrid). Infected sap from some of these plants was prepared in 50 mM potassium phosphate, pH 7.0, containing 3% (w/v) polyethylene glycol 8000 (Bouwen and Maat, 1992) and used to transfer the viruses into *Chenopodium quinoa* and/or *Nicotiana clevelandii* plants by mechanical inoculation. Virions were purified from the experimental hosts (Díez et al., 1998) and concentration was calculated assuming an extinction coefficient of 5 at 260 nm (as for *Carnation mottle virus*; Díez et al., 1998).

PFBV and PLPV cDNA cloning and in vitro *synthesis of digoxigenin (DIG) labelled RNA probes*

To obtain a PFBV-specific cDNA clone, total RNA was extracted from PFBV infected *C. quinoa* leaves and used as template for RT–PCR reactions with the Titan One Tube RT–PCR system (Roche) and primers CH1 (5′-TTCCCGGGCGGGTTAAGGTCTCCATC-3′), complementary to the 3′-terminal sequence of PFBV with some non-viral nucleotides (underlined), and CH2 (5′-ATGGTGGTAATGGGGGTTCTTGG-GTTG-3′), homologous to positions 2418–2444 of PFBV (Berthóme et al., 1998; Rico and Hernández, 2003). The resulting 1500 nt cDNA was eluted from the gel by the freeze-squeeze technique (Tautz and Renz, 1983) and ligated to the pGEM-T easy vector (Promega). This construct was digested with *Eco*RI to excise the viral specific 1500 nt fragment which was subcloned into the *Eco*RI digested pBluescript II KS+ vector, to yield pBPFB-1. A partial PLPV cDNA clone (PL-1030) was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). The plasmid contained an uncharacterised 1800 nt PLPV cDNA inserted into the pUC9 vector. Partial sequencing of PL-1030 fragment using an ABI PRISM DNA sequencer 377 (Perkin–Elmer) revealed that it was derived from a 3′ proximal region of the PLPV genome comprising part of the polymerase gene and the complete movement protein and coat protein genes. This cDNA was excised from plasmid PL-1030 with *Eco*RI–*Bam*HI and ligated to the *Eco*RI–*Bam*HI digested pBluescript II KS+ vector to yield pBPLP-1.

One microgram of each of the plasmids, pBPFB-1 and pBPLP-1, was linearised with the appropriate restriction enzyme and purified by phenol–chloroform extraction and ethanol precipitation. The synthesis of DIG labelled RNA probes complementary to the viral RNAs was carried out by *in vitro* transcription according to manufacturer's instructions (Roche).

Dot-blot hybridisation

Different procedures of sample preparation were assayed to optimise PFBV and PLPV detection in infected geranium plants. Three different extraction buffers were compared to prepare the plant extracts: 50 mM sodium citrate, pH 8.5, 0.5 M potassium phosphate, pH 7.0, or 200 mM potassium phosphate, pH 7.0, containing 0.1% Triton X-100, 5 mM DTT

and 10 mM 2-β-mercaptoethanol. In addition, several ratios between the weight of fresh tissue and the volume of extraction buffer were tested. Tissue from either healthy or virus-infected plants was triturated in a mortar or in plastic bags with the corresponding extraction buffer and the homogenates were clarified by centrifugation at 6000 rpm for 5 min. In some cases, an aliquot of the supernatant was used for partial purification of total nucleic acids by double phenol–chloroform extraction and ethanol precipitation. The clarified extracts or the total nucleic acid preparations were spotted (4 µl) onto positively charged nylon membranes (Roche) and the nucleic acids were bound by UV irradiation with a UV Stratalinker apparatus (Stratagene). Occasionally, samples were denatured by incubation at 60 °C for 15 min after adding an equal volume of denaturation solution, composed by 10× SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7.0) and 10% formaldehyde. To determine the sensitivity of the assay, clarified plant extracts and preparations of purified PFBV and PLPV were serially fivefold diluted in extraction buffer and applied to the membranes. Membranes were prehybridised for 2 h at 68 °C in a hybridisation buffer containing 50% formamide, 5× SSC, 0.1% (w/v) *N*-lauroylsarcosine, 0.02% (w/v) SDS, and 2% (w/v) blocking reagent for nucleic acid hybridisation and detection (Roche). The probe was denatured by boiling 5 min and added to fresh hybridisation buffer at a concentration of 0.2 µg ml^{-1}. After overnight hybridisation at 68 °C, the membrane was washed twice (5 min each) at room temperature with 2× SSC containing 0.1% SDS and twice (15 min each) at 68 °C with 0.1× SSC and 0.1% SDS. Chemiluminescent detection of the DIG-RNA probe using CSPD as substrate for anti-DIG conjugated alkaline phosphatase was carried out as described by the reagents supplier (Roche).

ELISA assay

Double-antibody sandwich ELISA was performed using commercially available PFBV and PLPV IgG and their derived alkaline phosphatase-conjugates (BIOREBA) according to the supplied instructions. Recordings were at 405 nm in a microtiter plate reader Biotek-Lx800 and samples were considered positive when the absorbance values were at least two times higher than those of the healthy controls.

Results

Detection of PFBV and PLPV by non-radioactive dot-blot hybridisation

The specificity of the synthesised DIG-RNA probes was examined by testing PFBV and PLPV-infected *C. quinoa* plants (Figure 1a). PFBV and PLPV DIG-RNA probes hybridised to PFBV and PLPV-infected leaf extracts, respectively. No hybridisation signals were observed in the healthy controls and the PFBV probe did not hybridise with PLPV-infected plant extracts or vice versa. To ascertain the level of sensitivity of the hybridisation method, unlabelled viral sense transcripts were produced by *in vitro* transcription and applied onto membranes in a dilution series. The detection limit of both the PFBV and the PLPV DIG-RNA probes, was about 0.03 pg of the complementary transcript (data not shown).

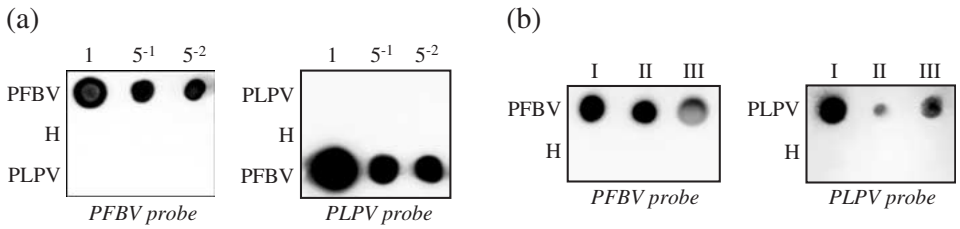

Figure 1. Chemiluminescent detection of PFBV and PLPV by non-radioactive dot-blot hybridisation. (a) Extracts were prepared by grinding healthy (H) and infected (PFBV or PLPV) *C. quinoa* leaves in four volumes of 50 mM sodium citrate, pH 8.5. Undiluted and fivefold dilutions (numbers on the top) of the extracts were applied onto nylon membranes and hybridised with the PFBV or the PLPV DIG-RNA probe as indicated. Films were developed after 15 min exposure. (b) Extracts were prepared by grinding healthy (H) and infected (PFBV or PLPV) geranium tissue in 20 volumes of: (I) 50 mM sodium citrate, pH 8.5, (II) 0.5 M potassium phosphate, pH 7.0 or (III) 200 mM potassium phosphate, pH 7.0, containing 0.1% Triton X-100, 5 mM DTT and 10 mM 2-β-mercaptoethanol. Undiluted samples were applied onto nylon membranes and hybridised with the PFBV or the PLPV DIG-RNA probe as indicated. Detection was carried out after 1 h exposure.

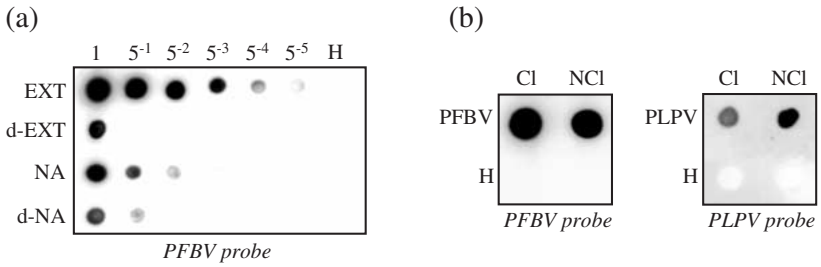

Figure 2. PFBV and PLPV detection in geranium samples subjected to different treatments. (a) Extracts (EXT) and total nucleic acids (NA) from PFBV-infected geranium leaves were applied onto a nylon membrane either directly or after formaldehyde denaturation (EXT-d and NA-d, respectively) together with fivefold dilutions (numbers on the top). Column H corresponds to undiluted healthy samples. Following hybridisation with the PFBV DIG-RNA probe, chemiluminescent detection was carried out after 1 h exposure. (b) Extracts from healthy (H) and infected (PFBV or PLPV) geranium were prepared by grinding tissue in 20 volumes of 50 mM sodium citrate, pH 8.5, and applied to nylon membranes either directly (NCl) or after 5 min centrifugation at 6000 rpm (Cl). Hybridisation was carried out with the PFBV or the PLPV DIG-RNA probe, as indicated. Films were exposed for 15 min.

When geranium tissue was used for viral detection, the best hybridisation signals were consistently observed with extracts obtained after homogenising the plant material in 20 volumes of 50 mM sodium citrate, pH 8.5 (Figure 1b), a buffer which had given good results in other virus–host combinations (Varveri et al., 1988; Más et al., 1993; Sánchez-Navarro et al., 1996; Hu and Wong, 1998). Neither partial purification of nucleic acids nor formaldehyde treatment of samples led to an improvement of the hybridisation signals (Figure 2a and data not shown). Indeed, denaturation caused a slight decrease in sensitivity (Figure 2a), a result which may be related to the type of membrane and/or to some steps required for non-radioactive detection since the same treatment had no effect in parallel radioactive hybridisations (data not shown). On the other hand, assays with clarified and non-clarified plant extracts indicated that centrifugation after grinding the tissue was not necessary since the non-clarified extracts gave signals similar, or even slightly stronger, than those obtained with clarified samples (Figure 2b) suggesting that an important amount of virus remained in the pellet after clarification as was confirmed by hybridisation analysis (data not shown). In all experiments, there was a total absence of non-specific reactions of the probes with healthy sap extracts (Figures 1 and 2).

Study of the distribution of PFBV and PLPV in geranium-infected plants

Since the distribution of a virus in an infected plant is often uneven, the choice of plant material to be sampled is of great importance for successful diagnosis (Hull, 2002). In order to select the most suitable tissue for PFBV and PLPV detection, the viral content of different parts of naturally-infected geranium plants was examined by dot-blot hybridisation (Figure 3). PFBV could be detected in all tissues tested although the highest titers were found in petioles and apical blade regions of fully expanded leaves as well as petioles of young leaves. In the case of PLPV, the results showed very irregular accumulation levels of this virus, in general, considerably lower than those of PFBV. The best signals were observed in extracts from petioles and apical blade regions of fully expanded leaves whereas signals in extracts from stems or from young leaves were very faint or negligible. A broader analysis showed detectable accumulation levels of the virus in petioles of young leaves (data not shown). Good hybridisation signals were usually obtained in extracts from petals (Figure 3) but the results with this plant material were more variable than those obtained using leaves (data not shown).

Comparison of the non-isotopic molecular hybridisation to the ELISA

Using non-radioactive hybridisation purified PFBV was detected down to a concentration of 0.1 pg μl^{-1}, whereas purified PLPV could be detected down to a concentration of 4 pg μl^{-1} (Figure 4). The above values correspond to a total of 0.4 pg of PFBV (in a 4 μl spot) and 16 pg of PLPV. In contrast, the detection limit of the ELISA was of 3 pg μl^{-1} (i.e., 300 pg in the 100 μl sample used per assay) and 100 pg μl^{-1} (10 ng per assay) for purified PFBV and PLPV, respectively. When virus-infected crude plant extracts were used, the

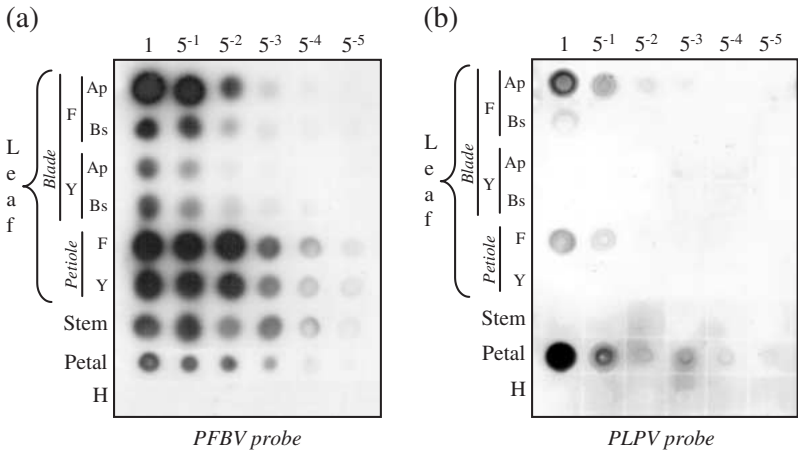

Figure 3. Analysis of the distribution of PFBV (a) and PLPV (b) in infected geranium plants by dot-blot hybridisation. Extracts were prepared from: apical (Ap) and basal regions (Bs) of blades and petioles of fully expanded leaves (F), apical and basal regions of blades and petioles of young leaves (Y), stems and petals. Undiluted extracts and fivefold dilutions (numbers on the top) were applied onto nylon membranes and hybridised with the PFBV and the PLPV DIG-RNA probes as indicated. Row H contains, consecutively, undiluted extracts from blades, petioles, stems and petals of healthy plants. Films were exposed for 15 min (a) and 1 h (b).

dilution endpoints for detection of PFBV and PLPV corresponded to 5^{-7} (equivalent to 2.5 ng of fresh tissue) and 5^{-6} (12.8 ng of fresh tissue), respectively, in the hybridisation approach, and to 5^{-4} (8 µg of fresh tissue) and 5^{-3} (40 µg of fresh tissue) in the ELISA test.

The hybridisation method was assayed for large-scale routine PFBV and PLPV detection in geranium plants and the results compared to those of the ELISA. A composite sample consisting of tissue from apical blade regions and petioles of two fully expanded leaves was prepared from each plant, and aliquots of it were used for viral detection by ELISA and non-isotopic dot-blot hybridisation. A total of 158 geranium cuttings collected from different areas of Spain were analysed. By ELISA, 13 and 21 samples tested positive for PFBV and PLPV, respectively. By dot-blot hybridisation, 17 and 80 samples were positive for PFBV and PLPV, respectively (Figure 5). All samples positive by ELISA gave positive results by molecular hybridisation.

Detection of PFBV and PLPV in a single hybridisation assay

In order to simplify the routine diagnosis of PFBV and PLPV in geranium, a mixture of the PFBV and PLPV DIG-RNA probes was used to assay the virus-infected material. Fivefold dilutions of extracts from plants infected either by PFBV or PLPV were applied to three membranes, two of which were hybridised with each specific DIG-RNA probe and the third with a mixture of them. In hybridisations with single probes, signals were observed clearly till dilution 5^{-4} in the PFBV-infected extracts, and till dilution 5^{-3} in the PLPV-infected ones. The mixture of probes detected the viruses in the same dilutions of the plant extracts than did single hybridisations showing that both approaches had identical sensitivity (Figure 6).

Extracts from the 158 geranium cuttings previously analysed by hybridisation with each specific probe were applied to nylon membranes and checked with a combination of the PFBV and PLPV DIG-RNA probes. Such combination detected exactly the same positive samples (Figure 5c) that were found in the individual hybridisations (Figure 5a,b) showing the potential of this procedure for large-scale indexing.

Discussion

Non-radioactive dot-blot hybridisation has been shown to be a reliable and sensitive method for detection of PFBV and PLPV in geranium plants. This method was able to detect purified PFBV and PLPV at concentrations 25 times lower than those required for detection by ELISA. Moreover, when sap extracts were used for comparison, the non-radioactive hybridisation was 125 times more sensitive for PFBV and PLPV detection than the ELISA assay. The higher increase of sensitivity observed with crude extracts may be due to the

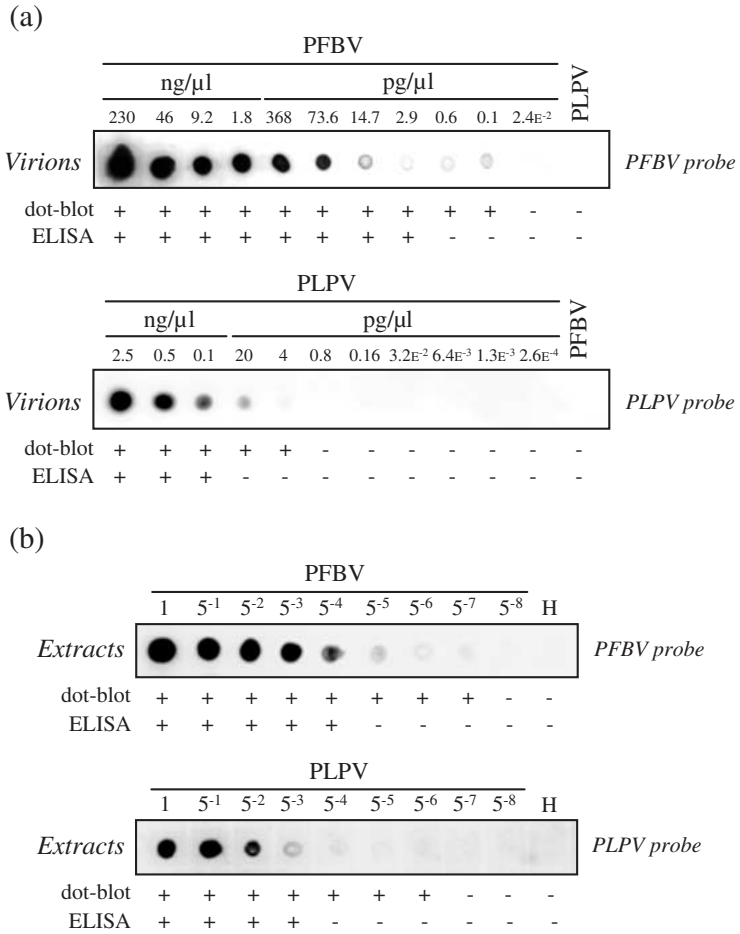

Figure 4. Non-radioactive dot-blot hybridisation of purified virions (a) and sap extracts (b). Numbers on the top indicate the concentration of the virus (PFBV or PLPV) preparations (a) or the dilutions performed from the original undiluted extracts (b). The last spots of the upper and lower membranes shown in (a) contain 10 ng of purified PLPV and PFBV, respectively. Sap extracts from healthy samples have been designated as H in (b). Hybridisations were carried out with the PFBV and the PLPV DIG-RNA probes as indicated at the right margin, and the films were developed after 1 h (a) or 15 min (b) exposure. The same samples were checked by ELISA in a parallel assay. Comparison of the results of the non-radioactive hybridisation with those of the ELISA is shown at the bottom of the panels where positive and negative samples have been designated as + and −, respectively.

presence of unencapsidated viral RNA in infected tissue which would not be detectable by the serological approach. In this context, it should be mentioned that we have used probes which can hybridise with all viral RNA species (genomic and subgenomic) in order to favour the detection process.

In spite of the fact that the minimal amount of complementary RNA detected by the PFBV-DIG RNA probe was identical to that required for the PLPV probe, as shown by the use of synthetic transcripts, the hybridisation method allowed to detect purified PFBV at a concentration 40 times lower (0.1 pg ml^{-1}) than that needed to detect PLPV (4 pg ml^{-1}) suggesting different accessibility of the two virus particles to the DIG-RNA probes. In any case, the sensitivity limits of the PFBV and PLPV DIG-RNA probes were similar (0.4–16 pg of purified virus or 0.03 pg of complementary viral RNA) to those reported for other viral non-radioactive probes (Roy et al., 1988; Fouly et al., 1992; Dietzgen et al., 1994; Sánchez-Navarro et al., 1996; 1999).

The application of the non-radioactive dot-blot hybridisation to the analysis of 158 geranium plants yielded 2% and 37% more positive samples

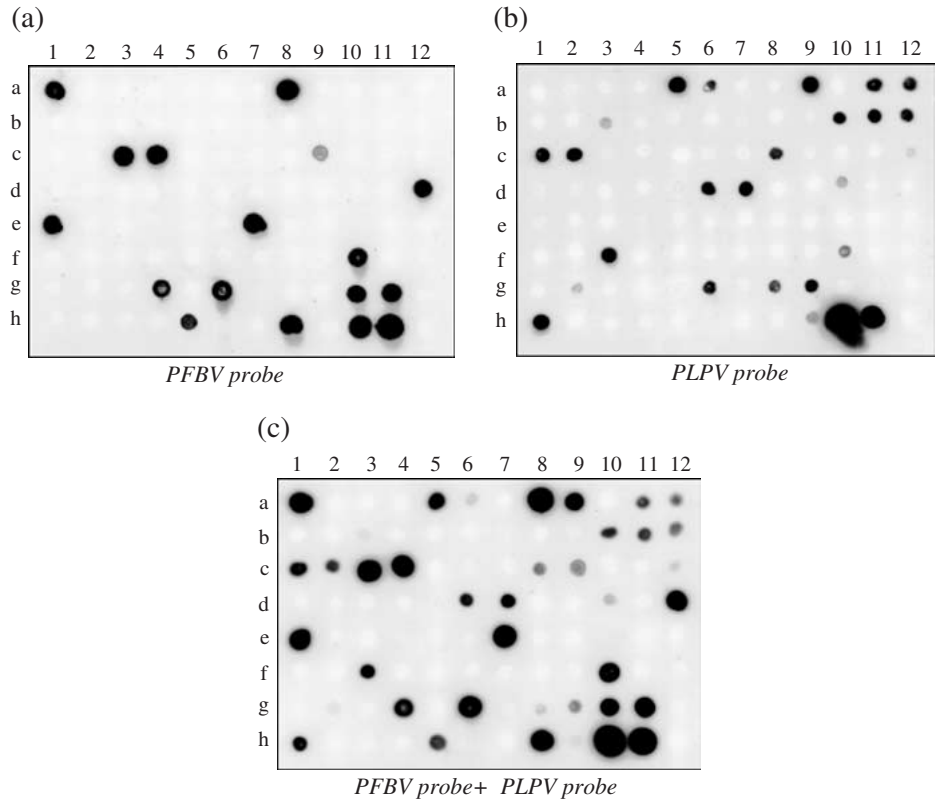

Figure 5. Application of the non-radioactive molecular hybridisation for routine large-scale analysis of geranium plants. Leaf extracts were applied onto nylon membranes and hybridised with the PFBV DIG-RNA probe (a), the PLPV DIG-RNA probe (b), or a combination of both (c). Chemiluminescent detection was carried out after 20 min exposure. In a parallel ELISA test, 11 samples (a1, a8, c3, d12, e1, e7, f10, g6, g10, g11 and h8) were positive for PFBV and only five (a11, a12, b12, c8 and g2) for PLPV. Positive controls correspond to samples h11 (for PFBV) and h10 (for PLPV). Sap extract from healthy plants was included in h12 as negative control. Negative ELISA values ranged from 0.014 to 0.177 whereas positive values ranged from 0.422 to 2.553.

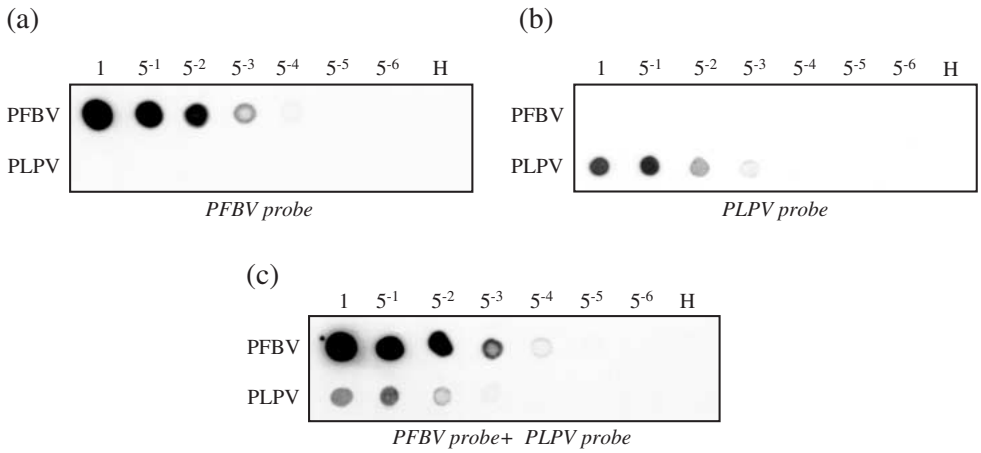

Figure 6. Non-radioactive dot-blot hybridisation of geranium samples. Undiluted and fivefold dilutions of extracts from healthy (H) and infected (PFBV or PLPV) geranium leaves were applied onto nylon membranes and hybridised with the PFBV DIG-RNA probe (a), the PLPV DIG-RNA probe (b), or a combination of both (c). Films were developed after 1 h exposure.

for PFBV and PLPV, respectively, than a parallel ELISA assay, confirming the higher sensitivity of the method described in this work. The improvement was especially remarkable in the case of PLPV most likely because its accumulation levels often do not reach the minimum required to be detected by ELISA. It is also worth highlighting that the time required to perform the hybridisation procedure in large-scale detection assays was similar or less than that needed to perform the ELISA test.

Seasonal fluctuations in the PLPV content in leaf blades and petioles have been reported, in certain months the viral titer was higher in the petioles than in the blades, and vice versa (Bouwen and Maat, 1992). Although we have not checked seasonal changes in the titer of PFBV and PLPV, results presented here indicate that the petioles and apical regions of blades of fully expanded leaves are the best source material since the highest levels of viral accumulation were consistently observed in these parts of the infected plants. To avoid false negatives, a composite sample of two leaves should be used discarding very old and young leaves. High concentrations of PLPV were detected in petals, however this is not a good choice for routine viral detection since geranium cuttings are not always in bloom and, moreover, we observed notable differences in PLPV titers between inflorescences.

Detection of several viruses in a single assay can dramatically reduce the diagnostic costs. In this study, we have shown that a mixture of specific probes can detect both PFBV and PLPV in geranium without loss of sensitivity with respect to individual hybridisations and without background increase. Similar approaches have been described for phytosanitary certification of tomato plants (Saldarelli et al., 1996), for large-scale indexing of carnation mother plants (Sánchez-Navarro et al., 1999) and for testing of three ilarviruses in stone fruit trees (Saade et al., 2000). In some of these practical examples as well as in the detection of PFBV with radioactive transcripts (Frank et al., 1996), the introduction of an RNase treatment was necessary to remove unspecific hybridisation of the probes, a step which could be omitted in our case because of the absence of non-specific background.

To summarise, the results presented here show that non-isotopic molecular hybridisation can be used for sensitive detection of PFBV and PLPV in geranium greatly improving the results obtained with immunological methods and allowing simple, fast and cost-effective testing of large number of samples.

Acknowledgements

We thank Pino Lago, Adolfo Fernández, Andrés Hernández, Yago Pérez, David del Pozo, José Antonio Segado and Cristina Sesma for technical assistance. This work was supported by grants AGL2000-0942 (to C.H.) and FIT-10000-13 (to M.B.) from the Ministerio de Ciencia y Tecnología de España, and by grant RF-2000-006 (to M.B.) from the Instituto Nacional de Investigaciones Agrarias.

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