Techniques

A Semi-Automated and Highly Sensitive Streptoavidin Magnetic Capture-Hybridization RT-PCR Assay: Application to Genus-Wide or Species-Specific Detection of Several Viruses of Ornamental Bulb Crops.

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ABSTRACT

A semi-automated, rapid and sensitive method that combines magnetic capture-hybridization and reverse-transcription polymerase chain reaction (MCH/RT-PCR) for the detection of plant viruses is described. The assay uses a target specific biotin-labelled oligoprobe for RNA capture and streptavidin-coated magnetic beads for subsequent RNA-oligoprobe hybrid isolation from plant lysate. Detection and specific identification was accomplished by RT-PCR. This approach was investigated for the specific detection of *Tobacco rattle virus* and for the detection of viruses within the potexvirus group in leaves, dormant bulbs and corms of flower bulbs of different species. Dilution series of TRV-infected tulip leaf sap showed that MCH/RT-PCR was 71,250 times more sensitive than enzyme-linked immunosorbent assay (ELISA) and was similar to that of RT-PCR. ELISA underestimated the infection levels of TRV in field samples compared to MCH/RT-PCR. The ability of MCH/RT-PCR to be performed in a microtiter plate on an automatic nucleic acid isolation station facilitates high throughput virus diagnostics. RNA isolation and purification was rapid, specific, sensitive, contamination free and reproducible making this method amenable for routine indexing of stock plants as part of a management plan to reduce the propagation of virus-infected plants.

Keywords: KingFisher automatic nucleic acid isolation station; *Potexvirus; Tobravirus*

1. Introduction

In plant pathogen diagnosis, polymerase chain reaction (PCR)-based techniques are widely used due to their sensitivity, specificity and the relatively short time it takes to complete the test. However, their application in routine and large scale application is somewhat limited (Eun and Wong, 1999; Henson and French, 1993; James at al., 2006). Several problems hamper the adoption of PCR in large scale testing. First of all, lack of automation of pre- and post-PCR processing steps to facilitate high-throughput sample analyses makes this technique less amenable for large scale testing (Eun and Wong, 1999; Garner, 1994; James at al., 2006; Pallas et al., 1998; Tullis, 1994). Secondly, PCR-based assays involve higher costs and the value of the testing material should be high enough to justify the additional cost. Finally, the isolation of nucleic acids from plant samples often requires laborious, time-consuming and expensive procedures, making them impractical for processing a large numbers of samples. Standard RNA isolation techniques (Chomczynski and Sacchi, 1987) are not suitable for routine application and require several handling steps. While silica gel-based nucleic acid isolation techniques (Boom et al., 1990), in the form of columns or free silica offer the convenience of fewer procedural steps, they are not practical in handling hundreds of samples. Introducing automation to process large number of samples that would provide templates for PCR would facilitate the use of PCR in routine and large scale testing of samples.

In this study, we evaluated a modified and semi-automated method of the magnetic capture-hybridization polymerase chain reaction (MCH-PCR) method (Chen et al., 1998; Doorn et al., 1994; Hsuih et al., 1996; Jacobsen, 1995; Little and Rezaian, 2006; Olsvik et al., 1991), previously described and referred to as MCH/RT-PCR (Miglino et al., 2006), for testing several RNA viruses of plants. The method uses streptavidin-coated magnetic beads and biotin-labelled

DNA oligonucleotide probes (oligoprobe), to simplify the target RNA isolation procedure and to facilitate the removal of potential PCR inhibitors. RNA isolation procedure was directly performed on plant lysate on a KingFisher automatic nucleic acid isolation station. This device is a small, affordable, bench-top workstation capable of processing up to 24 samples in about 17 minutes. This approach was tested for its applicability for both broad spectrum detection of members of the genus *Potexvirus* and for the specific detection of a virus species, *Tobacco rattle virus* (TRV) of genus *Tobravirus*. Additionally, we evaluated MCH/RT-PCR for its sensitivity and specificity in comparison with double-antibody sandwich (DAS) ELISA and to RT-PCR performed on total RNA extracts.

2. Material and methods

2.1. *Plant material and viruses*

Lily (*Lilium* hybrids), narcissus (*Narcissus* sp.) and tulip (*Tulipa* sp) plants infected with *Lily virus X* (LVX), *Narcissus mosaic virus* (NMV) and *Tulip virus X* (TVX) (Fauquet et al., 2005; Mayo and Horzinek, 1998), respectively, were obtained from the virus collection of the Dutch Flower Bulb Inspection Service (BKD, Lisse, The Netherlands).

Tulip plants doubly infected with TVX-TBV (*Tulip breaking virus*) or TVX-TRV, and lily plants doubly infected with LVX-CMV (*Cucumber mosaic virus*) or LVX-LSV (*Lily symptomless virus*) were obtained from the BKD virus collection and used as controls to evaluate the specificity of the MCH/RT-PCR technique. Tulip cultivar Blenda, infected with the TRV serotype TF, was provided by the Applied Plant Research Institute, (PPO, Lisse, The Netherlands).

Virus-free plants and corms of gladioli cultivars Cimarosa and Vedi Napoli were obtained from BKD experimental fields. Plants were grown on nematode-free soil and periodically tested by ELISA and RT-PCR to ensure freedom from viruses. These plants were used for calibrating ELISA data and as negative controls in both RT-PCR and MCH/RT-PCR assays.

Gladioli, tulip, crocus, narcissus, *Allium* and *Hyacinthus* cultivars used in this study were either sampled in commercial fields and propagation nurseries or grown on the BKD experimental fields during the years 2002-2005. Glycerol-suspended, homogenized leaf material of *Nicotiana benthamiana* and *N. hesperis* infected with *Potato virus X* (PVX) strain 9401794 and 20009019, Hosta sp. infected with *Hosta virus X* (HVX) (Park and Ryu, 2003), *N. glutinosa* infected with *Pepino mosaic virus* (PepMV) and *N. glutinosa* infected either with *Hydrangea ringspot virus* (HRSV), *Cymbidium mosaic virus* (CymMV) or *Cactus virus X* (CVX), and an unidentified potexvirus isolate no. 20006480 and isolate no. 20006631, were obtained from Ko Verhoeven, Dutch Plant Protection Service, Wageningen, The Netherlands.

Fresh plant material of *N. benthamiana* infected with PVX and PepMV, and *N. glutinosa* infected with CymMV, were obtained from Ko Verhoeven, Dutch Plant Protection Service, Wageningen, The Netherlands.

2.2. *Purification of TRV*

TRV isolate TF was purified according to the purification method of Lister and Bracker (1969) from infected tulip plants provided by the Applied Plant Research Institute, (PPO, Lisse, The Netherlands). After the last differential centrifugation step, the virus pellet was resuspended in 0.01 M PBS buffer, pH 7.0. Virus concentration, determined using a spectrophotometer (Biospec, Shimadzu Corporation, Kyoto, Japan), was estimated at 260 nm (A_{260}) assuming a specific absorbance of 3.0.

2.3. *Design of Biotin-labelled oligoprobe and primers*

Primers and oligoprobes were selected based on sequences available in EMBL and GenBank databases. Potexvirus universal primers, POTEX4-POTEX5, were designed from the conserved viral replicase encoding region, since this nucleotide sequence is highly conserved among the members of the genus *Potexvirus*. Potexvirus oligoprobe (BIOTIN-POTEX) has a 3´-terminal oligonucleotide sequence identical to the reverse primer POTEX4 which is complementary to the conserved viral replicase encoding region of the NMV RNA genome (GenBank accession no. **D13747**).

TRV-specific universal primers, TRV1 and TRV2, were designed from the 3´-untranslated region (3´-UTR) of the TRV RNA genome, since this nucleotide sequence is highly conserved among known TRV isolates (Visser et al., 1999). TRV oligoprobe (BIOTIN-TRV) has a 3´-terminal oligonucleotide sequence identical to the reverse primer TRV1 which is complementary to the 3´-untranslated region (3´-UTR) of the TRV RNA genome (GenBank accession no. **X 03686**). Both probes have a biotin moiety attached at the 5´-end capable of interacting with streptavidin-coated paramagnetic beads. CMV-specific primers, CMV1 and CMV2, were designed from the viral coat protein gene (GenBank accession no. AJ271416).

LSV-specific primers, LSV1 and LSV2, were designed from the viral coat protein gene (GenBank accession no. D43801).

Potyvirus generic primers, U335 and D335, as described by Langveld et al. 1991, were used.

The primers and oligoprobes sequences used for isolation, amplification and detection of potexviruses and TRV are listed in Table 1. Biotin-labelled oligoprobes and PCR primers were purchased from Eurogentec (Eurogentec EGT Group, Seraing, Belgium) and stored at –20 ºC.

2.4. *Total RNA extraction*

Total RNA was extracted from either 100 mg fresh plant tissue or 100 µl glycerol-suspended homogenized leaf material, by using the RNeasy Plant Kit (Qiagen GmbH, Germany). Plant samples were homogenized in500µl RLT Qiagen lysis buffer, containing guanidine isothiocyanate (GITC) as chaotropic salt, and total RNA was purified according to manufacturer's recommendations.

2.5. *RNA extraction by automated magnetic capture-hybridization*

An amount of either 100 mg fresh plant tissue or of 100 µl glycerol suspended homogenized leaf material was homogenized in presence of 500 µl of lysis buffer containing SDS (afterward indicated as Lysis buffer C), provided with the KingFisher mRNA purification kit, (Thermo Life Science, Hampshire, UK). A volume of 200 µl of supernatant was then transferred to a KingFisher microplate, and a volume corresponding to 200 µg of streptavidin-labeled magnetic beads (10 µg/µl dispersion) and 3 µl of 0.2 µmol/µl solution of oligoprobe were added according to the manufacturer's recommendations for the use of mRNA extraction kit. Microplates were placed on a KingFisher extraction processor and RNA purified according to the manufacturer's recommended protocol listed in the processor as mRNA2L file. The final step involved the release of hybrid capture probe-RNA from the streptavidin-coated beads in 30 µl of deionized sterile water. A volume of 1 µl of purified RNA was used in RT-PCR assay. For dormant gladioli corms, the above protocol was modified in order to reduce the inhibitory substances present in the corms homogenate which might affect the binding capacity of the magnetic beads (Stein and Loebenstein, 1988). Gladiolus (0.4 g) was sampled at the heel-end of corms with a razor blade. Each corm piece was ground in a mortar, adding 500 µl of sample extraction buffer (PBS buffer pH 7.4) containing 1% of cellulase (Onozuka R-10) and 1 % of SDS. Tuber extracts were transferred to 2.0 ml microfuge tubes (Eppendorf, UK) and incubated

for 10 min at 4 ºC. The tubes were centrifuged at 4000 x g for 30 sec, 30 µl of supernatant were transferred to a clean microtube and 160 µl of lysis buffer containing SDS, provided with the KingFisher mRNA purification kit, were added. The lysate samples were finally processed according to the above mentioned isolation procedure.

2.6. *Optimization of the MCH/RT-PCR assay*

In order to determine the optimum conditions for the MCH/RT-PCR analysis, the ability to form a RNA- oligoprobe hybrids in the presence of lysis buffers based on different types of chemistry were tested.

Lysis buffer A (Tris-buffered saline containing 2% lithium dodecylsulfate) and the washing buffers were obtained from the Roche mRNA Capture Kit (Roche Diagnostics GmbH, Germany). Lysis buffer B (Tris-buffered saline containing GITC) and the washing buffers were obtained from the Bilatec mRNA Streptavidin Capture Kit (Bilatec GmbH, Germany). Lysis buffer C (Tris-buffered saline containing SDS) and the washing buffers were obtained from the KingFisher mRNA Purification Kit (Thermo Life Science, Hampshire, UK). Healthy and TRV-infected tulip plants were homogenized in 7 volumes (w/v) of either buffer A, B, or C, and serially diluted (5-fold dilutions) in the same buffer. RNA from these samples was extracted using the KingFisher extraction processor by using 200 µg streptavidin magnetic beads and 0.6 µmol of BIOTIN-TRV oligoprobe and the protocol file mRNA2L, as described above. A volume of 1 µl purified RNA was used in RT-PCR assay performed by using the TRV1/TRV2 primer pair.

2.7. *RT-PCR assay*

RT-PCR amplification was carried out either on total RNA from infected plants or purified viral RNAs, by using the Reverse-iT^{TM} One-Step RT-PCR kit (ABgene House, UK). The RT-

PCR reaction mixture (10 µl) contained: 0.4 µl of 100 pmol/µl of either POTEX4/POTEX5 or TRV1/TRV2 primer pairs, 5 µl of a 2 x working concentration solution of RT-PCR Master Mix (enzymes and buffer to a final concentration of 1.5 mM $MgCl_2$, 0.2 mM each of dNTP, 1.25 units Thermoprime Plus DNA Polymerase and an optimised reaction buffer) and 1 µl of purified total RNA or viral RNA. The RT-PCR cycling profile, performed on a PTC 200 Thermal Cycler (MJ Research, Inc., USA), consisted of 45 min at 48 ºC (reverse transcription); 2 min at 94 ºC; followed by 45 cycles of amplification of 30 s at 94 ºC; 1 min at 60 ºC, and 1 min at 68 ºC. At the end of the program, the microtubes were held at 68 ºC for 10 min. The presence of a specific PCR product, 284 bp for the POTEX4/POTEX5, 250 bp for the TRV1/TRV2, 206 bp for the CMV1/CMV2, 296 bp for the LSV1/LSV2 and 335 bp for U335/D335 primer pairs, respectively, was verified by electrophoresis of 5 µl of each PCR reaction on a 1.2% agarose gel in TAE buffer containing 0.5 µg of Ethidium Bromide per ml. Bands were visualized by UV excitation and photographed using an AlphaImager digital photo documentation system (Alpha Innotech Corp., USA). RT-PCR procedure performed on total RNA will be referred to as the RT-PCR.

2.8. *ELISA*

Double-antibody sandwich ELISA (DAS-ELISA) performed as described by Clark and Adams (1977), was used for detecting TRV in different organs of several flower bulb plants. TRV gamma-globulins (IgG) and conjugates with alkaline phosphatase of antisera (coded TRV-TF, TRV-IG6, TRV-J, TRV-Y, TRV-PV, TRV-Sim, TRV-UM) prepared against different serotypes of TRV were purchased from the Applied Plant Research Institute, (PPO, Lisse, the Netherlands) and used according to recommendations. Briefly, polystyrene microtiter plates (type PS, Greiner Bio-one, NL) were coated for 20 h at 6 ºC with 150 µl per well of coating IgG

(1µg/ml) in 50 mM carbonate buffer, pH 9.6, with 0.02 % sodium azide. Seven different ELISA assays, one for each strain-specific antiserum were performed. Plates were washed twice with desalted tap water. Healthy and TRV-infected plant material was squeezed through a ribbed Pollähne roller press with the simultaneous addition of 5 volumes (w/v) of extraction buffer (0.137 M NaCl, 95 mM Na_2HPO_4, 13.2 mM KH_2PO_4, 0.1% Tween 20, 6.2 mM NaN_3, pH 7.4) and serially diluted (fivefold dilution) in the same buffer. Aliquots of 150 µl were added to each well and plates were incubated overnight at 6 ºC. Afterwards, plates were washed two times with softened tap water, incubated for 2 h at 37 ºC with 150 µl per well of alkaline phosphatase-conjugate TRV diluted 1:500 in conjugate buffer (0.137 M NaCl, 0.22 mM KH_2PO_4, 8.1 mM NaH_2PO_4, 3.0 mM NaN_3, 0.3% Tween 20, 0.5% non-fat-dried milk, pH 7.4), washed again and incubated for 2 h at 37 ºC with 150 µl of substrate (0.5 mg/ml of p-nitrophenyl phosphate in 10% dietanolammine, pH 9.8).Absorbance of samples at 405 nm (A_{405}) was read in an ELISA microplate reader (MKII, Titertek Multiskan Plus, ICN Biomedical, USA). A sample was considered positive, when its absorbance value at A_{405}, was higher than the mean plus three times the standard deviation (S.D.) of the healthy control ($\pm > 0.15$).

2.9. Comparison of sensitivity of ELISA and MCH/RT-PCR for TRV detection

Five-fold dilution of purified TRV-TF virus was used to determine the absolute sensitivity of DAS-ELISA and MCH/RT-PCR. A_{260}/A_{280} values of purified virus ranged from 1.00 to 1.15. An amount of 1mg/ml of purified virus was serial diluted in ELISA extraction buffer. From these dilution, aliquots of 150 µl for the ELISA assay and aliquots of 200 µl for RNA extraction and MCH/RT-PCR, were collected.

The relative sensitivity was determined by using TRV-infected tulip leaf material. Specifically, 100 mg of leaf material was homogenized in 700 µl of ELISA extraction buffer and

five-fold serial diluted in the same buffer. From these dilutions, aliquots of 150 µl for the ELISA assay and aliquots of 200 µl for RNA extraction and MCH/RT-PCR, were collected.

3. Results

3.1. *Optimization of the MCH/RT-PCR assay*

No differences in the detection limit were observed when the homogenization of plant tissue was made in lysis buffer containing SDS or lithium dodecilsulfate instead of GITC. In all cases, the detection limit was 1: 390625, dilution 5^{-8} (data not shown). Unless mentioned otherwise, further MCH/RT-PCR experiments were conducted with lysis buffer C, 200 µg magnetic beads and with 0.2 µmol of biotin oligoprobe on a KingFisher magnetic particle processor.

3.2. *Comparison of specificity of MCH/RT-PCR and RT-PCR for detection of potexviruses*

The ability to form a specific RNA- oligoprobe hybrid in the presence of lysis buffer C was analyzed by using the BIOTIN-POTEX capture probe and 13 different potexvirus- infected samples. Previous results (Miglino et al., 2006) showed that , for 12 of the 13 samples tested, MCH/RT-PCR gave a PCR amplicon corresponding to the expected size, 284 bp (Figs. 1A, and C). No PCR product could be detected for HVX. The results of MCH/RT-PCR were compared with the RT-PCR assay performed on total RNA extracted manually by the Qiagen system. The results indicated that the MCH/RT-PCR was as efficacious in detecting potexviruses as RT-PCR (Figs. 1B, and D). Samples 2, 3, 4, 5 and 6 (Fig 1D) were negative in RT-PCR. The discrepancy could be due to the glycerol present in the samples which might have interfered with the binding capacity of the silica gel column. This conclusion was based on the fact that the RT-PCR procedure was the same for both total RNA and viral RNA and that no discrepancy was observed

when PVX, CymMV and PepMV-infected fresh plant material was used in the experiments (Figs 1A and B).

3.3. *Specificity of MCH/RT-PCR*

In order to verify whether plant RNAs or non target viral RNAs could be non-specifically extracted together with the target viral RNA, several MCH/RT-PCR assays were performed using the BIOTIN-POTEX oligoprobe on tulip plants doubly infected with TVX-TBV (Fig. 2, lanes a1,a5, a9) or TVX-TRV (Fig. 2, lanes b2, b6, b10) and lily plants doubly infected with LVX-CMV (Fig. 2, lanes c3, c7, c11) or LVX-LSV (Fig. 2, lanes d4, d8, d12);. The extracted RNA was then subjected to a series of amplification reactions by using the following primer pairs: CMV1/CMV2, *Cucumber mosaic virus* specific primers (R. Miglino et al., unpublished results); LSV1/LSV2, *Lily symptomless virus* specific primers (R. Miglino et al., unpublished results); U335/D335, potyvirus group primers (Langveld et al., 1991); and POTEX4/POTEX5 and TRV1/TRV2 specific primer pairs (Table 1). Gel electrophoretic analysis of the amplification products after MCH/RT-PCR showed that when the potexvirus specific primers, POTEX4/POTEX5, were used, PCR amplicon of expected size (284 bp) was obtained for all the samples tested (Fig. 2, lanes a1, b2, c3, d4). No PCR amplicons could be detected when CMV1/CMV2, U335/D335, LSV1/LSV2 and TRV1/TRV2 primer pairs were used (Fig. 2, lanes a5, b6, c7, d8). RT-PCR assays, performed on total RNA, extracted by using Qiagen system from aliquots of the same samples yielded the expected PCR amplicon for CMV, LSV, TBV andTRV . This confirmed the multiple infections of the samples (Fig. 2, lanes a9, b10, c11, d12). The results showed that washing was extremely efficient and capable of eliminating all non-specific components present in the hybridization reaction. while only the specific hybridized RNA was efficiently captured. Uninfected material was negative in all the assays.

3.4. *Comparison of sensitivity of ELISA and MCH/RT-PCR for TRV detection*

Five-fold dilutions of purified TRV-TF showed that the absolute sensitivity limit was approximately 64 ng/ml for ELISA (Fig 3A) corresponding to a minimum amount of detected virus equivalent to 9.6 ng, and it was 4 pg/ml for MCH/RT-PCR corresponding to a minimum amount of detected virus equivalent to 40 fg (Fig. 4A). When using infected tulip leaves, the end point of dilution was 1:125 (dilution 5^{-3}), corresponding to an equivalent of 132 µg of fresh weight tissue (or 880 µg/ml) for ELISA (Fig. 3B). The same tissue material was used for direct comparison with MCH/RT-PCR method which was carried out with TRV1/TRV2 specific primers. The PCR product was electrophoresed in a 1.2% agarose gels and a single, specific product of 250 bp could be visualized in samples up to the 5^{-8} dilution, representing 2.4 ng of infected tulip tissue (concentration limit 250 ng/ml) (Fig 4B). When using non-infected material, no PCR amplicon was detected. No difference was observed, when the dilutions were made in healthy tulip plant extracts, instead of lysis buffer suggesting that there was no interference with the plant total RNA (data not shown).

3.5. *Detection of TRV in field-collected gladiolus samples*

To assess the robustness of the MCH/RT-PCR as a tool for routine screening of plant viruses, three sets of experiments were performed. In the first one, 175 gladioli plants cv. Peter Pears were collected from a commercial field. About 40% of them had symptoms suggestive of TRV infection. Fifty symptomatic and 125 asymptomatic plants were harvested with their intact corms and roots and their leaves were tested by ELISA, RT-PCR and MCH/RT-PCR assays. The results were verified in a grow-out test that assessed TRV infection by ELISA, MCH/RT-PCR and RT-PCR tests on leaves and corms from tested gladioli plants. After being dried and cleaned

from roots, the corms were put into storage at 5 ºC. Corms were finally planted on nematode-free soil in a non-climatized greenhouse. Leaf material was collected at different growing stages and analyzed by ELISA, RT-PCR and MCH/RT-PCR assays. At the end of the growing time, the plants were lifted, the corms were harvested and tested for TRV by ELISA, RT-PCR and MCH/RT-PCR assays. Gladioli plants were subjected to a visual inspection during the growing time. Results of the different methods are given in Table 2. ELISA test on leaves of gladioli plants underestimated the incidence of TRV compared to both MCH/RT-PCR and RT-PCR assays. The results after the grow-out test indicated that ELISA test was not efficacious in detecting TRV in leaf material when compared to both RT-PCR and MCH/RT-PCR tests and to a visual inspection. Furthermore, results showed that discrepancy was even higher, when gladioli corms were tested. ELISA test did not detect all infected corms, whereas results of tests on leaves and corms showed no discrepancy in case of RT-PCR and MCH/RT-PCR tests. In order to evaluate whether the lower efficiency observed in detecting TRV infection in gladioli plants by the ELISA assay was cultivar related, 15 symptomatic and asymptomatic plants of several gladioli cultivars were harvested with their intact corms from commercial fields. Gladioli cv Hunting Song, Vedi Napoli and Cimarosa were obtained from the virus collection of the BKD experimental fields. Plants were tested for TRV infection by ELISA, RT-PCR and MCH/RT-PCR assays. Results of the different methods are given in Table 3. ELISA test on leaves and corms of gladioli plants underestimated the incidence of TRV when compared to both MCH/RT-PCR and RT-PCR. Once again, the results of the tests on corms showed a high discrepancy between ELISA and MCH/RT-PCR assays. Uninfected plant material, cv. Vedi Napoli and Cimarosa, always scored as negative for TRV in all the assays.

3.6. *Detection of TRV in various bulb crops*

The potential of MCH/RT-PCR for TRV detection was evaluated by testing field-grown various bulb crops. Field samples of alliums, crocus, gladiolus and narcissus were collected in 2005. Ninety-two plants in total, collected from commercial fields and from the BKD virus collection experimental fields, were tested for TRV infection by MCH/RT-PCR and its performance was compared with ELISA and RT-PCR assays. Each time 6 plants were harvested with 5 of them displaying characteristic symptoms of TRV and 1 without symptoms as negative control. Results of the different testing methods are given in Table 4. The results showed that the ELISA assay underestimated the TRV infection in all the tested samples. All plants showing the TRV characteristic symptoms proved positive in a MCH/RT-PCR assay.

Healthy and TRV-infected tulips of cv. Flaming Parrot were harvested from the BKD greenhouse early in the growing season when the plants were about 15 cm tall. One of the selected groups of plants was over 90% infected and showed the characteristic symptoms of TRV, while the other group was free of symptoms of virus infection and was used as healthy control in this study. In this case ELISA was unable to detect the TRV infection (Table 4). When testing tulip plants cv. Blenda, infected with TRV serotype TF, ELISA could detect only 50% of the infected plants, while MCH/RT-PCR succeeded in detecting all of the infected tulip plants. A discrepancy between ELISA and MCH/RT-PCR was also observed, when testing different field-grown tulips cultivars, harvested upon symptoms observation (Table 4). In all of the above mentioned experiments no discrepancy was found between RT-PCR and MCH/RT-PCR results (data not shown).

4. Discussion

In this paper we have described a strategy that combines semi-automation, magnetic isolation of viral RNA-probe hybrids, target-specific hybridization of biotin DNA oligoprobes, and RT-PCR amplification of the captured RNA. Several applications of the magnetic bead-based isolation methods have been developed and implemented both in clinical and plant laboratories (Chen et al., 1998; Doorn et al., 1994; Hsuih et al., 1996; Jacobsen, 1995; Olsvik et al., 1991). Total DNA and RNA, mRNA, and PCR clean-up extraction procedures based on magnetic beads technology are widely used. It appears that no attempts have been made to adapt magnetic beads technology for the direct and specific isolation of viral RNA for use in diagnosis with the exception of one recent report of the detection of grapevine leafroll associated virus-1 (Little and Rezaian, 2006). However, in the approach reported by Little and Rezaian (2006), the magnetic hybridization step was performed by using a 80bp hybridization probe on a previously purified total RNA making it laborious and thus may not be suitable for automation and large scale testing of samples.

The MCH/RT-PCR-based one-step isolation of viral RNA with biotin-labelled oligoprobe and streptavidin paramagnetic beads described in this paper offer several advantages. Firstly, this technique simplifies nucleic acid purification since it can replace several centrifugation and precipitation steps with a single and rapid magnetic separation step eliminating complicated, laborious and expensive RNA extraction procedures. Furthermore this technique is amenable for automation allowing the processing of a large number of samples which will facilitate the use of PCR in routine testing. The use of the KingFisher automated nucleic acid isolation station allowed the processing of multiple samples simultaneously, thus reducing the variability linked to the handling of each sample.

The MCH/RT-PCR method was previously described for the detection of a novel potexvirus in Allium (Miglino et al., 2006) and successfully applied to a survey during April 2004 and April 2005 to identify viruses affecting *Crocus* spp. in the Netherlands (Miglino et al., 2005). An MCH/RT-PCR method was also designed in order to be able to specifically detect all TRV virus isolates. For viruses such as TRV, which consists of several isolates, the need for the use of different antisera presents a limitation for using ELISA (Harrison et al., 1983; Van der Vlugt et al., 1998) as well as immunocapture-reverse transcription-polymerase chain reaction methods (Nolasco et al., 1993; Rowhani et al., 1995; Schoen et al., 1996). TRV can be detected in the field by visual inspection since this virus produces characteristic symptoms (Asjes and Elbertsen, 1982). However, screening should be preferentially done with the bulbs in order to fit into a general quality inspection program. Previous experiments on the detection of TRV in gladioli corms and tulip bulbs during storage time revealed that both ELISA and cDNA hybridization methods provided erratic and unreliable results (Van der Vlugt et al., 1988). This may be partly due to technical problems such as the low concentration of the viruses, presence of inhibitory substances in the homogenate or the antigenic variability among tobraviruses (Harrison and Robinson, 1986; Visser et al., 1999). Moreover, RNA1, the largest of the two genomic RNAs of TRV, can establish an infection in plants in the absence of RNA 2. In such cases the CP gene was lost completely (Hernández et al., 1996;Visser et al., 1999). Such an infection cannot be detected with an antiserum against the virus, because the RNA-2-encoded coat protein is not produced. RNA1 infections and incomplete infections are known to occur in some potato stocks, NM-type isolates, (Harrison et al., 1983; Harrison and Robinson, 1986; Visser et al., 1999) but have not been observed in tulip or in gladioli yet.

When MCH/RT-PCR, performed using BIOTIN-TRV and TRV primers, was applied to large-scale testing of TRV in alliums, crocus, gladioli, narcissus and tulips from fields, 60 % more samples were found positive compared to ELISA (Table 4). In fact, even plants showing no clear TRV symptoms on their leaves and flowers were found positive by MCH/RT-PCR methods. Data obtained by using 175 TRV-infected and healthy gladioli plants cv. Peter Pears revealed that MCH/RT-PCR was able to detect the virus in 5% and 22% additional samples of leaves and corms respectively, compared to ELISA (Table 2). The grow-out test and the visual inspection confirmed that ELISA was unable to detect all TRV-infected plants. The highest discrepancy was found when testing gladioli corms which was in agreement with previous reports (Stein et al., 1988). In one other survey 7 gladioli cultivars were used to evaluate the robustness, sensitivity and specificity of the TRV detection by mean of MCH/RT-PCR method. Once again MCH/RT-PCR was able to detect more TRV infected leaf and corm material then ELISA (Table 3). Detection of TRV in dormant corms of several gladioli cultivar showed complete agreement in discriminating between positive and negative sample by the MCH/RT-PCR assay, RT-PCR and fields inspections. However, the testing time of gladioli corms was reduced from a minimum of 3 months for the current indexing methods to 1 day for the MCH/RT-PCR assay.

Secondly, MCH/RT-PCR can be performed using lysis buffer not containing harmful or hazardous chaotropic or organic salts. In fact, no difference in the detection limit was observed when using lysis buffers containing SDS or lithium dodecylsulfate instead of GITC. In all cases, the detection limit was equal to 250 ng/ml, corresponding to 2.4 ng of infected tissue.

Thirdly, the capture of RNA-probe hybrids on paramagnetic beads facilitated the removal of plant contaminants which might inhibit the RT-PCR reaction. The removal of un-hybridized

non-specific RNA and DNA present in the lysate significantly reduces the possibility of false-positives and allows the efficient concentration and purification of the target RNA from diluted mixtures. This is especially important for the detection of low amounts of target RNA in the presence of a large amount of host nucleic acids. Data obtained by using a purified virus preparation of TRV-TF showed that MCH/RT-PCR was sensitive enough to detect TRV at a concentration of 4 pg/ml (40 fg, detection limit), 15,625 times higher than DAS-ELISA (64 ng/ml, corresponding to 9.6 ng detection limit) and similar to RT-PCR (Figs. 3A and 4A). The limit of detection by ELISA was similar to the one described by Van der Vlugt et al., (1988) for the same TRV-TF, in which the observed limit of detection was equal to 14 ng for both ELISA and radioactive cDNA-hybridization techniques. The difference in the detection limit was even more remarkable when herbaceous hosts were tested. Data obtained by using TRV-infected tulip leaves revealed that MCH/RT-PCR technique was 71,250 times more sensitive than DAS-ELISA (Figs. 3B and 4B) and similar to the data obtained with the RT-PCR technique, which was more than expected. Furthermore, the possibility that non-specific hybridization could generate false-positive signal was not a concern, since our results showed that no PCR amplicon was generated when performing MCH/RT-PCR by using CMV, LSV, potyvirus, and TRV-specific primer pairs on RNA extracted by means of BIOTIN-POTEX probe and plant homogenates of tulip and lily leaf material infected with TVX-TBV, TVX-TRV, and LVX-CMV, LVX-LSV multiple infections, respectively (Fig. 2, lanes a5, b6, c7, d8). Only PCR amplicons of the expected size (284 bp) were detected when the POTEX4/POTEX5 primer pair was used in the amplification step on the extracted RNA (Fig. 2, lanes a1, b2, c3, d4).

In conclusion, the high sensitivity obtained by MCH/RT-PCR can overcome the problems related to localization, serotype variability, inhibitory substances, and incomplete or

low levels of infection as seen in case of TRV infection of selected bulb crops. The combined reliability, automation and the easiness of sample preparation make MCH/RT-PCR a good alternative to serological methods for the virus detection in certification and selection schemes. The MCH-RT-PCR offers the potential to develop a fully automated system requiring a minimum of laboratory manipulation. The combination of this method with SYBR Green- or Taqman-based real-time PCR can further decrease the number of manipulations and allow a fully automated virus indexing procedure.

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TABLE 1. Primers and biotin probes used for isolation and detection of Tobacco rattle virus and member of the genus *Potexvirus*

Primer/probe	Sequence	Length (nt)	Position (nt)	Type[a]
POTEX4	5′-agcatggcgccatcttgtgactg-3′	23	4252-4230	Reverse
POTEX 5	5′-ctgaagtcacaatgggtgaagaa-3′	23	3969-3991	Forward
BIOTIN-POTEX	5′-agcatggcgccatcttgtgactg-3	23	4252-4230	Probe[a]
TRV 1	5′-gggcgtaataacgcttacgtaggcgagg-3′	28	1905-1872	Reverse
TRV2	5′-attttaaattgttatctgtttctgtg-3′	26	1655-1680	Forward
BIOTIN- TRV	5′-gggcgtaataacgcttacgtaggcgagg-3′	28	1905-1872	Probe[a]
CMV 1	5′ -ccacccaacctttgtgggtagtg- 3′	23	161-184	Forward
CMV 2	5′ -cccacacggtagaatcaaatttcggc- 3′	26	367-342	Reverse
LSV 1	5′ -gcaactaccgagcagatggcta- 3′	22	325-346	Forward
LSV 2	5′ -gcttgccaatcagctggaggttg- 3′	23	596-620	Reverse

[a] The probe carries a biotin moiety attached at the 5′ terminal nucleotide.

TABLE 2. Detection of Tobacco rattle virus by magnetic capture-hybridization reverse-transcription polymerase chain reaction (MCH/RT-PCR) and enzyme-linked immunosorbent assay (ELISA) in leaves and corms of gladioli cv. Peter Pears and verification of infection by ELISA and MCH/RT-PCR on corms and leaves of the corresponding plants after the grow-out test.

Year	No. of plants tested	Positive by ELISA[a]		Positive by MCH/RT-PCR[b]		Positive by RT-PCR[b] on total RNA	
		Leaves	Corms	Leaves	Corms	Leaves	Corms
2004	175	40	n.t.[c]	50	n.t.[c]	50	n.t.[c]
2005 grow-out test	175	40	10	50	50	50	50
Total	175	40	10	50	50	50	50

[a] Samples were positive when A_{405} was higher than the mean plus three times the standard deviation (S.D.) of the healthy control. The following TRV antisera, TRV-TF,TRV-J, TRV-Y, TRV-Sim, TRV- PV, TRV- UM, TRV- IG6, were used in the ELISA assay.

[b] Samples in which a TRV-specific 250-bp amplicon band was obtained.

[c] Not tested.

TABLE 3. Detection of Tobacco rattle virus by magnetic capture-hybridization reverse-transcription polymerase chain reaction (MCH/RT-PCR) and enzyme-linked immunosorbent assay (ELISA) on leaves and corms of several gladioli cultivars.

Cultivar	No. of plants tested	Positive by ELISA[a]		Positive by MCH/RT-PCR[b]		Positive by RT-PCR[b] on Total RNA	
		Leaves	Corms	Leaves	Corms	Leaves	Corms
Cimarosa[c]	15	0	0	0	0	0	0
Hunting Song[d]	15	6	6	15	15	15	15
Nova Lux	15	3	0	9	9	9	9
Peter Pears	15	3	0	9	9	9	9
Plum tart	15	6	6	6	6	6	6
Trader Horn	15	3	3	9	9	9	9
Vedi Napoli[c]	15	0	0	0	0	0	0
Total	105	21	15	48	48	48	48

[a] Samples were positive when A_{405} was higher than the mean plus three times the standard deviation (S.D.) of the healthy control. The following TRV antisera, TRV-TF,TRV-J, TRV-Y, TRV-Sim, TRV- PV, TRV- UM, TRV- IG6, were used in the ELISA assay.

[b] Samples in which a TRV-specific 250-bp amplicon band was visualised by Ethidium Bromide after agarose gel electrophoresis.

[c] Healthy plants of gladioli cultivars.

[d] TRV-infected plant of gladioli as positive control.

TABLE 4. Detection of Tobacco rattle virus by magnetic capture-hybridization reverse-transcription polymerase chain reaction (MCH/RT-PCR) in field grown commercial samples and verification of infection by enzyme-linked immunosorbent assay (ELISA).

Plant	Cultivar	No. of plants tested	Positive by ELISA[a]	Positive by MCH/RT-PCR[b]
			Leaves	Leaves
Allium	Mars	6	4	5
Crocus	Ruby Giant	6	2	5
Gladiolus	Red Majesty	6	0	5
Narcissus	Altea	6	0	4
Tulip	Alpeldoorn	10	4	4
	Beauty Queen	4	0	0
	Blenda	8	4	8
	Bolroy Silver	4	0	4
	Flaming Parrot showing leaf necrosis symptoms	8	0	8
	Flaming Parrot showing no infection symptoms	4	0	0
	Lilac Lady	4	0	4
	Parade	6	0	6
	Seawerld	4	0	4
	Turkestanica	6	2	6
	Yellow Flight	10	0	4
	Total	92	16	67

[a] Samples were positive when $A_{405}$was higher than the mean plus three times the standard deviation (S.D.) of the healthy control. The following TRV antisera, TRV-TF,TRV-J, TRV-Y, TRV-Sim, TRV- PV, TRV- UM, TRV- IG6, were used in the ELISA assay.

[b] Samples in which a TRV-specific 250-bp amplicon band was obtained.

Figure Legends

Fig. 1. Gel electrophoretic analysis of the amplification product (280 bp) after magnetic capture-hybridization reverse-transcription (RT) polymerase chain reaction (MCH/RT-PCR) and total RNA RT-PCR of different potexviruses from infected fresh plant material (**A** and **B**) and glycerol suspended sap leaves material (**C** and **D**). The tested extraction conditions were: MCH/RT-PCR automatically performed in a microtiterplate on a KingFisher extraction processor (**A** and **C**); Total RNA manually extracted by RNAeasy plant kit (**B** and **D**). The amplification was performed on 1 µl of either viral RNA or total RNA. **A**, and **B,** Lane M, 100-bp DNA ladder (Eurogentec); lane 1, *Tulip virus X* (TVX); lane 2, *Lily virus X* (LVX); lane 3, *Narcissus mosaic virus* (NMV); lane 4, *Cymbidium mosaic virus* (CymMV); lane 5, *Potato virus X* (PVX); lane 6, *Pepino mosaic virus* (PepMV); lane H, healthy tulip control. **C** and **D**, Lane M, 100-bp DNA ladder; lane 1, TVX; lane 2, PVX strain 9401794; lane 3, PVX strain 20009019; lane 4, HVX; lane 5, *Pepino mosaic virus* (PepMV); lane 6, *Hydrangea ringspot virus* (HRSV); lane 7, potexvirus strain nr. 20006480; lane 8, potexvirus strain nr. 20006631; lane 9, PepMV; lane 10, CymMV; lane 11, *Cactus virus X* (CVX). (Figs 1A and 1C, Acta Hort. 722, ISHS 2006)

Fig. 2. Specificity of the BIOTIN-POTEX oligoprobe for the RT-PCR detection of members of the genus *Potexvirus.* Gel electrophoretic analysis of amplification products after magnetic capture-hybridization reverse-transcription polymerase chain reaction (MCH/RT-PCR), and reverse-transcription polymerase chain reaction (RT-PCR) of RNA extracted from tulip and lily virus infected plants.. The tested plant materials were: (**a**) TVX-TBV, (**b**) TVX-TRV, (**c**) tulip-infected plants with LVX-CMV, (d) LVX-LSV, lily-infected plants. The tested extraction

conditions were: (lanes 1 to 8**)** MCH/RT-PCR was automatically performed in a microtiterplate on a KingFisher extraction processor by using 0.2 µmol of BIOTIN-POTEX oligoprobe and 200 µg of streptavidin magnetic beads; (lanes 9 to 12) total RNA was manually extracted by mean of the Qiagen, RNeasy plant kit, silica gel columns. The primer pairs used in this assay were: POTEX4/POTEX5, potexvirus group primers (PCR amplicon 284 bp) ; U335/D335, potyvirus group primers (PCR amplicon 335 bp); TRV1/TRV2 *Tobacco rattle virus* specific primers (PCR amplicon 250 bp); CMV1/CMV2, *Cucumber mosaic virus* specific primers (PCR amplicon 206 bp); LSV1/LSV2, *Liy symptomless virus* specific primers (PCR amplico 296 bp). The amplification condition were: **a1**, TVX-TBV by using POTEX4/POTEX5; **b2**, TVX-TRV by using POTEX4/POTEX5; **c3**, LVX-CMV by using POTEX4/POTEX5; **d4**, LVX-LSV by using POTEX4/POTEX5; **a5**, TVX-TBV by using U335/D335; **b6**, TVX-TRV by using TRV1/TRV2; **c7**, LVX-CMV by using CMV1/CMV2; **d8**, LVX-LSV by using LSV1/LSV2; **a9**, TVX-TBV by using U335/D335; **b10**, TVX-TRV by using TRV1/TRV2; **c11**, LVX-CMV by using CMV1/CMV2; **d12**, LVX-LSV by using LSV1/LSV2. Lane **M**, 100-bp DNA ladder.

Fig. 3. Enzyme-linked immunosorbent assay (ELISA). Absorbance values obtained with purified Tobacco rattle virus (TRV) particles (**A**) and TRV-infected tulip material (**B**) using a fivefold dilution series in phosphate buffer. Each point is the mean of three replications. In **A**, the last dilution recorded as ELISA-positive corresponded to a virus concentration equal to 64 ng/ml. In **B**, the dilution endpoint corresponded to 1:125 (5^{-3}) for TRV-infected tulip plant tissue. Background values (buffer sample in **A** and uninfected tulip leaves in **B**) were 0.03 ± 0.005 and 0.03 ± 0.004.

Fig. 4. Agarose gel electrophoresis analysis of RT-PCR-amplified products (250 bp) after magnetic capture-hybridization reverse-transcription polymerase chain reaction (MCH/RT-PCR) of purified *Tobacco rattle virus* (TRV) particles (**A**) and TRV-infected material (**B**) using fivefold dilution series in lysis buffer C. The viral RNA was extracted by using the biotin labelled probe, BIOTIN-TRV, and streptavidin magnetic beads. In **A**, the last detected positive RT-PCR amplicon detected corresponded to a virus concentration equal to 4 pg/ml. In **B**, the endpoint dilution for which a clear positive RT-PCR amplicon was detected corresponded to 1:390625 (5^{-8}). Lanes M are DNA markers (100 bp DNA ladder, Eurogentec) ranging in size from 100-1000. Lanes H, in **A** and **B**, correspond to non-infected tulip material. Lane P, in **B**, correspond to TRV-infected tulip leaf material.

Figure 1 . R. Miglino. *Phytopathology*.

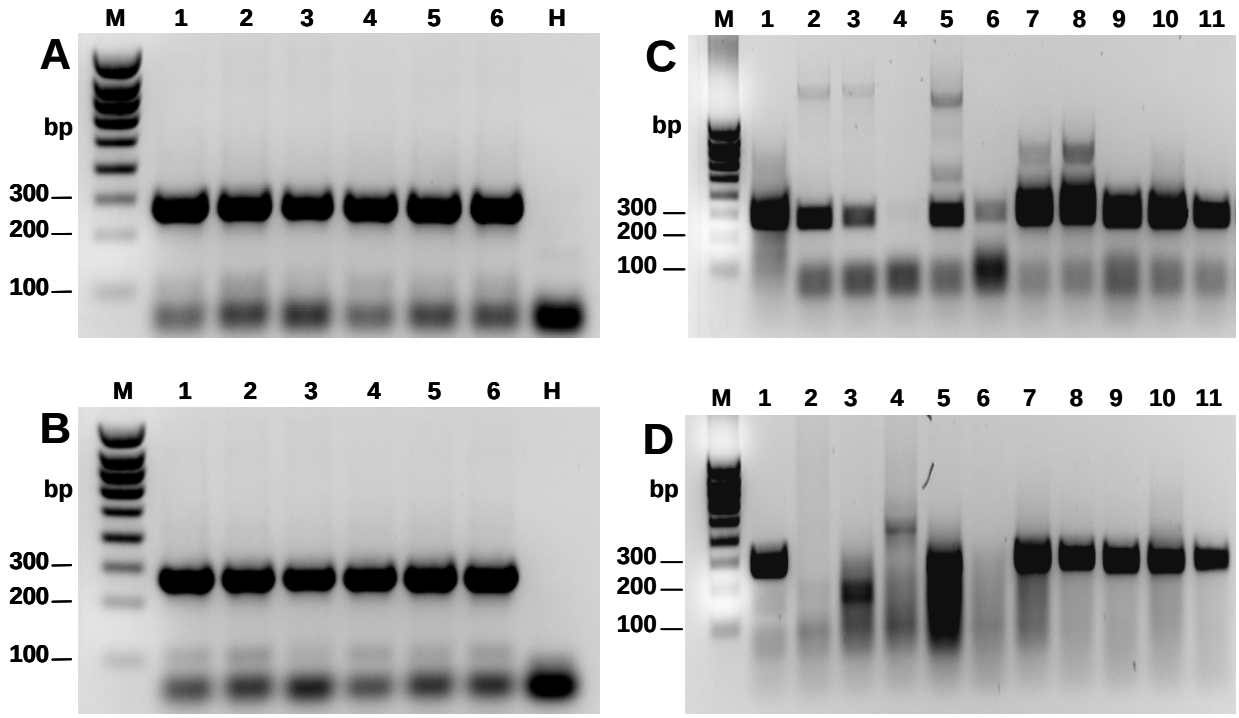

Figure 2. R. Miglino. *Phytopathology*.

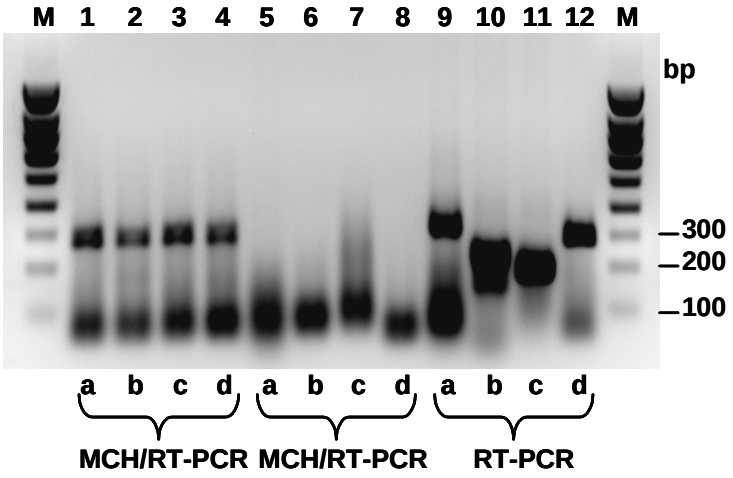

Figure 3. R. Miglino. *Phytopathology*.

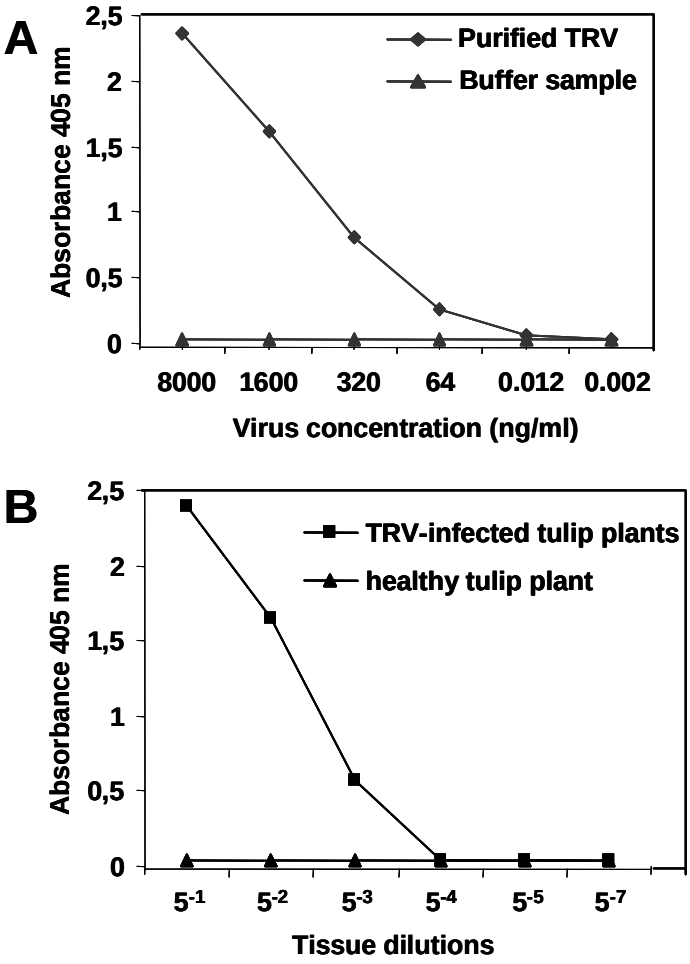

Figure 4. R. Miglino. *Phytopathology.*