European Journal of Plant Pathology (2005) 112:371–378
DOI 10.1007/s10658-005-6232-3

Improved PCR detection of potyviruses in *Allium* species

Pablo Lunello[1], Daniel Ducasse[2] and Vilma Conci[2,*]
[1]*Department of Biotechnology, INIA, Autopista A-6, km 7. 28040, Madrid, Spain;*
[2]*Instituto de Fitopatología y Fisiología Vegetal, Instituto Nacional de Tecnología Agropecuaria (IFFIVE-INTA), Camino 60 cuadras km 5½ (5119), Córdoba, Argentina; *Author for correspondence (Phone: +54 (0351) 4973636; Fax: +54 (0351) 4974343; E-mail: vconci@correo.inta.gov.ar)*

Accepted 25 April 2005

Key words: Alliaceae, garlic, indexing, Leek yellow stripe virus, molecular tests, Onion yellow dwarf virus, RT-PCR, Shallot yellow stripe virus, Turnip mosaic virus

Abstract

Protocols for producing virus-free *Allium* plants require an indexing system that is more sensitive than DAS-ELISA and can detect low virus concentrations in infected plants. In the present work, degenerate primers were designed and a one-step IC-RT-PCR protocol was developed to differentiate between Leek yellow stripe virus (LYSV) and Onion yellow dwarf virus (OYDV) in single and mixed infections in several *Allium* spp. A 566-bp band was observed for LYSV, a 489-bp band for OYDV in single infections, and two bands of the same sizes in mixed infections in different species of *Alliaceae*. A 508-bp band of Shallot yellow stripe virus and a 594-bp band of Turnip mosaic virus were also amplified with the same primers. RT-nested-PCR was also conducted directly in microtitre plate wells after negative or questionable reactions were produced in an ELISA experiment. The detection limit of the DAS-ELISA for LYSV was 16.5–27.3 ng ml^{-1}. The RT-nested-PCR done after DAS-ELISA was 10^2 times more sensitive than the DAS-ELISA alone. In parallel, an IC-RT-nested-PCR in microcentrifuge tubes was 10^4 times more sensitive than the DAS-ELISA. The DAS-ELISA-RT-nested-PCR enables the initial screening of samples by DAS-ELISA to eliminate a high percentage of virus-positive plants, considerably reducing the number of plants to analyze further by RT-PCR.

Introduction

Viruses infecting *Alliaceae* are widespread throughout the world and affect a great number of *Allium* species, causing significant yield losses. Some of the crops affected are *Allium sativum* var. *sativum* (garlic), *A. cepa* (onion), *A. ampeloprasum* var. *porrum* (leek), *A. ampeloprasum* var. *ampeloprasum* ('gigantajo' or great-headed garlic) and *A. sativum* var. *ophioscorodon* ('castaño' garlic). Among the potyviruses affecting *Allium* spp., Onion yellow dwarf virus (OYDV) and Leek yellow stripe virus (LYSV) have been detected worldwide (Conci et al., 1992; Van Dijk, 1993; Barg et al., 1997; Tsuneyoshi et al., 1998b; Chen et al., 2001). These are the most important viruses in terms of the damage, with garlic bulb weight reduced between 24 and 60% for OYDV and between 17 and 54% for LYSV (Canavelli et al., 1998; Lot et al., 1998). Shallot yellow stripe virus (SYSV) and Turnip mosaic virus (TuMV) have less frequently been reported in *Allium* spp. (Van Dijk, 1993; Gera et al., 1997; Van der Vlugt et al., 1999).

Because international health standards are steadily becoming stricter and product health is so closely linked to quality and thus marketing, the production of virus-free plants is of vital impor-

tance. The common method for producing virus-free plants is meristem-tip culture, sometime complemented with thermotherapy (Walkey et al., 1987; Conci and Nome, 1991; Verbeek et al., 1995). The success of these techniques is variable, depending on the virus to be eliminated, the garlic cultivar and the treatment used. In every case, each plant must be analyzed with a highly sensitive detection system in order to separate the healthy plants from those that are still infected with one or more viruses. Enzyme-linked immunosorbent assay (ELISA) has become a favoured technique for the rapid analysis of large numbers of samples. The double antibody sandwich (DAS)-ELISA, the most commonly used variant of ELISA, is not advisable, however, for the *in vitro* analysis of plants (obtained by meristem-tip culture); the viral concentration is usually below the detection limit of DAS-ELISA, frequently producing ambiguous results or false negatives (Conci, 1997).

Although immunosorbent-electron microscopy (ISEM) is a reliable technique to detect low concentrations of virus in plants (Walkey et al., 1987; Conci and Nome, 1991), the high cost of the electron microscope and its inability to screen large numbers of samples in a short time, preclude its use in routine screening. Reverse transcription (RT) and polymerase chain reaction (PCR) are highly sensitive methods to detect viruses in different species as well as in *Allium* spp. (Takaichi et al., 1998; Tsuneyoshi et al., 1998a, b; Dovas et al., 2001). These techniques are promising alternatives for detecting virus at low concentration, yet they too are costly and limited in the rapidity and number of samples that can be screened.

The present study reports the simultaneous detection of different potyviruses in *Allium* species, as well as the use of DAS-ELISA in conjunction with molecular techniques to more sensitively and rapidly test a large number of samples.

Materials and methods

Virus isolates, plant materials and antisera

An LYSV isolate from leek was obtained according to Lunello et al. (2002). The OYDV isolate from onion and SYSV isolate from shallot were kindly provided by Dr D. Lesemann from the Federal Biological Research Centre for Agriculture and Forestry, Braunschweig, Germany. The TuMV was obtained from an infectious cDNA clone and kindly provided by Dr F. Ponz from the Biotechnology Department, INIA, Madrid, Spain (Sanchez et al., 1998). Different *Allium* species with virus disease symptoms were also used. 15 *Allium sativum* var. *sativum* (garlic), six *A. ampeloprasum* var. *porrum* (leek), four *A. ampeloprasum* var. *ampeloprasum* ('gigantajo' or great-headed garlic), four *A. sativum* var. *ophioscorodon* ('castaño' garlic) collected from different production areas of Argentina (Mendoza, San Juan and Córdoba), and seven *in vitro* garlic plants, obtained by meristem-tip culture and shown to be infected with LYSV and/or OYDV through ISEM plus decoration (ISEM-D) (Milne and Luisioni, 1977). Virus-free garlic plants, obtained according to Conci and Nome (1991) and Conci et al. (2004) served as healthy plant samples. The LYSV and OYDV antisera were from the stock at IFFIVE-INTA and were produced as described by Lunello et al. (2002) and Conci et al. (1999), respectively.

Primer design

The degenerate primers P1 and P2 were designed based on the predicted amino acids sequence from the C terminus of the nuclear inclusion b (NIb) and the N terminus of the coat protein (CP)-coding regions of several garlic and leek potyviruses published in the NCBI GeneBank (LYSV: AB005610, AB005611, AB005612, AY007693, D11118, X89711, AB00561, AB000836, AB000472; OYDV: AB000473, AB000474, AB000837, AB000841, AB000843, AB000845, AJ293278, D73378, E13843, OYAB000836). Sequences were processed and analyzed using the DNAstar software. After determining the high homology regions at amino acid levels, degenerated primers were designed. For the detection of LYSV through nested-PCR, primer P1 and an oligo dT primer were used in the first reaction. Two LYSV-specific internal primers, P3 and P4, were designed to amplify an internal fragment from the previous reaction by a second stage of amplification. These primers were designed based on the CP gene of an Argentine isolate of LYSV (accession number: AY007693). Figure 1 shows the nucleotide sequence and position of the primer designs for this study.

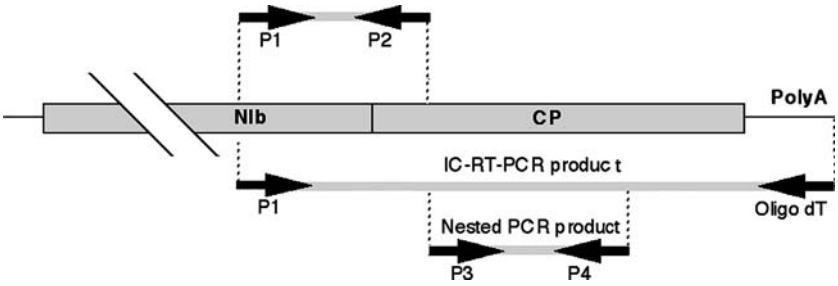

Figure 1. Position on the LYSV genome of the PCR primers used in this study. P1: 5′-CGGGGCCCTWGGNMAAGCRCCA TWYAT-3′, P2: 5′-CGGAGCTCTNCCRTTYTCRATRCACCA-3′, P3: 5′-CGGGGCCCGAGCTCATGCGAAACCAACCTT-3′, P4: 5′-ATCAAGATGGTGCATCCGTGC-3′.

RNA purification and RT-PCR

Total RNA was purified from 50 mg leaf tissue from virus-infected plants, using the RNeasy mini plant kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. An Access RT-PCR system kit (Promega, Madison, WI, USA) was used as indicated by the manufacturer. The reaction mix contained 200 ng of total RNA from infected and non-infected plants, 5 µl upper (P1) and lower (P2) primer (2.5 µM), 5 µl reaction buffer (10×), 1 µl AMV-RT DNA polymerase (*Avian myeloblastosis virus* reverse transcriptase) (5 U μl^{-1}), 1 µl of *Tfl* (*Thermus flavus*) DNA polymerase (5 U μl^{-1}), 5 µl dNTP's mix (2.5 mM) and nuclease-free distilled water up to a final volume of 50 µl. cDNA synthesis was carried out at 37 °C for 45 min. The cycling programme was as follows: 2 min at 94 °C; then 5 cycles of 94 °C for 30 s; 37 °C for 30 s and 68 °C for 1 min; then 40 cycles of 94 °C for 30 s; 50 °C for 30 s and 68 °C for 2 min 30 s and a final extension for 7 min at 68 °C. RT-PCR amplification products were viewed under UV light after electrophoresis in 2% agarose gel in TAE buffer and staining with ethidium bromide.

Immunocapture (IC)-RT-PCR

IC-RT-PCR was done in microtitre plates (Nunc Immuno Plate, MaxiSorp F96), using the ELISA buffers and conditions described by Clark and Adams (1977). IgG to LYSV and OYDV were both diluted to 0.4 µg ml^{-1} for coating of plates, and 100 µl per well was used for all incubation steps. For the simultaneous detection of LYSV and OYDV from dually infected plants, wells were coated with an equal mixture of the two virus-specific IgGs. Reverse transcription was done in the same ELISA plate for 45 min at 37 °C, using the reaction mixture described in the previous section. For the PCR reaction, the RT mixture was transferred to 0.5 ml microcentrifuge tubes. The cycle programme was the same as before.

DAS-ELISA-RT-nested-PCR and IC-RT-nested PCR

To obtain homogeneous material for comparison between DAS-ELISA-RT-nested-PCR in microtitre plates and IC-RT-nested PCR in microcentrifuge tubes, the ELISA plate and microcentrifuge tubes (0.5 ml) were prepared simultaneously, both were coated with 100 µl of 0.4 µg ml^{-1} IgG to LYSV in sterilized coating buffer, and kept at 37 °C for 4 h. After three washes with sterilized PBS-Tween 100 µl of the samples were added. Plants infected with LYSV were ground at a ratio of 1:10 (wt/vol) in buffer and starting from this extract, 20 double serial dilutions were prepared using a 1:10 dilution (wt/vol) of a healthy plant extract in sterilized PBS-Tween. The same samples were analyzed in parallel on the previously sensitized ELISA plate and microcentrifuge tubes. Purified virus of LYSV isolate (Lunello et al., 2002) of known concentration was also tested on the same ELISA plate to estimate the virus concentration in the different double serial dilutions of the infected plants. The ELISA plate and the microcentrifuge tubes were incubated overnight at 4 °C. For the DAS-ELISA test, alkaline phosphatase-conjugated IgG was diluted 1:4000 in conjugate buffer with 2-nitrophenyl phosphate as the substrate at 0.8 mg ml^{-1} substrate buffer. The

ELISA test followed the protocol in Clark and Adams (1977). After absorbance was measured at 405 nm, the ELISA plate was washed and the RT-PCR mixture was added. The reaction mixture for the RT contained: 5 µl (2.5 µM) oligo(dT) primer, 5 µl reaction buffer (10×), 1 µl AMV-RT DNA polymerase (5 U μl^{-1}), 5 µl dNTPs mixture (2.5 mM) and nuclease-free distilled water up to a final volume of 50 µl. cDNA synthesis was conduced at 42 °C for 1 h in a water bath. For the PCR reaction, 1 µl cDNA input, 5 µl (2.5 µM) oligo(dt) primer, 5 µl (2.5 µM) upper primer (P1), 5 µl reaction buffer (10×), 0.2 µl of *Taq* DNA polymerase (5 U μl^{-1}), 5 µl dNTPs mixture (2.5 mM) and nuclease-free distilled water up to a final volume of 50 µl were used. The cycling programme was as follows: 5 cycles of 94 °C for 30 s, 37 °C for 30 s and 72 °C for 1 min; then 40 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2 min 30 s and final extension for 7 min at 72 °C. Nested PCR was done using 1 µl of the PCR product as input DNA. The reaction mix was prepared by adding 5 µl (2.5 µM) upper primer (P3), 5 µl (2.5 µM) lower primer (P4), 5 µl reaction buffer (10×), 0.2 µl of *Taq* DNA polymerase (5 U μl^{-1}), 5 µl dNTP's mix (2.5 mM) and nuclease-free distilled water up to a final volume of 50 µl. The cycling programme was as follows: 40 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 2 min 30 s and a final extension for 5 min at 72 °C. Nested-PCR amplification products were viewed under UV light after electrophoresis in 1% agarose gel in TAE buffer and staining with ethidium bromide. All assays were run in triplicate and were repeated to confirm the results.

Results

IC-RT-PCR and RT-PCR

With both techniques, a band of 566 bp corresponding to LYSV was detected in samples from leek (Figure 2, lane 1). In onion infected with the OYDV isolate, a band of 489 bp corresponding to this virus was observed, and in the samples with mixed infections of garlic, 'gigantajo,' and 'castaño' garlic, bands corresponding to LYSV and OYDV were amplified simultaneously. The P1 and P2 primers also allowed the detection of a band of 594 bp of TuMV and a band of 508 bp of

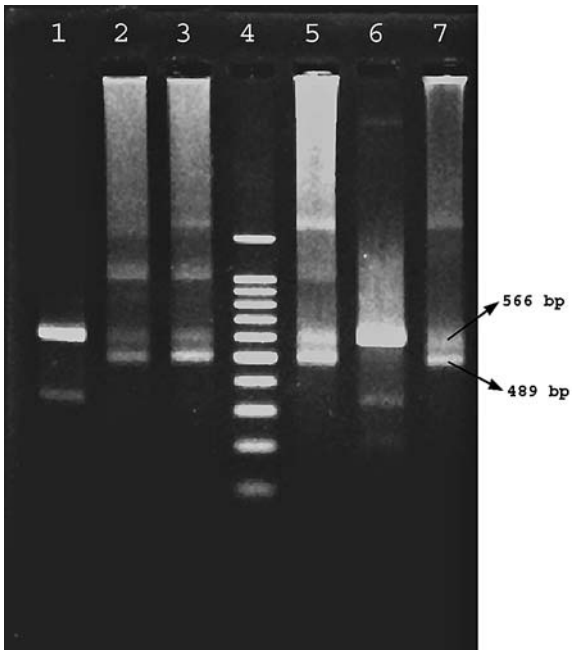

Figure 2. IC-RT-PCR detection of LYSV and OYDV in different *Allium* spp. Lanes 1, 2, 3, 5, 6 and 7 show the fragments amplified from virus-infected plants. Lane 1: leek infected with LYSV. Lane 2: garlic infected with LYSV and OYDV. Lane 3: 'castaño' garlic infected with LYSV and OYDV. Lane 4: molecular weight marker, 100-bp ladder (PROMEGA). Lane 5: 'gigantajo' infected with LYSV and OYDV. Lane 6: garlic infected with LYSV. Lane 7: garlic infected with LYSV and OYDV.

SYSV from shallot, other viruses reported infecting *Allium* spp. (Figures 2 and 3). The garlic plants obtained by meristem-tip culture, that had ambiguous values in DAS-ELISA but were confirmed by ISEM-D as infected with LYSV and/or OYDV, were positive in IC-RT-PCR tests in all cases (Figure 4). The identity of the PCR products was confirmed by sequence analysis of the cloned amplicons (data not shown). Samples of six different *Allium* species from diverse geographic areas of Argentina and other countries (onion with OYDV isolate and shallot with SYSV isolate provided by Dr Lesemann and TuMV provided by Dr Ponz) were used to test the efficiency of the primers to detect potyviruses in *Allium* spp.

Combination of DAS-ELISA with RT-nested PCR

In DAS-ELISA, extracts from LYSV-infected plants gave positive reactions up to dilutions 1:40, 1:320 and 1:640 in different tests. The absorbance

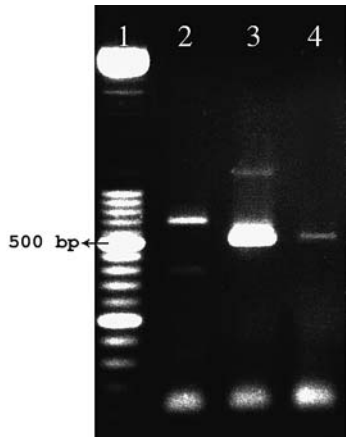

Figure 3. IC-RT-PCR detection of TuMV, OYDV and SYSV. Lane 1: molecular weight marker, 50 pb. Lane 2: TuMV isolate. Lane 3: OYDV isolate from onion. Lane 4: SYSV isolate from shallot.

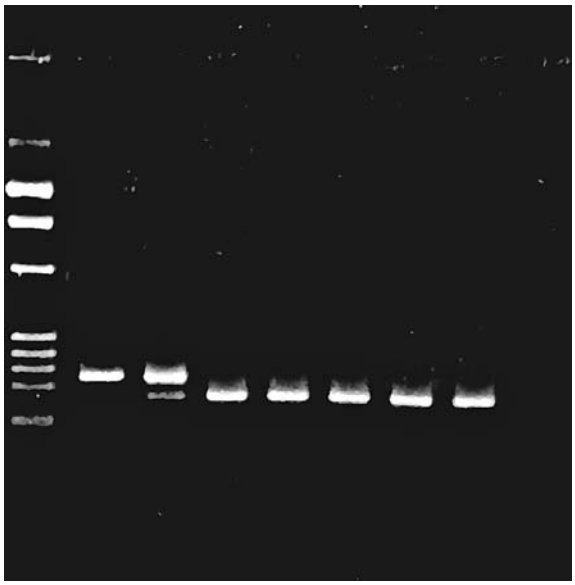

Figure 4. Fragments amplified by IC-RT-PCR of *in vitro* garlic plants following meristem-tip culture. Lane 1: Molecular weight marker (3200, 2200, 1420, 800, 700, 600, 520 and 400 bp). Lane 2–8: *in vitro* plants, 2 infected with LYSV, 3 infected with LYSV and OYDV and 4–8 infected with OYDV. Lane 9: healthy control.

value obtained for this dilution corresponded to 22.6, 27.3 and 16.5 ng ml^{-1} of LYSV, respectively, in different tests and/or with purified virus extractors. The RT-nested-PCR performed after the DAS-ELISA permitted amplification of the expected LYSV fragment from leaf extracts at up to seven dilutions more than DAS-ELISA, that is, 10^2 times more sensitive. The IC-RT-nested-PCRs done entirely in microcentrifuge tubes were markedly more sensitive, detecting LYSV in leaf extracts diluted up to 14 times more than for DAS-ELISA (10^4 times more sensitive) (Figure 5).

Discussion

The techniques developed in this study enabled the detection of *Allium* potyviruses at the low concentrations typical in plants grown from meristem-tip culture through the use of a set of primers that detect LYSV, OYDV, TuMV and SYSV simultaneously. Single-step RT-PCR and IC-RT-PCR techniques have been described for the detection of viral and subviral RNA of plant viruses (Nolasco et al., 1993). The increased sensitivity obtained through molecular methods is indisputable, but the high cost of the tests limits their widespread use. Therefore, detecting all viruses in one test will result in considerable savings and a more practical procedure.

As previously mentioned, OYDV and LYSV are the most frequent potyviruses infecting *Allium* sp., especially garlic. The possibility of differentiating both viruses with one set of primers also enables the differentiation of OYDV from LYSV in plants. Even though both viruses are important in terms of damage, OYDV is responsible for greater losses in yields (Conci, 1997; Canavelli et al., 1998; Lot et al., 1998) and is the only one of concern for the certification of garlic plants in several countries (e.g., Argentina, France). A single test that can detect the two most important viruses and differentiate between the two viruses in a plant is thus extremely useful both in selection and certification programmes.

With the set of primers, P1 and P2, it was possible to detect potyviruses in all the *Allium* spp. tested (garlic, onion, leek, shallot, 'gigantajo' and 'castaño' garlic) and in plants from various production areas in Argentina. The test may also have the capacity to detect different strains or isolates of the viruses. After analysis of the sequences of potyviruses that can infect *Allium* spp. available in the NCBI GeneBank, we can conclude that most of the viruses can be amplified using primers P1 and P2. All sequences analyzed (41) match perfectly with primer P2, and half of them (22) present

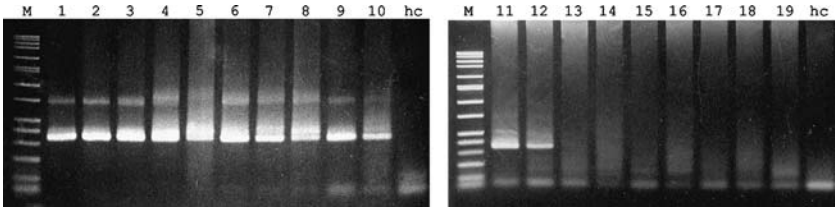

Figure 5. IC-RT-nested-PCR developed in microcentrifuge tube sensitivity assay. Amplification products (about 330 bp) from LYSV-infected leek plant using specific internal primers (P3 and P4). Lanes 1–19: infected leek plant in double serial dilutions from 1/320, M: molecular weight marker, DirectLoad™ (SIGMA), hc: healthy control.

several mismatches (from one to three mismatches) with the sequence of primer P1. It is difficult to predict whether amplification by RT-PCR using primers P1 and P2 will work on those sequences that do not match perfectly. Computer programmes do not correlate the mismatch tolerance of a PCR amplification with primer length under various conditions (Burpo, 2001). The only way of proving the effectiveness of the primers is to actually amplify those isolates. Based on the number and position of the mismatches, we have marked the sequences that probably will not be amplified properly with the designed primers (those with an asterisk in Figure 6).

A broader spectrum for primer P1 could be attained with a few changes such as the sixth base from the 3′ (A) by an N. In any case, this or other changes should be checked experimentally. The broad specificity of the primers is an important criterion for applying PCR as a diagnostic tool. Because variations in the nucleotide sequences of different *Allium* potyviruses have already been reported (Tsuneyoshi et al., 1998b), the use of degenerate primers designed in the present study could provide the means for detecting intraspecific variations in viruses because they were derived from highly conserved domains of the genome.

In many studies, there is a higher sensitivity of molecular methods over the classical techniques to detect plant viruses. Such is the case for *Prunus necrotic ringspot virus*, *Prune dwarf virus*, *Potato virus Y* and *Beet yellows virus*; an increase in detection was achieved with IC-RT-PCR or adding nested PCR after the IC-RT-PCR (Stevens et al., 1997; Sanchez-Navarro et al., 1998; Moury et al., 2000; Varveri, 2000; Helguera et al., 2001, 2002). Here we used this method at a crucial stage in the production of virus-free garlic plants, when the plants are still in the *in vitro* culture phase and the concentration of virus is generally very low. Such early detection is very useful because it enables infected plants to be discarded before starting *in vitro* micropropagation of the material. The results match those reported by Shiboleth et al. (2001) who used RT-PCR for the detection of *Potyvirus, Allexivirus* and *Carlavirus* from plants obtained by meristem-tip culture. At the same time, Dovas et al. (2001) showed IC-RT-PCR and RT-PCR techniques to be between 10^2–10^4 times more sensitive than DAS-ELISA for detecting

	PRIMER P1: WGGNMAAGCRCCATWYAT	Size of amplf fragment
01. LYSV-AJ409304	T	570bp
02. LYSV-AJ409305	G	570bp
03. LYSV-AJ409306	G	570bp
04. LYSV-AJ409307	G	573bp
05. LYSV-AJ409308	G T	573bp
06. OYDV-AB000838	C T	489bp *
07. OYDV-AB000839	T	489bp
08. OYDV-AB000840	G T G	489bp *
09. OYDV-AJ409309	T	489bp
10. OYDV-AJ409310	G T G	489bp *
11. OYDV-AJ409311	T	489bp
12. OYDV-AJ409312	C C	489bp
13. OYDV-AJ409313	G T G	489bp *
14. OYDV-AJ510223	C G C	489bp *
15. OYDV-NC_005029	C G C	489bp *
16. ScaMV-AJ310208	C	546bp
17. ScaMV-AJ316084	C	546bp
18. ScaMV-NC_003399	C	546bp
19. SYSV-AJ311370	G	492bp
20. SYSV-AJ488151	G	492bp
21. WoYSV-AB000842	T G	492bp *
22. WoYSV-AB000844	T G	492bp *

Figure 6. Alignment of sequences from the NCBI GeneBank. Each sequence was designated by its accession number. Sequences were tailored and aligned to assess their homology with degenerate primers P1 and P2. From 41 sequences analyzed, 19 had a perfect match (identified later) with both primers. The rest of the sequences are presented in the figure and had different degrees of mismatch with P1 primer. Only he mismatched bases are presented in the figure. All sequences had a perfect mach with primer P2. (Sequences with total homology with primers P1: LYSV: AB005610, AB005611, AB005612, AY007693, D11118, X89711, AB00561, AB000836, AB000472; OYDV: AB000473, AB000474, AB000837, AB000841, AB000843, AB000845, AJ293278, D73378, E13843, OYA-B000836.)

virus in garlic. Using a real-time fluorescent (Taqman®) RT-PCR assay, they increased the sensitivity 10^6 times over DAS-ELISA sensitivity for LYSV and OYDV (Lunello et al., 2004), indicating the possible utility of the assay in those laboratories with the necessary equipment. Nonetheless, the high cost of molecular techniques is often a limitation when a quantity of samples must be analyzed. For this, the DAS-ELISA-RT-nested-PCR, developed here, first screens out plants with higher virus concentrations using DAS-ELISA. Those plants that gave negative or ambiguous results can then be assayed in the same plate by RT-nested-PCR with a 10^2-fold increase in sensitivity. In this way, we can exploit the most important features of each technique: the practical nature and economy of the DAS-ELISA and the increased sensitivity of the IC-RT-nested-PCR.

When microcentrifuge tubes were used for IC-RT-nested-PCR, the sensitivity of virus detection was increased even more, up to 10^4 times more sensitive than DAS-ELISA. This large difference in sensitivity is probably due to the numerous preparatory steps required for the standard DAS-ELISA process. These steps (washing, temperature changes, incubation with conjugate, and substrate) probably affect the integrity of the virus particles adsorbed to the plate resulting in lower sensitivity. IC-RT-nested-PCR could be used in virus-free garlic production programmes to avoid erroneous conclusions that diseased plants are healthy, when in fact the viral concentration is below the detection limits of traditional methods. In previous studies, the concentrations of LYSV and OYDV varied throughout the year and at times were at very low concentrations, making their detection difficult (Conci et al., 2002). More sensitive detection systems, like those developed in this study, may enable the accurate detection of viruses at any time of the year.

Acknowledgements

This work was supported by grants from INTA, Agencia Córboba Ciencia, CONICET, FONCYT and CABBIO. Pablo Lunello was the recipient of a postdoctoral fellowship from CONICET for studies abroad. The authors wish to thank to Dr D. Lesemann from the Federal Biological Research Centre for Agriculture and Forestry, Germany for providing OYDV and SYSV isolates and Dr F. Ponz from INIA, Madrid, Spain for the TuMV isolate.

References

Barg E, Lesemann DE, Vetten HJ and Green SK (1997) Viruses of alliums and their distribution in different *Allium* crops and geographical regions. Acta Horticulturae 433: 607–616

Burpo FJ (2001) A critical review of PCR primer design algorithms and cross-hybridization case setudy. Biochemistry 218: 1–11

Canavelli A, Nome SF and Conci VC (1998) Efecto de distintos virus en la producción de ajo (*Allium sativum*) ''Rosado Paraguayo''. Fitopatología Brasilera 23 (3): 354–358

Chen J, Chen J and Adams MJ (2001) Molecular characterisation of a complex mixture of viruses in garlic with mosaic symptoms in China. Archives of Virology 146: 1841–1853

Clark MF and Adams AN (1977) Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. Journal of General Virology 34: 475–483

Conci VC (1997). Virus y fitoplasmas de ajo In: Burba JL (ed) 50 *temas sobre producción de ajo*. Vol 3 (pp 267–291) EEA-INTA La Consulta, Mendoza, Argentina

Conci VC and Nome SF (1991) Virus free garlic (*Allium sativum* L.) plants obtained by thermotherapy and meristem tip culture. Journal of Phytopathology 132: 186–192

Conci VC, Nome SF and Milne RG (1992) Filamentous viruses of garlic in Argentina. Plant Disease 76: 594–596

Conci VC, Helguera M and Nome SF (1999) Serological and biological comparison of *Onion yellow dwarf virus* from onion and garlic in Argentina. Fitopatología Brasilera 24: 73–75

Conci VC, Lunello P, Buraschi D, Italia RR and Nome SF (2002) Variations of *Leek yellow stripe virus* concentration in garlic and its incidence in Argentina. Plant Disease 86: 1085–1088

Conci VC, Cafrune EE, Lunello P, Nome S and Perotto C (2004) Producción de planas de ajo libres de virus. In: Echenique V, Rubinstein C and Mroginski L (eds) Biotecnología y Mejoramiento Vegetal. Parte VIII, Cap 6 (pp. 313–316) Ediciones INTA, Buenos Aires, Argentina www.argenbio.org/h/biblioteca/libro.php; www.inta.gov.ar/ediciones/2004/biotec/biotec.htm

Dovas CI, Hatziloukas E, Salomon R, Barg E, Shiboleth Y and Katis NI (2001) Comparison of methods for virus detection in *Allium* spp. Journal of Phytopathology 149: 731–737

Gera AD, Lesemann DE, Cohen J, Franck A, Levy S and Salomon R (1997) The natural occurrence of turnip mosaic potyvirus in *Allium ampeloprasum*. Journal of Phytopathology 145: 289–293

Helguera PR, Docampo DM, Nome SF and Ducasse D (2002) Enhanced detection of *Prune dwarf virus* in peach leaves by immunocapture-reverse transcription-polymerase chain reaction with nested polymerase chain reaction (IC-RT-PCR-nested PCR). Journal of Phytopathology 150: 94–96

Helguera PR, Taborda R, Docampo DM and Ducasse D (2001) Immunocapture reverse transcription-polymerase chain reaction combined with nested PCR greatly increases the detection of *Prunus necrotic ring spot virus* in the peach. Journal of Virological Methods 95: 93–100

Lot H, Chevelon V, Souche S and Dellecolle B (1998) Effects of *Onion yellow dwarf virus* and *Leek yellow stripe virus* on symptomatology and yield loss of three French garlic cultivars. Plant Disease 82: 1381–1385

Lunello P, Ducasse DA, Helguera M, Nome SF and Conci VC (2002) An Argentinean isolate of *Leek yellow stripe virus* from leek can be transmitted to garlic. Journal of Plant Pathology 84(1): 11–17

Lunello P, Mansilla C, Conci V and Ponz P (2004) Ultrasensitive detection of two garlic potyviruses using a real-time fluorescent (Taqman®) RT-PCR assay. Journal of Virological Methods 118(1): 15–21

Milne RG, Luisoni E (1977). Rapid immune electron microscopy of virus preparations. In: Maramorosch K and Koprowski H (eds) Methods in Virology. Vol 6 (pp 265–281) Academic Press, New York

Moury B, Cardin L, Onesto JP, Candresse T and Poupet A (2000) Enzyme-linked immunosorbent assay testing of shoots grown in vitro and the use of the immunocapture-reverse transcription-polymerase chain reaction improve the detection of *Prunus necrotic ring spot virus* in rose. Phytopathology 90: 522–528

Nolasco G, deBlas C, Torres V and Ponz F (1993) A method combining immunocapture and PCR amplification in a microtiter plate for the detection of plant viruses and subviral pathogens. Journal of Virological Methods 45: 201–218

Sanchez F, Martinez Herrera D, Aguilar I and Ponz F (1998) Infectivity of turnip mosaic potyvirus cDNA clones and transcripts on the systemic host *Arabidopsis thaliana* and local lesion hosts. Virus Research 55(2): 207–219

Sanchez-Navarro JA, Aparicio F, Rowhani A and Pallas V (1998) Comparative analysis of ELISA non radioactive molecular hybridization and PCR for the detection of *Prunus necrotic ringspot virus* in herbaceous and prunus host. Plant Pathology, 47: 780–786

Shiboleth YM, Gal-On M, Koch HD, Rabinowitch HD and Salomon R (2001) Molecular characterization of *Onion yellow dwarf virus* (OYDV) infecting garlic (*Allium sativum* L.) in Israel: Thermotherapy inhibits virus elimination by meristem tip culture. Annals of Applied Biology 138: 187–195

Stevens M, Hull R and Smith HG (1997) Comparison of ELISA and RT-PCR for the detection of Beet yellows closterovirus in plants and aphids. Journal of Virological Methods 68: 9–16

Takaichi M, Yamamoto M, Nagakubo K and Oeda K (1998) Four garlic viruses identified by the reverse transcription-polymerase chain reaction and their regional distribution in northern Japan. Plant Disease 82: 694–698

Tsuneyoshi T, Matsumi T, Deng TC, Sako I and Sumi S (1998a) Differentiation of *Allium* carlaviruses isolated from different parts of the world based on the viral coat protein sequence. Archives of Virology 143: 1093–1107

Tsuneyoshi T, Matsumi T, Natsuaki KT and Sumi S (1998b) Nucleotide sequence analysis of virus isolates indicates the presence of three potyvirus species in *Allium* plants. Archives of Virology 143: 97–113

Van der Vlugt RAA, Steffens P, Cuperus C, Barg E, Lesemann DE, Bos L and Vetten HJ (1999) Further evidence that shallot yellow stripe virus (SYSV) is a distinct potyvirus and reidentification of Welsh onion yellow stripe virus as a SYSV strain. Phytopathology 89: 148–155

Van Dijk P (1993) Survey and characterization of potyviruses and their strains of *Allium* species. Netherlands Journal of Plant Pathology 99: 1–48

Varveri C (2000) Potato Y potyvirus detection by immunological and molecular techniques in plants and aphids. Phytoparasitica 28: 2

Verbeek M, Van Dijk P and Van Well MA (1995) Efficiency of eradication of four viruses from garlic (*Allium sativum*) by meritem-tip culture. European Journal of Plant Pathology 101: 231–239

Walkey DGA, Webb MJW, Bolland CJ and Miller A (1987) Production of virus-free garlic (*Allium sativum* L.) and shallot (*A.* ascalonicum L.) by meristem-tip culture. Journal of Horticultural Science 62: 211–220