COMPARISON OF MOLECULAR ASSAYS FOR THE DETECTION OF ALLEXIVIRUSES IN GARLIC PLANTS

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Abstract

Allexiviruses and several Carla- and Potyvirus species are the most widespread viral pathogens of Allium crops world-wide. Simultaneous infection by these viruses causes significant reduction of bulb weight. Production of species-specific antisera against allexiviruses is complicated. Production of virus-free propagation material requires the use of highly specific, sensitive and rapid virus detection methods. The aim of the present study therefore was to examine primer sequences using RT-PCR and IC-RT-PCR method and testing the dot-blot hybridization for detection of allexiviruses. Electron microscope was also used for selective garlic plants infected with Allexiviruses.

Key words: *Allexivirus, RT-PCR, IC-RT-PCR, dot-blot hybridization*

INTRODUCTION

Garlic (*Allium sativum* L.) is one of the most important culinary plants widely cultivated throughout the world. This plant is vegetatively propagated, therefore viruses are transmitted from one crop cycle to another through infected cloves, as well as by vectors. From garlic plants more than 12 virus species were isolated and identified (Yamashita et al. 1996). Garlic plants are commonly infected by viruses belonging to three families: *Potyviridae* (genus, *Potyvirus*), *Betaflexiviridae* (genus, *Carlavirus*) and *Alphaflexiviridae* (genus, *Allexivirus*) (King et al. 2012). These viruses have similar morphological properties and often similar biology, thus, it is difficult to separate them according to these properties. Simultaneous infection by viruses causes significant reduction of bulb weight and growth (Lot et al. 1998).

Allexiviruses induces a yellowing and deformation on garlic leaves that can result in high bulbs yield reductions in infected plants, with corresponding economic consequences. Investigations carried out in Argentina showed that infection of garlic cultivars Blanco-IFFIVE and Morado-INTA with GarV-A resulted in decrease of bulb weight (14 to 32%) and reduction in their diameter (6 to 11%). In case of infection with GarV-C the bulb weight and diameter dropped by 15% and 5%, respectively (Cafrune et al. 2006). Similar data were reported by Perotto et al. (2010) who investigated the effect of *Garlic virus A* and *Garlic virus C* on the bulb weight of cultivar Blanco-IFFIVE for 4 years. They also demonstrated that in case of additional infection of plant material with *Onion yellow dwarf virus* (OYDV) and/or *Leak yellow strip virus* (LYSV) the bulb weight dropped by 25 to 43%.

Virions of *Allexivirus* species are very flexuous filamentous about 800 nm in length and 12 nm in diameter, have helical symmetry and a surface pattern of cross-banding. The genome is the positive sense, single-stranded RNA forming 12 nm wide and 800 nm long particles. Genes are translated from a total of 6 open reading frames (King et al. 2012).

Detection of these viruses in plants is difficult due to the lack potentiality of use of all available methods. Production of species-specific antisera against allexiviruses is complicated by the simultaneous occurrence of several serologically distinct *Allexivirus* species/types in *Allium* species (Barg et al., 1994). Meristem-tip culture is a method of obtaining virus-free propagation material. Production of such material requires the use of highly specific, sensitive and rapid virus detection methods. Reverse transcriptase (RT)-polymerase chain reaction (PCR) protocols, for large-scale routine diagnosis in production of virus-free propagation material in certification schemes, should allow detection of a wide range of virus isolates using a simple sample preparation procedure. The aim of the present study therefore was to examine primer sequences using RT-PCR and IC-RT-PCR method and testing the dot-blot hybridization detection of three different allexiviruses.

MATERIALS AND METHODS

Allexiviruses isolates were selected using DAS-ELISA test and RT-PCR technique from fields in different regions of Poland. Occurrence of GarV-A and GarV-D was confirmed using IC-RT-PCR technique. To confirmed presence of GarV-D and GarV-X used a dot-blot hybridization. In order to obtain homogenous material for all techniques, leaves were cut into small pieces, mixed and randomly put into small plastic bags for further processing.

Viral particles were observed under transmission electron microscope (Jeol JEM 1220, Japan). The virus preparations were prepared from plant sap, diluted 1:20 in phosphate buffer with 0.5% Na_2SO_3 addition, according to the 'sap-dip' EM technique described by Szyndel (1997). Preparations were stained with 2% uranyl acetate.

Total RNA was extracted from leaf and bulb tissue using the silica capture (SC) method described originally by Boom et al. (1990) and adapted to the diagnosis of plant viruses by Malinowski et al. (1997).

Immunocapture of virions was performed in 0.2 ml tubes (Axygen, USA), pre-coated with 100 µl of virus-specific IgG diluted with concentration 1:500 in carbonate buffer pH 9.6, incubated for 180 min at 37°C and subsequently washed three times with PBS-Tween. The tubes were filled with 100 µl of extract (0.250 g of leaf or bulb tissue in 2.5 ml of PBS-Tween containing 2% PVP and 0.2% albumine), incubated at 4°C overnight, followed by three washes with PBS-Tween prior to RT-PCR. For RT-PCR, Transcriptor One-Step RT-PCR Kit (Roche) was used with the primer designed by the authors (Chodorska et al. 2013). Single tube RT-PCR used a 30 min heating step at 50°C and a 7 min heating step at 94°C followed by 32 cycles of 10 sec melting at 94°C, 30 sec annealing at -°C (Chodorska et al. 2013), and 45 sec elongation at 68°C with a final extension of 7 min at 68°C. The reaction products were resolved by electrophoresis in the TBE buffer in 1.2% agarose gel.

DIG-labeled cDNA probe was synthesized using the DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche, Germany) according to the manufacturer's recommendations. Samples of RNA (5 µl) were separately spotted onto nylon membranes (Hybond-N+, Amersham, Buckinghamshire, UK) soaked beforehand in 2x standard saline citrate (SSC). The nucleic acids were fixed to the membrane by baking at 80°C for 2 hours. Pre-hybridization and hybridization of dot-blots were performed following the manufacturer's instructions (Roche, Germany). The hybridization solution contained 25 ng of probes for every milliliter of solution. Hybridization was carried out overnight at 42°C. The membrane was washed twice (for 5 min each time) in 2xSSC and 0.1xSDS at room temperature and twice (for 15 min each time) in 0.5xSSC and 0.1xSDS at 65°C. The hybridized probes were immunodetected with anti-digoxigenin-AP fab fragments and then made visible with CSPD (Roche, Germany) as the chemiluminescent substrate according to the manufacturer's recommendations (Roche, Germany).

RESULTS AND DISCUSSION

The severity of viral infection of garlic world-wide and the great economic losses caused, necessitate the production of virus-free propagative material. This strategy involves testing of explants produced by meristem tip culture (Verbeek et al. 1995), and routine testing of propagative material in the field. The development of a specific, sensitive and simple virus detection method is required for certification purposes. This study aimed at the adaptation of three methods according to the needs of the above strategy.

Firstly the visualization of viral particles was performed using electron microscope. In electron microscope preparations obtained from garlic leaves virus particles were observed. Based on the EM data, about 800-1000 nm in length of the rod shaped allexiviruses particles (Figure 1) were observed. During the observation we reported that different viruses were mixed within a garlic plants. The EM technique is low effective for allexiviruses detection. Diekmann et al. (1997) found that studies using electron microscopy are useful for distinction of *Allexivirus* and *Carla*- and *Potyvirus* virion particles, but it is impossible to distinguish the different species of these pathogens. Similarly Koo et al. (2002) ,

Cafrune et al. (2006) and Lee et al. (2007) used the electron microscope only to confirm the presence of allexiviruses without detailed identification of species. The results of the present study confirm the data given above.

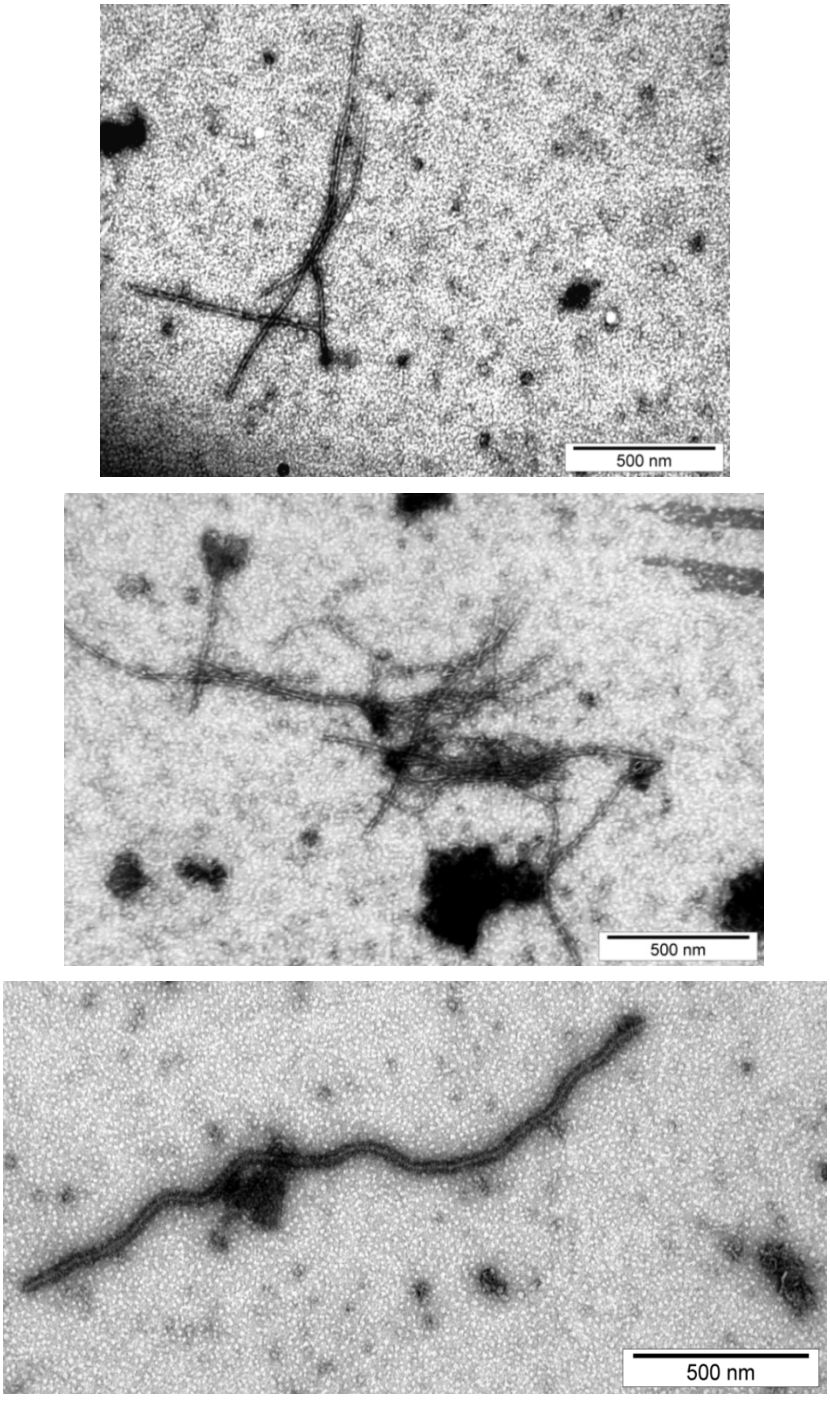


Fig. 1. The electron microscope image of garlic viruses particles

In many cases degenerate primers were used to detect and amplify cDNA of viruses of the genera *Allexivirus* from garlic plants (Yamashita et al. 1996, Chen et al. 2001, Chen et al. 2004). In our studies we designed primers specific to complete coat protein and nucleic acid binding protein genes. Extensive testing of primers for their polyvalence constitutes an important criterion for the application

of PCR assays as diagnostic tools (Hadidi et al. 1998). Samples of diverse geographic origin, were used to test the performance (polyvalence and specificity) of the primers designed in this study. In all cases, RT-PCR yielded the amplification of expected products. As expected, no product was amplified from total RNA of healthy garlic leaves. Figure 2 presents selected results of virus detection.

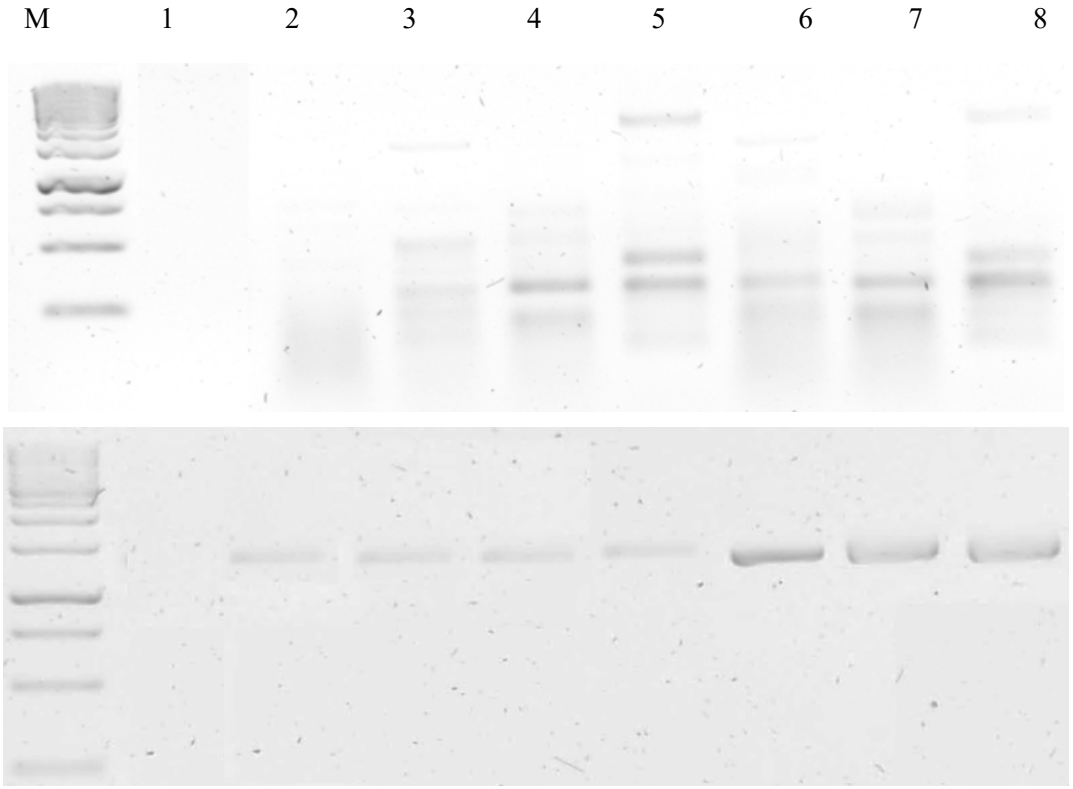


Fig. 2. Agarose gel analysis of GarV-A (upper fig.)/GarV-D (lower fig.) amplicons obtained by RT-PCR tests. Lane M: GeneRuler™ 1 kb DNA Ladder (Fermentas); lanes 1: healthy garlic plants; lanes 2-8: samples infected with GarV-A (1303 bp) / GarV-D (1344 bp)

RT-PCR method encountered several problems in epidemiological research of the viral diseases e.g. RNA extraction is troublesome and expensive, and RNA molecules are also readily degraded due to the ubiquitous presence of RNase. Also, the potential for contamination made the analysis of RT-PCR results more difficult. In contrast, the IC-RT-PCR method avoided the extraction of viral or plant total RNA, and was easily carried out in a single tube. Such procedures have been developed for detecting many plant viruses (Sefc et al. 2000; Mansilla et al. 2003). Here, we present results an IC-RT-PCR procedure for detecting GarV-A and GarV-D isolates. Application of IC-RT-PCR with the appropriate primers revealed the presence of GarV-A and GarV-D in garlic plants. The IC-RT-PCR procedure was optimized as to the dilution of coating antibodies. A dilution of IgG at 1:500 worked best for detection.

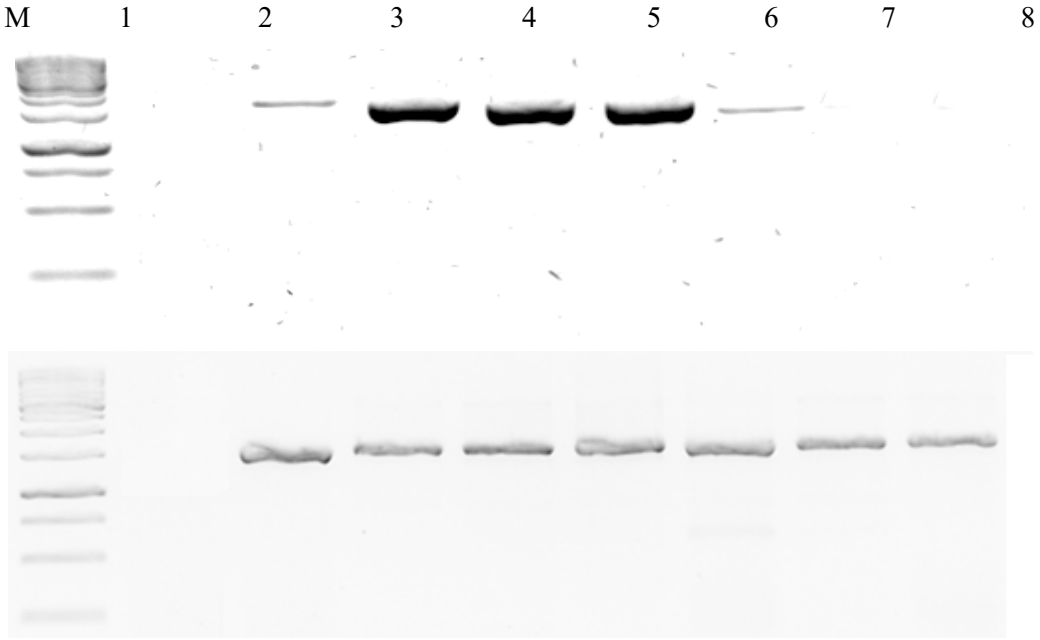


Fig. 3. Agarose gel analysis of GarV-A (upper fig.)/GarV-D (lower fig.) amplicons obtained by IC-RT-PCR tests. Lane M: GeneRuler™ 1 kb DNA Ladder (Fermentas); lanes 1: healthy garlic plants; lanes 2-7: samples infected with GarV-A (1303 bp) / GarV-D (1344)

Nucleic acid hybridization has proved to be a very reliable and sensitive technique in plant virus diagnosis. The most common method for molecular hybridization is the dot-blot hybridization technique which involves the direct application of a nucleic acid solution to a solid support, such as nitrocellulose or nylon membranes, and detection which appropriate probes. Dot-blot hybridization constitutes an easy technique which is currently used for pathogen detection (Widada et al. 2004). In order to establish its suitability and sensitivity, hybridization was first carried out using total RNA extracted from garlic plants tissues. Figure 3 shows results of comprehensive survey for GarV-D and GarV-X in garlic plants, as indicated by dot-blot hybridization analysis of 6 total nucleic acid samples from leaves and bulbs with DIG-11-UTP labeled GarV-D or GarV-X cRNA probe. Hybridization signals were seen in all total RNA extracted from garlic leaves and bulbs infected with GarV-D or GarV-X. No hybridization was seen with total RNA extracted from healthy garlic plants.

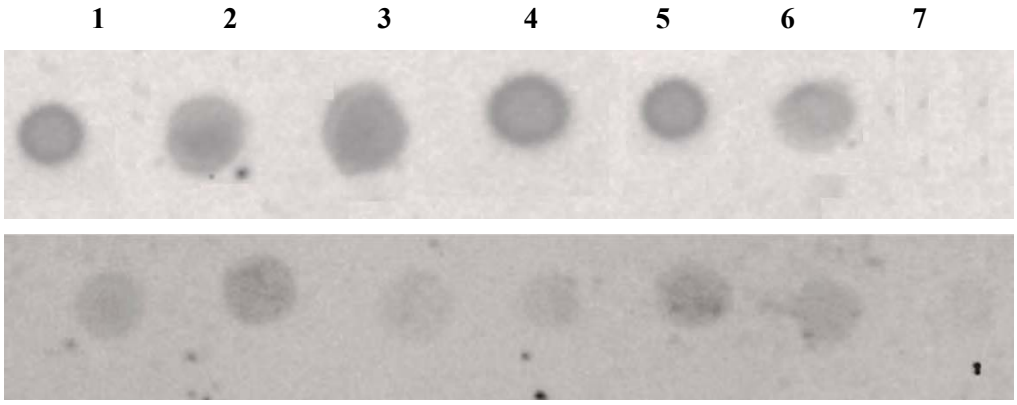


Fig. 4. Dot blot hybridization of a DIG-labeled GarV-D (upper fig.)/GarV-X (lower fig.) cRNA probe to total nucleic acids from garlic plants naturally infected with GarV-D/GarV-X. Samples from garlic plants (lanes 1-6), negative control (lane 7)

Garlic plants are infected by a wide range of viruses. Many cause devastation of garlic production field resulting in significant economic losses. Resources available for routine detection of allexiviruses tend to be limited, because for only few of the species are available kits for ELISA test. The results of this study indicate that RT-PCR, IC-RT-PCR and dot-blot hybridization were efficient for GarV-A, GarV-D and GarV-X detection, but the most sensitive method was IC-RT-PCR.

ACKNOWLEDGEMENTS

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