THE DETECTION AND IDENTIFICATION OF GARLIC VIRUSES IN POLISH GARLIC PLANTS

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Key words: garlic viruses, DAS-ELISA test, electron microscope, coat protein gene.

SUMMARY

Garlic (Allium sativum L.) can be infected by numerous viruses. The aim of our study was the detection and identification of 3 garlic viruses: GarV-A, GarV-B and GarV-C by serological DAS-ELISA test. A virus survey was conducted in 11 garlic production fields located in different regions of Poland. Leaf and bulb samples from 80 plants during the 2011-2012 growing season were tested by DAS-ELISA. Results indicated that 38 samples (47,5%) were infected with GarV-A, 49 samples (61,3%) with GarV-B and 14 samples (17,5%) with GarV-C. Furthermore, viral particles were observed under a transmission electron microscope (‘sap-dip’ technique). To confirme the occurrence of viruses total RNA extracted from leaves was used together with the primer pairs designed in the conserved region of ORF5 (coat protein) and ORF6 (nucleic acid binding protein) of GarV-A, GarV-B and GarV-C. Products of the expected size were amplified only from the DAS-ELISA-positive samples.

INTRODUCTION. Virus diseases of garlic (*Allium sativum* L.) are widespread throughout the world, causing serious damage to crop yields and quality. Garlic plants are propagated vegetatively, resulting in widespread infection by viruses. These infections cause severe yield losses that vary depending on the cultivars, but it some cases reach 60% (Conci et al. 2002). Garlic can be infected by many viruses, including members of the genus *Allexivirus* (family *Alphaflexiviridae*) (King et al. 2011). This genus contains viruses that have all been detected only in *Allium* spp., they have occur in multiple infections and are thought to be mite-transmitted (Lu et al. 2008). They are also mechanically transmissible to *Chenopodium murale*, *Ch. quinoa* and *Ch. foliosum*. In Korea this viruses caused mosaic or streak symptoms in cultivated garlic (Koo et al.

1998). Virions of the *Allexivirus* are very flexous filamentous about 800 nm in length and 12 nm in diameter, have helical symmetry and a surface pattern of cross-banding. The genome is a single-stranded positive sense RNA (size ~9.0 kb) and comprises six ORFs (Chen et al. 2004).

Our purpose of this work was the detection and identification of three garlic viruses: *Garlic virus A* (GarV-A), *Garlic virus B* (GarV-B) and *Garlic virus C* (GarV-C) in eleven garlic production fields located in different regions of Poland. The DAS-ELISA (*Double Antibody Sandwich – Enzyme Linked Immunosorbent Assay*) results was confirmed using molecular biology technique (*Reverse Transcriptase - Polymerase Chain Reaction* RT-PCR). Electron microscope was also used for selective plants infected with *Allexiviruses*.

MATERIALS AND METHODS

Survey of virus infection in garlic plants

Garlic plants were randomly collected from eleven garlic production fields located in different regions of Poland during the 2011-2012 growing season. Garlic leaves and bulbs (80 samples) were tested by DAS-ELISA using antibodies to GarV-A, GarV-B and GarV-C from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Germany), following the protocol described by Clark and Adams (1977). Extract from the investigated plants was obtained by adding 2,5 ml of sample extraction buffer (dilution 1:10) to 0,250 g plant tissue. Substrate hydrolysis was recorded using the Infinite® 200 PRO (Tecan) with the filter of 405 nm wavelength. Samples were considered positive if their optical density readings were at least twice of negative control.

Electron microscope (EM)

Viral particles were observed with a transmission electron microscope (Jeol JEM 1220, Japan). Virus preparations for electron microscope were prepared directly from plant sap diluted 1:20 in phosphate buffer with 0,5% Na_2SO_3 addition accordingly to sap-dip EM methods described by Szyndel (1997). Preparations were stained with 2% uranyl acetate and observed with a transmission electron microscope.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

Total RNA was extracted from 100 mg tissues of leaves of 9 infected (DAS-ELISA-positive) and 5 healthy garlic plants using the Spectrum^{TM} Plant Total RNA Kit (Sigma-Aldrich, Germany). RNA from each sample was eluted in 50 µl of RNAse free water. For RT-PCR, Titan One Tube RT-PCR System (Roche) was used with the primer pairs designed in the conserved region of the open reading frame ORF5 (coat protein) and ORF6 (nucleic acid binding protein) (Tabl. 1).

A primer pair specific to the ribulose 1,5-bisphosphate carboxylase chloroplast (*Rbc1*) gene (Rbc1-F/Rbc1-R 5'-TACTTGAACGCTACTGCAG-3' and 5'-CTGCATGCATTGCACGGTG-3') (Sanchez-Navarro et al. 2005) was used as the internal control to amplify the corresponding mRNA in garlic leaf tissue by RT-PCR. Single tube RT-PCR used a 30 min heating step at 50°C and a 2 min heating step at 94°C followed by 35 cycles of 30 sec melting at 94°C, 45 sec annealing at -°C (Tabl 1), 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 buffer TBE in 1,2 % agarose gel.

Table 1

RT-PCR primers for amplification of the coat protein (CP) and nucleic acid binding protein (NABP) genes of GarV-A, GarV-B and GarV-C together with nucleotide sequence and annealing temperatures

Virus	Primer	Nucleotide sequence	Tm (°C)	Annealing temperature (°C)
GarV-A	ACPF	5'-ATGTCGAATCCAACTCAGTCG-3'	52.4	52
	ACPR	5'-AGACCATGTTGGTGGCGCG-3'	55.4	
GarV-B	BCPF	5'-TGACGGGCAAACAGCAGAATAA-3'	53	50
	BCPR	5'-ATATAGCTTAGCGGGTCCTTC-3'	52.4	
GarV-C	CCPF	5'-TTGCTACCACAATGGTTCCTC-3'	52.4	51
	CCPR	5'-TACTGGCACGAGTTGGGAAT-3'	51.8	

RESULTS

Survey of virus infection in garlic plants

The incidence of virus infection in garlic plants collected from eleven production fields was surveyed by serological DAS-ELISA test. Results indicated that 38 samples (47.5%) were infected with *Garlic virus A*, 49 samples (61.3%) with *Garlic virus B* and 14 samples (17.5%) with *Garlic virus C*. Mosaic symptoms, deformation and yellow stripes were noted on leaves on DAS-ELISA-positive plants.

Electron microscope

To confirm occurrence of three allexiviruses in garlic plants preparations from leaves were observed under the transmission electron microscope. Based on the EM data, about 600-800 nm in length of the rod shaped *Allexiviruses* particles were observed

(Fig. 1). During the observation we reported that different viruses were mixed within a garlic plants.

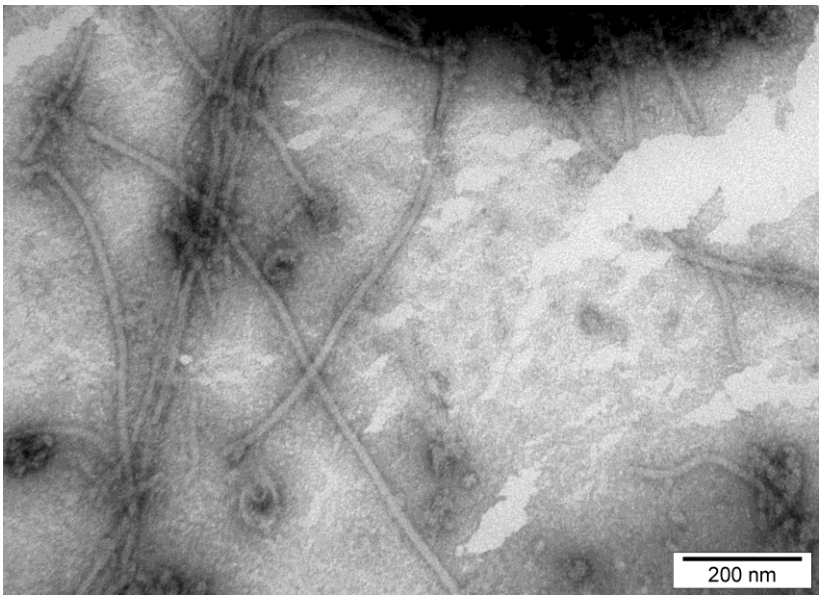

Fig. 1. An electron microscope image of garlic viruses particles.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The presence of GarV-A, GarV-B and GarV-C was confirmed by RT-PCR in nine leaf samples. Products of the expected size (444 bp for GarV-A, 576 bp for GarV-B and 679 bp for GarV-C) were amplified only from the DAS-ELISA-positive samples. As expected, no product was amplified from total RNA of healthy blueberry leaves while a 183 bp long fragment corresponding to the plant internal control Rbc1 was obtained (Figure 2).

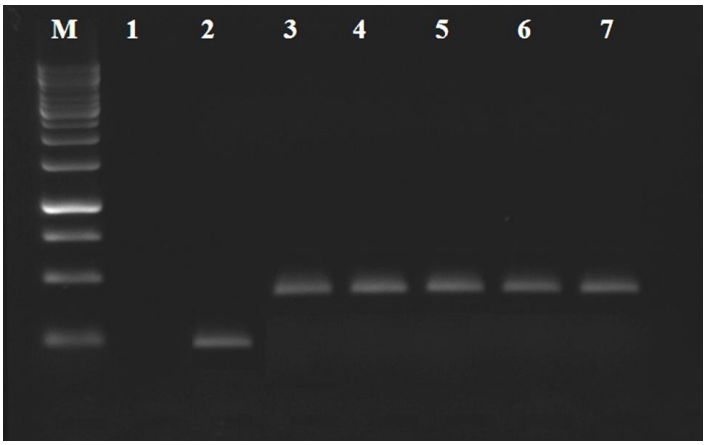

Fig. 2. Agarose gel analysis of Garlic virus A amplicons obtained by RT-PCR assays. Lane M: GeneRuler™ 1 kb DNA Ladder (Fermentas); lanes 1-2: healthy garlic plants (DAS-ELISA-negative samples); lanes 3-7: samples infected with GarV-A (DAS-ELISA-positive samples). The GarV-A amplicons are shown at 444 bp. The Rbc1 amplicon is shown at 183 bp.

DISCUSSION

Garlic is an economically important plant grown worldwide as a medicinal plant and spice. Growers traditionally produce their own garlic propagative material, therefore the observed heavy viral infection and implies a potentially high reduction in yield and quality of this crop.

The allexiviruses, *Garlic virus A* (GarV-A), *Garlic virus B* (GarV-B) and *Garlic virus C* (GarV-C) transmitted by *Aceria tulipae* (Van Dijk et al. 1991) which are reported in this study, induce a mild mosaic, deformation and yellow stripes or no symptoms in *Allium* species (Yamashita et al. 1996), viruses usually occur in mixed infections. *Garlic virus A* (47,5%) and *Garlic virus B* (61,3%) were detected at a high frequency in garlic plants from fields located in various region of Poland, while *Garlic virus C* (17,5%) was detected with a low frequency. However, this results suggested that these viruses are of considerable epidemiological significance.

In Europe. the occurrence of viruses infecting garlic has been reported only in France (Lot et al. 1998), Greece (Dovas et al. 2001) and the Czech Republic (Klukácková et al. 2004, Smékalová et al. 2010), but in Poland only limited data are currently available.

Although ELISA has often been employed for virus diagnosis in garlic (Conci et al. 2003, Shahraeen et al. 2008), RT-PCR is a more efficient and sensitive method of virus detection. Standard RT-PCR has been used to detect allexiviruses (Dovas et al. 2001). In our study we designed RT-PCR primers for amplification of CP and NABP genes of GarV-A, GarV-B and GarV-C. The amplicons of GarV-A, GarV-B and GarV-C were received only for DAS-ELISA-positive plants.

To confirm the occurrence of viruses and characterize virus by viral particle morphology, EM was performed. During the observation flexuous filamentous particles under the transmission electron microscope were found.

Studies involving other species of the genus *Allexivirus* and more production fields are currently continued in Poland.

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