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ASSESSMENT OF ALLEXIVIRUSES INFECTION IN GARLIC PLANTS IN POLAND

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Abstract. Garlic (*Allium sativum* L.) plants may be infected in the field by viruses of the genera *Potyvirus*, *Carlavirus* and *Allexivirus*. These viruses are transmitted by vegetative propagation and by vectors. Detection and identification of allexiviruses was carried out in 2011–2012. It was based on ELISA and RT-PCR assays. Samples of plant material were collected from 26 garlic production fields located in different regions of Poland. *Garlic virus D*, *Garlic virus B* and *Garlic virus X* were the most abundant viruses in all examined regions and were identified in 79%, 64% and 59% of all garlic samples, respectively. *Garlic virus A* and *Garlic virus C* were identified in all studied regions with low frequency. *Garlic virus E* was detected with 100% frequency in east-central Poland. None of the tested garlic samples were infected with *Shallot virus X*. Allexiviruses were always present in garlic plants in mixed infections.

Key words: garlic, allexiviruses, ELISA, RT-PCR

INTRODUCTION

Garlic (*Allium sativum* L.) is one of the most important culinary plants widely cultivated throughout the world. As the crop is vegetatively propagated, viruses are transmitted from one crop cycle to another through infected cloves, as well as by vectors. There are more than 12 virus species infecting garlic world-wide [van Dijk 1993, Sumi et al. 1993, 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.

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.

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The genome is a single-stranded positive sense RNA (size ~ 9.0 kb) and comprises six ORFs, encoding, in order, the replication-related proteins, the first two proteins of a triple gene block (TGB), a serine-rich protein of unknown function, the coat protein (CP) and a putative nucleic acid-binding regulatory protein [Koo et al. 2002, Chen et al. 2004]. The eight species recognized by the ICTV (International Committee on Taxonomy of Viruses) are designed *Garlic virus A* (GarV-A), *Garlic virus B* (GarV-B), *Garlic virus C* (GarV-C), *Garlic virus D* (GarV-D), *Garlic virus E* (GarV-E), *Garlic virus X* (GarV-X), *Garlic mite-borne filamentous virus* (GMbFV) and *Shallot virus X* (ShVX). The serious damage of viruses of the genus *Allexivirus* in garlic cultivations is mainly due to significant decrease of crop quality. 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%.

Information on infection of *Allium* crops by allexiviruses in Europe is limited [Dovas et al. 2001, Dovas and Volvas 2003, Tabanelli et al. 2004, Mavric and Ranikar 2005, Lanzoni et al. 2006, Klukácková et al. 2007]. In this study garlic samples of different origins in Poland were analyzed to determine the occurrence of economically important viruses by enzyme-linked immunosorbent assay (ELISA) and by reverse transcriptase-polymerase chain reaction (RT-PCR).

MATERIALS AND METHODS

In 2011–2012, a total of 321 garlic samples (leaves and bulbs) were collected randomly from 26 field sites located in five geographical districts of Poland: north (Pomerania province), east-central (Mazovia and Łódź provinces), west-central (Wielkopolska province), south (Małopolska and Silesia provinces) and south-western (Lower Silesia and Opole provinces). Viruses were detected by DAS-ELISA in garlic leaf and bulb tissue with specific antibodies against GarV-A, GarV-B, GarV-C and ShVX obtained from Leibniz Institut DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). Samples were prepared by grinding 0.5 g of fresh leaves or bulbs in phosphate buffer saline supplemented with 2% polyvinylpyrrolidone and 0.2% of egg albumin in the ratio of 1:10 (w:v) and tested according to the manufacturer's protocol. After 1 h of incubation at room temperature, substrate hydrolysis was measured as a change in absorbance at OD 405 nm using Infinite® 200Pro microplate reader (Tecan, Austria GmbH). Samples were considered positive if their optical density (OD 405 nm) readings were at least twice those of healthy controls. RT-PCR with total RNA and appropriate primers was used in order to confirm DAS-ELISA results and to detect and identify isolates of GarV-D, GarV-E and GarV-X in garlic plants. Total RNA was extracted from leaf tissue using the Spectrum™ Plant Total RNA Kit according to

the manufacturer's protocol with modification provided by Kalinowska et al. [2012]. For RT-PCR, Transcriptor One-Step RT-PCR Kit (Roche, Mannheim, Germany) was used with the primer pair, designed by the authors, specific to the segment of the GarV-A, GarV-B, GarV-C and GarV-D RNA encoded the coat protein (CP) and nucleic acid binding protein (NABP) genes. Primers pairs for detection of GarV-E, GarV-X and ShVX were designed in the open reading frame I (ORFI, replicase) (tab. 1). A primer pair specific to the ribulose 1.5-bisphosphate carboxylase chloroplast (Rbc1) gene (Rbc1-F/Rbc1-R 5'-TACTTGAACGCTACTGCAG-3' and 5'-CTGCATGCATTGCA CGGTG-3') [Sanchez-Navarro et al. 2005] was used as the internal control to amplify

Table 1. RT-PCR primer pairs specific to the segment of the GarV-A, GarV-B, GarV-C, GarV-D, GarV-E, GarV-X and ShVX RNA together with nucleotide sequence and annealing temperatures

Virus	Primer	Nucleotide sequence	T_m (°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	
GarV-D	DCPF	5'-AAGGAGCTACACCGAAGGAC-3'	53.8	52
	DCPR	5'-TAAAGTCGTGTGGATGCATCAGA-3'	53.5	
GarV-E	EF2	5'-TTGCTAGACCACCTCAGTATTGAGAA-3'	56.4	55
	ER2	5'-TAT TGG GCG TAC ATC GGT GAC TGT-3'	57.4	
GarV-X	XF	5'- GCGGTAATATCTGACACGCTCCA-3'	57.1	55
	XR	5'-ACGTTAGCTTCACTGGGGTAGAATAT-3'	56.4	
ShVX	ShVXF	5'-ACCGAAATCACAGTTAACTCCTTTGG-3'	56.4	54
	ShVXR	5'-TCTACGGTTGTCGATTTTGTGCGT-3'	55.7	

the corresponding mRNA in garlic leaf tissue by RT-PCR. Samples were subjected to a reverse transcription 30 min at 50°C, 2 min of denaturation at 94°C, followed by 35 cycles of 30 s of denaturation, 45 s annealing at -°C (variable), and 45 s 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.

RESULTS

In most investigated virus-infected garlic crops virus symptoms were observed. Virus infected plants were stunted with yellow stripe mosaic and streak symptoms. Based

on ELISA and RT-PCR tests, GarV-D, GarV-B and GarV-X were the most abundant viruses in all sampled regions of Poland. The incidence of these viruses was 79%, 64% and 59%, respectively.

GarV-A and GarV-C were detected and identified in five geographical districts of Poland. In east-central region all tested samples were infected with GarV-E, while in north and south part of country low incidence of this virus was noted. GarV-X was detected only in the north, south and south-western regions (95–100%). None of the tested garlic samples were infected with ShVX (tab. 2).

Table 2. Incidence of viruses in garlic crops in different regions of Poland

Geographical districts of Poland	Number of samples	GarV-A (%)	GarV-B (%)	GarV-C (%)	GarV-D (%)	GarV-E (%)	GarV-X (%)	ShVX (%)
North	41	13	75	16	100	33	100	0
East-central	90	69	62	35	100	100	0	0
West-central	25	70	100	20	0	0	0	0
South	125	33	63	20	95	43	95	0
South-western	40	20	20	40	100	0	100	0
Total	321	41	64	26.2	79	35.2	59	0

The majority of the tested plants were infected with a mixture of up to five different allexiviruses (tab. 3). Four viruses, GarV-B, GarV-D, GarV-E and GarV-X, represented the most frequent combination detected in mixed infections. The triple virus infection of GarV-B, GarV-D and GarV-X was found at a lower, but still significantly level.

Table 3. Percent of multiple infections of allexiviruses

Number of viruses in sample	%
Two viruses	6.45
Three viruses	51.61
Four viruses	38.71
Five viruses	6.45

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 garlic leaves while a 183 bp long fragment corresponding to the plant internal control Rbc1 was obtained (fig. 1).

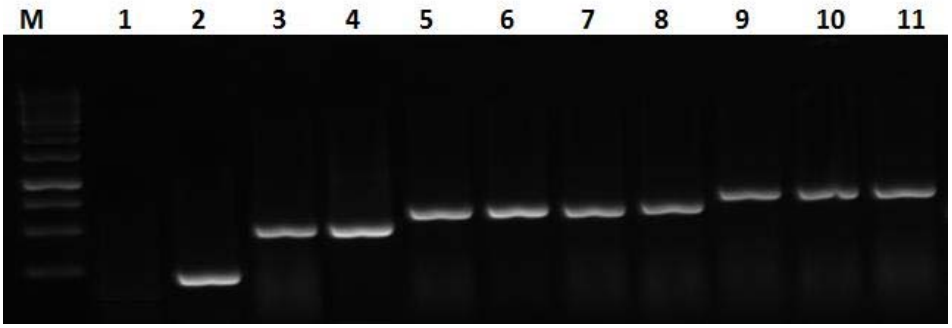

Fig. 1. Agarose gel analysis of GarV-A, GarV-B and GarV-C amplicons obtained by RT-PCR tests. Lane M: GeneRuler™ 1 kb DNA Ladder (Fermentas); lanes 1–2: healthy garlic plants (DAS-ELISA-negative samples); lanes 3–4: samples infected with GarV-A (444 bp); lane 5–8: samples infected with GarV-B (576 bp); lane 9–11: samples infected with GarV-C (679 bp). The Rbc1 amplicon is shown at 183 bp

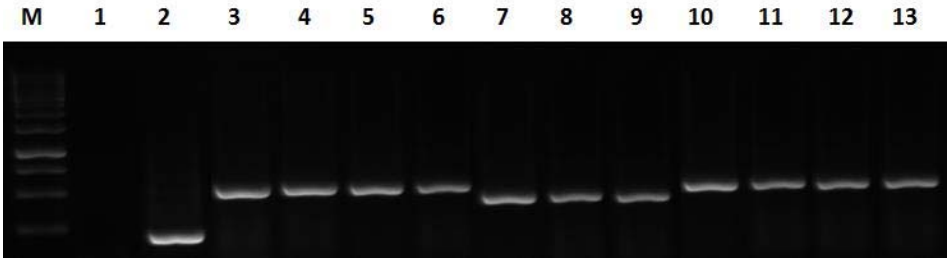

Fig. 2. Agarose gel analysis of GarV-D, GarV-E and GarV-X amplicons obtained by RT-PCR tests. Lane M: GeneRuler™ 1 kb DNA Ladder (Fermentas); lanes 1–2: healthy garlic plants (DAS-ELISA-negative samples); lanes 3–6: samples infected with GarV-D (456 bp); lane 7–9: samples infected with GarV-X (386 bp); lane 10–13: samples infected with GarV-E (458 bp). The Rbc1 amplicon is shown at 183 bp

RT-PCR with the appropriate primers also revealed the presence of GarV-D, GarV-E and GarV-X in garlic plants growing in different parts of Poland (fig. 2).

The Allexiviruses sequences determined in this study are available in GenBank as accession Nos JX628786.1-JX628790.1 (GarV-A); JX561093.1, JX561094.1, KC117180.1- KC117182.1, KC517076.1 (GarV-B); JX628791.1, KC491199.1 (GarV-C); KC491198.1 (GarV-D); KC491196.1 (GarV-E) and KC491197 (GarV-X).

DISCUSSION

Garlic is an economically important plant grown worldwide as a medicinal plant and spice. Growers traditionally produce their own garlic to propagate the successive crop, therefore viruses continue to persist, resulting in reduce of yield and product quality.

Allexiviruses, transmitted by *Aceria tulipae* [Dijk van 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]. These viruses usually occur in mixed infections. Our results showed that garlic crops in Poland are heavily infected with viruses. *Garlic virus D*, *Garlic virus B* and *Garlic virus X* were found to be the most abundant and widespread viruses in the country (up to 100%), while *Garlic virus A, Garlic virus C* and *Garlic virus E* were detected at a lower frequency. *Shallot virus X* has not been detected in tested garlic samples.

In Europe, 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 [Chodorska et al. 2012].

Although ELISA has often been employed for virus diagnosis in garlic [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 the amplification of CP and NABP genes of GarV-A, GarV-B, GarV-C and GarV-D and three primer pairs for the detection GarV-E, GarV-X and ShVX by amplification of replicase gene. The amplicons of GarV-A, GarV-B and GarV-C were obtained only for DAS-ELISA-positive plants.

The information concerning the incidence of garlic viruses is important for virus indexing for virus-free propagative material. Infected plants, including mother plants, comprise the pathogen source for the already existing and new plantations. What is more, some of the viral species described in literature may be transmitted by vectors, which also spread garlic viruses and the diseases they cause.

This report presents the results of the detection and identification of seven garlic viruses belonging to the genus *Allexivirus*. Furthermore, it is a source of important information concerning the protection of garlic crops against these pathogens as well as breeding for virus resistance of the new garlic cultivars to the tested viruses.

CONCLUSIONS

1. GarV-D, GarV-B and GarV-X were the most abundant garlic viruses in all examined region of Poland.
2. Garlic viruses always occurred in mixed infections.

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OCENA STANU PORAŻENIA PRZEZ ALLEXIWIRUSY ROŚLIN CZOSNKU W POLSCE

Streszczenie. Czosnek (*Allium sativum* L.) może być porażany przez wiele gatunków wirusów należących do rodzajów: *Potyvirus*, *Carlavirus* i *Allexivirus*, które przenoszone są z materiałem rozmnożeniowym, a w okresie wegetacji za pośrednictwem wektorów. Ba-

dania przeprowadzono w latach 2011–2012. Ich celem było wykrywanie allexiwirusów porażających rośliny czosnku w Polsce oparte na teście serologicznym ELISA oraz technice RT-PCR. Próby materiału roślinnego pobierano losowo z 26 pól produkcyjnych czosnku. Wirus D czosnku, wirus B czosnku oraz wirus X czosnku były wykrywane we wszystkich badanych rejonach w, odpowiednio, 79, 64 i 59% badanych prób. Wirus A czosnku oraz wirus C czosnku występowały w mniejszej liczbie prób. Wirus E czosnku został wykryty we wszystkich próbach pobranych z pól zlokalizowanych w centralno-wschodniej Polsce. W żadnej z badanych prób nie stwierdzono obecności wirusa X szalotki. Allexiwirusy w roślinach czosnku zawsze występowały w mieszanych infekcjach.

Słowa kluczowe: czosnek, allexiwirusy, ELISA, RT-PCR

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