FRESH ALLEXIVIRUSES ON THE POLISH MARKET

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Abstract

The aim of the research conducted was to detect and identify allexivuruses: GarV-A, GarV-B, GarV-C, GarV-D, GarV-E, GarV-X and ShVX in garlic plants from Australia, Egypt, Mexico, Turkey and Italy available on the Polish retail market by ELISA as well as RT-PCR. Allexiviruses of garlic plants have always occurred in mixed inflections. GarV-A, GarV-B, GarV-C, GarV-D, GarV-E and GarV-X were detected in garlic from Australia, Egypt and. The Mexican materials were infected with GarV-A, GarV-B, GarV-D, GarV-E and GarV-X. GarV-A and GarV-C were not detected in garlic from Turkey, whereas the garlic from Italy were GarV-C and GarV-E-free. As regards the material gathered in different parts of the world, the largest amount of onions were infected with GarV-D and GarV- X, whereas GarV-C was detected in few garlics from Australia and Egypt. ShVX was detected in none of the tests conducted.

Key words: *garlic plant, Allexivirus, DAS-ELISA, RT-PCR technique*

1. INTRODUCTION

Allium sativum L. belongs to the family *Liliaceae.* It is one of the oldest crop plants and it is not only a valued spice plant, but also a major pharmaceutical raw material due to its pro-health properties (Marjanowski 1988).

Allexiviruses comprise the most sizable group of viruses endangering garlic crops. 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 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%.

Allexiviruses in garlic crops were detected in Argentina (Helguera et al. 1997; Lunello et al. 2000; Conci et al. 2003), Australia (Wylie et al. 2014), Brazil (Melo-Filho et al. 2004; Fayad-Andre 2011), China (Chen et al. 2001), Czech Republic (Klukackova et al. 2007), Greece (Dovas et al., 2001), Spain (Tabanelli et al. 2004), Iran (Shahraeen et al. 2008), Japan (Yamashita et al. 1996; Sumi et al. 1999; Takaichi et al. 2001), South Korea (Song et al. 1998; Kwang-Sook et al. 2007; Lee et al. 2007), New Zealand (Ward et al. 2009), Italy (Dovas and Volvas 2003; Lanzoni et al. 2006), Slovenia (Mavric and Ravnikar 2005), Russia (Kanyuka et al.1992), the USA (Gieck et al. 2009).

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%.

The polish production of garlic maintains the level of circa 15-20.000 tons and is conducted on a surface of around 3.000 ha, mainly in the following voivodeships: Lodz, Masovian, Lesser Poland and Pomeranian. Apart from domestic garlic crops, material coming from different parts of the world is also available on the Polish retail market.

The research on allexiviruses in Poland has been conducted since 2010. Chodorska et. al. (2012; 2013; 2014) had detected *Garlic virus A*, *Garlic virus B*, *Garlic virus C*, *Garlic virus D, Garlic virus E* and *Garlic virus X* in bulbs of garlic from production fields located in the following voivodeships: Lodz, Masovian, Lesser Poland and Pomeranian. 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.

The aim of the research conducted was to detect and indentify seven virus species belonging to the *Allexivirus* genus: GarV-A, GarV-B, GarV-C, GarV-D, GarV-E, GarV-X and ShVX in garlic plants from different parts of the world available on the Polish retail market using ELISA test (enzyme-linked immunosorbent assay) as well as RT-PCR technique (reverse transcription polymerase chain reaction).

2. MATERIALS AND METHODS

The research was conducted in the Department of Plant Pathology WULS-SGGW, Warsaw in the years 2013-2014. The plant material (bulbs) originating from Australia, Egypt, Mexico, Turkey and Italy was available on the Polish retail market. A total of 49 bulb samples were tested on the presence of allexiviruses (Table 1).

Viruses were detected by DAS-ELISA in garlic bulb tissues 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 bulbs in phosphate buffer saline 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. 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). 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 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 (Table 2.).

Table 2. 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 GACTGT-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	

3. RESULTS AND DISCUSSION

The garlic bulbs used in the research by the authors of this work originated from Australia, Egypt, Mexico, Turkey and Italy and were available on the Polish retail market.

The presence of GarV-A, GarV-B and GarV-C was noticed by DAS-ELISA and confirmed by RT-PCR in bulb 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. RT-PCR with the appropriate primers also revealed the presence of GarV-D, GarV-E and GarV-X in tested garlic materials (Fig. 1., 2., 3.).

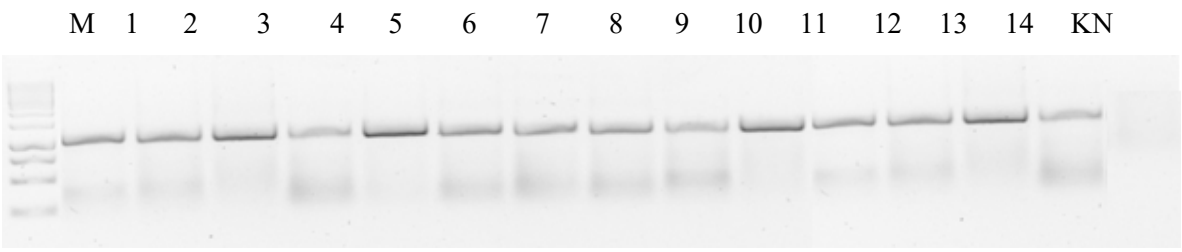


Fig. 1. Agarose gel analysis of GarV-X amplicons obtained by RT-PCR tests. Lane M: GeneRuler™1 kb DNA Ladder (Fermentas); lanes 1–14 samples infected with GarV-X, line KN healthy garlic plants (DAS-ELISA-negative samples).

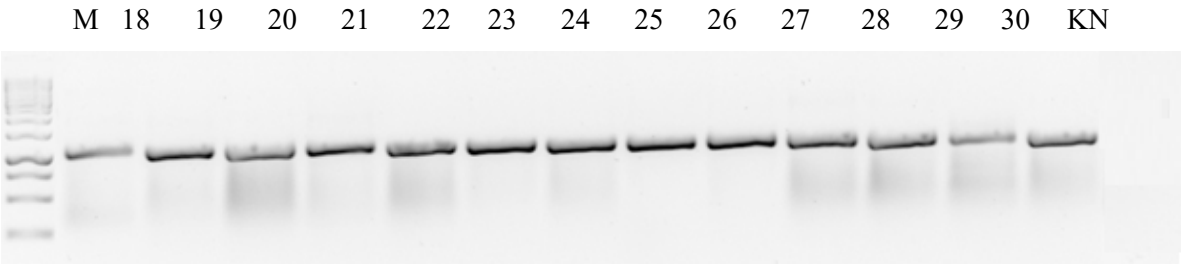


Fig. 2. Agarose gel analysis of GarV-D amplicons obtained by RT-PCR tests. Lane M: GeneRuler™1 kb DNA Ladder (Fermentas); lanes 18–30 samples infected with GarV-D, line KN healthy garlic plants (DAS-ELISA-negative samples).

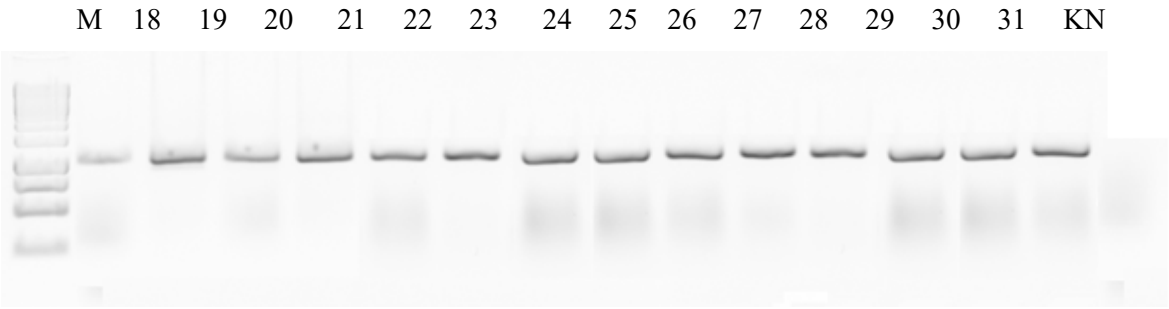


Fig. 3. Agarose gel analysis of GarV-B amplicons obtained by RT-PCR tests. Lane M: GeneRuler™1 kb DNA Ladder (Fermentas); lanes 18–31 samples infected with GarV-B, line KN healthy garlic plants (DAS-ELISA-negative samples).

The presence of GarV-A, GarV-B, GarV-C, GarV-D, GarV-E and GarV-X was detected in garlic from Australia and Egypt. Mexican garlic plants were infected with GarV-A, GarV-B, GarV-D, GarV-E i GarV-X. GarV-A and GarV-C did not occur in bulbs from Turkey while bulbs from Italy were not infected by GarV-C or GarV-E. The largest amount of bulbs from different parts of the word were infected with *Garlic virus D* and *Garlic virus X* whereas *Garlic virus C* was detected in only a few bulbs from Australia and Egypt. ShVX was not detected in any of the samples tested (Table 1).

The results presented correspond with the data published in literature on the subject of the occurrence the *Allexivirus* genus in garlic plants in different parts on the world (Helguera et al. 1997; Lunello et al. 2000; Conci et al. 2003; Wylie et al. 2014; Melo-Filho et al. 2004; Fayad-Andre 2011; Chen et al. 2001; Klukackova et al. 2007; Dovas et al., 2001; Tabanelli et al. 2004; Shahraeen et al. 2008; Yamashita et al. 1996; Sumi et al. 1999; Takaichi et al. 2001; Song et al. 1998; Kwang-Sook et al. 2007; Lee et al.2007; Ward et al. 2009; Dovas and Volvas 2003; Lanzoni et al. 2006; Mavric and

Ravnikar 2005, Kanyuka et al. 1992; Gieck et al. 2009). On the other hand, the research results are a source of new previously unpublished data.

In addition to GarV-A, GarV-B, GarV-C, GarV-D GarV-X detected by Wylie et al. (2014) in bulbs of Australian garlic, GarV-E was also detected therein.

Research concerning the occurence of allexiviruses in garlic crops cultivated in Egypt, Mexico and Turkey is yet to be conducted. The authors of this work found GarV-A, GarV-B, GarV-C, GarV-D, GarV-E, GarV-X in Egyptian garlic, GarV-A, GarV-B, GarV-D, GarV-E, GarV-X in Mexican garlic and GarV-B, GarV-D, GarV-E, GarV-X in Turkish garlic.

According to our knowledge, this type of research was conducted in Poland for the first time. Chodorska et al. (2012; 2013; 2014) detected and identified the *Allexivirus* genus in garlic crops from fields located in the following voivodeships: Lodz, Masovian, Lesser Poland and Pomeranian. Upon analyzing the results of ELISA and RT-PCR technique, they established that GarV-D, GarV-X and GarV-B were the most frequently occurring viruses. GarV-A and GarV-C were detected in the voivodeships listed above, albeit with a frequency lower than the other species. All the plants from Masovian and Pomerian voivodeships tested were 100% infected with GarV-D and GarV-E as well as GarV-D and GarV-X. ShVX was not detected.

The detection of allexiviruses in garlic available on the retail market in different parts of the world is of unquestionable significance for basic research. It provides the Main Inspectorate of Plant and Seed Inspection, whose main task is to carry out phytosanitary inspections of plants in places of their importation into Poland, with significant data as well as is of importance for Polish garlic producers. It is sometimes possible that batches of bulbs imported for human consumption are used for cultivation. Worse still, it is common for a large part of garlic available on the Polish retail market to be imported without compliance with applicable regulations. This can result in the introduction of new virus species as well as new isolates of viruses already present, which could constitute a real threat to domestic planting in the future. Therefore, the intense development of trade in plant materials, especially in propagating material, which contributes significantly to the spread of plant pathogens, should take place in compliance with the appropriate phytosanitary requirements.

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-CR 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 garlic viruses belonging to the genus *Allexivirus*. Furthermore, it is a source of important information concerning the protection of domestic garlic crops against these pathogens as well as breeding for virus resistance of the new garlic cultivars to the tested viruses.

Table 1. Incidence of *Allexivirus* in garlic plants in different parts of the world

Country	The number of the samples tested	The number of the samples infected with virus/The percentage of the samples infected with virus						
		GarV-A	GarV-B	GarV-C	GarV-D	GarV-E	GarV-X	ShVX
Australia	6	4 /66,67/	1 /16,67/	2 /33,33/	5 /83,33/	4 /66,67/	4 66,67	0 0
Egypt	10	9 /90/	8 /80/	3 /30/	8 /80/	4 /40/	10 /100/	0 /0/
Mexico	10	8 /80/	10 /100/	0 /0/	10 /100/	3 /30/	10 /100/	0 /0/
Turkey	8	0 /0/	4 /50/	0 /0/	4 /50/	1 /12,8/	4 /50/	0 /0/
Italy	15	2 /13.33/	1 /6.67/	0 /0/	1 /6.67/	0 /0/	1 6,67	0 0
Total	49	23 /46.94/	24 /48.98/	5 /10,20/	28 /57.14/	12 /24.49/	29 /59.18/	0 /0/

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