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Research Article

Molecular Identification of Important Viruses of Garlic in Egypt

Atef Mohamed* and Mohamed Hassan

Department of Plant Pathology, Faculty of Agriculture, Fayoum University, Egypt

***Corresponding Author:** Atef Mohamed, Department of Plant Pathology, Faculty of Agriculture, Fayoum University, Egypt.

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Abstract

Egypt is among the top four major garlic producing countries in the world. Garlic (Allium sativum L.) is infected by numerous viruses, which form a viral complex disease. Garlic-infecting viruses are transmitted by vectors like thrips, aphid, and eriophyid mites which heavily infest garlic in Egypt. In this study, major garlic viruses have been tested in virus-like symptoms samples, collected from three different governorates in Egypt. RT-PCR was performed using generic and specific primers followed by sequencing to evaluate the sanitary status of garlic in Egypt. Out of 120 garlic samples tested (75%) were infected with at least one virus. The newly released local garlic cultivar Sids-40 was marked less infected than cultivar Balady. The presence of Leeks Yellow Strip Virus (LYSV, Potyvirus), Onion Yellow Dwarf Virus (OYDV, Potyvirus), and Iris Yellow Spot Virus, Tospovirus) have been confirmed. The four allexiviruses, GarV-A, GarV-B, GarV-D, and GarV-X were detected for first time in Egypt. Most detected viruses were found in mixed infections and in various combinations. There was no correlation between the association of the virus species and the symptoms, proving that virus-like symptoms are not a reliable indicator of the viral status of garlic planting material. Phylogenetic and sequence analysis data obtained indicated that the Egyptian isolates are genetically diverse and not geographically segregated, although a limited number of samples were examined.

Keywords: Garlic; Allexivirus; Potyvirus; Tospovirus; detection; Egypt

Introduction

In Egypt, garlic is an economically important cash crop for domestic consumption and exportation. Egypt is among the world's top four garlic producing countries. Up to thirteen viruses belonging to four different viral genera have been reported to infect garlic in the Mediterranean region, including thrips-transmitted tospoviruses, aphid-transmitted carlaviruses, aphid-transmitted potyviruses, and mite-transmitted allexiviruses [1,2]. Of these, the most common are, Leek Yellow Stripe Virus (LYSV; Potyvirus), Onion Yellow Dwarf Virus (OYDV; Potyvirus) [3]; the allexiviruses, garlic virus A, B, C, D, E, and X (GarVA, -B, -C, -D, -E, and -X) [4-7]. In addition the thrips-transmitted tospovirus, Iris Yellow Spot Virus (IYSV) has been reported [8,9].

Globally, numerous studies have identified and characterized the virus's infecting garlic, including nearby countries to Egypt in Africa and Mediterranean basin [1,10-12].

In Egypt, available data is scarce on garlic viruses. Only Onion Yellow Dwarf Virus, Leek Yellow Stripe Virus and Iris Yellow Spot Virus have been reported in Egypt [13-15].

Egyptian garlic cultivars are suffering a decline indicated by lower bulb yield and smaller bulb size [16]. Vegetative propagation of garlic and viral transmission by arthropod vectors have significantly contributed to the accumulation and dissemination of garlic-infecting viruses often referred to as a 'garlic viral complex [17-19]. It is estimated that viruses can reduce garlic yield by up to 88% over time [20]. In addition, the bulb weight of virus-free garlic plants has been shown to be 32-216% higher than that of infected plants for most cultivars tested [21,22]. In the past, serology and host range tests have been used for plant virus identification. However, these methods are time-consuming, of limited sensitivity and of variable specificity. There have been reports of the successful deployment

of degenerate virus genus- or family-specific primers for reliable detection and characterization of several known or unknown viruses from their respective groups. At present, genus-specific degenerate PCR primers have been designed and published for about a dozen plant virus groups, including Potyvirus [23], Tospovirus [24,25], and Allexivirus [26].

Having recognized the lack of knowledge concerning the identification and distribution of garlic viruses in Egypt, the aim of this study is to develop and apply genus-specific degenerate primer-mediated RT-PCR assays to identify and characterize garlic viruses in Egypt.

Materials and Method

Sample collection

A survey was conducted in the main garlic growing areas of Fayoum, Beni Suef and Menia governorates. Sample collection was carried out during the growing seasons of 2020-2021 to collect. Fields were selected in the main garlic growing areas of the different zones. By moving in a zigzag pattern across the fields, a random sample of symptomatic and asymptomatic leaves was harvested from 8-10 plants, making a composite sample. A total of 120 young leaf samples exhibiting virus-like symptoms from the surveyed fields were collected. Samples were stored in a deep freezer at temperatures of -20°C until total nucleic acid extraction.

Extraction of total nucleic acids (TNAs)

According to Gibbs and Mackenzie [27], 0.5 gm of young leaves tissues were homogenized in 600 µl CTAB buffer (2% w/v CTAB, 1.4 M NaCl, 0.1 M Tris-HCl, pH 8.0, containing 0.5% β-mercaptoethanol). The mixture was incubated at 55 °C for 10 min. and 400 µl Chloroform, isoamyl alcohol (24:1 v/v), was added to the mixture and vortexed for emulsification. The mixture was then centrifuged at 14,000 xg for 10 min. and the aqueous top phase was transferred to a clean Eppendorf tube. Half volume of isopropanol was added to the Eppendorf tubes and mixed well then placed in the freezer at – 20°C for 15 min. Nucleic acids were pelleted by centrifuging the mixtures at maximum speed (14.000 xg) for 10 min. Supernatants were poured off and 1 ml of 70% ethanol was added, and the tubes were centrifuged for 1 min and the pellets were air dried for a maximum of 5 min. Then the nucleic acid pellets were dissolved in 50 µl sterile nuclease-free H2O. The samples were stored at -20°C for later use.

Detection of RNA viruses using genus-specific RT-PCR

To detect garlic infected potyviruses, a generic primers was used according to [28]. Genus-specific primers to detect allexiviruses developed by [26] was used. Universal tospoviruses reverse primer and IYSV specific primers was developed and optimized in this study (Table 2). To determine the virus species of detected allexiviruses, specific primers to GarV-A, GarV-B, GarV-C, GrV-D, and GarV-X were used according to [29,30]. One step RT-PCR protocol was used, such as Qiagen One Step RT-PCR system.

Cloning of RT-PCR products in TA cloning kits and nucleotide sequence determination:

Briefly, RT-PCR products were electrophoresed on 1% agarose gel to check their quality before use. amplicons were directly sequenced after cleaning through a QIAGEN PCR purification kit following the manufacturer's instructions. Subsequently, PCR products were cloned into a TA-cloning plasmid, pCR2.1 vector (Invitrogen, Carlsbad, CA). Clones with proper inserts were sequenced using an automated sequencer (Macrogen Company).

Sequence analysis and identification of virus, strains:

Sequences of detected viruses aligned with all known garlic viruses on the NCBI database using BLASTn available on the NCBI. Pairwise alignment of each detected virus was further analyzed and processed using Clustal-W implemented in BioEdit computer software [31]. Phylogenetic trees were constructed using MEGA-11 program, using Neighbor-joining method with 1000 boot-strap replication [32].

Results

Survey and symptoms

Garlic crops consisting of both local garlic cultivar (Balady) and improved cultivar (Sids 40) commonly cultivated in Egypt were surveyed. A total of 25 garlic fields were visited in various major growing locations in Egypt. The surveyed fields, located in three governorates, were distributed as follow: ten fields in Fayoum Governorate, twelve fields in Beni Suef Governorate and three fields in Menia governorate. A total of 120 plants were inspected and samples collected. Monitoring of virus symptoms indicated that 5% of the samples had virus-like symptoms. Virus-like symptoms such as leaf striping and mild mosaic yellowing were observed in only a few garlic fields during the survey and no clear virus-like symptoms were encountered in most fields. Symptoms appear not to be genotyped dependent. However, other nonspecific symptoms were observed in inspected fields, such as small and malformed bulbs, which could be caused by viral infection. These cloves were collected for further tests to ascertain if viral infections are the cause of such symptoms.

Detection of potyviruses

A total of 55 samples out of 120 were tested positive (45%) using the generic potyvirus primers pairs targeting the NIb-coding region [28]. 42 samples were positive from cultivar Balady (76%), whereas (24%) of samples tested positive from cultivar Sids-40, in all surveyed area. Nine representatives' positive amplicons were selected randomly and sequenced to ascertain the potyviruses identity. Five isolates showed the highest nucleotide identity with OYDV, whereas four isolates showed the highest identity with LYSV. OYDV isolates showed sequence identity (99%) between each other's. Whereas LYSV isolates showed sequence identity (98%) between the three isolates. To get the full amplicon sequences, representative amplicon of both viruses was cloned, and three clones sequenced. The consensus sequences of LYSV and OYDV were subjected to BLASTn analysis. Sequence LYSV-BS isolate from Beni Suef Governorate, revealed 92% identity to LYSV garlic isolate from Tunisia (MH890557). Nucleotide sequence of OYDV-Fy isolate from Fayoum Governorate showed 92% identity to OYDV infecting garlic from Sudan (KF623541).

Pairwise comparison and phylogenetic analysis revealed that both LYSV and OYDV are rather diversified from others detected in nearby countries (Figure 1). Both sequences have been submitted to Genbank and their accession number were (LYSV-Bs; OQ924131 and OYDV-Fy; OQ924132).

Phylogenetic analysis using partial nucleotide sequence of NIb-coding region of LYSV and OYDV with corresponding isolates retrieved from NCBI database and Yellow Oat Grass Virus (YOgV-Sb; NC024471) was used as out-group. The Egyptian isolate OYDV-Fy was clustered together with other OYDV isolates from Sudan (KF623541) and Argentina (JX433019 and JX433020) in the same cluster with high bootstrap value. Whereas the Egyptian isolate of LYSV clustered with isolate from Tunisia (MH890557) and India (MT731491) (Figure 1).

Detection of Allexiviruses using universal primers.

The reported genus-specific allexiviruses primer pair targeting the CP-NBAP coding region successfully amplified products of the expected band size about 750 bp from a total of 91 out of 120 samples tested (75%). Representative samples positive for allexiviruses subjected to RT-PCR using specific primers to Garlic Virus A (GarV-A), Garlic virus B (GarV-B), Garlic virus C (GarV-C), Garlic virus D (GarV-D), Garlic virus X (GarV-X) [29]. Virus-specific RT-PCR amplicon of the expected size was only obtained for GarV-A, GarV-B, GarV-D, and GarV-X. It was thus confirmed that 91 allexiviruses positive samples, seventy-four samples were infected with GarV-D,

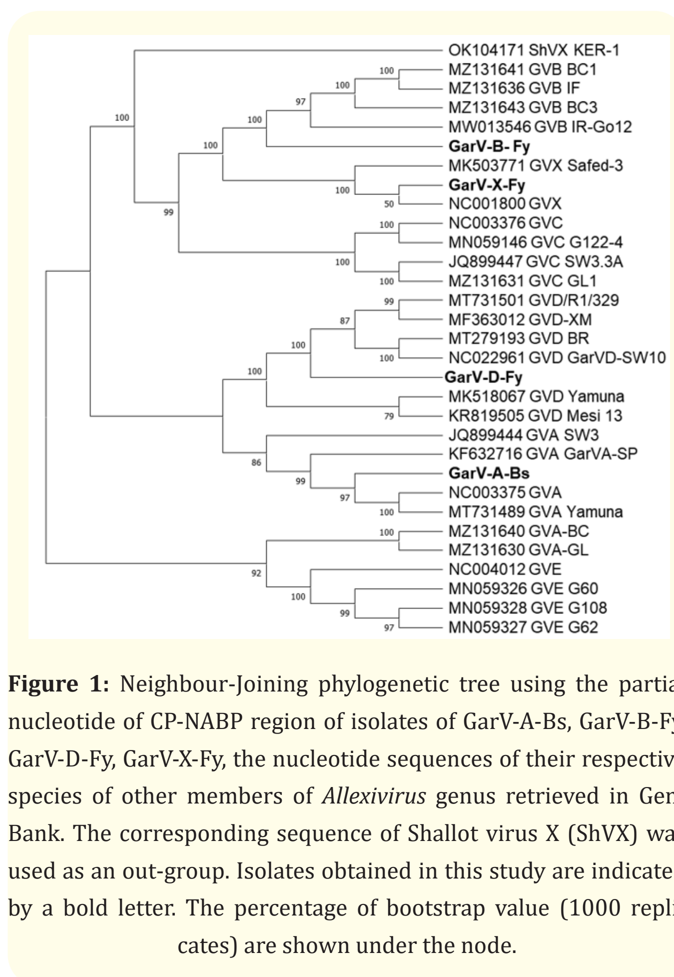

Figure 1: Neighbour-Joining phylogenetic tree using the partial nucleotide of CP-NABP region of isolates of GarV-A-Bs, GarV-B-Fy, GarV-D-Fy, GarV-X-Fy, the nucleotide sequences of their respective species of other members of *Allexivirus* genus retrieved in GenBank. The corresponding sequence of Shallot virus X (ShVX) was used as an out-group. Isolates obtained in this study are indicated by a bold letter. The percentage of bootstrap value (1000 replicates) are shown under the node.

fifty-six contained GarV-X, fifteen GarV-A and seventeen were positive for GarV-B. All allexiviruses positive samples were negative for (GarV-C) using specific RT-PCR, suggesting that these viruses are absent in tested samples. Mixed infection of two or more of detected allexiviruses in tested samples were found in Sids-40 and Balady cultivars collected from all surveyed areas in Egypt (Table 1). The sequences of Egyptian allexiviruses were deposited in GenBank (Accession numbers: OQ924126 for GarV-A; OQ924130 for GarV-B, OQ924127 for GarV-D and OQ924128 for GarV-X).

Comparison with other allexiviruses sequences available on GenBank revealed that, the Egyptian isolates shared identity to a range of orthologous. Using Blastn impeded in NCBI website https://blast.ncbi.nlm.nih.gov/Blast.cgi. GarV-A isolate-Bs shared 97% nucleotide identity with isolates from India and United Kingdom (MW653921; AJ551478). GarV-B isolate-Fy showed the highest nucleotide identity of 96% with an isolate from Sudan (KC207716). GarV-D isolate-Fy isolate showed 96% of nucleotide identity with an isolate from Poland (MN167132).

Genus/species	Primer name	Primer sequence 5' to 3'	Reference
Potyvirus	NIb2-F	GTITGYGTIGAYGAYTTYAAYAA	Zheng., *et al*. 2010
	NIb3-R	TCIACIACIGTIGAIGGYTGNCC	
Allexivirus	CP-F	TGGRCXTGCTACCACAAYGG	Chen., *et al*. 2004
	NABP-R	CCYTTCAGCATATAGCTTAGC	
Garlic virus A	GarV-AF	ATGTCGAATCCAACTCAGTCG	Chodorska., *et al*. 2014
	GarV-AR	AGACCATGTTGGTGGCGCG	
Garlic virus B	GarV-BF	GAGGAGAACTAACGCCACAC	Gieck., *et al*. 2009
	GarV-BR	ACGACCTAGCTTCCTACTTG	
Garlic virus C	GarV-CF	TTGCTACCACAATGGTTCCTC	Chodorska., *et al*. 2014
	GarV-CR	TACTGGCACGAGTTGGGAAT	
Garlic virus D	GarV-DF	GCTCACTCRGATGTGTTAGC	Gieck., *et al*. 2009
	GarV-DR	CGCGTGGACATAAGTTGTTG	
Garlic virus X	GarV-X(+)	GCGGTAATATCTGACACGCTCCA	Chodorska., *et al*. 2014
	GarV-X (-)	ACGTTAGCTTCACTGGGGTAGAATAT	
Tospovirus	UPA-TOS_R	GACCACGCGTATCGATGTCGACAGAGCAATC	This study
Iris yellow spot virus	IYSV-F	AGTTGCAAGAGTCATGGCAGC	
	IYSV-R	GACTCACCAATGTCTTCAACAATC	

Table 1: Generic and specific primer sequences used for detection and identification of garlic viruses in this study.

The phylogenetic relatedness of the studied allexiviruses was inferred using the partial nucleotide sequences of the CP-NBAP coding region. The CP-NBAP coding region has well-resolved clades each containing distinct allexivirus species (Figure 2). The four detected allexiviruses from Egypt (GarV-A, GarV-B, GarV-D, and GarV-X) clustered with high bootstrap value with corresponding species members. However, the most diverse allexivirus was GarV-B-Egy isolate which was distant from all other isolates and formed separated branch with high bootstrap value, sharing an ancestor with isolates from Iran (MW013546) and Republic of Korea (MZ131641). Similarly, GarV-D and GarV-A were distant from the closest corresponding allexiviruses isolates from other countries, suggesting Egyptian isolates are divergence.

Detection of IYSV using specific primers.

RT-PCR using Iris Yellow Spot Virus (IYSV) specific primers targeting nucleocapsid protein gene, amplicon of the expected band size (191 bp) was obtained from 26 samples out of 120 (21%). PCR fragments were purified and sequenced using IYSV specific primers. BLASTn analysis revealed that IYSV Egyptian isolates have the highest nucleotide identity of 98% to IYSV isolate infecting onion from Egypt (KC161369). The phylogenetic analysis show that the IYSV isolate infecting garlic from Egypt clustered together in one clade with high bootstrap value other IYSV onion isolates from Egypt (KC161369) (Figure 3).

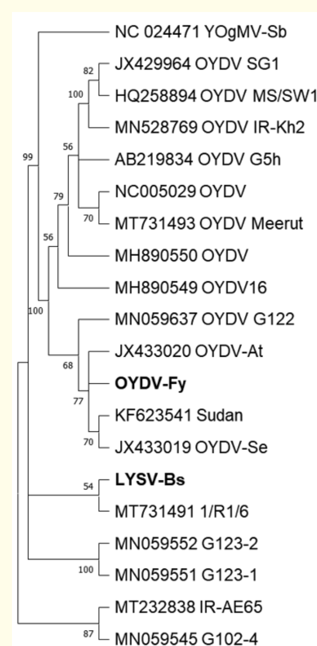

Figure 2: Phylogenetic tree based on partial nucleotide of NIb region showing the relationship of the different potyviruses isolates identified in this study with respected Allium infecting-potyvirus sequences retrieved in GenBank. The corresponding sequence of Yellow oat grass virus (YOgV) was used as an out-group. Isolates obtained in this study are indicated by a bold letters. The percentage of bootstrap value (1000 replicates) are shown under the node.

Discussion

Garlic is a crop that has been grown in Egypt since the time of the ancient Egyptians. Nowadays, Egypt is among the top four of major garlic producing countries. However, Egyptian garlic cultivars are reported to be suffering degeneration, as indicated by lower bulb yield and smaller bulb size, which represents a crucial problem [16]. Viral infection reported to cause the greatest economic losses in garlic production [20,22,33,34]. To date, there has been a lack of data on the viruses infecting garlic in Egypt [14,15].

Our results show that garlic is heavily infected with viruses in Egypt. GarV-D had the highest disease incidence of 61.5% among the total garlic samples collected from fields, while the lowest disease incidence was found in samples infected with GarV-A at 12.5%. However, the incidence percentages of plants infected with GarV-B and GarV-X were 14.1% and 46.6% respectively (Table 2). Also, table 2 revealed that IYSV was responsible for 21.6% of the total garlic samples' disease incidence.

Governorate	Variety	Samples number	*Allexivirus*				*Tospovirus*
			GarV-A	GarV-B	GarV-D	GarV-X	IYSV
Fayoum	Balady	49	9/49	11/49	39/49	26/49	9/49
	Sids 40	11	0/11	0/11	2/11	3/11	2/11
Total		60	9	11	41	29	11
Beni-Suef	Balady	31	4/31	5/31	20/31	18/31	8/31
	Sids 40	9	0/9	1/9	3/9	3/9	1/9
Total		40	4	6	23	21	9
Minia	Balady	11	2/11	0/11	7/11	4/11	5/11
	Sids 40	9	0/9	0/19	3/19	2/19	1/19
Total		20	4	0	10	6	6
Total		120	15/120	17/120	74/120	56/120	26/120

Table 2: Viruses detected using specific primers RT-PCR for from garlic (*Allium sativum*) plants collected from three different governorates in Egypt.

A total of seven viruses, reported to be prevalent in garlic elsewhere in the world, were detected in garlic samples from Egypt [10-12,17,26,35].

Interestingly, we found that the incidence of allexiviruses in Egypt (75%) is, on average, higher than that of potyviruses (LYSV and OYDV) (45%) or tospovirus (IYSV) (21%). This is contrary to the accepted view that potyviruses infect garlic more frequently than allexiviruses because of the more effective transmission of potyviruses by aphids [36].

The highest allexviruses incidence was reported from different parts of the world [37-39]. Whereas GarV-C was detected in only a few garlic samples from nearby countries [10,11,40].

It is possible that allexiviruses are mostly latent and most infection results from the vegetative propagation of poor-quality material resulting in escaping negative selection, which cannot be performed during garlic seed production process [38,41]. On the other hand, the variable distribution of individual virus species across the surveyed governorate and cultivars indicates multiple introductions of viruses into Egypt.

The occurrence of allexiviruses species in the tested samples appears to be incidental. Regardless of the tested cultivars or the geographical sampling, there is no domination of one virus species observed in tested samples, similar results reported by [17,18,37,42-44]. This is most likely associated with the transmission of viruses by eriophyid mites, *Aceria tulipae*, which occurs mainly during the storage of garlic bulbs These data are supported by research conducted by [2,38,45].

IYSV vectored by *Thrips tabaci,* is reported in garlic, onion, and leek plants produced in Egypt [14]. *Thrips tabaci* is an abundant pest heavily infesting *Allium* crops in Egypt where a high incidence of thrips insects was observed in all fields inspected with varying intensity. The incidence of IYSV was positively correlated to the population of *Thrips tabaci* [8].

Generic and specific RT-PCR results followed by sequencing have demonstrated that garlic samples collected in Egypt were severely infected by viruses. The high rate of infection and its homogeneous distribution in tested garlic varieties indicates the source

of infection is mostly through vegetative propagation [18,45]. Mixed infection in garlic is often designated as a "garlic viral complex" and associated with garlic low production and quality [18]. The coexistence of viruses may have a synergistic negative impact and cause even higher yield reduction [44,46].

This results probably attributed to movement of infected material imported from outside the country as well as new isolates of garlic viruses might be already infecting the Egyptian germplasm. Previous studies reported from other countries showed similar evolutionary results which attributed to international exchange of infected vegetative planting materials between countries and the obligatory nature of asexually propagation of garlic over generations [2,17,22,39,47].

Conclusion

In conclusion, the results obtained from this study describe the distribution of garlic viruses in Egypt. Also, provide a reliable approach for the detection of diverse individual virus species belonging to different viral taxa in limited time and efforts. Garlic is an important cash crop in Egypt. Therefore, the production of virus-free cloves is the most effective strategy to tackle garlic viruses, improving overall productivity and quality. This preliminary survey enabled us to monitor garlic viruses in Egypt, a prerequisite to solving epidemiological problems and devising integrated control practices, including reducing the use of harmful insecticides to control insect vectors. The local dissemination of the results obtained in this work should be recommended to farmers, producers, public agents, and all those involved in the garlic production chain in Egypt. Additionally, establishing virus-free garlic production programs is crucial for a country that is the fourth largest garlic producer in the world. A program like this could lead to significant economic benefits for producers, the general population, and the country's economy.

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