Full Length Research Article
Advancements in Life Sciences – International Quarterly Journal of Biological Sciences

ARTICLE INFO

Date Received: 30/08/2023;
Date Revised: 19/07/2024;
Available Online: 15/10/2024;

Author's Affiliation:
Plant Protection Department, College of Food and Agriculture Sciences, King Saud University, P.O. Box 2460, Riyadh - Saudi Arabia

***Corresponding Author:**
Mahmoud A. Amer
Email: ruamerm@ksu.edu.sa

How to Cite:
Amer MA, Amir M, Hussain K, Al-Shahwan IM, Al-Saleh MA (2024). Characterization of garlic virus A, garlic virus D, and onion yellow dwarf virus infecting onion. Adv. Life Sci. 11(4): 791-796.

Keywords:
Allexiviruses; Potyviruses; Serological Detection; *Allium cepa*; RT-PCR; Sequence

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Characterization of garlic virus A, garlic virus D, and onion yellow dwarf virus infecting onion

Mahmoud A. Amer*, Muhammad Amir, Khadim Hussain, Ibrahim M Al-Shahwan, Mohammed A. Al-Saleh

Abstract

Background: *Allium cepa* is one of the major vegetable crops cultivated in Saudi Arabia. This economically important crop faces several biotic stresses which adversely affect the quality and quantity of its production. Several virus groups (potyviruses, carlaviruses, tospoviruses, and allexiviruses) have been reported infecting *Allium* crops.

Methods: During the growing season of 2021-2022, a total of 81 onion samples exhibiting virus-like symptoms were collected from two different geographical regions in Saudi Arabia. The serological technique (ELISA) was used to detect the important allexiviruses and potyviruses. RT-PCR amplification of partial genome sequence was done using degenerate primers for allexiviruses and potyviruses and the phylogenetic trees were constructed using different bioinformatic tools.

Results: The results obtained from ELISA tests showed that 26% and 32% of onion samples were positive with both Garlic Virus A (GarV-A) and Onion yellow dwarf virus (OYDV) respectively. RT-PCR amplification and sequencing results showed that two allexiviruses, GarV-A, garlic virus D (GarV-D), and one *Potyvirus* (OYDV) were detected in both regions. Sequence data were deposited in the GenBank database with accession numbers, OQ397545, OQ397546 for GarV-A, OQ397547 for GarV-D, and OQ397548, OQ397549 for OYDV, sequentially. Phylogenetic tree analysis of these virus isolates showed making clades with closely related isolates of their respective viruses. Pairwise nucleotide sequence identity showed their similarity with GarV-A, GarV-D, and OYDV isolates reported earlier in the GenBank.

Conclusion: To the best of our knowledge, these two distinct allexiviruses (GarV-A, GarV-D) and one *Potyvirus* (OYDV) were isolated for the first time from an onion crop in Saudi Arabia.

Introduction

Among monocotyledonous plants, *Allium* is one of the largest genera which comprises almost 950 species [1]. Onion (*Allium cepa* L.) is one of the economically important crops which is grown worldwide [2]. Onion cultivation makes up around 24% of all vegetable production in the world (FAOSTAT, 2018). The top ten nations that cultivate onions are China, India, the United States, Egypt, Iran, Turkey, Pakistan, Brazil, the Russian Federation, and the Republic of Korea [3]. In Saudi Arabia, onion crops are cultivated over a 7650 ha area and its production reached up to 297974 tons according to APSB, 2019 [4].

Viruses, bacteria, fungi, and the cost of cultivation all have an impact on onion production. Unfortunately, several viral infections have an adverse impact on the quantity and quality of crops annually. In cases of severe infections, the losses increase up to 25–50% of the production. When multiple virus infections occur frequently in the onion crop, it is referred to as a "garlic viral complex" and plants show a variety of symptoms such as yellow stripes, stunted growth, leaf deformation, mosaics on leaves, and leaf striping [2, 5].

The *Allium* plants are affected by more than 20 viruses belonging to the four genera: *Potyvirus*, Carlavirus, Allexivirus, and Tospovirus that have been reported to lessen the yields and quality of garlic and onion crops significantly [5-12].

The viruses belonging to the genus *Potyvirus and Carlavirus* are transmitted by aphids in a non-persistent manner [13] whereas a *Tospovirus* is efficiently transmitted by thrips at the larval stage [14]. While eriophyid mites are responsible for the transmission of *Allexivirus*. All these four virus groups are also transmitted through mechanical inoculation [15, 16]. The host range for these four groups is limited to some indicator plants such as onions, garlic, shallots, a few ornamental *Alliums*, *Chenopodium amaranticolor, C. quinoa, Datura stramonium* and *Vigna unguiculata* [16, 17]. In Saudi Arabia, viruses exhibiting symptoms such as light and dark green patches, yellowing, leaf striping, stunted growth, and leaf deformation were observed in onion fields in two different geographical regions of Riyadh and Al-Baha. The aim of the present study is to identify the *Allexivirus* and *Potyvirus* infecting onion plants in Saudi Arabia using serological and molecular techniques.

Methods

Sample collection and ELISA

Onion plants that exhibited the symptoms of yellowing, dwarfism, yellow mosaic, and twisted leaves were observed in Riyadh and Al-Baha regions during field surveys in the years 2021-2022 (Figure 1). A total of 81 samples of onion plants were collected from the Al-Baha and Riyadh Regions (Table 1). Double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA) with specific antibodies obtained from LOEWE®, Biochemica, and DSMZ, Germany, were used to test the collected samples against the important allexiviruses including Garlic virus A (GarV-A), Garlic virus B (GarV-B), Garlic virus C (GarV-C), shallot virus X (ShVX), and potyviruses including Onion yellow dwarf virus (OYDV), Leek yellow stripe virus (LYSV) and Shallot yellow stripe virus (SYSV).

The procedure was performed and demonstrated according to the manufacturer's protocol using BioTek ELx808 ELx808IU Absorbance Microplate Reader96-Well. Samples were considered positive according to Clark and Adams,1977 [18] and Daryono et al., 2003 [19].

Figure 1: Symptoms observed on onion plants under field conditions showing mild mosaic, leaf striping, depicted yellowing, and deformation of leaves (A, B, C, and D).

Reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was extracted from all collected leave samples (100mg) using Gene JET Plant RNA Purification Mini Kit and One-step RT-PCR was carried out by following the instruction of the manufacturer's protocol of Thermo Scientific, USA using degenerate primer for allexiviruses (Allex-CP (+) and Allex-NABP (−) [20]. RT-PCR cycle was performed as follows: 60℃ for 10 min and 98℃ for 2 min followed by 35 cycles, 94℃ for 45 sec, 60℃ for 45 sec, 72℃ for 1 min, 72℃ for 10 min as final extension. For *Potyvirus*, the degenerate primers (Nib2-F and Nib3-R) were used [21] and the RT-PCR cycles were set to 42°C for 45 minutes, 95°C for 12 seconds, 40 cycles, 95°C for 15 seconds, 48°C for 20 seconds, 72°C for 30 seconds, and 72°C for 1 minute as the final extension.

The RT-PCR products were electrophoresed using 1% agarose gel in TBE buffer (Tris Borate EDTA) [22]. A Gel Documentation system was used to analyze the PCR-

amplified products. The expected molecular sizes have been determined using the 50 bp HyperLadder DNA (Bioline Ltd., USA) and the 100 bp DNA Ladder (GeneRuler, Thermo Scientific, USA).

Analysis of phylogenetic trees with nucleotide sequencing

Three and two RT-PCR products were obtained from onion samples by general primers of *Allexivirus* and *Potyvirus* groups, respectively. These amplified products were purified and sent to Macrogen Inc. Seoul, South Korea for two-directional Sanger sequencing of the DNA. Sequencing data was aligned using DNA star computer software and the National Centre for Biotechnology Information (NCBI). Using 1000 bootstrap replications and the Maximum-Likelihood method algorithm, the phylogenetic trees were constructed from ClustalW-aligned sequences by MEGA-X to analyze the phylogenetic relationship of the Saudi isolates with the other isolates reported from different countries. Pairwise nucleotide sequence identity percentages were determined using the MegAlign tool in the DNAStar software package by Lasergene and a graphical explanation of pairwise nucleotide percentage identity was presented by Sequence Demarcation Tool (SDT) [23].

Results

Field Observation and ELISA

Eighty-one symptomatic samples exhibited mosaic, dwarfism, yellowing, leaf striping, stunted growth, and deformation of leaves were collected from two main different onion growing regions (Al-Baha and Riyadh) in Saudi Arabia (Figure 1). The obtained results of ELISA from 81 onion plant samples indicated the occurrence of GarV-A and OYDV with a detection percentage of 26% and 32%, respectively. The obtained ELISA results showed that 47/81 (58%) onion samples were positive for at least one of the tested viruses. As far as a single and mixed infection of GarV-A and OYDV was concerned, 27% of the infected samples were singly infected with either GarV-A or OYDV and 55% of those had mixed infection of both viruses. The GarV-B, GarV-C, and ShVX were not detected in any of the tested samples (Table 1).

RT-PCR Reaction

For the detection of allexiviruses from onion samples using general primers, the amplified RT-PCR products have been of expected size 750 bp (Figure 2 A). In the case of *Potyvirus* amplification, the expected size of the amplicon was 350 bp (Figure 2 B). RT-PCR amplification was not observed with total RNA isolated from healthy plants as a negative control with any of the primer pairs used in this study. The outcome of these results revealed that ELISA-positive onion samples were infected with at least one of the viruses belonging to *Allexivirus* and *Potyvirus*. The presence of the exact virus infection was confirmed after Sanger DNA sequencing of these RT-PCR amplified samples.

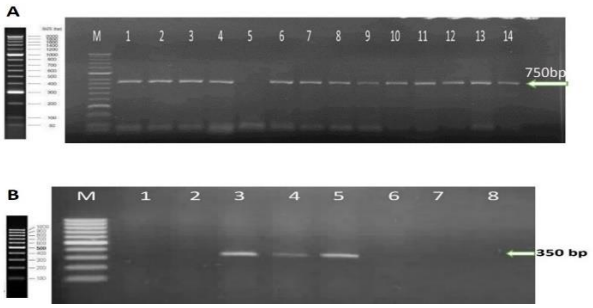

Figure 2: (A) Agarose gel (1%) electrophoreses depicted positive onion samples . Lanes 1,2,3 and 4 are positive samples from Al-Baha and 6-14 from Riyadh. All samples are amplified at 750 bp. However, sample 5 is negative in the RT-PCR reaction. Lane M represents 50bp HyperLadder DNA; (Bioline Ltd, USA). **(B):** Agarose gel (1%) electrophoreses showed potyvirus-positive samples (Onion) from Riyadh and the Al-Baha region. All the positive samples showed their bands at 350 bp. Lanes 1,2, 6 showed negative results as a negative control. lanes 3 and 4 (Riyadh) and lane 5 (Al-Baha) show a positive result, however. Lane M represents 100bp DNA Ladder; (GeneRuler), Thermo Scientific, USA.

Phylogenetic tree analysis with partial nucleotide sequencing

For general allexiviruses, two isolates of GarV-A and one isolate of GarV-D isolated from the onion samples showed a higher level of sequence identity with GarV-A and GarV-D based on their BLAST (basic local alignment search tool) analysis. After completing the analysis, sequences have been submitted to GenBank with the following accession numbers: OQ397545 (GarVA-O-Riyadh), OQ397546 (GarVA-O-AlBaha), and OQ397547 (GarVD-O-Riyadh) sequentially. The phylogenetic tree of GarV-A showed that our Saudi isolates grouped with the GarV-A isolates from Russia which were isolated from *Allium sativum* (JX682827), and China (LC097148) and our GarV-D isolate made a clade with GarV-D isolates from Argentine isolated from *A. sativum* (KF550407; Figure 3 A). As far as their nucleotide sequence identity percentage is concerned, GarV-A isolates from Riyadh and Al-Baha regions had 100% identity among themselves and 96.7% to 98.5% with the already published isolates in NCBI GenBank databases. The highest percentage identity was observed with GarV-A Chinese isolate (MN059312, MN059313), and the lowest percentage was observed with Poland isolate (KX889752; Figure 4 A). Moreover, GarV-D shared 80.6% to 83.6% of identity with the other available isolates in the NCBI GenBank. The highest percentage was observed with Poland isolate (KX889787), and the lowest percentage was also observed with Poland isolate (HQ681925; Figure 4 B).

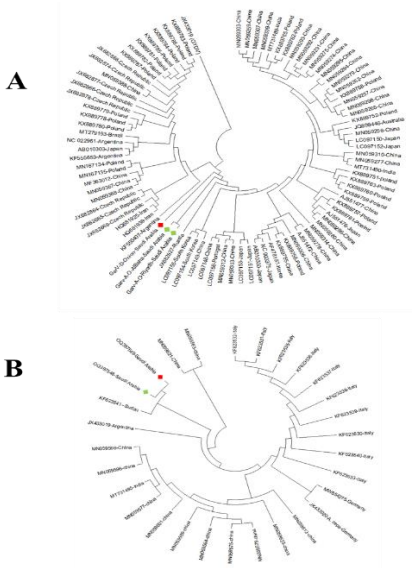

Figure 3: (A) Collective phylogenetic tree of two GarV-A and one GarV-D Saudi Arabian isolates (Riyadh and AlBaha) from Onion that show their similarity with the other respective isolates of different countries. **(B):** Phylogenetic tree of two Saudi isolates of OYDV (Riyadh and AlBaha) from Onion that show their similarity with the other respective isolates of different countries.

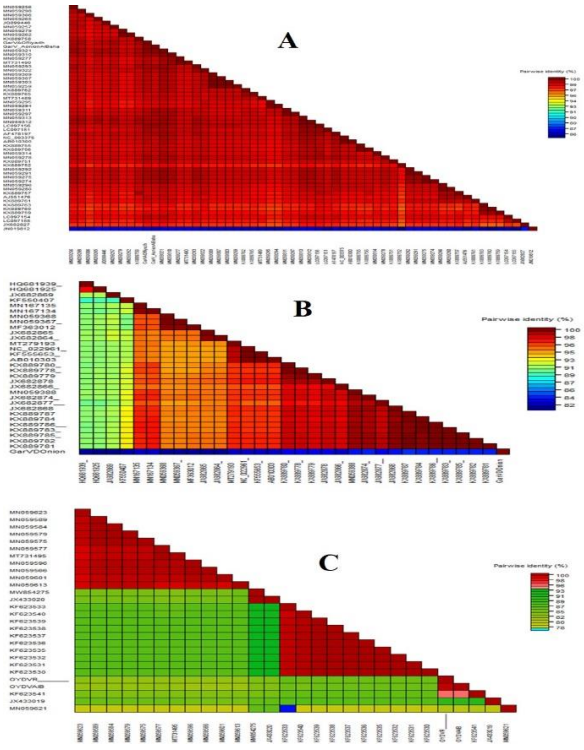

Figure 4: (A) Distance matrix shows the pairwise nucleotide identity percentage of two Saudi isolates of GarV-A (Riyadh and AlBaha), **(B)**: GarV-D (Riyadh), and **(C)**: OYDV with other isolates posted in the GenBank. Closely grouped isolates of GarV-A are highlighted with red color and blue color shows the least similar isolate and GarV-D are highlighted with red, light green, dark yellow, and light-yellow colors. Closely grouped isolates of OYDV are represented with red, light to dark green, and yellow-green colors.

For *Potyvirus*, the nucleotide sequence analysis of the two isolates of *Potyvirus* isolated from the onion samples in Riyadh and Al-Baha revealed that these sequences were related to OYDV. These sequences were submitted to GenBank with the accession numbers OQ397548 (OYDV-Riyadh) and OQ397549 (OYDV-AlBaha), respectively. The phylogenetic tree and SDT analysis of these two isolates of OYDV were constructed (Figures 3 B and Figure 4 C). Both isolates showed 99% to 100% identity between themselves. However, OYDV from Riyadh shared 80% to 95% identity with other isolates from NCBI GenBank, and OYDV from Al-Baha showed 80% to 95.5% similarity with the NCBI GenBank isolates.

Discussion

Onion is a major bulbous crop among vegetables and is of global importance [24, 25]. As a result of vegetative propagation, which causes viruses to accumulate and replicate in planting material, viruses are the primary issue in bulb crops like onion and garlic. Although viruses that infect *Allium* species have been intensively studied. Due to the multiple virus infections, serological and molecular studies were not easy to conduct for the detection of these viruses [26-28]. Viruses are ubiquitous worldwide and cause impacts in the form of crop production loss and reduction in quality [29, 30, 7]. It is tough to identify and estimate yield losses that are precisely attributable to each virus due to the incidence of mixed virus infections.

In eighty-one samples, 47 samples of onion (58%) were positive with GarV-A and OYDV. Hence, a large number of infected samples showed that the exchange of infected planting material from one region to another region could be the main reason for their occurrence in surveyed regions, but the presence of their vectors (aphids and mites) cannot be neglected because they play a significant role in their short distance disseminations within the fields.

Furthermore, in this study, two allexiviruses (GarV-A and GarV-D) and one *Potyvirus* (OYDV) isolated from onion were amplified by degenerate primers in the Riyadh and Al-Baha regions of Saudi Arabia. Interestingly, GarV-D could not be detected during the ELISA assay (unavailability of antisera) but got amplified in RT-PCR using general allexiviruses primers. This showed along with ELISA tests, a variety of other molecular techniques, RT-PCR, and hybridization must be incorporated into detection methods. Thus, a virus's entire genome can be identified by molecular analysis, and only a small number of viruses can be identified through serological investigation [31-36]. Therefore, molecular analysis is always chosen over serological testing due to their higher sensitivity and robustness.

Region	Total tested Samples	Total Infected Samples	Detected viruses							Singly infected	Mixed infection
			Allexivirus				*Potyvirus*				
			GarV-A	GarV-B	GarV-C	ShVX	OYDV	LYSV	SYSV		
Riyadh	45	31	15	o	O	0	14	0	0	7	15
Al-Baha	36	18	6	o	o	0	12	0	0	6	12
Total	81	49	21	o	o	0	26	0	0	13	27
%		60%	26%	0%	0%	0%	32%	0%	0%	27%	55%

Table 1: Plant viruses detected in onion samples in different Regions in Saudi Arabia during the survey period (2021–2022).

In RT-PCR, two Saudi Arabian isolates of GarV-A from Riyadh and Al-Baha regions and one Saudi Arabian isolate of GarV-D from Riyadh region showed their nucleotide identities of 96.7% to 98.5% and 80.6% to 83.6%, respectively with already published isolates in GenBank from different countries. Moreover, two Saudi Arabian isolates of OYDV from Riyadh and Al-Baha regions showed 100% nucleotide sequence identity with each other and 80.4% to 95.5% nucleotide identity with the other isolates of different countries. So, natural genetic variation could be the reason for the variation in the nucleotide identity of these isolates. Generally, phylogenetic analyses unveiled that Saudi Arabian onion isolates are normally grouped together, and the phylogenetic similarity relationship between them and their geographical roots is frequently unrelated. The great diversity and swift evolution of these viruses have been associated with the international exchange of vegetative planting materials between states, based on numerous studies conducted in other countries [37-40].

The results of this investigation would be useful in providing information about the evolutionary changes and diversity of these significant viruses. Also, this research may play a significant role in enhancing disease management procedures. Therefore, biological research is necessary to identify the transmission routes and assess the effects of isolated and combined infections of different isolates and different viruses causing mixed infections on onion crops. According to the best of our information, this is the first report of GarV-A, GarV-D, and OYDV infecting onion crops in Saudi Arabia, and this is also the first report of GarV-A from onion in the world.

Acknowledgments

The authors extend their appreciation to the Deputyship for Research and Innovation, Ministry of Education in Saudi Arabia for funding this research work through project no. (IFKSUOR3-556-2).

Author Contributions

Mohammed Amir, Mohammed Al-Saleh, and Mahmoud Amer designed the study, prepared the materials, collected the data, conducted the analysis, wrote and edited the manuscript. Ibrahim M. Al-Shahwan and Khadim Hussain conducted the data analysis.

Conflict of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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