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COLLECTION OF, AND SEROLOGICAL AND MOLECULAR VIRAL DISEASE DETECTION IN LOCAL GARLIC (*ALLIUM SATIVUM* L.) ACCESSIONS COLLECTED FROM NORTHERN CYPRUS

Nihat Yilmaz[1,*], Hakan Fidan[2]

[1]Department of Crop and Animal Production, Safiye Cikrikcioglu Vocational College, Kayseri University, Kayseri, Turkey
[2]Department of Plant Protection, Faculty of Agriculture, Akdeniz University, Antalya, Turkey

ABSTRACT

This study was conducted by collecting samples of garlic (*Allium sativum* L.) and studying its morphological characteristics to determine which viral diseases are causing an economic loss of yield in cultivation areas in Northern Cyprus. Considering their morphological characteristics, 87 garlic genotypes were collected from 6 different locations in Northern Cyprus, 83 of which were infected with one or more viruses. The isolates obtained were tested against Garlic common latent virus (GarCLV), Leek yellow strip virus (LYSV), Shallot latent virus (SLV), Cucumber mosaic virus (CMV), Tobacco mosaic virus (TMV), and Onion yellow dwarf virus (OYDV). For diagnoses, both double antibody sandwich enzyme-linked immunosorbent assay and reverse transcription polymerase chain reaction techniques were used. Eighty-seven garlic-leaf samples and 87 garlic clove samples were tested. The results from these samples were similar. Among the 87 plants tested, 38 (44%) were positive for GarCLV, 25 (29%) for LYSV, 15 (17%) for SLV, and 5 (5.7%) for OYDV. GarCLV was found to be the most common virus in the isolates.

KEYWORDS:
Garlic (*Allium sativum* L.), Garlic common latent virus, Onion yellow dwarf virus, Leek yellow stripe virus, RT-PCR technique.

INTRODUCTION

Garlic (*Allium sativum* L.) has been known since ancient times to be a miraculous vegetable throughout world and is among the oldest known horticultural plants. This plant was known as "garlic" in ancient times, especially in Egyptian and Indian cultures, and there was evidence that it was used by the Babylonians 4,500 years ago and by the Chinese 2,000 years ago [1]. Garlic is an indispensable part of the pharmaceutical, food, and spice industries as a result of its many health benefits, flavor, and therapeutic properties [2,3]. Garlic is a herb containing a very high amount of antimicrobial compounds, comprising 61 g per 100 g water, and is rich in chemical compounds, such as sulfur, essential oils, sucrose, and various vitamins [4,3].

Plants are an essential constituent of our diets and can shape the future of agriculture in terms of their importance in human nutrition and genetic codes. These indispensable agricultural commodities have adapted to areas where they have been grown for many years. After surviving several environmental and climatic storms, their genetic structure, which continues to survive, is described as "plant genetic resources".

The significance of plant genetic resources continues to increase. Together with increased consciousness about such resources, countries focus mainly on the collection and preservation of their local plant materials and generally have the support of international organizations. Old and valuable local cultivars and populations are highly adapted to local climatic and soil conditions, are quite resistant to pests and diseases, and have various other qualities. By using such materials, genotype resistant and/or tolerance to pests, diseases, abiotic stress factors, salinity, drought, and various other environmental phenomena could be developed. These materials may also constitute a valuable resource for presently unknown but prospective needs [5].

In Northern Cyprus, studies on plant genetic resources and the assessment of their resistance/tolerance to diseases are quite limited. Those studies conducted comprise mainly efforts with a limited number of plant species; however, except for those on a few species, there is no study on the collection of samples and viral diseases of plants already cultivated. Some genotypes, with their yields and positive responses to storage, are not able to compete with today's modern cultivars; thus, they face extinction. Because Northern Cyprus is located along a significant migration route, it has been host to several civilizations; therefore, it has become an area housing many genetically diverse plants species. Northern Cyprus is also located along the migration route

of wild birds, which have had significant contributions to that genetic diversity by spreading plants seeds.

Allium species belong to a large family of plants and are considered throughout the world to have significant health benefits. The worldwide production of garlic is 28.164.055 T, with China being the biggest producer with 22.160.465 T, followed by India with ~1.693.000 T [6]. According to 2017 data from the Turkish Republic of Northern Cyprus (TRNC), Northern Cyprus produces 123 T of garlic. The Morphou region produces 62 T, and Famagusta produces 60 T [7].

Garlic plants are affected by many abiotic and biotic factors. Biotic stress factors include viruses, bacteria, and fungal diseases. The main symptoms of disease in plants are mosaic line patterns, yellowing, and leaf deformations. Worldwide, most viruses are known to infect garlic as a natural host. *Potyvirus*, *Allexivirus*, and *Carlavirus* are among the most common that cause diseases among *Allium* species. *Potyviruses* include the Onion yellow dwarf virus (OYDV) and Leek yellow stripe virus (LYSV) [8] and are the most common diseases found in garlic as one of its host plants [9,10]. *Carlavirus* species, such as the Garlic common latent virus (GarCLV) and Shallot latent virus (SLV), are also found but are less common [11].

Potyvirus molecules have ssRNA ~9.7 -12 kb long and range from 680 to 900 nm and 11–13 nm in diameter. Only a few species of this virus can be transmitted by seed [10,12].

OYDV can be transmitted nonpersistent and mechanically by aphids (Aphidoidea). This viral disease, which affects onions, garlic, and leeks, causes serious losses in the plants from stunting their growth, yellowing and curling the leaves, causing irregular lines on the leaves, deteriorating the structure of the flower, and preventing seeding.

LYSV is also carried by aphid vectors but is not transmitted by seed [13]. Transmission through vegetative materials is considered the primary contamination route. Although the host range of LYSV is considerably wide, among *Allium* species, common garlic, leeks (*A. ampeloprasum* var. *ampeloprasum*), onions (especially, *A. ampeloprasum* var. *sectivum*), and some ornamental and wild plants can be listed as the most common hosts to be infected [14].

Other viruses observed on garlic are *Carlavirus* of the Betaflexiviridae family and *Allexivirus* belonging to the Alphaflexiviridae family. *Potyvirus* species usually cause yellow necrotic-chlorotic spotting on the leaves and stunted growth in plants; however, *Carlavirus* and *Allexivirus* species cause latent growth in plants and generally do not exhibit significant symptoms. Of the 43 *Carlavirus* species, GarCLV and SLV are the most common, with GarCLV considered the most common virus worldwide [10].

There are eight *Allexivirus* species in the Alphaflexiviridae family [10]. The hosts are *Allium* species and the viruses are transmitted by mites (Eriophyidae) [10]. Light-green strip formation on the leaves of the garlic plant can indicate the presence of *Allexivirus* species [15]. *Garlic virus*-A (GarV-A) and GarV-C are more commonly found in the garlic plant, but it has been reported that they can also reduce the total weight of *A. sativum* by 96% [16,17].

Garlic is a vegetatively propagated plant using the cloves from the flower structure. Cloves are used both as seed and production material [18]; therefore, viral infections are the most critical pathogens in these plants. Viral infections reduce clove size, weight, and diameter and are transmitted to future generations through the cloves [19,17]. The seed production protocol is critical because garlic is only vegetatively propagated. Virus-induced yield and quality losses are evident everywhere *Allium* species are grown. It has been indicated that *Carla-*, *Allexi-*, and *Potyvirus* species cause quality and quantity losses in these plants [20]. The infections of these viruses either alone or combined has caused losses in yield of 30–78% [21]. Few studies have been conducted in Northern Cyprus on these viruses in garlic; however, it has been reported that viral diseases are common in *Allium* species, and that these diseases significantly influence yield and quality; reduce size, weight, and diameter of the cloves; cause light lines or mosaics; and cause recessed growth and development [22].

These plant diseases limit the production of garlic in Northern Cyprus and affect the agricultural economy in the area. Complaints from the farmers about these diseases have prompted the initiation of this study. Widespread viral diseases that cause economic yield losses were assessed by collecting plant samples, determining morphological characteristics, and identifying the viral diseases.

MATERIALS AND METHODS

This study was conducted in two stages. First, 87 plant samples were collected from 6 different locations in Cyprus (Morphou, Lefka, Trikomo/ Karpas, Famagusta, Nicosia, and Kyrenia). The morphology was characterized were according to the International Union for the Protection of New Varieties of Plants (UPOV) criteria in 10 local garlic samples, which were different from the 87 genotypes collected. Ten different garlic materials collected were sown in vials containing 1:1 peat–perlite. Germination was not achieved from Sar-01, Sar-02, Sar-03, Sar-04, Sar-05, or Sar-10 genotypes. Seedlings of the four germinated genotypes were planted into experimental plots in 1-m rows and 25 cm apart.

TABLE 1
Specific primers used in virus testing.

Virus	Primer Name	Primer set	Length	Size (bp)
LYSV	LYSV(F)	CGCATATGCAGGGATGTTTCGGTT	24	316 (Fidan
	LYSV(R)	ATCAGCATTCAGGCTGCTTATACAC	25	2010) [1]
SLV	SLV(F)	TATGGCTAACGAAGAAGAAGAACTC	25	204 (Fidan
	SLV(R)	CGTTCACGCTAGACAATTCAGACAT	25	2010)
OYDV	OYDV(F)	CACCNTAYATAGCRGARACAGCTCT	25	602 (Fidan
	OYDV(R)	ACTGAAATGCGCCATTATYTGYCTA	25	2010)
GarCLV	GARCLV(F)	GCACCAGTGGTTGCGAATGA	20	481 (Fidan 2010)

Notes: GarCLV, Garlic common latent virus; LYSV, Leek yellow strip virus; SLV, Shallot latent virus; and OYDV, Onion yellow dwarf virus.

The morphologies were characterized until the flowering stage following UPOV criteria, and the morphological differences were identified among these four genotypes. Second, studies to screen the viruses were conducted on all collected 87 samples.

Tests were conducted using the double antibody sandwich enzyme linked immunosorbent assay (DAS-ELISA) method following the manufacturer's recommendations (Agdia Inc and Bioreba AG). Two samples of each positive DAS-ELISA result and a clear sample were verified with reverse-transcription polymerase chain reaction (RT-PCR) using the Dellaporta method of total nucleic acid extraction [23], running agarose gel electrophoresis with specific primers (Table 1), and imaging after staining with ethidium bromide. The Thermo Scientific Verso 1-Step RT-PCR ReddyMix Kit (#AB-1454/LD/A) was used for RT-PCR testing. The PCR cycles were arranged as for annealing with primers designed by multiplex RT-PCR (Lee et al. 2005) for 37 cycles at 55°C; cDNA synthesis (for reverse transcription) for 1 cycle at 50°C for 15 min; Verso inactivation for 1 cycle at 95°C for 2 min; separation at 95°C for 20 s; binding at 55°C for 30 s; extension at 72°C for 1 min; and final extension for 1 cycle at 72°C for 5 min.

RESULTS

The study was conducted by collecting 87 *Allium* samples from 6 different regions of Northern Cyprus (Morphou, Lefka, Trikomo/Karpas, Famagusta, Nicosia, and Kyrenia). The samples were tested serologically and molecularly using virus suspect samples with both the DAS-ELISA methods and RT-PCR techniques.

Among the germinated Sar-06, Sar-07, Sar-08, and Sar-09 genotypes, Sar-06 had a smaller clove and the skin's binding to the clove was weaker than that in the other genotypes. Thus, this genotype was separated from the others. Differences were not observed among the three remaining genotypes (Sar-07, Sar-08, and Sar-09). Of these, Sar-07 was preserved.

Virus symptoms were studied in garlic planted for testing and observation. The most common symptoms of viral infections in garlic are growth retardation, mosaic lines, yellowing lines, irregular line-shaped color opening in the leaves, small and amorphous clove formation, and small stem diameter between the middle veins of the leaf (Figure 1). In addition to the friability and inward curl of the leaves, narrowing of the angle between the leaf and stem was observed.

There were 87 samples with symptoms of a viral infection (stunting, yellowing, lines over the leaves, small cloves, and lighter color) that were collected from 6 different locations of garlic fields

TABLE 2
Test Results of the Distribution of Virus Species.

Regions	Samples	LYSV	OYDV	GarCLV	SLV	CMV	TMV
Morphou	19	6	2	8	2	-[a]	-
Lefka	13	4	-	6	2	-	-
Trikomo/Karpas	21	7	2	7	3	-	-
Famagusta	12	5	-	4	4	-	-
Nicosia	8	1	-	4	1	-	-
Kyrenia	14	2	1	9	3	-	-
Total	**87**	**25**	**5**	**38**	**15**	**-**	**-**

Notes: LYSV, Leek yellow strip virus; OYDV, Onion yellow dwarf virus; GarCLV, Garlic common latent virus; SLV, Shallot latent virus; CMV, Cucumber mosaic virus; and TMV, Tobacco mosaic virus.
[a]– no virus detected

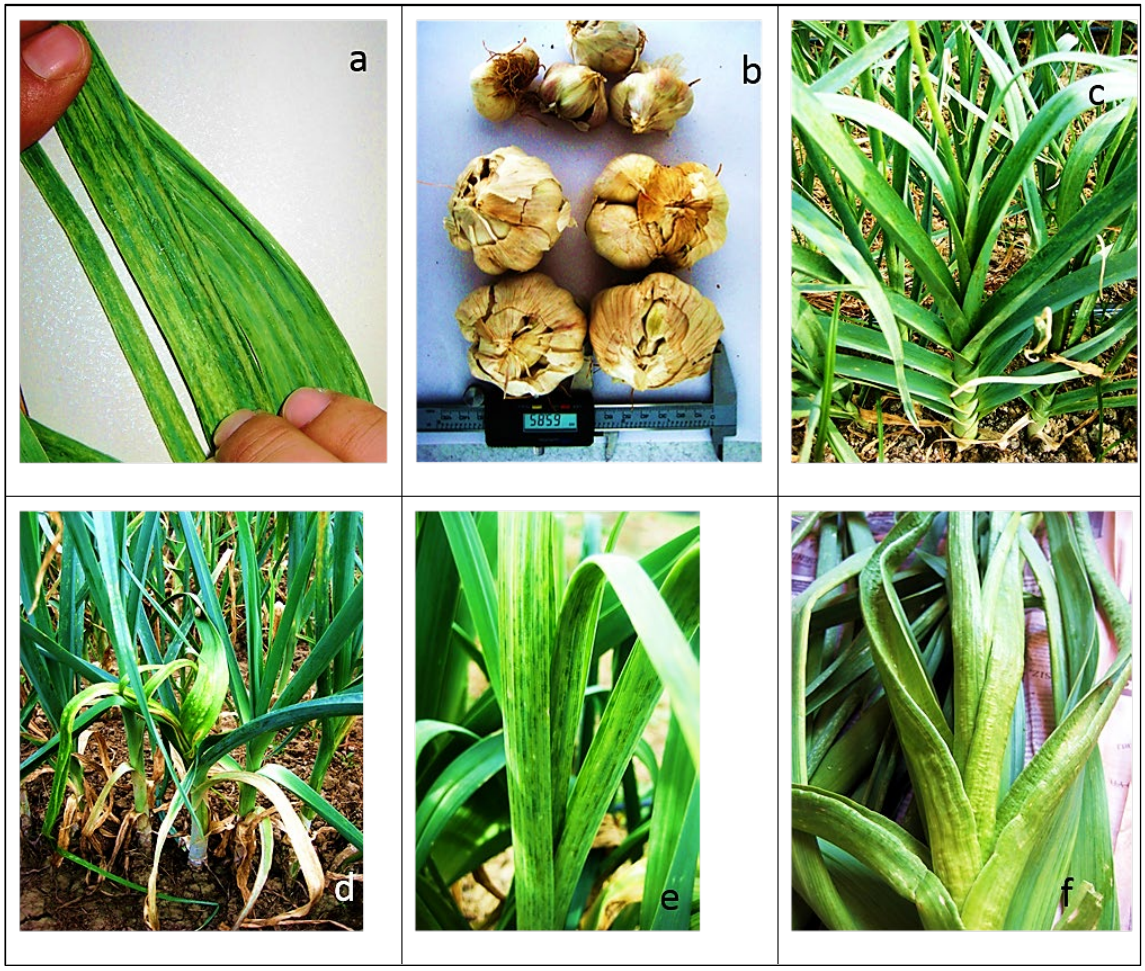


FIGURE 1
Symptoms of viral infections in garlic leaf, stem and clove. a) Line-shaped mosaics parallel to the central vein of the leaf, b) small and amorphous head and teeth formation, c) shortened between-node distance, d) general mosaic, and f) roughened leaves.

and home gardens in Northern Cyprus. Green herbage and cloves of the collected samples were assessed as separate samples. In the DAS-ELISA test, 87 leaf and 87 clove samples were tested.

In the DAS-ELISA tests, the same results were obtained from the cloves and leaves. In 83 of the collected 87 samples, at least 1 viral infection was identified. The viruses studied were not observed in the remaining four samples. Tests revealed that GarCLV (44%) was the most common virus in 38 of the 87 samples, followed by LYSV (29%) in 25, SLV (17%) in 15, and OYDV (5.7%) in 5 samples. Mixed infection of LYSV+GarCLV was observed in eight samples and SLV+LYSV were observed in five samples. Cucumber mosaic virus (CMV) and tobacco mosaic virus (TMV), which were previously identified, were not observed in any of the samples (Table 2).

According to the DAS-ELISA test, symptomatology works well in garlic plants. Certified seeds are not used in planting; the cloves are used as seeds. Four viruses with positive results from the DAS-ELISA test, two samples with mixed infections, and two samples with no viruses were subjected to RT-PCR, which revealed the product that proved the DAS-ELSA results.

SLV was identified in samples by their recessed development, irregular shapes, or small cloves, but generally, these samples had no symptoms (Figure 1). LYSV was identified in samples with irregular lightened lines over the leaves, mosaic lines, recessed development, small and irregular cloves, and narrow spaces between internodes (Figure 1).

DISCUSSION

There is limited information about viral infections in garlic plants grown in Northern Cyprus. This study identified the viruses that affect the production and yield of garlic in that area using both molecular and serological diagnostic methods, as well as data on the symptomological studies of these viruses. Phenotypes were observed from the information obtained within the scope of the study and serological and molecular analyses supported these data. The studies were conducted taking into consideration the

criteria of many researchers [24], which are the mosaic lines, irregular chlorotic or yellow lines on the leaves, irregular discoloration, deformations, irregular lines parallel to the central vein, stunting, general yellowing, growth retardation, small and amorphous head formation, narrowing trunk diameter, and plant deformity, all of which can be classified as suspicious in the samples. Field observations of the 87 collected samples were conducted using a commercial antisera by DAS-ELISA test. Symptomological characteristics of the collected garlic plants were taken into consideration and, during testing, it was decided to determine the presence of common viruses in these plants. The green parts and bulbs of the collected samples were determined to be the most suitable tissues for testing and were tested separately. Mixed infections were detected in 83 of the 87 samples collected from 6 different regions of Northern Cyprus. Two negatives (0.102 and 0.091) and two positives (0.522–0.749) controls were used for the DAS-ELISA test. Values near and above one-half of the value of the samples selected as positive according to the test were considered positive. ELISA readings of the samples obtained from the study were considered to be approximately six times higher (3.163-3.196-1.076). The reasons for this are believed to be that garlic viruses generally exist in plants but produce no symptoms and are not diagnosed. It is believed that garlic is not a seed-binding plant and the use of its clove as production material causes replication of the virus during each production period, leading to an increase in the viral titer. Notably, when *Allexivirus* and *Carlavirus* species produce a single infection in garlic, their symptoms remain hidden and difficult to diagnose, but if these viruses cause a mixed infection in plants, the expected yield from the plants is significantly reduced [25]. Serological data obtained from DAS-ELISA were supported by RT-PCR analyses. The presence of mix infections was detected in 83 of the 87 samples.

As a result of the analyses, 10 garlic genotypes tested were planted in pots for phenotypic observations and 6 of these genotypes did not germinate. The symptomatic characteristics of the viruses were to be interpreted through these samples. As a result of the observations, the most common virus symptoms in garlic were growth retardation with mosaic lines, yellowing, irregular lines and discoloration in the leaves, small and amorphous clove formation, shrinkage of plant stem diameter, and reduced spaces between the nodules (Figure 1). In addition, the friability of the leaves and inward curl and narrow angles between the leaf and stem were observed. Many researchers have emphasized that mixed infections are the most important factor that affects garlic plant yield. In mixed infections especially, growth retardation and line mosaics have been observed (Figure 1).

Line mosaics have been observed in *Allium* species, especially in the leaves and cloves of the garlic plants for an extended period of time, but because of insufficient identification, these symptoms are believed to be caused by unknown etiological factors [26]. As technological advances progressed, the diagnosis of viral diseases was facilitated using diagnostic-specific antibodies and primers, and identification of these diseases helped to identify the plants carrying this virus symptom and clarify the causes of these symptoms.

According to researchers, the most common factors that affect garlic yield are mixed infections. *Allexivirus* and *Carlavirus* species alone do not affect garlic production because they are usually latent in the plant; however, the presence of the *Potyvirus* family can cause both symptoms and a decrease in yields. Among the 87 plants tested, 38 (44%) samples had GarCLV, 25 (29%) had LYSV, 15 (17%) had SLV, and 5 (5.7%) had OYDV. GarCLV was the most common virus in the isolates with mixed infections. LYSV+SLV and LYSV+GarCLV, were the mixed infections; therefore, to prevent yield losses from viral diseases in garlic production areas, one of the most effective methods is to make cultural control to avoid mixed infections.

Although GarCLV and SLV are more common according to the research, it is indicated that *Potyvirus* species are an essential factor affecting garlic yield, especially in mixed infections [24,27]. In the studies conducted in Iran for GarCLV, despite regional differences, the intensity of the virus within the country varied between 60 and 86.2% [28]. In the Mediterranean area, Fidan and Baloğlu [29] have detected the first instance of viral disease in areas of garlic production in Turkey. Turkey's rate of becoming infected with isolates collected from different areas of regional garlic production changes. In Italy, differences in the geographical distribution of GarCLV infection at production sites has been reported, particularly in the southern areas, where the infection rate is 23% compared to that in the northern regions at 98% [30]. In addition, there are regional differences in GarCLV infection in Greece, and it has been reported that the rate of infection is significantly higher in the southern part of the country compared to that in the northern regions [31]. Pramesh and Baranwal's [32] study on GarCLV and its coat protein has indicated that there are several variations in the N-terminal region of this virus. In future studies, these directional variations in the coat protein of GarCLV, which is the most commonly detected virus in the garlic isolates from the Northern Cyprus region, can be detected on a genomic basis, and phylogenetic relationships with world isolates can be determined.

In the current study, garlic cloves were planted and observed in the different garlic genotypes to determine the symptoms of these viruses. The plants were tested to determine single and mix infections and their behavior in the plants. Although GarCLV has been reported in many garlic-producing areas

worldwide, there is little information on the molecular level of these isolates, such as the genetic diversity, phylogenetic relationship, or intra- or interspecies relationships; therefore, we believe that it is important to focus on these issues in future studies.

SLV is a viral disease carried by aphids that also carry GarCLV, and in this study conducted in Northern Cyprus, 17% of the samples collected were infected with this disease. SLV does not exhibit significant symptoms of infection, such as with GarCLV alone, but in some symptomological observations made by researchers, it has been stated that narrowing space between nodules causes shrinkage in the diameter and height of the garlic heads in direct proportion to growth retardation [22].

LYSV, a *Potyvirus*, was found at a rate of 29% in the isolates in the study. LYSV is also transmitted by aphids and is an economically important virus because it exhibits more significant symptoms than other viruses observed in garlic [9].

In our study, samples with virus-like symptoms, such as irregular color expansions parallel to the leaf veins, were collected and serologically and molecularly tested. The results showed that these samples were infected with LYSV, which could have been a single infection with LYSV or a mixed infection with GarCLV or SLV, and aphids are the contamination vector. In addition, Fidan et al. [33] have observed symptoms of LYSV in *Urginea maritime*, a herbaceous plant located near the garlic production sites and have confirmed using serological diagnosis that this plant was infected with LYSV. These weeds within the vicinity of the production areas are a major problem because they help to spread the viral diseases to the plants each period. Combining the information obtained from studies and potting trials, SLV is detected as a symptom in single infections, but growth retardation is detected in amorphous or small clove forming parts. LYSV was detected in samples showing signs of contraction.

In this study, 5.7% of the 87 samples collected from Northern Cyprus were infected with OYDV. Arya et al. [34] have reported that OYDV is one of the major viruses that cause diseases in onions and garlic. Because of the variations in the N-terminal region of the sheath protein in the OYDV genome, ELISA failed to diagnose the disease. An alternative method of diagnosis was attempted using fast and reliable RT-PCR. In these studies, both serological and molecular identifications of viruses were made. While studies in the Czech Republic showed a low rate of OYDV [9], those in Greece and Ireland have reported a high rate of the virus [30]. In Iran [27] and Italy [29], OYDV infections were reported to be 91.5 and 98% in garlic plants, respectively; however, OYDV is rarely detected in garlic grown in Brazil. The presence of OYDV at regionally different concentrations may be related to the presence of aphids, the vector, that carry the virus to the plants. In addition, in Brazil [35], the use of virus-free bee material has been reported to cause lower levels of infection.

In our study, RT-PCR and DAS-ELISA were used to determine whether the isolates showed mosaic symptoms and whether they were infected with CMV and TMV. Viruses such as CMV and TMV have an extensive host range, and inconsistent transmittal of CMV by aphids can cause infections in many plants. The results of our study suggest that these samples were not infected with CMV and TMV. There are few reports of CMV in *Allium* plants, which suggests that either the garlic plants may be infected with CMV or that tests against this virus on garlic are less common. Previous studies have confirmed that garlic plants are the hosts for CMV isolates [36]. In addition, in this study, the presence of viruses, such as CMV and TMV, in the garlic production areas in Northern Cyprus was not detected, but it is possible to encounter this virus using autumn surveys. Low temperatures are believed to aid the development of CMV infections.

Thermotherapy is the most effective method by which to prevent infections caused by viruses in garlic species. Studies on this factor confirmed this environmental stimulus. Fajardo et al. [11] have reported that the second elimination of the virus in the reproduction material during the second growing period belong to this virus.

Concive et al. [37], in their study on garlic infection by *Potyvirus*, *Carlavirus*, and *Allexivirus* species, have found that viral infections are usually manifested in the form of mixed infections, which results in ~78% loss in yield; therefore, to obtain the production material free of viruses, thermotherapy and meristem culture methods must be used.

The most significant factor for certified seed is virus-free garlic produced from plants without single or mixed viral infections. If we use disease-free garlic cloves for seed and consumption material, we could control these viral infections. Using diseased garlic cloves may increase the infection rates. In addition, virus vectors such as aphids, thrips, and mites, are also an effective method by which viral diseases are distributed in Northern Cyprus. Farmers are aware of the vector damage; however, there are no registered pesticides against these pests.

In garlic plants with mixed infections, significant visible damage to clove structure can be observed. Remarkably, these infected plants are differentiated from others in the garlic fields. Entire virus symptoms, such as dwarfism, mosaic patterns, lighter line colors, and yellowing can be observed on a single plant.

CONCLUSION

First, garlic-growing areas should begin to produce certified virus-free garlic seed, and this virus-

free plant material should be available to producers. Second, studies should be conducted with vector insects and their proper control methods in garlic-planting areas. Suitable pesticides, known for effective control, should be licensed for these vectors and appropriate training techniques should be organized for farmers. The present quarantine of plants propagated only by the clove and the quarantine regulations should be amended to test against detected viruses. New varieties should be developed and resistance in these varieties against viral diseases and vector pests should be determined.

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Informed consent All authors reviewed the manuscript and approved its submission to Fresenius Environmental Bulletin

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CORRESPONDING AUTHOR

Nihat Yilmaz
Department of Crop and Animal Production,
Safiye Cikrikcioglu Vocational College,
Kayseri University,
Kayseri – Turkey

e-mail: nhyl01@gmail.com
nihatyilmaz@kayseri.edu.tr