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RESEARCH ARTICLE

Monitoring Changes in Citrus Chlorotic Dwarf-associated Virus Titer under Natural Field Conditions

Doğal Arazi Koşullarında Turunçgil Klorotik Cücelik Virüsü Titresindeki Değişimlerin İzlenmesi

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ABSTRACT

The citrus chlorotic dwarf-associated virus (CCDaV), which was first observed in citrus orchards in Turkey, is one of the recently emerged disease of citrus. The molecular diagnostic and characterization of the virus was carried out in recent years. However, the information available on the etiology of the virus is extremely limited. This study aimed to determine the presence of CCDaV in different tissues, and seasonal and vertical changes of virus titer in terms of the etiology of the causal agent. The presence and changes in CCDaV titer were investigated from samples taken from different tissues and parts of a naturally infected lemon tree throughout the four seasons. As a result of conventional and real-time PCR tests, CCDaV was detected in leaves, flowers and fruits. Analysis of titer changes of CCDaV in different seasons and directions revealed that the virus titer was statistically at the highest level in the northward direction in spring season. However, it has been determined that routine CCDaV surveys can be performed from leaf samples at any direction of tree and time of the year with conventional PCR as well.

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ÖZ

İlk olarak Türkiye'de yetiştirilen turunçgillerde tespit edilen citrus chlorotic dwarf-associated virus (CCDaV), turunçgillerde son yıllarda ortaya çıkan hastalıklardan biridir. Virüsün moleküler tanısı ve karakterizasyonu son yıllarda yapılmış olsa da etiyolojisine ilişkin mevcut bilgiler oldukça sınırlıdır. Bu çalışmada, etmeninin etiyolojisi açısından CCDaV'nin farklı bitki dokularındaki varlığının ve virüs titre düzeyinin mevsimsel ve dikey değişimlerinin belirlenmesi amaçlanmıştır. Doğal olarak enfekteli bir limon ağacından dört mevsim boyunca farklı doku ve bitki kısımlarından alınan örneklerde CCDaV'nin varlığı ve titre değişimleri incelenmiştir. Konvansiyonel ve gerçek zamanlı PCR analizleri sonucunda CCDaV'nin yaprak, çiçek ve meyve dokularında bulunduğu tespit edilmiştir. Virüs titre düzeyinin farklı mevsimler ve yönler açısından değişimi incelendiğinde, istatistiksel olarak en yüksek titre seviyesinin ilkbahar aylarında ağacın kuzey yönünde olduğu belirlenmiştir. Bununla birlikte, konvansiyonel PCR yöntemi kullanılarak CCDaV'ye yönelik rutin sürvey çalışmalarının yılın herhangi bir zamanında ve ağacın herhangi bir yönünden alınan yaprak örnekleriyle de gerçekleştirilebileceği ortaya konmuştur.

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Introduction

It has been reported that there are nearly 80 viruses and virus-like diseases in citrus growing regions worldwide. While some of these viral agents cause unnoticeable damage to citrus trees, they infect, others may restrict citrus cultivation due to yield and quality losses. In fact, viruses can cause losses leading to death in sensitive species or rootstock-scion combinations. The most important of these diseases is citrus tristeza virus (CTV), followed by citrus psorosis virus (CPsV), satsuma dwarf nepovirus (SDV), and citrus exocortis pospiviroid (CEVd) (Roistacher, 1991; Shimizu et al., 2011; Achachi et al., 2014; Duran-Villa, 2016; Ganesh et al., 2018). Citrus chlorotic dwarf-associated virus (CCDaV) is considered one of the novel virus diseases of citrus for the world.

CCDaV is a viral agent with a ssDNA genome from the Geminiviridae family (Loconsole et al., 2012). While the presence of the causal agent was reported only in Eastern Mediterranean region (Adana, Mersin and Hatay provinces) in Turkey until 2019 (Korkmaz, 2000), it was reported from İzmir in the Aegean region and Antalya in the Western Mediterranean region in 2019 (Karanfil & Korkmaz, 2018; 2019). The presence of the virus outside of Türkiye has been reported only in China and Thailand in the world (Guo et al., 2015; Yang et al., 2020). Although limited studies were carried out about CCDaV, important information has been obtained regarding genome structure and the genetic diversity of CCDaV. As a result of these studies with Turkish and Chinese CCDaV isolates, it was determined that genetic diversity of isolates did not show any association with the host species or geographical origin (Karanfil & Korkmaz, 2018; 2019; Randa-Zelyüt et al., 2022). In addition, isolates obtained from Thailand, where CCDaV was last detected, were genetically closely related to Turkish CCDaV isolates (Yang et al., 2020).

The genetic diversity of the virus has partially been enlightened within the scope of studies, however; studies on the etiology of the virus are extremely limited (Korkmaz et al., 1994; Korkmaz & Garnsey, 2000). Therefore, changes in virus titer in various tissues and different sides of the infected tree were determined in different time periods. In this context, the seasonal changes in titer of CCDaV were determined in leaf samples from a lemon tree naturally infected with CCDaV in Mersin. Additionally, the presence of the virus in various tissues, such as flowers and fruits, was investigated in different phenological periods of the tree.

Materials and Methods

Determination of Citrus Tree Infected with CCDaV

An Interdonato (*Citrus limon*) lemon tree infected with CCDaV in Mersin province was selected according to Korkmaz (1997). The selected lemon tree was first tested by PCR using CPG F and CPG-R primers designed specifically for the CP gene of CCDaV (Table 1). After determining that selected lemon tree was with CCDaV, presence of the virus in different tissues was determined using conventional PCR and the virus titer and seasonal and directional changes were monitored throughout the year.

DNA Isolation and PCR Analyses

Total nucleic acid (TNA) was isolated from the tissue samples by CTAB method (Li et al., 2008). The concentrations and qualities of the TNAs were determined using a nanodrop spectrophotometer (Thermo Fisher Scientific, USA). The TNA concentrations were equalized and used in qPCR analysis.

PCR studies were carried out in a thermal cycler (Bio-Rad MJ Mini Personal Thermal Cycler, USA) with a total volume of 20 µl, using the PCR master mix from Takara (Japan) company in line with the manufacturer's recommendations. The PCR mix used in CCDaV infection diagnostic studies was prepared using 2 µl total DNA, 1 µl CPG F and CPG R (Table 1) primer mix (20 pmol) (Karanfil & Korkmaz, 2018), 10 µl 2x Emerald AMP MAX HS PCR master mix and 7 µl sterile distilled water. PCR reaction conditions for initial denaturation was set at 94ºC for 5 mins, 30 sec denaturation at 94ºC, 30 sec annealing at 53ºC, 45 sec at 72ºC for extension, for 40 cycles, and 5 mins at 72ºC for the final extension. The PCR products were separated in 1.5% agarose gel with DNA size markers at 100 volts for 45 mins, and stained with EtBr, imaged under ultraviolet light on the Major Science UVDI (Taiwan) device.

Table 1. Primers and TaqMan Probe Sequences

Analysis	Code	Sequence	Direction	Specific Gene and Product
PCR	CPG-F	5' ATGGTGAGTACCAGGAGTGG 3'	Forward	Coat Protein
	CPG-R	5' CAAATCTTGACCGTCCATTC 3'	Reverse	(812 bp)
qPCR	CCDaV F	5' CAGGGACAGGTTTATTGC 3'	Forward	Coat Protein
	CCDaV R	5' CTAACTCATCCAAGTCAAGC 3'	Reverse	(92 bp)
TagMan Prob Sequence				
5' FAM – TCACACTATGACGGTAGACCTGC - BHQ1 3'				

Monitoring of Seasonal Titer Changes

Leaf samples were taken from the specified Interdonato lemon tree every 3 months for one year, in spring, summer, autumn and winter from four different directions at the bottom and the top of the tree. Moreover, flower and fruit samples were also taken once in the blooming period (spring) and once in the ripe fruit period. The samples were brought to the laboratory in the cold chain and tested with qPCR to determine the presence and the titer of CCDaV.

Real-time PCR (qPCR) Studies and Establishment of Standard Curve

To determine the viral density in different tissues, a pair of real-time PCR primers and one TaqMan probe were designed and synthesized based on the CP gene sequences of CCDaV isolates in the gene bank (Table 1). In order to determine the CCDaV concentrations in the tissue samples taken, absolute quantitation was performed using the qPCR method, and the viral density in the samples was determined as number of particles (Kokkinos & Clark, 2006; Noris & Miozzi, 2015). First, a standard was established for this purpose. Tests were carried out in 2 repetitions for each sample.

The CCDaV CP gene obtained from the tree selected for sampling to create a standard was amplified with the PCR method, using the low-error-rate DNA polymerase enzyme. The amplified CP gene was cloned into the pGEM-T Easy plasmid vector by TA cloning method using T4 DNA ligase (Promega, USA). After the plasmids were transferred to strain *Escherichia coli* JM109 competent cells, colonies carrying the CP gene were first determined by blue/white selection and confirmed with colony PCR method. Plasmid was purified from two selected colonies; the cloning of the CP gene was verified by *EcoRI* digestion. The concentration of one of the plasmids containing CCDaV CP gene was determined and copy number was calculated and used as a standard. For absolute quantitation, eight different standards with $1x10^8$, $1x10^7$, $1x10^6$, $1x10^5$, $1x10^4$, $1x10^3$, $1x10^2$, and $1x10^1$ copy numbers were established by serial dilution of the plasmid DNA. The virus copy number in all samples was determined based on the standards given in Figure 1.

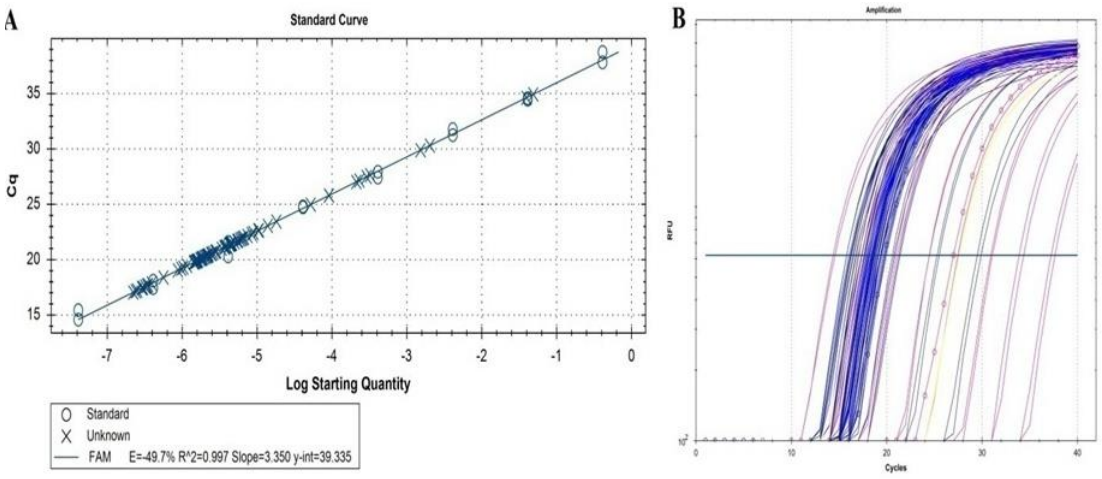

Figure 1. Standard curve (A) analysis and real time PCR amplification of CCDaV CP gene (B)

Detection and Quantification of CCDaV Different Parts of the Tree

Using the standards and the TNA preparations virus was detected, its titer was determined as copy number in different tissues. For this purpose, amplification and quantification were performed using the 100 ng TNA obtained from all tissue samples from the plant infected with CCDaV using a pair of primers specific to the CCDaV CP gene, one TaqMan probe and real-time PCR mixture (Light Cycler 480 Probes Master, Roche Diagnostics), and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). According to standards, the virus titer in each sample was determined as reported previously (Kokkinos & Clark, 2006; Noris & Miozzi, 2015). Statistical analyses were performed using threshold cycle (Cq) and copy number (Sq) levels.

Results and Discussion

At each sampling period, at least eight leaf samples were taken from the selected lemon tree at mid-season, from all four sides of the tree, from the bottom and top, as well as flower and fruit samples according to the phenological condition of the tree. In the conventional PCR test the presence of CCDaV was detected in all samples, with the exception of fruit and woody tissue samples.

In the qPCR tests, the presence of CCDaV was detected in all tested tissues, except wood tissue (Figure 1). While the presence of the virus could not be detected in fruit tissue using conventional PCR, it was detected in fruit tissue using qPCR. This result confirmed in parallel with the literature that qPCR is a much more sensitive method as compared to conventional PCR (Hongming et al., 2016). Some remarkable differences were observed regarding seasonal and vertical differences in titer of CCDaV among samples tested using qPCR. These differences were significant especially in copy number-based statistical analyses (Table 2 and 3).

Table 2. Analysis of variance for the threshold cycle numbers of different samples from the lemon tree

Variance Source	Error Degrees of Freedom	Sum of Squares	F Value	Pr (>F) *
Single	3	6.7963484E+08	84.12	<.0001
Direction	7	9.0279228E+07	11.17	<.0001
Season	3	1.0718028E+08	13.27	<.0001
Direction-Season	21	1.6032680E+08	19.84	<.0001
Error	93	7.5136047E+08		

*Significant at 0.01 level of error

Table 3. Analysis of variance table for the copy numbers determined by known standards

Variance Source	Error Degrees of Freedom	Sum of Squares	F Value	Pr (>F)*
Single	3	4.0037309E-9	3.71	0.0144
Direction	7	4.8519771E-9	4.49	0.0002
Season	3	3.3292797E-9	3.08	0.0312
Direction-Season	21	6.7834541E-9	6.28	<.0001
Error	93	1.0045136E-7		

*Significant at 0.01 level of error

Direction-based calculations of eight different directions sampled conducted by forming 3 and 4 statistical groups based on Cq value and particle number, respectively (Table 4 and 5). Analyses of statistical groups showed that the lowest virus titer was obtained from the samples taken from the western-bottom direction of the tree.

Table 4. Threshold cycle values of samples at the level of season and direction determined by qPCR

Direction	Spring	Summer	Autumn	Winter	Average
Western-Bottom	26.4 a	19.4 ba	19.5 cb	19.5 cd	21.2 A
Western-Top	17.8 dc	19.3 bac	19.1 cb	20.6 cbd	19.2 C
Eastern-Bottom	20.8 b	18.6 c	23.2 a	19.4 d	20.5 B
East-Top	18.9 c	19.2 bc	20.5 b	23.5 a	20.5 B
Southern-Bottom	17.0 d	19.4 ba	20.4 b	21.0 b	19.4 C
Southern-Top	20.8 b	19.4 ba	17.8 c	19.7 cd	19.4 C
Northern-Bottom	17.1 d	19.9 a	20.4 b	20.8 cb	19.6 C
Northern-Top	17.5 d	19.6 ba	19.8 b	20.3 cbd	19.3 C
Average	19.5 C	19.3 C	20.1 B	20.6 A	

For season-based statistical calculations, 3 statistical groups such as A, B and C, were formed based on Cq values similar to the directional statistical calculations. Winter and autumn were grouped as A and B, respectively, and spring and summer were grouped together in C as the result of season-based statistical calculations. The lowest level of CCDaV copy number was detected in winter, while the highest level was found in spring and summer seasons. As a result of the statistical analysis conducted based on copy number, 3 different statistical groups namely A, B and BA were formed. The statistical analyses performed based on both Cq and copy numbers showed that the samples taken in spring had higher CCDaV titer than that of seasons. Although, the lowest viral levels were observed in winter based on Cq values, no statistically significant differences in copy number were detected among seasons, except for spring.

Table 5. Copy numbers of samples at the level of season and direction determined by qPCR

Direction	Spring	Summer	Autumn	Winter	Average
Western-Bottom	1.84E-04 a	1.30E-06 b	1.57E-06 b	1.44E-06 b	4.71E-05 A
Western-Top	6.29E-07 b	1.23E-06 b	1.26E-06 b	4.61E-06 b	1.93E-06 C
Eastern-Bottom	3.84E-06 b	8.33E-07 b	1.26E-04 a	1.99E-06 b	3.31E-05 BA
Eastern-Top	1.55E-06 b	1.32E-06 b	3.37E-06 b	4.82E-05 a	1.36E-05 BC
Southern-Bottom	2.37E-07 b	1.32E-06 b	3.72E-06 b	3.88E-06 b	2.29E-06 C
Southern-Top	8.33E-06 b	1.42E-06 b	7.44E-07 b	1.71E-06 b	3.05E-06 C
Northern-Bottom	2.66E-07 b	2.75E-06 a	3.32E-06 b	3.63E-06 b	2.49E-06 C
Northern-Top	3.51E-07 b	2.01E-06 ba	1.77E-06 b	5.51E-06 b	2.41E-06 C
Average	2.49E-05 A	1.52E-06 B	1.77E-05 BA	8.87E-06 BA	

Statistical analyses considering the season-direction combinations determined that the spring-direction combinations formed 5 different statistical groups based on Cq value. Highest level of CCDaV was observed in samples taken from the southern-bottom, northern-bottom and northern-top directions in spring. Lowest concentration level in spring was obtained from the samples of western-bottom direction. Evaluation of the summer-direction combinations revealed that 5 different statistical groups were formed according to the CCDaV copy number in samples. In summer-directions combinations, the highest copy number level was obtained from the eastern-bottom direction, while the lowest level was found in the northern-bottom direction. In case of the evaluation of autumn-directions combinations, 4 different statistical groups were formed. In this combination, the highest concentration level was found in the southern-top direction, while the lowest concentration level was obtained from the samples of eastern-bottom direction. In winter-direction combinations, 6 different statistical groups were formed. The evaluation of winter-direction combination

showed that the highest CCDaV level was obtained from the eastern-bottom direction, while the lowest level was observed in the samples of eastern-top direction.

Statistical analyses demonstrated that the level of CCDaV titer in the samples from the northern directions in spring season stood out as compared to other season-direction combinations. However, in terms of changes in the virus titer, spring was the most heterogeneous season, while summer stood out as the most homogenous season.

The qPCR studies carried out to monitor the seasonal changes of CCDaV titer determined that the CCDaV concentration reached at the highest level in samples taken from the northern directions especially, in spring. It was observed that the lowest level of virus titer was obtained from the samples of the eastern-top direction during winter. However, it demonstrated that regardless of the seasonal level of virus titer and it is detected by PCR anytime in the year. This result indicated that the surveys and similar studies related to the virus could be conducted on leaf samples anytime in the year.

Conclusion

The result of this study demonstrated that the presence of CCDaV was detected in flower and fruit samples as well as in leaf samples. The results related to monitoring the seasonal changes of CCDaV titer, determined that the level of virus titer was statistically significantly higher in the western direction during spring. It has also been demonstrated that the routine CCDaV surveys could be carried out anytime in year. Additionally, the results revealed that the conventional PCR could successfully be used for the detection of CCDaV in leaf samples anytime in year.

Additional Information and Declarations

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