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Investigation of the Phytopathological Causes of Citrus Tree Trunk Foaming in Turkey

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Abstract
Foaming was observed in mandarin and orange species that were grafted on rootstocks trifoliata (*Poncirus trifoliata*) hybrids and was investigated in terms of phytopathological causes in the Cukurova region, where 80% of Turkey's citrus production takes place. Citrus exocortis viroid (CEVd) and citrus cachexia viroid (CCaVd) agents were screened by RT-PCR method by taking samples from different citrus rootstocks and cultivars showing foamy bark symptoms. It was determined that 17 of the 49 isolates were infected with CEVd and 14 of them were infected with CCaVd. There are 4 trees infected by both viroid agents at the same time. Based on a blast analysis, it was found that the reference genomes for CEVd and CCaVd in the NCBI GenBank had 95-100%. To check on the presence of bacterial and fungal agents, isolates were created from the foamy bark tissue of citrus trees. *Lasiodiplodia theobromae*, *Ilyonectria liriodendri*, *Fusarium pseudonygamai*, *Alternaria alternata*, and *Streptomyces spp*. were found as a result of the isolations. Analysis of soil samples taken from citrus plantations that had indications of foamy bark revealed a high calcareous soil structure with a pH above 7.5.
Keywords: Citrus, Foamy bark, Viroids, Cukurova region, Turkey
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1. Introduction

The citrus fruits agriculture today is one of the most important of the Turkey with an average production of 5.3 million tons. The Eastern Mediterranean region is the area where 80% of citrus agriculture in Turkey takes place (TUIK, 2022). There are many factors, especially biotic and abiotic, that negatively affect citrus cultivation in this area. One of the most important of these factors is virus and virus-like disease agents that do not have chemical control. To date, approximately 17 viral pathogens have been detected in the citrus growing areas of the Eastern Mediterranean region (Cinar et. al., 1993). The most important of these viral agents are citrus psorosis virus (CPsV), citrus tristeza virus (CTV), citrus chlorotic dwarf-associated virus (CCDaV), citrus yellow vein clearing virus (CYVCV), citrus exocortis viroid (CEVd) and citrus cachexia viroid (CCaVd).

The important factor in citrus cultivation is the selection of rootstocks suitable for the soil structure. The use of rootstocks in the evolution of citriculture has played a key role in the success of citrus industries worldwide. Rootstock has a strong influence on yield, fruit quality and tree size, and can provide tolerance to abiotic and biotic stresses. In commercial citriculture, sour orange (*Citrus aurantium*) has been a universal rootstock that is well-known for many attributes related especially to yield, fruit and juice quality, and tolerance to cold temperatures and various soil conditions (Castle, 1987). For these reasons, sour orange has become a popular rootstock in Turkey, especially among fresh fruit growers (Tuzcu et. al., 2001). Sour orange has one major weakness it is highly susceptible to decline isolates of CTV. Therefore, citrus growers in the Eastern Mediterranean region in Turkey are turning to alternative rootstocks despite having calcareous soils, as they fear the CTV virus from causing an epidemic. Macroscopic signs of some diseases, especially citrus viroid agents, have started to be observed more frequently with the trifoliata (*Poncirus trifoliata*) hybrids that have just started to be used in citrus production in the Cukurova region.

The aim of this study is to determine the presence, co-existence and symptom development of CEVd and CCaVd in trees showing foaming symptoms in citrus plantations grafted on trifoliate orange hybrids in Cukurova region. In addition, it was carried out to investigate the stress factors that may form the foamy bark at the bud union.

2. Materials and Methods

2.1. Plant material

In the context of this study, leaf and wood tissue samples were collected from 49 trees, 25 of which had symptoms of foaming and 24 of which served as controls from trifoliate hybrid citrus orchards in the province of Cukurova. These samples were obtained; 23 W.Murcott tangor (*C. reticulata* x *C.sinensis* cv W.Murcott), 10 Robinson mandarins (*C. reticulata* Blanco), 7 Interdonato lemons (*C. lemon* (L.) cv. Interdonato), 6 Tango mandarins (*C. reticulate* Tango), 2 Dobashi Beni mandarins (*C. reticulata* Blanco), and 1 Star Ruby grapefruit

(*C. paradisi* Star Ruby). 38 of the samples had their rootstocks grafted onto C35, 4 onto Carrizo, 4 onto C22, and 3 onto FA5.

2.2. Biological indexing studies

In biological indexing studies, C35 (*P.trifoliata* L. Raf. X *C.sinensis*. Osb. 'Ruby') rootstock was used for the detection of fungal agents, and tobacco (*Nicotiana tabacum* L.) was used to determine whether the bacterial agent was pathogenic.

2.3. Total nucleic acid extraction

Total nucleic acid (TNA) extraction was performed using CTAB (Cetyltrimethylammonium bromide) buffer solution to identify viroids in citrus cultivars grafted on trifoliata hybrids showing foaming disease. Extraction studies were applied modified according to Murray and Thompson (1980).

2.4. Reverse transcription-polymerase chain reaction and electrophoresis

RT-PCR studies were carried out in two stages according to the Onelge (1997). In the first step, the viroid RNA was translated into complementary DNA and then included in the PCR study. The 25 µl reaction mixture for conventional PCR contained 2 µl of cDNA, 12.5 µl of 2× DreamTaq Green PCR Master Mix (Thermo Fisher Scientifc, Waltham, MA, USA), 8.5 µl of RNase-free water, and 1 µl of each of the primers. A set of forward and reverse primers, CEVd (5'-ACCACAGGAACCTCAAGAAAG-3', 5'-GTGCTCACCTGACCCTGCAGG-3') and CCaVd (5'-TGCCCCGGGGCTCCTTTCTCAGGT-3', 5'-ACTCTTCTCAGAATCCAGCGAG-3') were used for reverse transcription PCR (RT-PCR) as described previously (Sano et. al., 1988). The PCR program consisted of one cycle of 94 °C for 3 min; 35 cycles at 94 °C for 30 sec, 57-60 °C for 30 sec, 72 °C for 1 min 30 s; and a final extension at 72 °C for 10 min. Electrophoresis method was used to analyze the PCR amplicons in 2% agarose gel with TAE buffer. cDNA was visualized by staining gel in ethidium bromide and photographed under UV light.

2.5. Sequence analysis and comparison of data

RT-PCR amplicons, in which the presence of viroid agents was confirmed as a result of gel imaging, were sent to the company for purification and determination of nucleotide sequences. The MEGA 11 program was used to view the base sequences of all samples whose sequencing had been finished. The base sequences were then matched using the BLAST method to the pertinent organisms included in the GenBank database of the NCBI (National Center for Biotechnology Information).

3. Result and Discussion

Within the scope of this study, plant tissue and leaf samples were taken from a total of 49 trees, as indicated in Table 1, from citrus plantations. It has been determined that the trees showing foaming symptoms are generally 10 years and older. These trees generally show stunting, back-drying of branches and cracks and signs of incompatibility and whitish foaming down at the bud union (Figure 1).

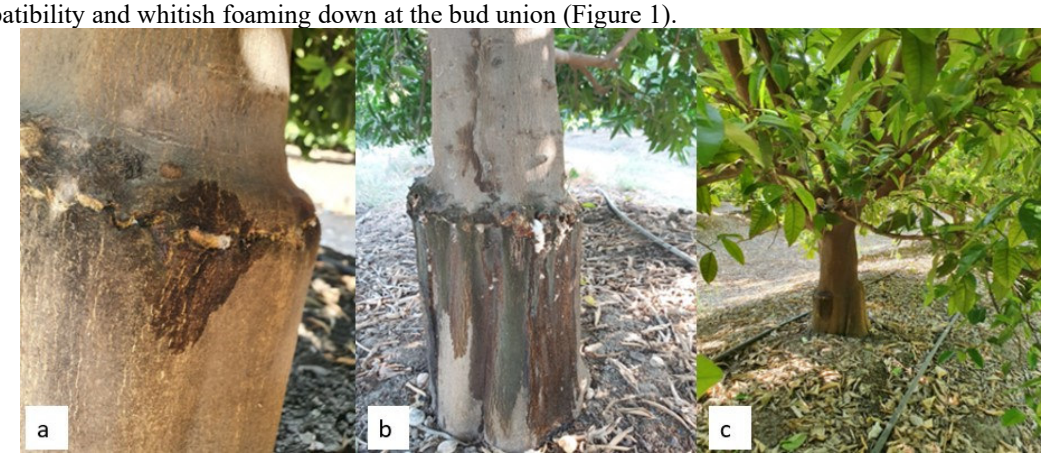

Figure 1. Foamy symptoms in W.Murcott tangor grafted on trifoliata hybrid rootstock (C35). a) Gum formation and wet appearance at the bud-union b) wet appearance of the rootstock due to the formation of gum and white foam formation at the bud union c) extreme leaf fall in W.Murcott tangor.

For this study, 38 of the samples collected from citrus plantations were grafted on C35, 4 on Carrizo, 4 on C22 and 3 on FA5 rootstock. It was observed that the mostly preferred rootstocks in the citrus orchards were Carrizo and C35 rootstocks (Table 1).

Table 1. Citrus Viroid Diseases Detected in Trees with Foamy Symptom

Location	Sample No	Rootstock	Scion	Foamy	CEVd	CCaVd
Akdam	ADA1	C35	Robinson	+	+	+
Köylüoğlu	ADA2	C35	W.Murcott tangor	+	-	+
Köylüoğlu	ADA3	C35	W.Murcott tangor	+	+	+
Akdam	ADA4	C35	Robinson	+	+	-
Akdam	ADA5	C35	Robinson	+	+	-
Akdam	ADA6	C35	Robinson	+	+	-
Köylüoğlu	ADA7	C35	W.Murcott tangor	+	-	+
Akdam	ADA8	C35	Robinson	+	+	-
Akdam	ADA9	C35	Robinson	+	-	-
Akdam	ADA10	C35	Robinson	-	-	-
Akdam	ADA11	C35	Robinson	+	-	-
Akdam	ADA12	C35	Dobashi Beni	-	-	-
Akdam	ADA13	C35	Dobashi Beni	-	-	-
Akdam	ADA14	C35	Star Ruby	-	+	-
Akdam	ADA15	C35	W.Murcott tangor	-	-	-
Akdam	ADA16	C35	W.Murcott tangor	-	-	-
Akdam	ADA17	C35	W.Murcott tangor	-	-	+
Akdam	ADA18	C35	W.Murcott tangor	-	+	+
Köylüoğlu	ADA19	C35	W.Murcott tangor	+	-	-
Köylüoğlu	ADA20	Carrizo	W.Murcott tangor	-	-	-
Köylüoğlu	ADA21	Carrizo	W.Murcott tangor	-	-	-
Köylüoğlu	ADA22	C35	W.Murcott tangor	+	-	+
Köylüoğlu	ADA23	C35	W.Murcott tangor	+	-	-
Köylüoğlu	ADA24	C35	W.Murcott tangor	+	-	-
Köylüoğlu	ADA25	C35	W.Murcott tangor	-	+	-
Akdam	ADA26	C35	W.Murcott tangor	-	+	-
Akdam	ADA27	C35	W.Murcott tangor	+	+	-
Köylüoğlu	ADA28	C35	W.Murcott tangor	+	-	-
Akdam	ADA29	C35	Tango	-	+	-
Köylüoğlu	ADA30	FA-5	Interdonato	-	-	+
Köylüoğlu	ADA31	FA-5	Interdonato	-	-	-
Köylüoğlu	ADA32	FA-5	Interdonato	-	+	+
Köylüoğlu	ADA33	C22	Interdonato	-	-	+
Köylüoğlu	ADA34	C22	Interdonato	-	-	+
Köylüoğlu	ADA35	C22	Interdonato	-	-	+
Köylüoğlu	ADA36	C22	Interdonato	-	-	-
Köylüoğlu	ADA37	C35	Tango	+	-	-
Köylüoğlu	ADA38	C35	Tango	+	-	+
Köylüoğlu	ADA39	C35	Tango	-	-	+
Akdam	ADA40	C35	Robinson	+	-	-
Akdam	ADA41	C35	Robinson	+	-	-
Akdam	ADA42	Carrizo	Tango	-	-	-
Akdam	ADA43	Carrizo	Tango	-	-	-
Köylüoğlu	ADA44	C35	W.Murcott tangor	+	-	-
Köylüoğlu	ADA45	C35	W.Murcott tangor	+	-	-
Köylüoğlu	ADA46	C35	W.Murcott tangor	-	+	-
Köylüoğlu	ADA47	C35	W.Murcott tangor	+	+	-
Köylüoğlu	ADA48	C35	W.Murcott tangor	+	+	-
Akdam	ADA49	C35	W.Murcott tangor	+	+	-

(+): Positive detection, (-): Negative detection, CEVd: Citrus exocortis viroid, CCaVd: Citrus cachexia viroid.

When the macroscopic symptoms at the trunk and bud union of these trees were examined, the presence of bark cracking was observed starting from the graft point to the soil level, especially in the rootstock with

macroscopic symptoms of CEVd. In addition, there were signs of gumming in the phloem tissue of the bark, which may be related to CCaVd (Child, 1950; Calavan et. al., 1961; Semancik and Roistacher 1991).

As a result of RT-PCR studies, it was determined that 17 of 49 samples showed bands at the level of 371 bp (Figure 2.a) and these trees were infected with CEVd (Table 1). In the RT-PCR studies carried out to determine the presence of CCaVd disease, 14 of 49 samples were found to form bands at 300 bp level and were infected with CCaVd (Figure 2.b). It was determined that CEVd and CCaVd were mixed together in the same tree in 4 of the examined samples (Table 1). In the study, 77.5% of the isolates were found to be contaminated with viroid agents as a result of RT-PCR studies. In trees with foam symptoms, CCaVd and CEVd, which are disease agents, were found together in very few mixtures. Researchers have reported that a synergistic effect can occur when the mix is infected with CEVd and CCaVd of citrus trees. The synergistic effect that could result from the coexistence of CEVd and CCaVd components, however, demonstrates that the RT-PCR findings in this study are not reliable for foam disease.

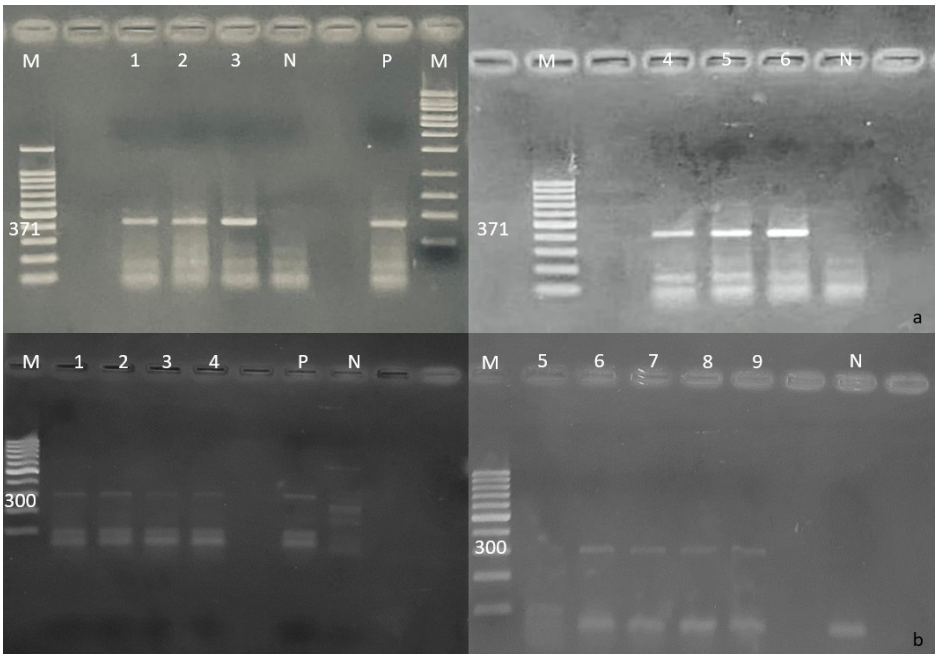

Figure 2. %2 agarose gel showing RT- PCR amplicons of foamy bark trees. a) M: 100 bp DNA size Marker, P: positive control CEVd (+), N: negative control CEVd (-), 1-3 CEVd infected W. Murcott tangor. 4-6 Robinson b) M: 100 bp DNA size Marker, P: positive control CCaVd (+), N: negative control CCaVd (-) 1-4 W. Murcott tangor, 5-9 Robinson.

The RT-PCR analysis identified a total of 8 isolates, 4 CEVd and 4 CCaVd, as having positive nucleotide sequences. BLAST analysis of nucleotide sequences was performed. CCaVd isolates showed approximately 96% similarity compared to other isolates registered in NCBI GenBank. Three separate groupings of CCaVd isolates were formed. Both ADA1 (Acc. No. OR344756) and ADA2 (OR344757) belonged to the same group as the isolates from Japan (AB054605.1) and Spain (X13000.1) and displayed 95% similarity. ADA3 (OR344755) isolate, on the other hand, were separated into a different group from other isolates and were found to be approximately 97% similar to Spain (X13000.1) and Italy (MT155390.1) and isolates. The ADA7 isolate selected from Murcott tangor belonged to the same group as the American and Sudanese citrus isolates, with 96% similarity (Figure 3).

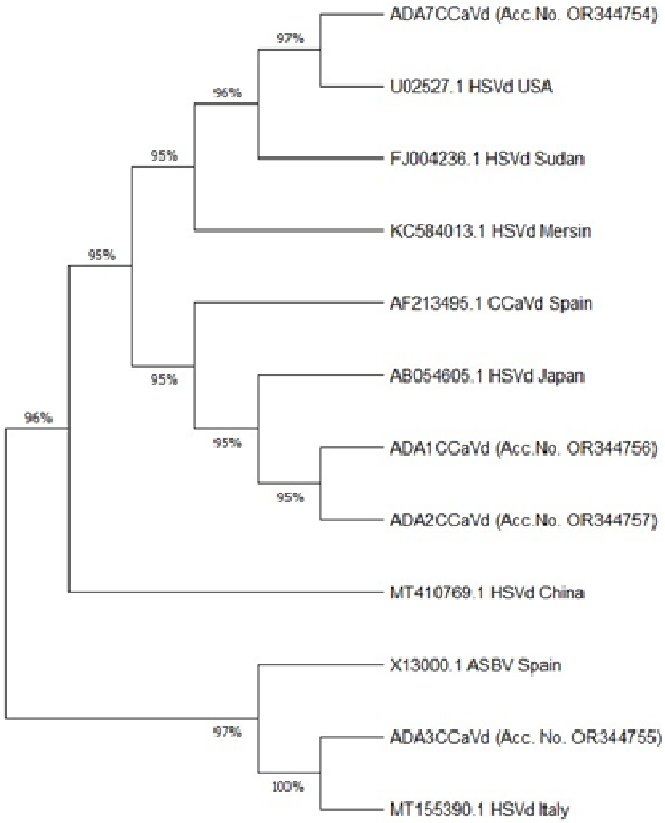

Figure 3. Phylogenetic tree of nucleotide sequence generated by the Maximum Likelihood method using the MEGA11 program. Comparison of ADA1 (OR344756), ADA2 (OR344757), ADA3 (OR344755), ADA7 (OR344754) isolates for CCaVd with NCBI GenBank data. Reference isolate X13000.1 Spain Avocado Sunblotch Viroid (ASBV) was used as out group.

CEVd isolates were found to be approximately 99% similar when compared to NCBI GenBank isolates. In terms of CEVd nucleotide sequence, the isolates ADA1 (OR344753), ADA6 (OR344751), and ADA8 (OR344752) shared %98 similarity with other isolates from the GenBank. In the other group, the ADA3 (OR344750) isolate displayed 100% similarity to the Australian isolate (MT917193.1) and 99% relationship to the American isolate (MZ330107.1). It was determined that CEVd and CCaVd isolates obtained from foaming citrus trees showed similar homology with other CEVd nucleotide sequences in NCBI (Figure 4).

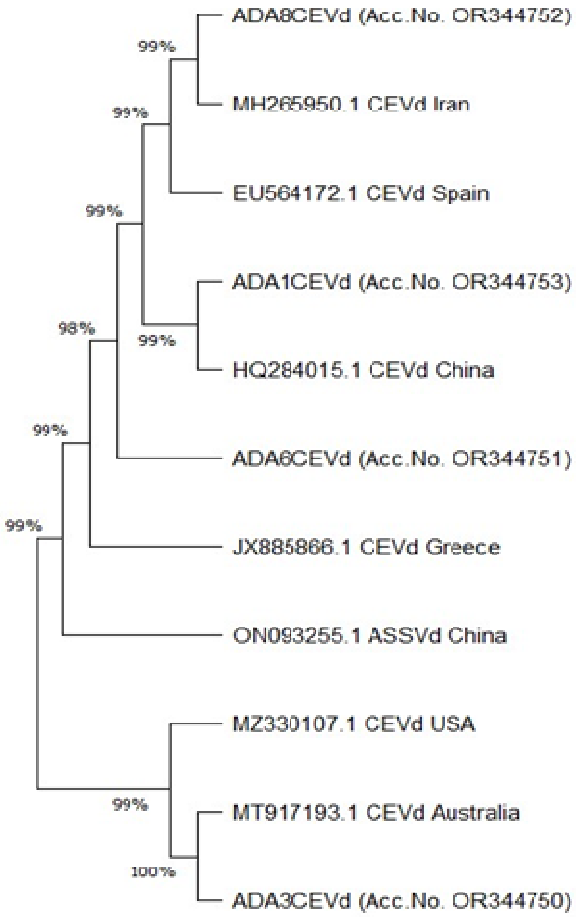

Figure 4. Phylogenetic tree of nucleotide sequence generated by the Maximum Likelihood method using the MEGA11 program. Comparison of ADA1 (OR344753), ADA3 (OR344750), ADA6 (OR344751), ADA8 (OR344752) isolates for CEVd with NCBI GenBank data. Reference isolate X13000.1 Spain ASBV was used as out group.

Isolations were carried out to investigate the status of fungal and bacterial agents in citrus trees with foam formation. As a result of the isolations, the agents *Lasiodiplodia theobromae*, *Ilyonectria liriodendri*, *Fusarium pseudonygamai*, *Alternaria alternata* were isolated from citrus trees showing foam symptoms (Table 2).

Table 2. Fungal and bacterial agents on citrus trees with foamy bark symptoms

Fungal Agents
Lasiodiplodia theobromae (Botryosphaeriaceae) (synonym: *Botryodiplodia theobromae*)
Ilyonectria liriodendri (Nectriaceae)
Fusarium pseudonygamai (Nectriaceae)
Alternaria alternata (Pleosporaceae)
Bacterial Agent
Streptomyces (Actinomycetota)

Among these agents, *L. theobromae* and *I. liriodendri*, which are thought to be pathogenic, were included in the pathogenicity test (Figure 5). C35 plant was inoculated from these pathogenic agents and as a result of inoculation, mild lesion signs were formed in the wood tissue. No signs of drying or death were observed in the plants.

Figure 5. Pathogenicity test result of *L. theobromae* and *I. liriodendri* agents on C35 plant.

As a result of bacterial isolations from trees with foamy symptoms, *Streptomyces spp.* was isolated. Pathogenicity test was performed for this factor and inoculations were made to tobacco plants. As a result of inoculation, no symptom development was observed in tobacco plants.

It has been reported that problems similar to foam formation are observed in citrus growing countries Indonesia (Dwiastuti and Aji 2021) and USA (Adesemoye et. al., 2011). In the study carried out in California, it was reported that serious losses occurred in Fukumoto navel orange trees grafted on C35 rootstock. The presence of foamy flows in citrus trees, where losses occur, has also been reported. Because of this foaming in the trees, the disease has been called 'foamy bark rot' (Adesemoye et. al., 2011). Many fungal and bacterial agents were detected, similar to the foamy bark seen in Cukurova citrus areas, but it was not specified which of these pathogens caused foaming. In another study conducted in Indonesia, it was reported that the fungal pathogen *L. theobromae* causes symptoms called wet diplodia (Wet Diplodia) in citrus orchards and creates foamy discharges on the trunk of citrus trees (Dwiastuti and Aji 2021). Although the pathogens determined in this study are similar to the agents obtained in both countries, *L. theobromae* seems closer to the result obtained in Indonesia as a pathogen. However, *L. theobromae* did not show any severe reaction in the C35 indicator plants in our inoculation studies.

It is known that the soil structure is very calcareous and the pH is high in Cukurova citrus growing areas. In the study, the soil structure of the citrus orchards where the isolates with foam symptoms were collected were examined. It is seen that the soil pH is 7.5 and above in 11 orchards and the lime rate is 18.3% (Table 3). Considering the citrus rootstocks that can be used for this soil structure, it has been reported by many researchers that it is not suitable especially for trifoliate orange and hybrids (Cooper et. al., 1956; Duran-Vila and Semancik, 2003). It has even been reported that trees grafted on C35 rootstock are uprooted in Australia due to the high pH of the soil structure (Khurshid, 2021). A similar situation is also observed in the Cukurova citrus fields. While there is no problem in Murcott tangor grafted on sour orange in the same plantation, foam formations are observed at the bud-union in mandarins grafted on C35. For this reason, it is seen as a possibility that foam formation develops due to soil and climate reasons and stress on trees.

Table 3. Analysis Results of Soil Samples Taken from Citrus Orchards with Foam Symptom

No	Rootstock	pH (6.5-7.5)	Location	Salinity % (0.0-0.15)	Lime % (5-15)	Organic matterr % (3-4)
1	C35	8,04	Cukurova 1	0,431	13,65	1,52
2	C35	7,68	Cukurova 2	0,01	21,11	1,33
3	C35	7,73	Cukurova 3	0,039	25,18	1,03
4	C35	7,62	Cukurova 4	0,032	19,39	0,5
5	C35	7,69	Cukurova 5	0,014	19,71	1,22
6	C35	7,67	Cukurova 6	0,013	23,62	1,1
7	C35	7,65	Cukurova 7	0,052	16,27	0,85
8	C35	7,64	Cukurova 8	0,03	12,98	1,41
9	C35	7,9	Cukurova 9	0,7	16,26	1,92
10	C35	7,5	Cukurova 10	2,6	14,3	2,21
11	C35	7,8	Cukurova 11	1	19,77	1,63

4. Conclusion

As a consequence of the investigation, RT-PCR analyses revealed that 38 of 49 samples obtained from citrus

species and varieties with tifoliata orange hybrids as rootstock were infected with viroid. This is an extremely high rate. However, in the Cukurova region, the use of certified seedlings is very limited, and the mechanical transport of viroid agents is extremely high. There were only four cases where CEVd and CCaVd were found in the same citrus tree. As a result, it is not possible to claim that the foam symptom is the result of a synergistic action. The phylogenetic study of viroids whose nucleotide sequences were identified revealed that they had approximately 97% similarity with the viroid agents recorded in the GenBank. The pathogens isolated from foamy trees were determined to be saprophytic agents in general, and *L. theobromae* was purified and transferred to C35, a trifoliata hybrid, and did not produce foaming symptoms alone. It was determined that the citrus orchards with the foaming symptom had extremely high calcareous soils and a pH level in excess of 7.5. Trifoliate hybrids can not be used as rootstock in these ratios because of the nature of the soil structure. Citrus trees are stressed by high pH and lime levels, in especially. As a consequence, it indicates that stress played a role in the foam production at the bud-union and that an abiotic factor is responsible for the problem.

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