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MOLECULAR DETECTION AND CHARACTERIZATION OF CITRUS PSOROSIS VIRUS IN CITRUS ORCHARDS IN NORTHERN CYPRUS

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Abstract

In this research, we detected the citrus plants molecularly in citrus orchards. The surveys were conducted in various regions of Northern Cyprus in an area of 3.000 da citrus orchard in 2022. In field surveys, leaf samples were taken from citrus trees suspected to be symptomatically infected with citrus psorosis virus. PCR techniques used for detection and results showed four samples were positive and infected by citrus psorosis virus in Northern Cyprus. After examining the degree of affinity with other CPsV isolates previously detected in different parts of the world, it was determined that the isolate found in Cyprus was the same as CPsV-NA8 (AY194905) from Italy and two isolates from Spain, CPsV-NA35 (AM235964) and CPsV NA4 (AM 235692). At the end of the PCR studies, four samples infected with Citrus Psorosis Virus (CPsV) were determined. The remaining 41 samples gave negative results.

Key words: Citrus, Genetic, Psorosis, RNA, Moleculer.

Introduction

Citrus fruit is one of the most important fruit products in the world. Citrus is produced in more than 100 countries in the world. Citrus, native to India, China and Southeast Asia, can be grown in tropical and subtropical regions in general lemon, grapefruit, orange and mandarin are the major spieces which produced on the World (Alas *et al.*, 2019). They have high economic value. The largest share of export products in Northern Cyprus belongs to agricultural products, and the most important of these is citrus fruits. However, in recent years, as well as the problems such as drought, water and soil salinity, input prices such as fertilizers, herbicides, pesticides, electricity and water costs, as well as the increase in labor costs of citrus cultivation has started to decrease compared to the past years. In addition to all these, one of the most important factors that negatively affect the yield and quality of citrus fruits are diseases and pests. Among these, virus disease cause damage in citrus production. Because viruses can be transmitted from plant to plant by mechanically, can be transmitted by grafting thus spreading to very large areas in a short time; moreover, the chemical management method which can be applied effectively in other diseases has not effect on viruses. Identification of citrus virus diseases have began with determination of Citrus psorosis virus in Florida in 1890 (Galipienso *et al.,* 2000; Belabess *et al.,* 2020). CPsV has a segmented genome consisting of negative-sense RNA (vRNA), three single-stranded, and ~48-kDa coat proteins (Velázquez *et al*., 2012; Moreno *et al.,* 2015). It is a citrus disease that causes a 5% annual decrease due to the Citrus psorosis virus. The disease can be found asymptomatically in citrus species. The most characteristic symptoms of the disease in adult trees are bark flaking on the trunk and main branches (Achachi *et al.,* 2014).

The first study to determine the citrus virus diseases in Turkey was investigated in 1950 in Eastern Mediterranean region. Stubborn, Impietratura, Exocortis, Cachexia and Psorosis virus diseases were reported in citrus orchards. The transmission of this virus from plant to plant is very easy with mechanically.

In recent years, it has been thought that the symptoms of chlorotic spotting on leaves and spalling on stem barks are commonly formed by CPsV in Northern Cyprus. It is known that this virus disease can be transmitted by grafting and transported mechanically easily. Grafting is a common management in citrus trees in Northern Cyprus, where most of the varieties have been grafted on sour orange; and grafting is among the major way of transmission of this virus, together with other mechanical practices (Zhou, 2018).

There are differences in the degree of infection between countries, especially in Mediterranean countries. For example, the spread of the disease in Italy is higher than in others (Alioto *et al.* 2000).

Virus diseases can be detected and identified with serological, biological and molecular methods. Serological tests, such as Enzyme-linked immunosorbant assay (ELISA), agar-gel spreading reactions and precipitation reactions might be used for detection and identification of viruses. Moreover, molecular methods such as hybridization, electron microscopy, dsRNA electrophoresis and Polymerase chain reaction can also be used (Clark *et al*., 1981; Luisoni & Boshia, 1994; Hull *et al*., 2004). After genetically sequences became available, molecular detection and characterization tests based on RT-PCR were also developed for Citrus Psorosis Virus (Barthe *et al*., 1998; Legaretta *et al*., 2000; Loconsole *et al*., 2009).

In previous studies, the symptoms of all citrus species and varieties in the Mediterranean Region, especially above 25 years old, were determined. Typical damages of this disease was reported by farmers in Northern Cyprus to be common and widespread on various citrus species, where all of them were grafted on the sour orange rootstock. Sour orange rootstock is highly susceptible to this virus. Since sour orange rootstock is used in almost all old orchards in Northern Cyprus, the symptoms can be seen quite easily.

In this research, different citrus species (orange, mandarin, lemon and grapefruit) tested by PCR technique molecularly to detect for Citrus Psorosis Virus in Northern Cyprus.

Material and Methods

Field survey and symptoms identification: The surveys were conducted in various regions of Northern Cyprus in an area of 3.000 da citrus orchard in 2022. The citrus trees which showed Citrus psorosis virus disease symptoms were determined in 45 different citrus orchards. 20 orange, 10 lemon, 10 grapefruits and 5 mandarin orchards were choosen for surveying the main materials of this study are: spalling on the main trunk and branches, gum flows on stems, yellows spots on the leaves that occur in the spring (March-April). Samples were taken from different species and cultivars in regions with high citrus production over 25 years of age showing the above symptoms (Table 1).

Table 1. Amount of collected and infected samples from different citrus species in Northern Cyprus.

Species	Collected samples	Infected samples
Orange	20	2
Mandarin	5	2
Grapefruit	10	0
Lemon	10	0
Infection (%)	9	

Collection of samples: In field surveys, leaf samples were taken from citrus trees suspected to be symptomatically infected with citrus psorosis virus. Each sample collected as plant material was identified and numbered. While choosing the trees to be taken as a sample, trees showed yellow band formation on the leaves, cracking, bark spalling and gum flow on the trunk were selected (Fig. 1).

Total nucleic acid extraction: Young leaves which showed virus symptoms were used in tNA isolation studies. Samples were extracted by diluting 1:4 (w/v) with extraction buffer (100mM Tris-HCl pH.8.0, 50mM EDTA b-pH. 7.0, 500 NaCl, 10mM 2. mercapto-ethanol (1/1000) and the plant sap was filtered through sterile cheesecloth. 1 ml of plant sap was taken and placed in eppendorf tubes. Samples were centrifuged at 4,000 rpm for 3 minutes. 50 µl of Sodium Dodecylsulfate (20%) was added to the c-Pellet and mixed in a vortex, and then the tubes were incubated in a water bath at 65 0C for 30 minutes. The samples were centrifuged at 13,000 rpm for 5 minutes to precipitate the RNAs. The total RNAs obtained were diluted with 50 µl of RNAse free distilled water. They were stored at -20 0C in eppendorf tubes (Astruc *et al*., 1996).

RT – polymerase chain reaction: First, the RT step was carried out to synthesize complementary DNA (cDNA). It was mixed with 3 µl total NA (200 ng/µl), 1 µl Reverse (R) and 13 µl H2O, incubated at 95°C for 3 minutes and then kept on ice for 5 minutes. Then, 10 µl of RT mixture (5 µl M-MLV RT buffer, 1 µl dNTPs (10 mm), 0.3 µl RT (200 units), 0.1 µl RNasin and 3.4 µl H2O) were added onto this mixture and cDNAs were obtained by keeping the samples in a thermocycle at 42°C for 1 hour. For subsequent PCR work, 4 µl cDNA, 5 µl Dream Taq Green Buffer (10X), 1 µl dNTP mix (10 mM), 1 µl CPV-1 (GCTTCCTGGAAAAGCTGATG) (10 pmol/µl,) primer, 1 µl CPV-2 (TCTGTTTTTGTCAACACACTCC) (10 pmol/µl,). A final volume of 50 µl was created with primer, 0.25 µl Dream Taq polymerase (5u/µl) and 37.75 µl ddH2O. PCR tubes were placed in the thermocycler, which was programmed according to the denaturation temperature (Thermal melting, Tm) values of the primer pairs used and the molecular weights of the expected PCR products using the primer pairs(600bp). In the thermocycle program applied to the samples, the bonding temperature within 35 cycles was applied by considering the Tm value of the primers and subtracting 3°C from the Tm value.

DNA Electrophoresis: Electrophoresis method was used to analyze the PCR products in 2% agarose gel at 45V with TAE buffer. DNA was visualized by staining gel in ethidium bromide and photographed under UV light.

Results and Discussion

PCR analysis results showed, samples numbered 33, 34, 35, 40 were positive about Citrus Psorosis Virus pathogen (Fig. 2). Electrophoresis results showed the positive samples were obtained in c, d, f, h lines at 600 bp (Fig. 3).

Fig. 1. Bark spalling on citrus stem which infected CPsV.

Fig. 2. Trees of samples which showed positive results.

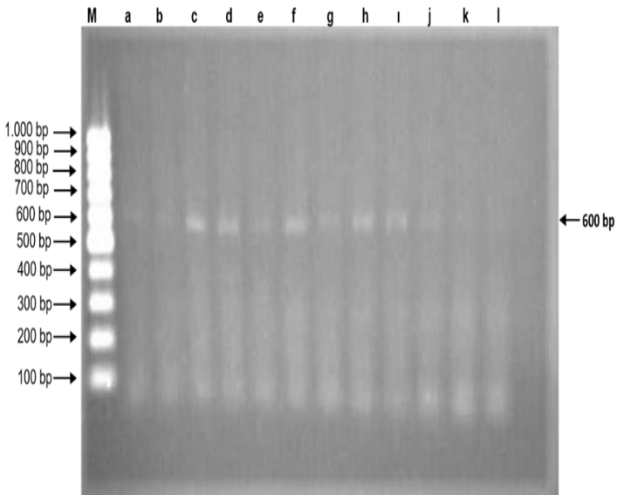

Fig. 3. Electrophoresis gel image of Citrus Psorosis Virus (CPsV) under UV after PCR analysis.

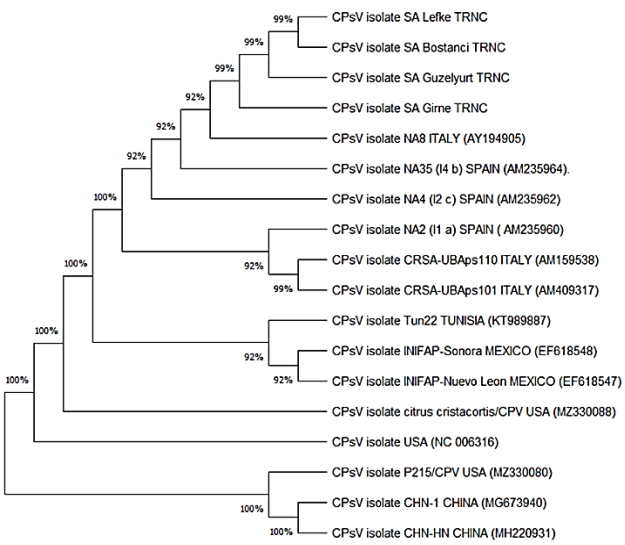

Fig. 4. Phylogenetic tree obtained as a result of comparing the degree of affinity between Cyprus isolates and other isolates in the world.

As a result of PCR and sequence analyzes of the samples found to be contaminated with CPsV were performed, nucleotide sequences were determined, and isolates obtained from Cyprus, CPsV-TRNC-SA Lefke (sample 33), CPsV-TRNC-SA Bostanci (sample 34), CPsV-TRNC-SA Guzelyurt (sample 35), and CPsV-TRNC-SA Girne (sample 40) were determined to be the same isolate (Fig. 2).

After examining the degree of affinity with other CPsV isolates previously detected in different parts of the world, it was determined that the isolate which found in Cyprus is the same as CPsV-NA8 (AY194905) from Italy; CPsV-NA35 (AM235964) and CPsV NA4 (AM 235692) from Spain which was degree of affinity determined % 92 on phylogenetic tree (Fig. 4).

Citrus psorosis virus is a species of the ophiovirus genus. Plant virus infection mostly causes a various disease symptoms that can have a dramaticaly impact on agricultural production (Reyes *et al*., 2016). A research conducted to assess the incidence of Citrus psorosis virus in Tunisia according to survey results shown that CPsV infects citrus varieties grown in Tunisia (Hamdi *et al*., 2017). Determination of CPsV by laboratory methods is an important requirement for researchers (Salem *et al*., 2018). RT-qPCR provides confidence in the rapid diagnostic procedure for monitoring the health status of citrus trees for the diagnosis of citrus psorosis virüs (Osman *et al*., 2015). PCR and RT-PCR tests, which are powerful tools for detection, offer advantages over ELISA due to their high sensitivity. Multiplex PCR and RT-PCR detect many pathogens, so using these types of methods saves labor, time and cost. It is generally beneficial in routine research and citrus bud tree certification programs (Yao *et al*., 2023; Meena *et al*., 2020). Therefore, PCR provides an effective method to detect citrus viruses, helping to diagnose many citrus plants simultaneously (Hyun & Jung, 2017). This is the mian reason to choose PCR analysis method to detect Citrus Psorosis Virus disease in Cyprus. Molecular detection of CPsV was performed using RT-PCR and primers approximately 600 bp in size in samples collected from citrus orchards (Falaki *et al*., 2020). In this research, the primers which used for RT-PCR were 600 bp size too.

Citrus psorosis virus (CPsV) is an important disease of citrus which psorosis is an casual agent. Sanitary and certification programmes helped reduce damages caused by psorosis in many citrus-growing regions (Simeone *et al.*, 2021).

Psorosis disease, which is commonly distributed all over the world, does not cause quick decline and yield losses, but leads to low productivity and gradual disappearance of the tree.

Virus diseases management method is sanitaiton and eradication to manage and prevent the transmission of virus diseases (Sedlak *et al.,* 2023)

This pathogen detected and reported in Cyprus, in next period, the positive trees have to be eradicated and positive orchards have to be sanitated to prevent the transmission of this disease.

In this study, leaf samples collected from 45 different orchards showed symptoms of psorosis virus in citrus fruits as a result of surveys in citrus orchards in northern Cyprus were molecularly diagnosed by PCR (Reverse Transcription- Polymerase Chain Reaction) method.

At the end of the PCR studies, 4 samples infected with Citrus Psorosis Virus (CPsV) were determined. The remaining 41 samples gave negative results.

Conclusion

Citrus existence on the island of Cyprus dates back to the 1300s. Many nations have established sovereignty over the island of Cyprus. Among them are Venetians, Ottomans and England (Kapari-Isaia *et al.*, 2002). People from there brought many plants and seeds with them when they came to the island. People who came to the island at that time brought young citrus saplings or bud scions. CPsV is transmitted with mechanically rapidly plant to plant. CPsV has carried to Cyprus from Italy with bud unions and scions.

In the upcoming period, scientists can investigate the effects of this virus factor on yield and plant. In addition, in order to make the molecular diagnosis of the virus easier, the periods in which the virus is in maximum concentration in plants should be determined in Cyprus conditions.

Cyprus, which has a natural biological isolation due to the fact that it is an island country, can easily be achieved with a production model that increases yield and quality with the researches to be made on many agricultural products, especially citrus, with the improvement of quarantine conditions and inspections. If these conditions are met, candidate entry of different virus diseases will be prevented.

As a result of this study, CPsV was reported for the first time in Northern Cyprus and its existence was proven.

References

Achachi, A., E. Ait Barka and M. Ibriz. 2014. Recent advances in Citrus psorosis virus. *Virusdisease*, 25: 261-276.

Alas, T., S. Baloglu, B.K. Caglar and A. Gunes. 2019. Detection and characterization of citrus tatter leaf virus (CTLV) and citrus yellow vein clearing virus (CYVCV) in citrus trees from Cyprus. *Saudi Journal of Biological Sciences*, 26(5): 995-998. https://doi.org/10.1016/j.sjbs.2019.02.001

Alioto, D., A. Troisi, A. Peluso, G. Quatrano, V. Masenga and R.G. Milne. 2000. Occurence of Citrus psorosis virus in Campania, Southern Italy. *Eur J Plant Pathol*., 106: 795-799.

Astruc, N., J.F. Marcos, G. Macquarie, G.T. Candresse and P. Vicent. 1996. Studies on the diagnosis of hop stunt viroid in fruit trees: İdentification of new host and application of a nucleic acid extraction procedure based on non-organik solvents. *European Journal of Plant Pathology*, 102: 837-846.

Barthe, G.A., G.L. Ceccardi, K.L. Manjunath and K.S. Derrick. 1998. Citrus psorosis virus: nucleotide sequencing of the coat protein gene and detection by hybridization and RT-PCR. *J. Gen .Virol*., 79: 1531-1537.

Belabess, Z., T. Sagouti, N. Rhallabi, T. Abdessalem, S. Massart, R. Tahzima, R. Lahlali and M.H. Jijakli. 2020. Citrus psorosis virus: current ınsights on a still poorly understood ophiovirus. *Microorganisms*. 6;8(8): 1197. doi: 10.3390/microorganisms8081197.

Clark, B.C., B.B. Jones and G.A. Jacubucci. 1981. Characterization of the hydroperoxides derived from singlet oxygen oxidation of (+) – limonene. *Tetrahedron Supp*., 1981;37(1): 405-409. https://doi.org/10.1016/0040-4020(81)85078-8

Falaki, F., S.V. Alavi, F. Rakhshandehroo and A. Mousavi. 2020. Partial biological and molecular characterization of citrus psorosis virus ınfecting satsuma mandarin (Citrus unshiu Marc) in the east of Mazandaran province. *Applied Entomology and Phytopathology*, 87(2): 195-207.

Galipienso, L., L. Navarro, F. Ballester-Olmos, J.A. Pina, P. Moreno and J. Guerri. 2000. Host range and symptomatology of a graft-transmissible pathogen causing bud union crease of citrus on trifoliate rootstocks. *Plant Pathology*, 49: 308-314.

Hamdi, I. and A. Najar. 2017. Incidence and molecular characterization of Citrus psorosis virus in Tunisia. *Tunisian Journal of Plant Protection*, 12: 135-147.

Hull R. Matthew. 2004. Plant Virology. Fourth ed. AcademicPress, 1001 p.

Hyun, J.W. and K.E. Jung. 2017. Development of multiplex PCR for simultaneous detection of citrus viruses and the incidence of citrus viral diseases in late-maturity citrus trees in Jeju Island. *The Plant Pathology Journal*, 33(3): 307.

Kapari-Isaia, T., G.J. Minas, D. Polykarpou, E. Iosephidou, S. Arseni and A.Kyriakou. 2002. Shoot-tip grafting *İn vitro* for elimination of viroids and citrus psorosis virus in the local arakapas Mandarin in Cyprus. *International Organization of Citrus Virologists Conference Proceedings*, 15(15):. https://doi.org/10.5070/C50kk5h9ff

Legarreta, G.G., M.L. Garcia, N. Costa and O. Grau. 2000. A highly sensitive heminested RT-PCR assay for the detection of citrus psorosis virus targeted to a conserved region of the genome. *J. Virol. Methods*, 84:15-22.

Loconsole, G., M.T. Fatone and V. Savino. 2009. Specific digoxigenin-labeled riboprobes for detection of Citrus prsorosis virus and Citrus variegation virus by molecular hybridization. *J. Plant Pathol*., 91(2): 311-319.

Luisoni, E. and D. Boshia. 1994. Serology course on plant virus diagnosis. Adana –Turkey. 15 –30 October, 1994; 29-66.

Meena, R.P., K. Prabha and V.K. Baranwal. 2020. Development of RT-PCR degenerate primers for the detection of two mandariviruses infecting citrus cultivars in India. *Journal of Virological Methods*, 275: 113753.

Moreno, P., J. Guerri and M.L. García. 2015. The psorosis disease of citrus: A pale light at the end of the tunnel. *Journal of Citrus Pathology*, 2(1): 1-18.

Osman, F., E. Hodzic, S.J. Kwon, J. Wang and G. Vidalakis. 2015. Development and validation of a multiplex reverse transcription quantitative PCR (RT-qPCR) assay for the rapid detection of Citrus tristeza virus, Citrus psorosis virus, and Citrus leaf blotch virus. *Journal of Virological Methods*, 220: 64-75.

Reyes, C.A., E.E. Ocolotobiche, F.E. Marmisollé, G. Robles Luna, M.B. Borniego, A.A. Bazzini and M.L. García. 2016. Citrus psorosis virus 24 K protein interacts with citrus miRNA precursors, affects their processing and subsequent miRNA accumulation and target expression. *Molecular Plant Pathology*, 17(3): 317-329.

Salem, R., I.A. Arif, M. Salama and G.E. Osman. 2018. Polyclonal antibodies against the recombinantly expressed coat protein of the Citrus psorosis virus. *Saudi Journal of Biological Sciences*, 25(4): 733-738.

Sedlák, J., J. Přibylová, I. Koloňuk, J. Špak, O. Lenz and M. Semerák. 2023. Elimination of *Solanum nigrum* ilarvirus 1 and apple hammerhead viroid from apple cultivars using antivirals ribavirin, rimantadine, and Zidovudine. *Viruses*., 15(8): 1684. https://doi.org/10.3390/v15081684

Simeone, M., C. Gómez, A. Bertalmío, E. Ruiz, C. Hauteville, L.G. Suarez, L. Blas Tito and M.L. García. 2021. Detection of citrus psorosis virus by RT-qPCR validated by diagnostic parameters. *Plant Pathology*, 70(4): 980-986. https://doi.org/10.1111/ppa.13341

Velázquez, K., J.A. Pina, L. Navarro, P. Moreno and J. Guerri. 2012. Association of citrus psorosis B symptoms with a sequence variant of the Citrus psorosis virus RNA 2. *Plant Pathology*, 61(3): 448-456.

Yao, S.M., M.L. Wu and T.H. Hung. 2023. Development of multiplex RT-PCR assay for the simultaneous detection of four systemic diseases ınfecting citrus. *Agriculture,* 13(6): 1227.

Zhou. C. 2018. Graft-transmissible citrus diseases in PR China-research developments. *Journal of Citrus Pathology*, 5(1):

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