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Combination of Cold Therapy and Chemotherapy for Eradication of *Citrus exocortis Viroid*

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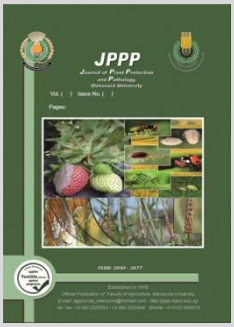

ABSTRACT

Citrus Excortis Viroid (CEVd) is California's first viroid disease described on citrus trees in 1948. It is the largest viroid of citrus with 371 nucleotides. The disease causes distinguished symptoms of bark scaling on rootstocks of citrus trees grafted onto trifoliate rootstocks causing severe stunting of trees grafted on these rootstocks. The use of indicator host plants for CEVd detection had been successful using clean matured fresh cuttings of specific indicators (Etrog lemon, Volkameriana lemon), which were grafted with the infected plants and cultured in plastic bags after a short dipping in IBA solution for 10 sec. Ideal clear symptoms were observed 18 days after grafted inoculation on new growing leaves of unique indicators. The obtained results of the biological indexing trail were confirmed using RT-PCR and transmission assays using some hosts such as *Solanum melongena*, *Piper nigrum*, *Gynura auranisa* and *Solanum esculentum* (Castel rock) with infectious sap. Results showed that cold treatment at 4 °C for 6 months companies with chemotherapy with 10, 20 and 30 mg/L Virazol and thiouracil gave plants free-viroid. viroid infection was reduced by using the highest concentrations of Virazol and thiouracil at 30 mg/L reaching 80 and 55%, respectively. The recovered plants were examined by RT-PCR. Cold therapy treatment and chemotherapy are effective and attractive techniques for eradicating CEVd from citrus trees in Egypt. Virazol has the greatest effect on the elimination of viroid because the viroid-free percentages by Virazol were higher than those obtained using thiouracil.

Keywords: CEVd; indexing; Cold therapy; Chemotherapy.

INTRODUCTION

Citrus Exocortis Viroid disease (CEVd) was first described by Fawcett & Klotz (1948). CEVd transmission was performed by grafting infected branches to healthy trees or mechanically (Lin *et al.*, 2015) using disinfected tools and infected buds. Because there are no chemicals to control this disease, the only method is prevention, using virus-free propagating material from genetic resource banks and greenhouses that are fully compatible with biological and molecular diagnostics. (Napo, 2013). This ailment is also known as "scaly skin" and "Rangpur Lyme disease" (Benton *et al.*, 1949). (Olson, 1952). Citrus plants that have been grafted onto sensitive rootstocks become small and produce fewer fruit. This disease is prevalent in most citrus growing regions with sensitive rootstocks. Although it is rare in North Africa and the United States, it is common in South America (particularly Brazil and Argentina), Australia, and the Mediterranean (especially Spain). In Japan, where there is no infection, commercial plantings are practically CEVd (Wallace, 1978). In studies relevant to infections, in vitro culture has proven to be an effective strategy for producing CEVd characteristic symptoms in commercially significant plants (El-Dougdoug *et al*., 2010). Rhizome sensitivity causes tree stunting and poor performance . The development of indicator species is typically weak, with brief leaf epinasty growth (Weathers, Greer & Harjung, 1967).

CEVd infecting trees Navelina Sweet orange cv. grafted onto Volkamariana lemon from field trials in Egypt which showed symptoms of gumming in the bark and wood. Biological indexing of the technique was carried out on the indicator host plant 'Etrog', Mexican lime and Volkamariana lemon. The infectious agent pathogen is composed of small naked single-stranded SSRNAs (about 371 nucleotides (nts)) that connect with the host's nucleus and membranes. There is no proof of vector transmission. The agent is easily moved mechanically. Many Rutaceae plants are impacted, as are some Solanaceae and Compositae species. It is ubiquitous in citrus-growing regions around the world. According to an unpublished study by J. S. Semank, Lycopersicon esculentum cv. (Castel rock) seeds can spread CEVd. Cuscutasubinclusa was utilized to spread citrus to citrus and citrus to petunias (Weathers, 1965a, 1965b). A few Solanaceae (Solanum tuberosum, Lycopersicon esculentum, Petunia hybrida) and Compositae (Gynura aurantiaca, G. saramentosa) species, as well as several Citrus (Rutaceae) species, are also vulnerable hosts. Mechanical sap inoculation from Gynura is easily propagated, whereas Citrus is difficult to spread (Weathers & Greer, 1968). Garnsey and Jones (1967). From 3 weeks to 6 months after grafting, there is epinasty and roughness of the leaves , cracking and browning of the vein undersides, and severe stunting (Calavan *et al*., 1964). Gynura aurantica or Lycopersicon esculentum. Some field isolates that are not transmissible to the herbaceous plants being examined perform well on Citrus medica (Etrog lemon).

Biological indexing by graft transfer using seedlings of indicator plants has been the main method of diagnosis for years. There are many limiting factors for the application of biological indexing, such as the need for expensive breeding

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facilities; high amount of indicator seeds; large space in the greenhouse; a long period for indicator plant production (1 year) and for development of symptoms after inoculation (1-12 months); high cost and labor and excellent plant management and symptom observation skills. In addition, any change that occurs during the indexing period can greatly jeopardize the good end of the test (Roistacher, 1991). Because of the multiple limiting aspects of graft transmission that are still required for the identification of most citrus viruses and virus-like illnesses, leafless Etrog citron cuttings treated with major citrus viroids are now utilized to index exocortis. viroid replication in general and citrus viroid replication (Abd-Elbacki *et al.*, 2005. The use of viroid-free propagation material, which can be produced by eradicating the pathogen from infected plants using the cold therapy technique (Morton *et al*., 1993), is critical to preventing or reducing the deleterious consequences induced by the viroid (Morton *et al.*, 1993). Momma and Takahashi (1983) used cold treatment at 10 C for 1-4 months to remove HSVd from hop plants.

Many scientists have tried chemotherapy to eliminate important viruses from potatoes grown in different regions of the world. Klein and Livingston (1982) determine efficacy of ribavirin (Virazole) treatment for potato eradication virus X (PVX) from cultivated potato shoots. Antiviral activity of Virazole was studied in several plant viruses. Virazole was found to be slows down and suppresses the systemic infection with Tomato Spotted Wilt Virus [TSWV] and tobacco plants if pretreated with virazole. It also decreases the concentration of CMV and Alfalfa Mosaic Virus [AMV] in tissue culture plant.

This study aims 1) to enhance the biological indexing test for detecting Citrus Exocortis viroid (CEVd) in citrus trees by using rooted inoculated cuttings of indicator plants.2) to investigate the impact of low-temperature treatment on the elimination of CEVd and its distribution in Etrog Citron tips.33) to assess the efficacy of various in vitro therapeutic interventions for the elimination of CEVd in citrus trees.

MATERIALS AND METHODS

***Citrus Exocortis viroid* CEVd source:**

Citrus Exocortis viroid infected trees of Navelina Sweet orange cv. grafted on Volkamariana lemon in Egypt that showed symptoms of gumming in the bark and wood and severe stunting have been used as a source of CEVd isolate (Fig.1). These positive sources were kept under screen house and greenhouse conditions.

Biological Indexing technique.

Traditional biological indexing was carried out by inoculation using the bud grafting technique of ten seedlings of lemon 'Etrog', lemon Volkameriana and mixcane lime with a positive source of CEVd. Grafted inoculated indicator seedlings were maintained at 32-38°C for 6 months to observe symptoms.

Inoculation of rooted cuttings.

Cuttings of indicator plants, six-month-old seedlings of Etrog lemon, Volkameriana lemon and mixcane lime were used to detect CEVd, which produces characteristic symptoms. (Bhusal *et al* 2001), 6-month-old seedlings of the above indicators were used as sources of stem cuttings. About 50 fresh cuttings of each indicator with 6 nodes were grafted-inoculated with bark tissue from the CEVd source (Figure 1). Grafts were sealed with parafilm and immersed in IBA (indole-3-butyric acid) solution for 10 seconds; then placed in a soil mixture containing peat and perlite inside a plastic bag. The grafted inoculated cuttings were then cultivated in a greenhouse at high temperatures (34-36°C). Five days after grafting, the plastic bag was opened from above and completely removed after 10 days for acclimatization (Fig. 3). Observation of symptoms was carried out starting with flushing of the shoots. Plants were kept for more than 3 months in the greenhouse in the same pots for further investigation (bark cracking symptoms). Symptoms were observed after 21 days in Etrog Citron infected with CEVd. Nevertheless, most symptoms developed after 25-30 days in V. citron cuttings.

Host Range

Some host species of the Solanaceae (*Solanum tuberosum, Lycopersicon esculentum* (Casttel rock cv.), *Nicotiana tabacum* and *datura metal*), Amaranceace (*Chenopodium amaranticoclor and Chenopodium quinoia*) and Compositae (*Gynura aurantiaca*) were inoculated with infected sap using razor slash inoculation. Citrus medica (*Etrog citron*). About 100 ng of viroid transcripts were mechanically inserted into the cotyledons of 12-day-old tomato seedlings. As previously disclosed, tomato plants were mock infected with 20 mM sodium phosphate buffer (pH 7.0). According to (Semank & Weathers, 1972c), the symptoms of Lycopersicon esculentum (tomato) cv. Rutgers were identical to Etrog lemon; apparent from 10 to 30 days after sap inoculation. The principal herbal indication is Gynura aurantiaca (velvet plant). Similar to Etrog citron, symptoms appear 10 to 30 days after sap inoculation (Weathers & Greer, 1967.

Molecular detection of CEVd.

According to Qiagen Inc.'s Mini kit RNeasy Plant instructions, CEVd-infected citrus plants' total RNA was extracted. In liquid nitrogen, a mortar and pestle pounded 100 mg of fresh leaves into a fine powder. Sterile microcentrifuge tubes contained pulverized sample powders. Vortex and incubate 450 µL RLC buffer at 56°C for 3 min. This lysate was centrifuged for two minutes at maximum speed using a purple QIA shredder spin column in a 2 ml collecting tube. Without disrupting the cell debris pellet in the collection tube, the flow-through fraction was moved from the QIA shredder to a new tube. The cleaned lysate received 0.5 vol of 96–100% ethanol, which was pipetted thoroughly. A 2 ml collection tube containing these samples—including any precipitate—was put onto an RNeasy mini column (pink) and centrifuged for 15 seconds at 10,000 rpm. After pipetting 700 µl of RWI buffer onto the RNeasy, it was centrifuged for 15 seconds at 10,000 rpm. The tubes used for flow and collection were thrown away. On to a 2 ml collecting tube went RNeasy. The RNeasy column was coated with 500 µL of RPE buffer, which was then centrifuged for 15 seconds at 10,000 rpm, discarding the supernatants. To dry the membrane, 500 µL RPE buffer was added to the RNeasy column and centrifuged at maximum speed for 2 minutes. The RNA was eluted with 50 µL of water and stored at -20 °C after the RNeasy column was transferred to a new 1.5 mL collection tube and 30 to 50 mL of RNase-free water was directly put over the membrane.

RNA preparations were subjected to reverse transcription polymerase chain reaction (RT-PCR) using the Reverse-iTTM One-Step RT-PCR Kit (ABgene®UK). Because of this, RT and amplification can be carried out

consecutively within the same tube. To be more specific, 1.25 U/50 µl of 2x RT-PCR Master mix was combined with 2.5 µl of target RNA in a mixing volume of 12.5 µl. (Taq Polymerase) 1.5 mM MgCl2, 0.0079 in each dNTP, 10 M of particular direct and reverse primers (Table 1), 0.5 µl Reverse-iTTM RTase Blend (50 U/µl), and RNase/DNase-free water up to a volume of 25 µl are the components that are required. The synthesis of cDNA was carried out at a temperature of 47 degrees Celsius for thirty minutes, and then it was denatured at 94 degrees Celsius for two minutes. Amplification was carried out for a total of 35 cycles at the following temperatures and times: denaturation at 94 degrees Celsius for 30 seconds, annealing at 62 degrees Celsius for 30 seconds, extension at 72 degrees Celsius for 60 seconds, followed by a final extension at 72 degrees Celsius for seven minutes. Agarose gel electrophoresis allowed for the detection of the results of amplification.

Table 1. Oligonucleotide primer pairs used for CEVd RT-PCR.

Primer	Sequence 5′-3′	(bp)	Reference
CEVd-F	>5' GGG GGA AGA AGT CCT TC < 3'	370	Rigden and Rezaian (1992)
CEVd-R	>5' CCG CCT CTT TTT TCT TTT CCT GCCTGC <3'		

Electrophoretic analysis

On 1.5 %agarose gels (6 x 8 cm) in TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.5), five µl aliquots of PCR products were examined at 120 volts. The buffer contained 89 mM Tris-HCl, 89 mM boric acid, and 2.5 mM EDTA. For determining the size of the PCR products, DNA molecular weight markers of 100 bp (ABgene, UK) were utilized. Ethidium bromide at a concentration of 10 g/ml was employed to stain the gels, and the results were observed under UV illumination (Bio-Rad) (Sambrook *et al*., 1989). After 75 minutes, the agarose gels were irreparably damaged and stained with ethidium bromide.

Assessment of plant growth regulators' hormones

Plant hormones IAA, IBA,GA3 and Abscisic (sa)4 acid were determined quantitatively by using the HPLC "high–performance liquid chromatography" according to the method of Koshioka, *et al.*, (1983), and Weiler and Zenk (1976) at the central lab. Of *Horticulture Research Institute* (HRI), Agricultural Research Centre (*ARC*), *Egypt*.

Anatomical changes:

For the purpose of this anatomical study, both healthy and diseased citrus leaves were utilized. Using a rotary microtome, thin sections measuring 15-17 m in thickness were cut out of the tissue, and the sections were then killed and fixed in FAA (10 ml formalin, 5 ml glacial acetic acid and 85 ml ethyl alcohols 70 percent). After being dehydrated in a progression of ethyl alcohols, the sections were washed in ethyl alcohol at a concentration of fifty percent (70, 90, 95 and 100 percent). Following that, it was infiltrated in xylene and embedded in paraffin wax, which has a melting point of between 60 and 63 degrees Celsius. After mounting the sections on slides, they were stained with aqueous Safranin (1 percent) and Fast Green (0.1 percent) (in 95 percent ethanol) following the description provided by Ruzin (1999). Histological signs of observable reactions brought on by infection were searched for by microscopically examining the sections of the tissue.

Cold treatment:

Cold treatment was performed to the infected plant material of citrus seedlings (Citron etrog). Infected seedlings were clipped and placed at 4°C for 6 months under a 16-h photoperiod and 5000 Lux in a growth chamber.

Chemotherapy:

After cold treatment at a low temperature, antiviral agents were used as leaf application: Ribavirin (1-B-D-ribofuransyl - 1, 2, 4-triazolecaboxamide, Virazole®) and thiouracil in different concentrations (10, 20 and 30 mg/l) to an evaluation of effectiveness and appropriate concentration to eliminate CEVd disease.

RESULTS AND DISCUSSION

Results

Symptoms of the manifestation of traditional indexing on indicator seedlings (Fig. 2) and rooted inoculated cuttings of indicator plants used in this experiment recorded in tables (2) including Epinasty after 35 days in the traditional indexing technique, while in the improved technique, epinasty symptoms were observed after 21 days in inoculated cuttings of indicator plans. These test plants showed positive results and were tested by RT-PCR. The results suggest that efforts are needed to increase and stimulate indexing programs, to maintain plant health, and develop sanitation programs aimed at limiting the spread of viroids and other graft-borne agents.

Figure 1. Symptoms of CEVd include bark peeling and gumming on the bark on the left and stunting (right).

Conventional biological indexing

Inoculated one-year-old seedlings of the tested indicators showed symptoms much later than in the case of inoculated indicator stem cuttings. Leaf epinasty was easily observed in the first growth spurt of all indicators within 4 weeks.

Figure 2.Traditional CEVd indexing: (A) left + positive plant, (B) right negative plant.

Inoculated rooted stem cuttings

As shown in Table 2, the graft success rate was relatively high in all indicators. Among the tested indicators, shoot flushing usually appeared after 8-10 days; symptom development varies in terms of symptom onset time and number of symptom plants. Symptoms could be observed after 18 days in Etrog lemon cuttings. However, most symptoms developed after 18-25 days. V. lemon first showed typical CEVd leaf symptoms a few days later the leaf. In the case of M. lime, which showed the same rate of symptomatic plants, but different week s as the period of development of symptoms (Figs. 3 and 4). Inoculated rooted cuttings of all indicators could easily be maintained in the medium for several months by supplying organic elements through leaf spray and irrigation water.

Table 2. Comparison between traditional indexing and rooted inoculated cuttings with CEVd.

Indicators	Rooted inoculated cuttings	Traditional indexing on seedlings
	Symptoms period/day	Symptoms period/day
Etrog citron	18 / day	Epinasty / 40 days
Volkamariana lemon	23/ day	Epinasty / 35 days
Mixcane lime	25/ day	Epinasty / 90 days

The obtained results verify the previous findings. The importance of temperature and humidity for the manifestation of symptoms, especially in rotten inoculated cuttings in young emerging shoots.

Figure 3. Storage of inoculated cuttings in plastic bags in a warm greenhouse (A, B and C). Root development (D & E). Development of new shoots.

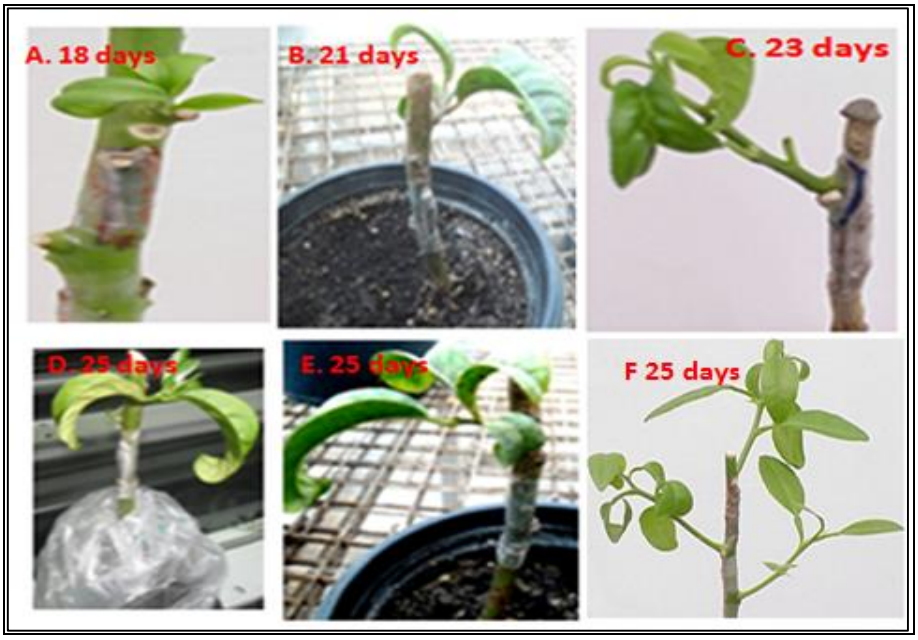

Figure 4. Symptom development on rooted inoculated Etrog cuttings (A, B, C, D and E). Mexican lime showing Epinasty (F).

Mechanical transmission:

Infectivity of Citrus Exocortis viroid isolated from lime Sp. has been confirmed by mechanical inoculation using a specific host such as Gynura. Abandonment symptoms induced by lime viroid consisted of slight epinasty at 3 months post-inoculation under greenhouse conditions (Figures 5 and 6).

Figure 5. Symptoms development on (A) Tomato solanum esculentum, (B) Nicotiana tabaccum, (C) Piper nigrum and (D) Solanum melongena.

Figure 6. Symptoms development on *Gynura aurantiaca*: healthy control (right), and typical epinasty in the new leaves (Left).

RT-PCR of CEVd isolate:

Total RNA of CEVd isolate was successfully amplified by the RT-PCR technique. An expected band size of approximately 370 base pairs was observed. DNA bands of a typical CEVd isolate were obtained as shown in Fig. (7). RT-PCR appears to recognize the Egyptian CEVd isolate.

The total RNA of the CEVd isolate was successfully amplified with the RT-PCR technique. The expected band size of approximately 370 base pairs was observed. DNA bands of typical CEVd isolate were obtained as shown in fig (7). The use of RT-PCR appears to be capable of recognizing the Egyptian isolate of CEVd and could be adapted for use on a larger scale. The method that was employed in this study to identify citrus Exocortis viroid infection is relatively uncomplicated, can be completed in a short amount of time, and is reliable.

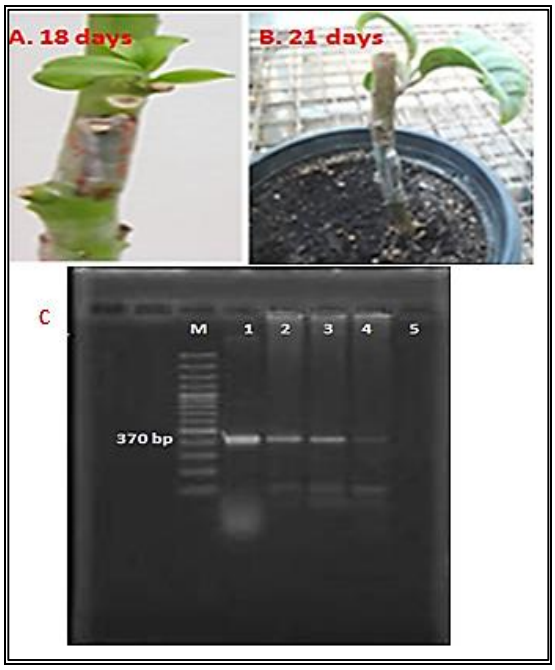

Figure 7. (A&B) typical epinasty symptoms of CEVd on Etrog citron rooted inoculated cuttings. (C) Agarose gel showing reverse transcriptase–polymerase chain reaction products amplified with specific primers for CEVd from viroid-infected citrus samples. M = 100 bp DNA ladder-, 1: positive control; 2-4, healthy citrus tested samples. Positive results were indicated by the appearance of bands at 370 bp position, 5 negative controls.

Anatomical studies on Exortis viroid-infected citrus leaves were determined using microtome sections. The obtained results show that in the tissue infected with CEvD, the mesophyll cells were compacted and the spongy cells were characterized as small and spongy tissues with reduced intracellular space, the pith, xylem, phloem and vascular bundle cells were normal with good compatibility in the healthy control as shown in Figures (8 and 9).

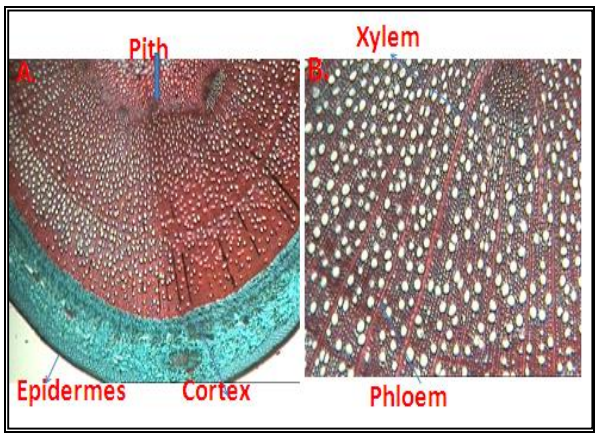

Figure 8. (A) stem section (grafting-budunion) of healthy grafted cuttings showing good compatibility. (B) healthy xylem vessels. (Mag. X50)

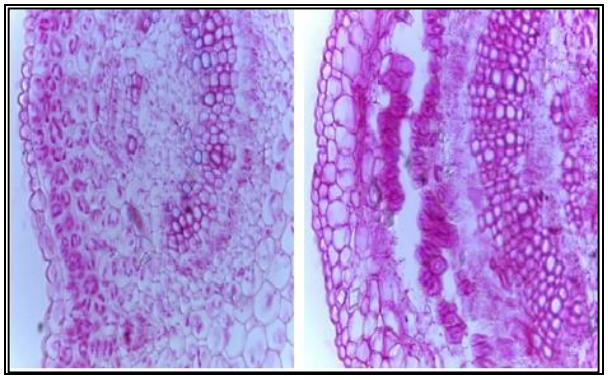

Figure 9. Transverse section of a leaf of a healthy Etrog Citron seedling showing healthy cells. (Mag. X40). A transverse section of a leaf of an infected Etrog lemon seedling shows necrosis and death of some xylem vessels. (Mag. X50).

Plant hormones:

We examined the amounts of IAA, IBA, and GA3 that were present in the leaves of rooted infected cuttings and seedlings to investigate the connection between endogenous plant hormones and the process of bud differentiation. Our results showed that the cuttings had significantly higher levels of GA3, ABA, and IBA compared to the seedlings, while the content of IAA in the cuttings' leaves was significantly lower than that of the seedlings. Additionally, our experiment revealed that IAA delayed seedling bud germination, whereas GA3, ABA, and IBA increased it in cuttings. Notably, the content of GA3 was higher in leaf cuttings than in seedlings. These findings suggest that endogenous IAA and ABA can influence inflorescence structure by affecting bud development, as evidenced by the increase in leafless inflorescence GA3 and the rise in ABA content and reduction in IAA content.

Table 3. Evaluation of plant growth regulator hormone content in the leaves of rooted inoculated cuttings and seedlings.

Hormon	Rooted cuttings	Seedlings
GA3	24.57µg/ 1g	22.40µg/ 1g
Abscisic (sa)4	3.84µg/ 1g	0.254µg/ 1g
3Indol acetic acid	0.10µg/ 1g	2.40µg/ 1g
3Indol butyric acid	0.09µg/ 1g	0.08µg/ 1g

Elimination of CEVd with cold therapy and chemotherapy:

When citrus seedlings infected with CEVd were treated with cold therapy at 4°C for six months, 65% of them were eliminated. The effectiveness of chemotherapy varied depending on the concentration of virazole and thiouracil used. As demonstrated in Table 4, the incorporation of Virazol® at concentrations of 10, 20, and 30 mg/L progressively increased the percentage of viroid-free citrus seedlings. Similarly, the percentage of viroid-free plants increased with the increasing concentration of thiouracil.The use of antiviral compounds, virazole, and thiouracil, also improved shoot growth differentiation. The percentage of CEVd elimination using Virazol® was 35%, 60%, and 80%, while the percentage of CEVd elimination using thiouracil was 15%, 25%, and 65%. The elimination of CEVd from citrus trees was evaluated using the RT-PCR method, in conjunction with cold therapy and chemotherapy.

Table 4. The percentage of CEVd- free plants treated with cold therapy and chemotherapy.

Treatment	CEVd elimination (%)
Cold therapy (4C)	65
Chemotherapy	
Virazole	
10 mg L	35
20 mg L	60
30 mg L	80
Thiouracil	
10 mg L	15
20 mg L	25
30 mg L	65

***The proportion was determined based on a total of twenty different plant materials. RT-PCR was used, and it corroborated the results.**

Discussion

According to these findings, CEVd can be detected successfully by utilizing inoculated indicator cuttings rather than seedlings as the test subject. Because the creation of stem cuttings in warm conditions overcomes the seasonal variability of rooting in most citrus species, this method might be utilized throughout the entire year. Stem cuttings can be taken from the top of the plant. It is possible to simply conclude the results of a bioassay utilizing indicator cuttings one month after grafting but before any transplantation. This strategy eliminates all of the drawbacks associated with conventional biological indexing in citrus without diminishing the accuracy of symptom expression. The IAA level of cutting leaves was significantly lower than that of seedlings, supporting Pennazio and Roggero's (1996) notion that viruses that cause plant disease have decreased auxin activity. Cuttings have significantly larger levels of GA3, ABA, and IBA than seedlings, but their leaves have significantly lower levels of IAA.It was discovered through the process of determining the hormones of plant growth regulators that the content of these hormones was higher in cuttings than it was in seedlings. As a result, the differentiation of new buds occurs earlier than it does in seedlings, and symptoms appear earlier than in seedlings. However, for a reliable sanitary assessment of citrus genotype, the use of this technique in tandem with laboratory tests is always suggested, particularly for cuttings that do not exhibit any symptoms of the disease. To extend the application of stem cuttings to other citrus indicator species, which is required for the biological characterisation of the virus, more studies should also be undertaken utilizing mild and moderate pathogen strains.

For the development of CEVd-free seedlings, sensitive diagnostic procedures are required. These methods must be able to identify the presence of viroids in infected plant tissue even when they are present in low concentrations. In this first stage of the diagnostic process, RT-PCR can be utilized to produce results that are both more accurate and confirmed. Citrus Exocortis viroid (CEVd)-free plants were obtained from infected materials by using a low-temperature treatment of infected plants for six months before chemotherapy using antiviral agents to eliminate CEVd from diseased plants. The same results were obtained (Adams *et al.*, 1995 ; Lizarraga *et al.*, 1980 and Paduch and Kryczynski, 1987). It is recommended to use the approach for viral particles that can withstand high temperatures (PSTVd, CSVd, HLVd). Hops plants that were infected with HLVd were subjected to a variety of low-

temperature treatments for varying amounts of time to eradicate the virus. The recovery rate of healthy plants was at its lowest when the plants were subjected to a very long cold treatment that lasted for up to 21 months. The plants that were kept at a low temperature for eight months produced the maximum number of HLVd-free plants. Our findings was agreed with those obtained by Grudzinski *et al*., (2006), who demonstrated that a cold treatment of plants for one month was sufficient to successfully remove HLVd from infected plants. Our findings were in agreement with those obtained by Grudzinski *et al*., (2006).

Cold therapy combined with chemotherapy can successfully remove CEVd from infected citrus plants in vitro, according to the current study's findings. It should be emphasized that success is strongly dependent on taking extra precautions when working with healthy plants and evaluating them on a regular basis.

CEVd-free plants were obtained from infected material by foliar application of antiviral compounds virazole and thiouracil to infected plants at various concentrations after cold treatment, which showed antiviral activity against CEVd plant infection.

CONCLUSION

In summary, both cold therapy and chemotherapy are effective techniques for eliminating CEVd from citrus trees. However, our results suggest that , a cold thermotherapy is a time-consuming approach. Therefore, a combination of in vitro cold therapy with chemotherapy may be a more attractive and alternative tool for reducing the risk of CEVd introduction into Egypt. Virazol was found to have the greatest impact on the elimination of viroids, with higher viroid-free percentages compared to thiouracil.

List of abbreviations:

ABA	Abscisic acid
Bp	base pair
CEVd:	*Citrus Exocortis Viroid*
GA3	Gebrilic acid
IBA	Indole-3-butyric acid
IAA	Indole acetic acid
RT-PCR	Reverse Transcription polymerase chain reaction
CV.	Cultivar

REFERENCES

Abd-Elbacki A.M., Milano R., Djelouah K., Brandonisio R., A.M. D'Onghia (2005). Improvement of detection of citrus exocortis viroid (CEVd) and citrus cachexia viroid (CCaVd). XVI conf of the IOCV, Monterrey 2004: in press

Adams, A.N., D.J. Barbara, A. Morton, P. Darby, (1996). The experimental transmission of hop latent viroid and its elimination by low treatment and meristem culture. Ann. Appl. Biol., 128: 37-44.

Adams, A.N., D.J. Barbara, A. Morton, P. Darby, C.P. Green, (1995). The control of hop latent viroid in UK hops. Acta-Horticulturae. 385: 91-97.

Benton, R.J., Bowman, F.T., Fraser, L. and Kebby, R.G. 1949. Stunting and scaly butt of citrus associated with Poncirustrifoliata rootstock Agr. Caz. NSW, 60: 521-526, 577582 641-645,654.

Bhusal R.C., Fusao M, Doo-Gyung M. and Kipkoriony L.R., (2001). Propagation of citrus by stem cuttings and seasonal variation in rooting capacity. Pakistan Journ. of Biol. Sc. 4 (11): 1294-1298.

Bitters, W. P., J. A. Brusca, and N. W. Dukeshire. (1954). Effect of lemon budwood selection in transmission of exocortis. Citrus Leaves 34(1):8-9, 34.

Calavan, E.C., Frolich, E.F., Carpenter, J.B., Roistacher, C.N. and Christiansen, D.W. (1964). Rapid indexing for exocortis of citrus Phytopathol., 54 1359- 1362

Chun-Yi Lin, Meng-Ling Wu, Tang-Long Shen, Hsin-Hung Yeh, and Ting-Hsuan Hung (2015). Multiplex detection, distribution, and genetic diversity of Hop stunt viroid and Citrus exocortis viroid infecting citrus in Taiwan. 015-0247.

D'Onghia A.M., El Sayed T., Djelouah K., and Brandonisio R. (2009). Improved biological indexing of Citrus tristeza virus (CTV).In :D'Onghia A.M. (ed.), Djelouah K. (ed.), Roistacher C.N. (ed.). Citrus tristeza virus and Toxopteracitricidus: a serious threat to the Mediterranean citrus industry. Bari : CIHEAM, p. 143-146

El Sayed T., (2005). Setting up of biological indexing of Citrus Tristeza Virus (CTV) and Citrus infectious variegation (CVV) using inoculated indicator cuttings. CIHEAM-IAMB MSc Thesis, Italy, Collection Master of Science 393: 23 p

Elbacki A.M., Milano R K., Brandonisio ., Djelouah R., and D'Onghia A.M., (2005). Improvement of detection of citrus exocortis viroid (CEVd) and citrus cachexia viroid (CCaVd). Proc. 16th Conf. of IOCV .

El-Dougdoug KHA, Osman ME, Hayam AS, Rehab DA and Reham EM. (2010). Elimination of Hop Stunt Viroid (HSVd) from infected peach and pear plants using cold therapy and chemotherapy. Australian Journal of Basic and Applied Sciences 4: 54-60.

Fawcett, H.S. & Klotz, L.J. (1948). Bark shelling of trifoliate orange Calif. Citrogr., 48-230.

Gergerich RC, Welliver RA, Gettys S, Osterbauer NK, Kamenidou S, Martin RR, Golino DA, Eastwell K, Fuchs M, Vidalakis G and Tzanetakis IE. (2015). Safeguarding fruit crops in the age of agricultural globalization. Plant Disease 99: 176-187.

Grande Valley Hort. Inst. 6:46–50. Garnsey, S.M.; Weathers, L.G. (1967). Factors Affecting Mechanical Spread of Exocortis Virus lnoculation Procedures.

Garnsey, S. M., and Jones, J. W., 1967, Mechanical transmission of exocortis virus with contaminated budding tools, *Plant Dis. Rep*. 51:410.

Grudziñska, M., E. Solarska, A. Czubacka, M. Przybyœ and A. Fajbuœ, (2006). Elimination of Hop latent viroid from hop plants by cold treatment and meristem tip culture. Phytopathol. Pol., 40: 21-3

Klein RE, Livingston CH. 1982. Eradication of potato virus X from potato by ribavirin treatment of cultured potato shoot tips. Am. Potato J. 59: 359-365.

Koshioka M, Harda J, Noma M, Sassa T, Ogiama K, Taylor JS, Rood SB, Legge RL andPharis RP, (1983): Reversed – phase C18 high performance liquid chromatography of acidic and conjugated gibbberellins. J. Chromatgr., 256:101-115.

Lin CY, Wu ML, Shen TL, Yen HH and Hung TH. (2015a). Multiplex detection, distribution, and genetic diversity of Hop stunt viroid and Citrus exocortis viroid infecting citrus in Taiwan. Virology Journal 12:11.

Lizarraga, R.E., L.F. Salazar, W.M. Roca, L. Schilde-Rentschler, (1980). Elimination of potato spindle tuber viroid by low temperature and meristem culture. Phytopathology, 70: 754-755.

Momma, T. and T. Takahashi, (1983).. Cytopathology of shoot apical meristem of hop plants infected with hop stunt viroid. Phytopath. Z., 106: 272-280.

Morton, A. D.J. Barbara and A.N. Adams, (1993). The distribution of hop latent viroid within plants of Humulus lupulus and attempts to obtain viroid-free plants. Ann. Appl. Biol., 123: 47-53.

NAPPO. (2013). Norma regional de medidas fitosanitarias 16. Medidas integradas para la movilización de material propagativo de cítricos. North American Plant Protection Organization. North Carolina, USA. Disponible en línea: http ://nappo. org/f iles/8914/5083/2412/RSPM16_10-09-2013-s.pdf.

Olson, E.O. (1952). Investigations of citrus rootstock diseases in Texas. Proc. Rio

Paduch-Cichal, E., S. Kryczyñski, (1987). A low temperature therapy and meristem-tip culture for eliminating four viroids from infected plants. J. Phytopathol., 118: 341-346.

Papayiannis LC. (2014). Diagnostic real time RT-PCR for the simultaneous detection of Citrus exocortis viroid and Hop stunt viroid. Journal of Virological Methods 196:93-99.

Pennazio, S. and Roggero, P. (1996): Plant hormones and plant virus diseases. The auxins.The New Microbiologica: 19(4):369-78

Rigden, J. E., Rezaian, M. A (1992). *In vitro* synthesis of an infectious viroid: Analysis of the infectivity of monomeric linear CEVd. Virol. 186, 201-206.

Roistacher CN, Pehrson JE, and Semancik JS (1991). Effect of citrus viroids and the influence of rootstocks on field performance of Navel orange. Page 234-239. In: Proceedings of the 11th Conference of the International Organization of Citrus Virologists, Riverside, Gainesville FL.

Roistacher, C.N., Calavan, E.C., Blue, R.L., Navarro, L. and Gonzales, R. (1977). A new more sensitive citron indicator for detection of mild isolates of citrus exocortis viroid (CEV). Plant Dis. Rep., 61: 135-139.

Ruzin, S.E. (1999). Staining techniques.In Ruzin SE, ed. Plant Microtechnique and microscopy. New York; Oxford University Press, 87-116.

Salibe AA, and Moreira S., (1965). Tahiti lime bark disease is caused by exocortis virus. Proceedings Conference of the International Organization of Citrus Virologists 3:143-147.

Sambrook, J.; Fritsch, E.F. and Maniatis, T. (1989). Molecular Cloninig: A laboratory Manual, edt., Cold Spring Harbor laboratory, New York. Sci Bull 70, 20 pp

Semancik, J. S., and Weathers, L. G., 1972, Exocortis disease: Evidence for a new species of "infectious" low molecular weight RNA in plants, *Nature* 237:242.

Wallace, J. M., 1978, Virus and virus-like diseases, in: *The Citrus Industry* ,Vol. IV (W. Reuther, E. C., Calavan, and G. E. Carman, eds.), pp. 67–184, Univ. Calif. Div. Agric. Sci., Berkeley.

Weathers, L. G., 1965, Transmission of exocortis virus of citrus by *Cuscuta subinclusa, Plant Dis. Rep*. 49:189.

Weathers, Greer and Harjung (1967). Transmission of exocortis virus of citrus to herbaceous plants, Plant Dis. Rep. 51:868..

Weathers, L.G. (1980). Exocortis. In Bové, J. M. & Vogel, R., eds. Description and illustration of virus and virus-like diseases of citrus. A collection of colour slides. Paris, I.R.F.A. SETCO- FRUITS.

Weiler, E.W., and Zenk, M.H., (1976). Radioimmunoassay for the determination of digoxin and related compounds in Digitalis lanata. Phytochemistry 15, 1537±1545

دمج طريقة العلاج بالبرودة والعلاج الكيميائي للقضاء على مرض اكسكورتز في الموالح.

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الملخص

يعد مرض اكسكورتز الموالح هو أول مرض فيرودي تم اكتشافه في عام ١٩٤٨ في كاليفورنيا على أشجار الموالح. وهو أكبر فيرويدات الموالح عند ٣٧١ نيوكليوتيدة. ويتسبب هذا المرض في ظهور أعراض تقشر اللحاء على جذر أشجار الموالح المطعمة بالبرتقال ثلاثي الأوراق وهجينه وقد يتسبب في تقزم الأشجار المزروعة على تلك الأصول. تم استخدام النباتات الكاشفة للكشف عن اكسكورتز الموالح بنجاح باستخدام عقل طازجة ناضجة من نباتات (Volkameriana lemon ، Etrog citron) بعد تلقيحها بالفيرويد وغمسها في محلول هرمون التجذير IBA ثم زراعتها في اكياس بلاستيكية بها مخلوط تربة من البيتموس والرمل والبرليت بنسبة ١:١:١. لوحظت أعراض واضحة نموذجية بعد ٢١ يومًا من العدوي على الأوراق الجديدة للنباتات الكاشفة. تم تأكيد نتائج الاختبارات البيولوجية باستخدام RT-PCR واختبارات النقل باستخدام بعض العوائل النباتات مثل الطماطم والجينيورا والفلفل والباذنجان بواسطة هذا الاختبار .أشارت النتائج إلى أن العلاج البارد عند ٤ درجات مئوية لمدة ٦ أشهر مصحوبة باستخدام العلاج الكيميائي بـالفيرازول والثيوراسيل بمعدل ١٠ و ٢٠ و ٣٠ ملجم / لتر أعطت نباتات خالية من الفيرويد حيث انخفضت الأصابه باستخدام اعلى تركيز من الفيرازول والثيوراسيل بنسبة ٥٥ و ٨٠٪ على التوالي. تم الكشف عن وجود الفيرويد في النباتات المعاملة بواسطة RT-PCR من خلال النتائج المتحصل عليها تبين أن العلاج البارد والعلاج الكيميائي من الأساليب الفعالة لاستئصال CEVd من أشجار الموالح حيث يبدو أنها طرق فعالة وبديلة لتقليل مخاطر هذا المرض إلى. للفيرازول تأثير أكبر على القضاء على الفيروسات أكثر من تأثير استخدام الثيوراسيل.