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Plant diseases associated with olive bark midge in West-Bank Palestine

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ABSTRACT

Olive tree is one of the most cultivated trees over the Palestinian territories, it is considered as the mainstay of rainfed agriculture in Palestine. Recently and due to the impact of the global warming, olive trees were infected with an outbreak of many pests and pathogens. In the last decades, both olive fruit fly, *Bactrocera oleae* (Diptera, Tephritidae) and peacock leaf spot, *Spilocaea oleagina* were reported with an unusual percentage of infections over the olive trees. During 2015-16, and throughout the field surveys to investigate any unusual diseased symptoms on olives; some trees exhibited pale yellowing on the newly formed branches, symptoms associated with viral infections such as OLYaV. Some other trees were noticed to get yellowing and later on developed branch dieback and stem canker and cracking syndromes. When the outer bark was removed, the affected tissue appeared dark brown, in contrast to the yellowish green of healthy inner bark. These symptoms were observed on both young and old trees in the northern part of Palestine. Field and laboratory investigation revealed a heavy infestation with larvae of *Resseliella oleisuga* Targ. (Diptera : Cecidomyiidae). The infestation rate reported ranged from 75 to 92 in some olive orchards. Pathogens were isolated and identified based on cultural morphology. Climate changes due to the global warming might be the cause of this outbreak; probably due to the changes in the environmental conditions favoured by the insects. To our knowledge, this is the first time this insect was reported to be widely spread of olive trees and causing damage in Palestine. In this study, several associated primary, secondary and saprophytic diseases were detected from the infected samples. Mainly *Botryosphaeria* spp., *Alternaria solani* Sor., *Aspergillus niger* v. Tieghem, *Cladosporium herbarum* Fr., *Fusarium solani* (Mart.) App., *Penicillium digitatum* Sacc., *Penicillium italicum* Wehmer, *Rhizopus stolonifer* (Her.) Vuill.

Key words : Olive bark midge, olive tree, pest management, pathogen, viruses

INTRODUCTION

Olive tree is the most important oil crop in Palestine, covering more than 460541 dounm, which covers more than 67.3% of the total agricultural lands in the West Bank and Gaza, and about 85% of the total cultivated fruit trees land area. This distribution area has steadily increased during the last decade. Olive trees are attacked by several insect-pests and diseases. In recent years, olive bark midge, *Resseliella oleisuga* (Targ.-Tozz.) (Diptera : Cecidomyiidae), a pest usually of modest importance for commercial olive crops, showed a considerable increase of its infections, providing significant concerns among nursery growers of this district. Infested shoots may decline and turn brown, developing leaves and fruits may dry, necrosis of the bark, infested branches are eventually died. In 2015-16,

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alarming incidence of wilting, twig dieback and death of young trees were reported by many farmers (Alkowni *et al.,* 2016). This problem, called 'Seca' in Spanish or Drying Syndrome (DS) by farmers and pest management technicians, has been diagnosed at the Plant Health Service in C´ordoba (PHSC) separately from Verticillium wilt, the most important disease affecting young olive trees in southern Spain (Rodriguez-Jurado *et al.,* 1993).

This study aimed at determining the insect-pests infection rate and disease incidence and identifying the various primary, secondary and saprophytic mold fungi that are usually associated with olive bark midge, for future and potential alternative control method.

MATERIALS AND METHODS

Field Surveys and Fungal Isolations

Field surveys were conducted from 2016 to 2017, throughout the main olive-production areas in West Bank, Palestine. More than 30 sites in the nine governorates (Table 1 and Fig. 1) were visited weekly, sampled for olive bark midge (OBM) and associate pathogens. Surveyed locations included semi-naturalized olive trees in local communities, private orchards, multiple gardens with different age plantations, trees present in highland areas and rural areas. In total, 4000 trees were examined and almost 1500 samples were collected from mature orchards. Samples collected included trunks, branches and twigs collected from trees showing characteristic dieback symptoms. Samples were collected from the most prevalent olive cultivars grown in the visited sites. Collection was carried out using two main methods, visual inspection and beating. Specimens collected were placed in labelled sealable plastic bags for later analysis. A list of the samples collected and descriptions of any symptoms were compiled on site.

Infected samples were kept in cold storage and brought to the PTUK laboratories for testing and pathogen isolations. Infected samples were first inspected for the presence of any fungal growth in the laboratory using a Carl Zeiss Stereo Microscope 47510-9904 (ZEISS Microscopy GmbH, Germany). After peeling off the outer bark of the olive samples, surfaces were sterilized by dipping in 0.5% sodium hypochlorite for 5 min then let air dry, infected tissues showing canker symptoms were sprayed with 95% ethanol, and briefly

Table 1. Field survey carried out through several villages in nine governorates in the West Bank, showing the % of infestation with OBM

Governorate	Village	Area	No. of trees	No. of infected trees	Per cent infestation
Hebron	Al Borg	3000	70	23	33
	Al Magd	8000	157	40	25
	Saka	2000	48	8	17
	Noba	2500	42	3	7
	Alramaden	6000	100	30	30
	Eata	4500	85	30	35
	Dora	2000	35	5	14
	BanyNiaem	5000	95	16	17
	Al Dahrey	3500	60	20	33
Bethlehem	Taqoue	30000	660	150	23
Ramalla	Bierzat	6000	100	36	36
	Surda	1500	25	8	32
Salfeet	Kofl Hares	60000	1300	350	27
	Farka	6000	140	20	14
Tulkarm	Tulkarm	5000	88	30	34
	Anabta	15000	300	100	33
	Balaa	14000	260	80	31
	Faroon	2000	40	10	25
	Shofe	1000	20	15	75
	Koor	5000	92	56	61
	Allar	6000	130	120	92
Tubas	Tubas	3000	55	20	36
	Agaba	1500	20	8	40
Jenin	Zababde	2000	36	15	42
	Kabatea	3000	50	22	44
Nablus	Nables	4000	70	30	43

Fig. 1. Field survey carried out through 26 sites in nine governorates in the West Bank, showing the per cent of infestation with OBM (Palestinian Central Bureau of Statistics, 2018).

flamed. Small pieces of the tissue (approximately 25 mm^2) were placed on 85-mm-diameter petri dishes containing potato dextrose agar (PDA) (Difco™, Becton, Dickinson Company, France). Cultures were incubated at room temperature (24±2°C) until fungal colonies were observed, then individually sub-cultured to fresh PDA-petri dishes. Pure cultures of the different fungi were obtained by hyphal tip from colony margins and placed on fresh PDA. Pure fungal colonies were then incubated at ambient laboratory light and temperature conditions.

Morphological Characterization

Fungal species were first identified based on colony characteristics (colour, mycelium growth speed, and type and shape of the colony) after 3 or 4 weeks' incubation. Pycnidia were mounted in water, and conidial masses were observed by bright field

microscopy using an Inverted Microscope – Optika XDS-2 Trinocular (AIPTEK international GmbH, Italy). Images were recorded with an AIPTEK HD1080P digital camera (AIPTEK international GmbH, Germany).

Extraction and Purification of dsRNA

A column of 0.6 ml tube was made and packed with 500 µl of 50% v/v slurry of Cellulose Powder D Advantec® Extraction. It was equilibrated with washing buffer (1X STE (100 mM NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8) containing 16% v/v Ethanol); that was subjected to centrifugation at 12200 rpm for 5 sec just before use. About 400-500 mg of leaf tissue were grinded with mortar and pestle in cold conditions, 1 ml of extraction buffer (2X STE containing 0.1% v/v β-mercaptoethanol and 1% w/v SDS) was added. The crude extract was transferred to 1.5 ml tube and approximately 10 mg of polyvinylpyrrolidone (PVP) was added to each crude extract. Five hundred µl of 25 : 24 : 1 Phenol-Chloroform-Isoamyl alcohol were added, vortexed for 1 min and then centrifuged at 13400 rpm for 10 min. This step was repeated if the supernatant remained cloudy. Four hundred µl of upper aqueous phase were transferred into a new 1.5 ml tube, after that, 80 µl of chilled absolute ethanol were added. The resulting mixture was centrifuged at 13400 rpm for 10 min, and the supernatant was transferred to the pre-prepared micro-spin column device, and centrifuged at 12200 rpm for 15 sec, the flow-through was discarded. Four hundred µl of wash buffer (1X STE containing 16% v/v ethanol) were added to the column and centrifuged at 12200 rpm for 15 sec, the flow-through was discarded (This step was repeated twice). After the last wash, the column was centrifuged at 12200 rpm for 30 sec.

The 0.6 ml tube was placed in a new 2.0 ml tube and 400 µl of Elution buffer (1X STE) were added to the column, centrifuged at 12200 rpm for 15 sec and the 0.6 ml tube was discarded. Precipitation of RNA was done by adding 40 µl of 3M sodium acetate, pH 5.2, and 1 ml of chilled absolute ethanol. The mixture was centrifuged at 13400 rpm for 10 min and finally the precipitated dsRNA samples were re-suspended in 20 µl of 2X STE.

One-Step RT-PCR (Reverse Transcription and PCR)

For 200-250 mg amount of tissue, were extracted by using PureLink™ RNA Extraction Mini Kit. 1.5 ml lysis buffer (containing 1% ?-mercaptoethanol) was added, and the tissue was grinded in pre-chilled mortar and pestle. The resultant extract was spun for 5 sec to eliminate the tissue paste, the supernatant was mixed with 0.5 volume of chilled absolute ethanol and then vortexed thoroughly. Approximately 700 µl of sample were transferred to the Spin Cartridge (with a Collection Tube) and centrifuged at 13400 rpm for 15 sec, the flow-through was discarded. The Spin Cartridge was re-inserted in the same Collection Tube. Seven hundred µl of Wash Buffer I were added to the Spin Cartridge and centrifuged at 13400 rpm for 15 sec, the flow-through was discarded and the Spin Cartridge was placed into a new Collection Tube.

Five hundred µl of Wash Buffer II with ethanol were added to the Spin Cartridge and centrifuged at 13400 rpm for 15 sec, the flow-through was discarded and the Spin Cartridge was re-inserted in the same Collection Tube. The last two steps were repeated once and then the tube was centrifuged at 13400 rpm for 1 min, and the Collection Tube was discarded. The Spin Cartridge was inserted into a Recovery Tube and 50 µl RNase-Free Water were added to the center of the Spin Cartridge and incubated at room temperature for 1 min. The tube was finally centrifuged at 13400 rpm for 3 min and the resultant solution was stored at -20°C.

RNA product-containing tube was first boiled at 95°C for 10 min, then directly placed on ice bath for at least 5 min. Meanwhile, a master mix containing 2.5 µl of 10X PCR Buffer, 2.5 µl of 10X Sucrose/cresol red, 1.25 µl of 0.1 M Dithiothrietol (DTT), 1.5 µl of 50 mM $MgCl_2$, 0.5 µl of 10 mM dNTPs mixture, 1.25 µl of each of forward and reverse primers, namely, OLYaV-F (5'-CGAAGAGAGCGGCTGAAGGCTC-3') OLYaV-R(5'-GGGACGGTTACGGTCGAGAGG-3'), 0.25 µl of MMLV-RT and 0.25 µl of Taq DNA polymerase was prepared, 8 µl of denatured RNA samples were added to a final volume of 25 µl (sterile distilled H_2O) in 0.6 ml tubes. The tube was then placed in thermocycler to conduct the following : first, creation of cDNA

(reverse transcription) at 52°C for 30 min, then 35 cycles of denaturation at 94°C for 30 sec, followed by annealing at 56°C for 45 sec and then extension at 72°C for 1 min. After that, a final extension was done at 72°C for 7 min and the product was stored overnight at 4°C.

Preparation of Agarose Gel Electrophoresis and Sample Loading

First, 1-2% Agarose Gel was prepared by dissolving 1-2 g of agarose in 100 ml Tris-Acetate EDTA (TAE) buffer, and 0.8% GelRed™ Biotium dye was added to it. Sample loading included mixing 8 µl of cDNA sample with 2 µl Bromophenol Blue dye (~10 µl/well), 3 µl of 1Kb DNA ladder (GeneDireX) with 2 µl Bromophenol Blue dye or 3 µl of 100 bp DNA ladder were also loaded along with RT-PCR product samples. The system was allowed to run for ~45 min at 100 mV. Finally, the gel was observed under UV-light detector.

All data on inhibition rates were analyzed using ANOVA test using the general linear models (PROC GLM) procedure (SAS Institute, 1998).

RESULTS AND DISCUSSION

During the study period, many complaints were recorded by olive farmers and olive nurseries due to the increasing sudden death of the newly transplanted olives and the branch dieback, stem canker and quick decline. Disease incidence and severity varied from low to high disease pressure among locations.

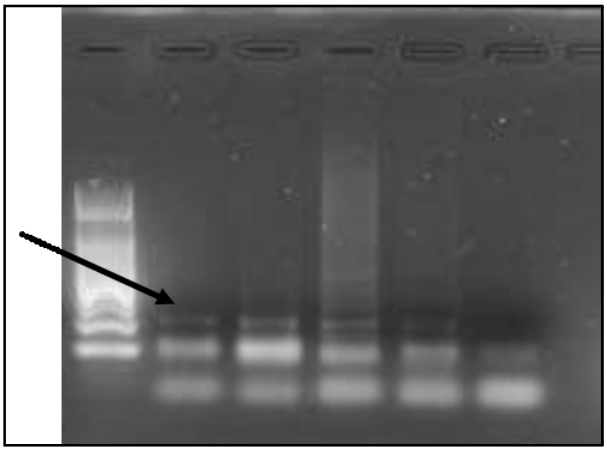

Fig. 2. Two step RT-PCR did not amplify viral fragment with 383 nt using 1 HSP 70 primers. Samples ran in 1.5% agarose gel. 100bp DNA ladder was used.

Infestation rate in the west bank ranged from 7-92% and the average was 35%. The highest infestation rate was recorded in Tulkarm governorate with 92, 75 and 61% in Allar, Shofe and Koor, respectively, while the least infestation rate was recorded in Hebron Governorate in Noba with 7%. The infestation with larvae of *R. oleisuga* was found highest at the temperature of 31°C with relative humidity at 57% and lowest population was found at 28°C relative humidity at 64% (according to the Palestinian Central Bureau of Statistics, 2018). The average temperature and relative humidity registered in Tulkarm was 22°C and 57%, respectively, while in Hebron was 17°C and 64%. An interaction was observed between locations and disease incidence; which might be due to the olive orchard density, weather conditions (lower temperatures and lower relative humidity in the southern part of west bank). Nevertheless, caution should be considered of transferring the OBM infestation from the northern part to the southern part due to non-regulated seedlings transfer.

Similar results were reported by the others (Williams and Liebhold, 2002), the effect of physical factors on the population dynamics of bark borer. While on OBM, the present results showed that the population of OBM increased with increase in temperature and vice versa (Tzanakakis, 2003) so that temperature and relative humidity were highly significant on OBM population. These finding were confirmed also by Nilsson (2008); he found the optimal conditions of temperature and relative humidity for the population build-up of OBM at the temperature of 30°C and relative humidity 60.75%.

Fungal isolate from infected olive samples examined in PTUK laboratories, showed that *Botryosphaeria* spp. was the primary pathogen isolated from infected olive samples (70%), especially from the olive orchard in the northern areas such as in Tulkarm and Jenin, were the average temperatures during the summer ranged from 28 to 30°C, respectively. Same results were found by Williamson and Hargreaves (1979) and Hernández *et al.* (1998). Other saprophytic fungi isolated from the infected olive tree were found in lesser degree such as *Alternaria solani* Sor. (10%), *Aspergillus niger* v. Tieghem (10%), *Fusarium solani* (Mart.) App. (20%), *Penicillium digitatum* Sacc. (30%) and *Penicillium italicum*

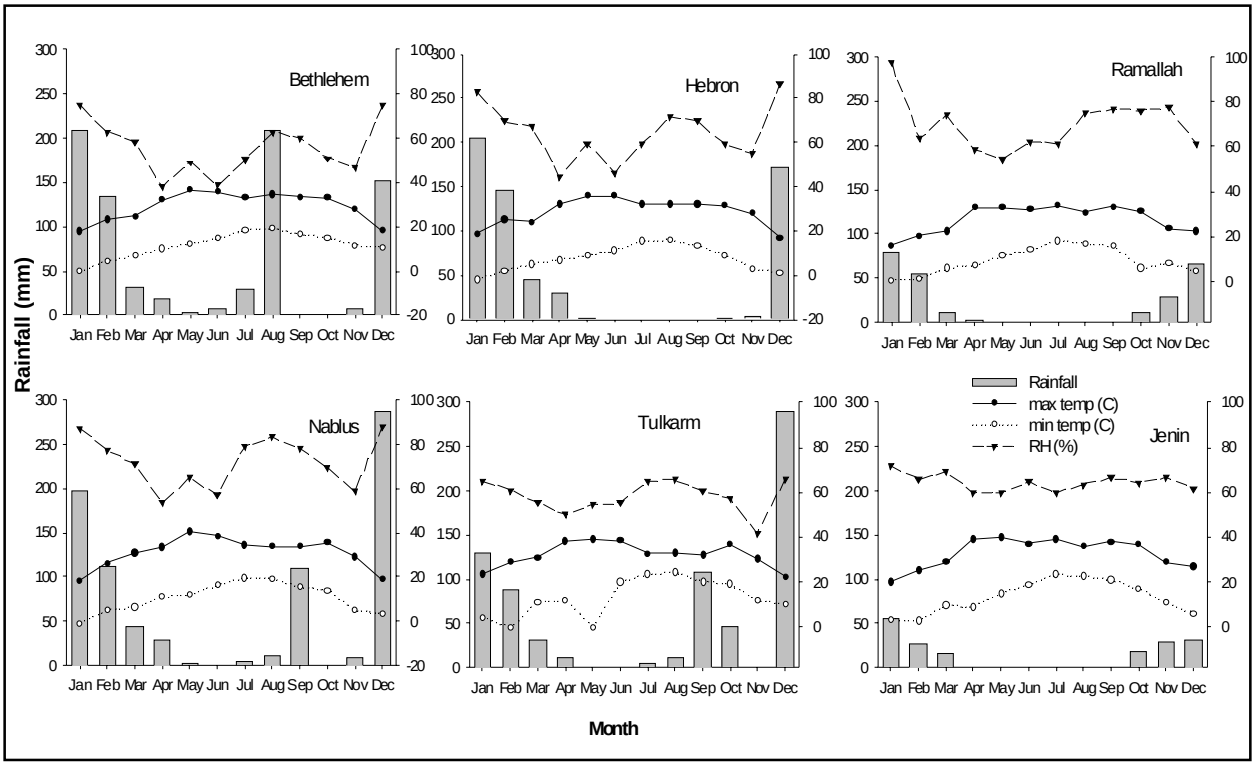

Fig. 3. Monthly temperature fluctuations, relative humidity and rainfall levels in Bethlehem, Hebron, Ramallah, Nablus, Jenin and Tulkarm governorate in West Bank 2016 (Palestinian Meteorological Authority, 2016).

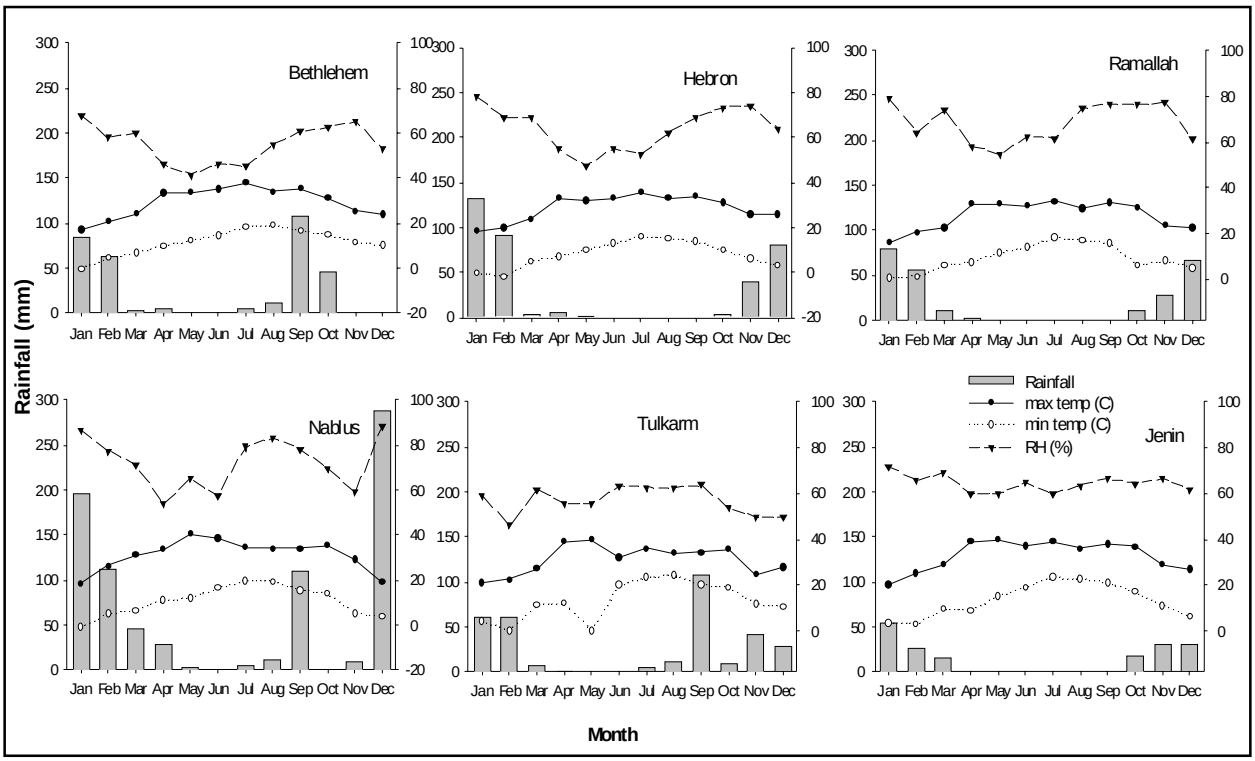

Fig. 4. Monthly temperature fluctuations, relative humidity and rainfall levels in Bethlehem, Hebron, Ramallah, Nablus, Jenin and Tulkarm governorates in West Bank 2017 (Palestinian Meteorological Authority 2017).

Wehmer (25%). Higher temperature and early rainfall during autumn season in the northern parts of the West Bank in Tulkarm and Jenin districts (Figs. 3 and 4), ended the long drought period of the summer might have enhanced the infestation of the OBM and the associated fungi. While late rainfall in the southern part including Hebron and Bethlahem might have delayed infestation with OBM and associated fungi. These results were also confirmed by the findings of Romero *et al,* (2005).

According to Morales *et al.* (2012), Botryosphaeriaceae species infected grapes had a significant conidial germination at higher temperatures that reached up to 35°C, while very low germination at low temperatures (even 5°C), this agreed with our findings. This could explain the lower infection rate in both Hebron and Bethlehem districts. It was also found that development of Botryosphaeria dieback was linked to temperature and humidity (Romero *et al.,* 2005). Extreme conditions as high temperature, accelerated light intensity and pH variations can enhance Botryosphaeria dieback symptoms on cob or grape clusters over short periods, especially in south China where serious fruit dropping resulted (Morales *et al.,* 2012).

Throughout the surveys, several samples were collected and subjected to lab investigation for isolation of any virus that could be associated with yellowing. Mechanical inoculations were applied for 30% of collected olive samples onto at least five different herbaceous plants, to detect any of graft transmissible viruses that might be associated with yellowing syndromes. Expectedly, all trials failed to isolate any virus from these samples, in accordance with many researches that reported the difficulties of olive infecting viruses to be transmitted onto herbaceous hosts. Molecular tests were carried out in two approaches : virus specific detection of OLYaV by RT-PCR and virus none specific detection via dsRNA analysis (Fig. 2). The total nucleic acids were purified from randomly selected symptomatic olives and the cDNA library was synthesized using random primer hexameres and PCR were conducted using specific primers of OLYaV. The RT-PCR resulted in giving 37.5% of tested samples as positive to this virus. This was similarly to what was previously reported for this virus on olives (40%) in most olives grown in the Mediterranean basin (Grieco *et al.,* 2000; Saponari *et al.,* 2002); as those obtained results were from both symptomatic and symptomless trees. Thus, the expectation of olive leaf yellow virus association was undertrained; since it should be higher than that. Ten of randomly selected olive samples which were free from OLYaV; were subjected to dsRNA analysis. Fibrous cellulose (CF-D) was imported from Japan through Sterlitech Corporation, Kent, USA and used to purify dsRNA from the virus infected samples. Surprisingly, none of these tested olives were virus infected. This was another indication that the yellowing could be referred to something other than viral cause.

Essential oil tested have shown to have variable antifungal activities. However, future work on these plants must be oriented to identify the active components in each essential oil and to explore the mechanism of actions and their toxicity levels on the bases of their applications.

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