TECHNICAL SHEET No. 11

Virus Detection: *Grapevine virus A* (GVA)

Methods: RT-PCR, and IC-RT-PCR

General

Virus detected: GVA from grapevine leaves, petioles, and stems.
General Methods are reverse transcription PCR (RT-PCR), Immuno-Capture reverse transcription PCR.

Developed by

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Goals

To develop sensitive methods for GVA detection in grapevines.

Introduction

Grapevine, which belongs to Vitidaceae family, is considered the second important fruit crop, after olives in Palestine. Based on the Palestinian Central Bureau of Statistics (1), the total fruit-trees cultivated area in Palestine is 1,118,075 dunums, of which 83,174 dunums are grapevine yards (7.4%). About three quarters of the grapevine area is located in Bethlehem and Hebron districts (1).

Until recently, 44 different viruses had been identified in grapevines worldwide, but not all of them cause serious diseases (2). Viruses causing the most important grapevine diseases in Palestine belong to three genera; Nepovirus, Closterovirus, and Trichovirus. Another disease detected in Palestine (grapevine fleck) is caused by a virus that has not been grouped yet (2).

A preliminary work conducted in 1995 (3) in an attempt to determine incidence of viral infections in viticulture in Palestine has shown that infection ranges between 50-98%. The most prevalent virus was GVA followed by Grape leaf roll association virus (GLRaV) 1,3, grapevine fleck virus, GLRaV-2, GVB, Grape fanleaf virus, and GLRaV-7. The highest infection with most viruses was in Bethlehem, Jenin, and Hebron areas. The GFLV and GLRaV-7 were the least common viruses among those detected in Palestine, and were restricted to Bethlehem, Jenin, and Hebron areas.

Grapevine A is a single stranded RNA virus belonging to the Trichovirus genus. GVA is one of the most common causes of the Rugose wood disease, particularly the syndrom known as Kober stem grooving disease (3). This disease is highly distributed in Bethlehem and Hebron areas, where infections reach 85% (3). The virus was first reported in *Vitis vinifera*; from Taranto, Italy; by Ciccarone. The symptoms caused by GVA include pits and grooves in the trunk. GVA is transmitted by a vector (very rarely); an insect; Pseudococcidae. The virus is also transmitted by mechanical inoculation (only to *Nicotiana glutinosa*); or by grafting but not transmitted by contact between plants; neither by seed or pollen. Grapevine A susceptible host species are *Nicotiana clevelandii*, *Nicotiana glutinosa*, *Vitis labrusca*, *Vitis rupestris, Vitis rupestris var. Rupestris, and Vitis vinifera* (4).

Materials and Methods

For both RT-PCR and IC-RT-PCR methods, the same primer set was used to detect GVA in the tested samples:

Primers

PCR Product: 430 bp of the coat protein

Primer 1, complementary sense primer: C995: 5'AAGCCTGACCTAGTCATCTTGG 3'.

Primer 2, antisense primer: H587: 5'GACAAATGGCACACTACG 3'.

RNA extraction from grapevine stems and petioles for RT-PCR

1. Cut the sample (0.5-0.7 g of the stems or petioles) into pieces by a scalpel and grind in liquid nitrogen with a mortar.
2. Add 5 volumes the plant weight of citric buffer (50 mM, pH 5.6) containing 2% PVP and 20 mM of DIECA.
3. Grind the samples very well with carborudpmax till you get green liquid homogenate.
4. Transfer the homogenate into a microfuge tube and centrifuge at 10,000 rpm for 10 min at 4ºC.
5. Distribute the supernatant into aliquots and store at –80ºC till use.
6. Dilute the extract to 40% in distilled water containing 1% of Triton X-100.
7. Incubate at 65ºC for 15 min.

cDNA synthesis (final volume 25µl)

8. Per 5 µl of the extracted RNA, add 15 µl of the RT mix (RT buffer 1X, dNTPs 0.4 mM, Hexa primer 100 ng, 0.1 M DTT, qsp with dH_2O).
9. Incubate at 100ºC for 2 min for denaturation.
10. Put on ice for 2 min.
11. Incubate for 10 min at room temperature (annealing temperature).
12. Add 1 unit of the RT enzyme (M-MLV) per reaction (diluted in 5 µl of H_2O).
13. Incubate at 37ºC for 1 h.
14. Store at –20ºC till used in the PCR.

IC-RT-PCR

Coating

1. Dilute the polyclonal antibodies obtained from Sanofi Kit, (make dilutions as recommended by Sanofi) in the coating buffer (carbonate bicarbonate) and deposit 100 µl in each well of the ELISA plate.
2. Incubate the plate at 37ºC for 2 h.

Extraction: (all the steps should be done at 4ºC)

1. Grind the 0.5-0.7 g of the petioles in liquid nitrogen.
2. Add 5 volumes of the extraction buffer (PBS containing 2% of PVP and 0.1% of Tween 20) and grind in the presence of carborundum.
3. Collect the homogenate and centrifuge at 10,000 rpm for 10 min at 4ºC.

4. Transfer the supernatant into a new tube.
5. At the end of the coating period, deposit 100 µl of the extract (supernatant) in each well.
6. Incubate the plate at 4ºC for overnight.

RT reaction

7. Prepare the RT mix (RT buffer 1X, DNTPs 0.4 mM, Hexa primer 100 ng, 0.1 M DTT, 1% Triton X-100, enough dH_2O as required) and heat at 65ºC for a few min.
8. Wash the plate 3 times with PBST.
9. Add 20 ml of the heated RT mix per each well, then incubate the plate at 65ºC for 15 min.
10. Put the plate on ice and disrupt the virus by 5-10 sec pipetting liquid in each well, then transfer the well content to a microfuge tube.
11. Incubate the tubes at 100ºC for 2 min, then at 4ºC (on ice) for 3 min, and finally at room temperature for 10 min.
12. Add 5 µl of dH_2O containing 1 unit of the RT enzyme (M-MLV) per each tube.
13. Incubate the tubes at 37ºC for 1 h.
14. Store at –20ºC till use for the PCR.

PCR conditions

5 µl of the cDNA, 0.2 mM dNTPs, 1X Polymerase buffer including 2.5 mM $MgCl_2$, 100 ng of each primer, and 1 unit of the *Taq* DNA polymerase. Complete to 50 µl with dH_2O. However, amounts less than 1.25 units of the *Taq* DNA polymerase failed to detect GVA. Hence we used 1.25 units per reaction for detection GVA.

PCR cycles

(Hot start: Add the *Taq* DNA polymerase while the reaction is heated at 94ºC): 94ºC for 5 min, then 5 cycles of 94ºC for 50 sec, 50ºC for 1 min, and 72ºC for 1 min. 30 cycles of 94ºC for 50 sec, 51ºC for 1 min, and 72ºC for 1 min. A final step of 72ºC for 5 min.

Results

M 1 2 3

Figure 1.
M: molecular weight marker.
1: IC-RT-PCR product of tested sample.
2,3: RT-PCR products of tested samples.

Discussion

The detection of GVA using the IC-RT-PCR method was much sensitive than the RT-PCR method. The results showed very faint bands in some of the tested sample using the RT-PCR method compared to the bands detected using the IC-RT-PCR method. Hence, we recommend the later technique for the detection of GVA especially in samples containing low titer of the virus, which could be previously determined by ELISA or other tests.

References

1. Palestinian Central Bureau of Statistics. Agricultural Statistics 1995/1996.
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3. Ra'ed Al-Kowni. (1997) Institute agronomique Mediterraneen De Bari. M. Sc Thesis.
4. http://life.anu.edu.au/viruses/Ictv/