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Isolation and identification of grapevine trunk diseases in Palestine and possible use of bacteria to control the disease

عزل وتشخيص أمراض جذع العنب في فلسطين وإمكانية استخدام البكتيريا في مكافحة المرض

Mariam Droubi [1], Mazen Salman[2*]

[1] مريم دروبي، [2*]مازن سلمان

[1,2] Department of Agricultural Biotechnology, Palestine Technical University-Kadoorie

[1,2] قسم التكنولوجيا الحيوية الزراعية، جامعة فلسطين التقنية-خضوري

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Abstract: The grapevine trunk disease (GTD) has been considered a real threat to grape production. This study aimed to identify the GTD causing agents in Palestinian grapevine orchards and to evaluate the efficacy of some bacterial isolates in controlling the GDT disease under in vitro conditions. Two fungal isolates; Fusarium solani isolate GR and Neofusicoccum parvum isolate GR3 were identified and diagnosed using PCR and BLASTn analysis. Pseudomonas fluorescence isolate ORS3 and Pseudomonas fluorescence isolate PFL showed very strong inhibition zones (> 10 mm, ++++) against both fungi under in vitro conditions. The other bacterial isolates were able to inhibit the fungi but the inhibition was less and varied among the bacteria. The effect of the bacteria on F. solani isolate GR was greater than that on N. parvum isolate GR3. Up to our knowledge, this study was the first of its kind in Palestine that identifies GTD. Further studies under field conditions are needed to evaluate the efficacy of the bacterial isolates.

Keywords: Grapevine trunk disease (GTD), Fusarium solani, Neofusicoccum parvum, bacteria, biological control.

المستخلص: يعتبر مرض جذع العنب (GTD) تهديدًا حقيقيًا لإنتاج العنب. هدفت هذه الدراسة إلى التعرف على العوامل المسببة لهذا المرض في بساتين العنب الفلسطينية وتقييم فاعلية البكتيريا لمكافحته في الظروف المخبرية. توصلت نتائج هذه الدراسة الى عزل الفطريات المسببة والتي تم تشخيصها باستخدام ال PCR ومن ثم تعريفها على انها Fusarium solani GR و Neofusicoccum parvum GR3 حسب بنك الجينات. تم بعد ذلك دراسة تاثير خمسة عزلات من البكتيريا على نمو هذه الفطريات. وأثبتت النتائج الى ان العزلات البكتيرية Pseudomonas fluorescence ORS3 و Pseudomonas fluorescence PFL لديها القدرة على تثبيط نمو الفطريات اكثر من غيرها من العزلات الأخرى. وقد كان تأثير هذه البكتيريا على فطر F. solani GR اكبر من تأثيرها على عزلة دفطرية N. parvum GR3. وحسب معلوماتنا فان هذه الدراسة هي الأولى من نوعها التي تم فيها تشخيص مسببات امراض جذع العنب في فلسطين. وعليه فان المزيد من الدراسات يجب اجراؤها لاختبار فعالية العزلات البكتيرية في الظروف الحقلية.

الكلمات المفتاحية: مرض جذع العنب، Neofusicoccum parvum ، Fusarium solani، بكتيريا، مكافحة حيوية.

* Corresponding Author E-mail: m.salman@ptuk.edu.ps

INTRODUCTION:

Grapevine is known as a host of a variety of fungal pathogens (Martelli, 2013) of which grapevine trunk diseases (GTDs) are the most important (Wilcox et al., 2015; Bertsch et al., 2013). The disease is considered of the important challenges to grapevine agriculture worldwide (Silva-Valderrama et al., 2021). The term GTD was established late in the 1990s and includes several symptoms that were observed on leaves and vascular tissues of grapevine plants. The GTD complex is caused by a group of fungal pathogens that primarily infect wounded grapevines and multiply in the woody tissues of plants (Bertsch et al., 2013). Symptoms of GTDs include decline and plant death within a short period of time (Fontaine et al., 2016) . High disease incidence and severity are commonly attributed to different factors such as expanded planting area and increased productivity, changes in cultural practices (Surico et al., 2004), and the banning of some chemical fungicides (e.g. sodium arsenite) used for disease management (Graniti et al., 2000).

The best control strategy for the disease is mainly achieved through disease prevention (Úrbez-Torres & Gubler, 2011). Spraying with fungicides is not always feasible due to human and environmental health complications. Until now, there are no effective measures that can provide complete eradication of the fungi once they become established within the plants. Alternative plant protection practices are becoming increasingly searched. Biological control agents (BCAs) of plant disease using nonpathogenic plant-associated microorganisms might provide a more suitable method for the control of GTDs (Van Loon et al., 1998).

Recently, some Palestinian farmers reported unknown symptoms in some vineyards. The disease caused severe losses in grapevine trees and no control measures have been tested to be effective and safe against the disease. The reported GTDs symptoms were not identified and farmers were using unsuccessfully different fungicides to control the disease. The aim of the present work was to identify and diagnose the causative agents of GTD disease in grapevine fields in Palestinian farm and to test effectiveness of some bacterial isolate GTD pathogens under in vitro conditions.

MATERIAL AND METHODS:

Cultivation and maintenance of antagonistic bacterial isolates

Five bacterial isolates (Pseudomonas fluorescens isolate ORS3, Pseudomonas fluorescens isolate PFL, Pseudomonas aeruginosa isolate SH1, Pseudomonas fluorescens isolate 1.2 and Bacillus atrophaeus isolate BAT) were obtained from the culture collection of Kadoorie Agriculture Research Center. Stock cultures of bacteria were grown on king'S B (King et al., 1954) and maintained at 4°C until use.

Fungal isolation, growth conditions and maintenance

Samples showing GTD symptoms were obtained from grapevine trees grown in Tamoun/Palestine. The samples were collected by farmers and sent to Kadoorie University where they were kept at 4^{O}C until use. For isolation of GTD fungi, infected stem segments (Figure 1) were surface sterilized in 1% (v/v)

sodium hypochlorite for 3 min and washed 5 times with sterile distilled water. The park tissue was removed from the segments, which were cut into 5 mm thick pieces, plated on PDA media and incubated for 7 days at 25°C (Salman et al., 2019). The isolated fungi were grown on PDA media and subcultured routinely every two weeks.

Figure (1). A cross section of grapevine stem showing symptoms of GTDs in infection.

Pathogenicity test of the isolated fungi

The pathogenicity of the isolated fungi was proofed under greenhouse conditions. For this, grapevine seedlings were wounded at the stem and inoculated with 5 mm diameter PDA disks grown with 5 days old fungal isolate. The wounds were wrapped with parafilm and the seedlings were grown for 4 weeks in the greenhouse. Control seedlings were inoculated with PDA disks without fungi The GTD fungi were then re-isolated from the infected seedlings and confirmed by PCR as mentioned below.

Molecular identification of fungal isolates

DNeasy plant mini kit (QIAGEN, Germany) was used to extract total genomic DNA from the fungi according to the manufacturer's instructions. PCR using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTA TTGATATGC-3') primers was performed to identify fungal isolates. The PCR reactions were carried out in 25 µL volumes containing 12.5 µL of Go-Taq® (2X) Master mix (Promega Cooperation), 1 µM of each primer, 1 µL DNA template and 9.5 µL nuclease-free water. Amplifications were carried out in a Thermal Cycler (Veriti™ DxThermo Fisher Scientific) according to the protocol of the following program: initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and a final extension cycle at 72°C for 7 min (White et al., 1990). PCR products were separated on 1% agarose containing 1 µl Gel Red DNA

stain. The PCR products were then sequenced and sequence alignment was done using BLASTn analysis at the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/).

In vitro antagonistic effect of the bacteria

Antagonistic efficacy of the bacterial isolates against the isolated fungi was determined using the dual culture assay (Salman, 2010; Salman et al., 2017). Each bacterial isolate was streaked at the center of a petri dish containing PDA medium and incubated at 25°C for 24 h. After that, two disks of PDA (7 mm diameter grown with 5 days old fungi) were placed about 3 cm apart from the bacterial streak and further incubated at 22°C for 5 days. Control experiments were done using sterile distilled water instead of bacteria (Salman, 2010). The effect of each bacterial isolate was determined by measuring the inhibition zone of mycelial growth. The rating scale was: -, no inhibition zone and growth of fungus over the bacterial streak; +, week inhibition, the growth of fungus was stopped at the bacterial streak line; ++, moderate inhibition with 1-5 mm inhibition zone; +++ strong inhibition with inhibition zone 5-10 mm and ++++, very strong inhibition with inhibition zone > 10 mm (Bardin et al., 2003). The experiment was carried out in triplicates and repeated three times.

RESULTS:

Isolation and identification of fungi

Two morphologically different fungal isolates (GR and GR3) were successfully isolated from the infected grapevine samples (Figure 2). Symptoms resemble GTD were also recovered after artificially infecting grapevine seedlings (Figure 3). Infection with F. solani showed browning of the apical vegetative part and collar rot. While infection with N. parvum showed blight and black coloring of the shoot and stem, recepectively (Figure 3). BLASTn search revealed 99% similarity of isolate GR to F. solani isolate VGFS15-5 and 96% similarity of isolate GR3 to N. parvum strain CMW20727 (Figure 4).

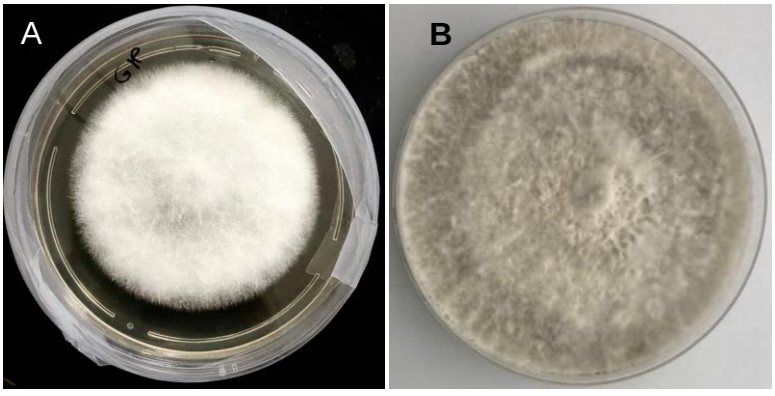

Figure (2). Fungal isolate from infected grapevine segments grown on PDA after 5 days at 25°C (A)F. solani isolate GR and (B)N. parvum isolate GR3.

Figure (3). Symptoms caused by fungal isolates GR (A and C) and GR3 (B and D) after artificial infection of grapevine seedlings grown in greenhouse.

Bacterial inhibition of fungal isolates

The effectiveness of the different bacterial isolates on inhibition of F. solani on PDA medium (Figure 5). Pseudomonas fluorescence isolate ORS3 and P. fluorescence isolate PFL were the most effective in inhibiting mycelial growth of F. solani isolate GR on PDA media with a very strong inhibition zone greater than 10 mm (++++) followed by P. aeruginosa isolate SH1 (strong inhibition zones (5-10mm, +++). The bacterial isolates P. fluorescence isolate 1.2 and B. atrophaeus isolate BAT showed little or no inhibition (+) of mycelium growth of F. solani (Table 1).

The bacterial isolates were showed also possible inhibition of N. parvum isolate GR3 (Figure 6 and Table 1). As shown in Figure 6, P. fluorescence isolate ORS3 was the most effective against the fungus (strong inhibition zone, +++). The bacteria P. fluorescence isolate PFL, P. aeruginosa isolate SH1 and P. fluorescence isolate 1.2 showed moderate inhibition (++). While B. atrophaeus isolate BAT showed little or no inhibition (-) of mycelium growth of N. parvum (Table 1).

A

Fusarium solani isolate VGFS15-5 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

Sequence ID: MF688672.1 Length: 505 Number of Matches: 1

Score	Expect	Identities	Gaps	Strand
560 bits(303)	5e-155	311/315(99%)	1/315(0%)	Plus/Plus

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Query  27   ACCCTGTGAAATACCTAAAACGTTGCTTCGGCGGGAACAGACGGCCCTGTAACAACGGGC  86
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    ACCCTGTGAAATACCTAAAACGTTGCTTCGGCGGGAACAGACGGCCCTGTAACAACGGGC  60

Query  87   CGGCCCCGCCAGCGGACCCCTAACTCTGTTTTTATAATGTTTTTCTGAKTAAACAAGCAA  146
            || ||||||||| ||||||||||||||||||||||||||||||||| ||||||||||
Sbjct  61   CGCCCCCGCCAGAGGACCCCTAACTCTGTTTTTATAATGTTTTTCTGAGTAAACAAGCAA  120

Query  147  ATAAATTAAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGA  206
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  121  ATAAATTAAAACTTTCAACAACGGATCTCTTGGCTCTGGCATCGATGAAGAACGCAGCGA  180

Query  207  AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACAT  266
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  181  AATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACAT  240

Query  267  TGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACAACCCTCAGGCCC  326
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  241  TGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACAACCCTCAGG-CC  299

Query  327  CCCGGGCCTGGCGTT  341
            |||||||||||||||
Sbjct  300  CCCGGGCCTGGCGTT  314
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B

Neofusicoccum parvum strain CMW20727 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence

Sequence ID: FJ752735.1 Length: 515 Number of Matches: 1

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            ||||||||||||||||| |||||||||||||||||||||||| ||||||||||||||||||
Sbjct  10   ATTACCGAGTTGATTCGAGCTCCGGCTCGACTCTCCCACCCAATGTGTACCTACCTCTGT  69

Query  288  TGCTTTGGCGGGCCGCGGTCCTCCGCAC-G-GCCCTTCGGGGG  328
            |||||||||||||||||||||||||||| | ||||||||||||
Sbjct  70   TGCTTTGGCGGGCCGCGGTCCTCCGCACCGCGCCCTTCGGGGG  112
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Figure (4). BLASTn similarity of the sequence identity of isolated fungi (A) F. solani and (B) N. parvum.

Table (1). Inhibition of mycelium growth of F. solani and N. parvum by different bacterial strains in dual culture assay on PDA medium.

Treatment	inhibition zone of mycelial growth	
	of F. solani	N. parvum
control	-	-
P. fluorescence isolate PFL	++++	++
P. fluorescence isolate ORS3	++++	+++
P. fluorescence isolate 1.2	-	+
P. aeruginosa isolate SH1	+++	++
B. artophagous isolate BAT	+	-

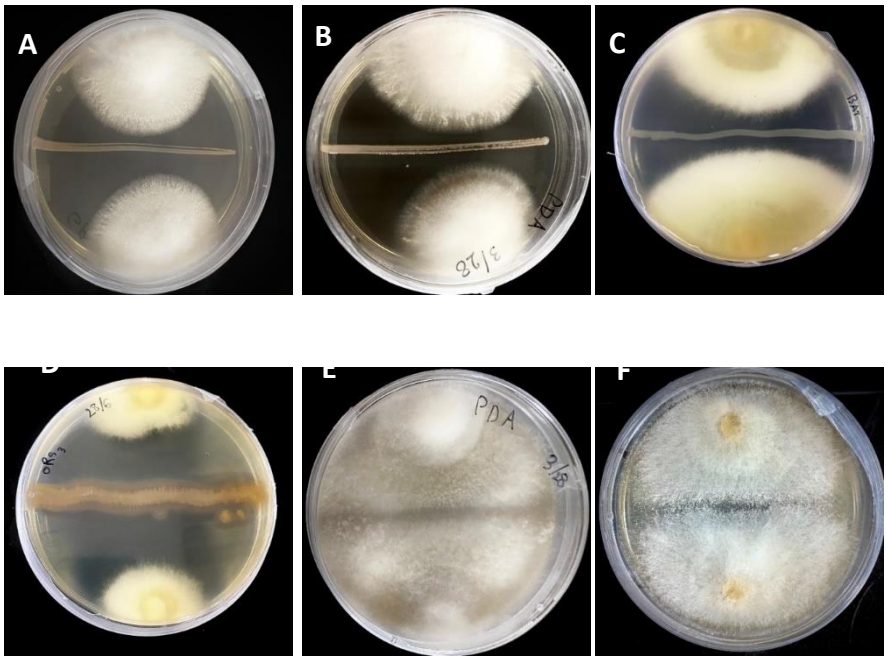

Figure (5). Effect of the different bacterial isolates, P. aeruginosa isolate SH1 (A), P. fluorescence isolate PFL (B), B. atrophaeus **isolate BAT (C), P. fluorescence isolate ORS3 (D), P. fluorescence isolate 1.2 (E) on the growth of F. solani isolate GR in dual cultures after 5 days of incubation in dark at 25°C.**

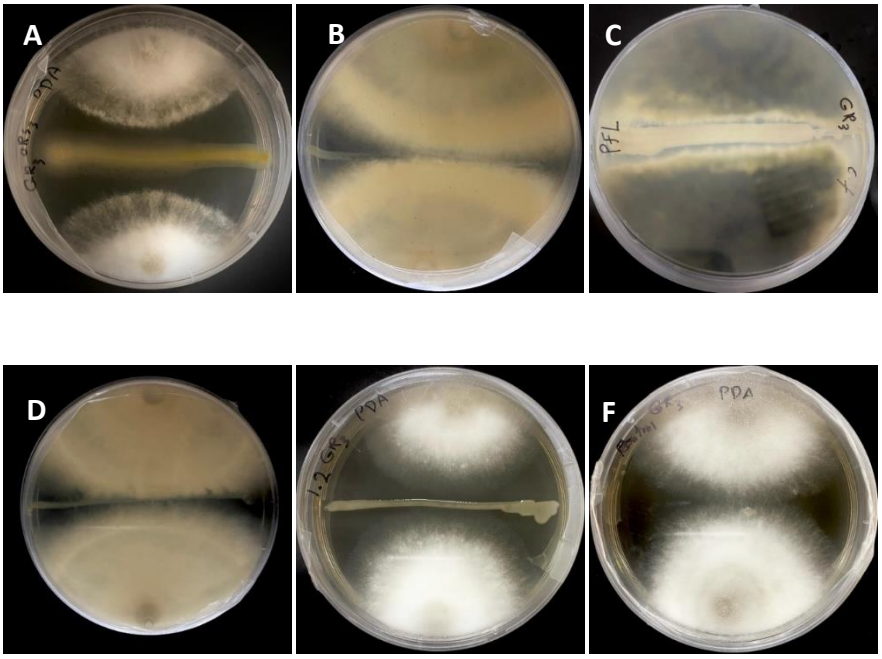

Figure (6). Effect of the different bacterial isolates of P. fluorescence isolate ORS3 (A), B. artophaous isolate BAT (B), P. fluorescence isolate PFL (C), P. aeruginosa isolate SH1 (D), P. fluorescence isolate 1.2 (E), and control sample (F) on the growth of N. parvum in dual cultures after 5 days of incubation in dark at 25°C

DISCUSSION:

Fusarium solani and N. parvum are considered the most important pathogenic fungi on the grapevine (Vakalounakis et al., 2019). Due to the lack of information about the management of the GTD in Palestine, it is very difficult to plan suitable strategies that could achieve proper control of the disease. The Biological control of plant pathogens by naturally occurring microbes or using integrated chemical and biological control is a well-known phenomenon (Cook, 1993). Up to our knowledge crop losses by the GTD disease were not sufficiently estimated. The application of fungicides in grapevine orchards in Palestine was also insufficient in controlling the disease . In this work antagonistic effectiveness of the bacteria against both fungal isolates (i.e F. solani and N. parvum) was done by testing the growth inhibition of fungi in dual culture assay.

The bacterial isolates P. fluorescence isolate ORS3, P. fluorescence isolate PFL, P. aeruginosa isolate SH1, P. fluorescence isolate 1.2 and B. atrophaeus isolate BAT varied in their efficacy against both fungal isolates with P. fluorescence isolate ORS3 was the most effective.

The ability of biocontrol agents to prevent or reduce the infection by GTD on grapevine should be further studied based on the restrictions that chemical fungicides are being banned and avoided in many countries. Thus, successful biological control of GTDs with antagonistic bacteria might be considered a practical alternative .

Studies on Biological control agents (BCAs) against plant pathogens to substitute or supplement chemical methods are limited to the grapevine endophytic fungal pathogens. Most of the studies that were conducted on grapevine fungi focused on bacterial endophytes (Bell et al., 1995) and much less on endophytic fungi (Deyett et al., 2017). For these reasons, there is a need to investigate the potential of some bacterial species as BCAs against the GTDs.

In recent years, the use of endophytic BCAs in the management of plant disease has gained much popularity as an alternative to chemical fungicide application (Hong & Park, 2016). For example, endophytes have mutualistic relationship with plants (Brader et al., 2017) and provide benefits to their host through promotion of plant growth, biocontrol of plant pathogens, enhancement of plant nitrogen fixation and phosphate solubilization (Rybakova et al., 2016).

Biocontrol depends on a wide variety of traits, such as the production by the biocontrol strain of various antibiotic compounds, iron chelators, and exoenzymes such as proteases, lipases, chitinases, and glucanases (Leong, 1986). In this study, we reported for the first time the isolation of GTDs from grapevine orchards in Palestine and the possible inhibition of the fungi using bacteria. Further studies are needed to better understand the severity and epidemics as well as the process of disease development and management strategies and to evaluate in more details the possibility of using the bacteria against the disease under filed conditions.

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