Integrated biological and chemical control of grapevine trunk diseases using bacteria and fungicides

By

Mariam Droubi

Supervisor

Prof. Mazen salman

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إعداد

مريم وحيد عبد الله دروبي

إشراف

أ.د. مازن سلمان

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Was successfully defended and approved on 10/2/2022

Examination Committee	**Signature**
Prof. Dr. Mazen Salman (Supervisor) **Plant Pathology**	**----------**
Dr Nasser Sholi, (Internal Examiner) **Assoc. Prof. of" Agricultural Engineering "**	**----------**
Prof. Dr. Raed Al Kowni, (External Examiner) **Molecular Plant Virology " (An Najah National University)**	**----------**

Palestine Technical University – Kadoorie

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Dedication

I dedicate this achievement to my family, thank you to my parents and my brothers all by their names and value in my heart, Mohammad, Hamad, Shorouq, Israa, Batool and Ayat, and my beloved aunt Najah om-Mohammad, who has always given me support.

It’s as well dedicated to my supervisor, Professor Mazen Salman, and my beloved friends who supported me.

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Abbreviation

meaning	abbreviation
Active ingredients	*a.i*
Analysis of variance	ANOVA
Azoxystrobin	Azo
Biological control agents	BCAs
Colony forming unit	CFU
Ethylenediamine tetra acetic acid	EDTA
Gravity	G
grapevine trunk disease	GTD
Hour	H
King’s Medium B	KB
Molar (mol/l)	M
Minute	Min
National Center for Biotechnology Information	NCBI
Optical density	OD
Polymerase chain reaction	PCR
Potato dextrose agar	PDA
Potato dextrose broth	PDB
Round per minute	Rpm
Standard deviation	SD
Tris-Borate EDTA buffer	TBE
World Health Organization	WHO

Integrated biological and chemical control of grapevine trunk diseases using bacteria and fungicides

Summary

The grapevine trunk disease (GTD) caused by *Fusarium solani* isolate GR *and Neofusicoccum parvum* isolate GR3 have a real threat on grape crops. This study aimed to evaluate and compare the efficiency of biological control agents in combination with low concentration of fungicides to prevent growth of fungi caused GTDs in vitro conditions.

Five bacterial isolates, *Pseudomonas fluorescence* isolate ORS3, *Pseudomonas fluorescence* isolate PFL, *Pseudomonas aeruginosa* isolate SH1, *Pseudomonas fluorescence* isolate 1.2 *and Bacillus artophaous* isolate BAT demonstrated to be antagonistic against *F. solani* isolate GR and *N. parvum* isolate GR3.

The isolated bacteria were evaluated as biocontrol agents alone and in combination with the fungicide Strubilurin (Azoxystrobin). *In vitro*, biocontrol agents were highly tolerant to the fungicide Azoxystrobin, commonly used to control GTD diseases. Azoxystrobin reduced disease symptoms by over 75% at 250 µg/ml, but it had little effect at lower concentrations on *F. solani*. Combination of the bacterial isolates and Azoxystrobin for *N. parvum* gave significant control of the disease up to 85% *in vitro* conditions. e.g: Application of Azoxystrobin at a low concentration (50 µg/ml) in combination with *P. fluorescence* isolate ORS3 inhibited disease symptoms to 84% on *N. parvum*, compared with a reduction of 66% obtained with the fungicide alone at 50 µg/ml and about 20% with the biological control alone for *N. parvum*. However, biological control alone inhibited *F. solani* to 50%. In this experiment, the treatment was also applied by volatiles produced by the different isolates of

bacteria, they gave a good inhibition on *F. solani* and slightly inhibition on *N. parvum*.

Trichoderma was also experimented as a biocontrol against GTD disease *in vitro* conditions, The results showed a good efficiency in both dual cultures assay and production of volatile antibiotics in inhibition of mycelium growth of *F. solani* and middle effect on *N. parvum*.

The average inhibition of fungus caused GTD by *Trichoderma* in dual culture assay about 70% for *F. solani* isolate GR and *N. parvum* isolate GR3 and 42% from volatile antibiotics by *Trichoderma* for *F. solani* but *N. parvum* didn't have any effect from volatile antibiotics.

:KeywordsGrapevine trunk disease **(**GTD), Strubilurin (Azoxystrobin).

Chapter One: Introduction

Introduction

General introductions

Grapevines is known as a host of a variety of fungal pathogens (Martelli, 1997) of which grapevine trunk diseases (GTDs) shown in figure 1 are the most important (Wilcox et al., 2015; Bertsch et al., 2013). The term GTD was established late in 1990s which includes several symptoms that were observed in foliage and vascular tissue of grapevines plants. The GTD complex is thought to be caused by a group of fungal pathogens that primarily infect grapes through wounds (Bertsch et al., 2013).

Fungal pathogens that cause GTDs include ascomycete species *Phaeomoniella chlamydospora*, *Phaeoacremonium minimum* and *Neofusicoccum parvum* as well as the basidiomycete species *Fomitiporia mediterranea* (Carter, 1991). These pathogenic fungi mostly damage wood tissue, causing various types of necrosis and, in most cases, the death of the plant.

Unfortunately, there are no effective control measures against the GTDs that provide a complete eradication of the fungi once they become established within the plants. spraying with fungicides is not always feasible due to human and environmental health complications. The best control strategy of the disease is mainly achieved through disease prevention and mitigation (Úrbez-Torres & Gubler, 2011). Increasing worldwide concern about pesticide use, in addition, the most effective preventative chemical products was lost because of high current restrictions and difficulties in used chemicals such as sodium arsenite or benzimidazoles, because of the risks for human health and the environment (Larignon et al., 2008). Alternative plant protection practices are becoming increasingly searched. Biological control agents (BCAs) of plant disease using non pathogenic plant-associated microorganisms might provide a more suitable method for the control of GTDs (Van Loon et al.,1998). BCAs are organisms selected for their intrinsic antagonism towards a particular pathogen or pathogen

groups, and that can function as effective alternatives to chemical pesticides (Wilson, 2003).

BCAs are diverse and include either generalist species of *Bacillus, Pseudomonas, Streptomyces, Trichoderma, Clonostachys Beauvaria*, or species in Agrobacterium, Ampelomyces, Fusarium or Aspergillus (Woo et al., 2014).

In Palestine, Grapevines are the second major crop after olives in terms of production and economic importance. It constitutes about 36.4% of total cultivated agricultural land with annual production estimated at 50,065 tons representing about 9% of agricultural production (Palestinian Ministry of Agriculture, 2013).

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 are not identified yet and farmers are using different fungicides to control the disease. The success in controlling the disease in the Palestinian grapevine is limited. In an attempt to reduce losses caused by GTDs, our study will focus on applying integrated systems of biological control including soil borne bacteria and fungi integrated with chemical fungicides that might be effective against the disease.

The main aim of the present work was to identify and diagnose the causative agents of GTD disease in grapevine trees in grapevine fields reported by the Palestinian farmers in the West Bank, and to find an effective bacterial and fungal isolate capable of inhibiting the fungal growth of a range of trunk disease pathogens under *in vitro* conditions.

1.2 Objectives

- Isolation and identification of the fungal pathogen causing GTD.
- Examining the antagonistic activity of some bacterial and fungal isolates against the fungus.
- Testing secondary metabolites from the bacteria that might be involved in the antagonistic activity of the bacteria against the fungus.
- Calculation concentration of active ingredient of appropriate fungicides in PDB and PDA medium which caused 50% reduction (ED_{50}) in mycelial growth of fungus.
- Evaluation of integrated control possibility of selected fungicides and bacteria against the disease *in vitro*.

Chapter Two: Literature review

2 Literature Review

2.1 Grapevine Trunk Diseases: review

In the last two decades, increased reports of losses and damages caused by grapevine trunk diseases (GTDs) have raised the concerns about these diseases that affect grapevine industries. The GTDs cause 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 GTDs include multiple diseases caused by different pathogenic fungi that grow in the woody tissues of grapevine plants. Esca disease, previously called "apoplexy" or "folletage'', was the first GTDs reported in many European and Mediterranean countries (Mugnai et al., 1999). Eutypa dieback, also called "eutypiosis" or "dying arm disease'' was first described on apricot, and then on grapevine (Carter, 1991). On young grapevines, Petri disease and black-foot are the most damaging diseases (Agustí-Brisach & Armengol, 2013). To date, up to 133 fungal species belonging to 34 genera have been described to cause GTD diseases worldwide, and most of them attacking grapevine trees (da Silva et al., 2017).

Grapevines can be affected by one or more GTDs at the same time since individual vines can be infected with different pathogens due to the multiple infection opportunities throughout a season and over the years.

2.2 Grapevine Trunk Diseases: Fungi Involved

Some of the important fungi *F. solani,* was isolated with the highest percent of the total Fusarium population. It has been also reported as the causal agent of many cankers and wilt diseases of forest tree nurseries, such as seed deterioration,

damping-off, cankers, and root rot of both conifers and hardwoods (Bloomberg, 1981). Another pathogenic fungi is *N. parvum*. It has been reported as the causal agent of branch cankers, dieback, leaf spots, shoot blight, fruit rot, and trunk diseases (Vakalounakis et al., 2019). The pathogen has been isolated from many other plants such as pome and stone fruit trees and grapevines (Laveau et al., 2009)

2.3 Epidemiology of Grapevine Trunk Diseases

Grapevine pathogens responsible for *Eutypa* dieback, *Botryosphaeria* dieback, Phomopsis dieback, esca, and grapevine leaf stripe diseases are primarly spread through the dispersion of airborne spores, and for Botryosphaeria dieback and esca pathogens can also be propagated through the use of infected cuttings. Depending on the fungal species, ascospores or conidia are released from perithecia or pycnidia embedded on the surface of dead grapevine wood (van Niekerk et al. 2010). However, each Grapevine Trunk Diseases can be differentiated individually as follows:

2.3.1 Botryosphaeria dieback.

Botryosphaeria dieback often presents as lack of spring growth from affected spurs with shoot dieback, bud and xylem necrosis (Úrbez-Torres 2011). Botryosphaeria dieback symptoms can appear in the field only 1 or 2 years after infections have occurred (Úrbez-Torres et al. 2006). However, cankers, dieback, and plant death have been recorded in 3- to 5-year-old table-grape vines (Úrbez-Torres et al. 2008). To date, 26 *Botryosphaeriaceaous* taxa in the genera Botryosphaeria, Diplodia, Dothiorella, Lasiodiplodia and *Neofusicoccum*, have been associated with Botryosphaeria dieback of grapevines (Yang et al. 2017).

2.3.2 Petri disease.

Petri disease can be recognized by the presence of dark-colored phenolic compounds in xylem vessels of the trunks, which exude out of the vessels when cut in cross sections and dark streaks in longitudinal section (Rooney-Latham et

al. 2005). The fungal species associated with Petri disease include: *Phaeomoniella chlamydospora*, 29 species of *Phaeoacremonium* and *Pleurostoma richardsiae* (Araújo da Silva et al. 2017).

2.3.3 Black foot.

Black foot can be recognized by black, sunken, necrotic lesions on roots and reddish brown discoloration in the base of the trunk of affected vines (Halleen et al. 2006). Up to 24 species in the genera *Campylocarpon*, *Cylindrocladiella* and *Dactylonectria*, have been reported to cause black foot disease (Agustí-Brisach and Armengol 2013).

2.4 Management of Grapevine Trunk Diseases

2.4.1 Cultural practices

Recently, there has been greater emphasis placed on the importance of pruning systems for managing GTD (Lee 2016) so there is a need to scientifically evaluate the variables of different pruning systems, such as proximity of wounds to the trunk, wound surface area, and blocking the flow of sap in vascular tissue.

In addition, Removal of dead wood and pruning debris in source blocks is strongly recommended since numerous fungal fruiting bodies can be retained in the vineyards and become a potential source of inoculum for new infections (Elena and Luque 2016).

2.4.2 Chemical control

The application of fungicides to control fungal trunk pathogens in the nursery process is difficult. Chemical dips and sprays used for the control of external pathogens do not penetrate grapevine cuttings sufficiently to control fungal pathogens inhabiting the vascular tissues (Waite and May 2005). However, the application of fungicides against trunk disease pathogens during the propagation

process is a common practice in grapevine nurseries worldwide, and there are many reports of varying effectiveness. Chinosol (Hydroxyquinoline sulfate) was reported to be the most commonly used fungicide (Gramaje et al. 2009).

In modern agriculture, the use of chemical pesticides, which raised production costs and led to environmental pollution is major problem An integrated management program that includes physical, chemical or biological control strategies have been suggested to reduce infections by plant pathogens in nurseries and field crops (Halleen & Fourie, 2016).

2.4.3 Biological control agents (BCA)

Investigation of BCA ability to prevent or reduce the development of GTD should be considered a research priority based on the restrictions that chemicals are facing in most countries around the world. Successful biological control of GTDs with antagonistic microorganisms is practiced to a rather limited extent.

In recent years, the use of endophytic BCAs in the management of plant disease has gained popularity as an alternative to chemical application (Hong and Park, 2016).

Another group of endophytes has mutualistic relationship with plants (Brader et al., 2017) and provide benefits to their host though promotion of plant growth, biocontrol of plant pathogens, enhancement of plant nitrogen fixation and phosphate solubilization (Rybakova et al., 2016). Some endophytes may also exhibit pathogenicity when conditions become favorable (Brader et al., 2017).

Recently, a commercial biofungicide Serenade, which contains a *Bacillus subtilis* strain (QST 713), was reported to be effective against various pathogenic fungi. This strain can inhibit mycelial growth, induce malformation of hyphae as well as reduce ascospore germination in vitro tests indicating a direct antibiosis effect of the strain (Compant et al., 2013).

The 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).

Much research reported on the use of the fungi *Trichoderma* spp. And *Gliocladium* spp. to control gray mold in grapevine (Elmer and Reglinski, 2006). For example, various *Trichoderma* strains had potential biocontrol agents for dieback (John et al. 2004).

Studies on Biological control agents (BCAs) against plant pathogens to substitute or supplement chemical methods are limited on 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 and *Trichoderma* as BCAs against the GTDs.

Chapter Three: Materials and Methods

3 Materials and Methods

3.1.1 Cultivation and maintenance of antagonistic bacterial isolates

The antagonistic bacterial isolates *Pseudomonas fluorescens* isolate ORS3, *Pseudomonas fluorescens* isolate PFL, *Pseudomonas aeruginosa* isolate SH1, *Pseudomonas fluorescens* isolate 1.2 *and Bacillus artophaous* isolate BAT were obtained from Prof. Salman collection.

Stock cultures of bacteria were prepared by inculating the bacteria in 125-ml erlenmyer flasks containing 25 ml king'S B liquid medium and kept on a rotary shaker (150 rpm) for 20 h at 28◦C.

3.1.2 Fungal isolation

Samples showing GTD symptoms were obtained from grapevine trees grown in Tamoun/Palestine and stored at 4°C until use.

Fusarium solani *isolate* GR, *Neofusicoccum parvum* isolate GR3 were isolated from infected grapevine samples on Potato Dextrose Agar (PDA). For isolation of GTD fungi, infected stem cuttings were surface sterilized in 1% (v/v) sodium hypochlorite solution for 3 min and washed with sterile distilled water. The outer park tissue was removed and the stems were cut with a sterile scalpel into 5 mm thick segments which were then plated on PDA media and incubated at 25°C for 7 days. The isolated fungi were maintained on PDA and subcultured routinely every two weeks.

3.1.3 *Trichoderma harzianum* cultivation

The fungus *Trichoderma harzianum* isolate Jn14 was obtained from Prof. Radwan Barakat, Hebron University, Palestine. The isolate was grown on PDA at 25°C for 7 days. After that, it was maintained on PDA media and subcultured routinely every two weeks.

3.2 Pathogenicity test of the isolated fungi

The pathogenicity of the two identified fungal isolates *F. Solani* and *N. parvum fungus* (see results sections) was explored under greenhouse conditions. For this, the seedlings were wounded and inoculated by placing 5 mm diameter PDA disks grown with five days old fungal isolate, wrapped with parafilm and grown for 4 weeks in the greenhouse. Control seedlings were inoculated with PDA disks without the fungi.

Fungi were then isolated from the seedlings and confirmed by PCR as mentioned before. The experiment was repeated twice.

3.3 Molecular Identification of fungal isolate

PCR was used for identification of fungal isolates. Total genomic DNA was isolated using the DNeasy plant mini kit (QIAGEN, Germany) according to the manufacturer instructions.

PCR reactions (25 µL volume) consisted of 1 µL of DNA template, 9.5 µL of water, 1 µM of each primer (ITS-1 5'-TCCGTAGGTGAACCTGCGG-3' forward and ITS-4 5'TCCTCCGCTTA TTGATATGC-3' Reverse), and 12.5 µL of Go-Taq DNA Amplification of DNA was carried out in a Thermal Cycler at 94 ℃ for 5 min, followed by 35 cycles of 94 ℃for 30 s, 55 ℃ for 30 s, and 72 ℃ for 30 s and a final extension at 72 ℃ for 7 min (White et al., 1990).

3.3.1 Gel Electrophoresis

Amplified products were analyzed by 1% agarose gel electrophoresis containing 2.5 µl Gel Red DNA stain and prepared with 0.5 × Tris-Borate EDTA buffer (TBE) and visualized on a UV Transilluminator. For estimating the size of the amplified DNA fragment, a 100bp DNA ladder was used as a molecular size marker. The PCR products were then sent to the sequencing facility at Bethlehem University/Palestine. Sequence alignment was done by BLASTn analysis at the

National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/).

3.4 In vitro screening of antagonistic bacteria

3.4.1 Dual culture assay

Antagonistic activity of the bacteria against the *F. solani isolate* GR, *N. parvum isolate* GR3 was determined using the dual culture technique. Each bacterial strain was streaked at the center of a PDA plate and incubated for 24 h at 25°C. Two disks of PDA grown with five days old fungal isolate were placed about 3 cm apart from the bacterial streak. Cultures were then incubated at 22°C for 5 days. Control experiments were done by using instead of bacteria sterile distilled water (Salman, 2010). The effect of each bacterial strain was determined by measuring the inhibition zone of mycelial growth in mm. The rating scale is: -, no inhibition zone and growth of GTS over the bacterial streak; +, week inhibition, the growth of GTD 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.

3.4.2 Production of volatile antibiotics

The method (Cliquet and Scheffer,1996) was applied with some modification for studying production of volatiles by bacterial isolates. One hundred µl of 24 h old bacteria suspension (1×10^9 cfu/ml, OD_{600} = 0.2)) were placed in the center of the one half of a petri dish containing PDA agar medium and a 7 cm PDA disks containing *F. solani isolate* GR or *N. parvum isolate* GR3 were placed in the center of the other PDA plate. The two plate halves were placed face to face sealed with parafilm and incubated at 22˚C for 5 days. Control experiments include bacteria without fungus and fungus in which the bacteria was replaced by

sterile water. Percentage of inhibition of mycelium growth was calculated as percent of control (equation below). The data were expressed as means of percent inhibition ±SD. Each experiment was done in triplicates and repeated 3 times.

Percentage mycelial growth inhibition = (C−T)/C×100

Where C = colony diameter (mm) of the control

T = colony diameter (mm) of the test plate

3.4.3 Extraction of secondary metabolites

Production of antibiotics will be assayed in broth cultures (Bonsall et al., 1997). A 50 ml of KB broth in 125-ml flasks are inoculated with bacteria and incubated on a rotary shaker (150 rpm) for 72 h at 28°C. Cultures including bacterial cells were centrifuged at 10000 g for 10 min and acidified with 1.0 M trifluoroacetic acid (TFA) to pH 2.0 and then extracted with 50 ml ethyl acetate (2 times). Extracts are dried with sodium sulfate and the solvent is evaporated by a rotary evaporator. Antibiotic activity was checked by the paper disk method (Salman, 2010).

3.5 In vitro effect of antagonistic fungi

3.5.1 Dual culture assay

Antagonistic activity of *T. harzianum* against the *F. solani isolate* GR or *N. parvum isolate* GR3 was determined using the dual culture technique. A disk of PDA grown with *Trichoderma* was placed about 3.0 cm apart from the center. *F. solani isolate* GR, *N. parvum isolate* GR3 were placed on opposite place of the plate. The plates were then incubated for 5 days at 25◦C. Zone of inhibition (in mm) was measured after 5 days of incubation at 25◦C. Control experiments are done by using instead of *Trichoderma F. solani isolate* GR and *N. parvum isolate* GR3. The inhibition of Fungus was determined by measuring the diameter of mycelial growth of *Trichoderma* in mm. The experiment was carried out in triplicates and repeated 3 times.

3.5.2 Production of volatile antibiotics

A PDA disk (7-mm) grown with *Trichoderma* was placed in the center of the one half of a petri dish containing PDA medium and a 7 mm PDA disk *F. solani isolate* GR, *N. parvum isolate* GR3 was placed in the center of the another PDA plate. The two plate halves were placed face to face, sealed with parafilm and incubated at 25◦C for 5 days. Control experiments include fungus in which the *Trichoderma* was replaced by the same fungus or sterile distilled water. Inhibition of mycelium growth was calculated as percent of control (equation below). The data were expressed as means of percent inhibition ±SD. Each experiment was done in triplicates and repeated 3 times.

Percentage mycelial growth inhibition = (C−T)/C×100

Where C = colony diameter (mm) of the control

T = colony diameter (mm) of the test plate

3.6 Chemical fungicide

3.6.1 In vitro measurement fungitoxicity

The fungicide Ortiva (25% Azoxystrobin a.i) was used to study the possibility to control GTDs. For this, the isolated fungi were grown on PDA media supplemented with different concentrations (0, 10, 50, 100, 250 µg/ml a.i) of the fungicide. Colony diameter was measured after 5 days of incubation at 22◦C in darkness. In addition to that, fungal growth was tested in PDB media containing the same fungicide concentration. For this, 250 ml Erlenmeyer flasks containing 25 ml PDB were inoculated with 7 mm diameter PDA disks grown with 5 days old. Flasks were incubated on a rotary shaker at 110 rpm and 22◦C for 5 days. Mycelia were collected on pre weighted Whatman #1 filter paper, dried for 24 h at 60◦C and mycelial dry weight was recorded. The ED_{50}-values (fungicide

concentration which retarred mycelial growth by 50% compared to the control) were calculated. The experiment was done in triplicates and repeated 3 times.

3.6.2 Compatibility test

The effects of the fungicide on bacterial growth were studied by growing the bacteria in KB media supplemented with the fungicide. A 50 µl of $1x10^9$ cfu/ml of each bacterial isolate was transferred to 250 ml flasks containing 25 ml KB liquid medium supplemented with different fungicide concentrations (0, 10, 50, 100, 250 µg/ml a.i). The flasks were incubated on a rotary shaker at 150 rpm for 24 h and 28◦C. Growth of bacteria was determined by measuring the absorbance of 1 ml bacterial culture using spectrophotometer at 600 nm wavelength and the results were compared with standard growth curve of the *P. fluorescence* isolate ORS3, *P. fluorescence* isolate PFL, *P. aeruginosa* isolate SH1, *P. fluorescence* isolate 1.2 *and B. artophaous* isolate BAT. The experiment was done in triplicates and repeated 3 times.

3.6.3 Effect of fungicide and bacterial combinations on dry weight of *N. parvum*.

Single 7 mm diameter agar disks grown with 5 days old were transferred into 125 ml Erlenmeyer flasks containing 25 ml PDB with concentration 50 µg/ml of Azoxystrobin (Azo) and 0.1 ml of either *P. fluorescence* isolate ORS3, *P. fluorescence* isolate PFL, *P. aeruginosa* isolate SH1, *P. fluorescence* isolate 1.2 *and B. artophaous* isolate BAT. Bacterial aliquots ($1x10^9$ cfu/ml) were added to the fungal cultures and incubated on a rotary shaker at 110 rpm and 22◦C. After 5 days of incubation mycelial dry weights were determined after filtration through pre weighted Whatman # 1 filter paper and dried overnight in oven at 60◦C.

3.6.4 Effect of fungicide and bacteria on mycelium growth of *N. parvum*.

Antagonistic activity of the bacteria in combination of fungicide against the *N. parvum* was also determined using the dual culture technique. The fungicide was added to PDA media at a concentration of 50 µg/ml. Inhibition of fungal growth was measured and determined as mentioned before.

Chapter Four: Results

4. Results

4.1 Isolation of fungal isolates

Two fungal isolates were successfully isolated from grapevine samples (Figure 2). The morphology and color of the isolates were different on PDA medium. *F. solani* had white color and fast-growing mycelium. It took 5 days to grow in PDA medium at 25◦C. *N. parvum* was fast-growing and formed many mycelia which were initially white then it turned over time to gray to black it took 5-6 days to grow in PDA medium at 25◦C.

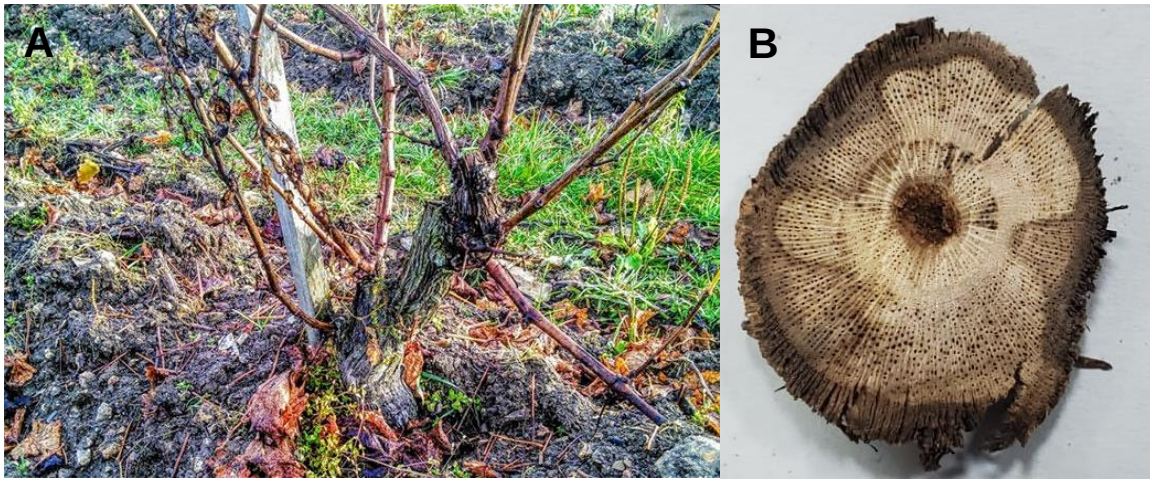

Figure 1: Symptoms of GTDs in infected grapevine (A) wilting of leaves and shoots of infected grapevine (Photo source: https://www.decanter.com/wine-news/opinion/jefford-on-monday/grapevine-trunk-disease-phylloxera-383975/) (B) Cross-section of shoot (Source: Author).

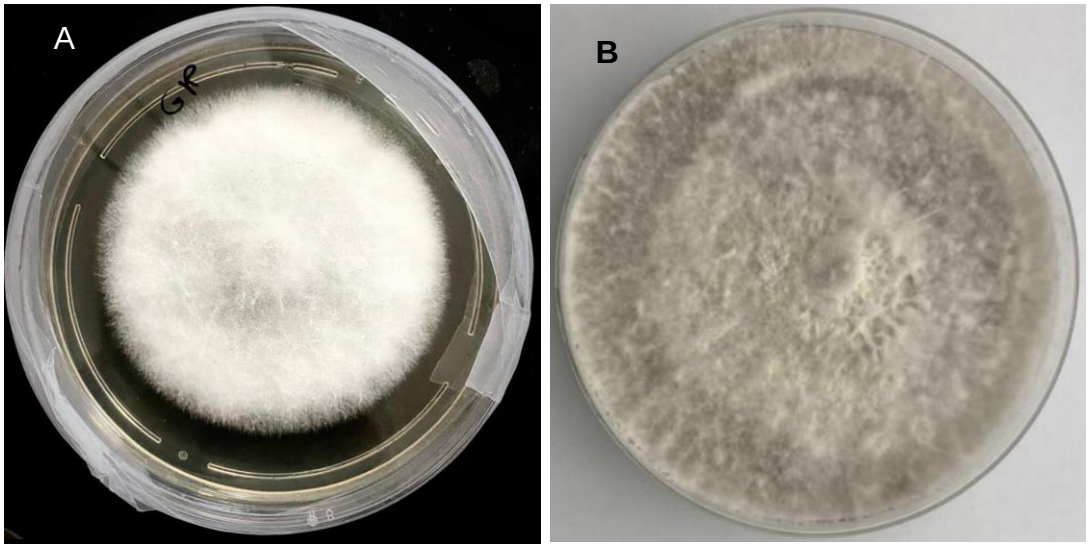

Figure 2: Fungal isolate (*F. solani* isolate GR *(A)* and *N. parvum* isolate GR3 *(B))* from infected grapevine from Tamoun city was cultured in PDA for 5 days at 25C. (source: the author)

4.2 Molecular identification of fungal isolation

Total genomic DNA from the fungal isolates from infected grapevines using DNeasy plant mini kit (QIAGEN, Germany) according to the manufacturer instructions. Amplified products were analyzed by agarose gel electrophoresis and visualized on a UV Transilluminator. The different sizes of amplified DNA fragments of *F. solani* isolate GR and *N. parvum* isolate GR3 are shown in figure 3 and the sequencing identify was done by BLASTn analysis (NCBI) database (figure 4-5).

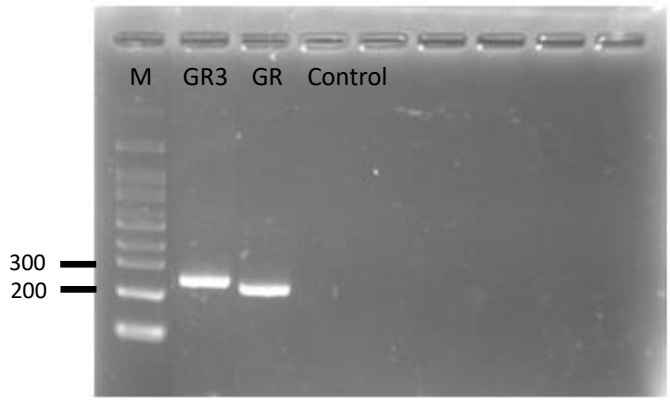

Figure 3: gel electrophoresis of PCR products amplified from DNA isolated from *F. solani* (GR) and *N. parvum* (GR3) from isolated sample from Tamoun city in 1.7% agarose gel.

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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

Range 1: 1 to 314 GenBank Graphics ▼Next Match ▲Previous Match

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
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Sbjct  1    ACCCTGTGAAATACCTAAAACGTTGCTTCGGCGGGAACAGACGGCCCTGTAACAACGGGC  60

Query  87   CGGCCCCGCCAGCGGACCCCTAACTCTGTTTTTATAATGTTTTTCTGAKTAAACAAGCAA  146
           || ||||||||| ||||||||||||||||||||||||||||||||| ||||||||||||
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           |||||||||||||||
Sbjct  300  CCCGGGCCTGGCGTT  314
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Figure 4: BLASTn similarity of the sequence identity of *F. solani* isolate GR isolated from Tamoun.

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

See 1 more title(s) See all Identical Proteins(IPG)

Range 1: 10 to 112 GenBank Graphics ▼Next Match ▲Previous Match

Score	Expect	Identities	Gaps	Strand
165 bits(89)	4e-36	99/103(96%)	4/103(3%)	Plus/Plus

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Query  288  TGCTTTGGCGGGCCGCGGTCCTCCGCAC-G-GCCCTTCGGGGG  328
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Sbjct  70   TGCTTTGGCGGGCCGCGGTCCTCCGCACCGCGCCCTTCGGGGG  112
```

Figure 5: BLASTn similarity of the sequence identity of *N. parvum* isolate GR3 isolated from Tamoun.

4.3 Pathogenicity test of the isolated fungi

Symptoms of grapevine artificiality infection in the greenhouse by *F. solani* namely browning of the apical vegetative part and collar rot. While infection by *N. parvum* presented in shoot blight and black color (figure 6).

The symptoms caused by the artificially inoculated pathogens were similar to those observed naturally and the control plants remained healthy.

Total genomic DNA from the fungal isolates from infections grapevines in green house prepared using DNeasy plant mini kit (QIAGEN, Germany) according to the manufacturer instructions.Amplified products from PCR reactions were visualized on a UV Transilluminator. Then compared the bands of *F. solani* isolate GR and *N. parvum* isolate GR3 with the bands that result of amplified DNA of the original sample of *F. solani* and *N. parvum.*

The requirements of Koch's postulates were fulfilled. The results showed that the bands of *F. solani* and *N. parvum* from both naturally and artificially inoculated pathogens infection samples have the same band size of base pair as shown in figure 7.

Figure 6: Symptoms of grapevine artificiality infection by *N.* parvum (A &C) and *F. solani* (B &D) in the greenhouse in PTUK. (source: the author)

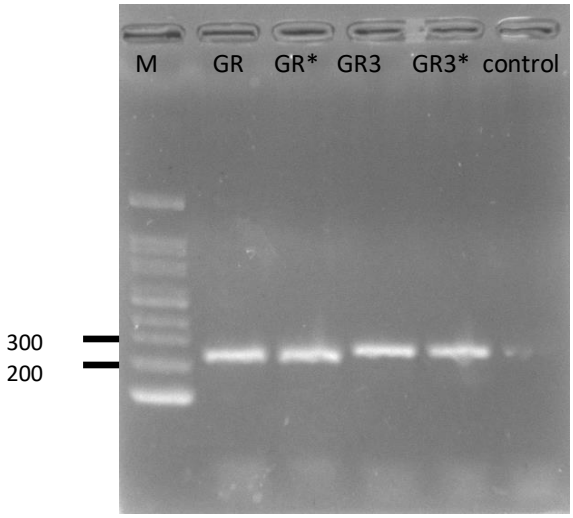

Figure 7: Agarose gel electrophoresis of PCR products amplified from DNA isolated from *F. solani* (GR) and *N. parvum* (GR3) fungi and from *F. solani* (GR*) and *N. parvum* (GR3*) cultured in the greenhouse.

4.4.1 Inhibition of *F. solani* isolate GR

The different bacterial isolates, *P. fluorescence* isolate ORS3, *P. fluorescence* isolate PFL, *P. aeruginosa* isolate SH1, *P. fluorescence* isolate 1.2 *and B. artophaous* isolate BAT. were able to inhibit the growth of *F. solani* on PDA medium after 5 days of incubation. *P. fluorescence* isolate ORS3 and *P. fluorescence* isolate PFL were the most effective in inhibiting mycelial growth of *F. solani*, the inhibition from both isolates bacteria reach to 35% and 40% respectively, exhibiting a very strong inhibition zone greater than 10 mm (++++), followed by *P. aeruginosa isolate SH1* with strong inhibition zones (5-10mm, +++). The bacterial isolates *P. fluorescence* isolate 1.2 and *B. artophaous* isolate BAT showed little or no inhibition (+) of mycelium growth of *F. solani* (Table 1). The effectiveness of the different bacterial isolates on inhibition of *F. solani* on PDA medium (Figure 8).

4.4.2 Inhibition of *N. parvum* isolate GR3

The different bacterial isolates, *P. fluorescence* isolate ORS3, *P. fluorescence* isolate PFL, *P. aeruginosa* isolate SH1, *P. fluorescence* isolate 1.2 *and B. artophaous* isolate BAT. were tested to inhibit the growth of *N. parvum* in PDA medium after 5 days of incubation, *P. fluorescence* isolate ORS3 was the most effective against GR3(+++). The percent of inhibition reach to 20%. While *P. fluorescence* isolate PFL, *P. aeruginosa* isolate SH1and *P. fluorescence* isolate 1.2 showed moderate inhibition (++), while *B. artophaous isolate* BAT showed little or no inhibition (-) of mycelium growth of *F. solani* (Table 1). The differential activity of various bacterial isolates on mycelium growth of *N. parvum* (Figure 9). Effect of bacterial strains on mycelium growth of *F. solani* and *N. parvum* in dual culture assay on PDA medium (figure 10).

Table 1:inhibition of mycelium growth of *F. solani*, and *N. parvum* by bacterial strains (*P. fluorescence* isolate ORS3, *P. fluorescence* isolate PFL, *P. aeruginosa* isolate SH1, *P. fluorescence* isolate 1.2 *and B. artophaous* isolate BAT)In dual culture assay PDA medium.

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

-, no inhibition zone; +, week inhibition growth of *F. solani* and *N. parvum* were 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.

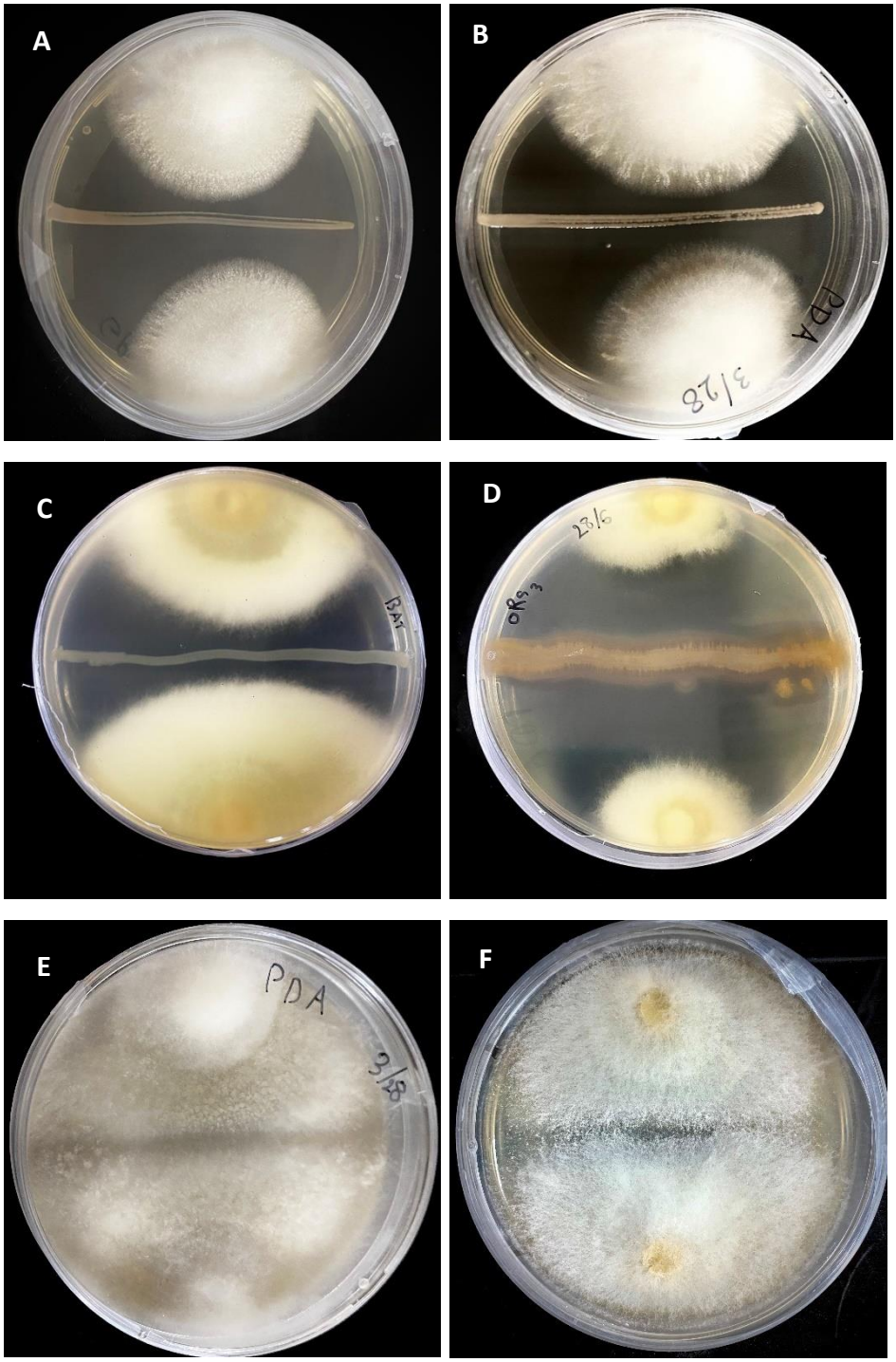

Figure 8: Effect of the different bacterial isolates, *P. aeruginosa* isolate SH1 *(A)*, *P. fluorescence* isolate PFL *(B)*, *B. artophaous* isolate BAT *(C)*, *P. fluorescence* isolate ORS3 *(D)*, *P. fluorescence* isolate 1.2 *(E)* on growth of *F. solani* in dual cultures after 5 days of incubation in dark at 25◦C.

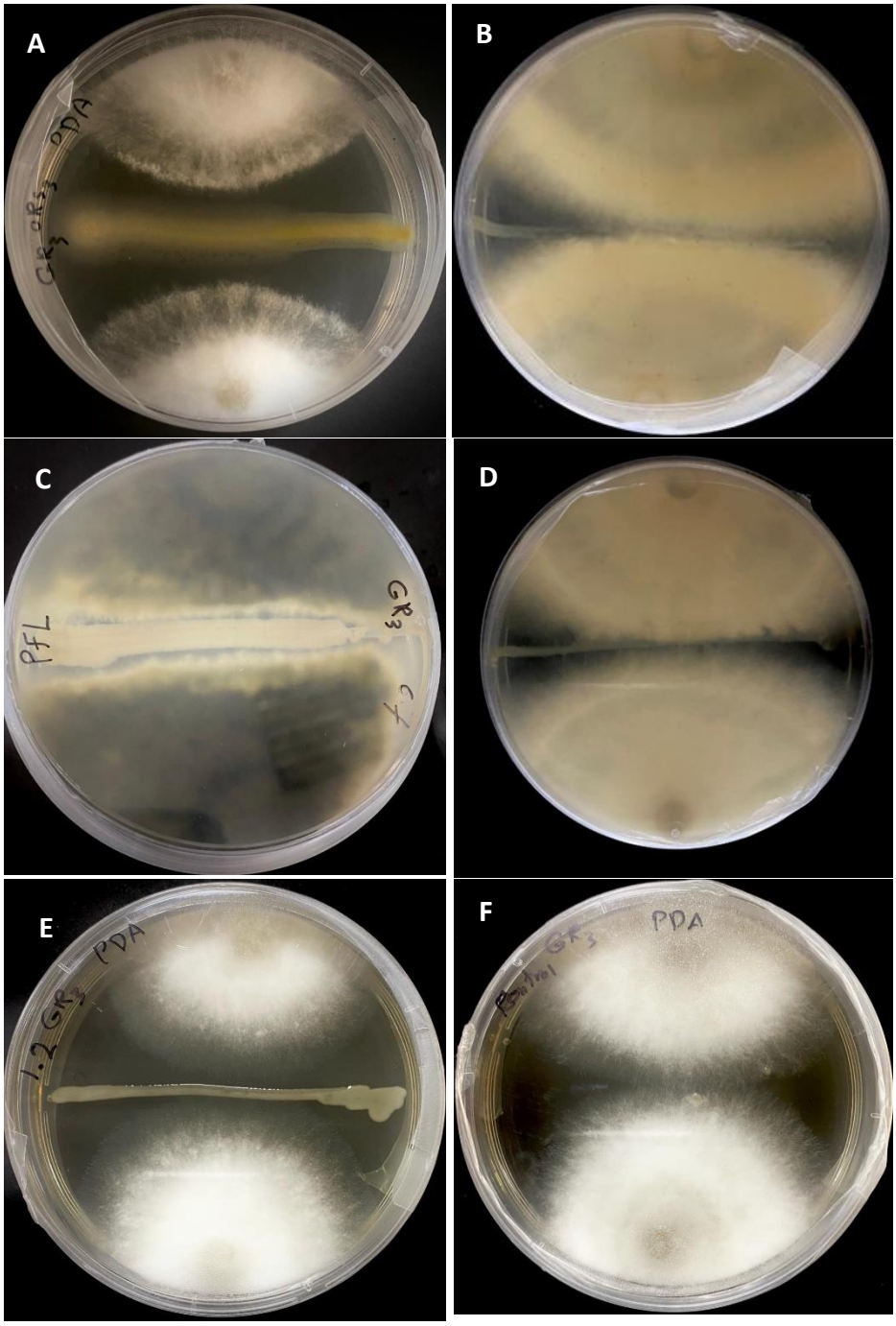

Figure 9: Effect of the different bacterial isolates of *P. fluorescence* isolate ORS3 *(A), B. artophaous* isolate BAT (B), *P. fluorescence* isolate PFL (C), *P. aeruginosa* isolate SH1 (D), *P. fluorescence* isolate 1.2 (E), and control sample (F) on growth of *N. parvum* in dual cultures after 5 days of incubation in dark at 25◦C.

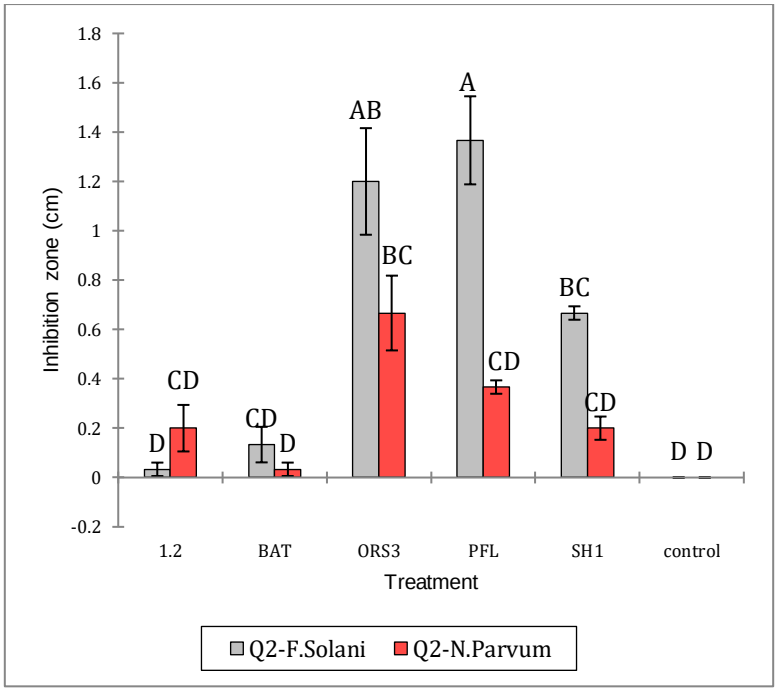

Figure 10: Effect of the different bacterial isolates, *P. fluorescence* isolate ORS3, *P. fluorescence* isolate PFL, *P. aeruginosa* isolate SH1, *P. fluorescence* isolate 1.2 *and B. artophaous* isolate BAT on mycelium growth of *F. solani*, and *N. parvum* in dual culture assay on PDA medium. Columns with different letters are significantly different after ANOVA using Tukeys HSD test at $p \leq 0.05$.

4.5 Production of volatile antibiotics

Colony size of *F. solani* and *N. parvum* was measured after 5 days of incubation in paired cultures with bacteria. The volatile compound which were produced by isolates *P. fluorescence* isolate ORS3, *P. fluorescence* isolate PFL, *P. aeruginosa* isolate SH1, *P. fluorescence* isolate 1.2 *and B. artophaous* isolate BAT reduced the radial mycelial growth of fungal isolates. The volatiles compounds come from *P. fluorescens* isolate ORS3, *P. fluorescence* isolate PFL, *P. fluorescence* isolate 1.2 *and B. artophaous* isolate BAT have the most effect in reduced the radial growth of *F. solani* mycelium. While *N. parvum* didn't show any inhibition of mycelium growth from any bacterial isolates. The most inhibition from volatile antibiotics by *B. artophaous* isolate BAT against *F. solani*, it reaches 30% compared with control sample. The inhibition of *F. solani* and *N. parvum* from 5 bacterial isolates in this experiment are shown in figure 11 and 12. The percent

of radial growth inhibition of *F. solani and N. parvum* caused by volatiles from bacterial isolates illustrated in figure 13.

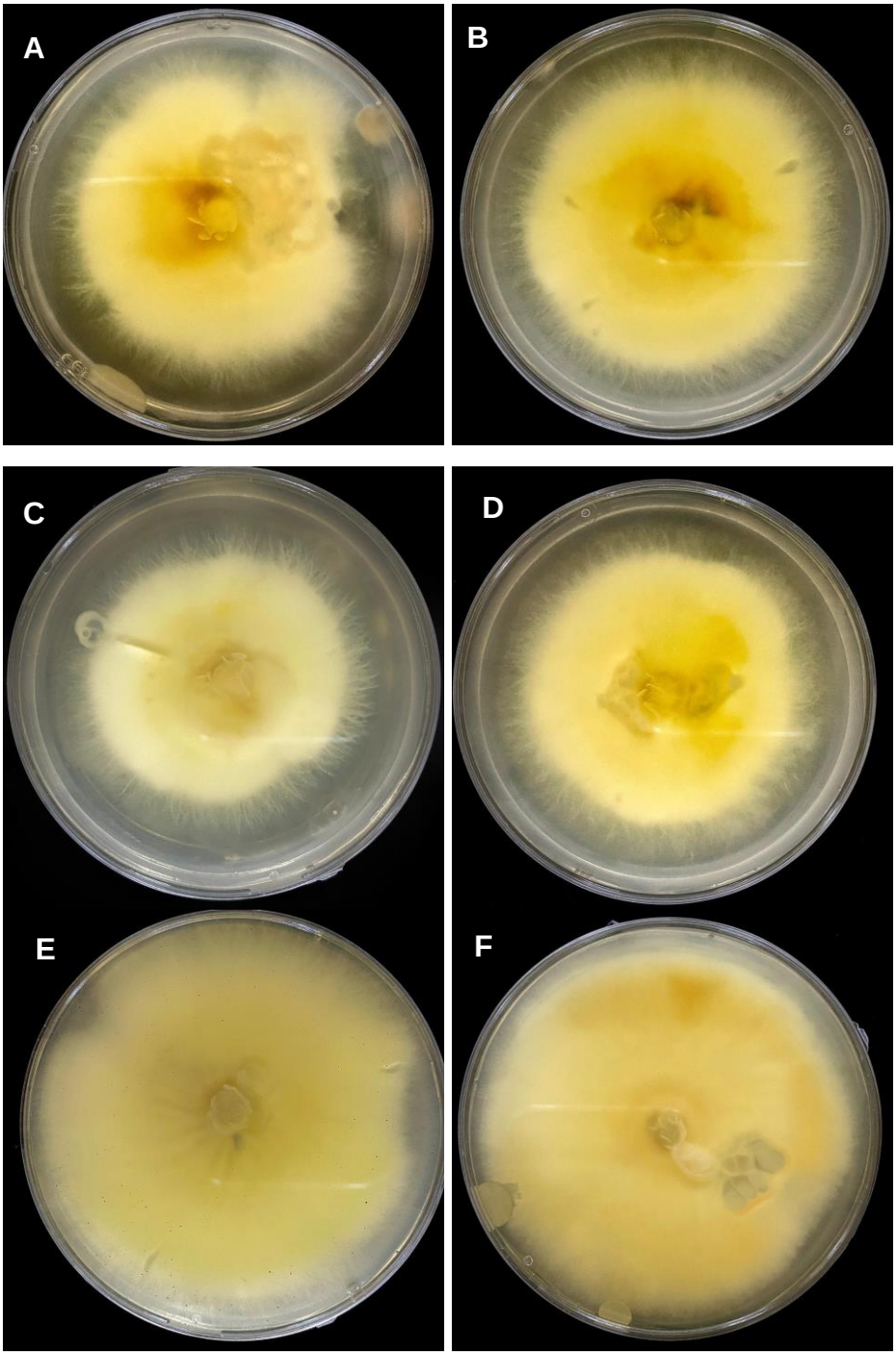

Figure 11: Effect of volatiles produced by the different isolates of *P. fluorescence* isolate 1.2 (A), *P. fluorescence* isolate PFL (B), *B. artophaous* isolate BAT (C), *P. fluorescence* isolate ORS3 (D), *P. aeruginosa* isolate SH1 (E) and control sample (F) on retardation of mycelial growth of *F. solani*. and incubated at 25◦C for 5 days.

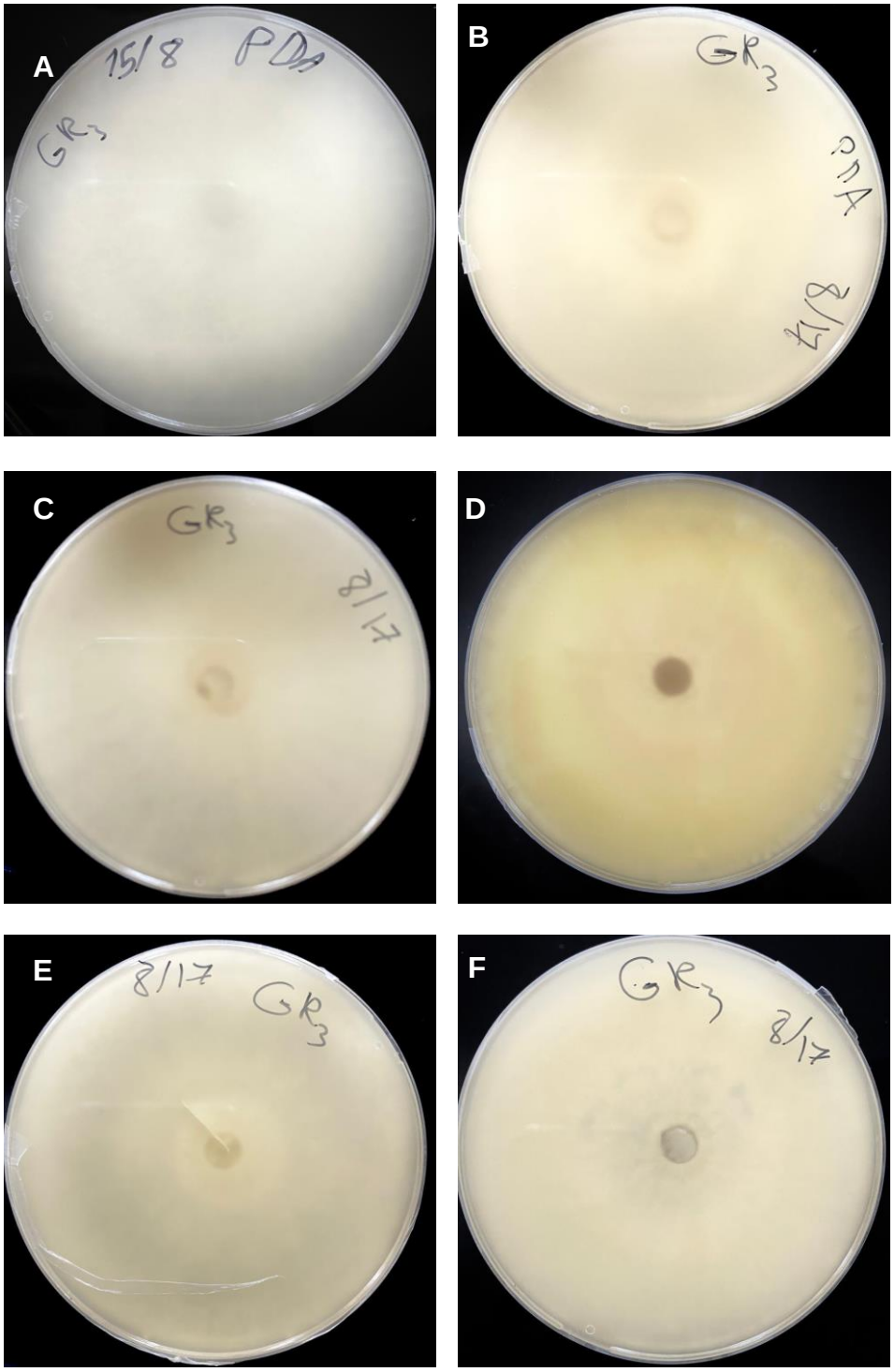

Figure 12: Effect of volatiles produced by the different bacterial isolates, *P. fluorescence* isolate 1.2 (A), *P. fluorescence* isolate PFL (B), *B. artophaous* isolate BAT (C), *P. fluorescence* isolate ORS3 (D), *P. aeruginosa* isolate SH1 *(E)* and control sample (F*)* on retardation of mycelial growth of *N. parvum*. and incubated at 25◦C for 5 days.

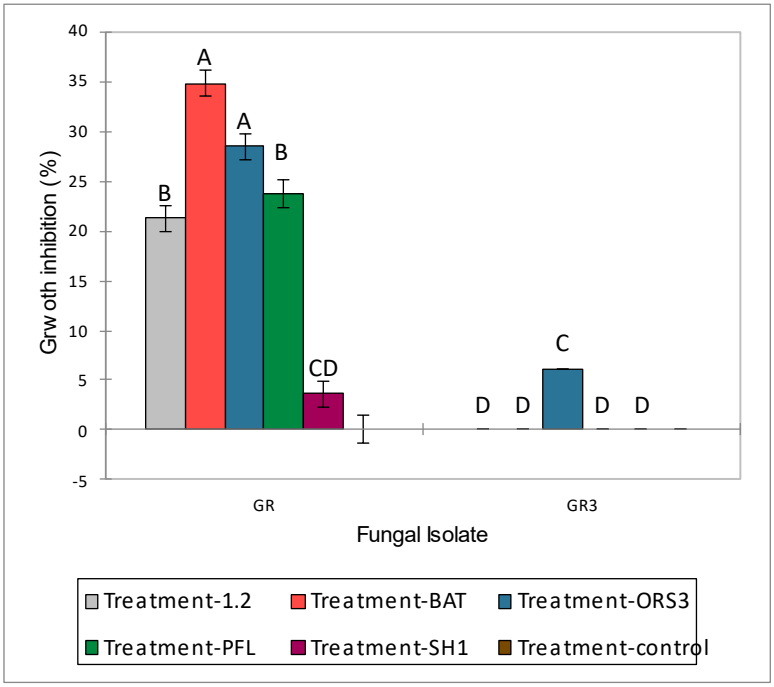

Figure 13: Effect of volatiles produced by different bacterial isolates, *P. fluorescence* isolate ORS3, *P. fluorescence* isolate PFL, *P. aeruginosa* isolate SH1, *P. fluorescence* isolate 1.2 and *B. artophaous* isolate BAT on retardation of mycelial growth of *F. solani* and *N. parvum* in dual culture on PDA medium, Columns with different letters are significantly different after ANOVA using Tukeys HSD test at $p \leq 0.05$.

4.6 Extraction of antibiotics by Bioautographic assays

Extracts were obtained after extracting bacterial culture filtrate. These extracts were tested against *F. solani*, and *N. parvum* on PDA medium (figure 14) *F. solani* and *N. parvum* were inhibited by extracts from *P. fluorescens* isolate ORS3, *P. fluorescence* isolate PFL and *P. aeruginosa* isolate SH1.

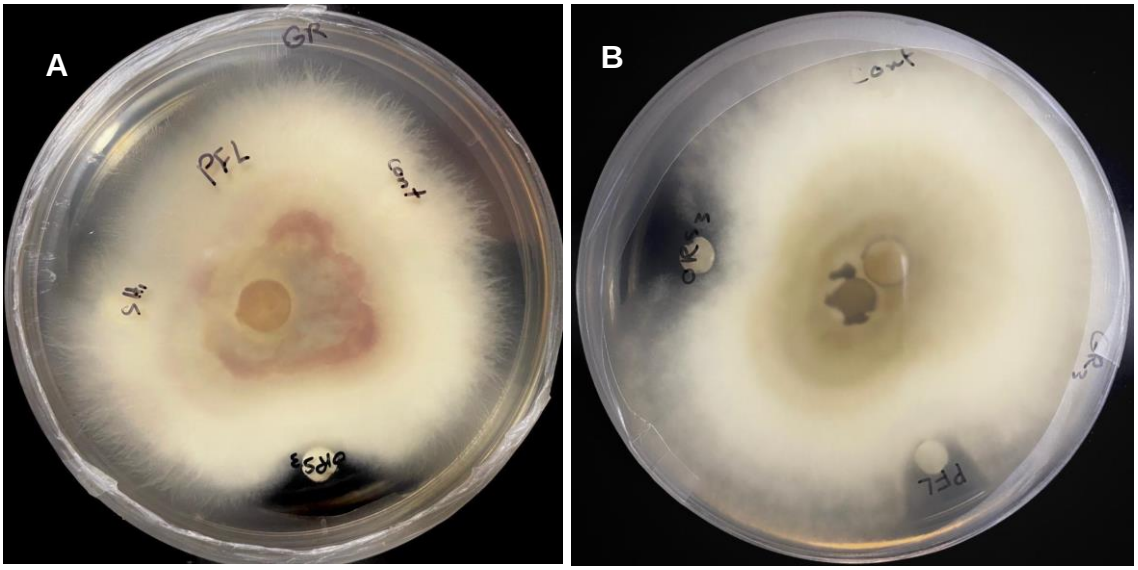

Figure 14: Effect of bacterial extracts from *P. fluorescens* isolate ORS3, *P. fluorescence* isolate PFL and *P. aeruginosa* isolate SH1*and P. fluorescence* isolate 1.2 on mycelial growth of *F. solani* (A), and *N. parvum* (B) on PDA medium.

4.7.1 Inhibition of fungus by *Trichoderma* in daul culture assay

Trichoderma Fungi was able to inhibit the growth of *F. solani* and *N. parvum* on PDA medium after 5 days of incubation. The inhibition by *Trichoderma* against *F. solani* and *N. parvum* reach to 76% and 70% respectively (Figure 15 and 16).

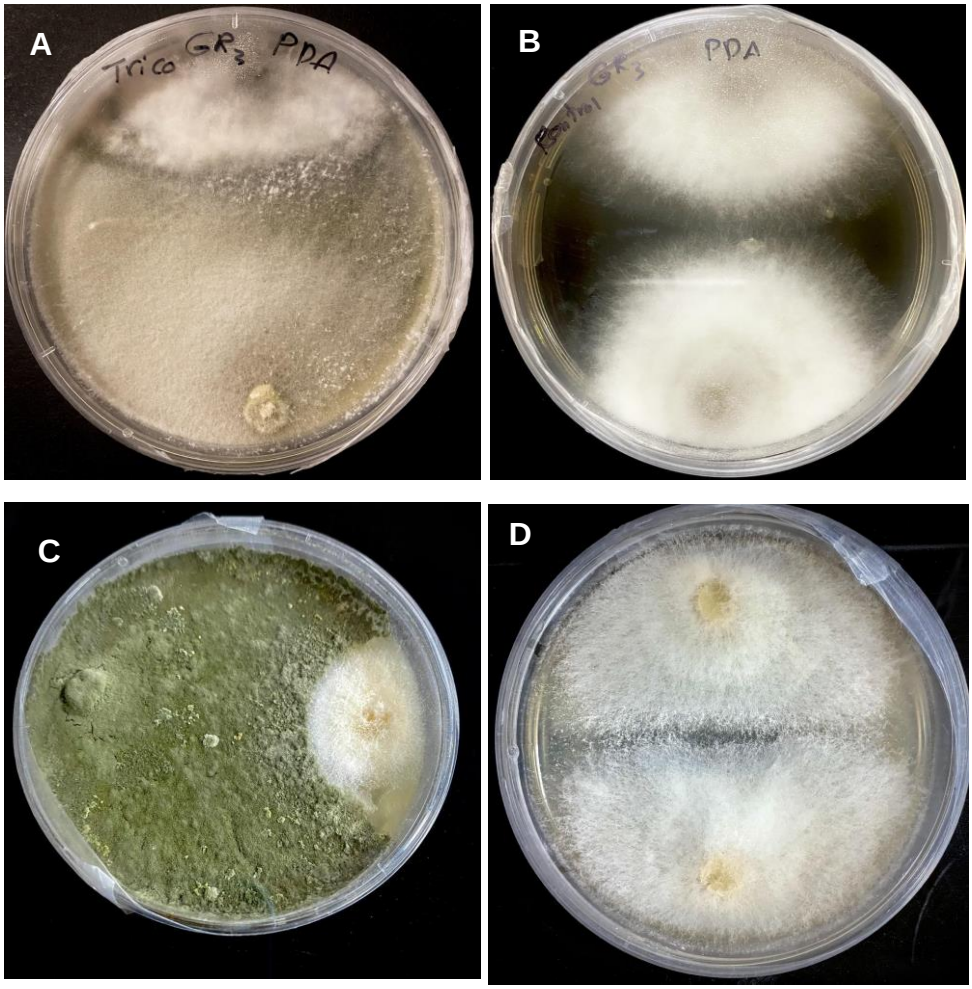

Figure 15: Effect of *Trichoderma* on growth of *N. parvum* (A), *F. solani* (C) and control samples (B & D) in dual cultures. One agar disks (7-mm diameter) of *Trichoderma* grown with 5 days old *F. solani* and *N. parvum* were placed on opposite place of plate. plates were then incubated for 5 days at 25◦C. Zone of inhibition (in mm) was measured 5 days after incubation at 25◦C.

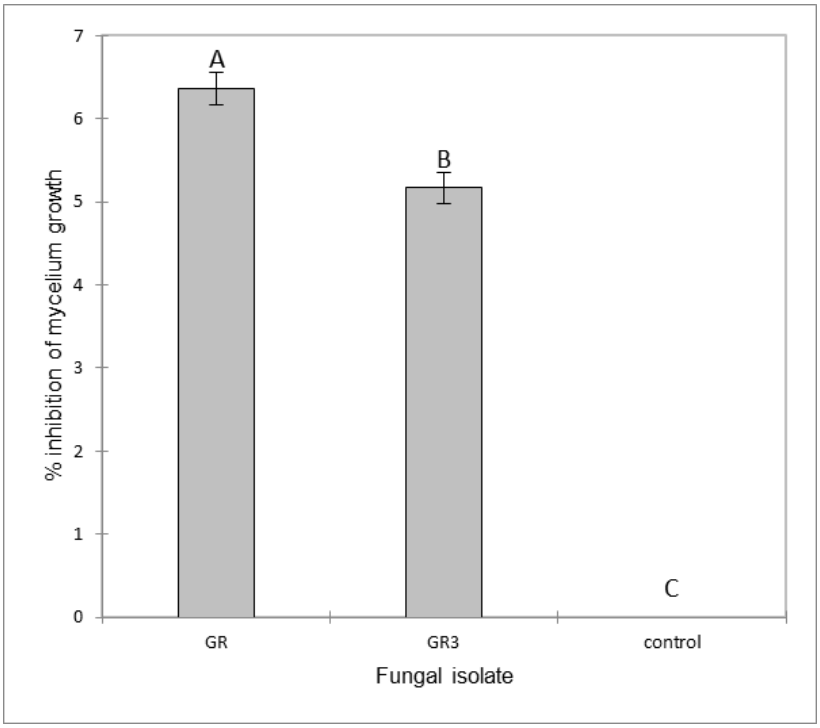

Figure 16: Effect of the isolate *Trichoderma* on inhibition of mycelium growth of *F. solani*, and *N. parvum*. in dual cultures assay on PDA medium, Columns with different letters are significantly different after ANOVA using Tukeys HSD test at $p \leq 0.05$.

4.7.2 Production of volatile antibiotics by Trichoderma

Colony size of *F. solani* and *N. parvum* were measured after 5 days of paired cultures with *Trichoderma*. The volatile compounds produced by *Trichoderma* fungi inhibition the radial mycelial growth of *F. solani* by 42% compared to the control sample. Where it hadn't any inhibition against *N. parvum*. The percent of radial growth inhibition of *F. solani* and *N. parvum* caused by volatiles from *Trichoderma* fungi shown in figure 17 and 18.

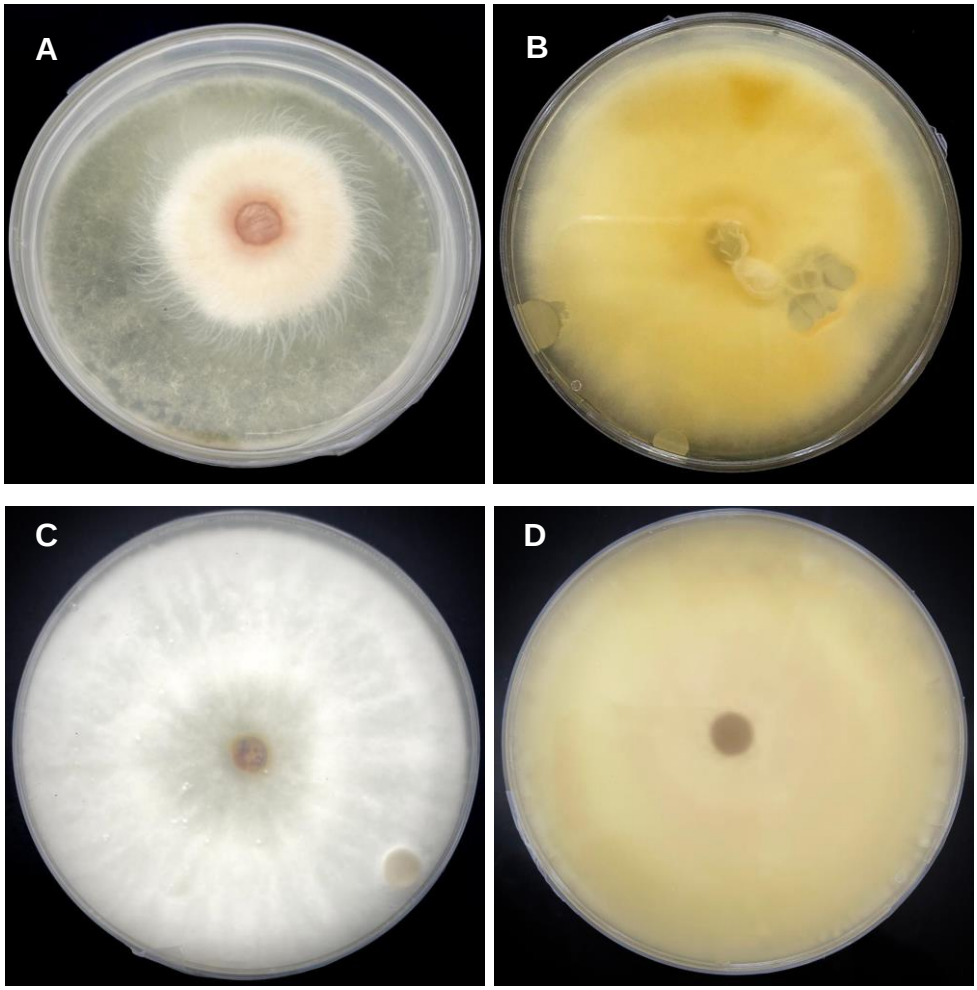

Figure 17: Effect of volatiles produced by the *Trichoderma* on retardation of mycelial growth of control *F. solani* (A), *N. parvum* (C) and control samples (B &D). The samples incubated at 25◦C for 5 days.

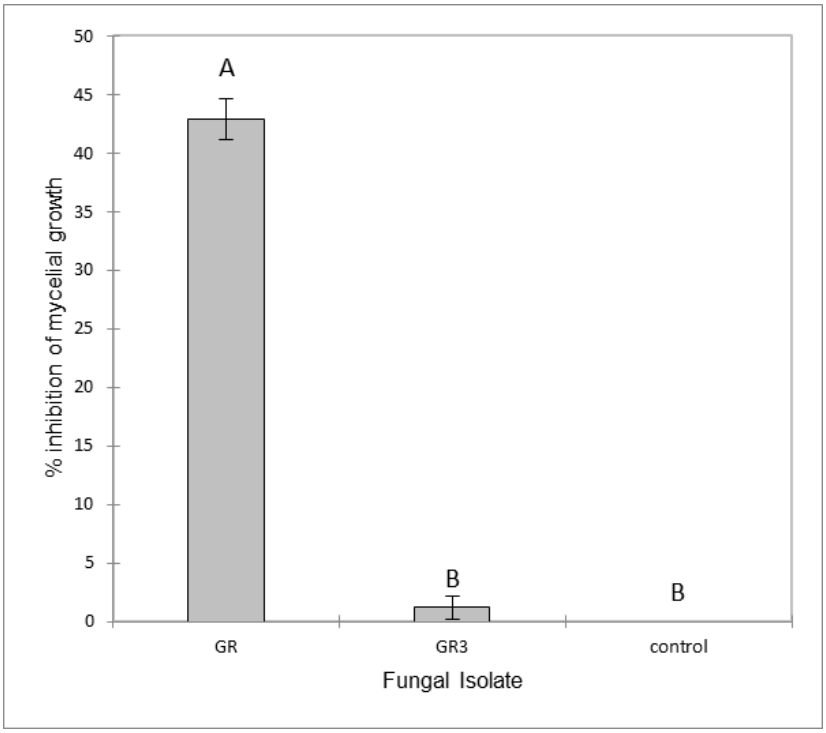

Figure 18: Effect of volatiles produced by *Trichoderma* on retardation of mycelial growth of *F. solani* or *N. parvum* compared to the control. The experiment was done in triplicates and repeated at least 3 times. Columns with different letters are significantly different after ANOVA using Tukeys HSD test at $p \leq 0.05$.

4.8 Antagonistic effects fungicide in vitro

The effects of different concentration of Azoxystrobin on mycelial growth of *F. solani* and *N. parvum* both on agar and in liquid medium were tested. ED_{50}-values were calculated by mycelial weight in PDB-liquid medium ED_{50}-values (table 2). There were evident differences regarding the sensitivity of *N. parvum* to the fungicide tested. So they highly sensitive to Azoxystrobin as indicated by relatively high ED_{50}-values, while *F. solani* had the least effect from Azoxystrobin on inhibition of mycelial growth.

On PDA medium the effects of the fungicide on *F. solani* and *N. parvum* growth were determined by measuring colony diameter and calculating the percent inhibition of mycelial growth (figure 19). Radial growth of *F. solani* and *N. parvum* were reduced by increasing fungicidal concentrations (figure 20- 21).

On PDB medium the effects of different fungicide concentrations on dry weight of *F. solani* and *N. parvum* were shown in figure 22 (table 3).

Table 2: Concentration of active ingredient of fungicides in PDB-liquid medium which caused 50% reduction (ED_{50}) in mycelial growth of *N. parvum.*

Fungicide (ai)	Concentration of Azoxystrobin (µg/ml)	mycelial growth of *N. parvum* on PDB (g)
Active ingredient	250	0.02
(ED_{50}) in mycelial growth	50	0.04

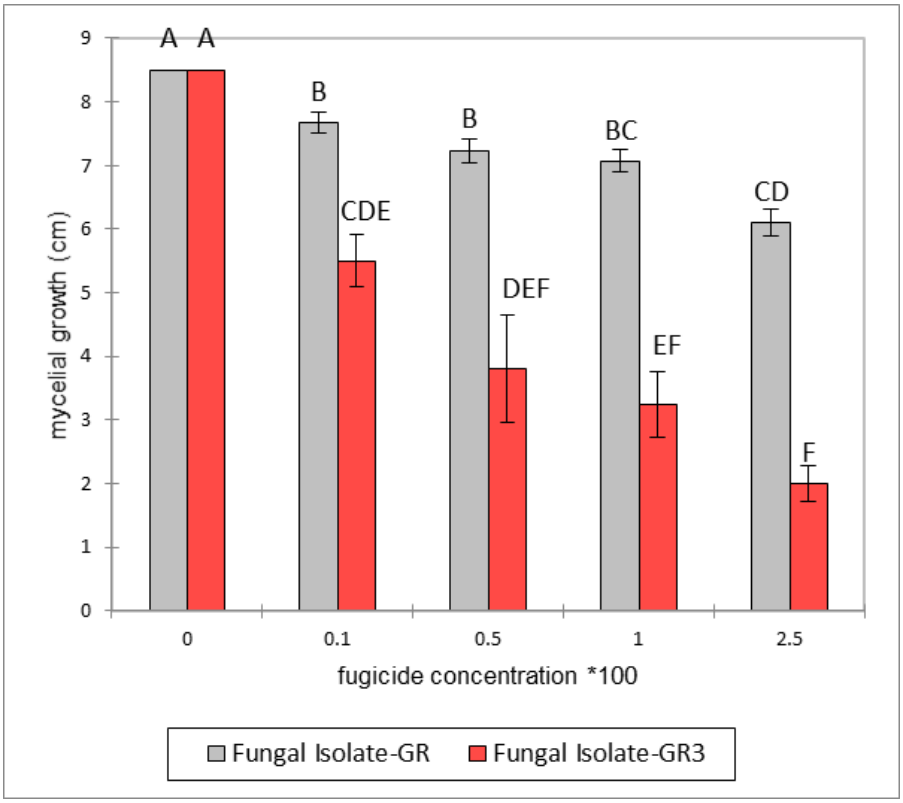

Figure 19: Effect Azoxystrobin at different concentrations (0, 10, 50, 100, 250 µg/ml) on colony diameter of *F. solani* and *N. parvum* in PDA, Columns with different letters are significantly different after ANOVA using Tukeys HSD test at $p \leq 0.05$.

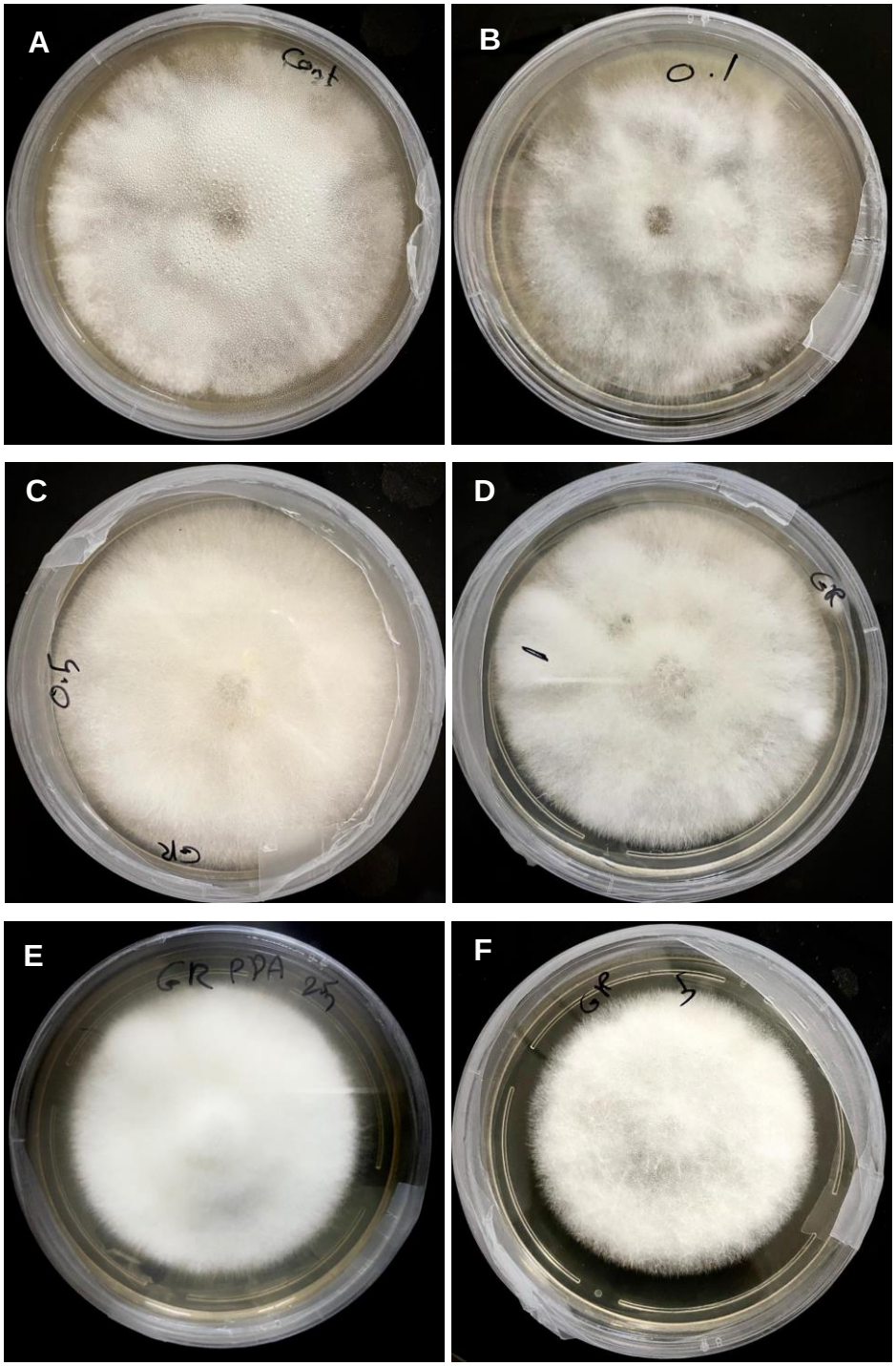

Figure 20: Effect Azoxystrobin at different concentrations 0 (A), 10 (B), 50 (C), 100 (D), 250 (E), 500(F) µg/ml) on mycelial growth of *F. solani* in PDA for 5 days at 25◦C.

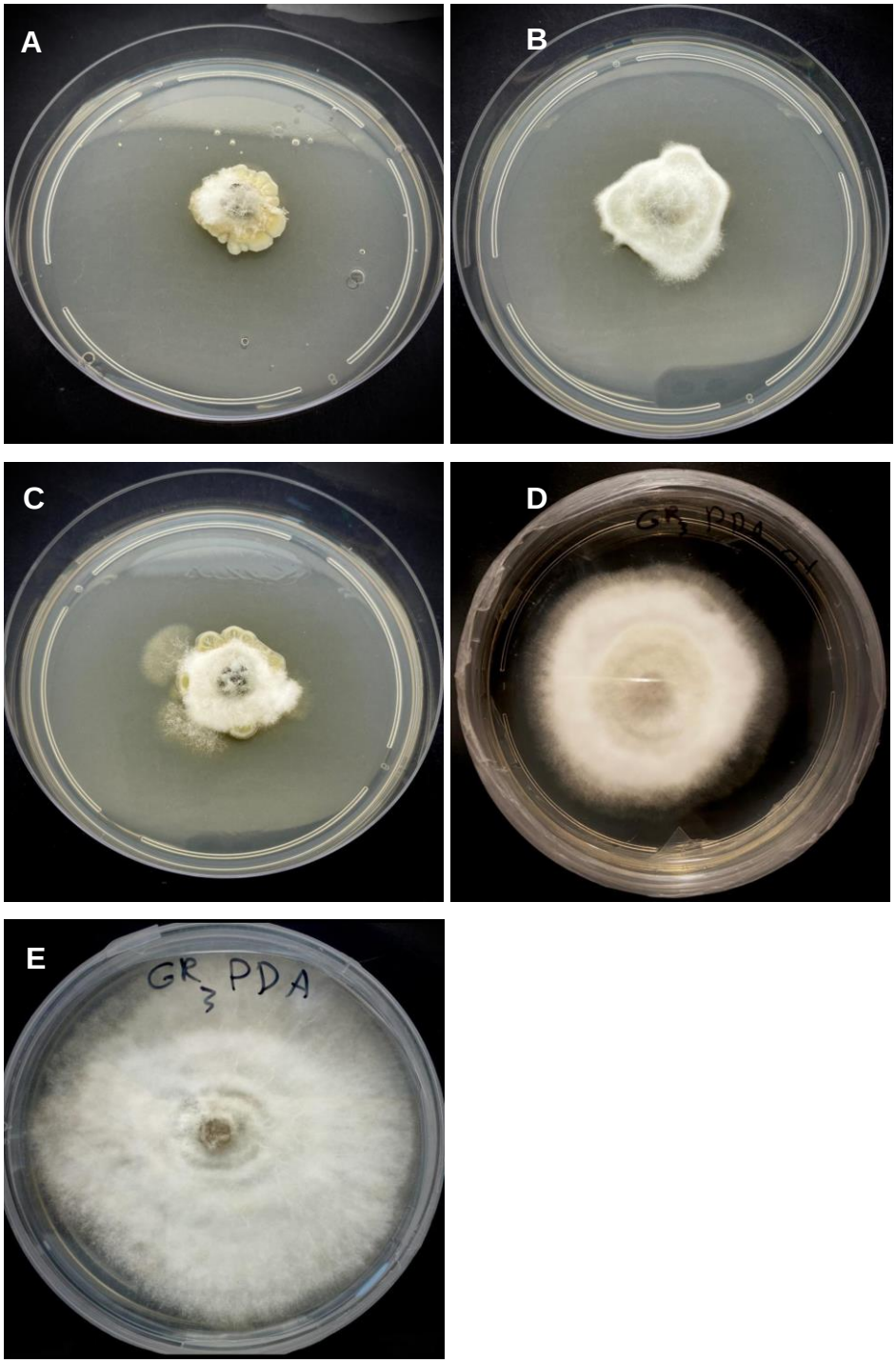

Figure 21: Effect Azoxystrobin at different concentrations 250 (A), 100 (B), 50 (C), 10 (D), 0 (E) µg/ml) on mycelial growth of *N. parvum* in PDA for 5 days at 25◦C.

.

Table 3: in vitro inhibition of *F. solani* and *N. parvum* on PDB medium with different concentrations of Azoxystrobin (0, 10, 50, 100, 250 µg/ml) the values presented the dry weight of *F. solani* and *N. parvum* in PDB medium.

Fungal isolate\fungicide conc. (µg/ml)	0	10	50	100	250
Dry weight of *F. solani* on PDB	0.068	0.503	0.040	0.041	0.0290
Dry weight of *N. parvum* on PDB	0.119	0.043	0.040	0.031	0.020

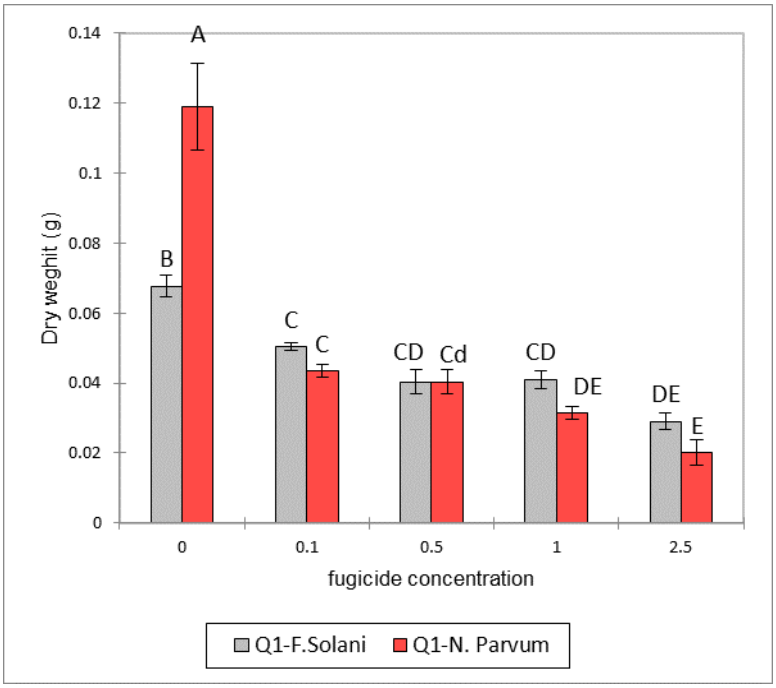

Figure 22: Effect Azoxystrobin at different concentrations (0, 10, 50, 100, 250 µg/ml) on mycelial dry weight of *F. solani* and *N. parvum* in PDB, Columns with different letters are significantly different after ANOVA using Tukeys HSD test at p≤ 0.05.

4.9 In vitro compatibility studies

Growth of *P. fluorescence* isolate ORS3, *P. fluorescence* isolate PFL, *P. aeruginosa* isolate SH1, *P. fluorescence* isolate 1.2 *and B. artophaous* isolate BAT in KB liquid medium was not inhibited by increasing fungicide concentrations up to 50 µg/ml. The compatibility test in vitro proved the ability to combine fungicides at 50 µg/ml concentrations with the bacterial isolates in controlling damping-off disease (figure 23).

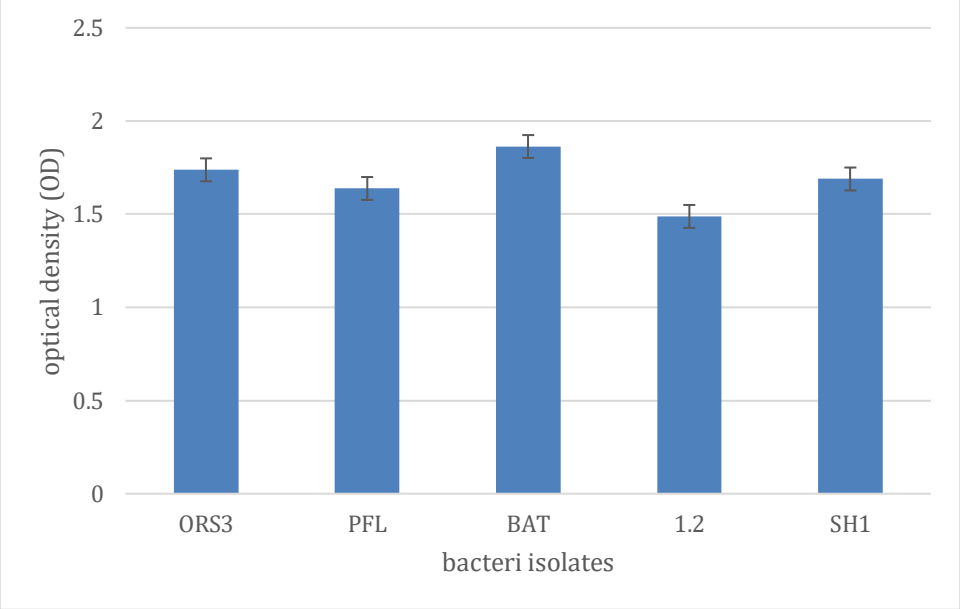

Figure 23: Effect of Azoxystrobin at 50 µg/ml in KB liquid medium on growth of bacterial isolates *P. fluorescence* isolate ORS3, *P. fluorescence* isolate PFL, *P. aeruginosa* isolate SH1, *P. fluorescence* isolate 1.2 *and B. artophaous* isolate BAT as determined by a pre calibrated spectrophotometer at 600 nm absorbance.

4.9.1 Effect of fungicidal concentration and bacterial isolates on PDA medium of *N. parvum*.

The application of bacterial isolates in combination with 50 µg/ml of Azoxystrobin (Azo) make a good inhibition on PDA medium in *N. parvum* treatments (table 4). As a result, *N. parvum* has highly inhibition from most bacteria in combination with Fungicide, especially inhibition from *P. fluorescens* isolate ORS3. The percent of inhibition from these bacteria combined with fungicide (50 µg/ml) reach to 55% compared to the control sample (figure 24).

Table 4: inhibition of mycelium growth of *N. parvum* by bacterial strains (*P. fluorescence* isolate ORS3, *P. fluorescence* isolate PFL, *P. aeruginosa* isolate SH1, *P. fluorescence* isolate 1.2 *and B. artophaous* isolate BAT) in combined with 50 µg/ml fungicide (Azoxystrobin) In daul culture assay PDA medium.

isolate bacteria	inhibition zone of mycelial growth of *N. parvum*
control	-
***P. fluorescence* isolate PFL**	++++
***P. fluorescence* isolate ORS3**	++++
***P. fluorescence* isolate 1.2**	++++
***P. aeruginosa* isolate SH1**	++++
***B. artophaous* isolate BAT**	++++

-, no inhibition zone; +, week inhibition growth of *F. solani* and *N. parvum* were 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.

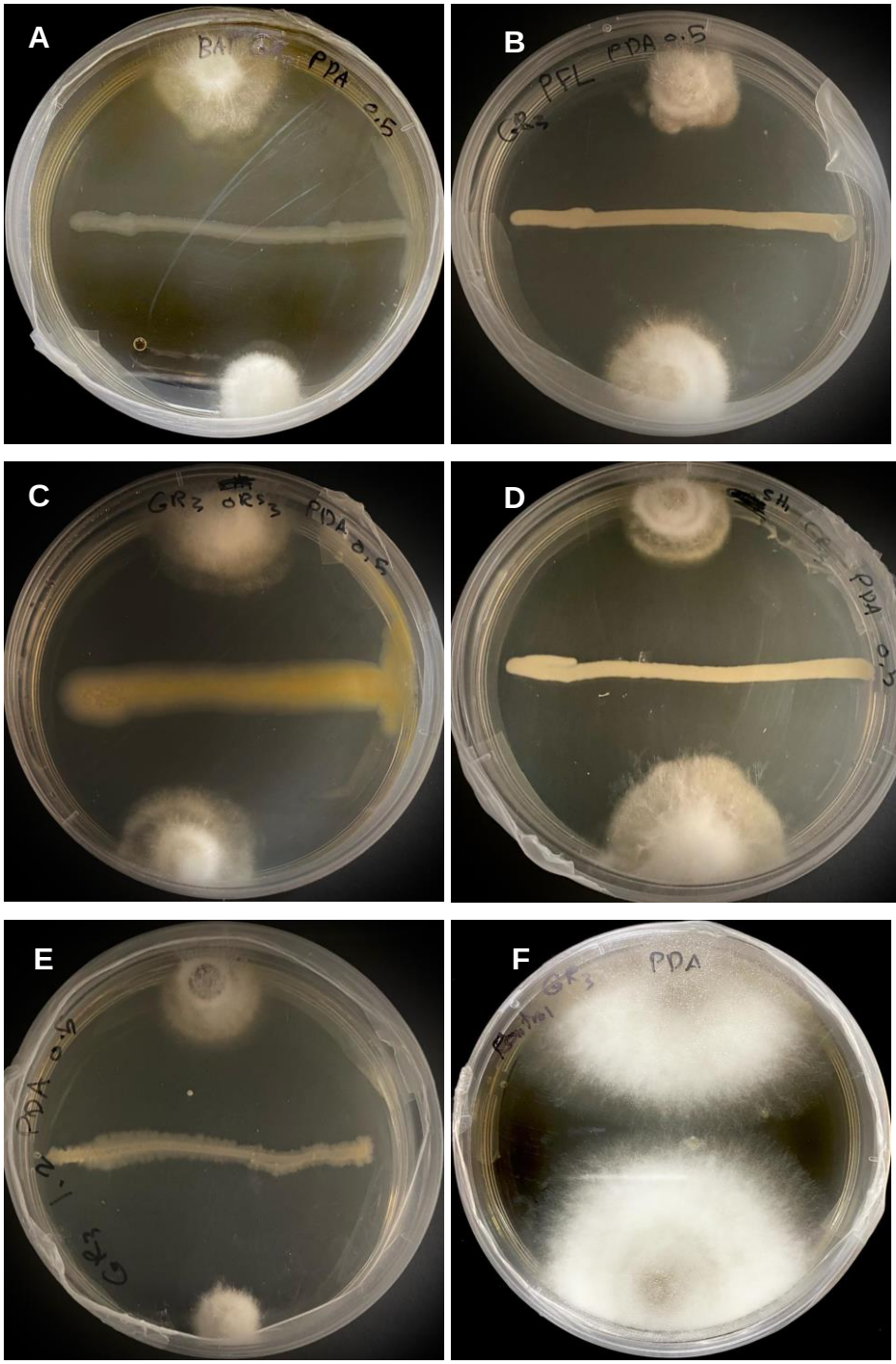

Figure 24: Effect Azoxystrobin (Azo) at concentrations 50 µg/ml in combination with different bacterial isolates *B. artophaous* isolate BAT (A), *P. fluorescence* isolate PFL (B), *P. fluorescence* isolate ORS3 (C), *P. aeruginosa* isolate SH1 *(D), P. fluorescence* isolate 1.2 (E), and control sample (F*)* on mycelial growth of *N. parvum* in PDA for 5 days at 25◦C.

4.9.2 Effect of fungicidal concentration and bacterial isolates on dry weight of *N. parvum*.

The effect of concentrations of fungicide and bacterial isolates applied in combination on *N. parvum* in PDB.

The results showed that all bacteria strains with 50 µg/ml concentration of Azoxystrobin (Azo) made a good inhibition on dry weight in *N. parvum* treatments. As results, *N. parvum* had high inhibition from *P. fluorescence* isolate ORS3 combined with fungicide (84% of inhibition) and moderate inhibition from other bacteria in combination with Fungicide. (figure 25).

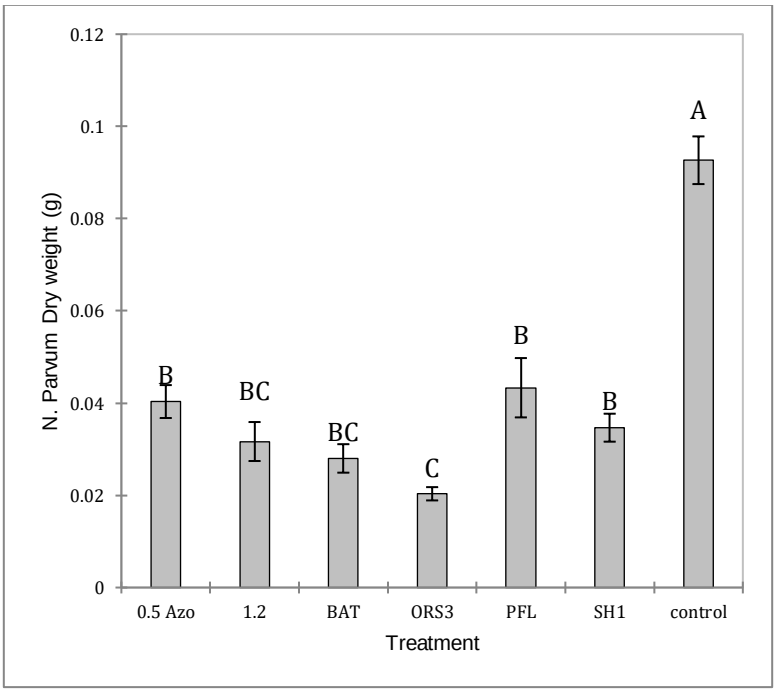

Figure 25: Influence of the different isolated bacteria, *P. fluorescence* isolate ORS3, *P. fluorescence* isolate PFL, *P. aeruginosa* isolate SH1, *P. fluorescence* isolate 1.2 *and B. artophagous* isolate BAT with fungicide (Azoxystrobin) on mycelial dry weight of *N. parvum.* Fungicide concentration: 50 µg/ml combined with isolated bacteria (isolated bacteria :1*10^9cfu/ml-1) in PDB for 5 days, Columns with different letters are significantly different after ANOVA using Tukeys HSD test at $p \leq 0.05$.

Chapter Five: Discussion

5. Discussion

In this study we reported for the first time the isolation of GTDs from grapevines orchards in Palestine. Up to our knowledge, the present study is also the first of its kind in Palestine that combined the efficacy of fungicide and BCA to control GTDs fungi .

The results showed that the both fungal isolates were considered of the most important pathogenic fungi on grapevine (Farr et at., 2018; Vakalounakis et al., 2019).

The Biological control of plant pathogens by naturally occurring microbes or using integrated chemical and biological control is well known phenomenon (Cook, 1993). Antagonistic effectiveness of bacterial isolates against *F. solani* and *N. parvum* was done by testing growth inhibition of fungi in dual culture assay. Results presented in this work showed the differential abilities of different bacterial isolates to inhibit growth of *F. solani* and *N. parvum in vitro.*

As revealed from dual culture assays, the bacterial isolates *P. fluorescence* isolate ORS3, *P. fluorescence* isolate PFL, *P. aeruginosa* isolate SH1, *P. fluorescence* isolate 1.2 *and B. artophaous* isolate BAT varied in their efficacy against both fungal isolates. The sizes of inhibition zones on PDA and mycelial dry weights on PDB was also significantly different. Interestingly, *P. fluorescence* isolate ORS3 was the most effective isolate among the other bacteria.

The size of inhibition zones varied between the bacterial isolates. *P. fluorescence* isolate ORS3 was the most effective in inhibition of mycelium growth of *F. solani* and *N. parvum*.

Also *P. fluorescence* isolate PFL had good effectiveness against *F. solani* and moderate inhibition to *N. parvum*. While *P. aeruginosa* isolate SH1 had a moderate inhibition to *F. solani* and *N. parvum*. Finally *B. artophagous isolate* BAT didn’t have effeciency against any type of three fungi in daul culture assay in PDA medium. As results, the bacterial isolates have varied efficacy against

fungal isolates. The isolate *P. fluorescence* isolate ORS3 was the most effective in inhibition of fungal isolates.

Volatiles from isolates had different effects on various fungi, *N. parvum* didn't affect from any bacteria volatiles, while *F. solani* had sometimes highly inhibition by bacteria isolates. For example *F. solani* had a highly inhibition by *P. fluorescens* isolate ORS3, *P. fluorescence* isolate PFL, *P. fluorescens* isolate 1.2 *and B. artophaous* isolate BAT.

There are many antifungal activities of plant-beneficial *Bacillus* against soil borne disease. *Bacillus* species are known for their capacity to produce a great variety of antifungal compounds to suppress or kill fungal pathogens (Chaurasia et al., 2005) based on gas chromatography/mass spectrometry (GC/MS) analysis, 29 volatile compounds produced by strain ZD01 were identified. Out of 29 identified VOCs, 9 VOCs showed complete growth inhibition activities against *Alternaria solani* (Zhang et al., 2020).

Extracts were obtained from bacterial isolates. These extracts were tested against *F. solani* and *N. parvum* on a PDA medium. *F. solani* and *N. parvum* were inhibited by extracts from *P. fluorescens* isolate ORS3, *P. fluorescence* isolate PFL *and P. aeruginosa* isolate SH1. So these isolates of bacteria can be a promising candidate for controlling the grapevine trunk disease.

The present study also indicated the prominent efficiency of Azoxystrobin against damping-off disease caused by *F. solani* and *N. parvum.* Azoxystrobin is effective in controlling growth of *N. parvum* but had little effect on *F. Solani* inhibition growth at different concentrations fungicides (0, 10, 50, 100, 250 µg/ml) On PDA or on PDB.

Results shown inhibition on diameter in *N. parvum* on PDA, and decreasing in dry weight of these fungi on PDB at low concentration of fungicide. The percent of inhibition at 50 µg/ml was about 66% obtained with the fungicide for *N. parvum*. while *F. solani* had the least effect from fungi at the same concentration, it had about 40% inhibition of mycelium growth in PDA and PDB medium.

So we tended to using bacterial isolates as a biological control in combination with azoxystrobin as a chemical control at the ED_{50}-values in order to decrease the usage of fungicide against *N. parvum*. Knowing that growth of isolated bacteria in KB liquid medium was not inhibited by increasing fungicidal concentrations up to 250 µg/ml.

Application of azoxystrobin at a low concentration (50 µg/mL) in combination with bacterial isolates revealed a highest inhibition from *P. fluorescens* isolate ORS3 against *N. parvum,* the inhibition of disease symptoms reached to 84% on *N. parvum*.

Experiments were also used *Trichoderma* as a biocontrol against GTD disease in vitro conditions, the results showed a good efficiency in both dual cultures assay and Production of volatile antibiotics by *Trichoderma* in inhibition of mycelium growth of GTDs.

In daul culture assay, *Trichoderma* was the most effective in inhibiting mycelial growth of *F. solani*, While moderate effective of the *Trichoderma* on inhibition of *N. parvum*. While in Production of volatile antibiotics by *Trichoderma*,

The volatile compounds which were produced by *Trichoderma* fungi reduced the radial mycelial growth of *F. solani*, but it hadn't any inhibition of growth mycelium of *N. parvum*.

The ability of *Trichoderma* spp. to grow quickly and compete for space contributes to its ability to inhibit the growth of the pathogens in dual cultures (Kucuk and Kivanc, 2004).

Moreover, the formation of inhibition zones without contact is most likely due to the ability of *Trichoderma* spp. to produce volatile (John *et al.*, 2004; Kucuk and Kivanc, 2004) and non-volatile (John *et al.*, 2004) substances.

The capability of *Trichoderma* spp. to produce a great number of volatile (e.g. pyrones, sesquiterpenes) and non-volatile secondary metabolites (e.g. peptaibols) has been reviewed recently (Reino et al., 2008). Volatile secondary metabolites

have been demonstrated to play a key role in mycoparasitism of *Trichoderma* as well as in its interaction with plants (Stoppacher et al., 2010).

Chapter Six: Conclusion and Recommendations

6.1 Conclusion

To conclude, this study highlighted the efficacy of fungicide (Azoxystrobin) of low concentration in combination with biocontrol or biological control alone to protect grapevine pruning wounds against *F. solani* and *N. parvum* infections in vitro conditions. Several conclusions can be made from previous work:

1. The bacterial isolates were capable of inhibiting *F. solani* and *N. parvum* on PDA medium.
2. The most effective bacteria *P. fluorescence* isolate ORS3, it might be a promising candidate for controlling the disease if combined with suitable chemical fungicides at low rates.
3. There were good results of inhibiting mycelium growth of *F. solani* infection from volatiles produced by some bacteria for example *P. fluorescence* isolate PFL and *B. artophaous* isolate BAT.
4. *Trichoderma*-based treatments showed higher efficacy against GTD (*F. solani*) both in PDA and volatiles produced from *Trichoderma* fungi.
5. The results that we had from experiments have been applied in the laboratory and it can have some change if it is applied in the environment.

6.2 Recommendations

1. It is possible to try the results of these experiment at infected grapevine in filed at different conditions.
2. The types of bacteria that inhibits the growth of the fungus have been determined, but the type of enzymes and volatiles substances produced by the bacteria responsible for inhibiting the growth of the pathogenic fungus were not determined.

الملخص

مرض جذع العنب (GTD) الناجم عن عزلة *Fuzarium solani* و عزلة *Neofusicoccum parvum* لهم تهديد حقيقي على محاصيل العنب. هدفت هذه الدراسة إلى تقييم ومقارنة كفاءة عوامل المكافحة البيولوجية جنبًا إلى جنب مع التركيز المنخفض لمبيدات الفطريات لمنع نمو الفطريات المسببة لأمراض جذع العنب في الظروف المختبرية .

خمس عزلات بكتيرية ، عزلة ORS3 *Pseudomonas fluorescence* ، وعزلة *Pseudomonas fluorescence* PFL ، وعزلة SH1 *Pseudomonas aeruginosa* ، وعزلة *Pseudomonas fluorescence* 1.2 وعزلة BAT *Bacillus artophagous* أظهرت أنها معادية لعزلة *F. solani* ، و*N. parvum*.

تم تقييم فعالية البكتريا المعزولة كعوامل للمكافحة الحيوية وحدها وعند دمجها مع مبيد الفطريات ستروبيلورين (Azoxystrobin). في المختبر، كانت عوامل المكافحة الحيوية شديدة التحمل لمبيد الفطريات Azoxystrobin ، الذي يشيع استخدامه للسيطرة على جذع العنب. قلل Azoxystrobin أعراض المرض بنسبة تزيد عن 75٪ عند استخدامه على تركيز 2.5 ميكروغرام / مل ، لكن تأثيره ضئيل عند التراكيز المنخفضة خاصة على *F. solani*.

أعطت العزلات البكتيرية و Azoxystrobin لعزلة *N. parvum* سيطرة جيدة على المرض تصل إلى 85٪ في الظروف المختبرية. على سبيل المثال: استخدام Azoxystrobin بتركيز منخفض50 µg/ml بالاشتراك مع ORS3 يثبط أعراض المرض إلى 84٪ على *N. parvum* مقارنة مع انخفاض بنسبة 66٪ و تم الحصول على 50٪ بمبيد الفطريات بمفرده عند50 µg/ml باستخدام المكافحة البيولوجية وحدها لـ . *N. parvum* ومع ذلك ، فإن المكافحة البيولوجية وحدها منعت *F. solani* بنسبة تصل إلى 50٪. في هذه التجربة ، تم تطبيق استخدام المواد المتطايرة التي تنتجها عزلات مختلفة من البكتيريا والتي أعطت تثبيطًا جيدًا لفطر *F. solani* من بعض البكتيريا وتأثيرا ضعيفا على *N. parvum*.

كما استخدمت التجارب *Trichoderma* كعنصر تحكم حيوي ضد مرض جذع العنب في ظروف المختبر ، وأظهرت النتائج كفاءة جيدة في كل من اختبار الزراعة المزدوجة وإنتاج المضادات الحيوية المتطايرة بواسطة *Trichoderma* في تثبيط نمو الفطريات ل *F. solani* وتأثير متوسط في تثبيط *N. parvum*. متوسط تثبيط الفطريات المسببة لـ GTDS بواسطة *Trichoderma* في اختبار الزراعة المزدوجة حوالي 70٪ لعزل *F. solani* ، *N. parvum* وما يصل إلى 42٪ من المضادات الحيوية المتطايرة لعزلة *F.solani*. ولكن *N. parvum* لم تحصل على أي تأثير من المضادات الحيوية المتطايرة.

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