Application of leaf extracts of *Ambrosia artemisiifolia* to control *Spilocaea oleagina* the causal agent of olive leaf spot disease on olive trees.

By

Fatema Shawqi Kleef

Supervisor

Dr.Mazen Salman

This Thesis was submitted in partial fulfillment of the requirements for the Master's Degree of Agricultural Biotechnology

Faculty of Graduate Studies

Palestine Technical University-Kadoorie (PTUK)

April, 2019

جامعة فلسطين التقنية خضوري

أنافاطمة شوقي خليف، أفوض جامعة فلسطين التقنية خضوري بتزويد نسخ من رسالتي / أطروحتي للمكتبات والمؤسسات أو الهيئات أوالأشخاص عند طلبهم حسب التعليمات النافذة في الجامعة.

التوقيع:

التاريخ: 2019/4/18

PTUK

I, Fatema Shawqi Kleef, authorize the PTUK to supply copies of my thesis / dissertation to libraries or establishments or individuals on request, according to the university of PTUK regulations

Signature:

Date: 18/4/2019

Committee Decision

This thesis / dissertation entitled (Application of leaf extracts of *Ambrosia artemisiifolia* to control *Spilocaea oleagina* the causal agent of olive leaf spot disease on olive trees),

Was successfully defended and approved on: 23/5/2019

Examination Committee	Signature
Dr. Mazen Salman (Supervisor)	……
Dr. Raed Al Kowni (External Examiner)	……
Dr. Daoud Abu Safieh (Internal Examiner)	……

Dedication

I would like to dedicate this thesis to my parents who gave me and still giving every support and encouragement to continue my education. I also dedicate this work to my doctor, Mazen Salman, and all those who have supported me with all respect and appreciation.

Acknowledgment

I would first like to thank deeply my great supervisor, Dr. Mazen Salman, for his fruitful advice and supervision all through my study period.

I would also like to extend my thanks and profound gratitude to the members of the discussion panel: Dr. Raed Al Kowni and Dr**.** Daoud Abu Safieh for the valuable remarks that gave me the impact of the thesis.

I am gratefully indebted to my parents and family for their support, love and comments throughout my thesis study.

I would also thank PTUK University for offering me the scholarship to achieve my master degree.

I would like to thank all the people who contributed directly or indirectly to this work.

Finally, I am very thankful to all who gave me advice, help and encouragement during those two years especially the lab technicians for their close cooperation.

Table of Contents

List of Tables

List of figures

List of Abbreviations

LE	Leaf extract
BLAST	Basic Local Alignment Search Tool
OLS	Olive leaf spot
PDA	potato dextrose agar
RH	relative humidity
T	temperature
H	hour
Conc.	Concentration
DW	distilled water
Sec	Second
Min	Minute
°C	Degree Celsius
%	Percentage
EDTA	Ethylene diamine tetra acetic acid
Rpm	Revolution per minute
PCR	Polymerase chain reaction
NaCl	Sodium chloride
Ml	Milliliter
G	Gram
&	And
e.g.	for example
i.e.	in other words
cu	copper
DNA	Deoxyribonucleic acid
Bp	Base pair
mM	Mill molar
cm	centimeter
GE	Gel electrophoresis
TAE	Tris acetate EDTA
UV	Ultra violet
w/v	Weight per volume
v/v	Volume per volume
V	Voltage
µl	Micro liter
Ng	nanogram

Application of leaf extracts of *Ambrosia artemisiifolia* to control *Spilocaea oleagina* the causal agent of olive leaf spot disease on olive trees.

Fatema Shawqi Kleef

Supervisor

Dr. Mazen Salman

Summary

Olive leaf spot caused by the fungus *Spilocaea oleagina* is one of the most common diseases attacking olive trees all over the world. The disease causes severe premature defoliation of the leaves resulting in severe loses in yield. Control of the disease is achieved by application of copper containing fungicides which might not be always feasible due to negative impacts on human health and environment. The use of plant extract against plant diseases is considered a safe alternative to harmful fungicides. *Ambrosia artemisiifolia* is an invasive plant with allelopathic properties. The aim of this work is to

study the efficacy of *A. artemisiifolia* leaf extract on inhibition of germination of the olive leaf spot spores isolated from different regions in Palestine. Specific primers 18SF forward and 18SR reverse were used to confirm the identity of *S. oleagina* isolates. Box PCR using the Box REPAIR primer was used to discriminate between isolates. Aqueous extract (10% w/v) from *A.artemisiifolia* dried leaves was prepared in water and different concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 5.0 and 10%) were tested against spore germination. The chemical fungicide Fungran was used in combination with the extract to study the efficacy of integrated control of the fungus. The PCR products revealed a 750 bp bands using the 18SF and 18SR specific primers.PCR identification of the isolated fungal stains confirmed that our isolates belong to *S. oleagina*. BLASTn analysis revealed 92-99% similarities with that the isolated fungal strains were to *S. oleagina* (Accession #. AF338393.1).The results showed that the extract could inhibit conidial germination at all concentrations. While maximum germination (80.33%) was obtained in control untreated spores, the germination rate of the spores was reduced significantly with increasing plant extract. Complete inhibition of spore germination was recorded in 10% plant extract. The lowest germination percent (2.8%) was achieved at 5% concentration. Interestingly, at low extract concentrations (0.5%), the germination rate did not exceed 50% of the

control. The results showed also that combination of fungicide and plant extract (1:1 v/v) reduced the germination rate of spores to less than 5% of the control.

Up to our knowledge, this work is the first of its kind that tested the efficacy of *A. artemisiifolia* leaf extract in combination with fungicides against the olive leaf spot disease. Further experiments are needed to produce a suitable formulation for use against the disease under field conditions.

Keywords: germination; olive leaf spot; control; leaf extract; PCR, BLASTn; Box PCR.

CHAPTER ONE: INTRODUCTION

1. General background

The Olive tree, *Olea europaea* L. is the most cultivated plant in the world with more than eight million ha of which 98%in the Mediterranean region (Peralbo-Molina and de Castro, 2013). Between 2014 and 2016, the land occupied by olive trees increased by 10% (International Olive Council, 2016). Nowadays, there are more than 2000 cultivars in the Mediterranean areas which exhibit enormous diversity based on pit size morphology and fruit morphology (Besnard et al., 2001).

In Palestine, olive tree covers approximately 45% of the agricultural lands. The tree is distinctly important in the economic and social life of the people comprises one of the main sources of income, reaching its contribution to the good years to about 13% of the annual agricultural production. Olive (*Olea europaea* L.) is one of the oldest agricultural trees which are cultivated over large areas in Palestine.

The number of olive trees is exceeding more than 10 million (about 67.3% of fruit trees) and occupying more than 45% of the agricultural area. Olive production contributes to about 12-13% of the national income (Jabi, 2007).

As olive cultivation in the West Bank tends to be low-input traditional plantations, with low chemical use, it is more environmentally sustainable than the intensive olive cultivation, which is often practiced in other oil

producing countries. Olive cultivation provides employment and income for around 100,000 farming families who are olive oil producers.

Olive orchards are usually run as small family business, utilizing unpaid family labor. More than 80% of olive farmers are small to medium scale farmers, owning olive orchards equal to or less than 25 donums in area. The sector creates thousands of jobs.

Olive production generates seasonal employment for laborers (around 3 million working days in a good year). Many of these laborers are share croppers (who contribute labor during harvest season and receive a share of the crop in return).

Employing large numbers of unskilled labor and approximately 15% of working women, olive oil has attracted much attention from international development agencies and non-governmental organizations (NGOs). This attention and funding persists today, providing a significant resource for development of the sector, as well as a tremendous challenge to avoid potential duplication and cross-purpose work.

In addition to that, in 2009, there were 490 paid employees working in some 235 olive presses operating throughout Palestine. The sector also provides jobs to those who work in nurseries and bottling plants as well as traders.

In recent years, olive production has been impacted by several factors such as alternate bearing, aging of trees, reduced tree management and pests and diseases.

The peacock disease (*Spilocaea oleagina)*, Verticillium wilt (*Verticillium dahlia*), the olive fly (*Bactrocera Olea* Gmel.) and olive moth (*Prays Olea*) are the most common destructive diseases and pests of olives in Palestine. Unfortunately, pest and disease management practices are not common in Palestine.

The olive leaf spot disease (OLS) is considered the most destructive disease on olive trees in Palestine. The disease causes severe defoliation of the tree (Figure 1) leading to yield losses that might approach 20%.

Figure 1: Symptoms of defoliation on an olive tree as a result of severe infection with OLS (source: The author).

The disease is controlled by application of chemical fungicides that might not be always suitable due to environmental and human health perspectives.

The aims of this study are:

1- Testing the effect of *A. artemisiifolia* against olive leaf spot.

2- Determining the optimal extract concentration for the control of the disease.

3- Integrate the bio-fungicide (extract) with chemical fungicide

4- Determining the genetic variation of *S. oleagina* in Palestine.

CHAPTER TWO: LITERATURE REVIEW

2.1 Olive tree pests and diseases

The olive tree is affected by many pests and diseases (Sergeeva *et al.,* 2008; Macdonald *et al.,* 2000; Sanei & Razavi, 2011).The catalogue of organisms potentially harmful to the olive tree includes over 255 species at the present time and the number is increasing with the identification of new organisms, especially mites, nematodes, and pathogenic microorganisms (Haniotakis, 2005).

In the majority of cases the disease is caused by: fungi, bacteria and viruses. However, only a small number of these are capable of causing damage of economic importance on the olive tree (Haniotakis, 2005).

In general, the Mediterranean climate doesn't favor the epidemic explosion of most olive disease caused by fungi and bacteria; due to the long periods of high temperature and drought in summer. However, olive trees are the least sprayed crops; because the olive has fewer natural enemies than other crops, and because the produced olive oil retains the residual chemicals (Ferguson *et al.,* 1994). One of the most serious diseases affecting the leaves of the olive tree is the olive leaf spot (OLS) also known as Peacock eye disease (Azeri, 1993).

2.2Olive leaf spot disease

Olive leaf spot (OLS)or the pea cock eye disease is one of the most important foliar diseases affecting olive in many parts of the world (Graniti, 1993; Obanor *et al.*, 2011; Gonzalez-Lamothe *et al.*, 2002; Obanor *et al.*, 2008). The disease is caused by the Deuteromycete fungus *S.oleagina* (Cast.) Hughes (syn. *Cycloconium oleagina*) (Azeri, 1993; Gonzalez-Lamothe *et al.*, 2002**)**.The disease is widespread in all olive growing regions of the world and causes yield losses in olives that are estimated to be as high as 20% (Salman *et al.*, 2011; Azeri, 1993; Graniti, 1993). The disease may affect the quantity of the olive and reduce the quality **(**Stewart, 2012).

The Olive leaf spot usually occur on the upper surface of the olive leaf (Figure 2). Lower side of the leaves does not show apparent symptoms, and young olive leaves are more susceptible to infection (Graniti, 1993; Khadari *et al.*, 2001).

The disease causes severe premature defoliation (Figure1), fruit blemishes, poor twig growth, and sometimes death of the whole plant (Miller, 1949; Azeri, 1993).

Infection and the life cycle of the disease (Figure 3) is normally associated with high humidity and winter conditions (mild to low temperature and low light), where high temperatures restrict spore germination and growth

(Obanor *et al.*, 2008; Al-Khatib *et al.*, 2010). Conidial production is optimal at temperatures ranging from 21 to 25°C and high humidity (Graniti, 1993; Obanor e*t al.*, 2008).The Conidia are disseminated by wind and rain (Lops *et al.*, 1993) and can, in turn, produce new lesions. Cultivars vary in their susceptibility, but all are subjected to infection (Abuamsha *et al.*, 2013).

Figure 2: Symptoms of olive leaf spot disease on infected olive leaves (source: The author).

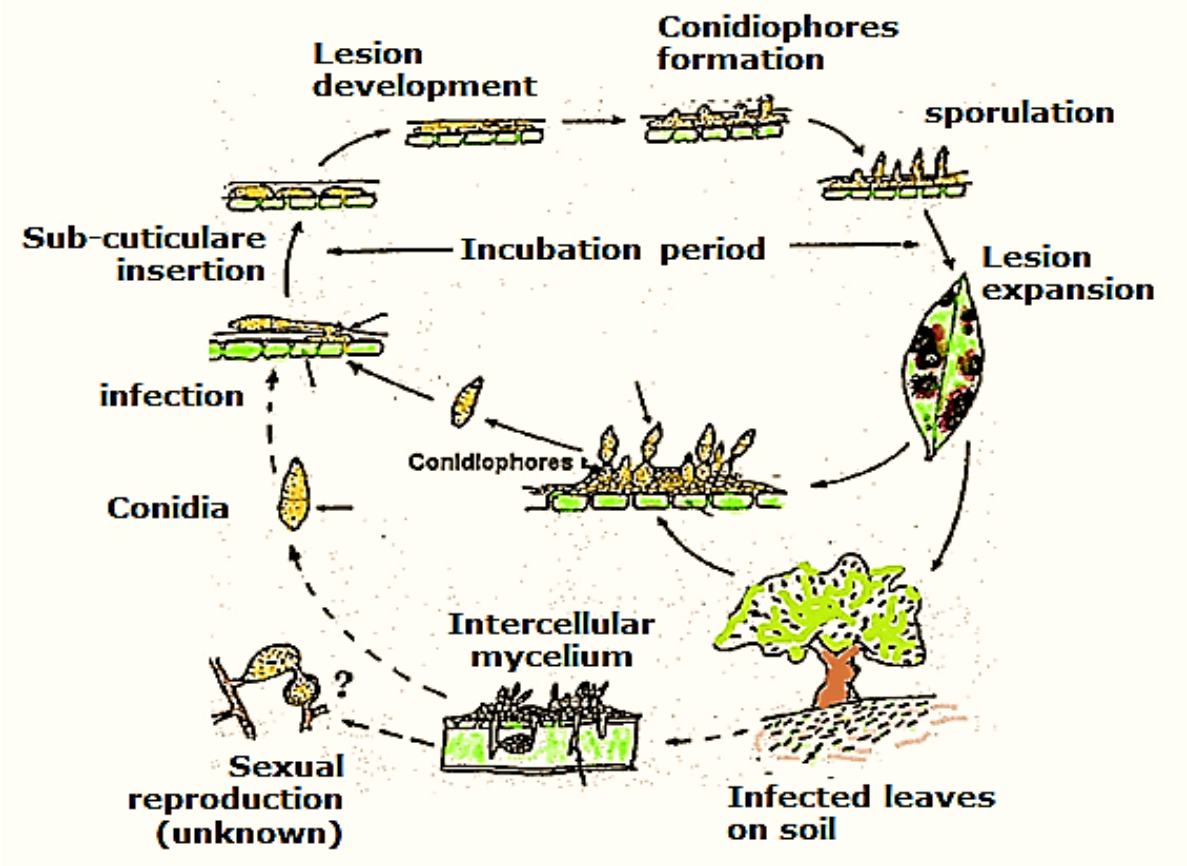

Figure 3: Life cycle of *S.oleagina* (source: Benkada *et al.*, 2015).

2.3 Control of OLS

The disease is chemically controlled by application of coppers fungicides directly after harvest season (Obanor *et al.*, 2008; Sistani *et al.*, 2009). The most commonly used fungicides include Bordeaux mixture, copper hydroxide, copper oxide and copper oxychlorides, although some long-persisting preventative fungicides such as chlorothalonil and dodine have also been used to control the disease (Sistani *et al.*, 2009). Chemical treatment

appears to be rarely effective (Graniti, 1993; Obanor *et al.,* 2008). Moreover, using chemical fungicides leads to the appearance of resistant pathogen races (Vanneste *et al.,* 2003; Carisse *et al.,* 2000) as well as disturbance of the plant metabolism following Cu accumulation in the soil (Obanor *et al.,* 2008).

In Palestine, the disease is wide common in many areas including Hebron, Bethlehem, Ramallah, Nablus, Qalqilia, Tulkarm, Salfit, Jenin and Tubas (Salman *et al.,* 2011). The Palestinian Ministry of Agriculture and several private institutions work on olive sector to provide guidance and information about improving olive crop production and protection. Application of chemical fungicides to control the disease in Palestine is, however, not acceptable because of the negative effects of these fungicide on human and environmental health as well as their effects on taste and quality of the olive oil which usually lead to low prices and low income of the yield crop.

In recent years, alternative techniques have been applied for the control of the disease. Genetic resistance represents an effective, economically feasible and ecologically sustainable mean to control the peacock disease (Rhouma *et al.,* 2012; Sanei & Razavi, 2011; Zine El Aabidine *et al.,* 2010). However, the level of susceptibility of olives to OLS is widely variable (Graniti, 1993; Sutter, 1994). Several cultivars (e.g. Leccino and Valatolina) are resistant to *S. oleagina* (Macdonald *et al.,* 2000; Sanei & Razavi, 2011). On the other

hand, some varieties such as Arbequine, Frantoïo, Barouni and Nabali are sensitive (Macdonald *et al.,* 2000; Sutter, 1994).

In response to environmental and health concerns about extended use of pesticides, there is a considerable interest in finding alternative control approaches for use in integrated pest management strategies for crop diseases. It seems inevitable that fewer pesticides will be used in the future and that greater reliance will be placed on biological technologies including the use of microorganisms as antagonists. Biological control using antagonistic microorganisms alone, or as supplements to minimize the use of chemical pesticides in a system of integrated plant disease management, has become more important in recent years (Mao *et al.,* 1997; Hwang *et al.,* 1993). Different microorganisms including bacteria can be used to control plant diseases. There has been a large number of literature describing potential uses of plant associated bacteria as biocontrol agents stimulating plant growth and managing soil and plant health (Blakeman & Fokkema, 1982). However; little is known about the use of bacteria against olive diseases.

2.4 Use of plant extract in control strategy

Disease management strategy needs to be shifted towards non chemical methods. Managing disease using biological method is less expensive,

permanent, and pollution free. This situation has led to an increased demand for environmentally-friendly products in order to reduce the effects of widespread fungicide utilization in crop protection (Coats *et al.*, 2003).Many plant species have been investigated for their antifungal activities (Eloff & McGaw, 2014; Raut & Karuppayil, 2014). Some plants can protect themselves against various phytopathogens by producing a variety of antimicrobials (e.g. phytoalexins and phytoanticipins) (Ribera & Zuniga, 2012).

The use of plant extract against plant diseases is considered a safe alternative to the use of harmful fungicides. Plant that are indigenous to the Palestinian agricultural flora, it is not expected to constitute any modification or threat to the ecosystem.

This application is not only safe to the environment and the community but also a sound economical alternative since it will save the cost of using expensive modern fungicides and fertilizers. Also the use of natural products together with chemical fungicides at low dosage in the framework of integrated pest management programs could achieve the aims of reducing costs and limiting the environmental impact of chemical fungicides.

2. 5*Ambrosia artemisiifolia. L*

Ambrosia artemisiifolia (of the family *Asteraceae*) is one of the widespread annual herbaceous weed that emerges in late spring (Figure 4), and is also commonly known as ragweed (Fumanal *et al.*, 2007;Kazinczi*et al.*, 2008; Smith *et al.*, 2013;Lambdon*etal.*,2008).

It is widely distributed across the world and has become the most noxious weed in agriculture. It can cause negative economic impact by decreasing crop yields, crop quality and efficiency of propagation and harvest (Reinhardt *et al.*, 2003).

Ambrosia artemisiifolia is a fast growing herb (Bassett & Crompton, 1975; Li *et al.*, 1989; Beres, 1994) because of some bioactive compounds (allelochemicals) (Bruckner e*t al.*, 2001) that is responsible for allopathic capacities which are the phenoloids and terpenoids (Bruckner, 1998).

Although, it was a traditional medicinal plant for Native American tribes (BRIT); and it is used in phytoremediation projects remediating soil pollution, for removing heavy metals such as lead from contaminated soil (Huang *et al.*, 1997).

Figure 4: *Ambrosia artemisiifolia* in fields near Tulkarm governorate (source: The author).

CHAPTER THREE: MATERIAL AND METHODS

Material and methods

3.1 Isolation and culture maintenance of *S. oleagina*

3.1.1 Collection of olive leaves

Infected olive leaves with obvious symptoms of OLS were collected from infected olive trees in six governorates in Palestine (Table 1). The samples were collected during February-March 2018.The leaves were placed in paper bags and transferred to the laboratory where they were stored at 4˚C until use.

Table 1: The coordinates of the regions where olive samples were collected.

Region	Coordinates
Qalqilia	32°11′25″N 34°58′07″E
Nablus	32°13′13″N 35°16′44″E
Tulkarm	32°18′42″N 35°01′38″E
Ramallah	31°54′N 35°12′E
Jenin	32°27′40″N 35°18′00″E
Salfit	32°04′55″N 35°10′56″E

3.1.2 Viability test

Viability of collected spores was tested by placing 20 µl distilled water (DW) on the spots of infected leaves which was then transferred to a glass slide in 9 cm Petri dish containing 3 ml DW. The plates were sealed with parafilm and incubated at 20°Cfor 24 h. Germination of conidia was checked under light microscope at 200X magnification and counted to evaluate the percent of germination. Conidia were considered germinated if the length of the germ tube exceeded half the length of the conidia.

3.1.3 Preparation of fungal spore solution

Spore suspension was prepared by collecting the spores from the spots of infected olive leaves with obvious sporulation symptoms. For that, a drop of DW (~ 50 µl) was placed on the spot and pipetted out into a one ml Eppendrof tube. This step was repeated to get 500-700 µl of conidial solution. The spore concentration was adjusted to ~ 10^4spore.ml^{-1}using hemocytometer.

3.1.4 Production of single spore culture

To obtain spores from infected leaves, a drop of autoclaved DW (20 µl) was pipetted on olive leaves with obvious sporulating lesions and transferred to

sterile glass slide. After that a one µl loop was used to transfer spores to Petri dish containing potato dextrose agar (PDA) media. The plates were sealed with parafilm and incubated at 20°C for 24 h. The cultures were examined under light inverted microscope at 200X magnification. Contaminated cultures were excluded and the germinated spores were transferred to a new PDA plates by cutting the agar block that bears the germinated spores. The plates were further incubated at 20°C until production of pure mycelia colony from each isolate. The cultures were maintained on PDA and subculture routinely every 2 months.

3.2 DNA extraction

Mycelium of each isolate was scrapped from the media and placed in a one ml Eppendrof tube containing 700µl extraction buffer containing 20M Tris-HCl, 250 mMNaCl, 25 mM EDTA, 0.5% SDS and 10µl proteinase K. The mycelia were ground using a pellet pestle homogenizer (Kimble).After grinding the mycelia; 500µl of phenol-chloroform was added to each tube and mixed by vortex for 5min before centrifugation at 13000 rpm for 20 min. The upper phase was transferred to fresh tubes and 30µl of 5M sodium acetate (NaoAc) and 1ml 70% ethanol were added. The tubes were centrifuged at 13000rpm for 10min and the pellets were washed with 70% ethanol. The

DNA was dissolved in 50µl TE buffer (pH 8) (containing 10 mM Tris-HCl and 1.0 mM EDTA) and stored at -20°C.

The isolated DNA was separated on 1% agarose gel in 1X TAE buffer (containing 2mMTris-HCl, 0.5mMEDTA and 1mM glacial acetic acid) for 60 min at 70 V and visualized under UV transilluminator.

3.3Confirmation of fungal species

Molecular identification of the fungus was done using specific primers (18SF 5'-GCTTGTCTCAAAGATTAAGCC-3' forward and 18SR 5'-CCTTG TTACGACGACTTTTACTTCC-3' Reverse) that amplify the 18S rDNA region (González-Lamothe *et al.,* 2002; Obanor *et al.*, 2010). The PCR amplification were performed in a final volume 20 µl containing 10 µl GoTaq® Green (2X) Master Mix (Promega Corporation), 1 µl of each primer (10 pmol), 1 µl Fungal DNA (~50 ng) and 7 µl nuclease-free water. The amplification was carried out in thermal cycler (Veriti™ DxThermo Fisher Scientific)using the following program: initial denaturation at 94˚C for 3 min, followed by 35 cycles of denaturation at 94˚C for30 sec, annealing at 55˚C for 30 sec and primer extension at 68˚C for 30 sec. A final primer extension at 68˚C was carried out for 7 min.

3.4 Genetic variation of *S. oleagina*

For the discrimination of fungal isolates, Box PCR using the Box REPAIR primer (5'-CTACGGCAAGGCGACGCTGACG-3') was used.

Box PCR is a rapid and highly reproducible molecular biologic technique for the discrimination of the genotypic variation among the different species. Its capacity to discriminate between similar but not identical isolates meets or exceeds that of other available molecular typing approaches (Koeuth *et al*., 1995).

PCR amplifications were carried out in 25 µl reactions. The reaction mixtures contained 3µl fungal DNA, 1.5 µM of Box primer, 12.5µl GoTag master mix and 8 µl nuclease-free water. Amplification conditions were as follows: initial denaturation at 94˚C for 3min, followed by 45 cycles of denaturation at 94˚C for 25sec, annealing at 52˚C for 60 sec and primer extension at 72˚C for 60 sec. A final extension at 72˚C was carried out for 8min. PCR products were separated and analyzed by TAE-agarose gel electrophoresis. Gel images were recorded for fingerprint profiles production. The images were recorded and imported into GEL Jv2 program. Fingerprint profiles from BOX PCR results were created for isolated sample and the resulting profiles were analyzed using the (Pearson) similarity matrix followed by cluster analysis using the

unweighted pair group method with arithmetic mean (UPGMA) to generate Phylogenetic tree.

3.5Plant material

Ambrosia artemisiifolia was collected from different fields in Palestine. The leaves were taken and dried in an oven at 65˚C for 48hr until constant weight. After that, the dried leaves were ground to fine powder using an electrical blender. The powder was stored in dark until use.

3.6Preparationof plant extract

Aqueous water extract (10% w/v) was prepared by soaking 10g of the powder in 90ml distilled water for 24h on horizontal shaker at 110rpm. The residue of the powder was removed from the extract by filtering the solution through a double layer of cheesecloth followed by filtrationthroughWhatmanNo.1 filter paper. The filtrate was stored in dark bottles at 4˚C until use.

3.7 Effect of plant extract on conidial germination

Different concentrations of plant extract (0.5, 1.0, 1.5, 2.0, 2.5, 5.0and 10.0%) were tested for their efficacy against spore germination of OLS. For this a mixture of conidial: extract suspension was prepared as mentioned in table (2).After that three 20µl droplets of the mixture (i.e. extract: conidia) placed

separately on glass slides. For control treatment, fungal conidia were only used. The slides were then incubated in a sealed 9 cm petridishcontaining3ml DW to provide high humidity and incubated upside down at 20˚C for 24h. Germination of 100 randomly selected conidia in each droplet was evaluated under light microscope at 200Xmagnification, and the mean percent germination was calculated for each concentration. Conidia were considered germinated if the length of the germ tube exceeded half the length of the conidia.

Table 2: The mixture of fungal conidia and extract

Extract conc.	Fungal conidia(µl)	10% Extract (µl)
control	**20**	**0**
0.5	19	1
1.0	18	2
1.5	17	3
2.0	16	4
2.5	15	5
5.0	10	10
10.0	0	20

3.8Effect of extract and fungicide on conidial germination

After determining the best concentration of the plant extracts that inhibit fungal growth which was 2.5%, it was mixed with fungicide (Fungran®-OH

50 WP) at different ratios (0.1:1, 0.25:1, 0.5:1, and 1:1) to prepare mixtures of one ml (Table 3). The fungicide was prepared by dissolving 0.025g in 10ml DW as mentioned by Salman *et al.*, (2014).

Three 20µldroplets from each solution including: control, fungicide alone, extracts alone and combined treatments were placed separately onto a glass slide and incubated at 20˚C for 24h. The mean percent of conidial germination was then counted.

Table 3: Ratio between extract and fungicide

Extract: Fungicide	**Extract(µl)**	**Fungicide(µl)**
1:1	500	500
1:0.5	666	333
1:0.25	750	250
1:0.1	900	100

3.9 Statistical analysis

Data were analyzed using Xlstat software (Adinosoft, USA).Significant differences among treatments were computed after Tukey's HSD test using ANOVA at $p<0.05$

CHAPTER FOUR: RESULTS

4.1 Germination and viability of fungal spores

All fungal isolates were subjected to germination test. The results showed high viability of conidia in all samples (Figure 5).The germination rates after 24 h of incubation at the 20°C ranged between 70-90% (Table 4). The lowest germination rate (70%) was recorded in spores from Nablus governorate and differ significantly from the highest germination rate (90.3%) in spores from Salfit governorate, but did not differ from those in the other regions (Figure 6).

Table 4: Viability of OLS spores.

Region	Percent of Germination
Qalqilia	80
Nablus	70
Tulkarm	75
Ramallah	80
Jenin	85
Salfit	90

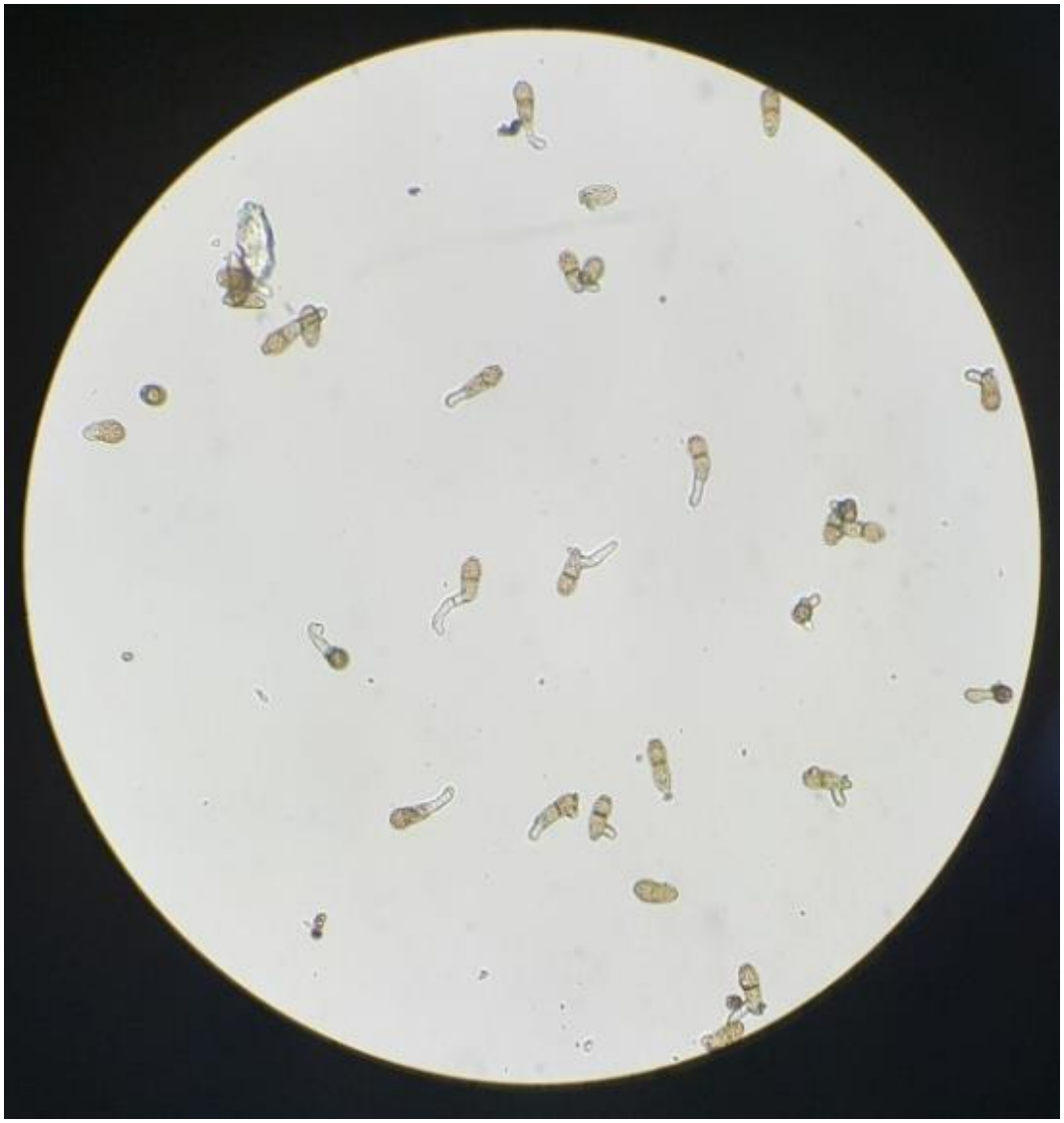

Figure 5: Spore germination after 24h of incubation at 20°C. The spores were examined under light microscope at 200X magnification.

.

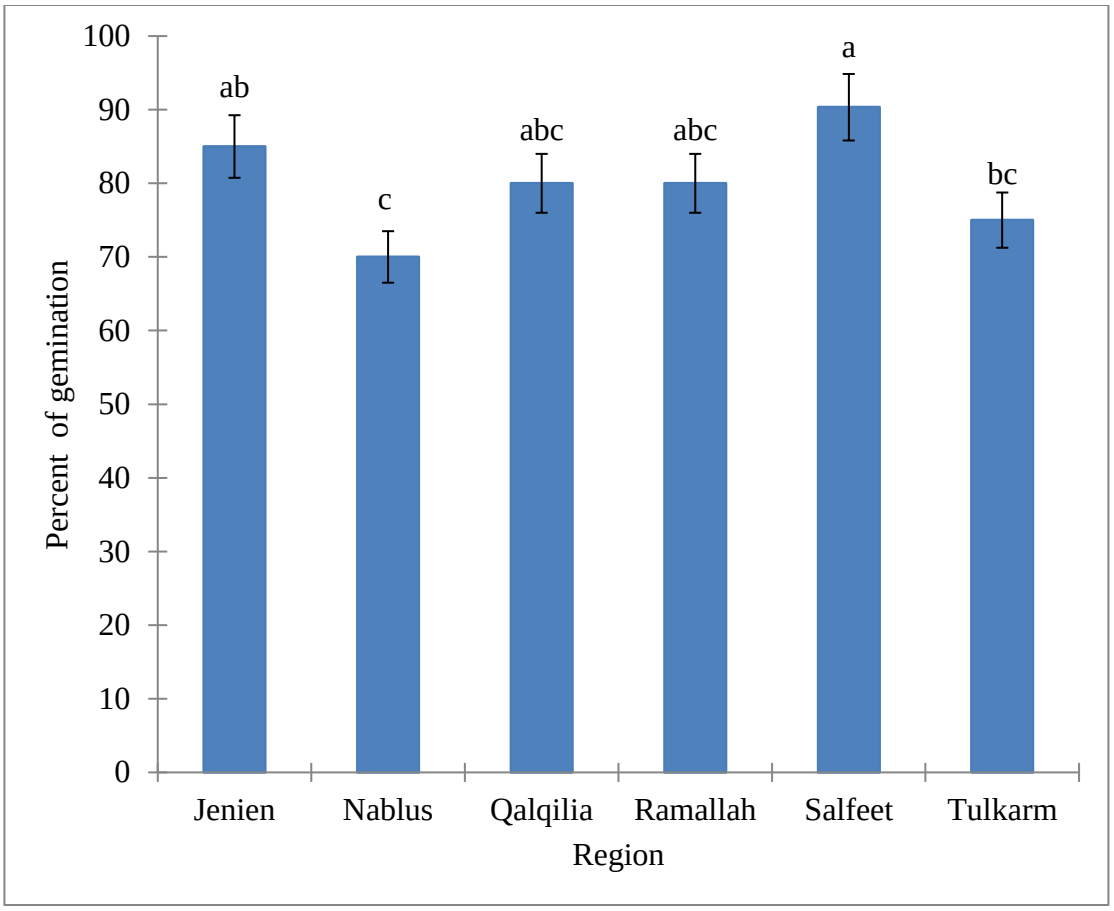

Figure 6: Percent of viability of OLS spores in different regions. Data with different letters are significantly different after Turkey's HSD test using ANOVA at $p<0.05$.

4.2Isolation and maintenance of single spore culture

Successful cultures were produced from single spores of OLS. Slow growing fungal cultures was obtained. The colonies were morphologically identical in all cultures from different regions. A hemispherical grayish green of about 1.5 cm in diameter were produced (Figure 7). The mycelium was very compact and did not produce conidia under in vitro conditions.

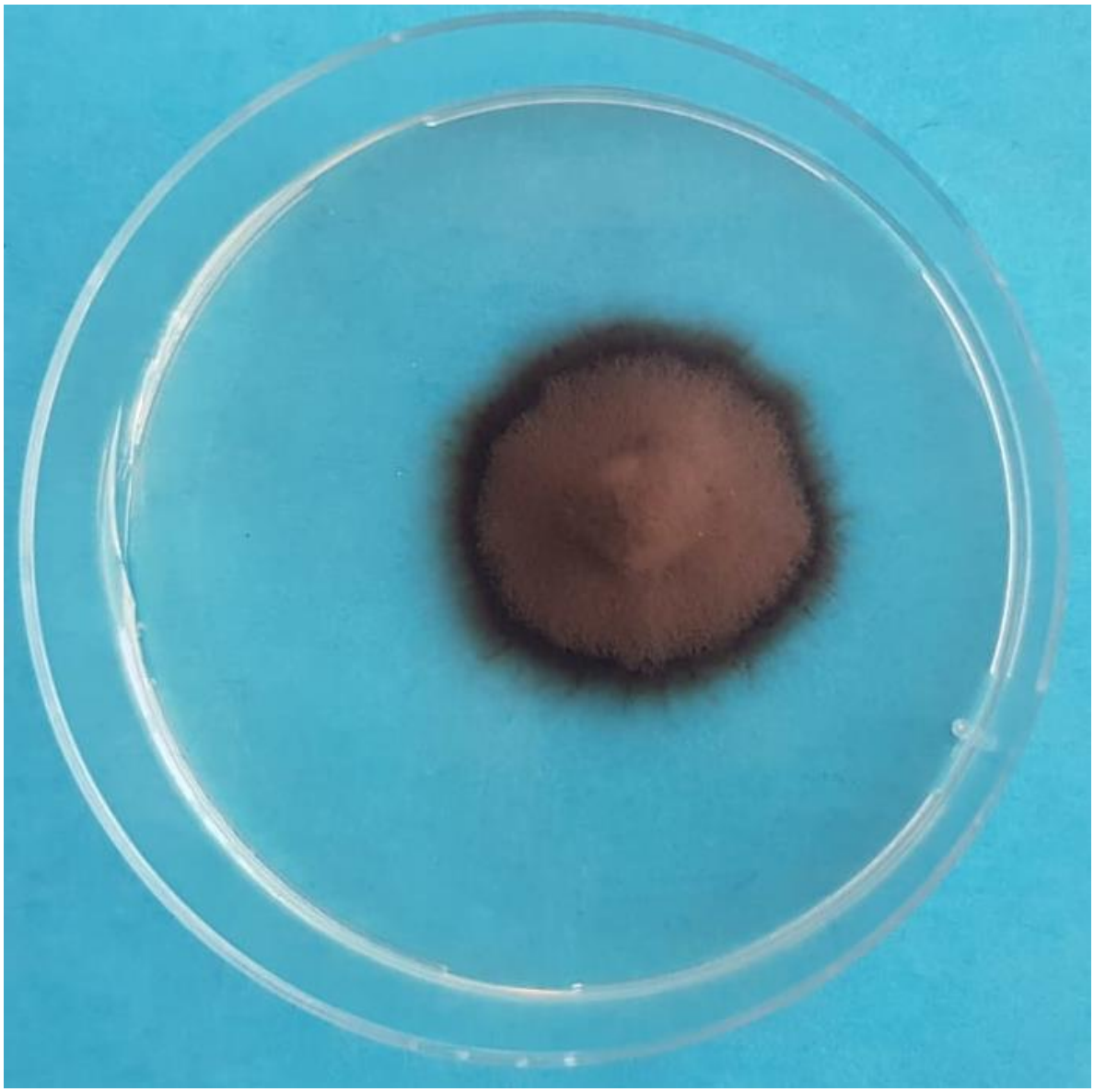

Figure 7: *S. oleagina* mycelia cultures on PDA media after 3 months of incubation at 20˚C.

4.3 Molecular biology

DNA extracted from *S. oleagina* isolates is an initial step towards developing an understanding of genetic diversity of *S. oleagina*. Figure (8) shows the DNA (molecular size > 10,000 kb) isolated from the six fungal samples.

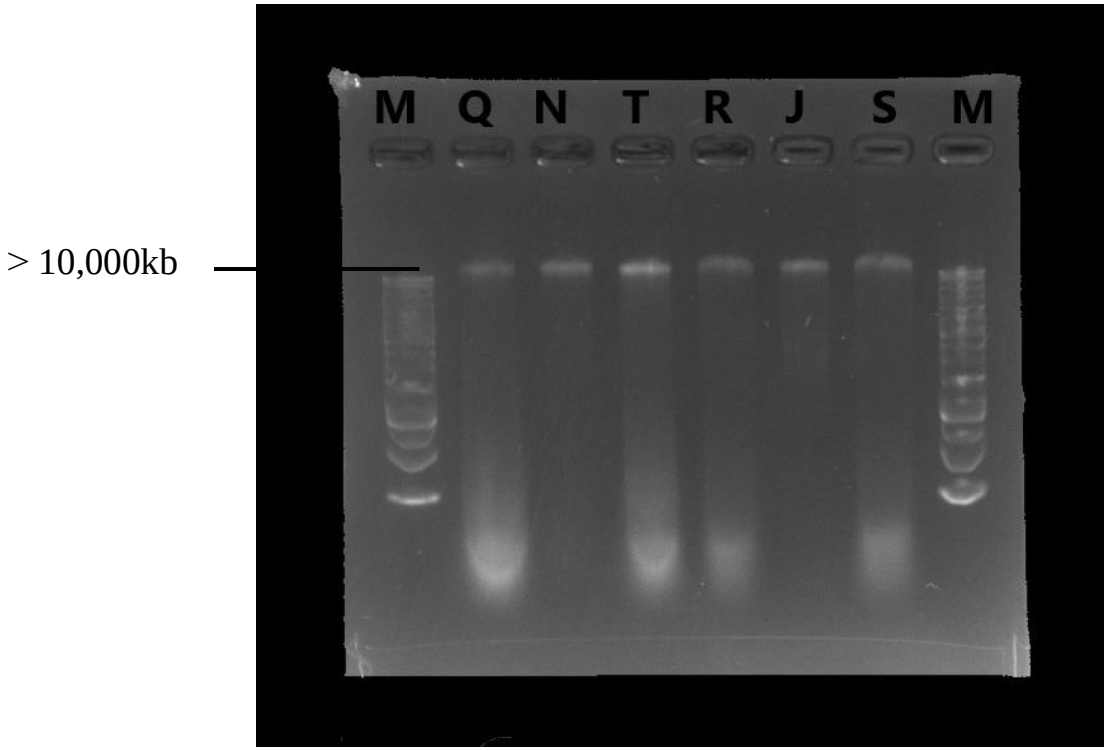

Figure 8: Gel electrophoresis of DNA extracted from *S. oleagina* isolated from different regions. Q; Qalqilia, N; Nablus, T; Tulkarm; R; Ramallah, J; Jenin, and S; Salfit, M: 1kb ladder.

4.3.1 Confirmation of fungal species

Six isolates of the fungus were subjected to PCR for confirmation of the fungal species. The PCR products revealed bands of about 750 bp using the

18SF and 18SR specific primers (Figure 9). BLASTn search of *S. oleagina* isolates from Qalqilia, Nablus, Tulkarm, Ramallah, Jenin and Salfit revealed similarities of 92, 99, 99, 99, 99 and 98% to *S. oleagina* (Accession #. AF338393.1), respectively (Figures 10-15).

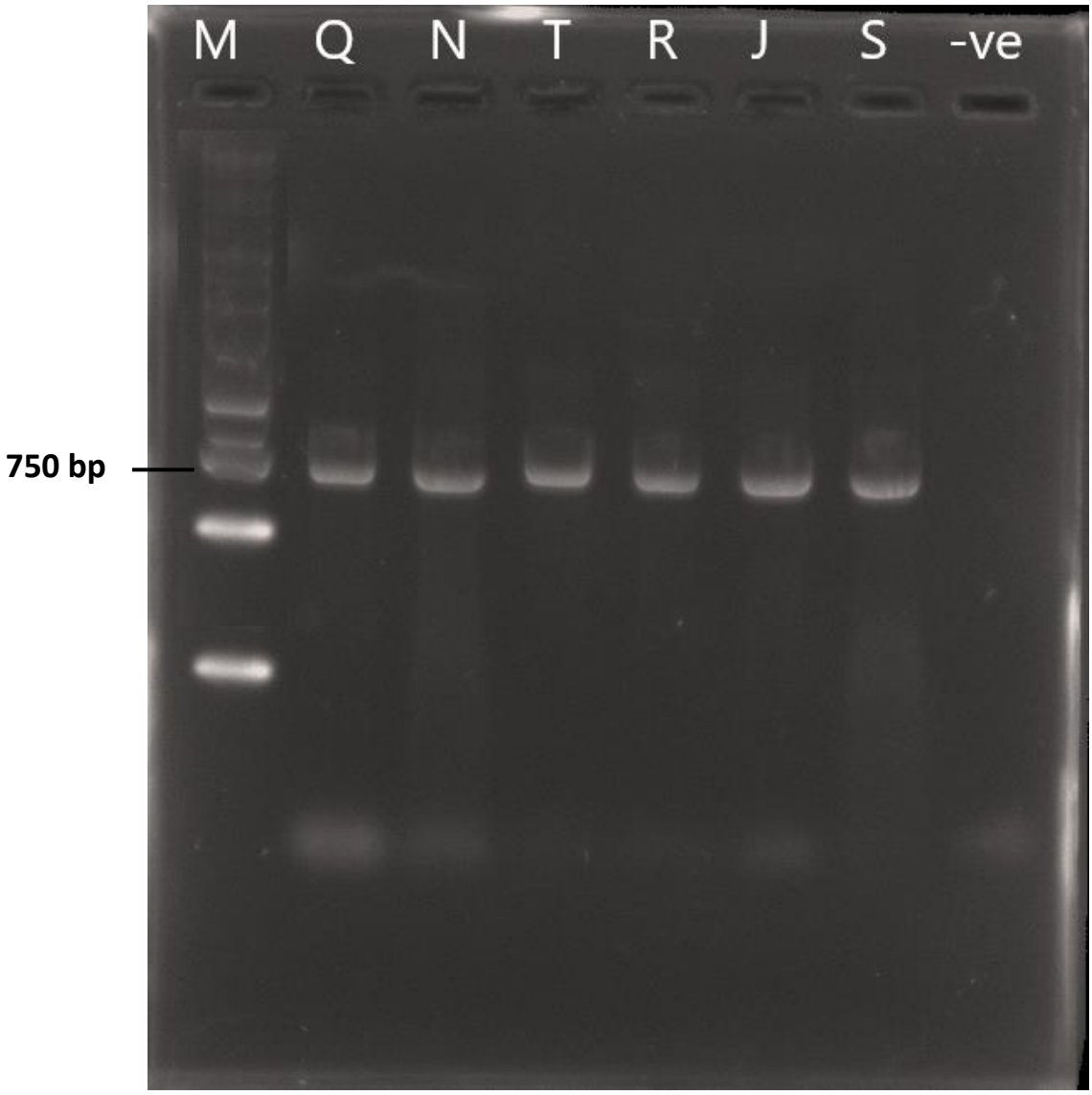

Figure 9: Gel electrophoresis of PCR products from *S. oleagina* isolated from different regions in Palestine: Q; Qalqilia, N; Nablus, T; Tulkarm; R; Ramallah, J; Jenin, and S; Salfit, M: 1kb ladder.

Spilocaea oleaginea 16S ribosomal RNA gene, partial sequence

Sequence ID: AF338393.1 Length: 1738 Number of Matches: 1

Range 1: 45 to 534 GenBank Graphics Next Match Previous M

Score	Expect	Identities	Gaps	Strand
747 bits(404)	0.0	452/491(92%)	2/491(0%)	Plus/Plus

```
Query  29   TATACTGTGCAARCTGCGAATGGCTCATTAMAT-MGTTATCGTTTATTTGATAGTACCTT  87
            ||||||||| || |||||||||||||||||| ||  ||||||||||||||||||||||||
Sbjct  45   TATACTGTG-AAACTGCGAATGGCTCATTAAATCAGTTATCGTTTATTTGATAGTACCTT  103

Query  88   ACTACTTGGATAACCGTGGTAATTCTAGAGCTAATACATGCGAAAAACCCCGACTTCGGA  147
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  104  ACTACTTGGATAACCGTGGTAATTCTAGAGCTAATACATGCGAAAAACCCCGACTTCGGA  163

Query  148  AGGGGTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGCTTCCTGGTGATTCATAAK  207
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  164  AGGGGTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGCTTCCTGGTGATTCATAAT  223

Query  208  AACTTAACGAATCGCATGGCCTTGCGCCGGCRATGGTTCATTCAAATTTCTGCCCTATCA  267
            |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct  224  AACTTAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTGCCCTATCA  283

Query  268  ACTTTCGATTGTMKAGTAGTGGTCTACAMTGGTTTCAACGGGTAACGGGGAATTAGGGTT  327
            |||||||||||  ||||||||||||| ||||||||||||||||||||||||||||||||
Sbjct  284  ACTTTCGATTGTAGAGTAGTGGTCTACAATGGTTTCAACGGGTAACGGGGAATTAGGGTT  343

Query  328  CGACTCCKGASAASGARCCTGAGAAACGGCTACTACATCCAAGGAAGGCAACACGCGCGC  387
            ||||||| || || || ||||||||||||||||||||||||||||||| || ||||||||
Sbjct  344  CGACTCCGGAGAAGGAGCCTGAGAAACGGCTACTACATCCAAGGAAGGCAGCAGGCGCGC  403

Query  388  AAATTACCCAMTCCCGACRCGGGGAGGTAGTGACAAGWMWTACTGATWCWSGGCTCTTTT  447
            |||||||||| ||||||| |||||||||||||||||    ||||||| |  |||||||||
Sbjct  404  AAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATACTGATACAGGGCTCTTTT  463

Query  448  GGGTCTTGTMATTGSAAYGASTACAAWTTAMMTCGCTTAACSARGAACAACTGGAGGAMA  507
            ||||||||| |||| || || ||||| |||  || |||||| | |||||||||||||  |
Sbjct  464  GGGTCTTGTAATTGGAATGAGTACAATTTAAATCCCTTAACGAGGAACAACTGGAGGGCA  523

Query  508  RATCTGGTGCC  518
              |||||||||
Sbjct  524  AGTCTGGTGCC  534
```

Figure 10: BLASTn similarity of the sequence identity of *S. oleagina* isolated from Qalqilia.

Figure 11: BLASTn similarity of the sequence identity of *S. oleagina* isolated from Nablus.

Spilocaea oleaginea 16S ribosomal RNA gene, partial sequence

Sequence ID: AF338393.1 Length: 1738 Number of Matches: 1

Range 1: 37 to 720 GenBank Graphics Next Match Previous Mat

Score	Expect	Identities	Gaps	Strand
1238 bits(670)	0.0	680/685(99%)	1/685(0%)	Plus/Plus

```
Query  18   TARGCAACCTATACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTTATTTGAT  77
            ||  ||||  ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  37   TAAGCAA-CTATACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTTATTTGAT  95

Query  78   AGTACCTTACTACTTGGATAACCGTGGTAATTCTAGAGCTAATACATGCGAAAAACCCCG  137
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  96   AGTACCTTACTACTTGGATAACCGTGGTAATTCTAGAGCTAATACATGCGAAAAACCCCG  155

Query  138  ACTTCGGAAGGGGTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGCTTCCTGGTGA  197
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  156  ACTTCGGAAGGGGTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGCTTCCTGGTGA  215

Query  198  TTCATAATAACTTAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTG  257
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  216  TTCATAATAACTTAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTG  275

Query  258  CCCTATCAACTTTCGATTGTAGAGTAGTGGTCTACAATGGTTTCAACGGGTAACGGGGAA  317
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  276  CCCTATCAACTTTCGATTGTAGAGTAGTGGTCTACAATGGTTTCAACGGGTAACGGGGAA  335

Query  318  TTAGGGTTCGACTCCGGAGAAGGAGCCTGAGAAACGGCTACTACATCCAAGGAAGGCAGC  377
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  336  TTAGGGTTCGACTCCGGAGAAGGAGCCTGAGAAACGGCTACTACATCCAAGGAAGGCAGC  395

Query  378  AGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATACTGATACAGG  437
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  396  AGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATACTGATACAGG  455

Query  438  GCTCTTTTGGGTCTTGTAATTGGAATGAGTACAATTTAAATCCCTTAACGAGGAACAACT  497
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  456  GCTCTTTTGGGTCTTGTAATTGGAATGAGTACAATTTAAATCCCTTAACGAGGAACAACT  515

Query  498  GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAGAAGCGTATATTAAAG  557
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  516  GGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAGAAGCGTATATTAAAG  575

Query  558  TTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTTGGGCCTGGCTGGCCGGTCCGCCTCACC  617
            |||||||||||||||||||||||||||||||||||||||||||||||||||| | |||||
Sbjct  576  TTGTTGCAGTTAAAAAGCTCGTAGTTGAATTTTGGGCCTGGCTGGCCGGTCCGCTTAACC  635

Query  618  GCGTGCACTGGTCCGGCCGGGCCTTTCCTCCTGGGGATCCGCATGCCCTTCACTGGGTGT  677
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  636  CCGTGCACTGGTCCGGCCGGGCCTTTCCTCCTGGGGATCCGCATGCCCTTCACTGGGTGT  695

Query  678  GTTGGGGAACCAGGACTTTTACTTT  702
            |||||||||||||||||||||||||
Sbjct  696  GTTGGGGAACCAGGACTTTTACTTT  720
```

Figure 12: BLASTn similarity of the sequence identity of *S. oleagina* isolated from Tulkarm.

Spilocaea oleaginea 16S ribosomal RNA gene, partial sequence

Sequence ID: AF338393.1 Length: 1738 Number of Matches: 1

Range 1: 44 to 720 GenBank Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Identities	Gaps	Strand
1216 bits(658)	0.0	673/680(99%)	3/680(0%)	Plus/Plus

```
Query  30   CTAWTACTGGTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTTATTTGATAGTACC  89
            ||| ||||  ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  44   CTA-TACT-GTGAAACTGCGAATGGCTCATTAAATCAGTTATCGTTTATTTGATAGTACC  101

Query  90   TTACTACTTGGATAACCGTGGTAATTCTAGAGCTAATACATGCGAAAAACCCCGACTTCG  149
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  102  TTACTACTTGGATAACCGTGGTAATTCTAGAGCTAATACATGCGAAAAACCCCGACTTCG  161

Query  150  GAAGGGGTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGCTTCCTGGTGATTCATA  209
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  162  GAAGGGGTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGCTTCCTGGTGATTCATA  221

Query  210  ATAACTTAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTGCCCTAT  269
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  222  ATAACTTAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTCAAATTTCTGCCCTAT  281

Query  270  CAACTTTCGATTGTAGAGTAGTGGTCTACAATGGTTTCAACGGGTAACGGGGAATTAGGG  329
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  282  CAACTTTCGATTGTAGAGTAGTGGTCTACAATGGTTTCAACGGGTAACGGGGAATTAGGG  341

Query  330  TTCGACTCCGGAGAAGGAGCCTGAGAAACGGCTACTACATCCAAGGAAGGCAGCAGGCGC  389
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  342  TTCGACTCCGGAGAAGGAGCCTGAGAAACGGCTACTACATCCAAGGAAGGCAGCAGGCGC  401

Query  390  GCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATACTGATACAGGGCTCTT  449
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  402  GCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATACTGATACAGGGCTCTT  461

Query  450  TTGGGTCTTGTAATTGGAATGAGTACAATTTAAATCCCTTAACGAGGAACAACTGGAGGG  509
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  462  TTGGGTCTTGTAATTGGAATGAGTACAATTTAAATCCCTTAACGAGGAACAACTGGAGGG  521

Query  510  CAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAGAAGCGTATATTAAAGTTGTTG  569
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  522  CAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAGAAGCGTATATTAAAGTTGTTG  581

Query  570  CAGTTAAAAAGCTCGTAGTTGAATTTTGGGCCTGGCTGGCCGGTCCGCCTCACCGCGTGC  629
            |||||||||||||||||||||||||||||||||||||||||||||||| | ||| |||||
Sbjct  582  CAGTTAAAAAGCTCGTAGTTGAATTTTGGGCCTGGCTGGCCGGTCCGCTTAACCCCGTGC  641

Query  630  ACTGGTCCGGCCGGGCCTTTCCTCCTGGGGATCCGCATGCCCTTCACTGGGTGTGTTGGG  689
            |||||||||||||||||||||||||||||||||||||||||||||||||||||||||| ||
Sbjct  642  ACTGGTCCGGCCGGGCCTTTCCTCCTGGGGATCCGCATGCCCTTCACTGGGTGTGTT-GG  700

Query  690  GGAACCMGGACTTTTACTTT  709
            |||||| ||||||||||||
Sbjct  701  GGAACCAGGACTTTTACTTT  720
```

Figure 13: BLASTn similarity of the sequence identity of *S. oleagina* isolated from Ramallah.

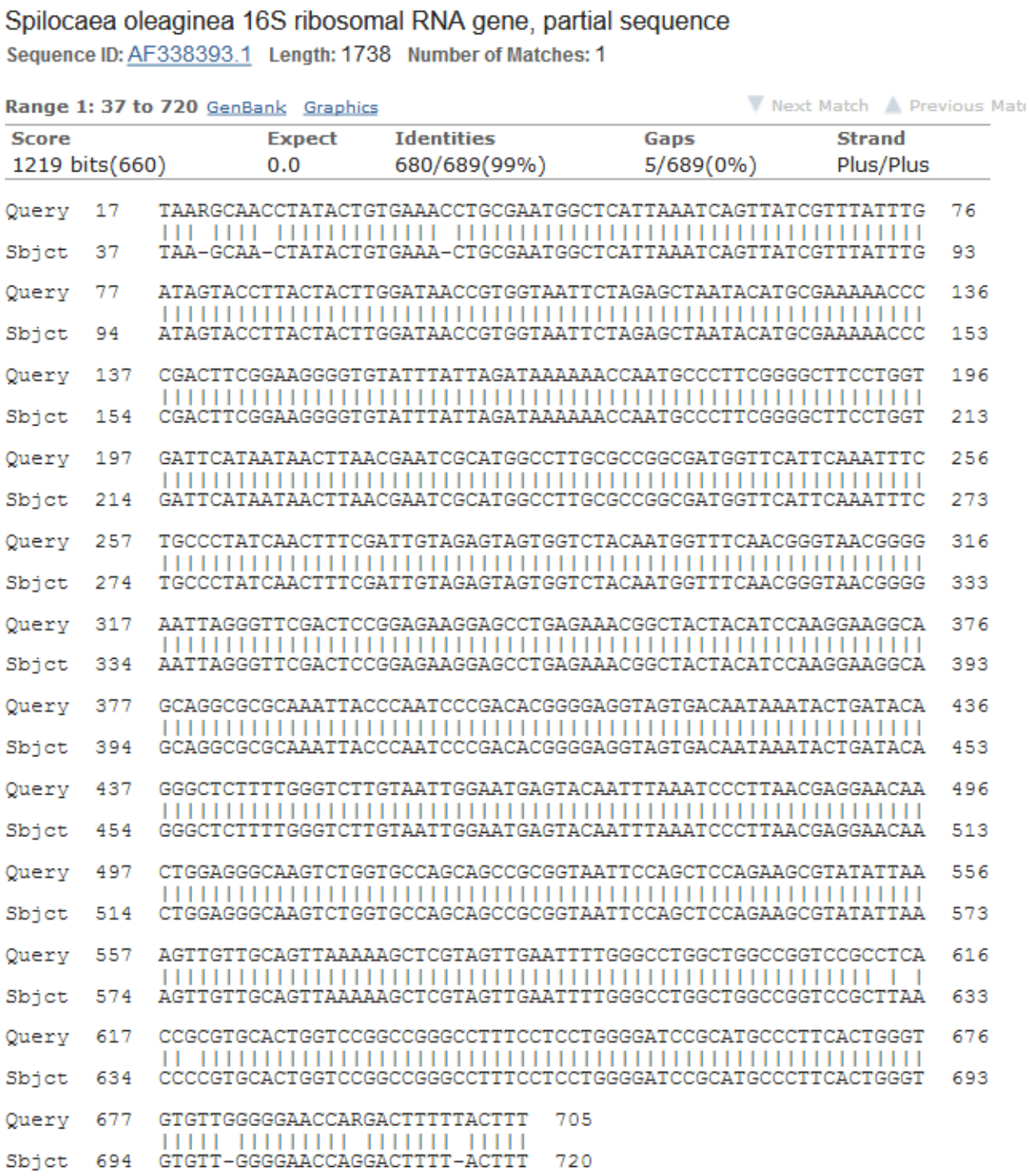

Figure 14: BLASTn similarity of the sequence identity of *S. oleagina* isolated from Jenin.

Spilocaea oleaginea 16S ribosomal RNA gene, partial sequence

Sequence ID: AF338393.1 Length: 1738 Number of Matches: 1

Range 1: 28 to 627 GenBank Graphics Next Match Previous Match

Score	Expect	Identities	Gaps	Strand
1055 bits(571)	0.0	589/602(98%)	3/602(0%)	Plus/Plus

```
Query  7    GTCT-ARTATARGTCAMCTATACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATCGT  65
            |||| | |||| | || ||||||||||||||||||||||||||||||||||||||||||
Sbjct  28   GTCTAAGTATAAG-CAACTATACTGTGAAACTGCGAATGGCTCATTAAATCAGTTATCGT  86

Query  66   TTATTTGATAGTACCTTACTACTTGGATAACCGTGGTAATTCTAGAGCTAATACATGCGA  125
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  87   TTATTTGATAGTACCTTACTACTTGGATAACCGTGGTAATTCTAGAGCTAATACATGCGA  146

Query  126  AAAACCCCGACTTCGGAAGGGGTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGCT  185
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  147  AAAACCCCGACTTCGGAAGGGGTGTATTTATTAGATAAAAAACCAATGCCCTTCGGGGCT  206

Query  186  TCCTGGTGATTCATAATAACTTAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTC  245
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  207  TCCTGGTGATTCATAATAACTTAACGAATCGCATGGCCTTGCGCCGGCGATGGTTCATTC  266

Query  246  AAATTTCTGCCCTATCAACTTTCGATTGTAGAGTAGTGGTCTACAATGGTTTCAACGGGT  305
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  267  AAATTTCTGCCCTATCAACTTTCGATTGTAGAGTAGTGGTCTACAATGGTTTCAACGGGT  326

Query  306  AACGGGGAATTAGGGTTCGACTCCGGAGAAGGAGCCTGAGAAACGGCTACTACATCCAAG  365
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  327  AACGGGGAATTAGGGTTCGACTCCGGAGAAGGAGCCTGAGAAACGGCTACTACATCCAAG  386

Query  366  GAAGGCARCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATAC  425
            ||||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  387  GAAGGCAGCAGGCGCGCAAATTACCCAATCCCGACACGGGGAGGTAGTGACAATAAATAC  446

Query  426  TGATACAGGGCTCTTTTGGGTCTTGTAATTGGAATGAGTACAATTTAAATCCCTTAACGA  485
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  447  TGATACAGGGCTCTTTTGGGTCTTGTAATTGGAATGAGTACAATTTAAATCCCTTAACGA  506

Query  486  GGAACAACTGGAGGRCAARTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAGAAGCGT  545
            ||||||||||||||| ||| ||||||||||||||||||||||||||||||||||||||||
Sbjct  507  GGAACAACTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAGAAGCGT  566

Query  546  ATATTAAAGTTGTTGCASTTAWWAAAGCTCGTAGTTGAATTTTGGGCCTGGCTGKYCGGT  605
            |||||||||||||||| |||  ||||||||||||||||||||||||||||||  ||||
Sbjct  567  ATATTAAAGTTGTTGCAGTTAA-AAAGCTCGTAGTTGAATTTTGGGCCTGGCTGGCCGGT  625

Query  606  CC  607
            ||
Sbjct  626  CC  627
```

Figure 15: BLASTn similarity of the sequence identity of *S. oleagina* isolated from Salfit.

4.3.2 Genetic diversity of the fungus

Discrimination between different isolates of *S. oleagina* was done using Box PCR. The distinct banding pattern (Figure 16) revealed differences after gel electrophoresis of the Box PCR product.

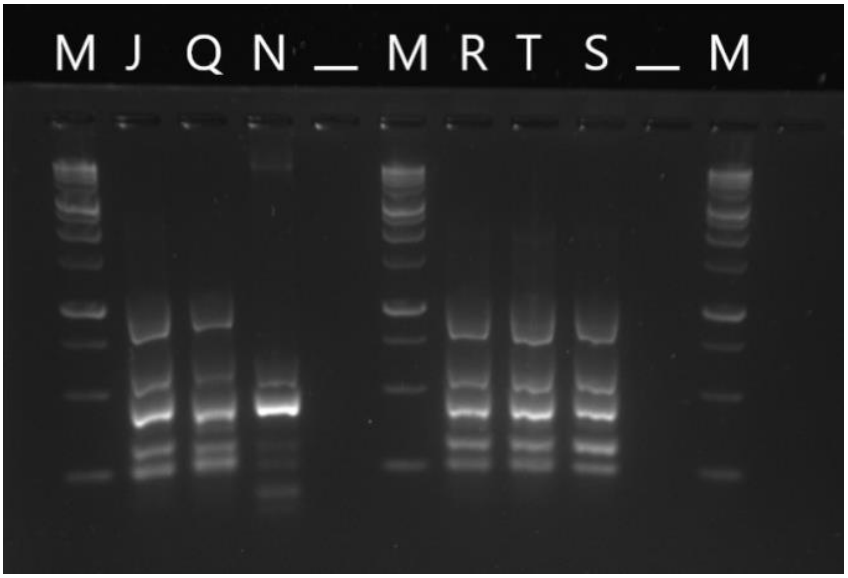

Figure 16: Separation of Box PCR products of six *S. oleagina* isolates. (M: 1Kb ladder. Lanes J, Q, N, R, T, and S are Jenin, Qalqilia, Nablus, Ramallah, Tulkarm, and Salfit, respectively).

4.3.3 Genetic diversity of *S. oleagina*

BOX-PCR amplification of *S. oleagina* isolates generated some distinct fingerprint patterns. Cluster analysis divided the fingerprints of the isolates into three groups (Figure 17). Group two (3 isolates) was the largest cluster.

The remaining groups, in decreasing number of isolates, were group 1 (2 isolates) and group 3 (1 isolate).

All three groups contained fungi isolated from host genotypes originally from different geographic regions. In group one *S. oleagina* samples were isolated from olive trees originating from Ramallah and Nablus, in group two, the samples were isolated from Salfit, Tulkarm and Qalqilia and in group 3; the samples were isolated from Jenin.

Based on analyses of the dendrogram obtained after Box PCR; Nablus and Ramallah are 64-69% identical; Qalqilia, Salfit and Tulkarm are 64-71%identical; Qalqilia, Salfit and Tulkarm with Nablus and Ramallah are 59-64%identical;Jenin and all share less than 59% identity.

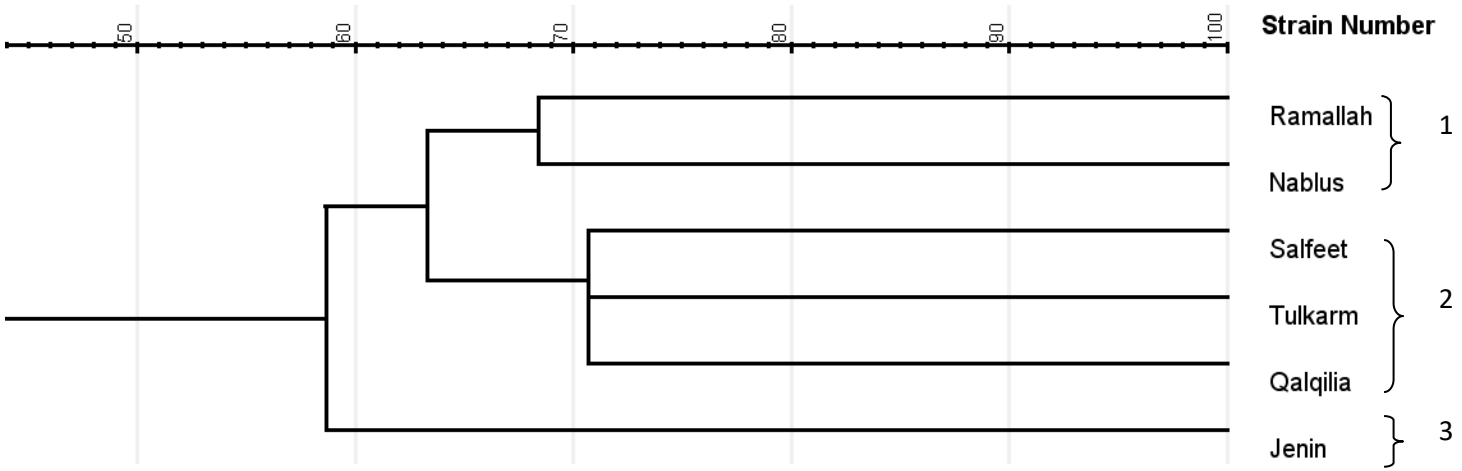

Figure 17: Phylogenetic tree indicating the relatedness among *S.oleagina* isolates based on Box-PCR analysis.

4.4Effect of plant extract on conidial germination

The result showed that inhibition of *S. oleagina* conidial germination and germ tube elongation was obvious in the presence of the different concentrations of the LE (Figure 18). In the control experiment, the length of the germ tube exceeded on average two times the length of the conidia. At 10%extractconcentration, no germination of the spore was recorded in all samples collected from the different regions.

Statistical analysis revealed that in the presence of 5%LEconcentration, the percent of conidial germination (2.75%) was significantly ($P < 0.05$) lower than that in the other concentrations. Moreover, germination rates did not differ significantly between the different concentrations 1.0, 1.5, 2.0, 2.5 and 5% (Figure 19). With increasing LE concentrations, the germination rates decreased significantly. The rates of germination were 49.5, 35.6, 25.57, 16.0 and 8.3% at 0.5, 1.0, 1.5, 2.0 and 2.5%extract, respectively.

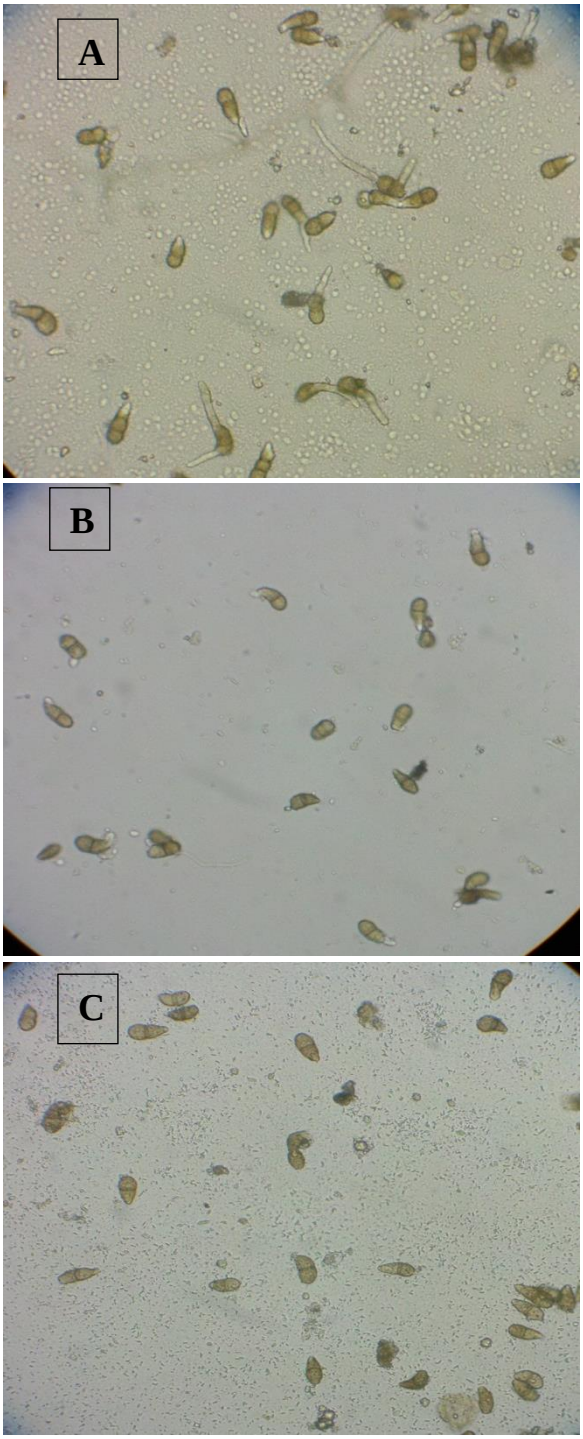

Figure 18: Effect of *A. artemisiifolia* on germination and germ tube length of *S. oleagina* after 24 h of incubation at 20°C, (A) control un treated spores, (B) spores treated with 2.5% extract and (C) spores treated with 10% extract.

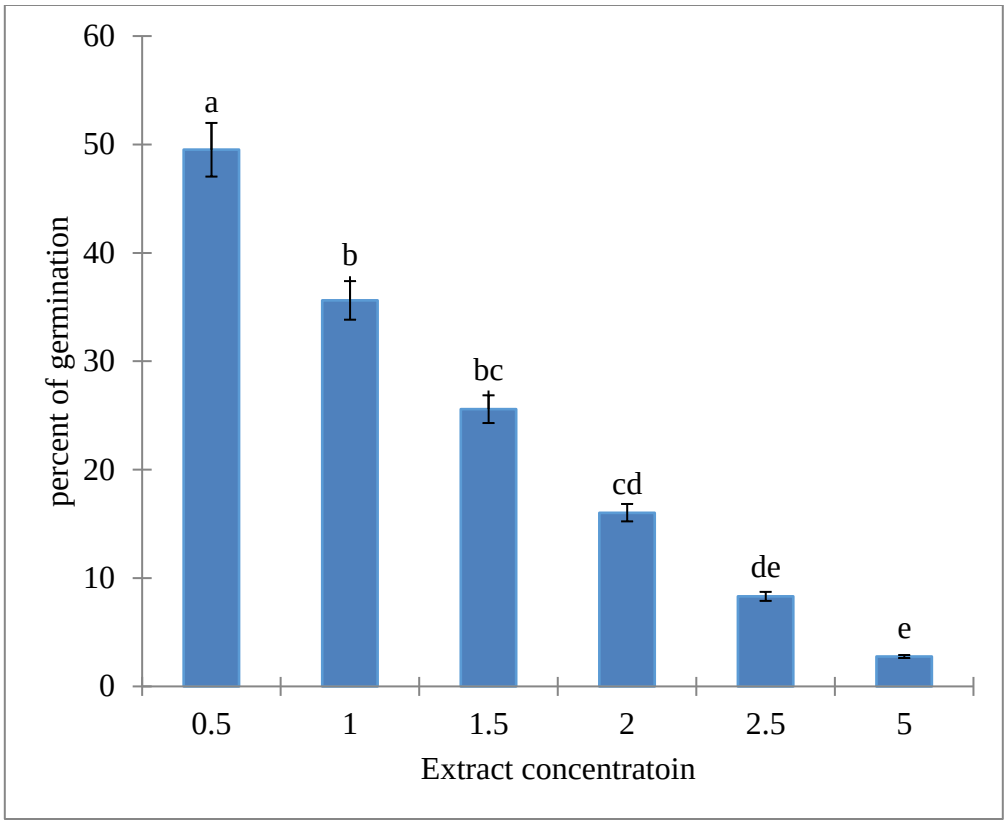

Figure 19: Effect of plant extract on conidial germination. Data with different letters are significantly different after Turkey's HSD test using ANOVA at $p<0.05$

4.5 Integrated control of conidial germination

As shown in figure (20), the germination rate using the fungicide treatment alone (0.439%) was significantly the lowest among that in the other treatments. The results also showed that the combination of LE and fungicides at1:1 (v/v) was the most effective combined treatment against conidial germination with a germination rate of 5.36% which was significantly lower than that in the other combinations.

At combination of 1:0.1 the percent of germination (16.93%) was significantly higher than that in the other combinations. Combined treatments at ratios 1:0.25 and 1:0.5 (v/v) also differ significantly from other treatments with germination rates (12.977 and 10.22 %, respectively).

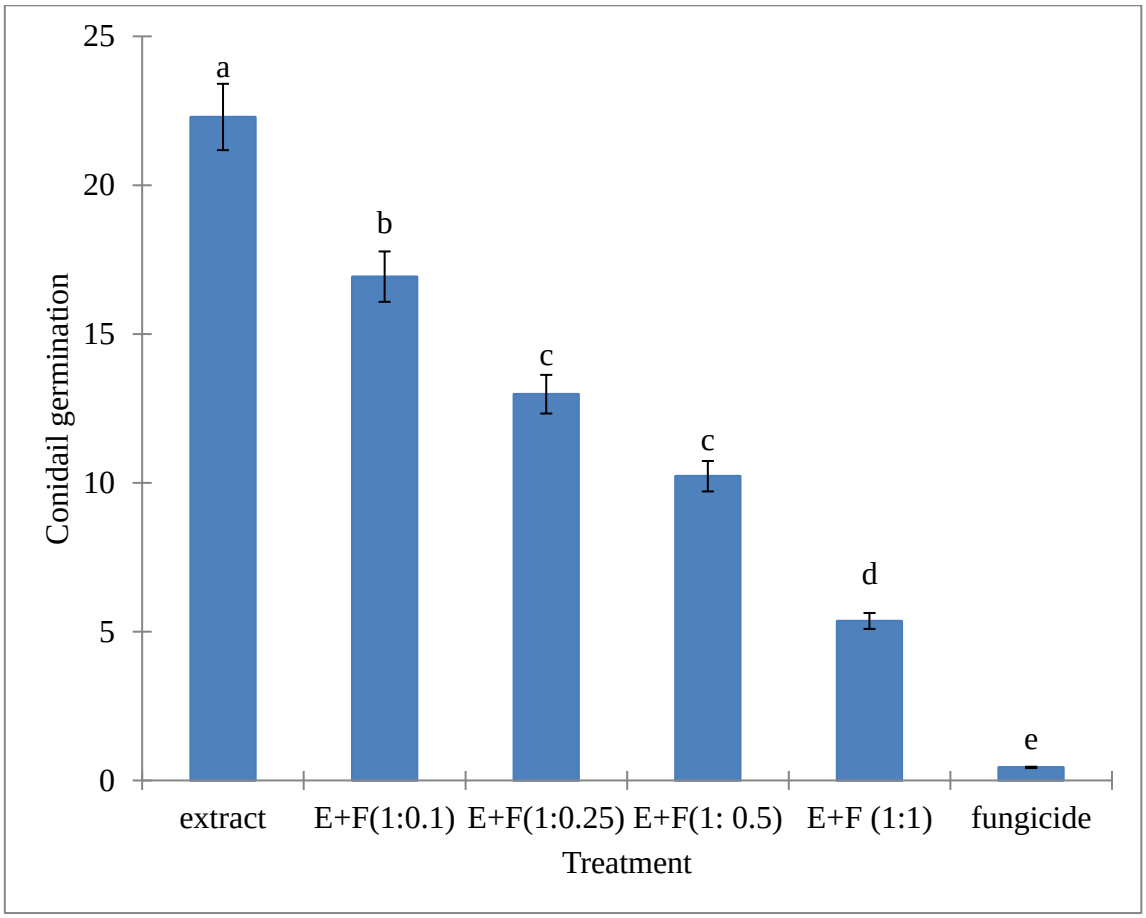

Figure 20: Percent of conidial germination in different treatments, E; extract and F; fungicide

CHAPTER FIVE: DISCUSSION

Discussion

The olive leaf spot is wide spread in Palestine (Salman *et al.,* 2011; Abuamsha *et al.,* 2013; Hajjeh *et al.*, 2014). Five regions (Qalqilia, Tulkarm, Nablus, Jenin and Salfit) were considered as highly affected by the disease (Salman *et al.*, 2011). The disease severity and index in these regions were higher than in other regions in Palestine (Salman *et al.*, 2011). This work showed that the germination of the spores of the fungus collected from these regions was highly variable. Despite the high viability of the spores, variations in germination rates were recorded as mentioned above in table (4). The samples were collected during March when the conditions (RH, T) are optimum for disease development. Salman *et al.,* (2011) showed that the latent infection and severity were maximum during the same month of the year. The differences in germination rate might be attributed to the microclimatic conditions that exist in these regions (Hajjeh *et al.,* 2014).Several studies proved that the fungus optimum growth conditions include high RH (> 85%) low temperature (15-21˚C) (Graniti, 1993; Guechi & Girre, 1994; Obanor *et al.*, 2008a).

In this study we found genetic variation among *S.oleagina* different isolates. Based on the clustering analysis after Box PCR, the isolates were grouped into three groups according to geographical regions. The differences could

also be due to the selection of pathogen genotypes by the environmental conditions and disease management in the different regions.

In this work, each group consists of regions that are similar in climatic conditions. For example, the first group (Nablus and Ramallah) are closely related compared to the second group (Tulkarm, Qalqilia and Salfit). Another explanation of the variation in pathogen populations might be generated by mutation, migration and recombination (sexual or asexual) (Obanor *et al.*, 2010). Based on our results it is possible that, these forces have not acted sufficiently produce genotypic variation among the same group of the Palestinian isolates. However, it might act to produce variation between the different groups of the isolates.

The low genetic diversity might result from asexual mode of reproduction that predominates among *S. oleagina* (Graniti, 1993).

The nucleotide sequences of the 16s region of rDNA were obtained from six *S. oleagina* isolates; except for the fungal isolate from Qalqilia, the comparative analysis of these sequences showed a high degree of similarity.

This study showed that the olive leaf spot disease could be controlled using the LE from *A. artemisiifolia*. Based on in vitro results, the different extract concentrations inhibited *S.oleagina* conidial germination and germ tube elongation. In this study, seven concentrations of the extract tested markedly

reduced the germination of *S. oleagina* conidia. The germ tube length of conidia exposed to extract was less than that of control conidia, indicating that the extract have an inhibitory effect on germination and elongation of the germ tube. The germination rate of the spores was reduced significantly with increasing plant extract because the effective material is increased.

All concentrations of the extract reduced percent of germinated conidia compared to the untreated control. The 2.5 and 5% concentrations were considered the most effective in preventing conidial germination and decreased significantly the mean percent of germination.

Application of copper-based fungicides is currently the main method of olive leaf spot control in many olive growing regions of the world (Obanor *et al.*, 2008; Sistani *et al.*, 2009). However, due to the negative effect of the fungicides on the human and the environment, the use of plant leaf extract in combination with chemical fungicides might be a realistic approach to decrease the amount of fungicides being used in controlling the disease.

Using a mixture of plant extract and fungicide against growth of the fungal conidia resulted in significant reduction in conidial germination. Although treatments of fungicide and extract reduced percentage of germinated conidia; the 1:1 combination of extract and fungicides was the most effective treatment against conidial germination (5.36%germination rate).

Several studies using natural products have demonstrated the possibility of their use to control pests and diseases. Researchers have identified other natural products (chitosan, β-aminobutyric acid, glucosinolates, propolis, fusapyrone, ethephon, microbial products, and plants extracts) that induce resistance against fungal pathogens. These products are used around the world to enhance quality and yields in agriculture setups (Tripathi & Dubey, 2004; Thakur &Sohal, 2013).

Copper fungicides are the chemicals most commonly used to control OLS in New Zealand olive groves and have also been showed to be effective in controlling OLS in California olive groves (Teviotdale *et al.,* 1989). Other chemical treatments including mixtures of difenoconazole (Score 25 EC) and mineral oil (Texaco Spraytex CT774) have been reported to reduce infection (Sistani *et al.,* 2009).

Obanor *et al.,* (2008) reported copper sulphate and a mixture of kresoxim-methyl and copper hydroxide were the most effective, reducing disease incidence by 85-96% and 63-93%, respectively.

Several alternative techniques have been applied to control OLS disease in recent years; Genetic resistance represents an effective, economically feasible

and ecologically sustainable mean to control the peacock disease (Zine El Aabidine *et al.,* 2010; Sanei & Razavi, 2011; Rhouma *et al.,* 2012).

A natural product of vegetable oil of *Brassica carinata* was used for the control of OLS, the vegetable oil reduced conidial germination 47, 20% compared with untreated experimental controls 56% (Rongai *et al.,* 2012).

A clear variation in the sensitivity against the fungus was revealed when 14 local and imported olive cultivars were subjected to 12 *S.oleagina* strains under artificial infection in the glass house (AL-Chaabi *et al.,* 2012).

However, the level of susceptibility of olives to OLS is widely variable (Graniti, 1993; Sutter, 1994). Several varieties (e.g. Leccino and Valatolina) are resistant while other varieties such as Arbequine, Frantoïo, Barouni and Nabali are sensitive (Sutter, 1994; Macdonald *et al.,* 2000; Sanei & Razavi, 2011). Experiments carried out by Vidotto *et al.,* (2012) showed that *A. artemisiifolia* inhibits the germination and growth of tomato (*Solanum lycopersicum*) by more than 50%.The same authors showed also a reduction in growth of *Brassica spp.*

In treatments with ragweed on oat (*Avena sativa* L.); the inhibition on the shoot development was higher than 80% according to Novak *et al.,*(2018). In addition to that, the shoot length of sunflower (*Helianthus annuus* L) was reduced by 43.09%after treatment with ragweed (Novak *et al.,* 2018).

Bruckner (1998) and Hodisan *et al.,* (2009) reported a significant reduction in the germination percentage of test-species in treatment with water extract of ragweed.

Choi *et al.,* (2010) determined that the inhibition of germination in three different test-species in treatments with leaf extract from ragweed was 68.3, 74.6 and 87.3%. The difference in the effect on germination in the reported study can be attributed to the different concentrations of extracts as well as different plant parts used for preparation of extracts.

Novak *et al.,* (2018) showed that ragweed was the species with the significantly stronger allelopathic effect compared to common cocklebur. Results recorded for ragweed by Novak *et al.,* (2018) are consistent with those reported by Bruckner (1998), Csiszár *et al.,* (2013) and Vidotto *et al.,* (2013).

This study is the first of its kind that used LE from *A. artemisiifolia* to control Olive Leaf Spot caused by *S. oleagina* in Mediterranean region. The data obtained led us to conclude that extract can be used to control *S. oleagina*. In addition to that, we determined that 2.5 and 5% were the best concentration suitable to obtain an effective inhibition of conidial germination of the olive leaf spot disease.

CHAPTER SIX: CONCLUSION AND RECOMENDATIONS

6.1 Conclusions

This study is the first of its kind to use leaf extract from *A.artemisiifolia* to control the olive leaf spot disease caused by *S.oleagina*. The aqueous extract has a high inhibitory effect on spore germination of the fungus under in vitro conditions. This study opens the way for development of bioactive natural products, with the added benefits of an environmentally safe and economically viable product.

Considering the extract; it can be viewed as the best choice for the control of the disease in Palestine. Further studies are needed to test the efficacy of this extract under field conditions for a better control planning of peacock disease in Palestine and to modify and develop its active ingredients.

This study also enabled us to obtain the best ratio between the extract and the fungicide (1:1, v/v), which reduces the effect of negative fungicide in the ecosystem.

Also, this study showed that different *S.oleagina* isolates had level of genetic diversity according to geographical regions, and this probably because asexual reproduction is the major method by which this fungus spreads its populations.

6.2 Recommendations

1. Avoid the use of fungicides alone because of their multiple negative effects.

2. Spray infected trees with the best combination of plant extract and fungicide (1:1) at the beginning of the rainy season in autumn before flowering.

3. Repeating spraying infected olive trees with plant extract several years to eradicate the disease.

4. Proper pruning and removal of weak branches annually for ventilation and entering the sun to reduce the spread of the disease.

5. Educating farmers about the harmful effects of fungicides and advising them to replace it with biocontrol means.

References

Abuamsha, R., M. Abueid, H. Hajjeh & M. Salman.(2013).Evaluation of the incidence and severity of olive leaf spot caused by *Spilocaea oleagina* in different olive cultivars in Palestine. Journal of Agriculture and Environment for International Development 107 (2): 201-212.

Al-Chaabi, S., L. Matrod, Y. Kutaefani, M. H. Safeih, J. Asmar, F. Alkaiem, S. Mohammed &R. Ali. (2012). Incidence of Peacock Spot Disease on Olive Trees in the Coastal Hills of Syria and Evaluation of Resistance among Local and Imported Olive Cultivars. Arab Journal of Plant Protection 30(1): 110-127.

Al-Khatib, M., K. Alhussaen, N. El-Banna&M. Zyadeh. (2010).Biological control of olive leaf spot (peacock spot disease) caused by *Cycloconium oleaginum* (*Spilocaea oleagina*). Journal of Microbiology and Antimicrobials 2: 64-67.

Azeri, T. (1993).Research on olive leaf spot, olive knot and Verticillium wilt of olive in Turkey. EPPO Bulletin 23: 437-440.

Bassett, I.J. & C.W. Crompton.(1975). The biology of Canadian weeds. 11. *Ambrosia artemisiifolia* L. and *A. psilostachya* DC. Canadian Journal of Plant Science 55(2):463-476.

Benkada, M.M., F. Belhoucine, N. Benhamed & A. Berrebbah.(2015).Isolation of monosporal strains of *Fusicladium oleagineum*, the fungal causal agent of olive leaf spot from North-Western Algerian groves. International Symposium of Applied Biology CIBA (1) 6: 64-6.

Beres, I. (1994). New investigations on the biology of *Ambrosia artemisiifolia* L. 46thInternational Symposium on Crop Protection 59:1295-1297.

Besnard, G., P. Baradat, C. Breton, B. Khadari & A. Berville.(2001).Olive domestication from structure of oleasters and cultivars using nuclear RAPDs and mitochondrial RFLPs. Genetics Selection Evolution. 33: 251-268.

Blakeman, J.P. & N.J. Fokkema.(1982).Potential for Biological Control of Plant Diseases on the Phylloplane. Annual Review of Phytopathology20(1):167-190.

Bruckner, D.J., A. Lepossa & Z.Herpai.(2001). Ragweed allelopathy: indirect interactions. Novenytermeles 50(2-3):231-236.

Bruckner, D.J. (1998).The allelopathic effect of ragweed (*Ambrosia artemisiifolia* L.) on the germination of cultivated plants. Növénytermeles, 47 (6): 635-644.

Carisse, O., A. Ouimet, V. Toussaint & V. Philion. (2000). Evaluation of the effect of seed treatments, bactericides, and cultivars on bacterial leaf spot of lettuce caused by *Xanthomona scampestri* pv. vitians. Plant Disease 84: 295-299.

Choi, B.S., D.Y. Song, C.G. Kim, B.H. Song, S.H. Woo & C.W. Lee.(2010).Allelopathic effects of common ragweed (*Ambrosia artemisiifolia* var. elatior) on the germination and seedling growth of crops and weeds. Korean Journal of Weed Science30 (1): 34-42.

Coats, J.R., C.J. Peterson, R. Tsao, A.L. Eggler & G.L. Tylka.(2003). Compound related to natural sources and their use as bio-pesticides. Patent no. US6, 545,043 B1.

Csiszár,Á., M. Korda, D. Schmidt, D. Šporčić, B. Teleki, V.Tiborcz, G. Zagyvai & D.Bartha.(2013) Allelopathic potential of some invasive plant species occurring in Hungary. Allelopathy Journal :31 (2): 309-318.

Eloff, J.N. & L.J. McGaw.(2014).Using African plant biodiversity to combat microbial infections. InGurib-Fakim A (Ed), Novel Plant Bio-resources: Applications in Food Medicine and Cosmetics.John Wiley.Pp 163-173.

Ferguson, L., G.S. Sibbett & G.C. Martin.(1994). Olive Production Manual. University of California, Division of Agriculture and Natural Resources: Publication pp33-53.

Fumanal, B., B. Chauvel & F. Bretagnolle. (2007).Estimation of pollen and seed production of common ragweed in France. Annals of Agricultural and Environmental Medicine 14: 233-236.

González-Lamothe, R., R. Segura, A. Trapero, L. Baldoni, M.A. Botella & V. Valpuesta.(2002).Phylogeny of the fungus *Spilocaea oleagina*, the causal agent of peacock leaf spot in olive. FEMS Microbiology Letters 210: 149-155.

Graniti, A. (1993). Olive scab: a review. EPPO Bulletin 23: 377-384.

Guechi, A. & L. Girre. (1994). Sources of *Cycloconium oleaginum* (Cast.) conidia for infection of olive leaves and conditions determining leaf spot disease development in the region of Se´tif, Algeria. Mycopathologia 125: 163-71.

Hajjeh, H., M. Salman, R. Abuamsha, M. Abueid, M. Jawabreh, A. Hawamda & B. Abu Rumaileh. (2014). Latent infection of olive leaf spot disease on Palestinian olives. Annual Research and Review in Biology 4: 2517-2524.

Haniotakis, G.E. (2005). Olive pest control: present status and prospects. In: Proceedings of the IOBC/WPRS.IOBC/wprs Bull. 28(9): 1-9.

Hodisan, N., G., Morar &C. M. Neag.(2009). Research on the allelopathic effect between the invasive species *Ambrosia artemisiifolia* L. ("FloareaPustei") and some agricultural crops. Bulletin of the University of Agricultural Sciences and Veterinary 66 (1): 554-563.

Huang, J.W., J. Chen, W.R., Berti& S.D. Cunningham. (1997). Phytoremediation of lead-contaminated coils: role of synthetic chelates in lead phytoextraction. Environmental Science and Technology 31:800-805.

Hwang, S.F., P. Chakravarty & D.Prevost. (1993). Effects of rhizobia, metalaxyl, and VA mycorrhizal fungi on growth, nitrogen fixation, and development of Pythium root rot of Sainfoin. Plant Disease 77: 1093-1098.

International Olive Council, (2016). http:// www.internationaloliveoil.org (accessed December 2016).

Jabi, F. (2007).The Olive Tree.Zain Press, Nablus.

Kazinczi, G., I. Béres, Z. Pathy & R.Novák. (2008) Common ragweed (*Ambrosia artemisiifolia* L.): a review with special regards to the results in Hungary: II. Importance and harmful effect, allergy, habitat, allelopathyand beneficial characteristics. Herbologia 9: 93-118.

Khadari, B., N. Moutier & F. Dosba.(2001). Molecular characterization of French Olive varieties: construction of a database of reference genotypes, 87.

Koeuth, T., Versalovic, J., Lupski, J.R(1995).Differential subsequence conservation: of interspersed repetitive Streptococcus pneumoniae BOX elements in diverse bacteria. Genome Res5:408–18.

Lambdon, P. W., P. Pyšek, C. Basnou, M. Hejda, M. Arianoutsou, F. Essl, V. Jarošík, J. Pergl, M.Winter, P.Anastasiu, P. Andriopoulos., I.Bazos, G.Brundu, L.Celesti-Grapow, P.Chassot, P.Delipetrou, M.Josefsson, S.Kark, S.Klotz, Y.Kokkoris, I.Kuehn, H.Marchante, I.Perglová, J.Pino, M.Vila, A.Zikos, D. Roy & P.E.Hulme. (2008).Alien flora of Europe: species diversity, temporal trends, geographical patterns and research needs. Preslia 80: 101-149.

Li, S., G. Dongchang & G. Guangqing.(1989). A study on the phenology of common ragweed and great ragweed. Journal of Shenyang Agricultural University 20(3):344-250.

Lops, F., S. Frisullo & V. Rossi.(1993). Studies on spread of the olive scab pathogen, *Spilocaea oleagina*. EPPO Bulletin 23: 385-387.

MacDonald, A.J., M. Walter, M. Trought, C.M. Frampton& G. Burnip.(2000). Survey of olive leaf spot in New Zealand. New Zealand Plant Protection53: 126-132.

Mao, W., J.A.Lewis, P.K. Hebber & R.D. Lumsden.(1997). Seed treatment with a fungal or a bacterial antagonist for reducing corn damping-off caused by species of Pythium and Fusarium. Plant Disease 81: 450-454.

Miller, H.N. (1949)**.**Development of the leaf spot fungus in the olive leaf. Phytopathology 39: 403–410.

Novak, N., M.Novak, K.Baric, M.Ščepanovic & D.Ivić. (2018). Allelopathic potential of segetal and ruderal invasive alien plants. Journal of Central European Agriculture19 (2):.408-422.

Obanor, F.O., M. Walter, E.E.J ones, J. Candy & M.V. Jaspers.(2010). Genetic variation in *Spilocaea oleagina* population from New Zealand olive groves. Australasian Plant Pathology 39: 508-516.

Obanor, F.O., M. Walter, E.E. Jones& M.V. Jaspers.(2011).Effects of temperature, inoculum concentration, leaf age, and continuous and interrupted wetness on infection of olive plants by *Spilocaea oleagina*. Plant Pathology 60: 190-199.

Obanor, F.O., M.V. Jaspers, E.E. Jones &M. Walter.(2008).Greenhouse and field evaluation of fungicides for control of olive leaf spot in New Zealand. Crop Protection 27: 1335-1342.

Peralbo-Molina, Á. &M.D.de Castro.(2013).Potential of residues from the Mediterranean agriculture and agrifood industry. Trends in Food Science and Technology 32:16-24.

Raut, J.S. & S.M. Karuppayil.(2014). A status review on the medicinal properties of essential oils. Industrial Crops and Products 62: 250-264.

Reinhardt, F., M. Herle, F. Bastiansen, & B.Streit.(2003).Economic impact of the spread of alien species in Germany. Federal Environmental Agency, Research Report: 201 86 211 UBA-FB 000441e. Germany: Federal Environmental Agency.

Rhouma, A., M. Chettaoui, S. Krid, H. Elbsir, M.Msallem& M.A. Trik.(2012). Evaluation of susceptibility of an olive progeny (Picholine × Meski) to olive leaf spot disease caused by *Fusicladium oleagineum*. European Journal of Plant Pathology 135: 23-33.

Ribera, A.E. &G. Zuniga.(2012).Induced plant secondary metabolites for phytopathogenic fungi control: a review. Journal of Soil Science and Plant Nutrition 12 (4): 893-911.

Rongai, D., C. Basti& C. Di Marco.(2012). A natural product for the control of olive leaf spot caused by *Fusicladium oleagineum* (Cast.)Ritscheland Braun. Phytopathologia Mediterranean 51(2): 276-282.

Salman, M., M.Jawabreh & B.Abu Rumaileh. (2014).The effect of local fungicides on conidial germination of *Spilocaea oleagina* in Palestine. PTUKRJ 2(1):26-28.

Salman, M., A.Hawamda, A.A.Amarni, M.Rahil, H.Hajjeh, B.Natsheh& R.Abuamsha.(2011). Evaluation of the incidence and severity of olive leaf spot caused by *Spilocaea oleagina on* olive trees in Palestine. American Journal of Plant Sciences 2: 457-460.

Sanei, S.J. & S.E.Razavi. (2011). Survey of *Spilocaea oleagina*, causal agent of olive leaf spot, in North of Iran. Journal of Yeast and Fungal Research 2: 33-38.

Sergeeva, V., N.G.Nair& R.Spooner-Hart.(2008).First report of flower infection in olives (*Olea europaea*) by *Colletotrichum acutatum* and *C. gloeosporioides* causing anthracnose disease. Australasian Plant Disease Notes 3: 81-82.

Sistani, F., S.S. Ramezanpour & S. Nasrollanejad.(2009). Field Evaluation of different fungicides application to control olive leaf spot. Australian Journal of Basic and Applied Sciences 3: 3341-3345.

Smith,M., L.Cecchi, C. A.Skjøth, G.S. Karrer & B.˘ikoparija. (2013). Common ragweed: athreat to environmental health in Europe. Environment International 61: 115-126.

Stewart, L. (2012): Pests and diseases of olive trees. Gar.Not.,pp 548.

Sutter, E.G. (1994).Olive cultivars and propagation. In: Ferguson L, Sibbett G.S, & Martin G (eds.). Olive Production Manual. University of California Division of Agriculture and Natural Resources, Oakland, pp. 23-25.

Teviotdale, B. L., G.S. Sibbett & D.L.Harper.(1989). Several Copper Fungicides Control Olive Leaf Spot. California Agriculture 43(5): 30-31.

Thakur, M. & B.S. Sohal.(2013). Role of elicitors in inducing resistance in plants against pathogen infection: A review. ISRN Biochemistry 1: 1-10.

Tripathi, P. & N.K.Dubey.(2004).Exploitation of natural products as alternative strategy to control post-harvest fungal rotting of fruits and vegetables. Postharvest Biology and Technology 32: 235-245.

Vanneste, J.L. & M.D. Voyle.(2003). Genetic basis of copper resistance in New Zealand strains of *Pseudomonas syringae*. Horticultural Pathology 109-112.

Vidotto, F., F.Tesio & A.Ferrero.(2012).Allelopathic effects of *Ambrosia artemisiifolia* L. in the invasive process. Crop Protection 54:161-167.

Zine El Aabidine, A., Y.Baissac, A.Moukhli, C.Jay-Allemand, B.Khadari & C.El Modafar. (2010). Resistance of olive-tree to *Spilocaea oleagina*is mediated by the synthesis of phenolic compounds. International Journal of Agriculture and Biology 12: 61-67.

استخدام مستخلص اوراق نبات *Ambrosia artemisiifolia* لمكافحة فطر *Spilocaea oleagina* المسبب لمرض عين الطاووس على اشجار الزيتون.

فاطمة شوقي خليف

المشرف: د. مازن سلمان

الملخص

تعاني أشجار الزيتون من عدة مشاكل تسببها الافات والامراض النباتية. ويعتبرمرض تبقع أوراق الزيتون أومرض عين الطاووس الذي يسببه فطر *Spilocaea oleagina* واحدا من أكثر الأمراض الخطيرة والإقتصادية المهمة التي تصيب أشجار الزيتون في جميع أنحاء العالم. يسبب المرض تساقطًا شديدًا للأوراق، والتي يمكن أن تقلل من نمو وإنتاجية شجرة الزيتون مما يسبب خسائر اقتصادية كبيرة في المحصول.يمكن مكافحة المرض من خلال استخدام مبيدات الفطريات الكيماوية المحتوية على مادة النحاس قبل هطول الأمطار في فصل الشتاء. ونظرا للآثار السلبية للمبيدات على البيئة وصحة الانسان فإنها تترك مخاوف كثيرة تؤدي للبحث عن طريقة جديدة وصديقة للبيئة.ان استخدام المستخلصات النباتية ضد الافات والأمراض النباتية يعتبر بديلاً آمناً يمكن استبداله بالمبيدات الكيماوية. تعتبر عشبة (*Ambrosia artemisiifolia*) من النبات المنتشرة في فلسطين والتي لها تأثير مثبط لنمو بعض النباتات الاخرى. يهدف هذا البحث الى دراسة فعالية مستخلص اوراق نبات*A.artemisiifolia* على تثبيط ومنع نمو ابواغ فطر عين الطاووس. تم في هذا البحث جمع الفطر من ستة مناطق في فلسطين وهي قلقيلية، نابلس، طولكرم، جنين، رام الله وسلفيت، حيث استخدم مستخلص اوراق العشبة بعد تجفيفها ونقعها بالماء بتراكيز مختلفة (0.5، 1.0، 1.5، 2.0، 2.5، 5.0 و 10%) لتبيان اثرها على نمو الابواغ. وبعد تحديد التركيز المناسب الذي يؤدي الى تثبيط نمو الابواغ، تم استخدامه مع مبيد فونجران النحاسي لدراسة اثر مزيج من المستخلص والمبيد الكيماوي لدراسة فعالية المكافحة المتكاملة ضد الفطر وعمل مقارنة بينهما للوصول الى افضل نسبة من شأنها مكافحة المرض.

اظهرت النتائج ان جميع تراكيز المستخلص لها القدرةعلى تثبيط نمو الفطر.ينما كانتاعلى نسبةلنمو أبواغ الفطر (80.33%) في العينات الغير معالجة بالمستخلص، تبين انه مع زيادة تركيز المستخلص قل نمو ابواغ الفطر بشكل متزايد. وعند تركيز 10% من المستخلص كان هناك تثبيط كامل للابواغ. كان اقل معدل لنموالابواغ(2.8%)عند تركيز 5%. ومن المثير للاهتمام،انه عند تركيز 0.5% لم يتجاوز معدل نمو أبواغ الفطر 50%مقارنة مع الابواغ الغير معالجة بالمستخلص.اظهرت النتائج ايضا انه عند استخدام المبيد الفطري مع المستخلص بنسبة (v/v 1:1) كان معدل نمو الفطر اقل بنسبة 5% من معدله في العينات الخالية من المستخلص.

تم في هذه الدراسة ولأول مرة في فلسطين توصيف الفطر المعزول من المناطق المختلفة باستخدام ال PCR مع البرايمر الخاصة لذلك. وبعد تحليل التسلسل الجيني للمادة الوراثية من الفطر تبين وجود تطابق بنسبة 92-99% مع عزلات الفطر *S. Oleagina* ذات الرقم التسلسلي(Accession No AF338393.1) الموجودةفي بنك الجينات.ومن أجل تحديد هوية العزلات المختلفة من الفطر تم استخدام الBox REPAIR primer لايجاد الفروق الجينية بين بين العزلات المختلفة. حيث وجد ان هذه العزلات قد انقسمت الى ثلاثةمجموعات حسب المناطق التي تم جمع الفطر منها.

بناءا على النتائج المتحصل عليها فإن هذه الدراسة تعتبر الاولى من نوعها في فلسطين من حيث التوصيف الوراثي لمرض عين الطاووس وكذلك على المستوى العالمي من حيث استخدام المستخلص النباتي لعشبة*A. artemisiifolia* ضد هذا المرض، ومن حيث تبيان الاختلاف الجيني بين عزلات الفطرباستخدامBox PCR