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Evaluation the Effect of *Paecilomyces lilacinus* as a Biocontrol Agent of *Meloidogyne javanica* on Tomato in Gaza Strip

تقييم كفاءة فطر الباسيلوميسيس في مكافحة نيماتودا تعقد الجذور الجاوية على محصول الطماطم في قطاع غزة

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إقـــــرار

أنا الموقع أدناه مقدم الرسالة التي تحمل العنوان:

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Evaluation the Effect of *Paecilomyces lilacinus* as a Biocontrol Agent of *Meloidogyne javanica* on Tomato in Gaza Strip

Abstract

The present study is based on the investigation of a soil hyphomyces, *Paecilomyces lilacinus*, an opportunistic bio-control agent. The egg pathogenic fungus *Paecilomyces lilacinus* is a unique strain with a wide range of activity against the most important plant parasitic nematodes. The aim of this study to evaluate the efficiency of *P. lilacinus* on parasitic nematodes *Meloidogyne javanica* on tomato plant in Gaza strip.The effects of *P. lilacinus* on the root-knot nematode *M. javanica* were examined under *in vitro* and greenhouse conditions. The fungal isolates were identified using morphological and moleculer primers. Infection ability of the fungal isolate on egg hatching and mortality of *M. javanica* juveniles (J2) were tested *in vitro.* Results indicated that nematode egg hatching inhibition and J2 mortality of the spore suspension of these fungi have variable effects on target nematode. Which shown 94%, reducing on hatching eggs nematode *M. javanica* especially when using a high concentration of *P. lilacinus* spores suspension 3000 spore/ml after 48 hours laying eggs in petri dishes and also led to killing 57% of *M. javanica* juveniles (J2) after 72 hours of incubation. In greenhouse experiment, the effects of *P. lilacinus* on tomato plant growth factors and nematode population were tested. The treatments were divided into six groups, namely Group 1- untreated control, Group 2- *Meloidogyne javanica* alone, Group 3- *P. lilacinus* alone, Group 4- *Meloidogyne javanica* and *P. lilacinus* simultaneously, Group 5- *Meloidogyne javanica* followed by *P. lilacinus* and Group 6- *P. lilacinus* followed *by Meloidogyne javanica* were arranged in a randomised complete block design. Pots were kept in greenhouse for two months. The data were recorded on plant length, fresh and dry weight, number of leaf per plant, number of galls, egg masses, and percentage of eggs infected was also estimated. Results showed that the group Inoculation of 2000J of *M. javanica* caused the significant reduction in various plant growth parameters and yield compared to untreated control. Use of *P. lilacinus* caused a significant increase in the growth and yield of tomato plants inoculated with of *M. javanica*. Application of *P. lilacinus* one week before nematode and simultaneous with nematode inoculation was more effective than other treatments. A significant enhancement was found in growth parameter of tomato and decrease the percentage of eggs and nematode populations. The experiments revealed that *P. lilacinus* effectively bio-controls root-knot nematode and can be applied in agricultural fields by farmers in the fields of tomatoes in Gaza Strip

Keywords: Root-knot nematodes, Biological control, *P. lilacinus, M .javanica,* Gaza Strip.

تقييم كفاءة فطر الباسيلوميسيس *في مكافحة نيماتودا تعقد الجذور على محصول الطماطم في قطاع غزة*

الملخص

هدفت هذه الدراسة الى تقييم كفاءة فطر *P. Lilacinus* في مقاومة مرض تعقد الجذور في نباتات البندورة المزروعة في قطاع غزة التي تحدث بسبب ديدان النيماتودا الديدان الثعبانية *M . javanica* حيث أن قطاع غزة يعتبر منطقة ضيقة كثيفه السكان وتمثل الزراعة ٣٠ % من الدخل العام, فكثافة الزراعة تقود الى كثافة استخدام للمبيدات والمخصبات لكيميائية التي تعتبر احد اهم اسباب تلوث البيئة وخصوصا المياه الجوفية مما يؤدي حتما الى تدهور الوضع الصحي العام للقطاع ,علما بان النيماتود تصيب معظم المحاصيل الزراعية المهمة اقتصاديا والتي يعتمد عليها السكان في غذائهم اليومي, والتي يستخدم لها اخطر المبيدات الكيميائية مثل النيماكور فهذا هو المصدر الاساسي لأهمية هذا البحث الذي يعتبر الاول من نوعه في هذا المجال, فقد تم عزل فطر *P. lilacinus* من مناطق مختلفة من قطاع غزة وتشخيصه والتعرف علية و ضبط الظروف الامثل لنموه وتم عزل مسببات مرض تعقد الجذور *javanica M.* والتعرف عليها وتشخيصها وقد بينت التجارب المختبرية قدرة فطر *.lilacinus. P* على خفض وتثبيط فقس بيض النيماتودا *M. javanica* بنسبة 94% وخاصة عند استخدام تركيز مرتفعة 3000 جرثومة/ ملم من راشح جراثيم الفطر *P.lilacinus* بعد 48 ساعة من وضع البيض في اطباق بتري داخل الحضانة ,وايضا أدى إلى قتل يرقات الطور الثاني للنيماتودا بنسبة 57% من اليرقات بعد72 ساعة من التحضين . آما في التجارب الحقلية داخل(الدفيئة الزراعية) فقد قسمت الى ستة مجموعات الاولى بدون معالجة, والمجموعة الثانية عولجت باضافة ديدان النيماتود لها فقط والمجموعة الثالثة تم اضافة الفطر لوحده والمجموعة الرابعة الفطر والديماتود معا والمجموعة الخامسة النيماتود ثم الفطر والمجموعة السادسة الفطر ثم النيماتود, وأظهرت نتائج معالجة اشتال الطماطم بالفطر*Lilacinus. P* في الحقل ومعالجتها قبل الاصابة وبعدها وعند تعريضها للإصابة لنيماتودا الممرضة (بتركيز 2000 يرقة) فادى إلى تحسين نمو النبات وزيادة طولة مع زيادة طول الجذور ووزن المجموع الخضري والجذري الطري والجاف وأيضا خفض دليل العقد الجذرية للجذور ويعود هذا إلى تثبيطها لفقس البيوض وقتل اليرقات النيماتود في النباتات المصابة، و حث النبات على زيادة ميكانيكية الدفاعية ضد مرض النيماتود. كما بينت النتائج ان المقاومة الحيوية لنيماتودا العقد الجذرية ممكنة باستعمال هذا النوع من الفطريات ويمكن تطبيقها في الحقول الزراعية من قبل المزارعين في حقول الطماطم في قطاع غزة .

كلمات مفتاحية: تعقد الجذور، النيماتود , الطماطم, قطاع غزة , المقاومة الحيوية

وَهُوَ ٱلَّذِىٓ أَنزَلَ مِنَ ٱلسَّمَآءِ مَآءً فَأَخۡرَجۡنَا بِهِۦ نَبَاتَ كُلِّ شَىۡءٍ
فَأَخۡرَجۡنَا مِنۡهُ خَضِرٗا نُّخۡرِجُ مِنۡهُ حَبّٗا مُّتَرَاكِبٗا وَمِنَ ٱلنَّخۡلِ
مِن طَلۡعِهَا قِنۡوَانٞ دَانِيَةٞ وَجَنَّٰتٖ مِّنۡ أَعۡنَابٖ وَٱلزَّيۡتُونَ وَٱلرُّمَّانَ
مُشۡتَبِهٗا وَغَيۡرَ مُتَشَٰبِهٍۗ ٱنظُرُوٓاْ إِلَىٰ ثَمَرِهِۦٓ إِذَآ أَثۡمَرَ وَيَنۡعِهِۦٓۚ إِنَّ فِي
ذَٰلِكُمۡ لَأٓيَٰتٖ لِّقَوۡمٖ يُؤۡمِنُونَ ٩٩

سورة الأنعام أية 99

DEDICATION

To those who made it possible for me.....

My Beloved Mother...·

My Dearest Father...·

My Brothers and Sisters...·

My wife and sons ...

My land Palestine as a weapon to vanquish our enemies...·

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List of Abbreviations

%	Percent
BCAs	Biological control agents
Cm	Centimeter
°C	Degree centigrade
G	Gram
GPS	Global Positioning System
Hr	Hour
J.	Journal
J5	Jabalia sample code
K1	Khan-Yunis sample code
LSD	Least Significant Difference
No.	Number
NS	Non-Significant
P.L.	*Paecilomyces lilacinus*
PCR.	Polymerase chain reaction
PDA	Potato Dextrose Agar
PDB	Potato Dextrose broth
pH	power of hydrogen
R	Replication
R1	Rafah- Morag sample code
RCBD	Randomized Completely Block Design
Res.	Research
RKN	Root-knot nematodes
N	Repeat number
SSF	Solid state fermentation
PCBS	Palestinian Central Bureau of Statistics

Chapter 1
Introduction

1.1 Overview

The tomato which belongs to the family *Solanaceae* and the species *Solanum lycopersicum* (Lucioli et al., 2014), is a major vegetable crop that has achieved tremendous popularity over the last century. World production of tomato exceeded 108.5 million metric tons in 2002 and occupied approximately 4.0 million hectares. World tomato production is valued at 5-6 billion US dollars with international trade amounting to 3-3.5 billion US dollars annually (FAO, 2003)

Aside from being tasty, tomato is very healthy as they are a good source of vitamins (A and C), minerals and carbohydrates. It plays a vital role in maintaining health. It has diversified uses such as fresh salad, cooked foods and in processed forms like ketchup, pickle and sauce. It is highly prized for its monitory gain and nutritional value especially for its richness in vitamins and minerals (Aberoumand, 2010). Hundred grams of edible parts of tomato contains 94.1g water, 0.9g protein, 0.1g fat, 3.5g carbohydrates, 15-20 calories energy, 500-1500 IU vitamin "A", 0.1mg thiamin, 0.02mg riboflavin, 0.6mg niacin, 20-25mg vitamin "C", 6-9 mg calcium and 0.1-0.3mg iron.

In the Gaza strip, tomato is one of the most important crops. It represents the second produced vegetable after cucumber and occupy the third cultivated area after cucumber and squash Palestinian Central Bureau of Statistics (PCBS, 2007/2008). More than 24.921 thousand dunums (1 donum = 1000m^2 = 0.1 hectare) is cultivated with tomato, with an annual production of about 207.559 thousand tonnes. It is grown both in large and small-scale farms in Gaza strip, in open and protected fields (green houses) as an important income generating crop for farmers (PCBS 2007/2008).

Tomato is often severely attacked by a number of pathogens like fungi, bacteria, viruses and nematodes. Nematodes are the most abundant multi-cellular organism on earth, occupying different ecological niches and living

as parasites of humans, animals and plants. Parasitic nematodes can devastate several economically important crops, causing significant losses in yield. These nematodes are obligate parasites, and they have developed different parasitic strategies and relationships with their hosts to attain enough nutrients for development and reproduction (Rybarczyk-Mydłowska et al., 2014).

At present, root knot nematode (*Meloidogyne* spp.) is one of the most economically important pests of crop plants that have world-wide distributions and extensive host ranges including several economically important crop plants including tomato. *Meloidogyne* are one of the most damaging genera of plant-parasitic nematodes on horticultural and field crops. Among more than 80 species of the genus, *Meloidogyne, M. incognita, M. Javanica, M. arenaria and M. hapla* were responsible for at least 90 % of all damage caused by root-knot nematodes (Castagnone-Sereno, 2002).

In Gaza strip, it is a combination of several factors, such as high temperature, sandy and warm soils that favour the nematode survival and multiplication in their host plant and soil. It has been estimated that some 18% - 20.7 % of tomato production is lost as a result of damage caused by these worms, which represents one third of the losses generally attributed to pest and diseases (Ministry of Agriculture Palestine 2013). Due to the continuous increase in population and the limited area of Gaza strip, it is necessary not only to maintain crop production, but also to increase it. This could be mainly achieved through increased crop productivity, which in turn is a function of pest management to reduce crop losses.

Moreover, pertaining to worldwide distribution, serious destruction and economic importance of nematodes, emphasis has been made to find out the most effective and feasible control measures.

Chemicals pesticides that are being used for controlling plant parasitic nematodes are costly and toxic in nature. In addition to killing the pests, they also have adverse effects on human beings, livestock and other living things,which come in contact directly or indirectly. Therefore, alternative

environment friendly measures are needed to be developed. Researchers all over the world are engaged in standardizing nematode management strategies by following non-chemical and eco-friendly approaches such as biological control agents to stabilize crop production (Sumathi et al., 2006).

Biological control is defined broadly as the use of natural or modified organisms, gene products to reduce the effects of undesirable organisms and support desirable beneficial organisms such as crops, trees, animals, and beneficial insects. It is simply the use of one or more organisms to maintain or to check population of another pest at a level where it ceases to be a problem (AgbeNiN, 2011). It depends on the knowledge of biological interactions at the ecosystem, organism, cellular and molecular levels and is often a more complicated management strategy than physical and chemical methods. It is less spectacular than most physical or chemical controls but it is usually more stable, longer-lasting and environmentally friendly.

Among the various biocontrol agents, the soil-inhabiting fungus *Paecilomyces lilacinus* is a unique strain with a wide range of activity against the most important plant parasitic nematodes. It is capable of parasitizing nematode eggs, juveniles and females, and reducing populations of plant parasitic nematodes in soil. Tests on potted plants and field plots have shown the fungus to control a range of nematode species including the root-knot nematode, *Meloidogyne* spp. on a number of crops. Its effectiveness was comparable to several chemical nematicides tested (Jatala, 1986). *P. lilacinus* is one of the most widely tested biological control agent used for management of plant-parasitic nematodes (Vasanthi & Kumaraswamy, 1999).

1.2 Objectives

1.2.1 General objective

The main objective of this study is to assess the effectiveness of using *P. lilacinus* as a biological control agent against tomato plant parasitic nematodes *M. javanica* in Gaza strip.

1.2.2 Specific objectives

The following specific objectives will be achieved:

1. Isolation and identification of locally *P. lilacinus* strain from the soil
2. Optimization of the growth conditions of *P. lilacinus*.
3. Evaluation *P. lilacinus* activity *in vitro* against eggs and juveniles of the root knot nematode.
4. Evaluation the effectiveness of application of *P. lilacinus* on growth parameters and identifying the best way of fungal application for effective control of the plant parasitic nematodes *M. javanica*

1.3 Significance of the study

The Gaza strip economy is partly agriculture based with more than 30% of its population deriving their livelihoods from agriculture. Moreover, in the Gaza strip, the agricultural sector accounts for about 25% of employment, about 38% of local trade and provides 70% of the country‘s food needs (Work Conditions Survey , PCBS, 2004).

The root knot nematodes *M. javanica* cause significant losses to a wide variety of crops in Gaza strip. Currently, control of root knot nematodes is primarily accomplished through chemical nematicides espacialy nimcore pesticide and methyl promide. These two methodes is very dangerous to the health and enviernement. Therefore, it is very important to prove scientifically that the use of biopesticide possible to be an alternative to chemical pesticides.

The importance of this idea is reflected in a distinct area like Gaza, where the density of population is high and there is an intensive agriculture in a narrow agricultural area.This situation forcing the farmers to excessive use

of chemical pesticide to compensate the deficiency of the agricultural land used.

Putting effective alternatives for these farmers is of utmost importance to begin to develop a strategy aimed to reduce the use of chemical fertilizers, while maintaining appropriate agricultural production. In addition, it is worth mentioning also that the only source of drinking water in this region of the world is the groundwater wells that are directly affected by the use of high amount of chemical pesticides.

This is the ultimate goal of such researches that fall within an integrated system aimed at preserving the environment, drinking water, health and at the same time providing a high agricultural productivity.

To best of our knowledge, this is the first study concerning the bio-control of plant parasitic nematodes in Gaza strip.

1.4 Limitations of the study

- Problems in obtaining kits, fungal media and chemicals for the practical part of this study.
- Difficulties in soil sample collection for fungal isolation.

Chapter 2

Literature Review

2.1 Root-knot nematodes

Root-knot nematode symptoms on plant roots are dramatic. As a result of nematode feeding, large galls or "knots" can form throughout the root system of infected plants. Severe infections result in reduced yields on numerous crops and can also affect consumer acceptance of many plants, including vegetables (Fig. 2.1, 2.2). The degree of root galling generally depends on three factors: nematode population density, *Meloidogyne* species and "race," and host plant species and even cultivar. As the density of nematodes increases in a particular field, the number of galls per plant also will increase. Large numbers of nematodes penetrating roots in close proximity also will result in larger galls (Barker, et al., 1998). *Meloidogyne hapla* (the northern root-knot nematode) produces galls less than half the size of those produced by *M. javanica* (root-knot nematode) on the same plant hosts. Finally, each crop responds differently to root-knot nematode infection (Fig .2.3, 2.4 and 2.5). Carrots typically undergo severe forking with galling predominantly found o lateral roots Root-knot nematode galls on lettuce are beadlike (Fig. 2.4). On grasses and onions, galls are usually small and barely noticeable, often no more than slight swellings. Depending upon the crop affected and the severity of infection, these symptoms can often result in significant economic losses to growers (France & Abawi, 1994)

Figure 2. 1 Numerous crops

(Mitkowski, N.A. and G.S. Abawi. 2003)

Figure 2.2 Carrots nematode galling

(Mitkowski, N.A. and G.S. Abawi. 2003)

Figure 2.3 Root nematode galling
(Courtesy G.S. Abawi 2003)

Figure 2.4 Roots with Root-knot nematode
(Courtesy G.S. Abawi 2003)

Figure 2.5 Grasses galling
(Smiley & Nicol, 2009)

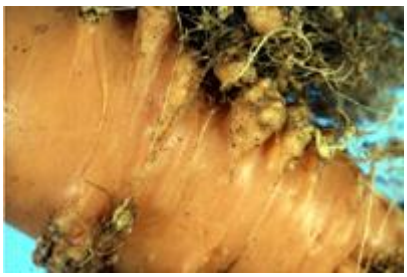

Figure 2.6 Carrots galls
(Affokpon et al., 2011)

Figure 2.7 Root-knot nematode infection
(Collange et al., 2011)

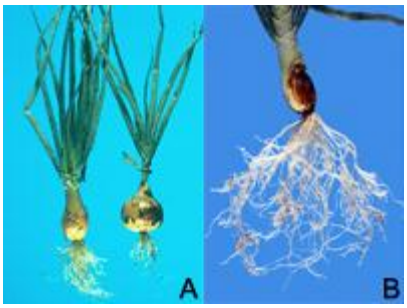

Figure 2.8 Onions Root-knot nematode
(Affokpon et al., 2011)

Most root-knot nematodes have a very wide host range. Thus, growers who have a root-knot nematode problem may find it difficult to control the nematode and its damage through crop rotation, although this is sometimes a viable option. Cotton growers who have an infestation of *M. javanicacan* often plant peanuts in subsequent years to reduce nematode populations. Unfortunately, peanut is an excellent host for a race of *M. arenaria* race 1, which can be found in fields that also contain *M. javanica* (Jepson, 1987**).** Growers who have a problem with *M.*

javanica can employ sweet pepper as a rotational crop, but not if they also have *M.javanica*. These two examples demonstrate the importance of understanding which *Meloidogyne* species is present. In addition to differences in pathogenicity on a specific crop, there can be an even greater degree of specialization. For example, *M. hapla* will not reproduce on grasses. In contrast, *M. graminis* only reproduces on grasses. Thus growers who experience problems with *M. hapla* can rotate corn and wheat into traditional vegetable production, provided they have the appropriate equipment available (Barker et al., 1998).

2.1.1 Pathogen Biology of the *Meloidogyne* nematode

Root-knot nematodes were first reported in 1855 by Berkeley, who observed them causing damage on cucumbers, which defined 4 species and one subspecies (*M. javanica*) within the genus *Meloidogyne*, the root-knot nematodes were all considered the same species. The name *Meloidogyne* is of Greek origin, meaning apple-shaped female (Chitwood, 2002). Approximately 100 species of *Meloidogyne* have been described. The most widespread and economically important species are *M. javanica, M. arenaria,M. hapla, M. chitwoodi and M. graminicola*. Root-knot nematodes are primarily tropical to sub-tropical organisms, however *M. hapla and M. chitwoodi* are well adapted to temperate climates (Viaene & Abawi, 1998).

Like all plant-parasitic nematodes, root-knot nematodes possess a stylet for injecting secretions as well as ingesting nutrients from host plant cells (Fig. 2.9). Nematodes have no internal skeletal framework, and their "skin" or cuticle acts against internal turgor pressure to maintain body shape and aid locomotion (Karssen, 2002) .

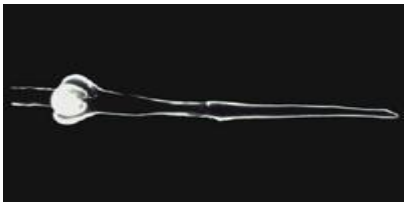

Figure 2.9 plant-parasitic nematodes
(Collange et al., 2011)

Unlike most other plant-parasitic nematodes, females nematode are sedentary at maturity. They range in length from 400 to 1000 µm. Once they establish a feeding site**,** they permanently remain at that location within the plant root. The root-knot nematode feeding site is actually a group of cells known as "giant-cells" (Figure 2.10). When a nematode initially penetrates a plant cell with its stylet, it injects secretory proteins that stimulate changes within the parasitized cells. Parasitized cells rapidly become multinucleate (contain many nuclei) as nuclear division occurs in the absence of cell wall formation. This process is considered to be "uncoupled" from cell division (Karssen, 2002)**.** Cells never actually divide into new cells; they just get bigger and contain more nuclear material. This allows the giant-cell to produce large amounts of proteins which the nematode will then ingest. Giant-cells also act as nutrient sinks, funneling plant nutrients to the feeding nematode. The root-knot nematode does not feed from the cells directly. It forms a feeding tube (from the esophageal gland cell secretions), secreted from the stylet into the plant cell cytoplasm, which acts as a sieve to filter the cytosol that the nematode ingests. As the name implies, giant-cells can grow very large in size (Jones et al., 2013). Triggered by nematode esophageal gland cell secretions, an increase in the production of plant growth regulators has been demonstrated to play a role in this increase in cell size and division. Root cells neighboring the giant-cells also enlarge and divide rapidly, presumably as a result of plant growth regulator diffusion, resulting in gall formatio (Jones et al., 2013)**.**

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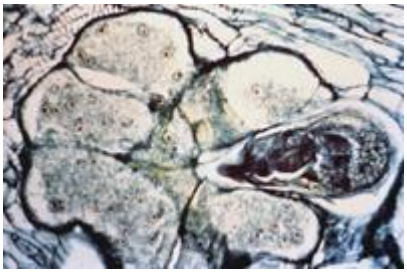

Figure 2.10 Giant-cells

(N.T, Powell, 2013)

As the female nematode enlarges, its posterior region may break the epidermis of the root, and the eggs are deposited into a gelatinous egg mass (Fig. 2.11, 2.12). Mature root-knot females (pearly white in color) can be observed without magnification. Second-stage juveniles (J2) and males can only be observed with the aid of a microscope. Generally, females have a globose body, with a short "neck," containing their stylet, (Fig. 2.12) metacorpus and esophageal gland cells (Viaene & Abawi, 1998).

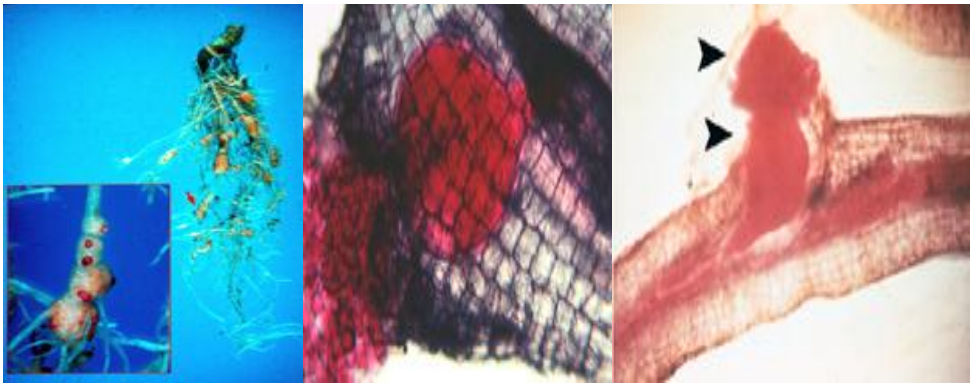

Figure 2.11 Red colure Gelatinous egg mass

(Postnikova et al., 2015)

Figure 2.12 Gelatinous egg

(Collange et al., 2011

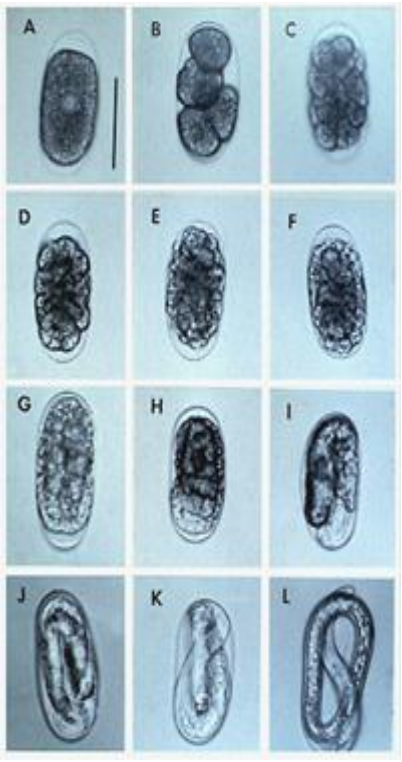

Figure 2.13 first-stage juvenile

.(T. Vrain, used by permission of Society of Nematologists)

Figure 2.14 Worm-shaped

(N. Mitkowski & Abawi, 2003)

Figure 2.15 Nematode males

(N. Mitkowski & Abawi, 2003)

The J2s of the root-knot nematode are most commonly encountered in soils and are vermiform (worm-shaped) (Figure 2.13, 2.14). They are usually no larger than 500 µm in length and 15 µm in width. This is the only infective stage (Jepson, 1987).

Root-knot nematode males also are vermiform and range from 1100 to 2000 µm in length (Figure 2.15). They have distinct lips and strongly developed stylets. In addition, they often have visible spicules, for mating, and a blunt, rounded tail.

Many *Meloidogyne* species are pathenogenic or facultatively pathenogenic. This means that males are not necessary to complete the nematode life cycle and viable eggs can be produced by female nematodes in the absence of fertilization. Because of this, males can be rare in a number of species and are only encountered when the nematode population is subjected to an environmental stress (France & Abawi, 1994).

Root-knot nematodes can be identified to species using a number of techniques, but one common method is perineal pattern analysis (Figure 2.15). The perineum (the region surrounding the vulva and anus) of female nematodes displays a pattern of ridges and annulations for each species. While some variation does exist among individuals, these patterns are quite consistent within a species (Dong et al., 2004). The analysis of isoenzyme electrophoretic profiles, often using esterase and malate dehydrogenase, is a common method for the diagnosis of *Meloidogyne* species in properly equipped labs. Likewise, DNA analyses can also be used to identify different species of root-knot nematodes (Karssen, 2002) .

Meloidogyne spp. are a major constraint to successful vegetable production all over the world, causing severe damage that leads to dramatic yield losses (Sikora & Fernandez, 2005). Control of root knot nematodes has been primarily accomplished through chemical nematicides (France & Abawi, 1994). However, due to the significant drawbacks of the chemical control including threats to human health and the environment, biological control has become one of the promising alternatives (Stirling & West, 1991). The egg-pathogenic fungus *Paecilomyces lilacinus* is the most widely tested biological control agent used for management of plant-parasitic nematodes.

2.1.2 Disease Cycle

Nematodes generally develop from the egg stage into four successive juvenile stages after which they become an adult male or female. Juveniles that hatch from the egg are J2 (Karssen et al., 2013).The infective J2 stages of *Meloidogyne* spp. penetrate the roots immediately after contact close to the root tip, by forcing their stylet through the root surface and producing cellulytic and pectolytic

enzymes for cell wall degradation.Once in the root, the J2 migrate intercellularly into the vascular cylinder, towards cortical tissue in the zone of differentiation. Here, they become sessile and induce the development of a feeding site, consisting of giant cells (G Gheysen et al., 2006). This feeding site provides the nematode with food throughout the further life stages and reproduction. Root galls are formed by hyperplasia and hypertrophy of the cells surrounding the feeding site and are the key instigators of restricted nutrient and water uptake by the host plant (Trudgill et al., 2000). After settling at a site, the nematode undergoes three more moults and reaches the adult life stage. The males become fili form, yet remain folded inside a sheath which is the moulted cuticle of the fourth-stage juveniles. Adult females enlarge as they mature and eventually attain a pyriform body shape (Denny, 2006). Except for the parthenogenetic species, the males escape from the sheath and the root before mating with the swollen females. Females then produce a gelatinous egg sac, into which they extrude hundreds of eggs, and which are then deposited on the surface of the root gall (Karssen et al., 2013).

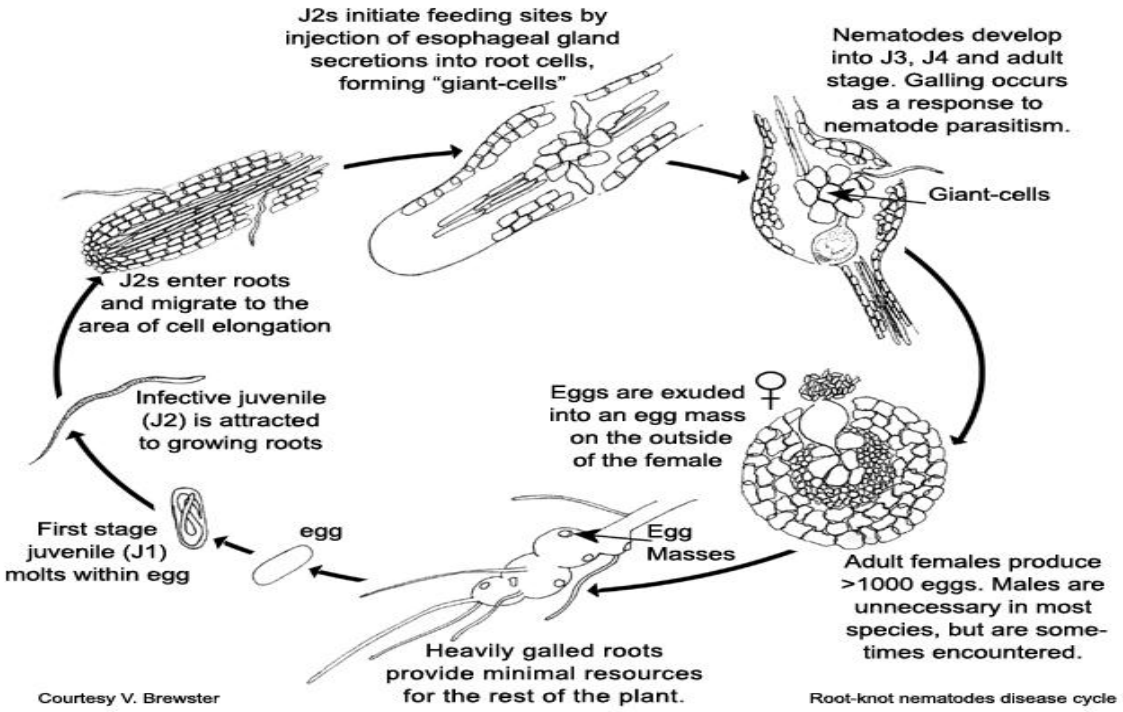

Figure 2.16 Root-knot Disease Cycle

2.1.3 Epidemiology

Root-knot nematodes begin their lives as eggs that rapidly develop into J1 (first-stage juvenile) nematodes (Fig. 2.16). The J1 stage resides entirely inside the translucent egg case.where it molts into a J2 nematode. The motile J2 stage is the only stage that can initiate infections (Figure 2.16). J2s attack growing root tips and enter roots intercellularly, behind the root cap (Figure 2.17). They move to the area of cell elongation where they initiate a feeding site by injecting esophageal gland secretions into root cells (Davis & Mitchum, 2005). These nematode secretions cause dramatic physiological changes in the parasitized cells, transforming them into giant-cells (Figure 2.10). If the nematode dies, so will the giant-cells upon which it .

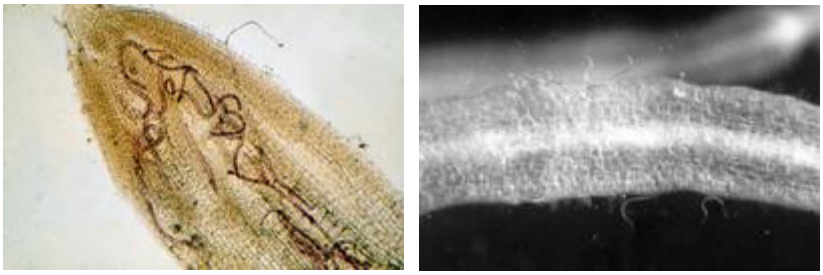

Figure 2.17: J2s attack growing root tips and enter roots intercellular (Postnikova, Hult, Shao, Skantar, & Nemchinov, 2015)

J2s do not possess reproductive organs. As with all nematodes, root-knot nematodes undergo four juvenile stages, each progressing through a "molting" process similar to that of insects. As a result of this process, juvenile root-knot nematodes have little resemblance to adult males and females. In the J4 stage, the progression from juvenile to globose adult females or to vermiform adult males becomes clearly visible (Viaene & Abawi, 1998). They emerge as adults from the J4 cuticle (Fig. 2.18). A single female nematode can produce 500 to more than 1000 eggs (Figures 2.19).

Figure 2.18 cuticle J2 nematode
(N. A. Mitkowski & Abawi 2003)

Figure 2.19 Nematode eggs
(Postnikova et al., 2015)

The length of a root-knot nematode life cycle varies among species but can be as short as two weeks. Nematodes in cooler regions typically have longer life cycles. Eggs may remain inside root tissue or may be released into the soil matrix. Eggs hatch at random, i.e. hatching does not require exposure to root exudates. Under favorable conditions, root-knot nematode eggs have been reported to survive for at least one year in the soil (Jones et al., 2013)

There is a great potential for genetic manipulation in tomato to enhance productivity through increasing pest and disease resistance, environmental stress tolerance and to study gene function and regulation (Rybarczyk-Mydłowska et al., 2014). The crop tomato is attacked by number of nematode pests. Globally, nematodes are common in almost all soils, with their distribution being determined by temperature, degree of moisture and soil particle size, along with the presence of acceptable food source (G Gheysen et al., 2006). Among the plant parasitic nematodes, Root knot nematode (RKN), *Meloidogyne javanica* is a serious pathogen hampering the productivity in tomato crop significantly throughout the world and has been found to be very widely distributed with wide host range and cause very serious damage specially in vegetables. In India, annual yield loss due to *M. javanica* has been estimated to as 27.21% in tomato

(France & Abawi, 1994). Nematode control using chemical nematicides has become environmentally unsafe and economically unviable due to removal of efficient fumigant nematicides from world market on environmental grounds. There is a need to develop environmentally and economically sound alternatives to nematicides for sustainable nematode management in tomato ecosystem. Biological control of plant parasitic nematodes using fungi and bacteria has been found to be a feasible option. The antagonistic fungi play an important role as biocontrol agent for many plant parasitic nematodes (Jatala, 1986). *Paecilomyces lilacinus* a saprophytic soil fungus has drawn many research attentions due to its promising effect in parasitizing and controlling population of phyto-nematodes (Jatala, 1986); (Hewlett et al., 1988); (Nagesh & Reddy, 1997); (Khan et al., 2004) Of these the use of pathogenic fungus *Paecilomyces lilacinus* is one the most widely tested biological control agent for management of plant parasitic nematodes. It has a high frequency of occurrence in the tropics and subtropic (Morgan et al., 1983) and can be found in most of agricultural soils (Brand et al., 2010).

2.2 Disease Management

Since 1950, the control of phyto-parasitic nematodes has been based on chemical pesticides, although several of them are being withdrawn from the market due to issues related to the environment health. (Eapen et al., 2005) Methyl bromide was widely used against nematodes, but now it has been withdrawn from the market because of its adverse effects on the ozone layer. (Eng, 2001) Nematodes also developed resistance against most of the known pesticides, and this triggered worldwide research for new alternative agents and methods for nematode control. Possible control measures change with climate conditions, socio-economical situation of the country, crop economy, availability of chemical pesticides, resistant cultivars, and the suitability of agricultural practices (Saad et al., 2012).

2.2.1 Resistant plants

Nematode resistance genes are present in several crops, and are an important component of various multiplication programs in tomatoes, potatoes, cotton, soybean, and cereals. Resistance to nematodes can be either broad with action against several species of nematodes or narrow against only selected specific biotypes113. Several resistance genes, dominant or semi dominant, were identified, cloned, and subjected to various studies (Brand et al., 2010).

2.2.2 Crop rotation

Crop rotation is an important method for maintenance and improvement of soil fertility, and for enhancing yield. In crop rotation, various crops are followed in a certain order in the same soil. With the same succession of crops reproducing in a regular time cycle, rotations can be biennial, triennial, and so on (Chen, et al., 1991). Crop rotation is a very good strategy that can always be adopted against nematode species with narrow ranges of plant-host, which is not the case of *Meloidogyne* sp. However, the order of plants and the time intervals between susceptible crops depend on the nematode species (Ogumo, 2014).

2.2.3 Chemical control

Plant-parasitic nematodes are more vulnerableas juveniles (J2) in soil, when searching for the roots of host plants. Once an endoparasitic nematode species penetrates a root, chemical control is more difficult as compounds have to be non-phytotoxic (Coventry & Allan, 2001). There are several nematicides that can be used effectively against nematode pests of many annual crops, but there appears to be little progress for management of nematodes in many susceptible perennial crops without repeated application of nematicides36 (Coleman & Crossley Jr, 1996). There are two kinds of chemical products that can be utilized against plant parasitic nematodes: soil fumigants and nematicides. Their application to soil depends on the form of the formulation, it can be by injection, spraying, mechanical means, or through irrigation pipes cuts are usually applied before planting and, in the case of pesticides, they are applied at the time of

planting. Fumigants are highly effective against nematodes, their efficacy is related to their high volatility at ambient temperatures (Jones et al., 2013). All fumigants have low molecular weights, and are available as gases or liquids. As they volatilize, the gas diffuses through the spaces between soil particles where the nematodes are killed. The most widely used fumigant is methyl bromide, which is mainly applied for high valued crops, such as strawberries and tomatoes, and in lesser amount to grains and commodities. However, methyl bromide has been banned in developed countries since 2005. In developing countries, substances with methyl bromide will be withdrawn from field application by the end of 2015.Other fumigants, such as chloropicrin, dazomet and meta sodium showed good activity against nematodes when applied (Gortari & Hours, 2008).

2.2.4 Bio-control methode

An eco-friendly pest management strategy that utilizes deliberate introduction of living natural enemies to lower the population level of a target pest.These enemies are commonly referred to use as Biological control agents (BCAs), which must demonstrate some characteristics for success in the field, including ability for rapid colonization of the soil, persistence, virulence (Dos Santos et al., 2013) predictable control below economic threshold, easy production and application, good viability under storage, low cost of production, compatibility with agrochemicals, and safety. In nature, it is observed that many natural enemies, such as viruses, bacteria, rickettsia's, fungi, and others, can attack plant parasitic nematodes, but in the search for suitable BCAs more attention has been given to fungi and bacteria (Jepson, 1987). Biological control can be either natural (i.e., when a natural population of a particular organism inhibits the growth and development of nematodes), or induced (i.e., when BCAs have been introduced artificially). There are two approaches for introduction: microbial pesticide application for rapid control of a pest, and the introduction or mass release of a biocontrol agent to provide long lasting control. The suppression can be specific or nonspecific, when only one or two organisms are involved. Researchers have made several attempts to utilize bacteria for nematode control (Dechechi, 1986).

2.3 *Paecilomyces lilacinus* fungi

Paecilomyces lilacinus is a common saprobic, filamentous fungus. It has been isolated from a wide range of habitats including cultivated and uncultivated soils forests, grass land, deserts, estuarine sediments and sewage sludge. It has also been found in nematode eggs, and occasionally from females of root-knot and cyst nematodes. In addition, it has frequently been detected in the rhizosphere of many crops. The species can grow at a wide range of temperatures – from 8°C to 38°C for a few isolates, with optimal growth in the range 26°C to 30°C. It also has a wide pH tolerance and can grow on a variety of substrates *P. lilacinus* has shown promising results for use as a biocontrol agent to control the growth of destructive root-knot nematodes (Domsch et al., 2007).

2.3.1 Taxonomy

P. lilacinus used to be classified with the Fungi I Deuteromycetes,fungi for which perfect (i.e., sexually reproducing) states have rarely been found. *Paecilomyces lilacinus* was classified in the section Isarioidea, for which perfect states have not been found (Inglis & Tigano, 2006)**.** Many isolates of *P. lilacinus* have been identified from around the world and it is accepted that variation exists within the species.It is now accepted that it should be placed in the family *Trichocomaceae* (Ascomycota). Phylogenetic analysis of *P. lilacinus* isolates show that it is more closely related *to Trichoderma, Gliocladium* and Hypocrea than to the other entomopathogenic *Paecilomyces* species in the Hypocreales (Anderson, 2003) **.**

Kingdom : Fungi

Division: Eumycota

Class: Deuteromycetes (Fungi imperfecti)

Order: Moniliales (Hyphomycetes)

Family: Moniliaceae

Subfamily: Hyalosporae

Genus: Paecilomyces BAINIER 1907

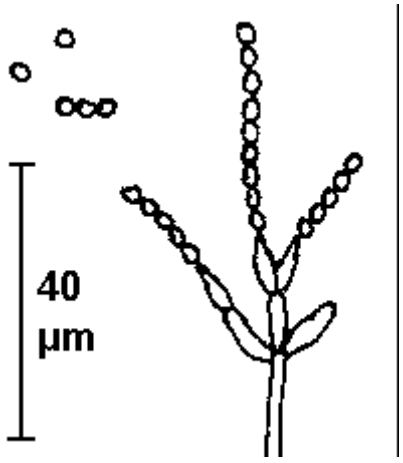

Fig 2.20 Conidiophore of *Paecilomyces lilacinus*

(System of anamorphs according to SACCARDC

Paecilomyces lilacinus strains have been repeatedly isolated from insect larvae. The first strain to be isolated from egg-pouches of *Meloidogyne* sp. (root knot nematode) was found in Peru. Isolates from eggs of other nematodes are also known (Jatala, 1986).The Conidiophores of the genus *Paecilomyces* ramify in grouped branches or irregularly. The one-celled conidia are separated from (fig 2.8) the phialides in form of chains (Samson, 1974). *P. lilacinus* shows fast hyphal groth. The conidiophores are up to 600 µm high and develop groups of lateral branches, from which each 2-4 bottle-shaped phialides grow. The ellipsoid, 2.5-3.0 µm long and 2.0-2.2 µm broad conidia are of lilac colour (Samson, 1974).In case of vibration or air movements they are whirled up in great quantities, so that the fungus spreads effectively. The facultative egg parasite is sometimes also able to infect mobile nematode stages or sedentary females, but it is most aggressive against eggs (Cabanillas & Barker, 1989).There have already been some successful trials for its utilization in biological plant protection, e. g. against *Meloidogyne javanica* in tomato or in potato (Jatala, 1986).

2.3.2 Description

P.lilacinus forms a dense mycelium , which gives rise to conidiophores. These bearphialides from the ends of which spores are formed in long chains. Spores germinate when suitable moisture and nutrients are available. Colonies on malt agar grow rather fast, attaining a diameter of 5–7 cm within 14 days at 25°C, consisting of a basal felt with afloccose overgrowth of aerial mycelium; at first white, but when sporulating changing to various shades of vinaceous. The reverse side is sometimes uncolored but usually in vinaceous shades. The vegetative hyphae are smooth-walled, hyaline, and 2.5–4.0 µm wide (Barker et al., 1998). Conidiophores arising from submerged hyphae, 400–600 µm in length, or arising from aerial hyphae and half as long.Phialides consisting of a swollen basal part,tapering into a thin distinct neck. Conidia are in divergent chains, ellipsoid to fusiform in shape and smooth walled to slightly roughened and chlamydospores are absent (Jepson, 1987).

2.4 *P. lilacinus* as Biocontrol agent

Plant-parasitic nematodes cause significant economic losses to a wide variety of crops. Chemical control is a widely used option for plant-parasitic nematode management (Stirling & West, 1991). However, chemical nematicides are now being reappraised in respect of environmental hazard, high costs, limited availability in many developing countries or their diminished effectiveness following repeated applications (Lysek, 1966).

2.4.1 Control of plant-parasitic nematodes

P. lilacinus was first observed in association with nematode eggs in 1966 and the fungus was subsequently found parasitising the eggs of *Meloidogyne javanica* in Peru. It has now been isolated from many cyst and root-knot nematodes and from soil in many locations. Several successful field trials using *P. lilacinus* against pest nematodes were conducted in Peru (Stirling & West, 1991).The Peruvian isolate was then sent to nematologists in 46 countries for testing, as part of the International *Meloidogyne* project, resulting in many more field trials on a range of crops in many soil types and climates. Field trials, glasshouse trials and in vitro testing of *P. lilacinus* continues and more isolates have been collected from soil, nematodes and occasionally from insects. Isolates vary in their pathogenicity to plant-parasitic nematodes. Some isolates are aggressive parasites while other, though morphologically indistinguishable, are less or non-pathogenic. Sometimes isolates which looked promising in vitro or in glasshouse trials have failed to provide control in the field (Jatala, 1986).

2.4.2 *P. lilacinus* Mode of Action

The fungus *P. lilacinus* in sufficient concentrations over 10^7 u.f.c/ml produce hyphae over eggs and larvae on anthropoids of the geneses *Meloidogyne*, Pratylenchus and Radopholus producing deformities in the embryo. The hyphae grow over the egg, while the tips swell and create deformities. A penetration peg grows from the bottom of the hyphae (aspersorium) into the egg (Jepson, 1987). The eggs swell and buckle. As penetration continues and the eggs split while the

hyphae, fill the egg completely. The fungus then emerges to the egg surface producing first vegetative growth. After 5 days, most of the eggs are infected. The young born infected soon die.

2.4.3 Enzymes and Eggs infection

Many enzymes produced by *P. lilacinus* have been studied. A basic serine protease with biological activity against *Meloidogyne* eggs has been identified. One strain of *P. lilacinus* has been shown to produce proteases and a chitinase, enzymes that could weaken a nematode eggshell to enable a narrow infection peg to push through. Before infecting a nematode egg, *P. lilacinus* flattens against the egg surface and becomes closely appressed tit (Lysek,1966). *P. lilacinus* produces simple appressoria anywhere on the nematode eggshell either after a few hyphae grow along the egg surface, or after a network of hyphae form on the egg. The presence of appressoria appears to indicate that the egg is, or is about to be, infected. In either case, the appressorium appears the same, as a simple swelling at the end of a hypha, closely appressed to the (Huang et al., 2004). Adhesion between the appressorium and nematode egg surface must be strong enough to withstand the opposing force produced by the extending tip of a penetration hypha. When the hypha has penetrated the egg, it rapidly destroys the juvenile within, before growing out of the now empty egg shell to produce conidiophores and to grow towards adjacent eggs (Cabanillas & Barker, 1989).

2.5 Solid state fermentation

Solid-state fermentation (SSF) can be defined as the growth of microorganisms in a moist solid substrate in the absence of liquid water. The water content in the moist solid substrate must be adequate to support growth and metabolism of microorganism.(Rybarczyk et al., 2014). SSF can be carried out in two types of matrices, either in a natural substrate acting as solid substrate and a source of nutrients or a nutritionally inert support, which must be impregnated with a liquid nutritive media. Several materials are utilized as inert supports for SSF, such as sugar cane bagasse, amberlite, vermiculite, polyurethane foam, and polystyrene

beads. SSF has several advantages over SmF, but the choice of the method should depend on the physiology of the microorganism and the end product (Johnson & Curl, 1972**).** Comparative evaluations of SSF and SmF indicated several advantages of SSF processes: simplicity of culture media; absence of liquid residues; reduction of contamination due to low water content; culture conditions mimic the natural environment; ease of aeration humid or dry (Jatala, 1986).

2.5.1 Growth physiology of filamentous fungi

Spore production of filamentous fungi is an important stage in its reproduction. Spore production consists of the formation and liberation of conidiospores. Lifecycle of imperfect fungi comprises five steps, which are dormancy of the spore, germination, development of apical mycelium, and conidiogenesis. Normal development of the mycelium and suitable conidiogenesis are the main conditions required for a successful sporogenesis. The conidiospore production is directly related to the quantity and nature of carbon and nitrogen sources available in a culture media, and it depends on several other factors including method of inoculation, media salinity, carbon/nitrogen ratio, aeration, water content, among others (Rombach, Aguda, Shepard, & Roberts, 1986). Conidiospores are characterized by a low water activity, absence of cytoplasmic movements, and reduced metabolic activity. Under favourable conditions, spore germination takes place through the formation of a vegetative tube, which will be the base of a future mycelium. A spore is considered as germinated when the length of the longest germ tube is greater than the dimension of the swollen spore. Different techniques, other than microscopic examinations can be used to assess spore germination. Gompertz equation and logistic function can be used for analyzing germination data. Determination of optimal culture conditions for the large-scale production of conidiospores of filamentous fungi, which are used as BCAs, is highly significant for commercial applications (Lysek, 1966).

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Equipment's

The equipment used in this research are listed in table 3.1.

Table 3.1: list of equipment and their manufacturers

#	Item	manufacture
1.	**Digital balance 0.0001 g**	**AE-adam-UK**
2.	**Autoclave**	**N-biotek-Korea**
3.	**Micro pipet 20-200µl**	**Jencons Scientific-USA**
4.	**Laminar flow cabinet**	**N-biotek-Korea**
5.	**Refrigerator**	**J.P. Selecta- Spain**
6.	**Biological incubator**	**N-biotek-Korea**
7.	**Compound microscope**	**LW- Scientific-USA**
8.	**Hotplate with stirrer**	**Heidolph- Germany**
9.	**Centrifuge 6000rpm**	**LW- Scientific-USA**
10.	**Digital camera omni**	**LW- Scientific-USA**
11.	**Neubauer counting champer**	**Boeco- germany**
12.	**Vortex mixer**	**bioRad- Germany**
13.	**Micro pipet 200-1000µl**	**Jencons Scientific-USA**
14.	**Micro pipet 200-1000µl**	**Jencons Scientific-USA**

15.	**Mixture**	**Moulinex –France**
16.	**Sonicator**	**DESCO – Germany**
17.	**Inverted microscope**	**Lw Scientific- USA**
18.	**Digital balance 0.001 g**	**BOECO-Germany**
19.	**Digital balance 0.0001 g**	**AE-adam-UK**
20.	**PCR**	**Biometra- D-37079 Gottingen, Germany**
21.	**Horizontal Gel Electrophoresis**	**Biometra, Germany**

3.1.2 Chemical reagents and disposable

Table 3.2: list of chemical reagent and disposable and their manufacturers

#	Item	Manufacture
1-	**Potato dextrose agar**	**Himedia – India**
2-	**Yeast extract**	**Himedia – India**
3-	**Lacto phenol blue stain**	**Himedia – India**
4-	**Ethanol alcohol**	**Himedia – India**
5-	**Tween 20**	**Himedia – India**
6-	**Petri dish plate 90mm**	**Miniplast- Palestine**
7-	**Sterile plastic cups**	**Meheco corb-china**
8-	**Inoculating needle**	**Himedia – India**
9-	**Microscopic slide**	**China**
10-	**Polyethylene bags**	**Local market**
11-	**Centrifuge tube**	**Himedia – India**
12-	**0.45 µm filer paper**	**PALL-USA**
13-	**Parafilm tape**	**Pechiny plastic-USA**
22.	**Centrifuge tube**	**Himedia – India**
23.	**0.45 µm filer paper**	**PALL-USA**

24.	Primer DNA	Pechiny –USA
25.	Sieve	Chempal Company-Palestine
26.	Flask	Germany(TGI)
27.	Needle	Changzhou Hekang Medical Instrument
28.	Incubator	CNW- China
29.	Gauze	DB GmbH- India
30.	SDA media	Himedia – India
31.	Sodium hypochloride	Himedia – India
32.	Label	Himedia – India
33.	Funnel	Germany(TGI)
34.	Potato Dextrose Broth	Himedia – India
35.	Cotton Wool	Mensucat San .Ticaret A.S.
36.	Vernier caliper	Dalya Company in China
37.	Swab	Himedia – India
38.	Aluminum Foil	Local market
39.	DNA extraction kit	Promiga
40.	Phloxine B	Sigma-Aldrich

3.1.3 Organisms

Table 3.2.1: list of Organism and their manufacturers

#	Name	Manufacture
1-	Fungi *P. lilacinus*	Localy Isolated
2-	Nematode *Meloidogyne javanica*	Localy Isolated
3-	Tomato type 593 coated seeds	(Hazera Genetic Ltd .Netherlands.)

3.2 Methods

3.2.1 Sample collection

3.2.1.1 Fungal collection, isolation and maintenance

Soil samples were collected from seven different greenhouses located on farms in Rafah, Khan Yunis, Abbasan, Al Shikh Ejleen, Gaza city, Jabalia and Bait Lahya of the Gaza strip (Fig. 3.1). The soil samples were taken from greenhouses which haven't been treated with any of the fungicide within the last 7 days. About 25 grams of top soil (after removal of 2 cm of soil surface) were collected in sterile cups, labelled with date and source of collection. The samples were transported to the laboratory and processed within 2 hours of collection (Babu et al., 2000).

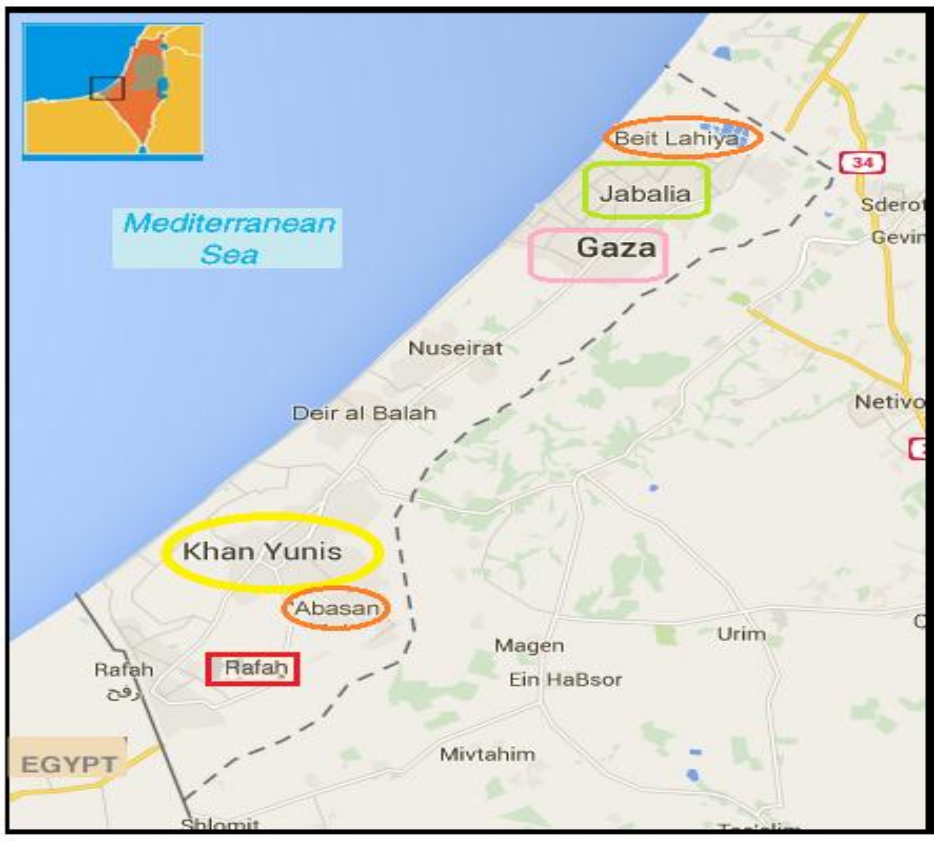

Fig. 3.1 Gaza Strip location map

For each location, where the soil sample was collected, the coordinates (latitude and longitude) were taken using a Global Positioning System (GPS) receiver (Table 3.3)

Table 3.3: GPS locations of the collected soil samples

Soil number	Location	GPS
1	**Rafah**	31°18'23.5"N 34°16'14.2"E
2	**Khan-Yunis**	31°22'15.2"N 34°19'11.1"E.
3	**Abbsaan**	31°18'58.8"N 34°20'46.0"E
4	**Khan-Yunis -a**	31°23'23.4"N 34°19'52.7"E
5	**Jabalia**	31°29'23.8"N 34°25'50.0"E
6	**Bait Lahya**	31°33'50.6"N 34°29'15.7"E
7	**Gaza**	31°29'23.8"N 34°25'50.0"E

For isolation from soil, serial dilution and pour plate technique was used. Dilution at 10^{-1}, 10^{-2} and 10^{-3}, were subsequently spread onto Semi-selective Medium plates and incubated at 25 ± 1°C for 14 days (Gaugler & Bilgrami, 2004).The fourteen days old of fungal culture was cut into small plug of mycelium using sterile borer and transferred to a new plate of Potato Dextrose Agar (PDA). Sub-culturing was repeated several times in order to get a pure culture (Fig. 3.2). The pure cultures were maintained on PDA plates at 4°C.

Fig 3.2 *Paecilomyces lilacinus* isolated on PDA Petri dishes

3.2.2 Semi-selective Medium for Isolation of *P. lilacinus* from Soil

Isolation medium was prepared by combining 10 g NaC1, 50 mg pentachloronitro-benzene, 50 mg benomyl, 39 g potato dextrose agar and deionized water to bring the final volume to 1 liter. After the medium was autoclaved for 15 minutes at 15 psi and cooled to 45-50°C, 100 mg of streptomycin sulfate, 50 mg of chlortetracycline hydrochloride , and 1 ml of Tergitol were added (Mitchell et al., 1987).

3.2.3 Fungal Identification

3.2.3.a Morphological Identification

Purified cultures (from seven days old fungal culture) of the isolated fungi were examined macroscopically and microscopically in order to identify the strain on the basis of their morphological traits and cultural characteristics of the fungi such as mycelium growth, colony texture, spores production and other characteristics (Andrews, 2006)

Microscopic examination for the purpose of identification was carried out by transferring a portion of the mycelium to clean microscopic glass slides. For better analysis of morphological characteristics, the slides was stained with Lactophenol blue solution (0.05g Aniline blue, 25g phenol, 25ml Lactic acid,50 ml glycerol) to enhance contrast. The prepared slides were examined microscopically at 40× magnification.

3.2.3.b Molecular identification of *P. lilacinus*

In order to confirm the morphological identification of fungi resulting positive in the semi-selective media, identification based on polymerase chain reaction (PCR) was applied.

3.2.4 DNA extraction and PCR amplification

Deoxyribonucleic Acid (DNA) was isolated from mycelia taken from the surface plate of overnight cultures on PDB at 30°C. The DNA was recovered from scrapped mycelia using (E.Z.N.A.® Fungal DNA Mini Kit) according to manufacturer's instructions. This Extraction Kit is designed for rapid and

sensitive isolation of DNA from a fungus. Full details of DNA extraction procedure from *P. lilacinus* is given in Appendix I. The quality of genomic DNA was evaluated using a Nano-drops spectrophotometer (Implen GmbH NanoPhotometer Spectrophotometer).

Primers used for Polymerase Chain Reaction (PCR) amplification of fungal 16/18S rDNA have the following composition: TW81, forward, (5'-GTT CCG TAG GTG AAC CTG CGC-3'), and AB28, reverse (5'-ATA TGC TTA AGT TCA GCG GGT-3') (White et al., 1990).

P. lilacinus gene amplified according to the procedure described by (Atkins, Clark, Pande, Hirsch, & Kerry, 2005).The 25 µl PCR reaction mixture contained 12.5 µl of PCR Master Mix, 1.0 µl of forward primer, 1.0 µl of reverse primer, 2.5 µl of DNA template and 12.5 µl of nuclease free water. The PCR was performed using 2720 PCR Thermo Cycler followed the standard procedure: initial denaturation at 94 °C for 1 min followed by 35 cycles for each denaturation (95 °C for 40 seconds), annealing (52 °C for 1 min) and extension (72 °C for 40 sec.). The last stage was the final extension at 72 °C for 5 min and cooling to 4 °C. Then, these PCR products were analysed on electrophoresis using 1% (v/v) agarose gel that was run at 100 volts, 400 mA for 30 min. The gel was stained with ethidium bromide and a band was photographed on a UV light trans-illuminator.

3.2.5 Preparation of spore suspension of *P. lilacinus*

Fifteen days before the experiment, the fungal was cultivated on PDA media plates in order to produce viable spores in sufficient numbers to allow a range of tests to be conducted on spores of the same age and life history. Spore suspension was obtained by washing the ascospores that formed on the surface of plates with 10ml of 0.01% sterile Tween 20. The suspension was collected in a sterile 100 ml Erlenmeyer flask and loosened by shaking with sterile glass beads for 2 hr (Fig 3.3). The density of spore suspension was adjusted with 0.01% sterile Tween 20 to correspond to a final concentration of approximately 5×10^7 spore/mL. The number of spores was quantified using a Neubauer counting chamber and a compound microscope

(400×),(Chandra, 2006). The suspension is then filtered through a gauze and stored in refrigerator at a temperature of 4 ° C until use.

Fig 3.3 Preparation of spores suspension of *Paecilomyces lilacinus*

3.3 Collection of the nematode *Meloidogyne javanica*

3.3.1 Egg Collection

Nematode eggs were extracted from heavily galled tomato roots originally obtained from a greenhouse cultured with tomato according to procedure described by (Reimann, Hauschild, Hildebrandt, & Sikora, 2008). Galled roots were washed free from soil under tap water, cut into 1 cm pieces and macerated in a Warring blender at high speed for 20 s and collected in a glass bottle. Sodium hypochlorite (NaOCl) was added to a final concentration of 1.5% active chlorine to separate eggs from their surrounding gelatinous matrix. The bottle was shaken vigorously for 3 min

and the suspension was thoroughly washed with tap water through a sieve combination (250, 100, 45 and 25 µm) to remove the NaOCl. Eggs were collected **(**Fig 3.3.1) on the 25 µm sieve and washed with tap water into a beaker and used directly for assay after an aliquot was removed to estimate the number of eggs per milliliter.

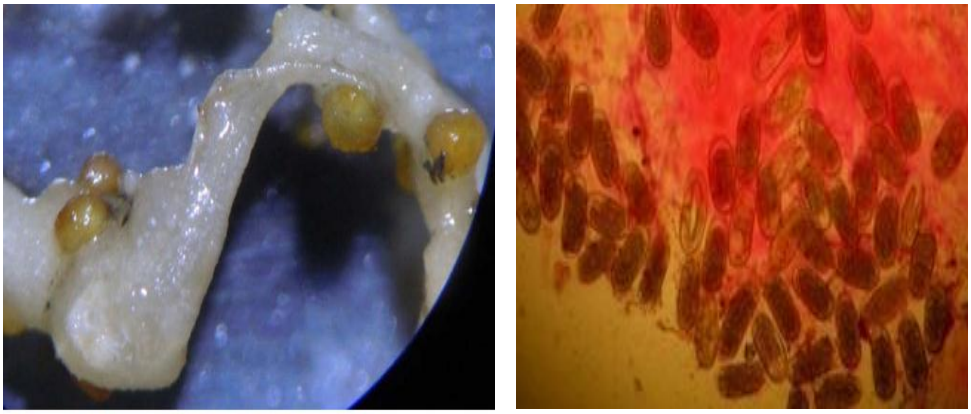

Fig 3.3.1 The *Meloidogyne* Egg mass

3.3.2 Nematode juvenile collection

Eggs were surface disinfested with sodium hypochlorite as described above and washed with sterile filtered 1% sodium thiosulfate for 3 minutes followed by sterile water for 5 minutes (Fig 3.4.) The eggs were then transferred for hatching onto nylon screens (30-µm-pore size) in sterile water. J2 that passed through the filter within 72 hr were collected and used immediately for assays.

Fig 3.4 Larvae isolated from the soil sample.

3.3.3 Nematode Identification

Nematode identification was performed by experts from the Laboratory of Plant Pathology at Ministry of Agriculture, Palestinian Authority. Based on morphological and morphometrical characteristics specimens were identified as the root-knot nematode, *Meloidogyne javanica*.

3.4 Study location

The laboratory work was carried out in biotechnology lab at the Islamic university of Gaza. The greenhouse experiment was done in greenhouse designated for the gardening purposes at the Islamic University of Gaza. The experiment was carried out during the period from January to May 2014.

3.4.1 Plant's temperature conditions in greenhouse experments

The experimental plants were kept in the green house where the temperature was ranged from 30 ± 2º C during the day to 21±2º C during the night with an average temperature of 28 ± 2º C.

3.4.2 Type of Tomato used

Tomato of 593 coated seeds type (Hazera Genetic Ltd .Netherlands.) was purchased from by local Agricultural Company in Gaza city.

3.4.3 Soil mixture preparation

Soil, sand and compost were collected from agronomy farm at Islamic University composited and mixed well in a ratio of 6:2:1 respectively (Johnson & Curl, 1972). The mixture was autoclaved at 121ºC for 15 minutes at 15 psi. The sterilized soil was allowed to cool at room temperature and used for filling the plastic tray and pots used for seedlings raising.

3.4.4 Preparations of pots

Plastic pots of 1000 cm^3 were cleaned, washed, dried up properly and sterilized with 70% ethanol. Each pot was filled with 1000 g of sterilized soil mixture for later use**.**

3.5 The laboratory work (*In-vitro*)

3.5.1 The effect of spore suspension on egg hatch

To test hatch rate, 1.0 ml of surface disinfested (with sodium hypochlorite) freshly prepared egg suspension (100 eggs) was combined with 9.0 ml of PDB in 5 cm diameter Petri dish plate. Eggs, in plates, were combined with fungus spore suspensions of 1500 and 3000 spore/ml, and with sterile water in controls (0 spore/ml). Plate lids were sealed with parafilm, and placed in 28 °C incubators. After 48 and 72 h, eggs were examined with the use of an inverted microscope (Olympus, ×2 objective, total magnification ×20) for sign of hatching. Each treatment was replicated five times and the average number of hatched eggs per each treatment was computed. The entire procedure was conducted in aseptic condition.

Egg hatch rate were determined by counting all eggs and J2 under a stereomicroscope and calculated according to the following formula:

Egg hatch rate= 100 x J2/(total no. of eggs)

3.5.2 The effect of spore suspension on juvenile mortality

To test for effects of *P. lilacinu*s on juvenile mortality, one hundred juveniles of *Meloidogyne* sp. were transferred in 0.2 ml of water into 5 cm Petri dish containing PDB medium. The plates were inculcated with spores (Zero, 1500 and 3000 conidia/ml) and incubated at 28°C. After 2 and 3 days of initiation Juveniles were observed under stereomicroscope (45×) **(**Fig 3.5**).** Juveniles were considered dead if they became rigid and did not exhibit any response after probing the tail with eyelash attached to a toothpick. Treatments were each replicated five time and the percentage of death per each treatment was calculated according to the following formula:

Juveniles mortality =100 x dead Juveniles /total no. Juveniles

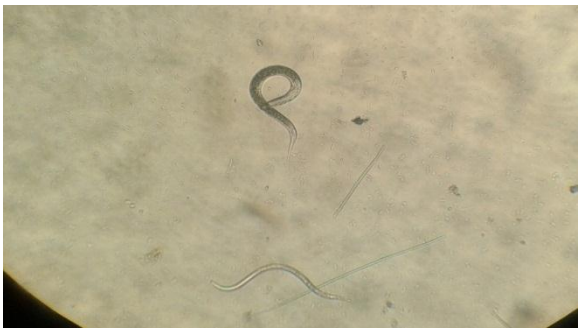

Fig 3. 5 Nematode juvenile

3.6 Greenhouse Experiment.

A greenhouse experiment was conducted during March to May, 2014 at greenhouse designated for the gardening purposes at the Islamic University of Gaza. Tomato seeds (593) were surface sterilized by soaking in a 75% ethanol solution for 1 min and then in a 1.5% NaOCl solution for 3 min. Seeds were then washed with demineralised water and transferred to sterile soil mixture in a seedling box for germination in greenhouse conditions (Ehlers, 2011). The culture substrate was a sterile soil mixture.

Fig 3.6 The Tomato pots were distributed randomly group

Twenty-cm-diameter (2 L) plastic pots were filled with 1000 g of sterile soil mixture and placed on greenhouse at 20-30 cm spacing (**Fig 3.6)**. Uniform four-week old tomato seedlings were transplanted into pots containing the soil mixture. Six treatments, namely **Group 1**- untreated control, **Group 2**-

Meloidogyne javanica alone, **Group 3**- *P. lilacinus* alone, **Group 4**- *Meloidogyne javanica* and *P. lilacinus* simultaneously, **Group 5**- *Meloidogyne javanica* followed by *P. lilacinus* and **Group 6**- *P. lilacinus* followed *by Meloidogyne javanica* were arranged in a randomised complete block design, with 10 replicates for each experiment. Nematode inocula were prepared by extracting juveniles of *Meloidogyne* sp. from infected soil and tomato roots by using Baermann funnel method (Viglierchio & Schmitt, 1983) . A 30-ml-plastic syringe was used to place juveniles into 3-cm-deep holes around the root system, while plants without nematodes each received a 20 ml filtrate from the nematode aliquot in order to establish microbes associated with nematodes. Juveniles were applied at a rate of 2000 per pot, while *P. lilacinus* was applied at about $3x10^4$ spores per each pot. According to the recommended proportion of this crop, plants were irrigated with 50 ml twice a day.

3.7 Data collection

After two months of initiating the experiment, plants were harvested and the follwing parameter were measured

1. Plant length (root + shoot);
2. Plant fresh weight (root + shoot);
3. Plant dry weight (root + shoot),

Plant length , fresh and dry weights were determined and mean values were then calculated. Shoots and roots were oven-dried at 52°C for seven days and weighed (dry weight). Plant length (measured from the soil surface to the terminal end of the flag leaf) was measured. Fresh weight of shoots was determined. Root systems were removed from pots, immersed in water to remove soil particles, blotted dry and weighed (fresh weight).

4. Number of leaves
5. Gall index; (GI)

For the assessments based on root galling, the numbers of galls were counted, and the root systems were rated on a scale of 0-5, where 0= 0, 1

= 1-2, 2 = 3-10, 3 = 11-30, 4 = 31-100, and 5 = >100 egg masses per root system, was used (Taylor and Sasser, 1978).

6. Egg mass index (EMI)

After counting the numbers of galls, the roots were placed in beakers containing approximately 300 ml Phloxine-B for 5 min to stain egg masses a bright red color so the number of egg masses per root system could be determined visually (Holbrook et al., 1983). Egg mass index was on a scale of 0 to 5 as described for gall index.

7. Number of eggs per egg mass

Egg masses were collected and Egg number per egg mass then calculated.

8. Percentage of eggs infected with *P. lilacinus*.

To determine the percentage of eggs infected with *P. lilacinus*, randomly selected Egg masses from the roots of plants were transferred gently onto a clean glass slide. The Egg mass was pressed under a cover slip, so that the eggs were separated and spread. The number of eggs infected with *P. lilacinus* was counted under a light compound microscope and the percentage of infected eggs was calculated after their comparison with a non-treated control group.

3.8 Statistical analysis

The data were subjected to analysis of variance (ANOVA), and mean comparison was conducted using the least significant difference (LSD) test at 5% level of probability. Pearson's correlation coefficients were calculated on the plant parameters and nematode indexes. Differences between means were compared using statistical package for the social sciences (SPSS) version 18.

Chapter 4

Results and Discussion

4.1 Isolation and identification of the *P.lilacinus*

Only three fungal isolates namely, *P.lilacinus*, *Aspergillus flavus* and *Aspergillus niger*, from Rafah, Khan-Yunis-a and Jabalia respectively, were grown on the semi-selective Medium (Table 3.3). No other fungi species were successfully grown on the medium even after 14 days of incubation. One of the successfully grown fungi has ascospores which is characteristic feature of *P. lilacinus*. Growth of colony on PDA plate formed a dense mycelium which gives rise to conidiophores **(**Gerdemann & Nicolson, 1963**).** These bear phialides from the ends of which spores are formed in long chains.

4.1.1 Morphological Identification

Growth of *P. lilacinus* on PDA are rather fast, attaining a diameter of 5–7 cm within 14 days at 28°C ± 2, consisting of a basal felt with a floccose overgrowth of aerial mycelium (Abubakar et all., 2005). Conidial heads appeared first white but gradually became light brownish when sporulated (Fig. 4.1). This colour change is consistent with species of *P. lilacinus* reported by (Samson, 1975). Microscopic observation (400-1000x) revealed smooth walled, hyaline and 2.5–4.0 µm wide vegetative hyphae. Phialides consisting of a swollen basal part, tapering into a thin distinct neck. Conidia are in divergent chains, ellipsoid to fusiform in shape, and smooth walled to slightly roughed. Conidiophores arise from aerial hyphae, have rough wall, and bore vermiculate short branches with whorls of 2-4 phialides. The hyphae was hyaline and bear flask shaped phialides (B) with huge numbers of conidia (A) attached (Fig. 4.2) Chlamydospores are absent agreement with (Ahmad & Jairajpuri, 1993). The incorporation of 3% NaCl to PDA gave a suppressive effect on other fungal species, while maintaining the growth and sporulation of *P. lilacinus*. Streptomycin sulphate and chlortetracycline hydrochloride antibiotics were added to kill a wide range of Gram-negative and Gram-positive bacteria.

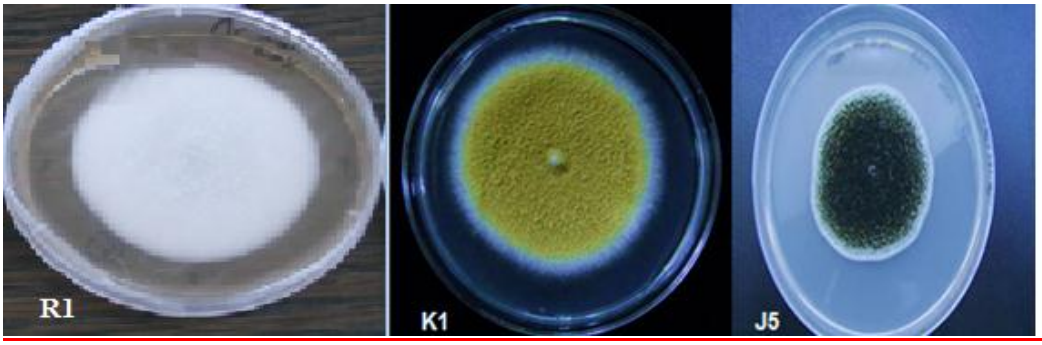

Fig. 4.1: Morphological appearances of *P.lilacinus*, *Aspergillus flavus* and *Aspergillus niger* isolated from soil samples collected from Rafah, R1, Khan Younis, K1 and Jabalia, J5 grown on semi-selective Medium agar plate

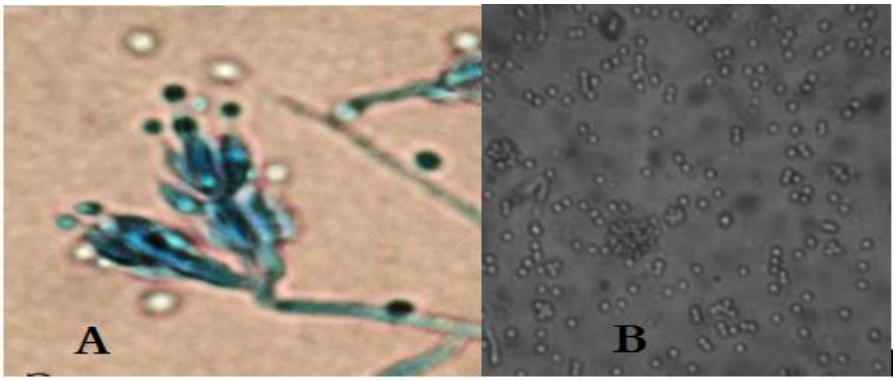

Fig. 4.2: *P.lilacinus* (A) Hyphae bear phialides (B) spores

4.1.2 Molecular identification

The identification of fungal species by classic taxonomy is based mainly on the use of morphological markers. However, it is difficult to depend on these markers especially by non-expert persons. The development of molecular biology techniques for the genetic differentiation of species has resulted in substantial advances in taxonomy due to their sensitivity and specificity. The amplification of certain genomic segment by the polymerase chain reaction, combined with sequencing of the amplicon and analysis of similarity between the sequences obtained and those already deposited in the gene bank, has been frequently employed for identification of fungal species. In this study, the morphologically identified *P.lilacinus* isolated was assayed for amplification using the primer pair TW81 and AB28. These primers, corresponding to a the region of the ribosomal

repeat ranging from the 3' end of the 16/18S ribosomal DNA (rDNA) to the 5' end of the 28S rDNA flanking the ITS1, the 5.8S rDNA and ITS2 sequences, successfully amplified a segment of approximately 500 bp from the genomic DNA of *P.lilacinus* isolates (Figure 4.3). This result agreement with (Inglis & Tigano, 2006) who found PCR products of about 500 bp for some entomogenous *Paecilomyces* isolates by using ITS1-5.8S-ITS2 (TW81-AB28) primers.

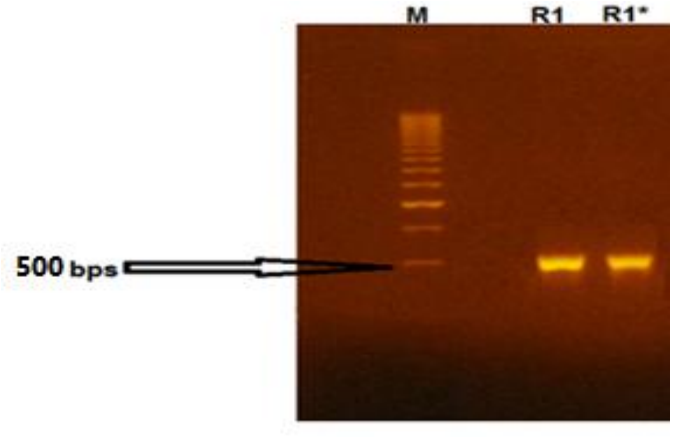

Figure (4.3) Gel electrophoresis of PCR products amplified from DNA extracted from *P. lilacinus* isolated from soil samples collected fro Rafah (R1 and R1*). The PCR products (500 pb) were amplified by the primers ITS1 and ITS4. M = DNA ladder

4.2 The laboratory work (*In-vitro*)

4.2.1 The effect of spore suspension on egg hatch

Egg hatch was not significantly different among the two spore densities (1500 and 3000 spore/ml) after 48 and 72 h of exposure but both densities were significant ($P< 0.05$) with the control (Table 4.1). At 72 h recorded higher egg hatch than 48 h of exposure. Hatching of nematode eggs incubated in the two spore suspension, 1500 and 3000 spore/ml of *P. lilacinus* for two and three days were significantly reduced; where 14 and 18%, and 6 and 7% of eggs were hatched only as compared to control (79 and 88%) after two and three days respectively (Fig. 4.5).

Table (4.1) The effect of different concentrations of fungus spores. *P lilacinus* nematode eggs to hatch

No.	Conc.of *P.lilacinus* spore/ml	Number of eggs *M .javanica* placed in a petri dish -	Number of hatched eggs*	
			After 48 hours	After 72 hours
1-	0 control	100	79[a] ±1.4	88 [a]±0.9
2-	1500	100	14 [b]±1.4	18 [b]±1.8
3-	3000	100	6 [b]±4.3	7 [b]± 5.2

*number are averages of five repeat .Similar letters in each column are not significantly different at (P<0.01) ± standard Deviation.

Eggs treated with spore suspension appeared shrunken, deformed and with multiple vacuoles (Fig.4.4). Most eggs in control treatment appeared empty, with many J2 appeared outside the eggs, indicating successful hatching. This result is in agreement with (Sun et al., 2006) who reported average 58% egg hatch inhibition for their 186 *P. lilacinus* isolates, (Pau et al., 2012) reported that culture filtrate of *P. lilacinus* grown in Czapek broth greatly reduced egg hatching of *Meloidogyne incognita*. According to (Bonants et al., 1995), hatching of eggs containing mature J2 appeared to be stimulated when incubated in culture filtrate of *P. lilacinus* but development of immature eggs appeared to be disrupted. Pau et al.,(2012), found that hatching of nematode eggs incubated in spore suspension of three indigenous isolates of *Paecilomyces lilacinus* namely PLA, PLB and PLM for seven days were significantly reduced; 88-89% of eggs were hatch-inhibited as compared to control (26%).

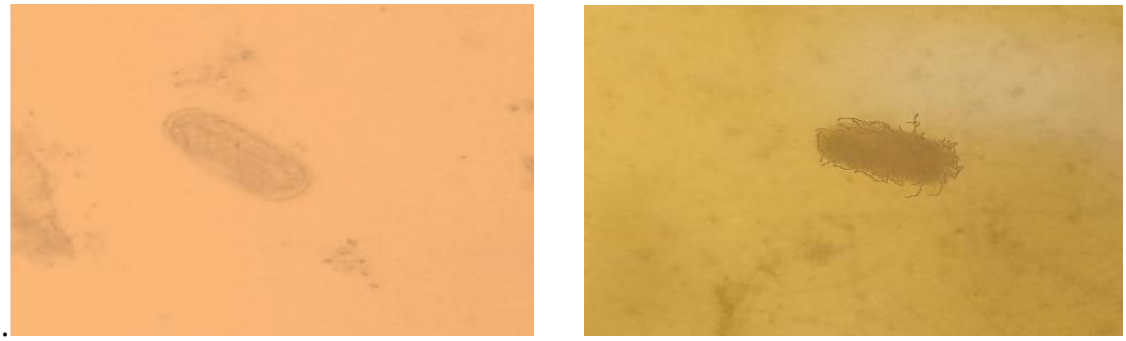

Fig. 4.4 Hyphae emerged from a deformed shape egg with disintegrated embryo after 3 days of incubation (1000 x). It also penetrated into the egg and consumed the egg content.

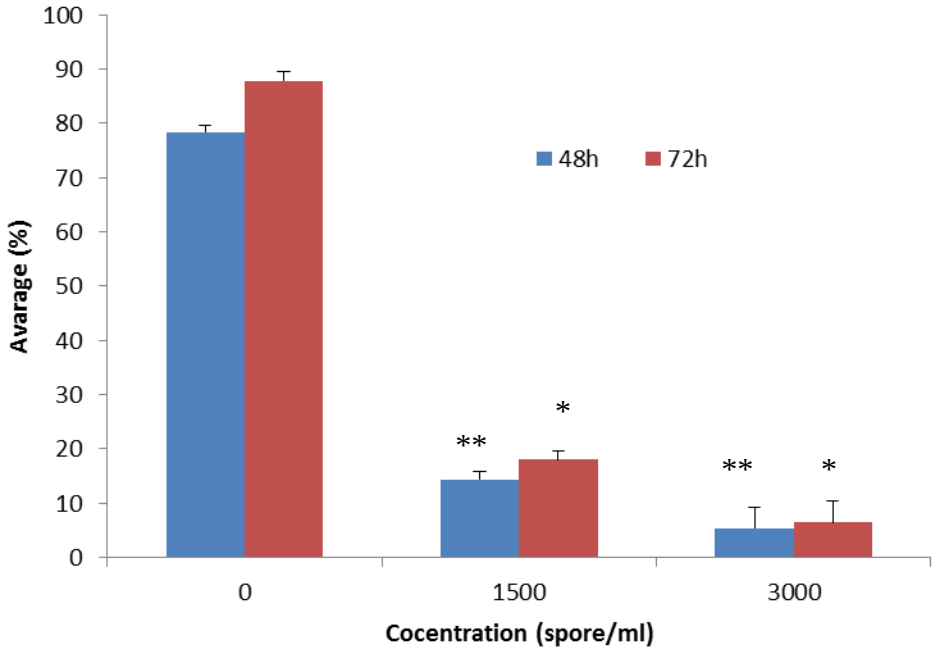

Fig. 4.5 Graph of the effect of different concentrations of fungus spores. *P. lilacinus* nematode eggs to hatch**:** deviation bars = standard deviation.* indicate significant differences when compared with control treated (P <0.05) ** indicate high significant differences when compared with control treated (P <0.01)

4.2.2 The effect of spore suspension on juvenile mortality

In this study, spore suspensions of 1500 and 3000 spore /ml of *P. lilacinus*, (Fig 4.7 **)** demonstrated a significant increase in mortality on juvenile (P< 0.05) as compared with control, with 57% of juvenile mortality after 48 and 72 hours of treatment respectively **(**Table 4.2**)**. This confirmed the findings of(Sun et al. 2006) who reported a high, in vitro nematicidal effect of *P. lilacinus* strain YES-X-2-14 on J2 of *Meloidogyne hapla*. Similarly, Al Kader (2008) reported a high

nematicidal effect of their *P. lilacinus* culture filtrate on J2 of *M. incognita*, with 99% of J2 immobilized after 2 days of treatment. It is suggested that different strain of *P. lilacinus* exhibit different nematicidal effect on J2. To infect juvenile, *P. lilacinus* needs to overcome the cuticle of nematode. The cuticle is a non-cellular layer production of the hypodermis, which consists of keratin, collagen and fibers (Huang et al., 2004). Once the cuticle is penetrated by fungus hypha (Fig. 4.6), the nematode get paralyzed, invaded and digested .

Fig.4.6 Colonization *P. lilacinus*. fungi on female nematode.Hundreds of conidio spores radiating from the body surface.

Table 4.2 the effect of different concentrations of fungus spores *P. lilacinus.* On death nematode larvae newly hatched

No.	**Conc. of *P.lilacinus* spore/ml**	**No. of juvenile M.javanica placed in a petri dish -**	**Number of death nematode juvenile averages of five repeat**	
			After 48 hours	**After 72 hours**
1-	**0 control**	**100**	**4**± 2.4	**7**± 3.1
2-	**1500**	**100**	**24**[a]±0.8	**35**[a]±1.7
3-	**3000**	**100**	**32**[a]±6.7	**57**[a]±5.8

a: indicate significant differences when compared with control treated (P<0.05)± standard Deviation

P. lilacinus is one of the most studied nematophagous fungi species. In the context of its role as biological control it functions either as obligate parasites or facultative parasites (Godoy et al.,1983). The obligate parasites attack the host as

a spore (Hallman et al., 2009). The fungal spores are either adhere or are ingested by a feeding nematode. An infectious hypha grows directly from the spore and penetrates the host through the cuticle or the gastrointestinal tract. On the other hand, the facultative parasites can grow in soil or rhizosphere as saprotrophs and from that state develop specialized spores, conidia or hypha that trap or adhere to nematodes and infect them (Barron, 1977). Many fungal species are known to infect nematodes but only a few are considered suitable as biological control agents (Siddiqui and Mahmood, 1996).

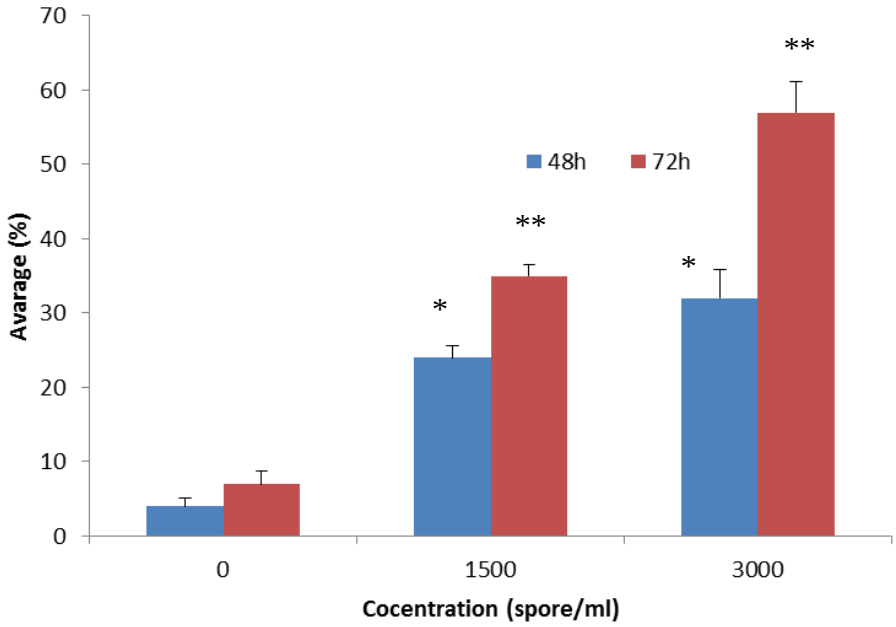

Fig 4.7 graph the effect of different concentrations of fungus spores *P. lilacinus* on death nematode larvae newly hatched.* indicate significant differences when compared with control treated (P <0.05) ** indicate high significant differences when compared with control treated (P <0.01)

Moreover, the fungus suppressed root-knot nematodes regardless of the nematode stage used and the quantity of spore inoculum, although suppression was somewhat greater with eggs than with juveniles and as the inoculum quantity increased. Similar results were obtained by Pau et al., (2012) who found 5.5 – 6% of J2 mortality as compared with 88.2 - 89.4% of egg hatch inhibition for their *P. lilacinus* isolates (PLA, PLB and PLM) on *M. incognita*.

4.3 Greenhouse Experiment.

4.3 .1 Plant length (root and shoot);

Tomato plants inoculated with *M. javanica* (group 2) showed significant reduction in their growth (Table 4.3). Plant lengths were significantly short ($P< 0.001$) in comparison to un-inoculated control (group 1). When tomato plants were inoculated with *P. lilacinus*, there was no significant difference in lengths of the plants in comparison to un-inoculated control (Figure 4.8). In simultaneous inoculation of *M. javanica*, and *P. lilacinus* plant length significantly differed ($P< 0.001$) from the control. When compared to plants inoculated with *M javanica* alone, plant length was significantly greater.

When *P. lilacinus* preceded *M javanica* by one week (group 6), plant lengths differed significantly from the control ($p=0.001$). However, when *M. javanica* preceded *P. lilacinus* (group 5), significant reduction was observed as compared to control and plant length differed from plants inoculated with *M. javanica* alone (group 3) (Figure 4.8).

Table 4.3 also shows the effect of *P. lilacinus* and variuos treatments on the number of leaves of Tomato after two months of planting. There was significant difference in the number of leaves between the plants inoculated with *M. javanica* alone (group 2), *M. javanica* and *P. lilacinus* simultaneously (group 4), *M javanica* preceded *P. lilacinus* (group 5), *P. lilacinus* preceded *M javanica* (group 6) and those of the control (group 1). Treated plants (group 4, 5 and 6) had significantly higher number of leaves than the untreated plants (group 2).There was no significant differences in the number of leaves between the control (group 1) and plant treated with *P. lilacinus* alone (group 3). When *P. lilacinus* preceded *M javanica* by one week (group 6), the number of leaves differed significantly from those groups inoculated with *M. javanica* and *P. lilacinus* simultaneously (group 4) and *M. javanica* preceded *P. lilacinus* (group 5).

Table 4.3 Effects of *P. lilacinus* on shoot and root lengths of tomato

Group no.		Shoot length (cm)	Root length (cm)	Plant length (root + shoot)	Numberof leaves/plant)
1-	Control	57.8 [a]	25.6[a]	83.4	69.1[a]
2-	*M.javanica* alone	23.7 [c]	16.2[b]	39.9	39.7[b]
3-	*P.lilacinus* alone	60.1 [a]	29.5[c]	89.6	70.2[a]
4-	*M.javanica* + *P. lilacinus*	47.2 [b]	18.7[d]	65.9	49.6[c]
5-	*M.javanica* then *P. lilacinus* after 7 day	41.6 [d]	15.7[b]	57.3	45.9[c]
6-	*P. lilacinus* then *M.javanica* after 7 day	49.6 [b]	24.3[f]	73.9	58.9[d]

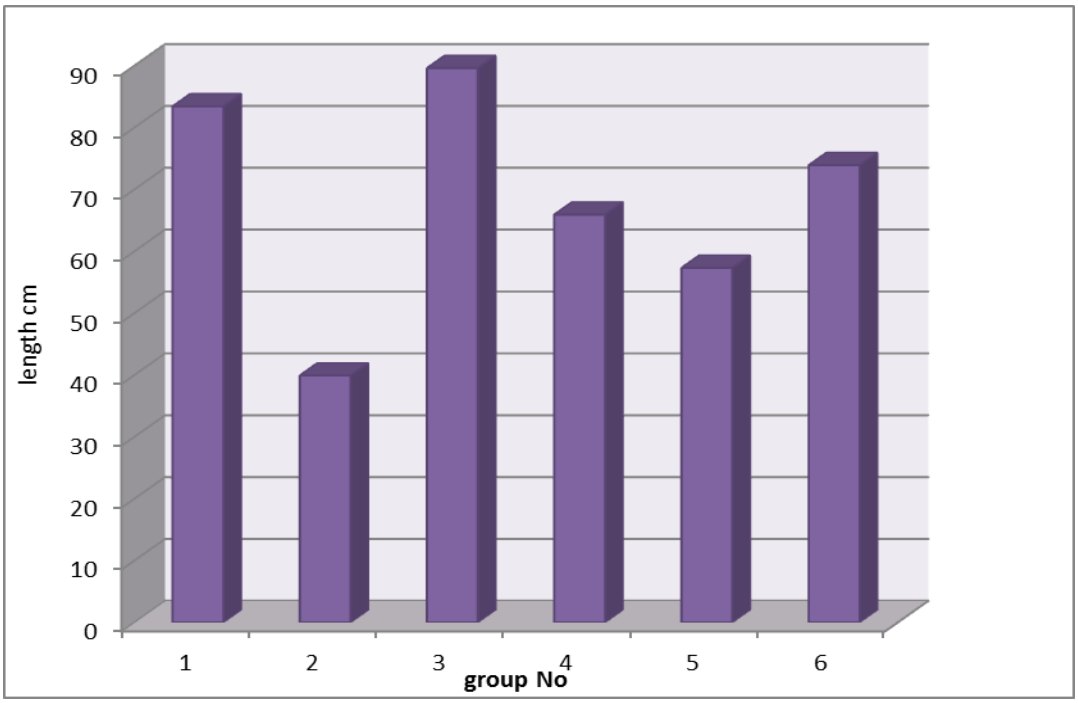

Figure 4.8 graph show the effects of *P. lilacinus* on tomato plant length

4.3.2 The fresh and dry weights of plants

The various treatments showed a similar trend as length. Plant fresh and dry weights were significantly poor (P= 0.05) in comparison to un-inoculated control **(**Table 4.4). When tomato plants were inoculated with *P. lilacinus* (group 3), there was no significant difference in fresh and dry weights of the plants in comparison to un-inoculated control.

Significant reduction (P< 0.001) in fresh and dry weights however occurred due to the infection of *M javanica* (group 2). When *M. javanica* and *P. lilacinus* were inoculated simultaneously (group 4), fresh and dry weights of plants were significantly greater than plants inoculated with *M. javanica* alone (group 2) and the weights differed significantly from the control (group 1). In sequential inoculations, when *M javanica* was inoculated one week prior to *P. lilacinus* (group 5), fresh and dry weights of plants did not differ from plants inoculated with *M javanica* alone. On the other hand, when *P. lilacinus* preceded *M javanica*, fresh and dry weights were significantly greater than plants inoculated with *M javanica* alone and *M javanica* followed by *P. lilacinus* (Figure 4.9). There was also a high correlation between fresh and dry weights and two of nematode indexes (p=0.01) (Table 4.4).

Table 4.4 Effects of *P. lilacinus* on fresh and dry weights of shoot and root of tomato

		DW		FW	
Group no.		**Shoot**	**Root**	**Shoot**	**Root**
1-	**Control**	**6.3** [a]	**3.4** [a]	**37.4** [a]	**15.2**[a]
2-	***M.javanica* alone**	**3.2** [b]	**1.2**[c]	**15** [c]	**8.4**[b]
3-	***P.lilacinus* alone**	**6.8** [a]	**3.9** [a]	**36.5** [a]	**16.5**[a]
4-	***M.javanica* + *P. lilacinus***	**3.9** [c]	**2.4** [b]	**31.2** [b]	**12.3**[c]
5-	***M.javanica* then *P. lilacinus* after 7 day**	**3.7** [b]	**1.8** [c]	**23.8** [c]	**9.3**[b]
6-	***P. lilacinus* then *M.javanica* after 7 day**	**5.8** [a]	**3.2** [d]	**33.4** [f]	**13.7**[c]

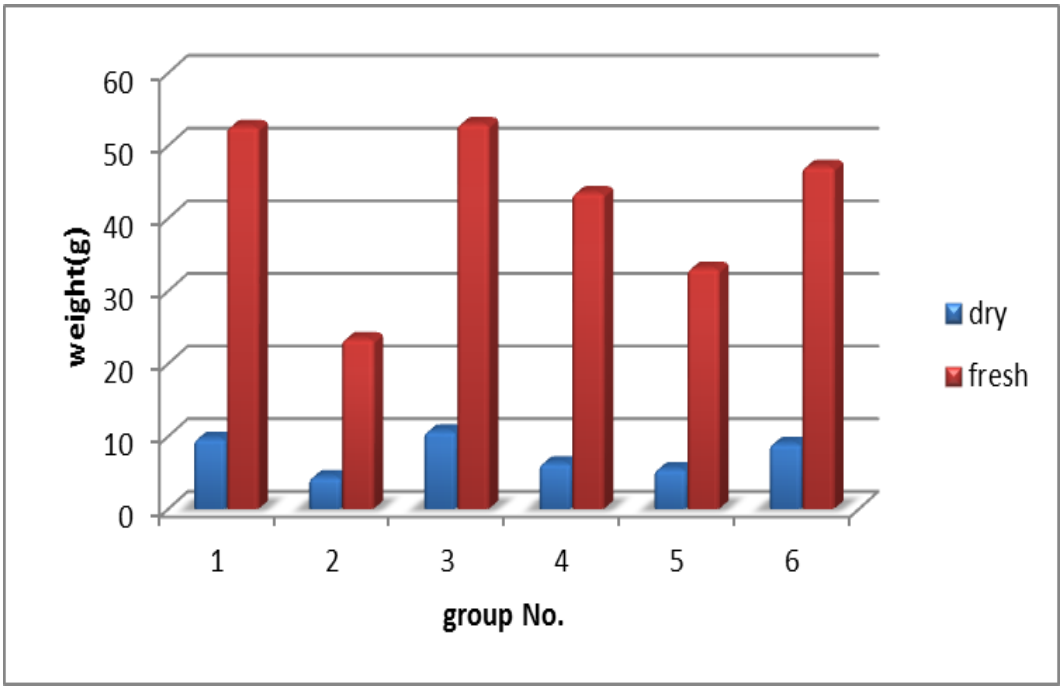

Figure 4.9 graph show the effects of *P. lilacinus* on fresh and dry weights of tomato plant

M.javanica readily infected tomato, retarded its growth, and reduced the fresh and dry weight of the plants. In inoculation, where *P. lilacinus* preceded *M. javanica* the adverse effects of *M. javanica* were greatly reduced and plant growth was as good as un-inoculated plants. Apparently, *P. lilacinus* was effective in suppressing *M javanica*, but time of application was important. The fungus was effective in reducing the resulting population of *M. javanica*. This proved the abilty of *P. lilacinum* as a bio-control agent of *M. javanica*. Several studies indicated that *P. lilacinus* exhibited a clear enhancement on plant growth (Khalil, 2013).

4.3.3 Root-galling and Egg mass production:

The inoculation of *P. lilacinus* reduced root-galling and Egg mass production of the nematode as indicated by gall index (GI) and egg mass index (EMI) as compared to plants inoculated with *M. javanica* alone (group 2). In simultaneous inoculation of *M. javanica* and *P. lilacinus* (group 4) GI/EMI were 4/4 in comparison to 5/5 in *M javanica* inoculated plants (Fig 4.9.1). Similar reduction

in GI and EMI values were observed, when *P. lilacinus* preceded *M. javanica* (group 6) in sequential inoculation (GI / EMI = 4/3). In other sequential inoculations, when *P. lilacinus* followed *M. javanica* (group 5) GI / EMI were slightly reduced (5/4) (Table 4.5).

Table 4.5 Effects of *P. lilacinus* on nematode (*M. javanica*) indexes (gall and egg mass indexes), number of egg per egg mass and percentage of infected eggs

Group no.	Group description	No. of Gall/root	GI	No. of egg masses/root	EMI	No. of egg/egg mass	Percentage of egg infected
1-	**Control**	0.0^{a}	0	0.0^{a}	0	0.0^{a}	0.0^{a}
2-	***M.javanica* alone**	145.4^{b}	5	99.8^{b}	5	178.2^{b}	0.0^{a}
3-	***P.lilacinus* alone**	0.0^{a}	0	0.0^{a}	0	0.0^{a}	0.0^{a}
4-	***M.javanica* + *P. lilacinus***	63.7^{c}	4	39.4^{c}	4	60.3^{c}	66.1^{b}
5-	***Meloidogyne* then *P. lilacinus* after 7 day**	105.3^{d}	5	67.5^{d}	4	93.4^{d}	47.5^{c}
6-	***P. lilacinus* then *M.javanica* after 7 day**	50.1^{c}	4	28.8^{c}	3	43.5^{c}	75.5^{b}

GI = Gall index; EMI = Egg mass index

The performance of plants in relation to root-galling and egg mass production was significantly better in simultaneous inoculation or in inoculation, where *P. lilacinus* preceded *M javanica*. Kiewnick and Sikora (2006) recorded that the pre-planting soil treated with *P. lilacinus* strain 251 (PL251) reduced root galling by 66% and number of egg masses by 74%. *P. lilacinus* was significantly reduced the galls number, egg masses and eggs per egg mass. Prior inoculation of *M. javanica* followed by *P. lilacinus* however, was not that effective. Simultaneous inoculation of tomato plants with *M. incognita* and *P. lilacinus*, and in

inoculation where *P. lilacinus* preceded *M javanica* (group 6) may provide biological protection of root surfaces from invasion by *M. javanica*. The low numbers of galls (63.7 and 50.1 galls/root) and the low numbers of egg masses per root in the tow treatments (60.3 and 43.5), as compared with those with the nematode without the fungus (145.5 galls/root and 99.8 egg masses/root), can be attributed to the colonization of eggs by *P. lilacinus* which apparently inhibited egg hatching and prevented root penetration by *M. incognita* juveniles (Dunn et al., 1982; Cabanillas et al., 1988). The ability of the fungus to grow on the host surface as an epiphyte, or within the host cells as an endophyte makes it a good candidate as an agent for biological protection of plant surfaces. This approach seems to offer the greatest potential use for commercialization for both field and high value ornamental and fruit crops (Cabanillas et al., 1988).

Fig 4.9.1 Nodes root on plant tomato group

A low percentage (47.5%) of Eggs was infected (Table 4.5), though the root-galling and Egg mass production were not poor (Table 4.5). This was primarily because the fungus was not present in the soil at the time the juvenile penetrated the roots. Consequently, plant growth suffered, but the population growth of the nematode was suppressed.

For the fungus to be able to control a plant parasitic nematode population it has to colonise the rhizosphere of the nematodes' host plant (Bourne et al., 1996). The efficacy of the fungus as an agent for biological protection however appear to depends on the correct application time of *P. lilacinus*. Its presence in the rhizosphere of roots at the time of penetration may reduce the number of juveniles that could ingress the roots (Nasr et al., 2007).This finding is in agreement with Holland et al, who stated that *P. lilacinus* colonized the root and protects its surface from root knot nematode attacks (Holland et al., 2003) .

4.3.4 Infections of eggs:

In all the treatments, where *M. javanica* and *P. lilacinus* were added simultaneously or sequentially (Table 4.5), a large number of eggs were infected with *P. lilacinus*. The highest percentage of infected eggs (75.5%) was found when P. lilacinus was added prior to *M. javanica* (group 6). This was followed by the treatment, where both were added simultaneously (66.1%). The lowest percentage of infected eggs (47.5%) was noticed when *M. javanica* was followed by *P. lilacinus* in sequential inoculation. In the present study, it was found that *P. lilacinus* penetrated the eggs and developed profusely inside and over the eggs completely inhibiting juvenile development **(**Figure 4.9.2). Infected eggs contained mycelium inside as well as over their surface. Most of the infected eggs were devoid of juveniles. In some eggs, juveniles were present, but showed various degrees of deformity and abnormal development. A number of juveniles that emerged from the eggs were infected and showed mycelia growth over their body.

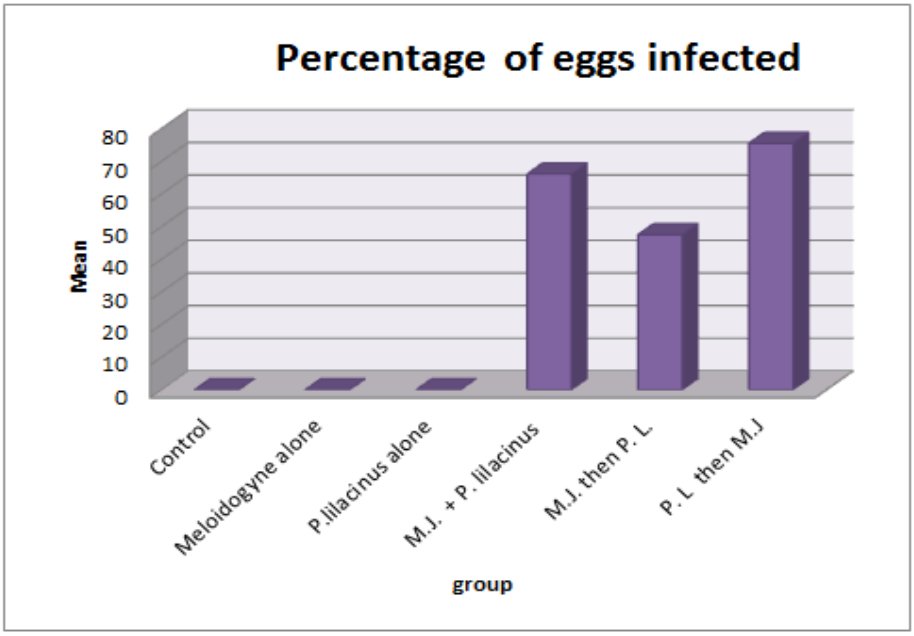

Figure 4.9.2 Comparison of the effect of *P. lilacinus* on of percentage of eggs infected. Vertical bars represent mean at ($P < 0.05$).

P. lilacinus, a saprophytic soil-inhabitant is not expected to cause any harm to plant roots in general and is not a plant entophyte, as was true in these trials too. But, when *M. javanica* eggs, Egg mases and juveniles were present, it attacked and destroyed them to a great extent, thereby ameliorating plant growth. It is clear that, fungal hyphae of *P. lilacinus* penetrate eggshells of *M javanica* with enzymes and pressure following the formation of a simple appressorium. The entire contents of the egg are then used as a food resource by the fungus, completely destroying the embryo/larva in the process. Eggs containing embryos or larvae can then become infected by the fungus (Alamgir et al., 1997).

4.4 Pearson's correlation coefficient of plant parameters and the two nematode indexes

Table 4.6 showed very high significant correlations (P=0.01). Therefore, it is plausible to expect that the presence of *P. lilacinus* before the nematode attack would offer greater protection to plants. Similar conclusions were drawn by (Nasr Esfahani, 2007) who investigate the effects of application of *Paecilomyces lilacinus* on the pathogenesis of *Meloidogyne javanica* on tomato plant growth parameters.

Table 4.6 Pearson's correlation coefficient of plant parameters and nematode indexes

Parameters	Plant length	Plant fresh weight	Plant dry weight	GI	EMI	Infected eggs	eggs/egg mass	No. of leaves
Plant length	1	.812**	.763**	-.852-**	-.800-**	-.077-	-.864-**	.836**
Plant fresh weight		1	.667**	-.779-**	-.697-**	.021	-.827-**	.734**
Plant dry weight			1	-.815-**	-.743-**	-.161-	-.743-**	.744**
Gall index				1	.963**	.318*	.837**	-.885-**
Egg mass index					1	.492**	.750**	-.837-**
Infected eggs						1	-.045-	-.239-
eggs/egg mass							1	-.779-**
No. of leaves								1
****. Correlation is significant at the 0.01 level.**								
***. Correlation is significant at the 0.05 level.**								

GI = Gall index; EMI = Egg mass index

The action of *P. lilacinus* against plant parasitic nematodes was interpreted in multitude investigations. Khan, et al.,(2006) recorded the directed penetration of fungal hypha to the female cuticle of *M. javanica* by transmission electron microscopy. While, Park et al.,(2004) reported that *P. lilacinus* could be produce leucino toxin and other nematicidal compounds. In the laboratory test this fungus infested eggs of *M. incognita* and destroys the embryos within 5 days because of simple penetration of the egg cuticle by individual hypha aided by mechanical and/or enzymatic activities, in addition to killing juveniles and females of *M. incognita* and Globodera pallida (Jatala, 1986). It was mentioned that *P. lilacinus* caused substantial egg deformation in *M. incognita,* these deformed eggs never matured or hatched (Jatala et al., 1985). The serine protease produced by *P. lilacinus* might play a role in penetration of the fungus through the egg shell of the nematode (Bonants et al., 1995).

Although our isolate of *P. lilacinus* shows some potential as a bio-control agent, but we must continue to study the conditions necessary to increase its efficacy.It is also important to know the fate of the bio-control agent after it has been applied to the soil (Nasr Esfahani and Ansari Pour, 2007). Estimation of the persistence of *P. lilacinus* after application to the soil indicated that, the levels

fall after application and it disappears from the soil after a few months of inoculation. This suggests that *P. lilacinus* will only cause short-term disturbances to the soil biota and will not have any long-term effect as other bio-control agents do (Kerry & de Leij, 1992; Lackey et al., 1994). Therefore, the fungus is a potential bio-control agent attacking the infective units of the root-knot nematodes (eggs and juveniles), checking the initiation of the disease and reducing the inoculum potential for successive crops. This two-pronged effect of the fungus is its most significant attribute.

Chapter 5

Conclusions and Recommendations

Conclusion

- *Paecilomyces lilacinus* is a soil-inhabiting fungus that is capable of parasitizing nematode eggs, juveniles and reducing soil --populations of plant parasitic nematodes
- The results obtained from our experiments revealed that *Paecilomyces lilacinus* effectively controls root-knot nematode. Accordingly, it is to design control program based on this fungal that will successfully manage this pest.
- The results revealed that the size of the inoculums is important which is reflected from the different effect by using different concentration.
- The results support the applicability of this fungal as commercial product in controlling root-knot nematode.

6.2 Recommendation

- It is recommended to use the *Paecilomyces lilacinus* as a biological control of nematode *Meloidogyne javanica* eggs, juveniles and females rather than chemical insecticide.
- We strongly recommend ministry of agriculture and farmers to use this fungal as safe product in biological control of nematode plant disease rather than chemical insecticides to protect tomato crops after more faild experimets.
- It is recommended to carry out training courses for farmers to use this fungal as biological control of *Meloidogyne javanica* in Gaza Strip.
- We also, recommend carrying out additional research work on this fungal as biological control on other economically important plants.

Chapter 6

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Appendix 1: Protocol of fungal DNA isolation

E.Z.N.A.® Fungal DNA Mini Kit Protocols

E.Z.N.A.® Fungal DNA Mini Kit Protocol - Short Protocol

This simplified method allows rapid isolation of DNA from fresh, frozen, or dried specimens for use in PCR reactions. The procedure limits the amount of starting material, so that DNA yields will generally be lower than those obtained with the previous protocols. The short protocol is not recommended for Southern analysis or cloning work, as in most cases there will be insufficient material.

Materials and Equipment to be Supplied by User:

- Microcentrifuge capable of at least 10,000 x *g*
- Nuclease-free 2 mL microcentrifuge tubes (Cat# SSI-1310-00)
- Water bath capable of 65°C
- Isopropanol
- 100% ethanol
- Liquid nitrogen
- β-mercaptoethanol
- Optional: 3M NaOH
- Optional: Sterile deionized water

Before Starting:

- Prepare the DNA Wash Buffer according to the instructions on Page 4
- Heat the sterile deionized water and Elution Buffer to 65°C
- For dried specimens: use a maximum of 10 mg ground tissue
- For fresh/frozen specimens: use a maximum of 40 mg ground tissue
- Prepare an ice bucket

1. Prepare tissue in a 2 mL microcentrifuge tube.

2. Add 600 µL FG1 Buffer and 5 µL RNase A. Vortex vigorously to mix. Make sure to disperse all clumps.

3. Let sit for 1 minute.

4. Add 10 µL β-mercaptoethanol. Vortex to mix thoroughly.

5. Incubate at 65°C for at least 5 minutes. Mix sample once during incubation by inverting tube.

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6. Add 140 µL FG2 Buffer. Vortex to mix thoroughly.

7. Let sit on ice for 5 minutes.

8. Centrifuge at 10,000 x *g* for 10 minutes.

9. Transfer supernatant to a new microcentrifuge tube, making sure not to disturb the pellet or transfer any debris.

   Note: In most cases 600 µL supernatant can easily be removed. The volume of supernatant will vary, and is usually lower with dried samples.

10. Add 0.5 volumes FG3 Buffer and 1 volume 100% ethanol. Vortex to mix thoroughly.

    Note: A precipitate may form upon addition of ethanol; it will not interfere with DNA isolation.

11. Insert a HiBind® DNA Mini Column into a 2 mL Collection Tube.

Optional Protocol for Column Equilibration:

1. Add 100 µL 3M NaOH to the HiBind® DNA Mini Column.
2. Let sit for 4 minutes.
3. Centrifuge at maximum speed for 60 seconds.
4. Discard the filtrate and reuse the Collection Tube.

12. Transfer 800 µL sample from Step 10 (including any precipitate that may have formed) to the HiBind® DNA Mini Column.

13. Centrifuge at 10,000 x *g* for 1 minute.

14. Discard the filtrate and reuse the Collection Tube.

15. Repeat Steps 12-14 until all of the sample has been transferred to the HiBind® DNA Mini Column.