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Genotyping and Thermotolerance Characterization of Several Isolates of Entomopathogenic Fungi from Palestine

By

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In Partial Fulfillment of the requirements for the Degree Master of Science

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The undersigned hereby certify that they have read and recommend to the Faculty of Scientific Research and Higher Studies at the Palestine Polytechnic University and the Faculty of Science at Bethlehem University for acceptance a thesis entitled:

"Genotyping and Thermotolerance Characterization of Several Isolates of Entomopathogenic Fungi from Palestine"

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In partial fulfillment of the requirements for the degree of Master of Science in Biotechnology

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"Genotyping and Thermotolerance Characterization of Several Isolates of Entomopathogenic Fungi from Palestine"

By Isra' Omar Ahmad Al_qadi

ABSTRACT

Some of the Entomopathogenic Fungi (EPF) belonging to the genera; *Metarhizium* and *Beauveria* are currently used as biocontrol agents and substitute the harmful chemical pesticides. Four EPF isolates were collected from the Midwest area of the West Bank and identified as *Metarhizium anisopliae* and *Beauveria bassiana* based on morphological traits of the conidia, colony and conidiophores. Also, the infectivity of these isolates to mammalian ectoparasites was investigated in other studies. The main objective of the reported work was to apply molecular-based techniques to assess the morphological-based identification and to differentiate between isolates of the same species in order to have diagnostic tools that could be useful for studying the infectivity of several strains applied simultaneously to the same host. These techniques include sequence analysis of β-tubulin (BT) gene, the Internal Transcribed Spacers (ITS) 1 & 2 of the rDNA, construction of Restriction Fragment Length Polymorphism (RFLP) patterns in an attempt to create an isolate fingerprint, and Inter-Simple Sequence Repeat (ISSR) analysis. Another objective of this work included characterization of the thermotolerance of each Palestinian isolate for the purpose of selecting a proper isolate to control mammalian ectoparasites.

For molecular characterization, sequences of ten isolates of EPF were analyzed for comparison purposes, four of which are *B. bassiana*: B-Bot, the Israeli isolate B-Bug, the Palestinian isolates B-151 and B-Med, and six isolates of *M. anisopliae*: the Palestinian isolates M-Th2, M-153, the Israeli isolates Ma-7, M-2004, M-WG and the Ethiopian one PPRC.

Sequencing data of ITS confirmed six isolates of *M. anisopliae* while the other four were *Beauveria bassiana* isolates. The ITS sequences of the Palestinian *B. bassiana* and *M. anisopliae* isolates were similar to the corresponding sequences of the same species available in the GenBank. Restriction digestion of ITS2 sequences of *M. anisopliae* isolates was able to produce a fingerprint for M.a.153. The ITS and BT sequences failed to differentiate between *Beauveria* isolates except the B-Bot genotype, but ITS discriminated three *Metarhizium* isolates: Ma-7, M-153, and PPRC. However, ISSR analysis detected high level of polymorphism among *Beauveria* (80%) and *Metarhizium* (90%) isolates.

Results of thermotolerance assessment showed that the isolates of the same species varied in their response to elevated temperatures in regard to spore germination and mycelial growth. All Palestinian isolates were capable of growth at temperatures (25 - 32°C) with apparent differences among isolates in their tolerance to the maximal temperature 32°C. At this temperature, the B.b.151 isolate showed high germination rate (99%), and the highest mycelial growth diameter value (18.13 mm $\pm$ 1.56 SD) that is significantly higher than the isolates; B-Med, M-Th2 and M-153 (U=360.0, 130.5, 0.00, P=.041, P<.001, <.001, respectively). In view of the present results, the isolate B-151 appears to be a good candidate for use as biopesticide taking into account its thermotolerance and virulence as documented in other study.

It is recommended to explore larger region of the BT gene and apply additional molecular-based techniques that may produce more variations. Additional research could be done in exploring naturally occurring thermotolerant isolates from the Jordan Valley, where the temperature is higher. Also, efforts should be put to test the ability of an isolate to germinate under optimal temperatures upon exposure to heat shock treatment, and to study the expression of the heat shock proteins genes in the different isolates studied.

Key words: *Metarhizium anisopliae*, *Beauveria bassiana*, morphological, ITS, RFLP, β-tubulin, ISSR, thermotolerance.

"تحديد النمط الجيني ومدى مقاومة الحرارة لعدة سلالات من الفطريات الممرضة للحشرات من فلسطين"

اسراء عمر احمد القاضي

ملخص

يتم حاليا استخدام بعض الفطريات الممرضة للحشرات والتي تنتمي للجنسين: *Metarhizium* و *Beauveria* كعوامل للمكافحة الحيوية كبديل لاستخدام المبيدات الحشرية الكيماوية. في هذه الدراسة، تم عزل اربع من هذه السلالات من شمال الضفة الغربية، ولقد تم تحديد هويتهن على انهن *Metarhizium anisopliae* و *Beauveria bassiana* بناء على الصفات الشكلية لكل من ابواغ التكاثر اللا جنسي والمستعمرة والاجسام الثمرية. بالاضافة الى ذلك، لقد تم – في دراسة اخرى – فحص نجاعة عدوى هذه السلالات للطفيليات الخارجية التي تصيب الثدييات.

هدفت هذه الدراسة الى تطبيق بعض التقنيات الجزيئية للتحقق من هوية هذه السلالات المحددة سلفا على اساس الشكل. كذلك هدفت الى تمييز سلالات النوع الواحد عن بعضها من اجل تطوير ادوات تشخيص جزيئية التي يمكن ان تفيد في فحص نجاعة عدوى عدة سلالات فطرية يتم استخدامها لمكافحة نفس العائل في آن واحد. تشمل هذه التقنيات: تحليل تسلسل ال DNA لكل من؛ الجين (BT) β–tubulin، والفاصل الداخلي المستنسخ Internal Transcribed Spacer (ITS) الاول والثاني في منطقة ال DNA الرايبوسومي، وتقنية انتاج قطع حصرية متعددة الطول Restriction Fragment Length Polymorphism (RFLP) بحيث تشكل بصمة خاصة بسلالة معينة، بالاضافة الى تحليل تكرار التسلسل البسيط المتداخل Inter Simple Sequence Repeat (ISSR). كما هدفت هذه الدراسة الى تحديد مدى مقاومة السلالات الفلسطينية للحرارة كعامل هام في اختيار السلالة المناسبة للسيطرة على الطفيليات التي تعيش خارج اجسام الثدييات. لقد تم تحليل تسلسل ال DNA لعشرة من السلالات الفطرية الممرضة للحشرات بهدف مقارنة تركيبة ال DNA، اربع من هذه السلالات هي *B. bassiana* (B-Bot، السلالة الاسرائيلية B-Bug، والسلالتان الفلسطينيتان B-151 وB-Med) ، وست سلالات هي *M. anisopliae* (السلالتان الفلسطينيتان M-Th2 و M-153، والسلالات الاسرائيلية Ma-7 و M-2004 و M-WG، والاثيوبية PPRC).

كشف تحليل منطقة ال ITS عن وجود تشابه بين تسلسل ال ITS لسلالات ال *B. bassiana* و ال *M. anisopliae* الفلسطينية وتسلسلات ال ITS المخزونة في بنك الجينات GenBank والتابعة لسلالات من نفس النوع. بالاضافة الى ذلك،

كشف تكسير تسلسل ال ITS2 لسلالات ال *Metarhizium* بتقنية ال RFLP عن وجود بصمة للسلالة M-153. لقد فشل تسلسل ال ITS وكذلك ال BT في التمييز بين سلالات ال *B. bassiana* ما عدا ال B-Bot، لكن استطاع ال ITS التمييز بين ثلاث سلالات من ال *M. anisopliae* هي: Ma-7 و M-153 و PPRC، وعلى العكس من ذلك فان تحليل ال ISSR كشف عن اختلافات بنسبة عالية بين سلالات ال *Beauveria* (80%) وكذلك بين سلالات ال *Metarhizium* (90%).

كما بينت الدراسة اختلاف السلالات الفلسطينية التابعة لنفس النوع في استجابتهن لدرجات الحرارة العالية من حيث انبات البوغ و نمو المستعمرة. استطاعت كل السلالات الفلسطينية النمو حتى درجة حرارة 32 ° م مع وجود اختلافات واضحة بينهن في مقاومتهن لهذه الحرارة، فقد سجلت السلالة B-151 نسبة عالية من انبات الابواغ (99%)، وكذلك سجلت اعلى قيمة لقطر المستعمرة (SD 1.56 $\pm$ 18.13 ملم) والتي هي اعلى بكثير من كل من: B-Med، M-Th2، M-153 (*U*=360.0، 130.5، 0.00، *P*=0.041، *P* <001.، <001.، على التوالي). في ضوء هذه النتائج، يبدو أن السلالة B-151 مرشحة جيدة لاستخدامها كمكافح حيوي آخذين بعين الاعتبار مقاومتها للحرارة و فعاليتها التي تم توثيقها في دراسة اخرى.

وفي الختام، استكمالا لهذا العمل يتطلب اجراء مزيد من الدراسات التي تشمل استكشاف اجزاء اخرى من الجين BT، تطبيق المزيد من التقنيات الحيوية التي تكشف عن المزيد من الاختلافات الجينية، بالاضافة الى استكشاف السلالات التي هي بطبيعتها مقاومة للحرارة، او تحفيز مقاومة السلالات المفحوصة للحرارة، ودراسة قدرتها على استئناف النمو بعد التعرض لدرجات حرارة عالية، ودراسة التعبير الجيني لبروتينات الصدمة الحرارية في كل من هذه السلالات.

DECLARATION

I declare that the Master Thesis entitled "Genotyping and Thermotolerance Characterization of Several Isolates of Entomopathogenic Fungi from Palestine" is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgement is made in the text.

Name and signature: Isra' Omar Ahmad Al_qadi _____

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Signature: Isra' Omar Ahmad Al_qadi _____

Date: _____

Dedication

To

My Dear Husband, Children, Parents, Sisters, Brother and My Sister-in-Law

For their patience, support and

encouragement, with love and respect

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List of Abbreviations:

AFLP	Amplified Fragment Length Polymorphism
BLASTn	Basic Local Alignment Search Tool-nucleotide
BT	β-tubulin
EPF	Entomopathogenic Fungi
IGR	Intergenic Region
ISSRs	Internal Simple Sequence Repeats
ITS	Internal Transcribed Spacer
MSA	Multiple Sequence Alignment
NCBI	National Center for Biotechnology Information
Nct.	Nucleotide
NEB	Number of Exclusive Bands
NG	Number of different Genotypes
NPB	Number of Polymorphic Bands
P %	Percentage of Polymorphic bands
PCR	Polymerase Chain Reaction
PDA	Potato Dextrose Agar
RAPD	Random Amplified Polymorphic DNA
rDNA	Ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
SCAR	Strain-specific sequence-Characterized Amplified Region
SDA	Sabaroud Dextrose Agar
SSRs	Simple Sequence Repeats
TNB	Total Number of Bands
UBC	University of British Columbia
UPGMA	Unweighted Pair Group Method using Arithmetic average

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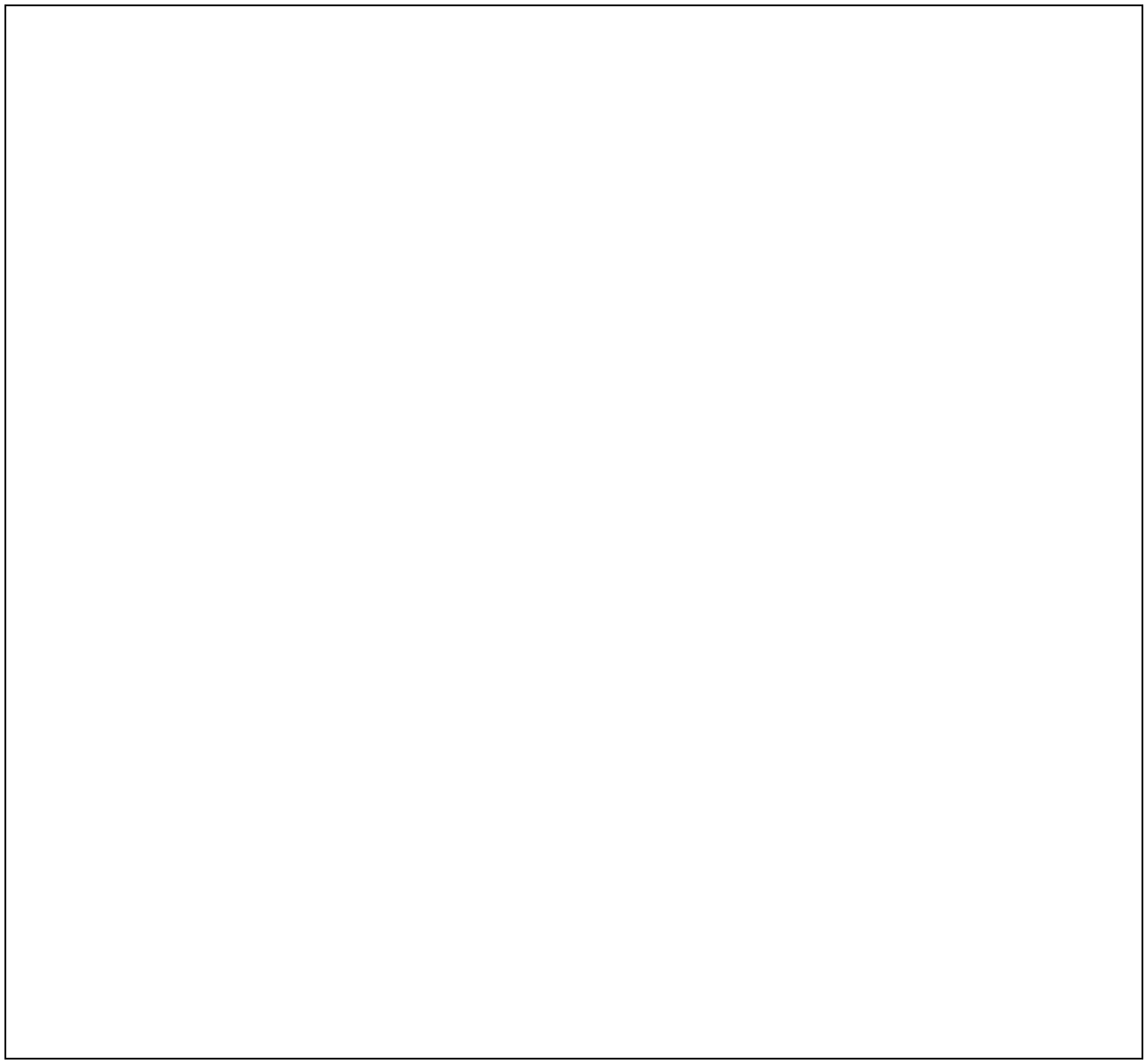


CHAPTER 1

Introduction

1.1 General Introduction To Fungi

1.1.1 Fungi Nutrition and Inhabitation

Fungi are eukaryotes and most are multicellular. They are heterotrophs that acquire their nutrients by absorption. Small organic molecules are absorbed directly from the surrounding, while complex ones are decomposed by hydrolytic enzymes secreted by the fungus. Based on the mode of nutrition, fungi can be grouped into three categories: saprobic fungi that absorb nutrients from non-living organic materials, parasitic fungi that absorb nutrients from the cells of living hosts, or mutualistic fungi that absorb nutrients from a host organism but they reciprocate functions beneficial to their partners (Campbell et al., 1999).

Most fungi inhabit terrestrial habitats, but some inhabit aquatic environments. Most fungi survive successfully in aerobic conditions if supplied with glucose, ammonium salts, inorganic ions, few growth factors, macronutrients (carbon, nitrogen, oxygen, sulphur, phosphorus, potassium and magnesium), and micronutrients (calcium, copper, iron, manganese, and zinc). They also grow successfully on complex carbon-rich media such as malt extract, or potato-dextrose agar, at the acidic pH range (Walker and White, 2005).

1.1.2 Fungal Morphology

The vegetative bodies of fungi consist of mycelia; net-like collections of branched hyphae adapted for absorption. Hyphae are minute long threads, composed of tubular walls surrounding plasma membranes and cytoplasm. Most fungi have their hyphae partitioned into cells by septa, with pores allowing cell to cell continuity. The fungus

concentrates its energy and resources on extending hyphal tips and so increasing the overall absorptive surface area. Most fungi have cell walls made of chitin and the cytoplasm contains the usual eukaryotic organelles.

1.1.3 Fungi Growth and Reproduction

Fungal growth involves transport and assimilation of nutrients, followed by their integration into cellular components leading to biomass increase and an eventual cell division. Mycelial fungi grow as the extension of the hyphal tip occurs. Natural substances like yeast extract and malt extract, promote favorable fungi growth as they contain sugars, proteins, vitamins and minerals, which could be readily assimilated. Fungal culture in the lab involves mainly Potato Dextrose Agar (PDA) and Sabaroud Dextrose Agar (SDA). The simplest method for assessing fungal growth is the linear measurement of the change in the culture radius over a period of time (growth rate). This method is nondestructive and allows repeated observations of the same mycelium. Physical requirements for fungal growth are: high water activity, medium with acidic pH range, light and optimum temperature.

Most fungi grow well at temperatures around 25 $^{\circ}$C. However, when the temperature increases beyond the optimal level, growth rate declines as fungi have no means of regulating their internal temperature. High temperatures decrease enzymatic activity and affect the synthesis of vitamins, amino acids, or other metabolites. Thermotolerance relates to the transient ability of cells subjected to high temperatures to survive subsequent lethal exposures to elevated temperatures. Thermotolerance can be intrinsic that is observed following a sudden heat shock (50 $^{\circ}$C), or induced that occurs only if cells are preconditioned by a brief exposure to sublethal temperatures (37 $^{\circ}$C for 30 minutes) prior to exposure to a more severe heat shock (Walker and

White, 2005). The vast majority of fungi inhabiting temperate regions are mesophiles; thriving at moderate temperatures (10-40 °C) (Moore-Landecker, 1996). Different fungal species as well as different strains of the same species respond differently to temperature (Moore-Landecker, 1996).

Regarding light, most fungi are exposed to alternating cycles of daylight and darkness. While others like parasitic fungi are likely to grow under total darkness.

Fungi reproduce by releasing spores that are produced either sexually (teleomorph or perfect fungi) or asexually (anamorph or imperfect fungi) from specialized hyphal structures. Under favorite conditions, fungi grow rapidly and clone themselves by producing large number of spores asexually. These spores are disseminated usually by air currents, but also by water or animals, and germinate on a moist place giving rise to mycelium. On the other hand, sexual cycle involves cell fusion (Plasmogamy) and nuclear fusion (Karyogamy) with intervening dikaryotic stage (with two haploid nuclei). The diploid phase is a short-lived stage and rapidly undergoes meiosis to produce haploid spores.

1.1.4 Fungi Genetics

The genome of fungi is relatively of small size, ranging from 12-88 Mba (Griffin, 1994). The number of chromosomes is difficult to determine due to their small size and lack of condensation. However, estimates of chromosome number in fungi range from 2-18 chromosomes in haploid cells (Moore-Landecker, 1996). Despite the small size of the fungal genome, fungi are characterized by a broad range of diversity.

1.1.5 Fungi Classification and Diversity

There are about 100,000 fungal species that are already known. In addition, it is estimated that another 1.5 million species are available worldwide (Campbell et al., 1999). Based on the mode of reproduction, fungi are classified mainly into four

divisions: Chytridiomycota, Zygomycota, Ascomycota, and Basidiomycota. The latter three reproduce sexually while forming fruiting bodies known as; zygosporongia, ascocarps, and basidiocarps, respectively. Fungi belonging to Ascomycota, maintain their sexual stages in macroscopic fruiting bodies or ascocarps. Also, they reproduce asexually by producing conidia (naked spores) at the end of hyphae in long chains or clusters called conidiophores. Fungi are distributed world wide due to light spores that can be carried by air for very long distances.

A rapidly growing and asexually reproducing fungus is known as mold. But same fungus may go through sexual reproduction in its life cycle, which constitutes the basis for its classification. However, there are molds that cannot be classified as zygomycetes, ascomycetes, or basidiomycetes as they have no known sexual stage. Those molds have been traditionally called deuteromycetes, or imperfect fungi.

Most of deuteromycetes are the non-sexual stages, or anamorphs, of the sexually reproducing fungi that belong mostly to Ascomycota. The reproduction of deuteromycetes relies on conidia, which are produced by specialized cell called conidiogenous cell that is supported by specialized hyphae called conidiophore. Conidiophores are usually morphologically distinct from the somatic hyphae. They are light or dark, unbranched or branched in a complex manner. These conidiophores are arranged as single, cluster or in succession. Conidia are often round, ellipsoid, sickle- shaped or coiled. Moreover, conidia come in a variety of colors: white, blue, green, yellow, brown or black.

Later on, molecular biology assists in assigning an unknown isolate to its appropriate species. This is of particular importance in case of isolates from deuteromycetes and yeasts, where data about sexual reproduction is absent which complicates species delimitation. The study of the taxonomy of fungi is extremely important as it allows

their identification and classification which are major requirements for applying a biocontrol system.

1.2 Biocontrol

1.2.1 Chemical Pesticides versus Biocontrol

Pests like nematodes, weeds, and insects have the potential to decrease yield of crop plants. Till several decades ago, the main measures used to control these pests were chemical pesticides due to their rapid effect. However, there are several drawbacks (Wainwright, 1992; Moore-Landecker, 1996) to the extensive use of these toxic chemicals. First, the use of these chemicals is expensive and their continued release into the environment raises concerns about their hazards to ecosystems. Second, these chemicals usually have an indiscriminate toxicity and can harm beneficial insects, some of which could be an endangered species. Third, toxicity effects could reach animals, as well as humans through food chains. Finally, chemical pesticides are not always effective, and many pests develop resistance to them.

A promising substitute for the hazardous chemical pesticides is the use of biological control. This method involves the application of environmentally benign natural enemies of pests to reduce the pest population to a level below the threshold that causes economic damage. There are many environmentally safe organisms that can be used as biocontrol agents. Among them are: nematodes, viruses, bacteria, insects and some of the entomopathogenic fungi (EPF). In contrast to the indiscriminate toxicity of chemical pesticides, biocontrol agents are host-specific and do not harm other beneficial insects. Further, in contrast to chemical pesticides, biocontrol agents do not cause production of resistant insects.

A fungal biocontrol agent must be applied in a suitable formulation to ensure its dispersal and adherence to the host. For example, *Metarhizium* formulation is a

mixture of spores, diluent, wetting agent and a sticker (Samson et al., 1988). The fungi *Beauveria bassiana* and *Metarhizium anisopliae* have been known to possess capacities to control a wide range of insects. Example of these insects is *Alphitobius diaperinus* (Geden and Steinkraus, 2003; Rohde et al., 2006; Gindin et al., 2009).

The use of native EPF strains to control pests in a given area will be of a greater advantage over the use of exotic strains. This is mainly because of the fact that local strains are adapted to the climatic conditions prevailing the concerned area. In Palestine, there are three different climates; Mediterranean, semiarid and arid. Hence, there is a need for isolating biocontrol agents from each of these areas to allow a broad application of this biotechnology. Moreover, the introduction of exotic fungi strains could be politically as well as ecologically problematic (Bidochka, 2001).

1.2.2 Biocontrol in Palestine

Biocontrol research and application in Palestine stills in its infancy. The most part of this activity is research, while commercial production and field application have not been achieved yet. Statistically, 12.2% of agriculture holdings in the Palestinian Territory use some biological control regimes, while 38.5% use pesticides (Palestinian Central Bureau of Statistics, 2005).

A study that may benefit the field of biocontrol application in Palestine, was what had been done by Ali-Shtayeh and his colleagues (2002). They studied the distribution, occurrence and characterization of entomopathogenic fungi in an agricultural soil in the Palestinian area. They found that *Conidiobolus coronatus* is the most abundant entomopathogenic species recovered. Moreover, it had a wide tolerance to agricultural practices, and was characterized by high mycelial growth rate, conidial production and germination. The authors claimed that this fungus could be a good candidate for pest control in agricultural soils. In addition to this kind of

studies, several laboratories in Palestine focus their research mostly on testing the efficiency of entomopathogenic fungi as biocontrol agents.

For instance, the Faculty of Agriculture at Hebron University had studied the potential of *Trichoderma* spp. isolates, recovered from Palestinian agricultural fields, to control the soil-borne phytopathogen *Rhizoctonia solani* (Barakat et al., 2007). Some isolates had been reported to be very efficient. Moreover, other *Trichoderma* spp. isolates from Palestine were found to be effective in controlling the phytopathogenic *Sclerotium rolfsii* (Barakat et al., 2006).

Also, researchers from the Faculty of Agriculture at An-Najah University had studied the bioprotection of stored grains from harmful insects by application of mycoinsecticides like the entomopathogenic fungi *Metarhizium anisopliae* (Batta, 2005) and *Beauveria bassiana* (Batta, 2008). Also, bioprotection of fresh produced fruits and vegetables from fungal rot pathogens was obtained by application of mycofungicides like the antagonistic fungus *Trichoderma harzianum* (Batta 2006, 2007).

Finally, at the UNESCO Educational and Research Center at Bethlehem University, seventy-six soil samples from West Bank were surveyed for the presence of entomopathogenic fungal species using *Galleria*-baiting method (Mahassneh, 1999). Seventeen strains belonging to seven fungal species were isolated, and their thermotolerance as well as their infectivity to *Galleria* were characterized. In addition, four Palestinian isolates of *Beauveria bassiana* and *Metarhizium anisopliae* were evaluated for their capacity to control the cattle tick *Rhipicephalus annulatus* (Ment et al. 2009a, b).

Most of the studies on entomopathogenic fungi in Palestine focused on assessing their infectivity to pests. However, studying the taxonomy and genotyping of these biocontrol agents at the molecular level has never been conducted.

1.3 Entomopathogenic Fungi (EPF)

Some of the EPF have been licensed as biocontrol agents since they act as insect parasites without causing any damage to the environment. A typical life cycle of an EPF involves: first, the attachment of the spores to host cuticle, then under permissive conditions like high humidity and optimal temperature, these spores germinate, grow as hyphae, penetrate the insect's cuticle by mechanical pressure and hydrolytic enzymes, eventually they bore through it and reach insect hemocoel (body cavity). After some time the insect is usually killed (sometimes by fungal toxins) and new spores are formed in/on the insect if environmental conditions are again permissive (Samson et al., 1988).

For many insects there is at least one antagonistic fungus. Furthermore, such fungi have also been evaluated as biological control agents for more than 200 economically important insects species (Tanada and Kaya, 1993; Maurer et al., 1997; Zurek and Keddie, 2000). They are generally non-specific in their action, and so can infect wide range of pests. Moreover, they can survive and resist adverse environmental conditions for long time as resting bodies, and so no need for reinoculation.

There are over 400 fungal species that can attack insects and mites (Wainwright, 1992). In 1990, in contrast, only four fungal species have been used commercially as biocontrol agents against arthropods and were registered properly (Ferron et al., 1991). The selection of a given EPF strain for biocontrol use depends mainly on the aggressiveness of the fungus to the targeted pest but not to the beneficial ones. Also, it must have high level of sporulation capacities. So far, the most intensively studied and

field applied EPFs belong to the genera *Beauveria* and *Metarhizium*, in the order Hypocreales of the Ascomycota (Figure 1.1).

Figure 1.1: Green vegetable bug infected with isolates of *Metarhizium* (to the left) and Mirid infected with isolates of *Beauveria* (to the right). Source: (Entomology Team (Field Crops), 2010).

1.3.1 *Beauveria*

Muscardine is a devastating disease afflicted the European silkworm industry in the 18th and 19th centuries. This disease transforms insects into a white mummy resembling sugared almond. Also, it is caused by *Beauveria bassiana* which is perhaps the most widely studied and used as EPF. It infects more than 700 insect species, most of them are economically important pests (Goettel et al., 1990; Moore-Landecker, 1996). This species is regularly used to control the pine caterpillar in about 100,000 acres in the 13 southern provinces in China (Wainwright, 1992). Also, there are at least 140 tons of oil-based fungal inoculums produced annually in this country (Moore-Landecker, 1996).

Chromosome number and nuclear genome size have been determined for several different isolates of *B. bassiana*. Pfeifer and Khachatourians (1993) observed eight chromosomes and estimated a nuclear genome size of 40.6 Mb for a single *B. bassiana* strain. On the other hand, Viaud et al. (1996) estimated that there are seven or eight chromosomes per haploid genome, depending on the strain. Genome size estimates vary between 34.1 and 44.1 Mb among the seven strains examined. These

estimates of genome size are similar to those reported for *Metarhizium anisopliae* (Shimizu et al., 1992).

1.3.2 *Metarhizium*

Metarhizium causes a disease in insects known as green muscardine. *Metarhizium* species are also known to infect more than 200 insect species, many of which are major agricultural and forest insect pests such as spittlebugs, sugar cane borers, termites, scarab grubs, and grasshoppers (Leger, 1993). Application of *Metarhizium anisopliae* was effective in reducing the population of leafhoppers and froghoppers in pastures and on sugar cane in Brazil (Moore-Landecker, 1996). Also it was used to control a serious pest of coconut in the Pacific (Wainwright, 1992).

The conidia of *Metarhizium* tend to aggregate into a palisades layer that can be blown about as one unit. The conidia are hydrophobic and tend to readily detach from colonies in dry air. Some *Metarhizium* strains are specific to a certain insect host, while other strains show a wide host range. This fungus could be distributed through infected insects, or transported by humans on fruit and vegetables.

1.4 Morphological Characterization

The Study of biological aspects of natural populations requires use of reliable tools of species identification. In the case of entomopathogenic fungi, researchers have traditionally utilized morphological characteristics, such as shapes and sizes of conidia, to distinguish between species of the two genera *Beauveria* (Glare and Inwood, 1998) and *Metarhizium* (Welling et al., 1994).

1.4.1 *Beauveria*

Beauveria is easy to distinguish morphologically. Its most distinctive characteristics are sympodial to whorled clusters of short-globose to flask-shaped conidiogenous

cells that produce a succession of one-celled, rounded to ovoid, sessile, hyaline, holoblastic conidia on a progressively elongating zig-zag rachis as shown in Figure 1.2 (Barnett, 1962; Kirk et al., 2001). In culture, *Beauveria* species typically produce white mycelium and conidia. Colony growth tends to be rapid, and mycelium texture is typically lanate to woolly (Kirk et al., 2001). Conidia in culture can often be copious, frequently creating a chalky, mealy, or powdery appearance at the colony surface.

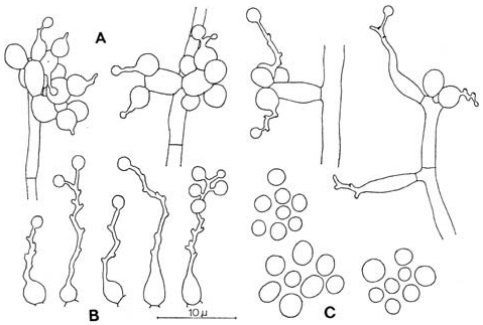


Figure 1.2: Forms of *Beauveria bassiana* asexual-reproduction microstructures. A. conidial structures; B. conidiogenous cells; C. conidia. Source: (de Hoog, 1972).

As *Beauveria* species solely reproduce by production of conidia, they were traditionally classified as hyphomycetous asexual fungi (Deuteromycetes or Fungi Imperfecti). But later on, the genetic data gathered by molecular methods allowed placing them in the sexually reproducing counterparts that are members of the *Cordyceps* genus. Despite the conduction of intensive taxonomic analyses of *Beauveria* during the last century, significant problems in the identification, taxonomy, and nomenclature of species in this genus still exist. *Beauveria* species identification is problematic because of a lack of informative morphological variation. The conidial form is the only morphological feature of *Beauveria* that is useful for species delimitation. Conidia vary from globose, ellipsoid, cylindrical, reniform, to vermiform and range in diameter from 2 to 4 µm (DoctorFungus, 2007). Unfortunately, overlapping variation in the size and shape of conidia among many of *Beauveria* species has complicated routine species identification.

1.4.2 *Metarhizium*

Metarhizium conidia appear as olive-green when being in mass, one-celled, produced in basipetal chains, compacted into columns, and long-ovoid to cylindrical in shape. Conidiophores are branched, erect, closely or loosely grouped, forming sporulating layer. Sporogenous cells (phialides) borne singly, in pairs or in whorls (Barnett, 1962).

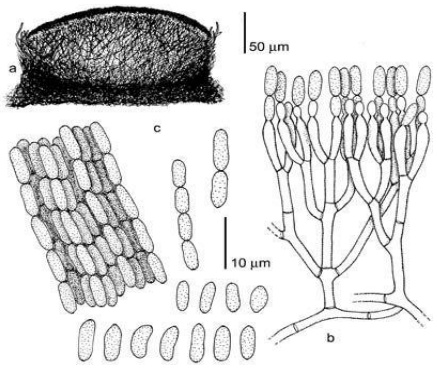


Figure 1.3: Forms of *Metarhizium anisopliae* asexual-reproduction microstructures. A. Sporodochium; B. conidiophores; C. Conidia. Source: (Crous et al., 2004).

The classification of *Metarhizium* has been based on morphological characteristics like conidial lengths, which range from 5-8 µm (Tulloch, 1976), the presence or absence of a subhymenial zone, formation of prismatic columns by laterally adhering conidia, and the color of the conidia and the fungal colony (Humber, 1997).

1.5 Molecular Characterization

When implementing biological control using entomopathogenic fungi, it is very important to have an efficient system of identification to identify the applied fungal isolates (Coates et al., 2002a). The identification is necessary for measuring their efficacy and detecting multiple isolate infections in the host. The morphology-based identification has been shown a non-reliable method in some cases. For example, Glare et al. (1996) found that phialide morphology could vary depending on the growth medium. Recent evidence suggests that morphological features are often ambiguous and that the genera contain cryptic species, both in *Beauveria* (Rehner, 2005) and *Metarhizium* (Bischoff et al., 2006).

However, DNA-based techniques for identifying species and varieties are accurate and widely used (Driver et al., 2000; Entz et al., 2005). Several kinds of molecular techniques have been used to study genetic diversity, for example, labeled probes to detect restriction fragment length polymorphism (RFLP) (Hegedus and

Khachatourians, 1993; Maurer et al., 1997), and methods based on the polymerase chain reaction (PCR) such as the use of random amplified polymorphic DNA (RAPD) (Fegan et al., 1993; Bidochka et al., 1994; Piatti et al., 1998; Freire et al., 2001; Jensen et al., 2001; Gaitan et al., 2002), internal transcribed spacer (ITS)-rDNA sequence and RFLP analysis (Lee and Taylor, 1992; Rakotonirainy et al., 1994; Buscot et al., 1996; Coates et al., 2002b; Gomes et al., 2002; Becerra et al., 2007). These different techniques have been successfully used for genotyping approaches. They show differences in their ability to reveal polymorphism between related individuals of the same species. Each one has its own advantages and disadvantages as it will be discussed in the subsequent sections.

1.5.1 Restriction fragment length polymorphism (RFLP)

It's one of the most widely used techniques for genotype identification. This technique is based on the digestion of DNA fragment with specific endonucleases known as restriction enzymes. The digested DNA is then separated by electrophoresis on an agarose gel. The polymorphism by this technique originates mainly from point mutations, insertions or deletions within the recognition sequence, which results in an altered pattern of restriction fragments that may distinguish between different genotypes. This technique is highly specific, its markers are co-dominant that could check homogeneity state of the genome, and its results are highly reproducible. Therefore, RFLP is considered to be highly informative and suitable for genotype identification. However, this technique is expensive, laborious and time consuming.

In the PCR- based RFLP, the PCR product of a specific DNA region will be digested with restriction enzymes. These PCR-RFLPs have been used for the characterization

of both *Beauveria* (Couteaudier and Viaud, 1997) and *Metarhizium* species (Bidochka, 2001). Although the method is specific and should, in principle, be explicit and reproducible, the scoring of fragment size is subjective. Furthermore, only few variables are obtained from a single restriction enzyme, thus several of these must be used for each target region to increase variability.

1.5.2 Random amplified polymorphic DNA (RAPD)

It is based on the use of short general primers that anneal to unspecified regions in the template DNA. The two primers should bind to complementary sequence in opposite directions with distance not exceeding 2kb in order to apply successful amplification. PCR products are then separated by agarose gel electrophoresis, stained with ethidium bromide, and visualized under UV light. Point mutation in the primer binding site, or length variations in the amplified fragments cause polymorphism in different genotypes. This technique is simple, rapid (PCR-based), multi locus approach, and prior sequence information is not needed which makes this technique applicable to many different genomes. But, RAPD has many drawbacks like reproducibility problems and being dominant marker; which decreases its informativeness. Besides, the method tends to be subjective, can be unreliable and requires pure cultures of the fungus for DNA extraction since the method by nature is unspecific.

The PCR-RAPD analysis has been extensively used to determine the genetic diversity of various entomopathogenic fungi (Bidochka et al., 1994; Neuvéglise et al., 1994) including *Metarhizium* (Fegan et al., 1993) and *Beauveria* (Williams et al., 1990; Maurer et al., 1997; Berretta et al., 1998).

1.5.3 Microsatellites

Microsatellites, or tandemely repeated simple sequences, are highly repeated DNA elements of eukaryotes; they consist of basic repeat units of 2-8 base pairs (Tautz and

Renz, 1984). These repeats are highly variable and dispersed throughout genomes of different organisms. Because microsatellite markers do not require prior sequence information and high level of polymorphisms are encountered at these loci, microsatellites are currently the most widely used population genetic markers. Examples of microsatellites are: simple sequence repeats (SSRs) and internal simple sequence repeats (ISSRs).

1.5.3.1 Simple sequence repeats (SSRs)

Simple Sequence Repeats markers have been used as powerful molecular markers for the study of the population genetics of fungi (Enkerli et al., 2001; Coates et al., 2002a) including *Beauveria bassiana* (Belkum, 1999). The SSR markers being particularly useful because they are highly polymorphic (multi-allelic) between strains due to the variation of the number of repeats, co-dominant and highly reproducible compared with other techniques. The main disadvantage of SSR markers is that the development of primers is time-consuming and expensive, but this can be offset by the fact that transferability of primer sequences within genera is high and allows primers to be shared between laboratories.

1.5.3.2 Internal simple sequence repeats (ISSRs)

The ISSRs are PCR products obtained using primers based on dinucleotide, trinucleotide, tetranucleotide and pentanucleotide repeats (Zietkiewicz et al., 1994). It is based on the amplification of specific regions between inversely oriented, closely spaced microsatellites. These regions are of variable lengths between the repeats, and so, multiple band profiles are possible to obtain with PCR-based ISSR. Microsatellite markers hold good potential to learn more about reproduction in ecosystems and the

tracking of isolates in the environment. This is possible if a unique allele profile can be established for the isolate. The ISSRs are reliable, quick technique as it's PCR based, dominant inheritance markers, and can generate large numbers of highly informative and reproducible alleles. They have been used for cultivar identification in maize (Kantety et al., 1995), wheat (Nagaoka and Ogihara, 1997), and potatoes (Prevost and Wilkinson, 1999). The markers will also have practical uses for tracking the fate of strains released for biological control of certain insects.

A single microsatellite marker that can be amplified by PCR from *B. bassiana*, *B. brongniartii*, *B. amorpha*, and *B. caledonica* was described by Coates et al. (2002a). Fingerprinting *Beauveria* isolates with this method has recently been applied (de Muro et al., 2005; Wang et al., 2005). Estrada and his colleagues (2007) have used the inter-microsatellite PCR (ISSR-PCR) markers to identify and examine the genetic diversity of *Beauveria bassiana* isolates. Estrada et al. have found that ISSR was highly informative marker, as it allowed the identification of all the *B. bassiana* isolates studied.

1.5.4 Nucleotide sequence data of β-tubulin gene (BT)

The β-tubulin gene codes for tubulins that constitute the structural components of microtubules. Microtubules have straight, hollow tubes structure. Their wall consists of 13 columns of globular tubulin molecules. Each tubulin molecule consists of two similar polypeptide subunits, α-tubulin and β-tubulin. Microtubules are known to have important functions in the cell: maintenance of cell shape, cell motility (as in cilia or flagella), chromosomes separation during cell division, and organelle movements.

Tubulins are amongst the most highly conserved eukaryotic proteins (Wade, 2007). However, tubulin genes are intron-rich. Therefore, they are good candidates for studying genetic diversity, and are potentially useful in fungal phylogeography

(Ceresini et al., 2007; Kauserud et al., 2007). Also, it has been used for studying genetic diversity for *Beauveria bassiana* (Devi et al., 2006).

1.5.5 Nucleotide sequence data of Internal transcribed spacer of ribosomal DNA (ITS-rDNA)

The nuclear-encoded ribosomal RNA genes (rDNA) of fungi exist as a multiple-copy gene family comprised of highly similar DNA sequences (typically from 8-12 kb each). ITS region is perhaps the most widely sequenced DNA region in fungi.

Comparisons of rDNA nucleotide sequence provide a tool for analyzing phylogeny over high and low taxonomic levels. The rDNA consists of highly conserved regions interspersed with variable regions, making it an ideal candidate for molecular evolutionary studies (White et al., 1990). The ITS region evolves fast and may vary among species within a genus or among populations (Jorgensen and Cluster, 1988; Gardes et al., 1991). This region was found to be highly variable at the intra species as revealed by Hirata and Takamatsu (1996) while studying the nucleotide sequence diversity of rDNA of powdery mildew fungi. The sequence data will facilitate the design of DNA probes specific to a group of closely related species, single species, or even single strains. These probes should be useful in taxonomic, ecological, and population-level studies.

Nuclear ribosomal DNA sequences, especially the two internal transcribed spacers (ITS1 and ITS2), have demonstrated differential rates of nucleotide changes that allow intra-specific comparison in fungi (Neuvéglise et al., 1994; Buscot et al., 1996). The RFLP technique has been used to investigate the inter- and intra-specific diversity of the ITS sequences of entomopathogenic fungi (Coates et al., 2002b; Becerra et al., 2007). Shih et al. (1995) reported a moderate level of mutation in the ITS1 region of *Beauveria bassiana,* although ITS2 was invariant in this species. However, *Beauveria*

brongniartii, isolated from a single host, showed an inverse trend (Neuvéglise and Brygoo, 1994). At least 5 RFLPs have been detected among *Beauveria* species (Neuvéglise et al., 1994; Glare and Inwood, 1998).

Driver et al. (2000) revised the genus *Metarhizium* based primarily on internal transcribed spacer (ITS) region and 28S ribosomal DNA (rDNA) sequence data and found some discrepancy between the nucleotide data and conidium morphology.

1.6 Thermotolerance characterization

The optimum environmental conditions for fungi development and pathogenicity can vary for different strains (Welling et al., 1994). Temperature is a major factor influencing; the fungi–insect interaction, fungal pathogenicity and survival in insect habitat, which can determine the success of pest control under natural conditions (Thomas et al., 1996; Fragues and Luz, 2000). The optimal temperatures for the development of pathogenicity of most entomopathogenic fungi are 25-28°C.

Most previous studies on temperature tolerance of entomopathogenic fungi involved selection of isolates with higher tolerance to extreme environmental temperatures in the field (Rangel et al., 2005; Fernandes et al., 2009; Li and Feng, 2009). Several strains from the genera *Beauveria* and *Metarhizium* differ in their response to elevated temperatures (Fargues et al., 1992; Ouedrago et al., 1997; Thomas and Jenkins, 1997; Devi et al., 2005). The tolerance of EPF to extreme temperatures is not limited to the genus or species level. For example, *B. bassiana* and *M. anisopliae* strains differ in their response to higher temperatures (Devi et al., 2005; Alexandre et al., 2006). Physiological aspects of heat tolerance that were studied include; mycelial growth and spore germination. These physiological parameters were investigated in *Beauveria bassiana* F-263 in an attempt to determine the optimal culture conditions and the tolerance of this strain to higher temperatures (Shimazu, 2004).

Identification of heat tolerant EPF is important for controlling mammalian ectoparasites. Heat tolerant EPF are important for controlling ticks while feeding on the warm skin of mammals. The common ambient temperature on animal's body skin will be a little bit less than 37°C. The temperature on cattle surface, for example, fluctuates in shade between 31°C and 35°C, reaching on the ears and spine; 35 and 41°C, respectively (Polar et al., 2005).

Various strains of *Metarhizium anisopliae* var. *anisopliae* are known to differ in their thermal characteristics (Polar et al., 2005; Leemon and Jonsson, 2008). The maximal temperature for conidial germination and mycelia growth is 35-37°C (Walstad et al., 1970), however, thermal death point for their conidia varied from 49 -60°C (Zimmermann, 1982; Fargues et al., 1992) . A study conducted by Ment et al. (2009b) on *Metarhizium anisopliae* showed that in order for any isolate to be suitable for application on cattle, they must be able to recover the germination and virulence capacity after exposure to temporal rise of temperature above the optimal level.

1.7 Problem Description

Four isolates of Entomopathogenic Fungi (EPF) were isolated from the northern part of the West Bank by researchers of the UNESCO Biotechnology Educational and Research Center at Bethlehem University. Based on morphological characteristics, two of these isolates were identified as *Metarhizium anisopliae*, while the other two as *Beauveria bassiana*. However, in some cases, morphological-based identification may fail to discriminate between two species of the same genus (Tymon et al., 2004). Moreover, in the course of studying the kinetics of infection of an insect by several strains of the same species, the use of morphological-based techniques to assess the penetration of a given strain would become worthless. Consequently, there is a need for more reliable and easy-to-apply methods for rapid identification and

discrimination between two isolates of the same species, especially during dual application in infectivity experiments. The entomopathogenic effects of *M. anisopliae* and *B. bassiana* against various developmental stages of *Rhipicephalus (Boophilus) annulatus* were confirmed in other work (Pirali-Kheirabadi et al., 2007) . The Palestinian isolates have been tested for their capacity to control the cattle tick *R. annulatus* (Ment et al. 2009a, b). In this work, several molecular-based approaches have been used to establish reliable methods for the identification of these four EPF isolates. These approaches include; analysis of RFLP, ISSR, and DNA sequence analysis of the ITS and β-tubulin gene. Moreover, this study aimed to identify thermotolerant fungal isolate as an important criterion for choosing strains for controlling ticks while feeding on the warm skin of mammals.

We hypothesize that the genetic characterization could be a helpful method to develop molecular tools for the identification of the four Palestinian isolates. Also, the study of their heat tolerance would facilitate selection of strain with an enhanced heat tolerance that could be useful in biocontrol application.

CHAPTER 2

Objectives

2.1 Overall Objectives

To apply molecular-based identification methods and strain-specific diagnostic techniques for the Palestinian entomopathogenic fungal isolates, and so to assess the previous partial classification of these fungi. In addition, the study aimed at screening for thermotolerance among these isolates.

2.2 Specific objectives

A. To assess the previous morphological characterization of the four Palestinian fungal isolates.

B. Using several molecular-based approaches to establish reliable methods for identification of the Palestinian isolates along with other six externally supplied EPF isolates (added for comparison purpose), these methods include:

   B.1 Sequence analysis of Internal Transcribed Spacers of rDNA to:

   - Assess the morphology- based identification
   - Construct RFLP patterns to create an isolate fingerprint

   B.2 Sequence analysis of β-tubulin gene

   B.3 Analyzing the ISSR marker to evaluate its variability among the isolates.

C. To characterize the thermotolerance of each Palestinian isolate in regard to spore germination and mycelium growth at different temperatures: 25 °C as a control, 30, 32, 34, and 36 °C.

CHAPTER 3

Methodology

3.1 Isolates Collection

Four Palestinian Entomopathogenic fungal isolates were collected from Nablus and Tulkarem areas. Two of them are *Metarhizium anisopliae* (M-TH2 and M-153) and the other two are *Beauveria bassiana* (B-Med and B-151) (Table 3.1). These isolates were subjected to morphological, molecular and thermotolerance characterization. For comparison purpose, the molecular characterization included another two isolates of *Beauveria bassiana*: B-Bug and B-Bot, and four isolates of *Metarhizium anisopliae*: Ma-7, PPRC, M-2004, and M-WG (Table 3.1).

3.2 Protocol for EPF isolation

The protocol used for isolation of the entomopathogenic fungi (from the infected insects):

1- The culture medium that could be used for isolation of the entomopathogenic fungi especially the two species: *Metarhizium anisopliae* and *Beauveria*

bassiana is either Potato or Sabouraud Dextrose Agar medium (Becton, Dickinson and company) amended with antibiotic Chloramphenicol at a concentration of 250 mg/ liter

2- If infected insects (cadavers) are used for isolation of the entomopathogenic fungi, the insects should be superficially disinfected with 70% ethyl alcohol then left aseptically for being dried before cut open. Small pieces of internally infected tissues should be taken with a scalpel under aseptic conditions then transferred onto the plate surface of the culture medium. Incubation of plates is at 25 °C and 16 h of photoperiod for seven days before being evaluated.

3- Evaluation of the plates is to be done according to the appearance of fungal colonies on the plate surface. Preliminary identification of the fungus species is to be carried out according to the characteristics of conidia and conidiophores of the fungus observed. Subculturing of the fungus colonies was conducted to ensure the purity of the fungal culture.

Table 3.1: The source of entomopathogenic fungal isolates.

Isolated species of entomopathogenic fungi	***Designation of the isolates***	***Host/ Isolated from***	***Area of collection***	***Collected by***
Metarhizium anisopliae	PAL-M01 (M-153)	Murky ground beetle (*Harpalus caliginosus)*	Nablus-Beit Eba/ Palestine	UNESCO Biotechnology Center at 2008
	PAL-M02 (M-TH2)	Cockchafer (*Melolontha hippocastani*)	Tulkarm-Shwekeh/ Palestine	
Beauveria bassiana	PAL-B01 (B-151)	European spruce bark beetles (*Ips typographus*)	Tulkarm-Shwekeh/ Palestine	

	PAL-B02 (B-Med)	Emerald ash borer (*Agrilus planipennis*)	Tulkarm-Shwekeh/ Palestine	
Metarhizium anisopliae var. anisopliae	Ma-7	Unidentified coleopterous	Israel	
	PPRC	*Pachnoda interrupta* (Coleoptera: Scarabaeidae)	Ethiopia	
	M-2004	Tick *(Rhipicephalus annulatus)*	Negev	Volcani center /Bet Dagan
	M-WG	Whitegrab insect (*Maladera matrida*)	Bet-Hashitta	
Beauveria bassiana	B-Bug	Bug	Bet Dagan	
	B- Bot	Botanigard*: unknown origin		

***** Botanigard is a commercial product that contains a *Beauveria bassiana* isolate called GHA. This biopesticide is a produce of Laverlam company – USA.

3.3 Fungi rearing and morphological characterization

Fungi isolates cultures were maintained in the laboratory on Sabaroud dextrose agar (SDA) media at 25°C in the dark for 2-3 weeks after the subculture; to allow formation of sufficient amounts of spores. The mycelium colony color, shape and conidia color can be easily observed by the naked eye.

In order to obtain spore suspension, conidia were harvested using a loop then immersing the spores in an aqueous solution of sterile deionized water prepared with Tween 80 (0.01%). The concentration of conidia in the suspension was determined by

hematocytometer under light microscope at a total magnification of 400x. The desired conidial concentration (10^6 spore/ml) was obtained by diluting the conidia suspension with the harvesting solution. Then conidia shape can be determined using the light microscope at 400x magnification. *Metarhizium* conidia lengths and *Beauveria* conidia diameters were determined using DinoCapture software and the average length or diameter of 10 conidia per isolate was calculated.

The microscopic observation of conidiophores is possible only if the hyphae bearing them are maintained at a very low density. A thin culture of hyphae bearing conidiophores was obtained as follows:

- Fungal mycelium from five days old culture was inoculated on a thin layer of SDA media in a 5.5 cm petridish (the thin layer was obtained by using 1.5 ml media per petridish). The inoculation was made on one half of the petridish.
- Using a scalpel, the non inoculated half of the solid medium was cut and removed.
- Two replicates were prepared for each culture.
- The inoculated petridishes were incubated at 25°C, in the dark, for 6 days till the appearance of hyphal extensions bearing conidiophores on the empty half of the petridish.
- Conidiophores of isolates were observed using the inverted microscope at 400x magnification, and photos were taken by DinoCapture Digital camera.

3.4 Molecular Characterization

3.4.1 Genomic DNA extraction

For each of the ten fungal isolates, about 50 mg of mycelium and spores mixture was used for DNA extraction using DNeasy Plant Mini Kit (QIAGEN), following the manufacturer's instructions. Extracted DNA samples were stored at – 20°C till used.

3.4.2 PCR amplification of ITS

In each amplification reaction, the final volume of 25 µl consisted of 3 µl of total genomic DNA, 0.5 µl of each primer (forward and reverse), and 21 µl of ultra-pure distilled water (Biological Industries). Then, all components were added to *AccuPower*® PCR PreMix tube (Bioneer Corporation - Hylabs). For each isolate, PCR amplification of ITS1, ITS2 and the whole region of ITS (ITS1+5.8S+ITS2) were performed in a thermocycler (PTC-200) with the following conditions (Hirata and Takamatsu, 1996): an initial denaturing step at 95°C for 2 min; thermocycling for 30 cycles, where each cycle consisted of 30 s at 95°C followed by 30 s at 52°C for annealing, and 30 s at 72°C for extension, and a final extension cycle of 7 min at 72°C. For more clarification, the schematic diagram (Figure 3.1) illustrates the amplification process of ITS region and shows the primers. Sequences of primers used in PCR amplification are shown in Table 3.2. These primers have been successfully used for identification of different fungal isolates in previous studies (Hirata and Takamatsu, 1996). The PCR amplification was assessed by gel electrophoresis, using 1.5% agarose gel in 0.5x TBE buffer, then PCR products were visualized by UV illuminator. Each amplification reaction was conducted twice to confirm the obtained results. Moreover, all PCR products were sequenced at Hylabs Company.

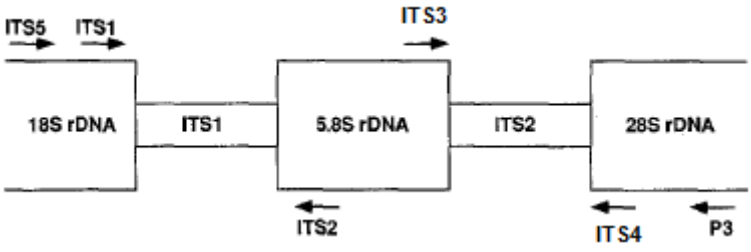


Figure 3.1: Schematic diagram showing the primers used for amplification of ITS1, ITS2, and the whole region (ITS1+5.8S +ITS2). Primer sequences are shown in Table 3.2.

3.4.3 PCR-RFLP analysis

Nebcutter software was used to screen the ITS sequences for restriction sites and the corresponding restriction enzymes. The restriction digestion of ITS2 PCR-products from all *Metarhizium* isolates was carried out using the endonuclease *TseI*; produce of New England BioLabs Inc. (Figure 3.2). The 20 µl of the RFLP reaction mixture included; 0.42 µl of the enzyme, 2 µl of its buffer 10x NEBuffer 4, 15.58 µl of ultra pure H_2O and 2 µl of the PCR product from each isolate. The components were mixed in a PCR tube held on ice. Incubation was at 65°C for 1hr. The resulting restriction fragments along with the non treated sample of PCR products were separated on 2.5% agarose gel in 0.5x TBE buffer at 90 V for 2 h. The RFLP technique was conducted twice to confirm the obtained results.

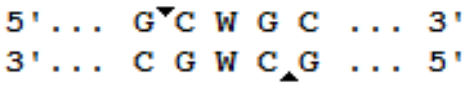


Figure 3.2: The recognition site for the restriction enzyme *TseI*. W: A or T.

3.4.4 PCR amplification of β-tubulin

β-tubulin gene sequence was amplified using the primers sequences presented in Table 3.2. In each amplification reaction, the final volume of 25 µl consisted of 3 µl of total genomic DNA, 0.5 µl of each primer (forward and reverse), and 21 µl of ultra-pure distilled water (Biological Industries). All components were added to *AccuPower*® PCR PreMix tube (Bioneer Corporation - Hylabs). For each isolate, PCR amplification of β-tubulin gene was performed in a PTC-200 thermocycler with the following conditions successfully used by Devi et al. (2006): an initial denaturing step at 94°C for 3 min; thermocycling for 35 cycles, where each cycle consisted of 1 min at 94°C followed by 1 min at 57°C for annealing, and 2 min at 72°C for extension and a final extension period of 5 min at 72°C. Sequences of primers used in PCR amplification are shown in Table 3.2. The separation of PCR products was conducted

on a 1.5% agarose gel in 0.5x TBE buffer and visualized by UV illuminator. A 100 bp DNA ladder was run side by side with the PCR products to estimate fragment sizes. Each amplification reaction was conducted twice to confirm the obtained results. All PCR products were sequenced at Hylabs Company.

3.4.5 PCR amplification of ISSR

Seven primers having dinucleotide and tetranucleotide repeats were used (Table 3.2). These oligonucleotides were used by Estrada and his colleagues (2007) when they studied the molecular diversity of different isolates of *Beauveria bassiana*. The primers were obtained from UBC (University of British Columbia) primer set # 9. In each amplification reaction, the final volume of 25 µl consisted of 3 µl of total genomic DNA, 1 µl of the corresponding primer, and 21 µl of ultra-pure distilled water (Biological Industries). All components were added to *AccuPower*® PCR PreMix tube (Bioneer Corporation - Hylabs).

For each isolate, PCR amplification of ISSRs was performed in a PTC-200 thermocycler and PCR conditions were adopted from Zietkiewicz et al. (1994) protocol: an initial denaturing step at 94 °C for 5 min; thermocycling for 45 cycles, where each cycle consisted of 30 s at 94 °C followed by 45 s at 52 °C for annealing, and 2 min at 72 °C for extension, and a final extension cycle of 6 min at 72 °C. The PCR products were stored at 4 °C until their analysis by electrophoresis using 2% agarose gel in 0.5x TBE buffer, stained using ethidium bromide, and then running at 90 V for 1.5 hours. Each PCR reaction with a given ISSR primer was repeated twice for all isolates to assess the consistency of the band profiles.

3.4.5.1 Analysis of the amplification profiles

The ISSR markers were scored for the presence (1) or absence (0) of homologous bands (bands with the same size) for all the isolates. The total number of bands

(TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (P%), number of different genotypes (NG), and number of exclusive bands (NEB) were calculated.

Table 3.2: The primers sequences used in ITS, β-tubulin, and ISSR analysis.

Primer name	***Sequence 5'…3'***	***Amplified region***	***Reference***
ITS1	5'TCCGTAGGTGAACCTGCGG3'	ITS1	(White et al., 1990)
ITS2	5'GCTGCGTTCTTCATCGATGC3'		
ITS3	5'GCATCGATGAAGAACGCAGC3'	ITS2	
ITS4	5'TCCTCCGCTTATTGATATGC3'		
ITS5	5'GGAAGTAAAAGTCGTAACAAGG3'	ITS1 + 5.8S + ITS2	(Kusaba and Tsuge, 1995)
P3	5'GCCGCTTCACTCGCCGTTAC3'		
Bt2a	5' GGTAACCAAATCGGTGCTGCTTTC 3'	β-tubulin	(Glass and Donaldson, 1995)
Bt2b	5' ACCCTCAGTGTAGTGACCCTTGGC 3'		
808	$(AG)_8C$	ISSR	UBC (University of British Columbia) primer set
809	$(AG)_8G$		
810	$(GA)_8T$		
818	$(CA)_8G$		
821	$(GT)_8T$		
828	$(TG)_8A$		
873	$(GACA)_4$		

3.4.6 Sequence and phylogenetic analysis

BLASTn (Basic Local Alignment Search Tool-nucleotide) was run, using all ITS sequences, against public databases at the National Center for Biotechnology Information (the NCBI website). To estimate the identity of nucleotide sequence data among the isolates, ITS1, ITS2 and 5.8S rDNA and β- tubulin gene sequences were subjected to multiple alignment using T- Coffee at EBI website using the default parameter sets. Based on β- tubulin, ITS and ISSR data, a dendrogram for the ten isolates was constructed using UPGMA (Unweighted Pair Group Method using Arithmetic average) provided by MEGA 4.0 software. *Metarhizium pingshaense* and *Metarhizium brunneum* with accession numbers (HM055447 and EU248826 respectively) were used as outgroups to construct *Metarhizium* ITS and BT trees, respectively. Two isolates of *Beauveria brongniartii* with accession numbers (AB027381 and EU604121) were also used as outgroups to construct *Beauveria* ITS and BT trees, respectively. To evaluate support for the branches, a bootstrap analysis (Felsenstein, 1985) using 1000 replicates was performed.

3.5 Thermotolerance Characterization

The spore germination and mycelium growth capacities of the fungal isolates were determined at five temperatures: 25 ºC (control), 30, 32, 34 and 36 ºC as follows:

A. For determination of spore germination rate:

1. Spores were collected from 2-3 weeks-old culture, using a loop, and transferred to 10 ml sterile water containing 0.01% Tween 80.
2. Using a hematocytometer, spores were counted in a sample from the harvested spores and the concentration was adjusted to 10^6 spore/ml.

3. A volume of 100 µl spore suspension was spread on a thin layer of SDA medium (1.5 ml medium in a 5.5 cm petridish). Plates were sealed with parafilm and incubated at the appropriate temperature.
4. Spore germination was monitored, under the microscope, after 18-24 hrs from inoculation. A sample of a total number of 100 spores, chosen randomly from different fields of the plate, was used to count the number of germinated spores. A spore was considered germinated if the germ tube length reached at least the length of the conidium. The spore counting was performed in four replicates for each treatment and the average was calculated.

B. For determination of mycelium growth rate:

1. Plates were lined with thicker layer of media (5 ml medium in a 5.5 cm petridish). The mycelium growth was monitored by measuring the diameter of the mycelium extending from a filter paper disk supplemented with 5 µl spore suspension (10^6 spore/ml) and placed on the center of plate which was then sealed with parafilm. The measurement of the mycelium growth was done, using a ruler, along two axes drawn at the bottom of the plate.
2. Four replicates were prepared for each treatment and incubated at the appropriate temperature.
3. Mycelium growth was recorded after 48 hrs from inoculation and the measurement continued every 24 hrs for eight days. The recorded mycelium diameters along the two axes were averaged, the filter paper diameter (~ 6 mm) was then subtracted from the average value. Radial mycelial growth rate was calculated in mm/day.

C. The whole heat tolerance experiment was conducted twice to confirm the obtained results.

3.6 Statistical Analysis

The results of thermotolerance characterization experiments, either the germination rate or mycelium diameter, were analyzed statistically using SPSS software program version 13.0. As the data was not normal (even after transformation), the non-parametric test "Kruskal-Wallis Test" had been used to test if there was any significant difference in the mean response among the four isolates at a given temperature. In case of any significant difference, Mann-Whitney Test was used to find which of the isolate responses was different from the others. Since the data of the two thermotolerance experiments were related, Sign test was used to compare these data in order to ensure that the results were not obtained due to chance.

CHAPTER 4

Results

4.1 Morphological characterization

Several morphological traits were examined in this study: the mycelium colony color and shape**,** conidia shape, color, length or diameter, and conidiophores shape**.**

4.1.1 Mycelium colony color and shape

The initial growth of all Palestinian isolates showed white mycelium. Later on, they turned to show different colors. The two *Beauveria* isolates (B.b.151 and B.b.Med) showed white mycelium with flat powdery and cotton-like appearance, respectively.

While the two *Metarhizium* isolates (M.a.153 and M.a.Th2) showed yellow-beige and green mycelium with a domed-like and flat powdery appearance, respectively (Figure 4.1 and Table 4.1).

4.1.2 Conidia shape, color, length or diameter

The two *Beauveria* isolates; B.b.151 and B.b.Med, have white and rounded conidia with averaged diameters of 2.06 µm ± 0.17 SD and 2.21 µm ± 0. 2 SD, respectively. While the two *Metarhizium* isolates; M.a.153 and M.a.Th2, have green and cylindrical conidia with averaged lengths of 5.04 µm ± 0.32 SD and 4.43 µm ± 0.36 SD, respectively (Figures 4.1, 4.2 and Table 4.1).

olor) of the four
B.b.151, and *M.*

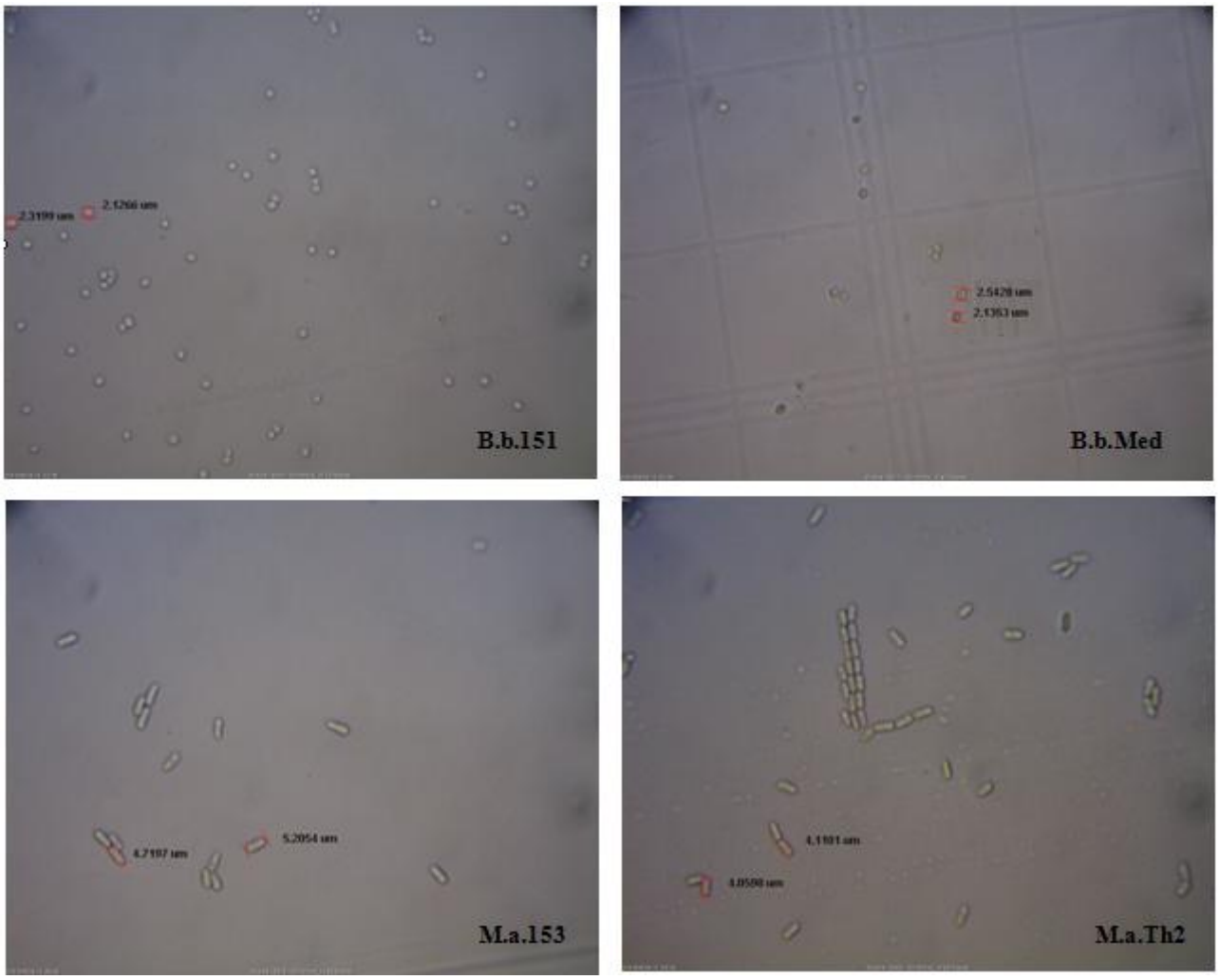
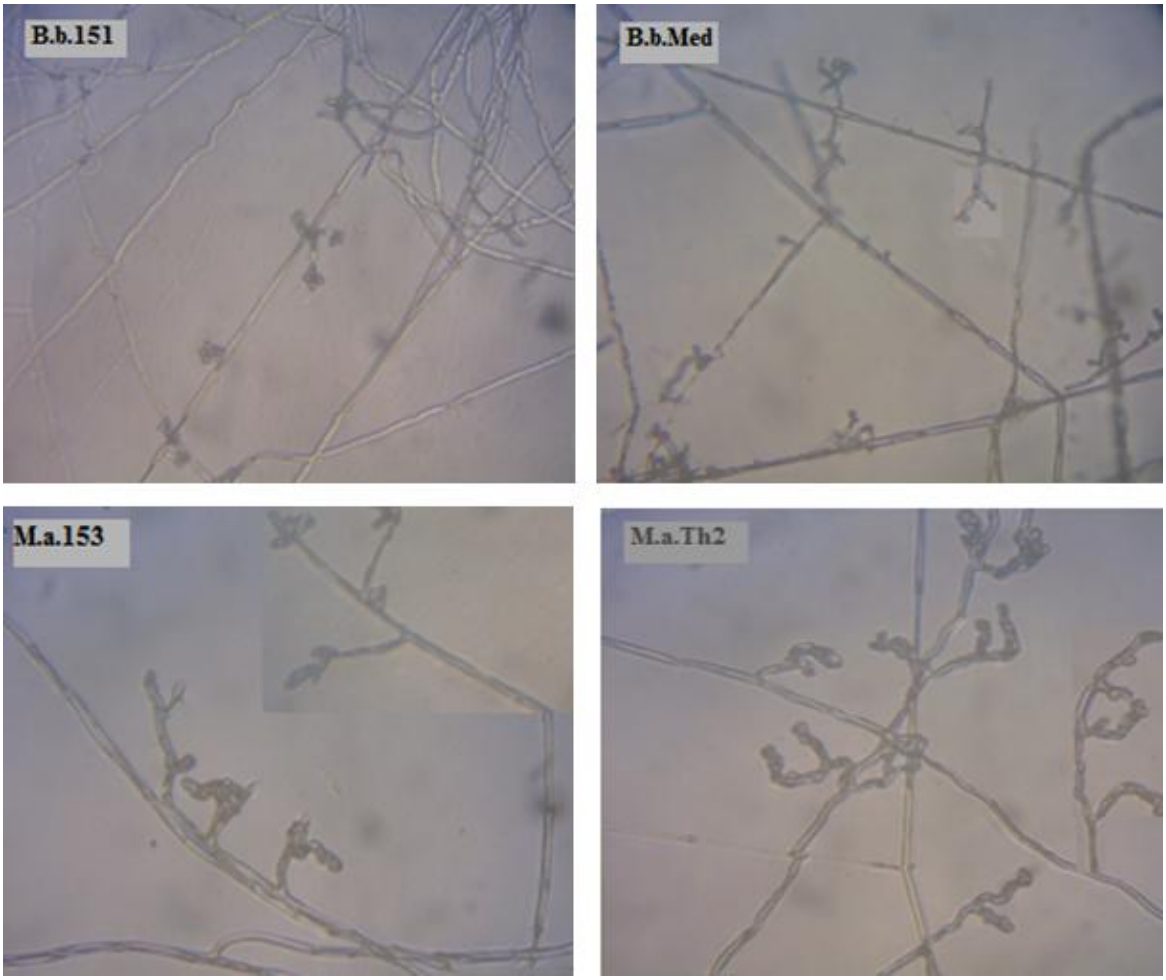


Figure 4.2: Some morphological features of conidia (shape and length or diameter) of the four Palestinian EPF isolates. The rounded conidia shown on the upper side are of the *B. bassiana* isolates while the cylindrical ones shown on the bottom are of the *M. anisopliae* isolates. *Metarhizium* conidia length and *Beauveria* conidia diameter are shown for the ones bordered by red lines.

4.1.3 Conidiophores shape

Beauveria bassiana isolates occur in whorled clusters of flask-shaped conidiogenous cells with inflation at the base and narrow zigzagging filaments at the apex. In contrast, the conidiophores of the *Metarhizium anisopliae* isolates are branched, erect, and loosely grouped, forming sporulating layer (Figure 4.3 and Table 4.1).


B.b.151
B.b.Med
M.a.153
M.a.Th2

olates. The whorl
on the upper side,
the bottom.

Table 4.1: Summary of the macro- and microscopic morphological features of the four Palestinian EPF isolates examined in this study.

Trait	***Isolates***			
	B.b.151	***B.b.Med***	***M.a.153***	***M.a.Th2***
Colony color	White	White	Yellow-Beige	Green
Colony shape	Flat powdery	Cotton-like mass	Domed-like	Flat powdery
Conidia color	White	White	Green	Green
Conidia shape	Rounded	Rounded	Cylindrical	Cylindrical

The average conidia diameter (d) or length (l)	2.06 µm ± 0.17 SD (d)	2.21 µm ± 0. 2 SD (d)	5.04 µm ± 0.32 SD (l)	4.43 µm ± 0.36 SD (l)
Conidiophore shape	Whorled clusters of flask-shaped conidiogenous cells with an inflation at the base and narrow zigzagging filaments at the apex	Whorled clusters of flask-shaped conidiogenous cells with an inflation at the base and narrow zigzagging filaments at the apex	Branched, erect, loosely grouped, forming sporulating layer	Branched, erect, loosely grouped, forming sporulating layer

4.2 Molecular characterization

4.2.1 PCR amplification of ITS

The ITS1, ITS2 as well as the whole ITS region (ITS1 + 5.8S + ITS2) were successfully amplified for all fungal isolates. There was no difference in fragment size between isolates of the same species when either the ITS1 or the ITS2 regions were compared. In contrast, there were substantial differences in fragment size when isolates of different genera were compared. For example, the length of the ITS1 region in *B. bassiana* was greater than that of the *M. anisopliae* (241 and 215 bp, respectively). Whereas, the ITS2 fragment in *M. anisopliae* was longer than that of the *B. bassiana* (363 compared to 348 bp). The fragment sizes of all ITS regions, as obtained by gel electrophoresis, are presented in Figure 4.4.

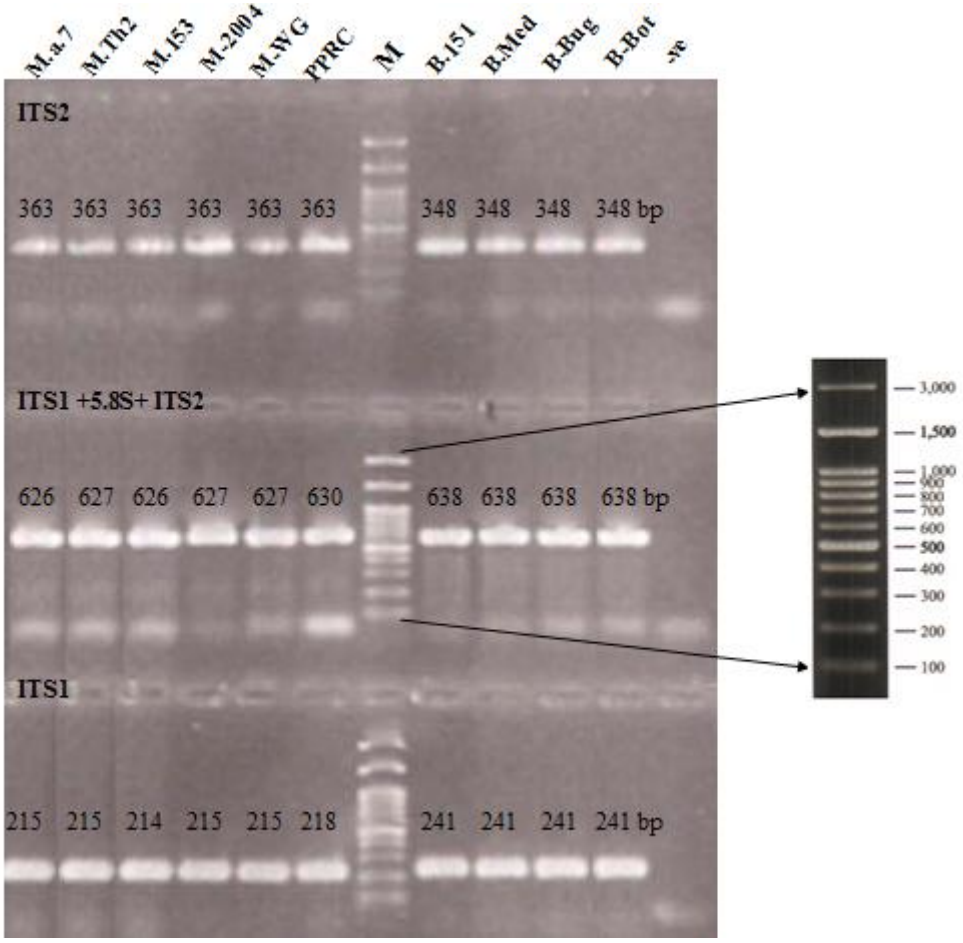


Figure 4.4: Fragment sizes of the PCR-amplified ITS regions as obtained by gel electrophoresis. In the middle of the photograph, bands from a DNA ladder scale (M) are shown. On the left side of this scale, bands from three ITS regions are presented for six *M. anisopliae* isolates. On the right side, bands from the corresponding ITS regions of four *B. bassiana* isolates are shown. Figures on the top of each band designate the fragment size in base pairs (bp).

4.2.2 PCR amplification of β-tubulin

Part of β-tubulin gene was amplified successfully for all isolates. The size of this part in *M. anisopliae* isolates was found to be larger than the corresponding one in *B. bassiana* isolates (386 compared to 356 bp, Figure 4.5).

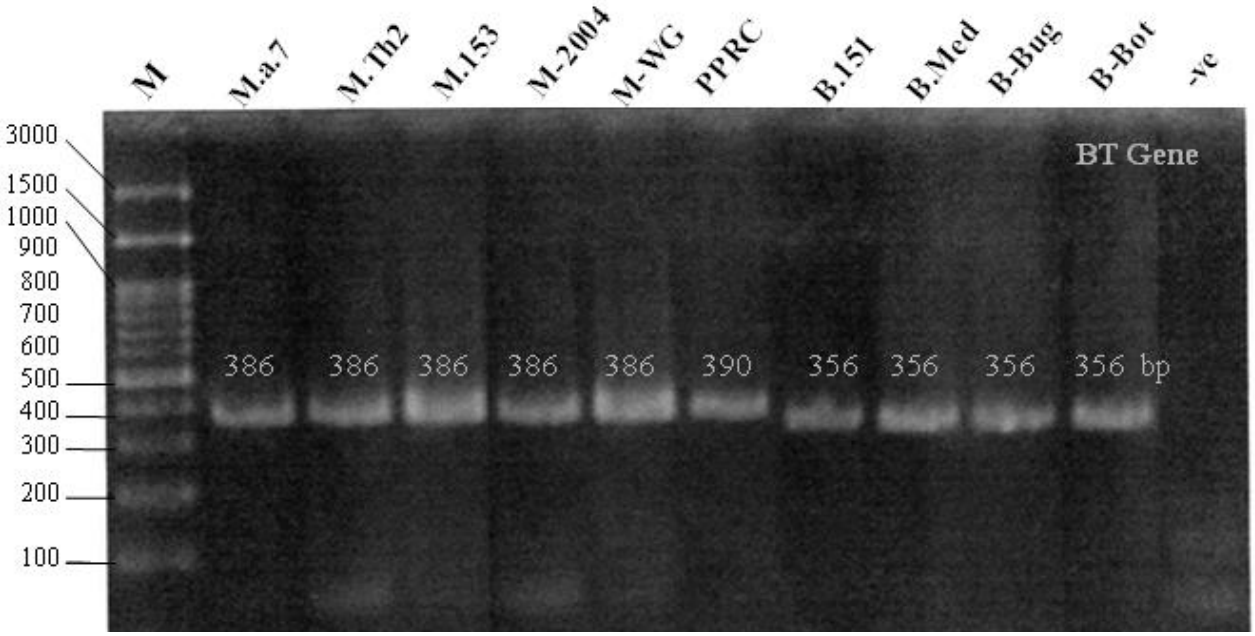


Figure 4.5: Gel electrophoresis bands obtained from the PCR-amplified part of the β-tubulin gene in *M. anisopliae* and *B. bassiana* isolates. Bands from left to right are; DNA ladder (M) followed by six *M. anisopliae* isolates and another four *B. bassiana* isolates, and finally negative control (-ve). Figures on the top of bands designate fragment size in base pairs (bp).

4.2.3 Sequence analysis

4.2.3.1 BLASTn

The ITS sequences obtained from the ten fungal isolates studied in this work were compared to the corresponding sequences available in the GenBank using BLASTn. In each case, the best match was selected and used for identification (Table 4.2). The DNA-sequencing data of these isolates confirmed that six of them match with *Metarhizium anisopliae*. While three of the remaining four isolates (B.b.151, B.b.Med, and B.b.Bug) showed a 99% homology with *Cordyceps* sp. (GenBank accession number AB044636), the fourth isolate, B.b.Bot, showed a 100% homology with *Beauveria bassiana* species (GenBank accession number AB576868). The difference in ITS sequences between the *Cordyceps* sp. and the other three *B. bassiana* isolates is attributed to a one transversion event involving replacement of the nucleotide T by G at position 392 from the 5' end of the query sequence.

The ITS sequences of the four *Metarhizium anisopliae* isolates; M.a.WG, M.a.2004, and the two Palestinian genotypes; M.a.Th2, M.a.153 showed a 100% homology with the corresponding sequences of *Metarhizium anisopliae* available in the GenBank. The ITS sequence of the other *Metarhizium* isolates; M.a.7 and PPRC, showed a best match of 99% homology with the corresponding sequences of the same species deposited in the GenBank. This difference is attributed to the occurrence of one Indel event involving presence of a gap after nucleotide 605 in the query sequence of M.a.7. Also, there are two Indels: first and second gap correspond to nucleotides 234 (G) and 339 (C) in the query sequence of PPRC. Query coverage was 100% for all significant alignments (Table 4.2).

Table 4.2: Summary of BLASTn results of ITS sequences for all EPF isolates.

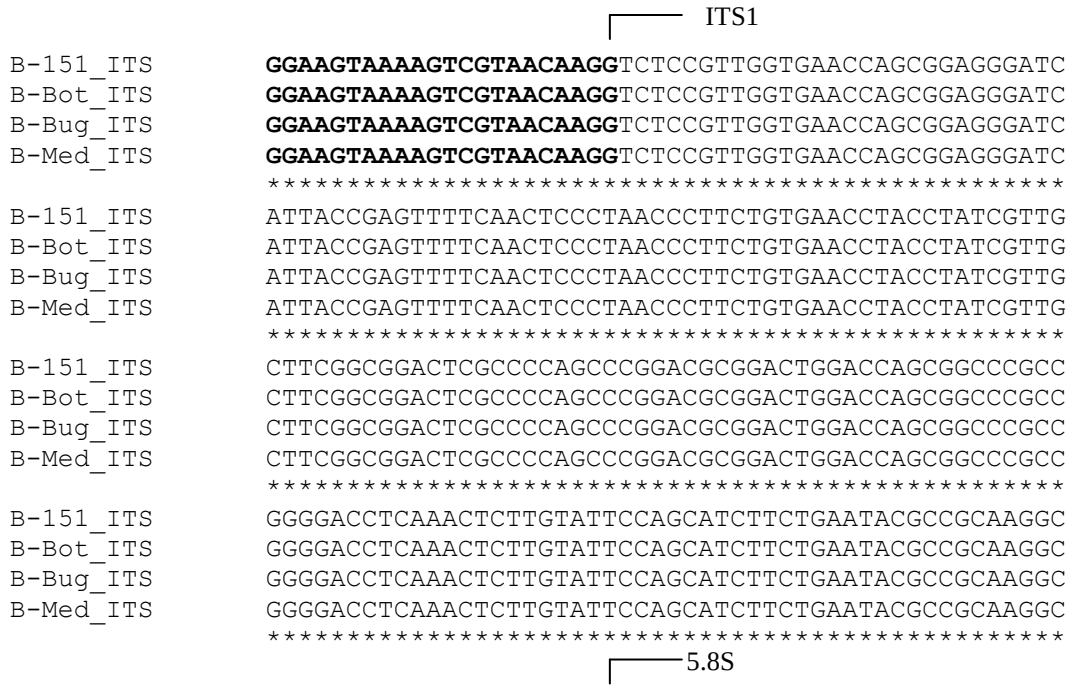
Isolates		***Isolate Identification***	***GenBank Accession number***	***Homology (%)***	***SNP Type***	***Query Coverage***
Beauveria	**B.b.151 B.b.Med B.b.Bug**	*Cordyceps* sp.	AB044636	99%	Transversion event: G392T	100%
	B.b.Bot	*Beauveria bassiana*	AB576868	100%	__	100%
Metarhizium	**M.a.7**	*Metarhizium anisopliae*	FJ545328	99%	Indel event: Gap after nct. number 605 in query	100%
	M.a.Th2 M.a.WG M.a.2004	*Metarhizium anisopliae*	FJ545328	100%	__	100%
	M.a.153	*Metarhizium anisopliae*	EU307929	100%	__	100%
	PPRC	*Metarhizium anisopliae*	FJ545329	99%	Two Indel events:	100 %

			First and second gap correspond to nct. number 234 (G) and 339 (C) in query

4.2.3.2 Multiple Sequence Alignment (MSA)

4.2.3.2.1 The results of MSA for the complete ITS of *Beauveria* isolates

The results of the MSA for the complete ITS of *Beauveria* showed that the Palestinian isolates; B.b.151 and B.b.Med, in addition to B.b.Bug were identical along the whole sequenced region. Whereas the isolate B.b.Bot was molecularly differentiated from the other *Beauveria* isolates by four substitutions (Figure 4.6). One substitution located at nucleotide 270 in the 5.8S region, and the other three were found at nucleotides 392, 397 and 472 in the ITS2 region. No differences were detected in the ITS1 region. It should be pointed out that all nucleotide positions were assigned in relation to the 5' end of the sequence.



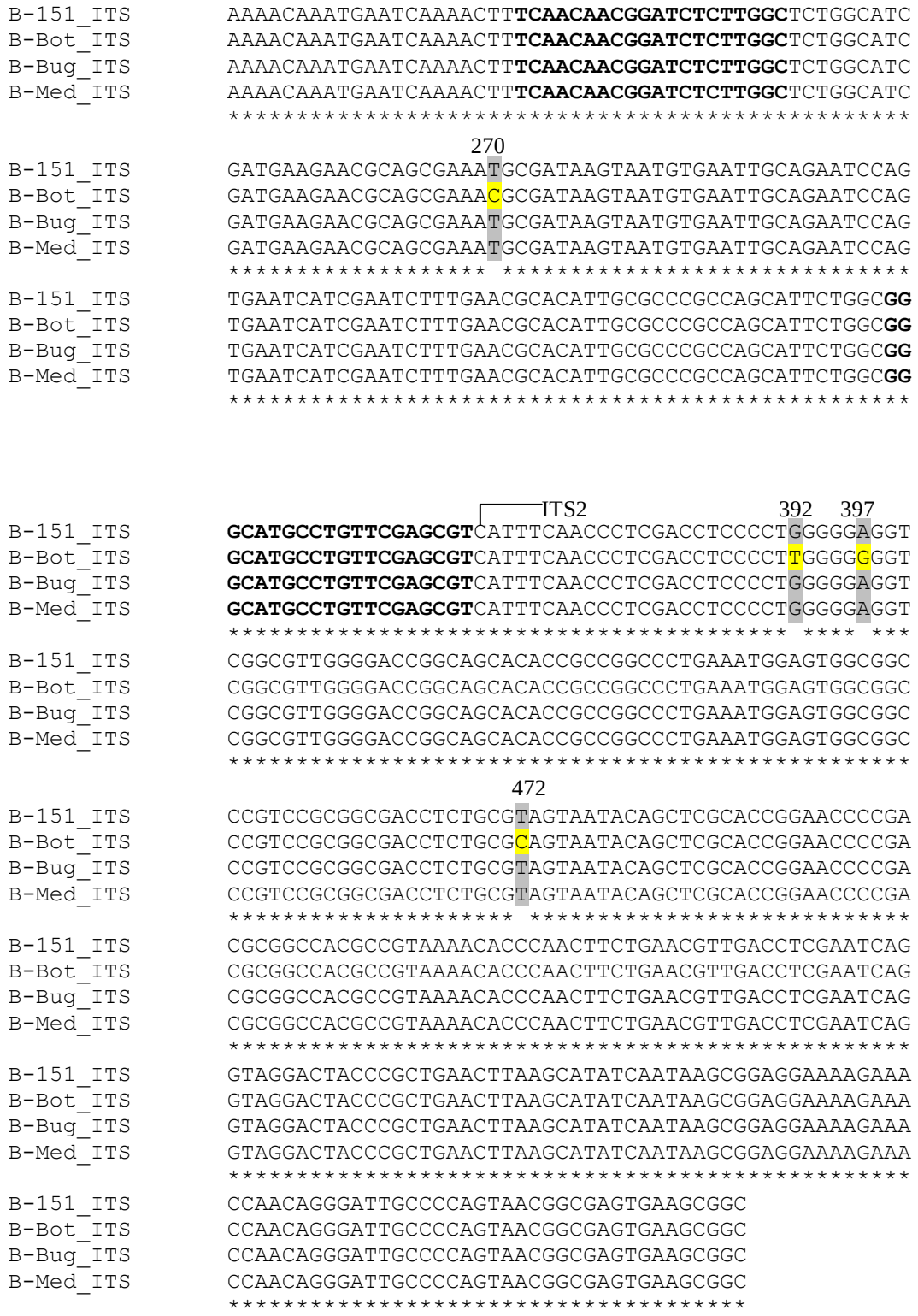
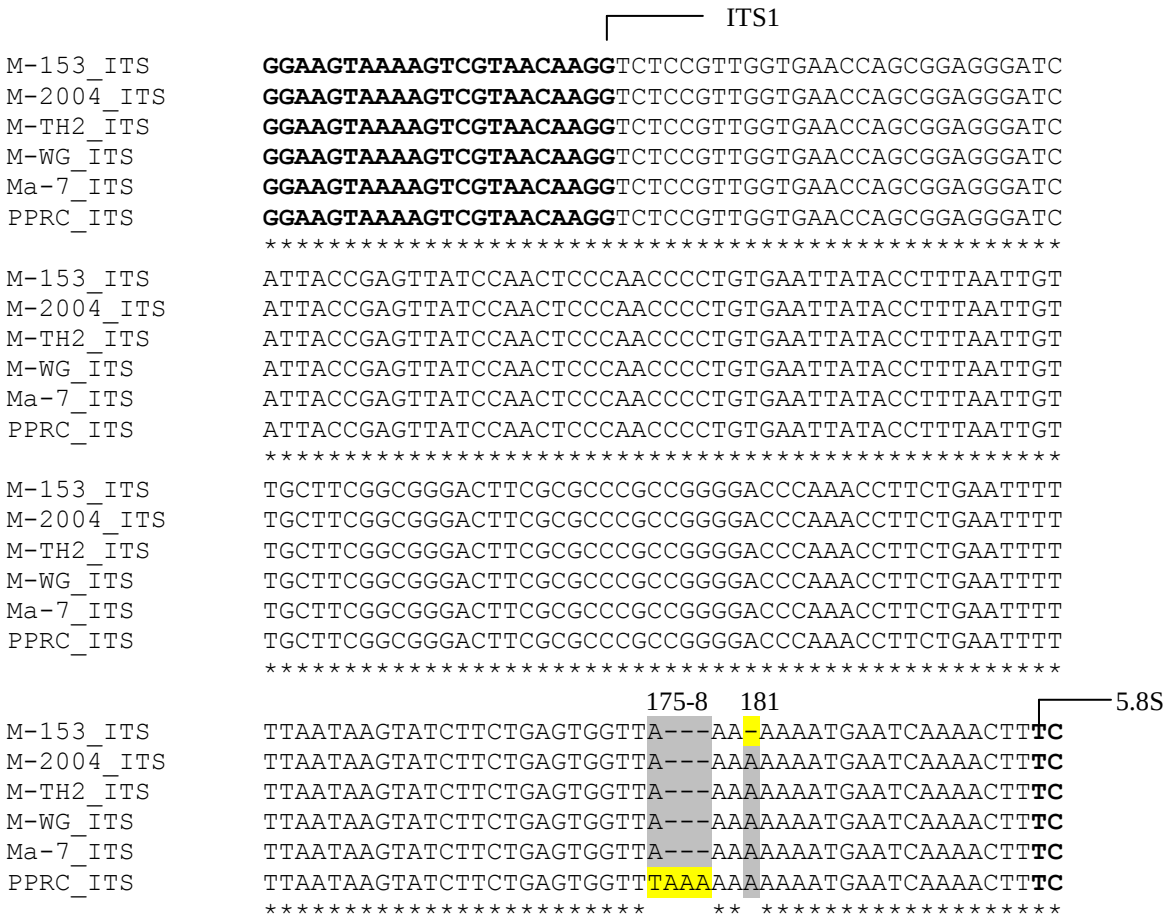
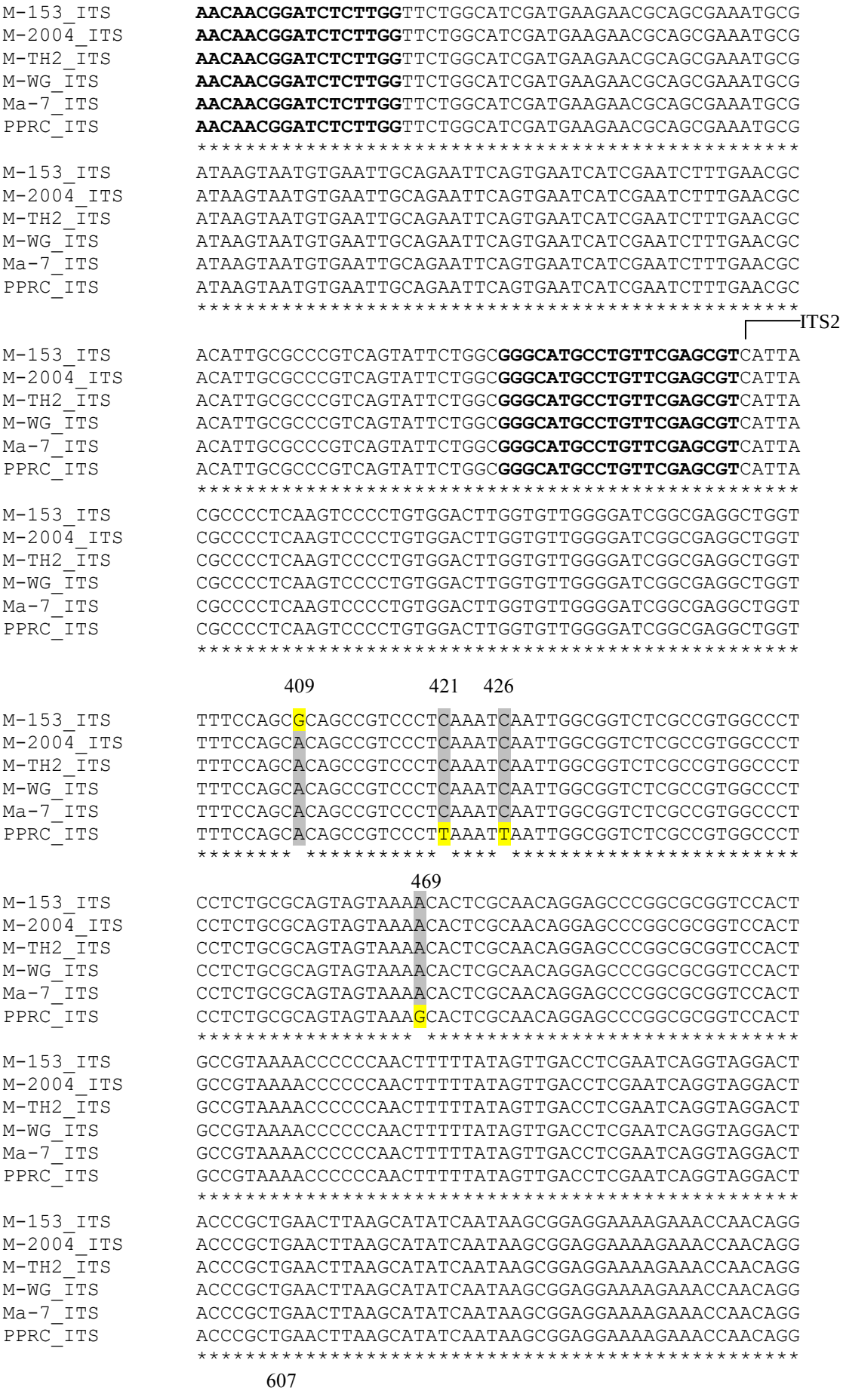


Figure 4.6: Multiple Sequence Alignment (MSA) results for the complete ITS of *Beauveria* isolates. Gray color indicates similar nucleotides while yellow color indicates the different one.

4.2.3.2.2 Results of the MSA for the complete ITS of *Metarhizium* isolates

The MSA results for the complete ITS of *M. anisopliae* isolates indicated that the Ethiopian isolate PPRC was the most variant in relation to the other isolates of this species. The variation is expressed in four substitutions and three indels; one substitution and three indels occur in the ITS1 region at positions 175-8, while the other substitutions occur in the ITS2 at nucleotides 421, 426, and 469. The Palestinian isolate M.a.153 differs from its sister isolate, M.a.Th2 and the other isolates by one indel and one substitution in the ITS1 and ITS2 regions at nucleotides 181 and 409, respectively. Moreover, the MSA revealed that M.a.7 differs from the rest isolates by one indel at the end of the ITS2 sequence (nucleotide 607). However, the Israeli isolates M.a.2004, and M.a.WG were identical to the Palestinian one M.a.Th2, in all ITS sequences (Figure 4.7).




M-153_ITS AACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCG
M-2004_ITS AACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCG
M-TH2_ITS AACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCG
M-WG_ITS AACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCG
Ma-7_ITS AACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCG
PPRC_ITS AACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCG
M-153_ITS ATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC
M-2004_ITS ATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC
M-TH2_ITS ATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC
M-WG_ITS ATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC
Ma-7_ITS ATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC
PPRC_ITS ATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGC
ITS2
M-153_ITS ACATTGCGCCCGTCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTA
M-2004_ITS ACATTGCGCCCGTCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTA
M-TH2_ITS ACATTGCGCCCGTCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTA
M-WG_ITS ACATTGCGCCCGTCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTA
Ma-7_ITS ACATTGCGCCCGTCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTA
PPRC_ITS ACATTGCGCCCGTCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTA
M-153_ITS CGCCCCTCAAGTCCCCTGTGGACTTGGTGTTGGGGATCGGCGAGGCTGGT
M-2004_ITS CGCCCCTCAAGTCCCCTGTGGACTTGGTGTTGGGGATCGGCGAGGCTGGT
M-TH2_ITS CGCCCCTCAAGTCCCCTGTGGACTTGGTGTTGGGGATCGGCGAGGCTGGT
M-WG_ITS CGCCCCTCAAGTCCCCTGTGGACTTGGTGTTGGGGATCGGCGAGGCTGGT
Ma-7_ITS CGCCCCTCAAGTCCCCTGTGGACTTGGTGTTGGGGATCGGCGAGGCTGGT
PPRC_ITS CGCCCCTCAAGTCCCCTGTGGACTTGGTGTTGGGGATCGGCGAGGCTGGT
409
421
426
M-153_ITS TTTCCAGCGCAGCCGTCCCTCAAATCAATTGGCGGTCTCGCCGTGGCCCT
M-2004_ITS TTTCCAGCACAGCCGTCCCTCAAATCAATTGGCGGTCTCGCCGTGGCCCT
M-TH2_ITS TTTCCAGCACAGCCGTCCCTCAAATCAATTGGCGGTCTCGCCGTGGCCCT
M-WG_ITS TTTCCAGCACAGCCGTCCCTCAAATCAATTGGCGGTCTCGCCGTGGCCCT
Ma-7_ITS TTTCCAGCACAGCCGTCCCTCAAATCAATTGGCGGTCTCGCCGTGGCCCT
PPRC_ITS TTTCCAGCACAGCCGTCCCTTAAATTAATTGGCGGTCTCGCCGTGGCCCT
469
M-153_ITS CCTCTGCGCAGTAGTAAAACACTCGCAACAGGAGCCCGGCGCGGTCCACT
M-2004_ITS CCTCTGCGCAGTAGTAAAACACTCGCAACAGGAGCCCGGCGCGGTCCACT
M-TH2_ITS CCTCTGCGCAGTAGTAAAACACTCGCAACAGGAGCCCGGCGCGGTCCACT
M-WG_ITS CCTCTGCGCAGTAGTAAAACACTCGCAACAGGAGCCCGGCGCGGTCCACT
Ma-7_ITS CCTCTGCGCAGTAGTAAAACACTCGCAACAGGAGCCCGGCGCGGTCCACT
PPRC_ITS CCTCTGCGCAGTAGTAAAGCACTCGCAACAGGAGCCCGGCGCGGTCCACT
M-153_ITS GCCGTAAAACCCCCCAACTTTTTATAGTTGACCTCGAATCAGGTAGGACT
M-2004_ITS GCCGTAAAACCCCCCAACTTTTTATAGTTGACCTCGAATCAGGTAGGACT
M-TH2_ITS GCCGTAAAACCCCCCAACTTTTTATAGTTGACCTCGAATCAGGTAGGACT
M-WG_ITS GCCGTAAAACCCCCCAACTTTTTATAGTTGACCTCGAATCAGGTAGGACT
Ma-7_ITS GCCGTAAAACCCCCCAACTTTTTATAGTTGACCTCGAATCAGGTAGGACT
PPRC_ITS GCCGTAAAACCCCCCAACTTTTTATAGTTGACCTCGAATCAGGTAGGACT
M-153_ITS ACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGG
M-2004_ITS ACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGG
M-TH2_ITS ACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGG
M-WG_ITS ACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGG
Ma-7_ITS ACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGG
PPRC_ITS ACCCGCTGAACTTAAGCATATCAATAAGCGGAGGAAAAGAAACCAACAGG
607

```
M-153_ITS       GATTGCCCCAGTAACGGCGAGTGAAGCGGC
M-2004_ITS      GATTGCCCCAGTAACGGCGAGTGAAGCGGC
M-TH2_ITS       GATTGCCCCAGTAACGGCGAGTGAAGCGGC
M-WG_ITS        GATTGCCCCAGTAACGGCGAGTGAAGCGGC
Ma-7_ITS        GATTGC-CCAGTAACGGCGAGTGAAGCGGC
PPRC_ITS        GATTGCCCCAGTAACGGCGAGTGAAGCGGC
                ****** **********************
```

Figure 4.7: Multiple Sequence Alignment (MSA) results for the complete ITS of *Metarhizium* isolates. Gray color indicates similar nucleotides while yellow color indicates the different one.

4.2.3.2.3 Results of the MSA for partial sequence of the BT gene of *Beauveria* isolates

All partial BT gene sequences for *Beauveria* isolates, including the Palestinian ones, were identical except the isolate B.b.Bot which differed from the other isolates by four substitutions at nucleotides 174, 186, 203, and 209 (Figure 4.8).

```
B-151_BT        GGTAACCAAATCGGTGCTGCTTTCTGGCAGACCATCTCTGGCGAGCACGG
B-Bot_BT        GGTAACCAAATCGGTGCTGCTTTCTGGCAGACCATCTCTGGCGAGCACGG
B-Bug_BT        GGTAACCAAATCGGTGCTGCTTTCTGGCAGACCATCTCTGGCGAGCACGG
B-Med_BT        GGTAACCAAATCGGTGCTGCTTTCTGGCAGACCATCTCTGGCGAGCACGG
                **************************************************
B-151_BT        CCTCGACTCCAGCGGTGTTTACAATGGCACTTCTGAGCTTCAGCTCGAGC
B-Bot_BT        CCTCGACTCCAGCGGTGTTTACAATGGCACTTCTGAGCTTCAGCTCGAGC
B-Bug_BT        CCTCGACTCCAGCGGTGTTTACAATGGCACTTCTGAGCTTCAGCTCGAGC
B-Med_BT        CCTCGACTCCAGCGGTGTTTACAATGGCACTTCTGAGCTTCAGCTCGAGC
                **************************************************
B-151_BT        GCATGAATGTCTACTTCAACGAGGTTTGTTGTGCCCTCCCAACGCGTTGC
B-Bot_BT        GCATGAATGTCTACTTCAACGAGGTTTGTTGTGCCCTCCCAACGCGTTGC
B-Bug_BT        GCATGAATGTCTACTTCAACGAGGTTTGTTGTGCCCTCCCAACGCGTTGC
B-Med_BT        GCATGAATGTCTACTTCAACGAGGTTTGTTGTGCCCTCCCAACGCGTTGC
                **************************************************
                                          174         186
B-151_BT        TTGATTTCGTTGTGGATACTGACCGCGATTTTCCATAGGCCTCCGGCAAC
B-Bot_BT        TTGATTTCGTTGTGGATACTGACTGCGATTTTCCAAAGGCCTCCGGCAAC
B-Bug_BT        TTGATTTCGTTGTGGATACTGACCGCGATTTTCCATAGGCCTCCGGCAAC
B-Med_BT        TTGATTTCGTTGTGGATACTGACCGCGATTTTCCATAGGCCTCCGGCAAC
                ********************** *********** **************
                  203     209
B-151_BT        AAATATGTACCTCGCGCCGTCCTCGTCGATCTTGAGCCCGGTACCATGGA
B-Bot_BT        AAGTATGTTCCTCGCGCCGTCCTCGTCGATCTTGAGCCCGGTACCATGGA
B-Bug_BT        AAATATGTACCTCGCGCCGTCCTCGTCGATCTTGAGCCCGGTACCATGGA
B-Med_BT        AAATATGTACCTCGCGCCGTCCTCGTCGATCTTGAGCCCGGTACCATGGA
                ** ***** ****************************************
B-151_BT        TGCTGTCCGTGCCGGTCCCTTCGGTCAGCTCTTCCGTCCCGACAACTTCG
B-Bot_BT        TGCTGTCCGTGCCGGTCCCTTCGGTCAGCTCTTCCGTCCCGACAACTTCG
B-Bug_BT        TGCTGTCCGTGCCGGTCCCTTCGGTCAGCTCTTCCGTCCCGACAACTTCG
B-Med_BT        TGCTGTCCGTGCCGGTCCCTTCGGTCAGCTCTTCCGTCCCGACAACTTCG
                **************************************************
B-151_BT        TTTTCGGTCAGTCCGGTGCCGGCAACAACTGGGCCAAGGGTCACTACACT
B-Bot_BT        TTTTCGGTCAGTCCGGTGCCGGCAACAACTGGGCCAAGGGTCACTACACT
B-Bug_BT        TTTTCGGTCAGTCCGGTGCCGGCAACAACTGGGCCAAGGGTCACTACACT
B-Med_BT        TTTTCGGTCAGTCCGGTGCCGGCAACAACTGGGCCAAGGGTCACTACACT
                **************************************************
```

```
B-151_BT        GAGGGT
B-Bot_BT        GAGGGT
B-Bug_BT        GAGGGT
B-Med_BT        GAGGGT
                ******
```

Figure 4.8: Multiple Sequence Alignment (MSA) results for the partial BT gene sequence of *Beauveria* isolates. Gray color indicates similar nucleotides while yellow color indicates the different one.

4.2.3.2.4 The MSA Results for the partial BT gene sequence of *Metarhizium* isolates

All partial BT gene sequences for *Metarhizium* isolates, including the Palestinian ones, were found to be identical except the isolate PPRC which differed from the other isolates by three substitutions at nucleotides 42, 48, 363 and four indels at nucleotides 175-7 and 185 (Figure 4.9).

```
                                                          42      48
M-153_BT        GGTAACCAAATCGGTGCTGCTTTCTGGCAGACCATCTCTGGCGAACATGG
M-2004_BT       GGTAACCAAATCGGTGCTGCTTTCTGGCAGACCATCTCTGGCGAACATGG
M-TH2_BT        GGTAACCAAATCGGTGCTGCTTTCTGGCAGACCATCTCTGGCGAACATGG
M-WG_BT         GGTAACCAAATCGGTGCTGCTTTCTGGCAGACCATCTCTGGCGAACATGG
Ma-7_BT         GGTAACCAAATCGGTGCTGCTTTCTGGCAGACCATCTCTGGCGAACATGG
PPRC_BT         GGTAACCAAATCGGTGCTGCTTTCTGGCAGACCATCTCTGGTGAACACGG
                **************************************** ***** **
M-153_BT        CCTCGACAGCAATGGTGTCTACAACGGTACCTCTGAGCTCCAGCTCGAGC
M-2004_BT       CCTCGACAGCAATGGTGTCTACAACGGTACCTCTGAGCTCCAGCTCGAGC
M-TH2_BT        CCTCGACAGCAATGGTGTCTACAACGGTACCTCTGAGCTCCAGCTCGAGC
M-WG_BT         CCTCGACAGCAATGGTGTCTACAACGGTACCTCTGAGCTCCAGCTCGAGC
Ma-7_BT         CCTCGACAGCAATGGTGTCTACAACGGTACCTCTGAGCTCCAGCTCGAGC
PPRC_BT         CCTCGACAGCAATGGTGTCTACAACGGTACCTCTGAGCTCCAGCTCGAGC
                **************************************************

M-153_BT        GTATGAGCGTCTACTTCAATGAGGTAAGTTTGAATGAGCCGAATCCTAGG
M-2004_BT       GTATGAGCGTCTACTTCAATGAGGTAAGTTTGAATGAGCCGAATCCTAGG
M-TH2_BT        GTATGAGCGTCTACTTCAATGAGGTAAGTTTGAATGAGCCGAATCCTAGG
M-WG_BT         GTATGAGCGTCTACTTCAATGAGGTAAGTTTGAATGAGCCGAATCCTAGG
Ma-7_BT         GTATGAGCGTCTACTTCAATGAGGTAAGTTTGAATGAGCCGAATCCTAGG
PPRC_BT         GTATGAGCGTCTACTTCAATGAGGTAAGTTTGAATGAGCCGAATCCTAGG
                **************************************************

                                          175-7      185
M-153_BT        ATGTTGAGGAACCATAGGTTGTTA---TCCATGG-GGAGATCAAAGACTG
M-2004_BT       ATGTTGAGGAACCATAGGTTGTTA---TCCATGG-GGAGATCAAAGACTG
M-TH2_BT        ATGTTGAGGAACCATAGGTTGTTA---TCCATGG-GGAGATCAAAGACTG
M-WG_BT         ATGTTGAGGAACCATAGGTTGTTA---TCCATGG-GGAGATCAAAGACTG
Ma-7_BT         ATGTTGAGGAACCATAGGTTGTTA---TCCATGG-GGAGATCAAAGACTG
PPRC_BT         ATGTTGAGGAACCATAGGTTGTTATCATCCATGGGGGAGATCAAAGACTG
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                  ************************   ******* ***************
M-153_BT          ACGCGGGGACTCCCCTCAAAAGGCCTCCGGTAACAAGTATGTTCCTCGCG
M-2004_BT         ACGCGGGGACTCCCCTCAAAAGGCCTCCGGTAACAAGTATGTTCCTCGCG
M-TH2_BT          ACGCGGGGACTCCCCTCAAAAGGCCTCCGGTAACAAGTATGTTCCTCGCG
M-WG_BT           ACGCGGGGACTCCCCTCAAAAGGCCTCCGGTAACAAGTATGTTCCTCGCG
Ma-7_BT           ACGCGGGGACTCCCCTCAAAAGGCCTCCGGTAACAAGTATGTTCCTCGCG
PPRC_BT           ACGCGGGGACTCCCCTCAAAAGGCCTCCGGTAACAAGTATGTTCCTCGCG
                  ************************************************
M-153_BT          CCGTCCTTGTCGATCTTGAACCTGGCACCATGGATGCCGTCCGTGCCGGT
M-2004_BT         CCGTCCTTGTCGATCTTGAACCTGGCACCATGGATGCCGTCCGTGCCGGT
M-TH2_BT          CCGTCCTTGTCGATCTTGAACCTGGCACCATGGATGCCGTCCGTGCCGGT
M-WG_BT           CCGTCCTTGTCGATCTTGAACCTGGCACCATGGATGCCGTCCGTGCCGGT
Ma-7_BT           CCGTCCTTGTCGATCTTGAACCTGGCACCATGGATGCCGTCCGTGCCGGT
PPRC_BT           CCGTCCTTGTCGATCTTGAACCTGGCACCATGGATGCCGTCCGTGCCGGT
                  ************************************************
M-153_BT          CCTTTCGGTCAGCTTTTCCGTCCCGACAACTTCGTCTTTGGTCAGTCTGG
M-2004_BT         CCTTTCGGTCAGCTTTTCCGTCCCGACAACTTCGTCTTTGGTCAGTCTGG
M-TH2_BT          CCTTTCGGTCAGCTTTTCCGTCCCGACAACTTCGTCTTTGGTCAGTCTGG
M-WG_BT           CCTTTCGGTCAGCTTTTCCGTCCCGACAACTTCGTCTTTGGTCAGTCTGG
Ma-7_BT           CCTTTCGGTCAGCTTTTCCGTCCCGACAACTTCGTCTTTGGTCAGTCTGG
PPRC_BT           CCTTTCGGTCAGCTTTTCCGTCCCGACAACTTCGTCTTTGGTCAGTCTGG
                  ************************************************
                                    363
M-153_BT          TGCTGGCAACAACTGGGCCAAGGGTCACTACACTGAGGGT
M-2004_BT         TGCTGGCAACAACTGGGCCAAGGGTCACTACACTGAGGGT
M-TH2_BT          TGCTGGCAACAACTGGGCCAAGGGTCACTACACTGAGGGT
M-WG_BT           TGCTGGCAACAACTGGGCCAAGGGTCACTACACTGAGGGT
Ma-7_BT           TGCTGGCAACAACTGGGCCAAGGGTCACTACACTGAGGGT
PPRC_BT           TGCTGGCAACAATTGGGCCAAGGGTCACTACACTGAGGGT
                  ************ **************************
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Figure 4.9: Results of the Multiple Sequence Alignment (MSA) for the partial BT gene sequence of *Metarhizium* isolates. Gray color indicates similar nucleotides while yellow color indicates the different one.

4.2.4 PCR-RFLP analysis

The ITS2 sequences of all *Metarhizium* isolates were digested by *TseI* enzyme. This enzyme cuts all sequences after nucleotide number 16, but this cleavage is affected by CpG methylation. ITS2 sequence for M.a.153 has another restriction site for *TseI* after nucleotide number 187 producing 5' overhang end. This produced unique RFLP pattern for M.a.153. Two bands of size 176 and 187 bp were produced for M.a.153. While in the other *Metarhizium* isolates only one band of size 363 bp was obtained (Figure 4.10).

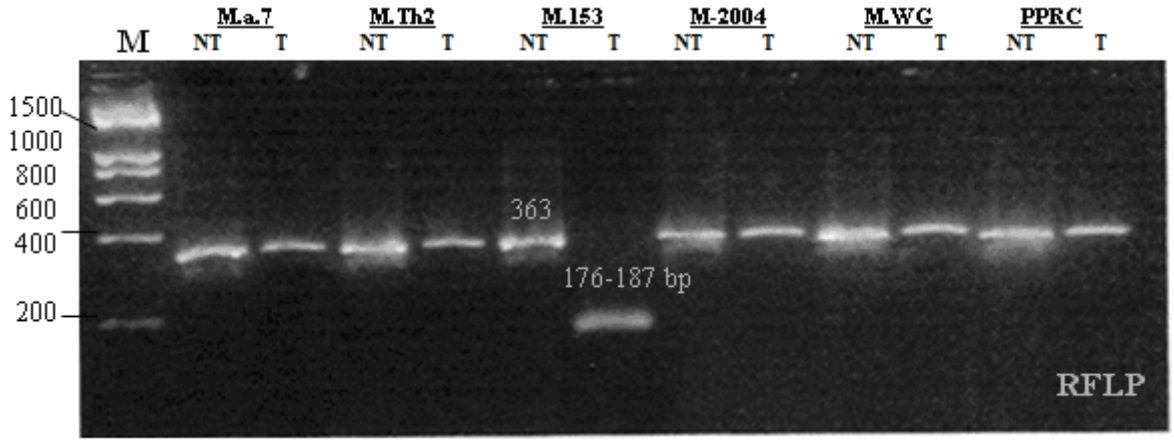
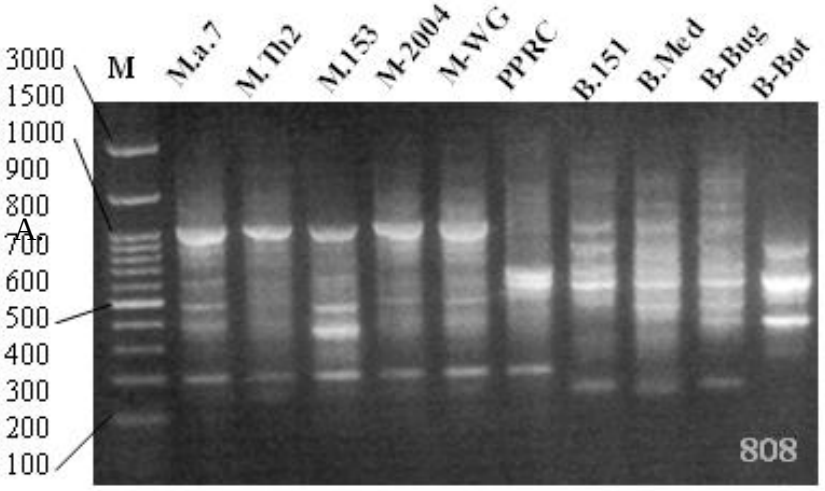


Figure 4.10: Gel electrophoresis bands of RFLP obtained from the ITS2 region of the *Metarhizium* isolates. T: treated with restriction enzyme, NT: not treated with restriction enzyme.

4.2.5 PCR amplification of ISSR

Only four primers out of seven produced clear and reproducible bands through PCR amplification of the ISSR. These primers are 808, 809, 810, and 828. The amplification profiles for both *Metarhizium* and *Beauveria* isolates are shown in Figure 4.11.



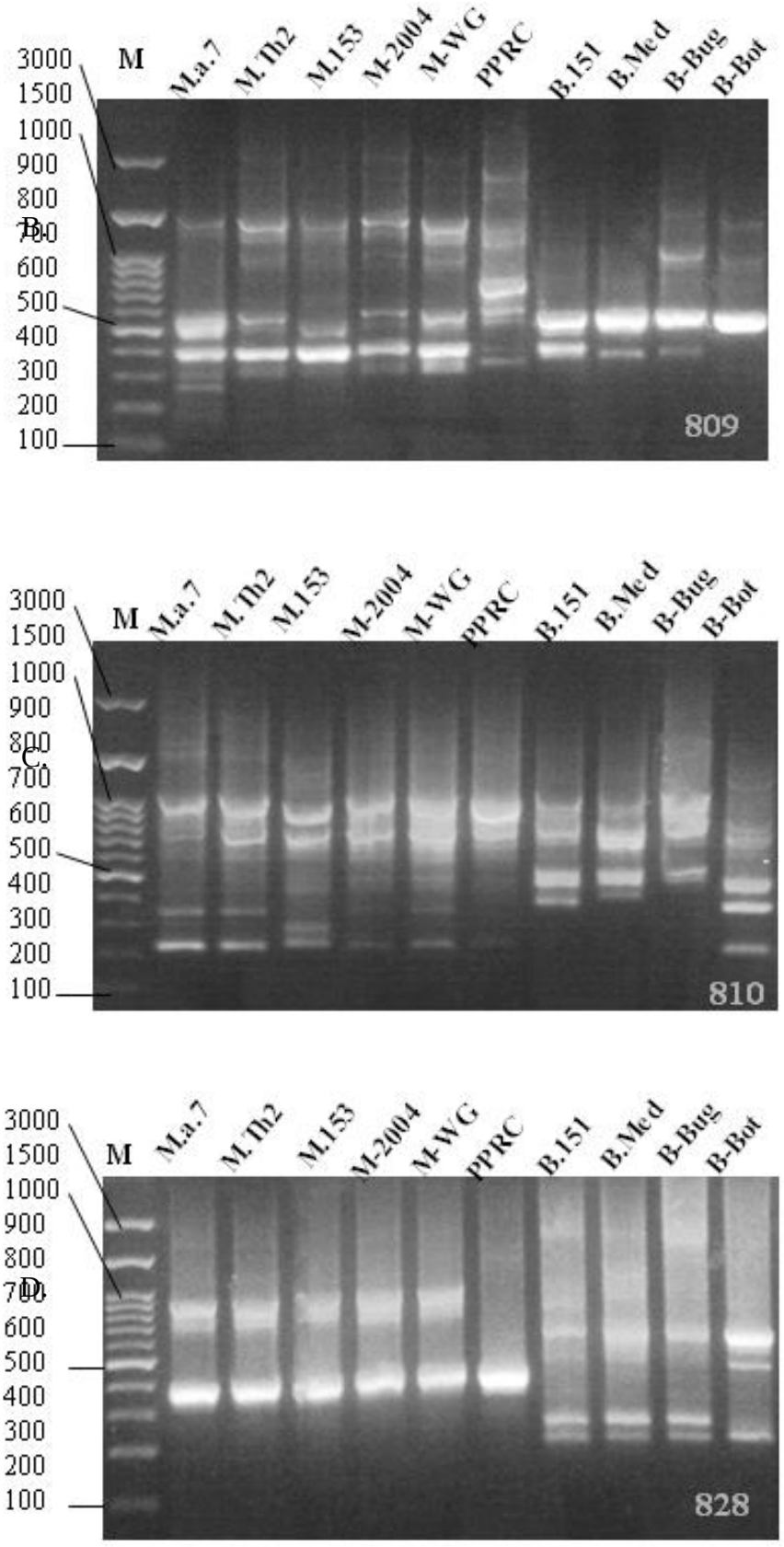


Figure 4.11: Amplification profiles of ISSR obtained from the ten *Beauveria* and *Metarhizium* isolates studied. Using different ISSR primers: A. 808, B. 809, C. 810 and D. 828. M = the molecular marker.

4.2.5.1 Analysis of the amplification profiles

The total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (P%), number of different genotypes (NG), and number of exclusive bands (NEB) obtained with each primer are shown in Table 4.3. The total number of amplified products was 42 for *Metarhizium* (ranging from 200 to 1500 bp) and 39 for *Beauveria* isolates (ranging from 200 to 2500 bp) with 38 (90.5%) polymorphic DNA fragments for *Metarhizium* and 32 (82%) for *Beauveria*. The maximum number of amplified products was 15 (primer 809) for *Metarhizium* and 13 (primer 810) for *Beauveria.* While the minimum was 4 (primer 828) for *Metarhizium* and 6 (primer 809) for *Beauveria* (Table 4.3). All *Beauveria* isolates in addition to the four different *Metarhizium* isolates (PPRC, M.a.153, M.a.2004, and M.a.7) showed a total of 13 and 11 exclusive bands, respectively. All primers were able to distinguish between all *Beauveria* isolates, while primer 809 was the only one able to distinguish between the five *Metarhizium* isolates as the maximum number of different genotypes identified (Table 4.3).

Table 4.3: The total number of bands (TNB), number of polymorphic bands (NPB), percentage of polymorphic bands (P%), number of different genotypes identified (NG) and number of exclusive bands (NEB) obtained with each ISSR primer.

Primer	TNB		NPB		P%		NG		NEB	
	M*	B*	M	B	M	B	M	B	M	B
808	12	10	11	9	91.7	90	4	4	4	1
809	15	6	14	5	93.3	83.3	5	4	4	3
810	11	13	9	10	81.8	76.9	4	4	3	5
828	4	10	4	8	100	80	3	4	0	4
Total	42	39	38	32	90.5	82	16	16	11	13

*M: *Metarhizium* isolates
*B: *Beauveria* isolates

4.2.6 Phylogenetic analysis

Phylogenetic relationships within *Beauveria* and *Metarhizium* isolates were constructed based on the ribosomal ITS1-5.8S-ITS2, β- tubulin sequences and ISSR data for the ten EPF isolates. To obtain a robust dendrogram, all ISSR markers, obtained with all primers, were used.

4.2.6.1 *Beauveria* Phylogeny

The two trees, constructed based on ITS and BT sequences, have the same topology. The three isolates B-Med, B-Bug and B-151 form one cluster with high bootstrap value; 99% for ITS and 98% for BT. The B-Bot, on the other hand, appears as a unique isolate (Figure 4.12). However, the dendrogram obtained based on ISSR markers, indicates that the Palestinian isolates, B-151 and B-Med are grouped together in one cluster with more than 50% bootstrap value. The B-Bug is the sister isolate for this cluster, while B-Bot still appears as a unique isolate (Figure 4.12).

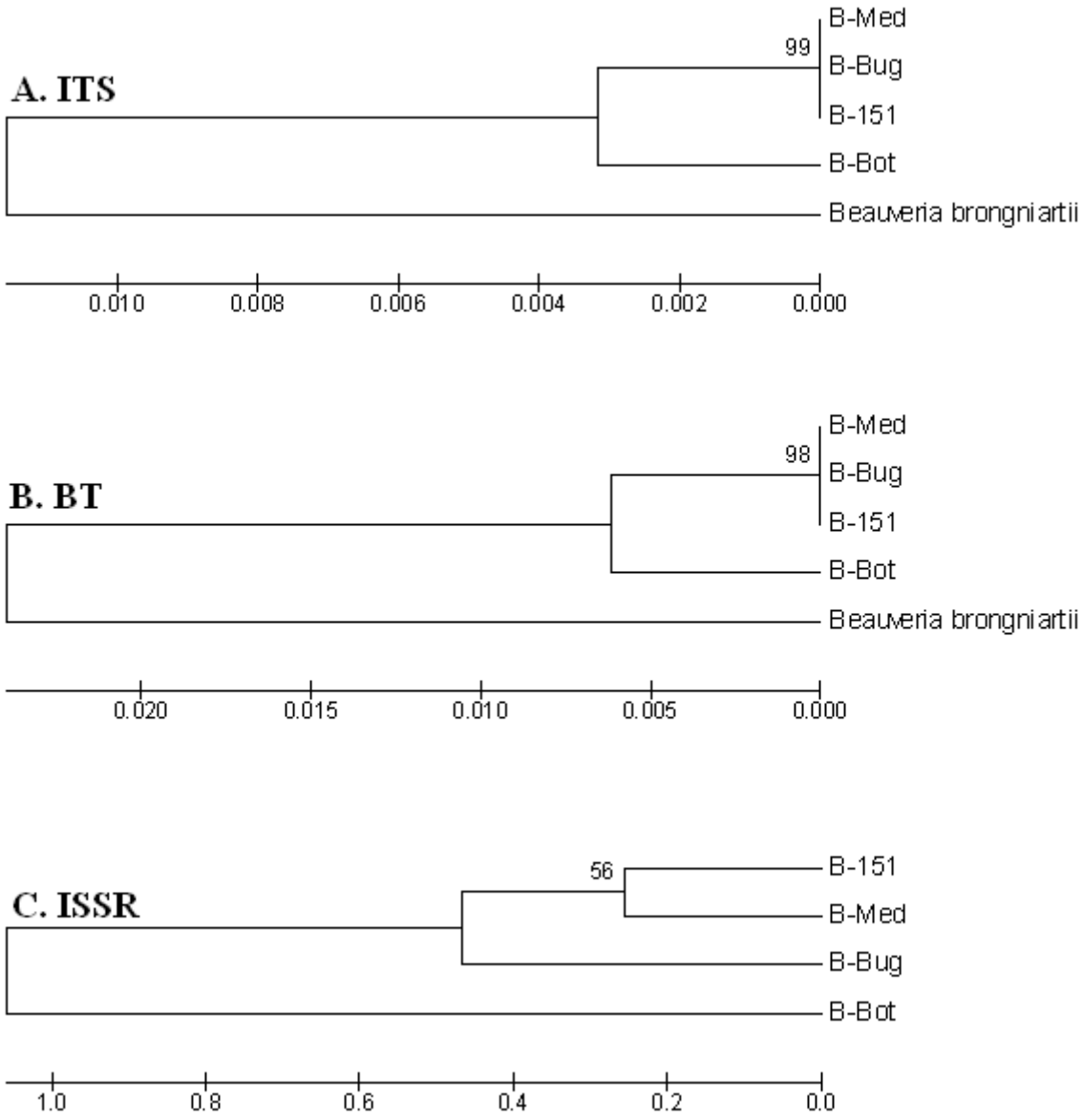


Figure 4.12: The UPGMA dendrograms showing the genetic relationships between *Beauveria* isolates based on: A. ITS, B. BT gene and C. ISSR-PCR markers. Numbers on branches indicate bootstrap (%) support for 1000 replicates.

4.2.6.2 *Metarhizium* Phylogeny

According to ITS and BT trees, the Israeli and Palestinian isolates (M-2004, M-WG, Ma-7 and M-Th2) form one cluster supported by more than 50% bootstrap values: 62% and 92%, respectively. The Palestinian isolate M-153 appears as sister isolate to the aforementioned cluster by high bootstrap value (86%) based on the ITS tree. However, it was clustered within this group according to BT tree (Figure 4.13). The

PPRC isolate was unique isolate according to both trees, in addition to the ISSR tree, and it was the farthest to the main cluster. According to ISSR tree, the Israeli isolate Ma-7 and Palestinian isolate M-Th2 were grouped together as one subcluster with moderate bootstrap value (75%). Another subcluster includes two Israeli isolates (M-2004 and M-WG) with 60% bootstrap value. The two subclusters are joined as one main cluster but with low bootstrap value of 47%. The Palestinian isolate M-153 appears as the sister isolate to this main cluster.

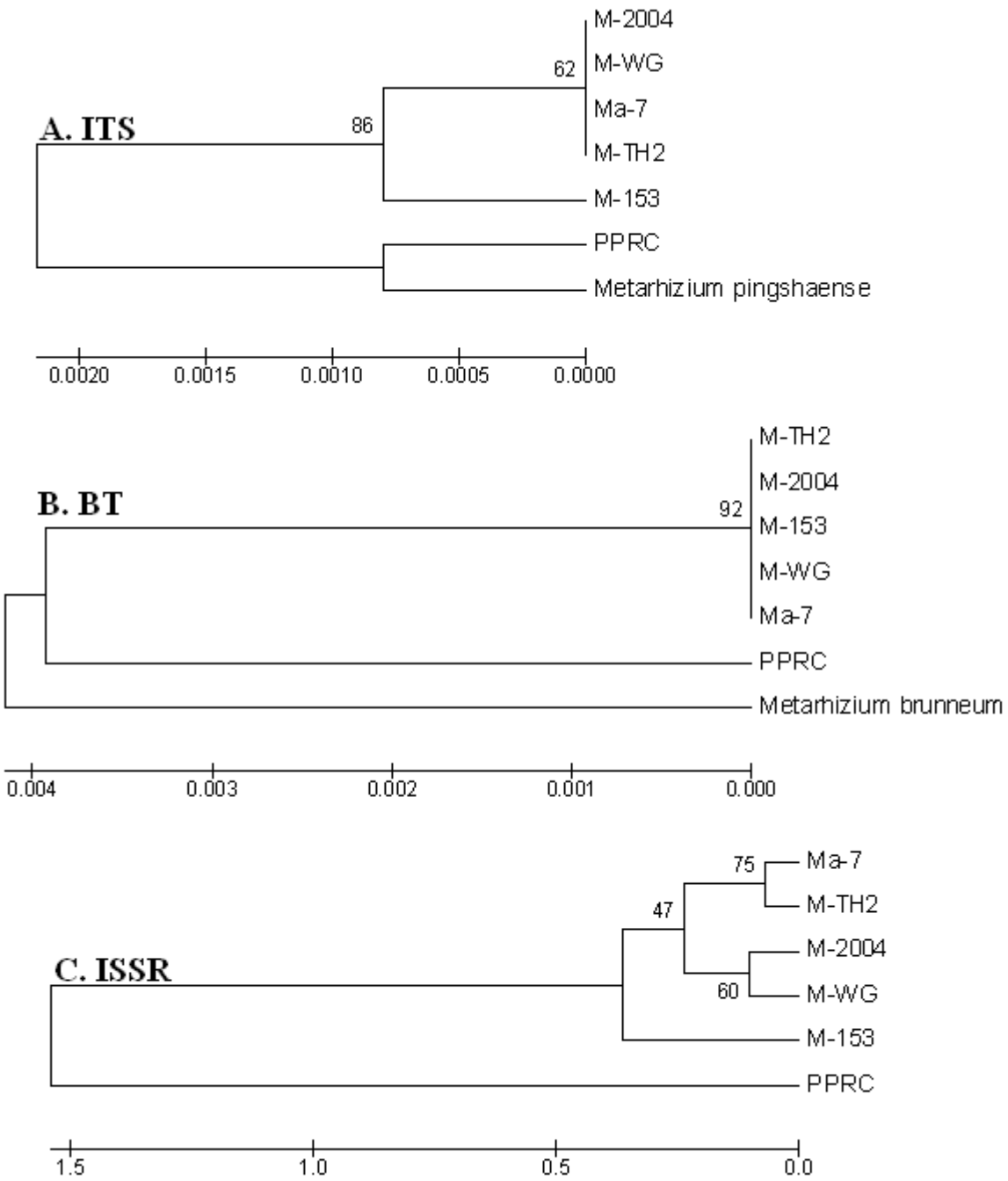


Figure 4.13: The UPGMA dendrograms showing the genetic relationships between *Metarhizium* isolates based on: A. ITS, B. BT gene and C. ISSR-PCR markers. Numbers on branches indicate bootstrap (%) support for 1000 replicates.

4.3 Thermotolerance characterization

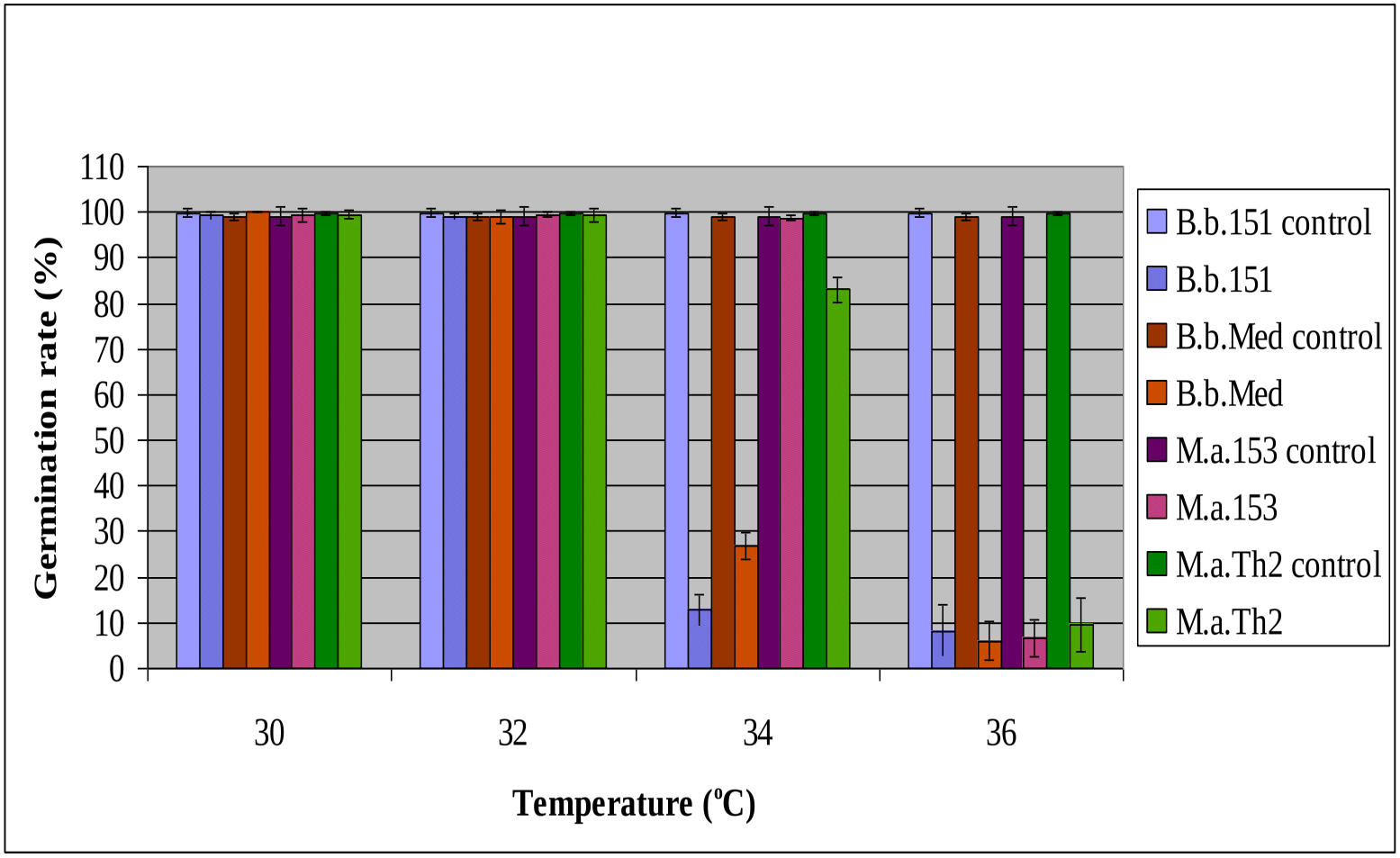
The characterization of thermotolerance involved determination the rates of mycelial growth and spore germination. The experiments in both cases were performed twice. In all statistical analysis, significance is related to *P* values below .05.

4.3.1 Spore germination rate

The results of spore germination for the two experiments were compared. A Sign test result indicated that the germination rates for both experiments were none significantly different under all temperatures (Z=0.00, P= 1.00). So, data of any of these two experiments can be considered for further analysis.

At 25, 30, and 32°C, all isolates exhibited high germination rates of 90-100 % (Figure 4.14). The statistical analysis of spore germination at these temperatures as well as at 36°C, using Kruskal-Wallis test indicated that the mean ranks of the spore germination rates for all isolates are not significantly different (H=2.74, 2.071, 1.024, 1.08, 3 d.f., P=.433, .558, .795, .781, respectively). However, they differed significantly among the isolates at 34°C (H=14.22, 3 d.f., P= .003). The M.a.153 isolate, gave the highest value of spore germination at 34°C that is 98.75 % $\pm$ 0.5 SD, while the B.b.151 gave the lowest one (12.75 % $\pm$ 3.59 SD) (Figure 4.14).

At 34°C, a Mann-Whitney test results revealed that M.a.153 isolate has mean ranks of germination rates significantly higher than the isolates B.b.151, B.b.Med, and M.a.Th2 (U=0.00, P=.018, .018, .017, respectively). M.a.Th2 has also mean ranks of germination rates that is significantly higher than that for B.b.151 and B.b.Med (U=0.00, P=.02). The latter two isolates are significantly different from each other with higher mean ranks are related to B.b.Med (U=0.00, P=.021). All isolates showed ability of spore germination at the highest tested temperature (36°C). However, the spore germination rates for all isolates were severely declined compared to other temperatures (Figure 4.14).



ure 4.14: The effect of temperature on spore germination of four Palestinian EPF isolates. Undashed columns correspond to mean spore mination rates at the control temperature (25 °C). Vertical lines denote standard deviations.

4.3.2 Radial colony growth rate

As for the investigation of spore germination, the work on radial growth was also conducted in two experiments. The results of radial colony growth for the two experiments were compared. Although Sign test revealed that the radial colony growth rates from both experiments were significantly different (Z=7.82, P<.001), same conclusions were drawn when their statistical analysis was performed individually except one difference concerning M.a.Th2 at 30°C. The following analysis is related to the first experiment.

At 25, 34 and 36°C, all isolates showed similar response. The application of the Kruskal-Wallis test showed that the mean ranks of the radial colony growth at these temperatures are not significantly different among all the isolates (H=4.36, 0.00, 0.00, 3 d.f., P=.225, 1.00 and 1.00, respectively) (Figure 4.15). Exposing the isolates to the temperatures 34 and 36°C totally suppressed the mycelial growth in all isolates. However, the isolates responded significantly different when exposed to the temperatures 30 and 32°C (H=11.92, 83.45, 3 d.f., P=.008, P<.001 respectively) (Figure 4.15 and 4.16).

The application of Mann-Whitney test revealed that at 30°C, the isolates M.a.Th2 and B.b.151 have mean ranks of radial colony growth that are not significantly different from one another (U=484.5, P=.712). However, these growth rates are significantly higher than the corresponding rates for both B.b.Med (U=355.5, 311.0, P=.036, .007,

respectively) and M.a.153 (*U*=346.5, 311.5, *P*=.026, .007, respectively). The B.b.151 and M.a.Th2 isolates showed the highest value of radial growth zone diameter after 9 days of incubation at this temperature (23.75 mm ± 0.54 SD, 29.25 mm ± 0.29 SD, respectively), while the isolates M.a.153 and B.b.Med exhibited the lowest one (22.06 mm ± 0.31 SD, 17.69 mm ± 0.59 SD, respectively) (Figure 4.16). The latter two isolates showed no significantly different mean ranks of radial growth rates when one is compared to the other (*U*=494.5, *P*=.814).

At 32°C, the B.b.151 isolate showed a significantly higher mean ranks of growth rate when compared to B.b.Med, M.a.Th2 and M.a.153 (*U*=360.0, 130.5, 0.00, *P*=.041, *P*<.001, <.001 respectively). In addition, the isolate B.b.Med shows significantly higher mean ranks of radial colony growth rate when compared to isolates M.a.Th2 and M.a.153 (*U*=202.5, 16.0, *P*<.001, respectively). The isolate M.a.Th2 has also mean ranks of radial colony growth rate significantly higher than that of the M.a.153 isolate (*U*=128.0, *P*<.001). At this temperature, B.b.151 showed the highest value of radial growth zone diameter after 9 days of incubation (18.13 mm ± 1.56 SD), while M.a.153 gave the lowest one (0.63 mm ± 0.32 SD) (Figures 4.15 and 4.16). The maximal temperature that allowed mycelial growth for all isolates was 32 °C.

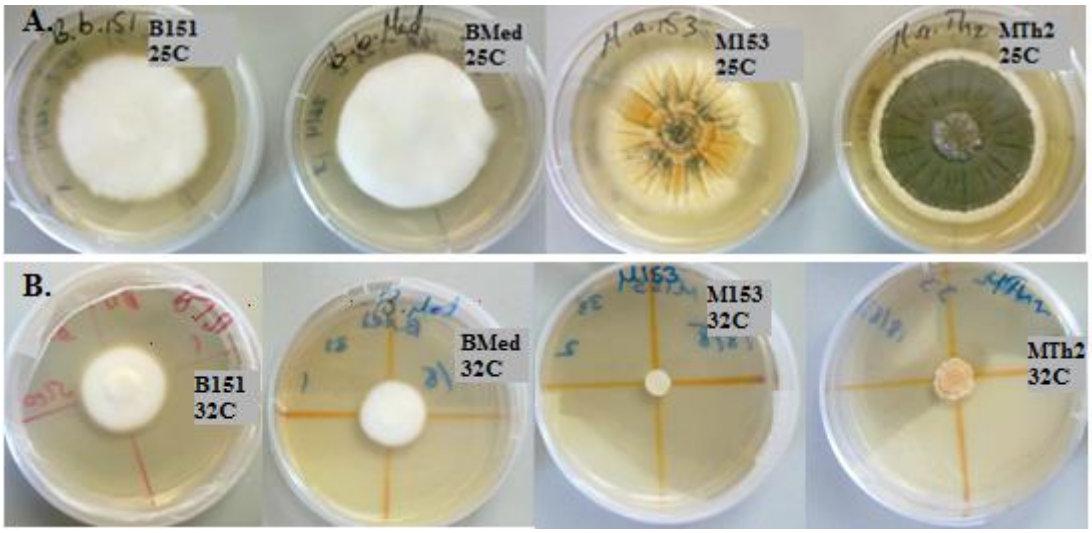
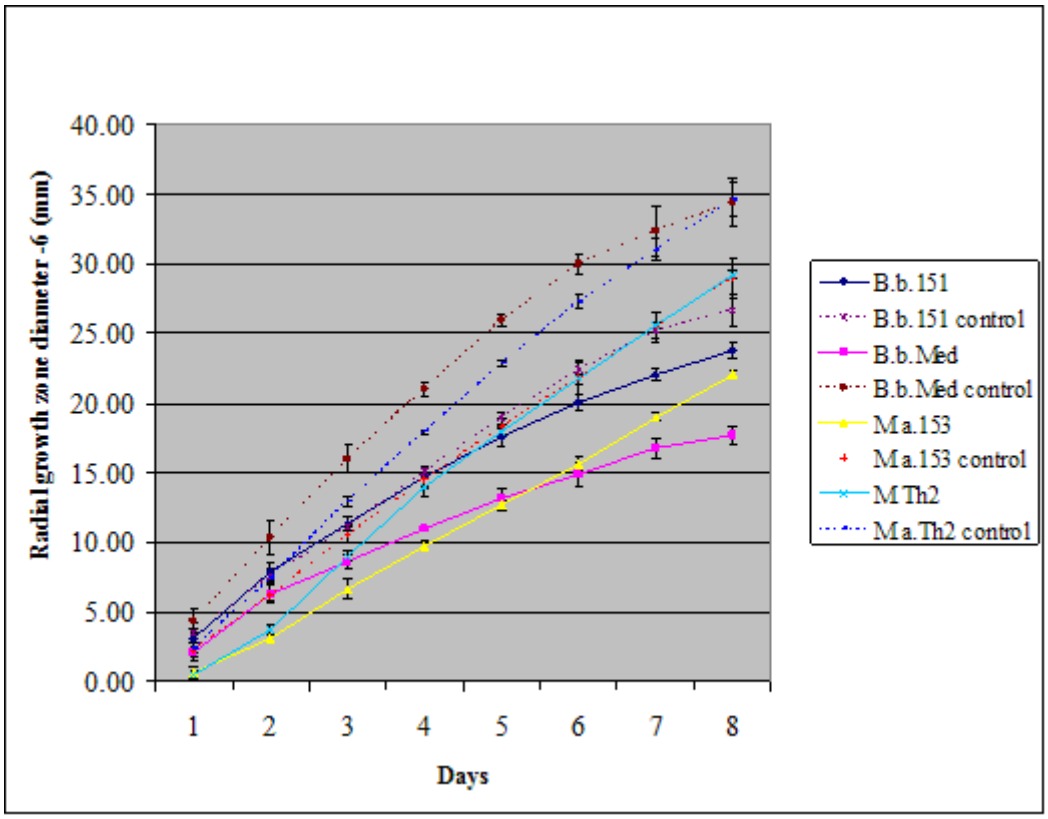


Figure 4.15: Radial colony growth of two isolates of *Beauveria* sp. and two isolates of *Metarhizium* sp. EPF isolates after 9 days incubation at 25 °C (A) and 32 °C (B).

A.



B.

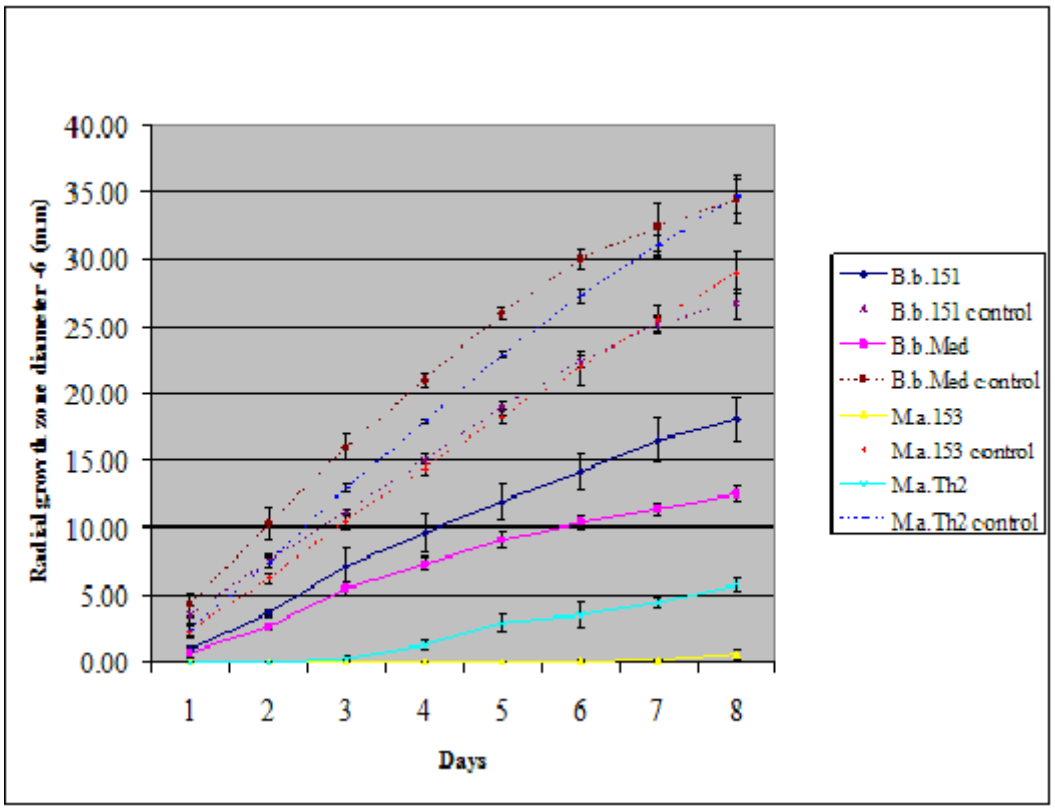


Figure 4.16: The effect of temperature on mycelium growth rate of the four Palestinian EPF isolates. A: the effect of 30 ˚C, B: the effect of 32 ˚C. Dashed lines correspond to mean growth rates of isolates under the control temperature 25 ˚C. Vertical lines denote standard deviations. No growth was observed under 34 & 36 ˚C.

CHAPTER 5

Discussion

Morphological characterization is a prerequisite step towards identification of EPF isolates as one important requirement for biocontrol applications. Conidia and conidiophores shape, as well as conidia color and diameter or lengths successfully discriminated between the different genera *Beauveria* and *Metarhizium*. However, there was an overlapping between the observed morphological traits for isolates belonging to the same species. This overlapping is obvious when comparing the conidial diameter or length, conidial shape and color, and conidiophores shape among isolates of the same species. The results of morphological characterization obtained in this study were in accordance with what had been stated by Tymon et al. (2004) that utilizing morphological characters is successful only in discrimination between different genera as *Beauveria* and *Metarhizium* and not between isolates belonging to the same species. This difficulty stems mainly from the lack of informative morphological variation.

Morphological measurements of conidia of the *Metarhizium anisopliae* and *Beauveria bassiana* putative isolates confirmed that all belong to *M. anisopliae* and *B. bassiana*, respectively. The measured conidial lengths for M.a.153 isolate were similar to those reported by Tulloch (1976) and Yip et al. (1992). However, on average, the conidia dimensions of the isolate M.a.Th2 were slightly smaller than the lower limit (5 µm) described by aforementioned authors. Whereas conidial diameters for *Beauveria* isolates were in accordance to what had been found by Glare and Inwood (1998) and Hussein et al (2010).

Different molecular markers were employed to study the genetic variability and to identify distinct isolates of *M. anisopliae* and *B. bassiana*. Genetic materials may allow distinguishing between isolates that are very similar in morphology. The highest score of ITS BLAST results was used as tool for isolate identification based on molecular data (Table 4.2). The isolates B-151, B-Med and B- Bug were initially identified as *Cordyceps* species. *Cordyceps* and *Beauveria* genera are closely related. They have different names to distinguish between the sexual species (*Cordyceps* sp.) and the asexual ones (*Beauveria* sp.). In fact, the same *Cordyceps* species could produce *Beauveria* anamorphs. As taxonomy relies on the sexual reproduction, both *Cordyceps* and *Beauveria* species are classified within the *Cordyceps* genus. However, the putative *Beauveria* isolates were asexually reproduced when cultured at the lab, as there must be two different mating types to reproduce sexually; a condition that can be only found in nature. So, these putative *Beauveria* isolates were later identified as *Beauveria bassiana* isolates. This is apparent as the just next 8 high alignments scores belonged to *B. bassiana* isolates. Moreover, when ITS1 and ITS2 were blasted individually against the GenBank sequences, the best match results were also related to *B. bassiana*. There is evidence supporting the direct links of *Beauveria* to the teleomorph genus *Cordyceps*. Shimazu et al. (1988) and Li et al. (2001) described species of *Cordyceps* (e.g., *C. brongniartii* and *C. bassiana*) that produced *Beauveria* anamorphs. They found that anamorphs of each species correspond morphologically to *B. brongniartii* and *B. bassiana*, respectively. Huang et al. (2002) provided additional confirmation of the *C. bassiana–B. bassiana* relationship by showing that *C. bassiana* was nested within a clade of *B. bassiana* isolates in phylogeny based on ITS sequences.

Both Palestinian *Beauveria* isolates have sequences that are not available in the GenBank with 99% homology with the best match sequences in the GenBank. This may indicate the occurrence of two new isolates that have never been reported before. This distinction is supported by the fact that the observed SNP corresponds to a transversion event.

Approximately, the same ITS size was recorded for isolates belong to the same species in both genera. In fungi, ITS length polymorphisms are not frequent within the same species; in most cases, variation in the ITS size does not exceed a few nucleotides among species of the same genus (Nazar et al., 1991). Gaitan et al. (2002) confirmed these observations while studying the genetic variability of *Beauveria bassiana* isolates.

The ITS2 sequence was more informative than ITS1 for *Beauveria* (Figure 4.6) as well as for *Metarhizium* isolates (Figure 4.7). These observations were in accordance with other findings, where *Beauveria brongniartii* showed higher sequence variation in the ITS2 region (Neuvéglise and Brygoo, 1994). However, a different trend was reported by Shih et al. (1995) who found a moderate level of mutation in the ITS1 region of *Beauveria bassiana*, although the ITS2 region was invariant.

The size of the partially amplified BT gene in our *Beauveria* isolates was around 350 bp. This size is larger by 10 bp than other BT genes amplified, using the same primers, in a worldwide sample of *Beauveria bassiana* isolates (Devi et al., 2006). The β-tubulin gene sequence was able to distinguish only one *Beauveria* isolate; B-Bot (Figure 4.8) as well as the *Metarhizium* isolate, PPRC (Figure 4.9). Only part of the gene sequence was investigated. The best match for the BT sequences of *Beauveria* isolates obtained, when BT sequences were blasted against GenBank sequences, was referred to *Beauveria bassiana* (accession no. AJ312228). The BT

sequence contains 5 exons and four introns, and our sequence aligned only with the region extending from nucleotide number 303 to 603. This region contains only one intron located between the exons 3 and 4.

Based on BT and ITS sequence analysis, the fungal isolate B.b.Bot was molecularly differentiated from the other *Beauveria* genotypes by four substitutions (Figure 4.6 and 4.8). One of them is located in the 5.8S region. As it is a rare event to have variation in the 5.8S region among isolates of the same species, this single polymorphism could differentiate between the *Beauveria bassiana* species that solely reproduce asexually; like *B. bassiana* (B-Bot), and other *B. bassiana* species (B-151, B-Med and B-Bug) that might be produced from teleomorphs. The B.b.Bot isolate differed from the other *Beauveria* isolates perhaps due to its unknown geographical and biological origin. On the other hand, the limited genetic variation found among the three *Beauveria* isolates is due to amplifying mostly conserved BT gene regions (two exons and one intron). In addition, the isolates shared the same insect host range, which was Coleoptera, and approximately the same geographical region. However, Wang et al. (2003) suggested that the genetic relatedness of *B. bassiana* strains was more associated with geographical location than with insect host species. This observation was confirmed by another study of Wang et al. (2005). It should be pointed out that all of the observed nucleotides differences were not a result of misreading the DNA sequence, because sequence results of two PCR clones supported these polymorphisms.

In our case, about 90% of the bands generated using ISSR were polymorphic for *Metarhizium* isolates and 80% for *Beauveria* genotypes (Table 4.3). This demonstrated the high level of genetic variation existing among the tested isolates. The same level of genetic variation was observed in the study conducted by Estrada et

al. (2007). The polymorphisms were detected in *Metarhizium* more than in *Beauveria* fungal isolates. This could be explained by the fact that a larger number of *Metarhizium* isolates were analyzed in this study (six versus four), in addition to the inclusion of the Ethiopian isolate PPRC which accounted for most of the variations. According to this study, ISSR markers proved to be an efficient marker system because of their capacity to reveal several informative bands in a single amplification (a mean of 9.5 and 8 bands per primer for *Metarhizium* and *Beauveria*, respectively). Furthermore, it was possible to identify all *Beauveria* isolates with a single primer (Figure 4.11: A, B, C and D). The maximum number of amplified products was produced by primer 810 for *Beauveria* isolates (Figure 4.11: C). This finding agreed with Estrada et al. (2007) finding while studying the molecular diversity for different *Beauveria* isolates using ISSR. There was genetically distinct variation among the four *Beauveria* isolates using all ISSR primers, while none of them was able to discriminate between all *Metarhizium* isolates (Figure 4.11). This is an expected result, since these primers were originally used for investigating *Beauveria* and not *Metarhizium* species. Investigating *Metarhizium* using ISSR is not well established yet. All of the tested EPF isolates, except M.a.Th2 and M.a.WG, have exclusive bands. These bands are very important for identification as they constitute isolates fingerprints.

The ITS sequence analysis, as well as BT sequence and ISSR analysis, of *Metarhizium* isolates indicated that the Ethiopian isolate PPRC was the most divergent from other isolates. This might be attributed to the distant geographical location from which it was isolated in comparison to the rest of the *Metarhizium* isolates, which are inhabitants of approximately the same geographical region. The latter factor may account for the little information provided by the molecular markers,

especially in the ITS and BT regions, concerning these isolates. The M.a.153 was distinct compared to all other *Metarhizium* isolates by two variations in the ITS sequence. This might indicate that the origin of this isolate is a region different than Palestine/Israel. This variation, even the single SNP, was very important as it allowed the discrimination of this isolate from the others. Consequently, this SNP constitutes a fingerprint for M.a.153 identification (Figure 4.7).

The ITS and BT markers have the same level of informativeness in discriminating *Beauveria* isolates (Figure 4.6 and 4.8). While ITS was more informative than BT in discriminating *Metarhizium* isolates as ITS marker distinguished M.a.153 from other isolates whereas BT could not. In comparison, ISSR marker was the most informative marker as it discriminated all *Beauveria* and most of *Metarhizium* isolates.

The ITS and BT dendrograms showed the same structure for both genera except the clustering of the isolate M.a.153 (Figure 4.12 and 4.13). In contrast to other markers, the ISSR marker was capable to resolve the cluster containing the four *Metarhizium* isolates M-WG, M-2004, M-Th2 and Ma-7 into two well supported subclusters (Figure 4.13).

In the ITS- and ISSR-based dendrograms (Figure 4.13), the isolates; T-2004, Ma-7, and M-Th2 were clustered as one group while the M-153 and PPRC isolates appeared clearly different from the rest of the *Metarhizium* isolates. The observed genetic variation could be of a particular importance, especially in light of the fact that the former three isolates, as well as the *Beauveria* isolate B-151, showed higher virulence against the cattle tick *Rhipicephalus annulatus* than the latter two genotypes (Ment et al., 2009a). Exclusive bands for the isolates: T-2004, Ma-7 and B-151 were obtained, and in the future it will be possible to identify specific molecular marker for these important isolates.

Although three molecular markers were used, none of them could genetically differentiate between the M-WG and M-Th2 isolates. On the other hand, in our study it was possible to distinguish between the four *Beauveria* and five *Metarhizium* isolates, which were expected to be genetically close, with only one primer per genus. This might be attributed to the limited number of studied isolates.

ITS and BT data did not provide sufficient resolution to clarify the relationships at the intra-species level. Highly conserved ITS regions have been demonstrated in other fungal studies (Skouboe et al., 1999; Tymon et al., 2004). However, ISSR revealed more variations than the BT and ITS could since it screens the whole genome.

The RFLP results showed that only few variables can be obtained from a single restriction enzyme. Thus several restriction enzymes must be used for each target region to increase variability. PCR-RFLP and DNA sequence alignment of the ITS regions of *Metarhizium* isolates identified polymorphic restriction endonuclease site for *TseI* (Figure 4.10). The identification of M.a.153 by RFLP technique was the only example we utilized to produce an isolate fingerprint for specific identification of fungal variants. All of the variations produced from different isolates can be examined either for facilitating the design of DNA probes specific to single strains, or producing a unique RFLP profile like what had been done with the M.a.153 isolate in this study. Furthermore, the exclusive bands produced by ISSR can be purified, sequenced, which would then allow designing specific primers for identifying the isolate that having the distinguished band. These probes would be useful in taxonomic, ecological, and population-level studies.

Unfavorable temperatures are one of the important factors that often determine the success of entomopathogenic fungi in controlling arthropods. Fungi thermotolerance to high temperatures is especially important when selecting fungal strains for

controlling ectoparasites while they feed on mammalian hosts. Pathogenicity of most entomopathogenic fungi is usually performed at 25–28°C. However, isolates with an optimal growth temperatures below these levels may not be successful for application on cattle skin where the temperature is higher (30 – 35°C) as shown by Polar et al. (2005). Consequently, the isolates were tested for their growth in vitro at temperatures ranging from 25 to 36°C. It appeared that all Palestinian EPF isolates were capable of growth at up to 32°C with apparent differences among isolates in their tolerance to this temperature.

It is known that the spore is much less susceptible to adverse environmental conditions like elevated temperatures than is the mycelium. This is an evident in our study as the maximal temperature for spore germination was 36°C for most isolates, which was in accordance with what was found by Walstad et al. (1970) and Fargues et al. (1992), while it was 32°C for mycelial growth for all isolates. All Palestinian isolates were isolated from temperate regions, so they were expected to be mesophiles that could grow at temperatures up to 32°C.

The Radial growth measuring method fails to account for differences in aerial mycelium, so inaccurate conclusions could be drawn about an isolate like B.b.Med which has obvious aerial cotton-like mass (Figure 4.15). Methods that account for all vegetative growth may reveal that this isolate tolerates high temperature more than or approximately the same as the isolate B.b.151, which showed the highest mycelium growth at the maximal temperature.

The only difference in analysis outputs between the two experiments was observed when comparing the results of radial colony growth of M.a.Th2 at 30°C. This isolate was not significantly different from both isolates B.b.Med and M.a.153 in the second experiment (*P*= .068, .098, respectively). This was because sample mean of the radial

growth diameter for this isolate decreased from 21.8 mm in the first experiment to 21.21 mm in the second one. This difference didn't affect our conclusions as we were interested in the difference between isolates in their tolerance to the higher and maximal temperature which was 32 rather than 30°C.

Now it is evident for all isolates that when temperature increases beyond growth-optimal levels, growth rate declines. The two *Metarhizium* isolates tolerated relatively higher temperatures like 34°C more than the two *Beauveria* isolates in terms of spore germination. However, the two *Beauveria* isolates showed more tolerance to the relatively high temperature 32°C than the *Metarhizium* isolates in terms of mycelium growth. In reality, both life stages shall be considered to judge the thermotolerance capability for each fungal isolate. In our experiment, for example, M.a.153 and B.b.151 isolates tolerated 32°C more than the other Palestinian isolates in terms of spore germination and mycelial growth, respectively. However, M.a.153 was the most sensitive regarding mycelial growth. The isolate B.b.151 gave high germination rate (99%) at the maximal temperature of 32°C, and the highest mycelial growth after 9 days incubation (18.13 mm). Also, this isolate was found to be highly virulent toward the cattle tick *Rhipicephalus annulatus* (>50% mortality) as reported recently (Ment et al., 2009a). In light of these facts, this isolate might be considered to have a promising potential for biocontrol applications.

As mentioned previously, the temperature of mammalian skin varies and is influenced by the environment. During midday, the skin temperature may be relatively high for several hours. Therefore initial selection of thermotolerant strains for tick control must be based not only on the ability of the conidia to germinate and to grow at constant temperature but also on their ability to retain their virulence and germinate following temporary exposure to high temperatures that exceed its maximal growth

limit (Jaronski, 2009). In this regard, part of the work was already done for some of the Palestinian EPF isolates in another study (Ment et al., 2009b). Six hours increasing of temperature to 37°C didn't influence the germination rate and radial growth of M.a.Th2 isolate following cultivation at 25°C.

CHAPTER 6

Conclusions and Recommendations

Since morphological characterization is not useful tool to discriminate between isolates of the same species, it helps as the first step of fungus identification that can be further confirmed by molecular tools.

The molecular methods applied in this study were successful to reveal genetic variation among the various tested isolates. According to the best of our knowledge, this study is the first attempt to discriminate between these local EPF isolates using advanced molecular techniques.

Sequencing data of ITS confirmed six isolates of *M. anisopliae* while the other four were *B. bassiana* isolates. The ITS sequences of the Palestinian *B. bassiana* and *M. anisopliae* isolates were similar to the corresponding sequences of the same species available in the GenBank.

As one important finding, the Palestinian isolate M.a.153 differed from its Palestinian sister isolate M.a.Th2 and other isolates by one substitution at nucleotide 409 in the ITS2 region. Using RFLP-PCR of this region was able to produce a fingerprint for M.a.153.

The techniques used here indicated that ITS and BT had limitations in identifying intra-specific variations in *B. bassiana* and *M. anisopliae*. However, ISSR markers detected a high level of polymorphism among *Beauveria* (80%) and *Metarhizium* (90%) isolates.

In this study, the produced sequence data will allow designing specific primers or specific RFLP profile to identify individual isolate in subsequent studies. Moreover, ISSR fingerprints provided a useful tool for establishing a rapid and rational approach for differentiation among isolates of entomopathogenic fungi as they provided genetic profiles that identified all *Beauveria* and most *Metarhizium* isolates. Then, ISSR markers may be converted into strain-specific sequence-characterized amplified region (SCAR) markers. SCAR markers can be used to track the fate of experimentally released strains.

Exploring larger region of BT gene, contains more than one intron, could be more informative. Also, exploring other parts of the genome like Intergenic region (IGR) or using Amplified Fragment Length Polymorphism (AFLP) and RAPD techniques may explore additional variations.

Isolates of the same species varied in their response to elevated temperatures in regard to spore germination and mycelial growth. All Palestinian isolates were capable of growth at temperatures (25 - 32°C) with apparent differences among isolates in their tolerance to the maximal temperature 32°C. At this temperature, the B.b.151 isolate showed high germination rate (99%), and the highest mycelial growth diameter value

(18.13 mm ± 1.56 SD). From the present study, this isolate appears to be a good candidate for use as biopesticide taking into account its thermotolerance and virulence documented in other study.

The next step of strain selection for use on warm-blooded animals is to test the ability of a strain to germinate following temporary exposure to high temperatures that exceed its maximal growth limit. This was already done for M.a.Th2 but not for B.b.151 which is of high potential as biocontrol agent. As none of the isolates succeeded to show mycelial growth beyond the temperature 32°C, additional research should be done to explore naturally occurring thermotolerant strains. The Jordan Valley or Jericho areas are examples for warm regions. Future work can be done by continuous cultivation of isolates that grew well at 32°C under gradually increasing temperatures in order to select lines that tolerate temperatures higher than 32°C. Another possibility is to induce thermotolerance in these isolates by preconditioning and exposing them to a mild heat shock (e.g. 30 minutes at 37°C) prior to a more severe heat shock. Moreover, thermotolerance variations among the different isolates studied could be attributed to a variation in the expression of the heat shock proteins genes. This could be verified by a quantitative determination of heat shock proteins produced by these isolates.

Finally, the results indicate that there are genetic and thermotolerant variations among isolates of the same species which could be a good basis for further study to achieve a detailed discrimination among the genomes. This achievement would, in turn, facilitate the use of these fungi as biocontrol agents.

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