Chapter 1

General Introduction

1.1. The economic importance of *Helicoverpa (Heliothis) armigera* (Hubner)

The corn earworm, *Helicoverpa (Heliothis) armigera* (Hubner) is a polyphagous insect attacking many crops, and is regarded as a serious pest in different geographical regions with world wide distribution. These regional populations are adjusted to regional environment, and to the prevalence of host. Since it is a generalist feeder, currently control of this insect mainly relies on applications of chemical insecticides. *Helicoverpa (Heliothis) armigera* developed considerable resistance to pesticides because of successive generations moving from one crop to another and exposed to a number of chemical applications of pesticides therefore the insect become one of the most difficult insect pests to manage due to high levels of insecticides resistance (Ranga & Shanover, 1999). Due to acquired resistance to pesticides, high expensive production process, and ecological pollution, several alternatives have been suggested, one of which is the biological control using the bio-agent *Nomuraea rileyi* (Farlow) Samson.

The larvae of *H. armigera* attacks tomato, pepper, cabbage, pea, kidney bean, corn, alfalfa, clover, tobacco, cotton, and peanuts. This insect pest may cause extremely serious economic damage and is widely distributed in many parts of the world. It is found in Africa, Asia, Europe, and Australia, and it is an economic insect pest in Middle East (Hariri, 1984).

1.1.1. Life cycle of *H. armigera*

Adults of *H. armigera* mate shortly after emergence. The males live for only few days. The females lay from 300-3000 eggs with an average of 700 eggs during their life span which may last 3-weeks in summer. The eggs are laid on stems, leaves and fruits either singly or in clusters. Under favorable conditions eggs hatch 3-5 days after oviposition, and the larvae starts feeding on the host plant specially leaves.

After 3-5 days the larvae starts borrowing into the fruits, and may move from one fruit to another. The larvae reach maximum size under favorable conditions within 2-3 weeks, after that the larvae leave the fruit to penetrate the soil where they pupate; in few cases it may pupate inside the fruit. The pupa development lasts 1-2 weeks during summer, while in the winter season it may last 4-5 weeks. The insects complete their development within 25-40 days during summer, but in the cold seasons it may require 6-7 months. The overwintering stage is pupa at a depth of 3-8 cm in the soil. In Palestine the insects have four generations a year (Sharaf, 1993; Avidov & Harpaz, 1969)

1.2. The fungus *Nomuraea rileyi*

1.2.1. Taxonomy

Nomuraea rileyi (Farlow) Samson a deuteromycete fungus (*Fungi Imperfectii*) belongs to the order Moniliales. This entomopathogen is dimorphic with invasive hyphal stage and a yeastlike vegetative stage. Each phenotype has unique characteristics that contribute to pathogenicity of the fungus towards insect hosts (Pendland & Boucias, 1997).

The penicillate head of the genus *Nomuraea* produces cylindrical phialides and greenish, one celled conidia in short, dry chains. Most of these features indicate a close relationship with genus *Penicillium* (Shigeru & Shun, 2002). The key difference is seen at the apex of the phialide

which is characterized by the absence of distinct neck (Shigeru & Shun-ichi, 2002). In addition, most species of *Nomuraea* are entomogenous (Tzean *et al*., 1993), whereas *Penicillium* species are known as widespread and omnivorous.

The entomopathogenic fungus *Nomuraea rileyi* (Farlow) Samson, originally described as *Botrytis rileyi* (Farlow) and later as *Spicaria rileyi* (Farlow) Charles, was redescribed by Kish *et al.* (1974) and transferred to the genus *Nomuraea* Maublanc.

The genus *Nomuraea* was established by Maublanc (1903). Kish *et al*. 1974, considered *Botrytis rileyi* (Farlow) identical with *N. pracina*, and simultaneously they proposed, for reasons of priority, the new combination *N. rileyi* (Farl.) Samson, as the type species of *Nomuraea*. Watson was the first to report the effectiveness of *N. rileyi* (=*Spicaria rileyi*) in Florida as the etiological agent of the disease known as "Cholera", to velvetbean caterpillar, *Anticarsia gemmatalis* (Hubner) during September and October of 1914 and 1915 (Kish et al., 1974). Hinds and Osterberger (1931) reported that *Spicaria prasina* (Maulb.) (= *Spicaria rileyi)* was an important factor in suppressing the final fall generation of the velvetbean caterpillar during September & October of 1929 and 1930 in Florida.

1.2.2. Life cycle of the fungus

The fungus *Nomuraea rileyi* (Farlow) Samson grows either in mycelial hyphal phase or in yeast-like hyphal body phase. The hyphae and hyphal body (blastospoes) phases often occur simultaneously during growth of the fungus (Pendland & Boucias, 1997).

Conidia attach to the insect cuticle via hydrophobic interaction (Boucias *et al*., 1988). After 12-24 h the conidia germinate, and the germ tubes penetrate the epicuticle and grow through the cuticular and epidermal region to the hemocoel; in hemocoel, the growth of the fungus switches

from hyphal phase to the yeast-like hyphal body phase (Pendland & Boucias, 1997). The yeast-like hyphal body replicate extensively by budding and septation within the hemolymph (Pendland & Boucias, 1997). At 5-7 days the noninvasive hyphal bodies, which fill the host hemocoel, synchronously convert to an invasive mycelial stage (mycelial cells) which forms conidiophores and conidia.

The mycelia grow on mummified cadaver, produce conidiophores & conidia and the cycle is repeated when the fungus disperses via asexual hydrophobic spores (conidia) and contact new host insects (Pendland & Boucias, 1991; 1997).

Numerous numbers of conidiophores emerge, producing a confluent white fungal mat over the entire surface of the host larvae and by 9–10 days, pale green conidia develop, making the larval body green, (Kumar *et al*., 1997). The fungus is believed to maintain itself at low densities in most lepidopterous population during the mild winters, and adult moths, migrating in the spring, potentially carry and help disperse conidia (Allen and Kish, 1978). Conidia from previous seasons remain viable in the soil and are able to initiate epizootics the following season (Ignoffo *et al*., 1975, 1978).

It has been indicated that *N. rileyi* has potential to overwinter as sclerotia in the form of hyphal bodies in larval cadavers (Sprenkel & Brooks, 1977). Furthermore, the fungus withstands environmental stress of winter in conidial form (Kish & Allen, 1976; Ignoffo *et al*., 1978). Pseudosclerotia formed during the host pupal stages also may offer considerable overwintering protection. So the body wall of infected pupae may aid in protecting the fungus from ultraviolet light, desiccation and other environmental stresses (Bechinski & Pediogo, 1983). Thick–walled intra hyphal hyphae, clamydospores and larval resting bodies formed in unfavorable conditions, when conidiogenesis is inhibited can resume

metabolic activity when conditions become favorable and hence produce conidiophores and conidia again (Boucias and Pendland, 1982).

1.3. Ecology of *N. rileyi*

1.3.1. Distribution and occurrence of *N. rileyi*

Nomuraea rileyi (Farlow) Samson has been found in several areas all over the world and frequently causes epizootics in Lepidoptera larval population. *N. rileyi* epizootics in insect populations have been reported frequently in several American states (Allen *et al*., 1971; Ignoffo *et al*., 1975). It has been found that the fungus *N. rileyi* known to be the causal agent of late season epizootics of several caterpillar pests of soybeans at Columbia, Missouri (Ignoffo *et al*., 1975), and reservoirs of inocula may exist in the soil to infect larvae in the next season (Ignoffo, *et al*., 1978). The fungus maintain itself at low densities in lepidopterous host population during the mild winters in southern Florida and the Gulf Coast; migrating adult moths, potentially carry and help disperse conidia (Allen and Kish, 1978). The importance of *N. rileyi* as a natural control agent of the cabbage looper was cited by Getzin (1961) in Texas and by Behnke & Paschke (1966) in Indiana. Canerday and Gudauskas (1966) in Alabama reported that the fungus frequently associated with soybean looper.

This fungus has been reported in the hottest regions of Palestine (costal plain and the Jordan Vally area) on *S. littoralis* larvae on alfalfa (Kenneth & Olmert, 1975, Ben-Zeev, 1993). Surveys conducted by Ben–Zeev, (1993) revealed that the activity of *N. rileyi* in Palestine occurred during summer and early autumn seasons when the hosts of the fungus are also peaking (Avidov & Harpaz, 1969).

It was reported that there was no activity for *N. rileyi* below 5.6 cm of the soil, and practically all activity was confined to the top 2 cm of soil,

(Ignoffo *et a*l., 1976). In addition, prevalence of disease declined after heavy rainfall (Fuxa, 1984).

1.3.2. Effect of physical factors on growth and development of *N. rileyi*

Temperature

High temperature, low RH and solar radiation are known to be major factors limiting development and field persistence of entomopathogenic fungi in nature (Uziel & Shtienberg, 1993). Inspite of these facts, *N. rileyi* has been isolated in Palestine during summer, under relatively hot conditions (Kenneth & Olmert, 1975).

Virulence of entomopathogenic fungi is always affected by environmental factors such as temperature, humidity and light (Walsted *et al*., 1970; Ignoffo *et al*., 1985 and Hajeck *et al*., 1990). Spore survival, persistence and sporulation are influenced drastically by temperature (Walsted *et al*., 1970; Ignoffo *et al*., 1985 and Hajeck *et al*., 1990).

It has been found that the optimal temperature for infection of *N. rileyi* was 20ºC which resulted in 95% host larval mortality and LT_{50} of 5.8 days; temperature below 26ºC decreased the development rate of the infection process; lower or higher temperatures than 26ºC caused lesser mortality and recorded higher LT_{50} values (Boucias *et al*., 1984).

Conidial germination rates were best at 20 and 25 ºC (Tang & Hou, 2001). The optimal temperature for germination and penetration into insect body was at 20-25ºC (Gardner & Storey, 1985). Incubation temperature also seems to affect development of mycosis in insect hosts (Boucias *et al*., 1984; Carruther *et al*., 1985; McDonal & Richard, 1995).

Light

It has been found that *in vitro* sporulation was severely inhibited by dark conditions (Glare, 1987). Furthermore, light was essential to *N. rileyi* for conidial production on *Heliothis* cadavers (Glare, 1987). The fungus produced fewer conidia on younger cadavers kept continually in the dark. However, there was no significant difference in sporulation of the fungus on larvae between the full and half light incubation period, despite the fact that sporulation rate was lower under full darkness (Tang & Hou, 2001). Furthermore, larval mortality of the 5th instars larvae of *H. armigera* when incubated under full and half light was not significantly different but was higher under full darkness (Tang & Hou, 2001).

Larval mortality however was not affected by photoperiods; sporulation of *N. rileyi* on the cadavers was not significantly different between full and half light. Spore survival may be reduced under field conditions after exposure to solar radiation (Daoust & Peeira, 1986; Carruthers *et al*., 1988).

Relative humidity

Sporulation was found to occur only from those cadavers that were incubated under 95% and 100% RH; no conidia formed under lower humidity condition (Tang & Hou, 2001). High humidity is essential for spores of entomopathogenic fungi to germinate and penetrate into hemocoel after attachment to cuticle (Allen *et_al*., 1971; Ignoffo *et al*., 1977; Hajek *et al*., 1990). It has been found that *N. rileyi* is able to infect 3rd instar larvae of *Helicoverpa armigera* effectively after inoculation for only 4 h under high humidity (Tang & Hou, 2001). In addition, spore survival and viability after storage are influenced by humidity (Daoust and Roberts, 1983; Ignoffo *et al*., 1985). Fungal sporulation on insect cadavers also requires high humidity (Ferron, 1977; Ramoska, 1984).

1.3.3. Growth and development of *N. rileyi in vitro*

The fungus *N. rileyi* is dimorphic as already mentioned earlier, growing either in a hyphal or mycelial phase or in a yeast-like hyphal body phase that produce conidia (blastospores). *In vivo*, the transition from one developmental phase to another is highly synchronous (Pendland *et al*., 1994); synchronous growth patterns can also occur when the fungus is cultured *in vitro* (Pend land & Bocias, 1997).

Formation of each type of structure *in vitro* is regulated by medium composition; in complex media hyphal body form (Bell, 1975; Pendland & Bocias, 1997) whereas less complex media, such as Vogel's glucose medium, induce the formation of mycelia (Pendland & Bocias, 1997) .

Since *N. rileyi* has been considered for potential biocontrol, studies involving *in vitro* culture of the fungus have focused mainly on the production of large quantities of conidia (Bell, 1975; Vimala, 1994)

The rate of germination of fungus conidia is isolate and nutrient dependant, (Boucias & Pendland, 1984). On water agar, only limited germination was observed, in contrast to high level achieved on Saboraud's Maltose Agar with Yeast extract (SMAY).

Supplementing various nutrient substrates with crude lipid extracts or with diacylglycerol fraction of *A. gemmatalis* larvae increased and accelerated germination of certain isolates of *N. rileyi*, but the rate of *in vitro* germination was not correlated with virulence (Bocias & Pendland, 1984). In media supplemented with cuticle, chitinases and proteases were produced; this phenomenon was not observed on non amended media (El-Sayed *et al*., 1993).

Fungal growth and sporulation has been investigated under sabourad's Maltose broth; the medium allowed fungal growth and sporulation within 2-4 days (Holdom & Van Deklashorst, 1986). Rapid

hyphal growth and vast sporulation also took place on crushed sorghum with yeast extract (Vimala, 1994). Furthermore, maltose as a carbon source is required for sporulation and peptone as a nitrogen source is required for mycelial growth.

Protoplasts are not naturally occurring in *N. rileyi*; Boucias & Pendland (1982), however have developed a method of producing protoplasts from *N. rileyi* hyphal bodies (yeast like cells formed in hymolymph or culture media). Great numbers of conidia can be obtained on inexpensive culture media incubated in subdued light, e.g. agar containing brewer's yeast and soluble starch (Holdom & Van De Klashost, 1986), and on bran (Shenhar, 1977). Inoculation of Whitman's filter paper sheets with hyphal bodies along with nutrition sources (e.g. Sabouraud maltose broth), allowed fungal growth and sporulation within 2 to 4 days (Holdom & Van De Klashorst, 1986). The fungal morphology, biology, infectivity and pattern of germination may be altered due to continuous culturing *in vitro* (Morrow *et al*., 1989). However, it has been reported that no change in fungal virulance was observed after 12 times of culturing *in vitro* (Ignoffo *et al*., 1982).

The fungus *N .rileyi* can be cultured directly on cuticle based media indicating the ability to synthesize hydrolytic enzyme required for degradation of cuticle's complex substrate (El-Sayed *et al*., 1993).

1.3.4. Host range

This fungus has fastidious growth requirements, possesses a narrow host range, and does not have a saprophytic phase in soil. *N. rileyi* is recognized as the key mortality factor in certain noctuid populations under certain environmental conditions.

There are over 30 species of Lepidoptera listed as susceptible to *N. rileyi* (Ignoffo, 1981). Caterpillars belonging to the family Noctuidae are

included among its most sensitive hosts, such as *Trichoplusia ni, Anticarsia gemmatatis, Pseudoplusia includens, Helicoverpa armigera*, *Helicoverpa zea*, *Helicoverpa virescens, Spodoptera littoralis*, *Spodoptera exigua*, *Spodoptera ornithogalli*, and *Spodoptera litura*. (Vimala, 1994).

1.4. Epidemiology

The fungus *N. rileyi* may persist in the nature during harsh environmental conditions as thick-walled hyphae, chlamydospores and resting bodies in mummified larvae (Pendland, 1982). Unlike conidia, viability of hyphae did not extend throughout winter (Thorvilson *et al*., 1985).

The fungus *Nomuraea rileyi* disperses via asexual hydrophobic spores (conidia) (Pendland & Boucias, 1997). Since conidia produced on dead larvae over winter in soil, the soil was the major source of the inoculum that initiate new epizootics, (Ignoffo *et al*., 1975 and 1978); this source of inoculum is sufficient to contaminate soil each autumn provided by extremely low population of infected caterpillars.

The conidia which are produced however, on larvae throughout the field are dispersed by wind or rain (Ignoffo *et al*., 1977); the dead larvae infected with the fungus fall to the ground and provide inoculum for next season epizootic. (Ignoffo *et al*., 1977). Therefore, the load of overwintering inoculum, early availability of host, proper environmental conditions, and extent of dispersal of conidia early in the season, largely determine the epizootic peak in the season (Ignoffo *et al*., 1977). It has been found that the movement of diseased larvae was a factor in spreading *N. rileyi* over plants (Ignoffo *et al*., 1976).

The epizootics usually occur late in the growing season (Kish *et al*., 1976). The fungus is believed to maintain itself at low densities in lepidopterous host populations during mild winters; heavy rainfall reduces

prevalence of the disease (Allen and Kish, 1978). It has been found that the progress of the epizootic was related to density of larvae and availability of the fungus; although the pathogen occurs naturally, it required several generations before sufficient spores become available to control insect populations (Allen & Kish, 1978).

The conidia of *N. rileyi* placed on the soil surface in cotton- plugged shell vials or directly on the surface of soil were still infectious for 3rd-instar larvae of the tobacco budworm, *H. virescens*, after 209 and 138 days, respectively (Sprekel & Brooks, 1977). Moreover, *N. rileyi* on buried cadavers or cadavers placed on the soil surface survived 285 and 268 days respectively (Sprekel & Brooks, 1977).

Fungicides applications in early growing season directed against phytopathogenic fungi can interfere with the natural occurrence of *N. rileyi* or other beneficial fungi (Johnson *et al*., 1976; Horton *et al*., 1980).
A practical strategy to avoid this interference is to apply fungicides with low impact on *N. rileyi* inocula, as sulfur or carbendazim based products. Fungicides as benlate present high inhibition of *N. rileyi* and can delay its epizootics in the field (Johnson *et al*., 1976; Horton *et al*., 1980). Some herbicides, as lactofen & fomesafen are strong inhibitors for *N. rileyi* growth "*in vitro*" (Johnson *et al*., 1976; Horton *et al*., 1980), but their effects under field conditions are undetermined.

1.5. *Nomuraea rileyi* biocontrol potential

Nomuraea rileyi, have potential for control of noctuids (Ignoffo, 1981; Vimala, 1994). The fungus was found to be safe to human beings and other nontarget organisms including insect parasites and predators (Ignoffo, 1981). Under laboratory conditions, the fungus was not able to infect lady beetle and *Hippodamia* (James & Lighthart, 1994).

Control of insect hosts with entomopathogenic fungi are usually affected by different biotic and abiotic factors in nature (Ferron, 1978). Spore survival and fungal infection suffer from inhibition by pesticides under field conditions (Ignoffo *et al*., 1975; Loria *et al*., 1983; Gardner & Storey, 1985; Hsiao & Lin, 1995). However, synergistic effects are possible if applied with insecticides properly (Anderson *et al*., 1989).

The entomopathogenic fungus, *N. rileyi*, caused 90.5–100% mortality in fourth-instar larvae of the corn earworm, *H. armigera*, when applied at 10^7 conidia/ ml to corn silks, leaves of soybean, tomato and chrysanthemum; the LT_{50} was 5.9–6.7 days (Li-chang & Roger, 1998).
The fifth-instar larvae of *H. armigera* showed a mortality of 94.6% on soil with 20% water content, and 41.7% on soil with 10% water content when the soil surface was sprayed with 10^8 conidia/ml suspension of *N. rileyi* (Li-chang & Roger, 1998).

It was found that injection or spraying of (10^5-10^7) conidia /ml suspension into corn ears was effective in controlling larvae of corn earworm resulting in 75.9%-85.8% marketable ears, and was not statistically different from carbofuran treatment (Li-chang & Roger, 1998). Virulence of insect pathogens however, can be reduced by leaf texture and antifungal substances produced by plants (Smirnoff, 1972; Hare & Andreadis, 1983 and Boucias *et al*., 1984). *N. rileyi* conidia were able to cause over 90% mortality of 4^{th} instar larvae when sprayed onto different plant parts. Fungal preparation in aqueous solution mixed with surfactants can be suitable for corn earworm control under field conditions (Li-chang & Roger, 1998).

In India, *S. litura* larvae exhibited >80% mortality, 19 days after spraying of castor plants with aqueous suspension of *N. rileyi* (Vimala, 1994). In Taiwan spraying of corn plants with aqueous suspensions of the

fungus significantly reduced *H. armigera* larvae, resulting in significant increase of marketable corn ears (Tang & Hou, 1998).

In Ecuador's humid costal plain, application of aqueous sprays of *N. rileyi* against *Anticarsia gemmatalis* and *Pseudoplusia includens* in soybean revealed limited increase in larval mortality (Stansly & Orellana, 1990).

It has been reported that eggs, pupae and adults of *Spodoptera littoralis* were not infected by *N. rileyi* (Boucias *et al*., 1984), but larvae emerging from contaminated eggs became infected by the fungus (Fargues, 1984).

In net house studies, initial mortality for *Spodoptera litura* by the fungus was seen after 7-8 days, but the cumulative mortality was high at 10 days; the mortality percentage had direct correlation to the dosage and was significantly high at 10^{11} and $5x10^{11}$ conidia/ liter (Vimala, 1994).

In the field, initial mortality of larvae was obtained at 9 days after spraying, and significant mortality of the larvae of *Spodoptera litura* was observed at 12 days after spraying (Vimala, 1994).

Commercial products of *N. rileyi* (Ago-Biocontrol *Nomuraea* 50) were registered for control of Lepidoptera in flowers and vegetables in Columbia; there is no other commercial product of *N. rileyi* (Shah & Goettel, 1999).

Virulence of *N. rileyi* isolates against the target insect pests is the most important criterion for commercial exploitation of the fungus. It has been found that virulent isolates of *N. rileyi* are necessarily fast in germination; possess high infectivity coupled with amenability to multiplication on various media (Vimala *et al*., 2003).

1.6. The infection process

Research regarding the mechanisms of fungal invasion and pathogenesis has revealed that a number of extacellular enzymes, toxins,

pigments and physical factors may be involved. (Fargues, 1984; Moore & Prior, 1993; Hajek & St. Leger, 1994). Hyphal bodies did not infect various noctuid larvae when applied topically, in contrast to the infectious effect of conidia (Bell, 1975). Furthermore, in terrestrial habitats, the infectious propagule of the fungus is the conidium.

The fungal pathogenesis in insects indicates that it occurs via a series of integrated, systematic events progressing from spore attachment to germination, penetration, growth and proliferation within the body of the host, interaction with insect defense mechanisms and finally death of the host (Moore & Prior, 1993).

1.6.1. Attachment & germination

Prerequisite to the initiation of infection is the attachment of the spore to the insect cuticle (Moore & Prior, 1993). The spores of the fungus are dispersed passively via air currents or free water over substrata inhabited by host insects. Upon contact, the spores attach preferentially to the cuticle, produce penetrating germ tubes which breach the host cuticle, and invade the host hemocoel. The conidium-cuticle interaction may involve a complex of specific (glycoprotein) and nonspecific (electrostatic or hydrophobic) recognition mechanisms (Fargues, 1984).

It has been found that *N. rileyi* possess a hydrophobic rodlet layer (a layer of highly organized surface protein) which may provide protection against dehydration and microbial attack and a mean of dispersal in air currents. However, a major role of the rodlet layer present on this fungus is believed to be for attachment of conidia to insect cuticle, a process which is mediated by a hydrophobic interaction. While this interaction does not appear to be specific to a particular host, it does provide a mean for preferential binding to insect epicuticle (Boucias *et al*., 1988).

Furthermore, it has been revealed that more conidia appeared to be trapped by and tightly adsorbed to the cuticular surface of 1st instar larvae of *H. armigera* than those adsorbed to the cuticular surface of the 4th instar larvae (Boucias *et al*., 1988).

The spore germination of the fungus *N. rileyi* is highly affected by temperature and humidity; the optimum temperature for germination is 20-25 ºC (Gardner, 1985). High humidity is also essential for spore germination and penetration into the hemocoel after attaching to the cuticle (Getzin, 1961; Allen *et al*., 1971; Hajek *et al*., 1990).

It has been found that the uptake of water, resulting in swelling of the spore, was required for synthesis of protein which is needed for emergence of a germ tube (Dillon & Charnley, 1990).

It has been found as well that an important aspect of host specificity is thought to be associated with insect cuticle, interaction related to adhesion and to cuticle substrate utilization and resistance to host – specificity inhibitory compounds (Boucias & Pendland, 1984).

1.6.2. Cuticle penetrations

The insect cuticle is composed of protein, chitin, lipids and phenolic compounds which act as a barrier preventing invading microorganisms (St. Leger *et al*., 1986). One important feature of the entomopathogenic fungus *N. rileyi* is its ability to make contact and penetrate through any part of its host's body, whereas most other entomopathogenic fungi have to be eaten to establish infectivity. Penetration of the insect integuments, by the fungus *N. rileyi* has been characterized morphologically (Mohamed *et al*., 1978), ultrastructurally (Boucias & pendland, 1982), and histochemically (Gabriel, 1968; Brey *et al*., 1986). These studies suggested a combination of enzymatic degradation and mechanical pressure resulting in penetration and then infection. Several cuticle

dissolving enzymes have been detected in entomopathogenic fungi (St. Leger *et al*., 1986). It was reported that attachment and penetration of fat body and other internal organs of the insect by the germ tubes (produced by the hyphal bodies & hyphae) is via laminin-binding sites that interact with components of the basement membrane of the host (Pendland *et al*., 1994). It has been found that chitinases enzymes were produced in significant amount by virulent isolates of *N. rileyi*, but not by avirulent isolates (El-Sayed *et al*, 1989).

A rapid amplification in the rate of increase in chitinase enzyme only occurred in the virulent isolates, at the onset of blastospore stage. Therefore, high levels of chitinase enzyme were present at a stage critical to penetration of insect's integuments (El-Sayed *el al*., 1989). The susceptibility of insects to these enzymes has been shown to depend on the infection technique (Ignoffo *et al*., 1975). It has been found that proteolytic enzymes that accompany the germinating conidia of *N. rileyi* appear early and in high quantities; this probably aids in the early penetration and subsequent infection of the larvae by the germinating conidia (El- Sayed *et al*., 1993).

The expression of proteolytic enzymes was detected almost immediately after conidia were in contact with the cuticle; the cuticular species also may influence the quantity of enzyme expressed by *N. rileyi.* Chymoelastase and aminopeptidase's expression in particular were far greater on *Trichoplusia ni* cuticle than on *Helicoverpa virescens* or *Helicoverpa Zea* cuticle (El- Sayed, *et al*., 1993). Differences in proteolytic activity between cuticular substrate may be due to differences in enzymatic inhibitors (phenolic compounds and melanin) present in the cuticle of each species (Kuo & Alexander, 1967).

Protease inhibitors were present in the hemolymph of the late instar of *Anticarsia gemmatalis* larvae which were inhibitory to *N. rileyi* germination and growth (Boucias & Pendland, 1987).

The expression of chitinolytic enzymes was probably a specific response to contact with a cuticular surface since little or no activity was expressed on non-host cellulose substrate. Therefore, chitinases in conjunction with other enzyme (e.g. proteases) may be important in penetration of the larval integument by germinating conidia of *N. rileyi* (El-Sayed *et al*., 1989).

Protease, capable of digesting cuticle, and chymoelastase was important in the degradation of cuticular proteins (St. leger, *et al*., 1986).

The early appearance and expression of enzymes by germinating conidia is part of a series of complex events to mycosis in lepidopterans larvae. These sequential events probably include the initial recognition and adhesion of the conidia to the cuticular integument, germination and germ tube formation, germ tube penetration of the larval integument, and general invasion of the hemocoel by blastospores budding from the germ tubes, and eventual larval mycosis and death (El-Sayed *et al*., 1993). These blastospores circulate freely within the host without being recognized by the insect immune system (Loperz & Boucias, 1994).

1.7. Histopathology of infection

Once inside the hemocoel, the fungus proliferates and rapidly spreads throughout the body by means of the insect's open circulatory system. Hyphal bodies fill the hemocoel at the time of insect death with tissues being invaded and degraded to varying degrees (Zacharuk, 1981).

The development of *N. rileyi* within the host hemocoel has been studied in details (Boucias & Pendland, 1982). When the hyphal bodies (yeast-like cells) reach maximum number in the infected larvae, they germinate,

forming germ-tubes that can attach to and penetrate various organs (Pendland *et al*., 1994).

This end in mummification and conidiophores production, where the insect succumbs and dies; the internal organs and cuticle are totally degraded during emergence of hyphae from the cadavers. Hyphae eventually emerge from the cadaver producing aerial spores (conidia) capable of initiating the cycle once again (Pendland *et al*., 1994).

Microscopic examination of *Spodoptera exigua* injected with $5x10^3$ hyphal bodies per larvae showed that *N. rileyi* replicated only as freely circulating hyphal bodies during the initial 72-h period. Examination of hemolymph samples revealed no association between the budding of hyphal bodies and the circulating hemocytes. The number of these fungal cells increased at an exponential rate reaching densities of $2.24x10^5$ hyphal bodies per micro liter hemolymph. By hyphal bodies multiplication, a reduction in the relative hemocytes count was observed. After 72 hr, infected larvae contained 74% fewer hemocytes than the saline challenged larvae (Lopez, & Boucias, 1994).

1.8. Role of *N. rileyi* metabolites

Most species of entomopathogenic fungi overcome their host by invasion of the hemolymph leading to physical disruption and digestion of internal organs. Furthermore, fungi have the ability to kill their hosts after only limited growth making the possibility of insecticidal toxins intriguing (Gillespie & Claydon, 1989).

Many fungi produce metabolites, which acts on other organisms, sometime causing inhibition of growth, disease and even death. Such metabolites includes aflatoxins produced by some *Aspergillus flavus* strains (Diener & Davis, 1969), destruxins A and B, cyclic depsipeptides produced by the fungal pathogen of insect *Metarhizium anisopliae* (Taylor,

1970). Fungi such as *Beauveria bassiana*, *Paecilomyces fumosoroseus* and *Fusarium moniliforme* produce cyclodepsipeptides, including beauvericin and the enniatin complex (Grove & Pople, 1980).

It has been reported that the fungus *N. rileyi* produce metabolites active against insects (Ignoffo *et al*., 1976; Wasti & Hartmann, 1978; Kucera & Sansinakova 1968; Mohamed & Nelson, 1984); some of these metabolites showed toxic activity against the larvae of *Helicoverpa zea* and *Helicoverpa virescens* (Mohamed & Nelson, 1984). Furthermore, Mohamed and Nelson (1984) reported that *Nomuraea rileyi* crude extracts caused after 42, 48 and 72 hours mortality rate of 23.3, 44.5, and 68.9 % for *H. virensens* larvae and 28.7, 53.8, and 78.3 % for *H. zea* larvae, respectively.

1.9. Influence of *N. rileyi* on insect physiology and behavior

It has been found that noctuid larvae infected by *N. rileyi* tend to aggregate, compared to noninfected larvae (Fuxa, 1984). Furthermore, it has been shown that larval infection with *N. rileyi* was associated with some behavioral changes, mainly in food consumption (Vimala, 1994). *N. rileyi* reportedly secretes a proteinaceous substance inhibiting larval molt and metamorphosis in the silkworm *Bombyx mori.* (Makoto *et al*., 2003). Furthermore, Mikuni and Kawakami (1975) found that the conditioned medium of *N. rileyi* culture contained a proteinaceous factor that inhibited pupation when injected into 5th instars *Bombyx mori* larvae and moth emergence when injected into pupae. In contrast, its injection into 3rd or 4th instar larvae induced precocious pupal metamorphosis. (Shikata *et al*., 1998), suggesting that *N. rileyi* secretes an enzyme that inactivates hemolymph ecdysteroids and disrupts host development.

1.10. Isolation of *N. rileyi*

The entomopathogenic fungi, *Metarhizium anisopliae*, *Beauveria bassiana* and *N. rileyi* are known to be overwintering in the soil mostly as conidia (Bell, 1975; Ignoffo *et al*., 1978). The entomopathogenic fungi can be isolated indirectly from the soil by live baiting with insects such as larvae of *Galleria* spp (Zimmermann, 1986), and/ or directly by extraction using a dilute plate technique, in conjunction with selective medium (Beilharz *et al*., 1982). Beilhatz *et al*. (1982) introduced dodine to their selective medium based on oatmeal agar, which improved the selectivity of medium. Furthermore, the dodine concentration has been reduced to 0.46 gram / l; benlate also has been added (0.38mg / l). This medium increased the recovery of the entomopathogenic fungi, *Metarhizium anisopliae* and *Beauveria bassiana*. Another reduction in dodine concentration has been introduced by Senh, (1991), in wheat germ selective agar medium (WGSA); 30 g of wheat germ in 1 litre of water, 0.25 g chloramphenicol, 0.8 mg benlate (50% benomyl), 0.30 g dodine , 10 mg crystal violet, and 15 g agar.

There are no specific selective media for the isolation of *N. rileyi* from soil. The standard medium used to day for multiplication is Saboraud's Maltose Agar Yeast extract medium (SMAY) (Bell, 1975; Bell *et al*., 1982).

N. rileyi has been isolated from various noctuids and other lepidopterans found in the various regions in the world (Boucias *et al*., 1984; Ignoffo & Boucias, 1992). Surveys throughout the populations of *H. armigera* and *S. littoralis* conducted by Ben-Zeev, (1993), revealed that the activity of the fungus *N. rileyi* occur during summer and early autumn, when the hosts of *N. rileyi* (*H. armigera*) are peaking (Avidov & Harpaz, 1969).

1.11. Formulation and mass production of *N. rileyi*

In any mycoinsecticide, production of sufficient quantities of good quality inoculum is essential to its success. The inoculum produced must be both consistent and with intended formulation and application methods. Many scientific papers have been written on the subject of mass production of fungi. The majority of these investigations, dealt with optimization of conidial production by specific fungal isolates on a given substrate, but rarely goes further than laboratory scale investigation (Ibrahim & Low, 1993).

Several substrates can be used for production of aerial conidia of Hyphomycetous fungi. The fungus *N. rileyi* can be a potential mycoinsecticide for the use against several noctuid pests (Ignoffo, 1981), Conidia of this fungus can be produced on Sabouraud's maltose agar with 1% Yeast extract (SMAY) which is the standard medium used for its multiplication (Bell, 1975; Bell *et al*., 1982).

Because production of *N. rileyi* conidia on SMAY is expensive, there is a need for cheaper alternatives to be used for mass production of the fungus on commercial bases. Several attempts have been made to multiply this fungus using semi synthetic media and solid substrate in order to reduce the cost of production. It has been found that maltose and peptone could be effectively replaced with 2% barley extract and 1% soybean extract respectively. However, replacement of yeast extract with dry yeast result in lower spore yields (Vimala *et al*., 2000). It has been found that the sporulation of *N. rileyi* was highest on crushed sorghum with addition of yeast extract, which produced visible conidia on the 5^{th} day (Vimala, 1994).

Numerous investigations have shown the advantages of oil based formulations (Burges, 1998). Sunflower oil, groundnut oil, cotton seed oil and paraffin oil were investigated both as pure carriers as well as in

mixtures. The fastest and most complete germination was observed with pure sun flower oil, (Burges, 1998).

1.13. Objectives

1. To develop selective media to isolate the fungus *N. rileyi* from the soil.
2. To study growth parameters of *Nomuraea* native isolates.
3. To isolate strains of the fungus *Nomuraea rileyi* adapted to local conditions.
4. To test the pathogenicity of *N. rileyi*, Isolates ARSEF 539 and ARSEF 1972 on *H. armigera*.
5. To test the efficiency of different formulations and application methods of the fungus *N. rileyi* to be used as bioagent.
6. To test the effect of serial culturing on *N. rileyi* virulence.
7. To test the pathogenicity of *Nomuraea* native isolates on *H. armigera*.

Chapter 2

Materials and Methods

2.1. Development of selective media for the isolation of *N. rileyi* from soil

2.1.1. Isolates

The two isolates of the entomopathogenic fungus *N. rileyi* used in this study were obtained from ARSEF: Agriculture Research Service (ARS) collection of Entomopathogenic Fungi, USDA, Ithaca, NY, USA. The first isolate ARSEF (1972) was isolated from larvae of *S. frugiperda* in Brazil in 1985. The second isolate ARSEF (539) was isolated from larvae of *S. exigua* in Thailand in 1980.

2.1.2. Selective media

Saboraud's maltose agar yeast extract medium (SMAY), developed for multiplication of *N. rileyi* (Bell, 1975; Bell *et al*., 1982), was used in this study.

The following experiments were conducted to modify the SMAY media to be selective for the isolation of *N. rileyi* from soil, by adding the fungicides Dodine and / or Benlate.

2.1.2.1. Effect of different concentrations of dodine on germination of the fungus *Nomuraea rileyi*

Fist experiment (Isolation from the soil)

The purpose of this preliminary study was to evaluate which concentration of the fungicide Dodine to be included in the SMAY medium to be selective for isolation of the fungus *N. rileyi* from soil. SMAY medium is composed of 40g Maltose, 10g Peptone, 10g Yeast

extract, 20g Agar and 0.25g Chloramphenicol. All ingredients were added to 1 liter Erlenmeyer flask containing 990 ml deionized water and dissolved on hot plate, before it was autoclaved for 20 min. Ten ml of 10% Tween 80 solutions already autoclaved and stored in refrigerator was added aseptically to the flask. The fungicide dodine was then added at the following concentrations (100mg/l, 200mg/l, and 300 mg / l). The fungicide was mixed well in the medium before all finally poured into 90 mm Petri dishes.

One hundred grams of soil sample were placed in each of four 250ml beakers, two of which contained autoclaved soil and two contained non-autoclaved soils. The two autoclaved soil samples were inoculated with 10^4 spores / g of one of the two *N. rileyi* isolates (ARSEF 539 and ARSEF 1972); the non-autoclaved soil samples were treated the same. Soil dilutions were made out of the above mentioned samples according to dilution plate technique. Five grams of each soil sample were placed in 100 ml flask; 45 ml of 0.1% agar solution was added to each flask and shaked for 30 minutes. Three hundred (300) micro liters of the dilution 10^{-2} spores/ml were seeded on each of the dodine amended SMAY plates used in the experiment, and incubated at 25 ± 1ºC in the dark for three days before it was incubated under light for sporulation.

The experiment was completely randomized (CRD) with five replications. Plates were inspected on a daily bases and the final readings were documented after 15 days

Second experiment

This experiment was conducted to evaluate the effects of different concentrations of dodine on germination of *N. rileyi*. Modified SMAY media were prepared in which 0.1% tween 80 and the different concentrations of dodine (100, 90, 80, 70, 60, 50, 40, 30, 20 and 0 mg/l) were added, as in the first experiment.

Twenty one day old cultures of the isolates ARSEF 539 and ARSEF 1972 were harvested in 0.05% tween 80. The numbers of conidia were determined by haemocytometer. Two hundred (200) micro liters of 10^2 conidia /ml suspension were seeded on each plate. Plates were incubated at 25±1ºC in the dark for three days before they were transferred to light. The conidial germination percentages were evaluated after 6, 8, 12, 14 and 16 days. The experiment was completely randomized (CRD), with four replicates for each treatment.

Third experiment

The effect of different concentrations of dodine on germination of *N. rileyi* was repeated for second time in this experiment. Twelve days old cultures were used and harvested with 0.05% tween 80. Three hundred (300) micro liter of 10^3 spores / ml of isolate ARSEF 1972 and two hundred (200) micro liter of $5x10^2$ spores / ml of isolate ARSEF 539 were seeded on each petri dishes.

The experiment was completely randomized, with four replicates per each treatment in case of the isolate ARSEF 539 and with five replicate per each treatment in case of the isolate ARSEF 1972. The percentage of spore germinations was determined after 8 days.

2.1.2.2. Effect of soil microbiota on growth of *N. rileyi*

This experiment was conducted to evaluate the CFU recovery of *N. rileyi* from autoclaved and non-autoclaved soils. Twelve day old cultures of both isolates were harvested, and conidial concentration was determined by haemocytometer. One hundred grams of soil were placed in each of four 250 ml beakers. Two of them contained autoclaved soil and the other two contained non-autoclaved soils. The two autoclaved soil samples were inoculated with 600 micro liters of $2x10^7$ spores /ml suspension of one of the two *N. rileyi* isolates (ARSEF 539 and ARSEF 1972); the non-

autoclaved soil samples were treated the same. Five grams of each soil samples were placed in 100 ml flask; 45 ml of sterile distilled water was added to each flask and shaked for 30 minutes.

Two soil dilutions were made (10^{-2} & 10^{-3}) out of the above mentioned samples by using 0.1% tween 80. One thousand (1000) micro liters of each dilution (10^{-2} and 10^{-3}) were seeded on each of the 90 mg dodine amended SMAYT petri plates used in the experiment. Plates were incubated at 25±1 ºC in the dark for three days before light was provided for sporulation.

The experiment was completely randomized (CRD) with four replications for each treatment. The final readings were documented after 13 days. The percentage of *N. rileyi* CFU recovering was calculated. CFU of other fungi were also documented.

2.1.2.3. The use of Dodine-Benlate combinations in SMAYTD medium to enhance their selectivity to *N. rileyi*.

First Experiment

A preliminary study was conducted to test the suitability of Benlate to be included in SMAYTD (SMAY +0.1% Tween80 + 90mg/l dodine), in order to further improve the selectivity of the medium. Four different concentrations of Benlate (0.8mg, 1mg, 2mg and 4mg) per liter medium were added to SMAYTD medium after it was cooled to 45ºC. The medium were then poured into 90 mm Petri dishes.

Fourteen day old cultures of (isolate ARSEF 539) were harvested in 0.1% Tween 80; concentration of conidia was determined using haemocytometer. Two hundred (200) micro liters of 10^4 spores/ml suspensions were seeded on SMAYTD medium plates amended with (0.8mg, 1mg, 2mg and 4mg) Benlate per liter medium (2000 spores were seeded on each plate).

The experiment was completely randomized with four replicate for each treatment. Germination percentages were recorded after 12 days of incubation at 25 ± 1 ºC.

Second Experiment

This experiment was conducted to test the suitability of using Benlate at the concentration of 0.8 mg/l (suggested by previous trials) and Dodine-Benlate combination for isolation of *N. rileyi* from soil. Four types of media were tested, (SMAYT as control, SMAYTD, SMAYTD + 0.8mg/l Benlate, SMAYT+ 0.8mg/l Benlate). The different media were then poured in 90 mm Petri dishes. Twelve day old cultures of both isolates (ARSEF 539 and ARSEF 1972) were harvested in 0.1% Tween 80. The harvested conidia were homogenized before the concentrations of conidia were calibrated using haemocytometer.

One hundred grams of soil were placed in each of the four 250 ml beakers. Two of the beakers contained autoclaved soils and the other two contained non-autoclaved soils. The two autoclaved soil samples were inoculated with 10^6 spores /g of one at a time of the two *N. rileyi* isolates (ARSEF 539 and ARSEF 1972); the non-autoclaved soil samples were treated the same. Soil dilutions were made out of the above mentioned samples according to the dilution plate technique. Five grams of each soil sample were placed in 100 ml flask; 45 ml of 0.1% tween 80 was added to each flask and shaked for 30 minutes. Two hundred (200) micro liters of soil dilutions (10^{-3}) were seeded on each of the above mentioned modified SMAY media plates. The plates were then incubated at 25 ± 1ºC in dark for three days before they were incubated under full light for sporulation.

The experiment was completely randomized (CRD) with four replicates. The final readings were determined twelve days after inoculation, and the percentages of CFU recovered per gram soil were calculated.

2.2. Isolation of *N. rileyi* from the Soil

2.2.1. Samples collection

Soil samples (300 g each) were collected from 210 different irrigated and non irrigated tomato growing fields in the West Bank (WB) during August 2003/2004. Samples were collected at soil depth of 2 -15 cm deep and under tomato plant canopies. In addition, samples were usually collected from soil under tomato plants that showed damage due to *H. armigera*. Soil samples were placed in plastic bags immediately at the time of collection and mixed by hands. Samples were collected from various areas in the WB: 120 from Hebron area, 28 Jericho, 40 Jenin, 10 Tulkarm, and 20 from Bethlehem.

2.2.2. Isolation Procedures

Five grams of each collected soil sample were suspended in 45ml 0.1% Tween 80 solution and shaked for 30 minutes. Two soil dilutions (10^{-2} & 10^{-3}) were made using 0.1% Tween 80. From each dilution 300 micro liters were seeded on SMAYTD medium petri plates with four replicates for each dilution. Plates were then incubated at 25 ±1 ºC under dark for three days before they were transferred to full light. Plates were inspected daily for *N. rileyi* growth.

2.3. Growth parameters of native *Nomuraea* isolates

Four native *Nomuraea* isolates were found in four samples collected from the Jenin area. Growth rate, sporulation and spore germination of the four isolates were evaluated under different temperatures 15, 20, 25, 30, and 35 ºC.

2.3.1. Growth rate

For measuring the mycelium growth rate, seven plates of SMAY medium were inoculated with single spore of each isolate taken from 21-day old cultures. Four plates were incubated under light condition and three incubated under dark condition. Readings of colony diameters were measured at 7 and 9 days after inoculation.

The experiment was completely randomized with four replicates under light condition and three replicates under dark condition.

The rate of increase in diameter was calculated by the formula:

Rate = $D_2 - D_1 / T_2 - T_1$ were D_2 the second reading of the growth diameter, D_1 the first reading of the growth diameter, T_2 the time of the second reading, T_1 the time of the first reading.

2.3.2. Spores germination

For measuring spores germination, conidia were harvested in distilled water from 30-day old cultures of the four native isolates. Spores concentrations were calibrated to 660 spores/ ml. For each native isolate, 500 micro liters of the above dilutions were seeded on seven SMAY medium plates. The experiment was completely randomized with four replicates under light condition and three replicates under dark condition. Number of colonies was determined at six and ten days after inoculation.

2.3.3. Sporulation

For measuring spore production, the same plates used in the spore germination experiment in (section 2.3.2) were used but after 17 days from inoculation. Mycelial disks (0.196 cm^2) were taken from each plate (replicate) and homogenized in 10 ml of 95% ethanol. Haemocytometer was used to determine spores production per unit area of the plate.

The experiment was completely randomized with four replicates per each treatment under light condition and three replicates under dark condition.

2.4. Bioassay (infectivity experiments)

2.4.1. Insect rearing and artificial diet

H. armigera cultures were established from a primary colony obtained from the lab of Prof. S. Applebaum of the H U J. The neonate larvae were fed individually on an artificial diet as described by (Vimala Devi, 1994; Applebaum, personal communication).

Two hundred fifty grams of *Phaseolus vulgaris* (beans) were soaked overnight in 1 liter of deionized water. The soaked beans were then placed in biosafety bags, and autoclaved. After that, the following ingredients were added to the autoclaved mixture.

The ingredients are:

- Dried alfalfa meal or pellets (102 g)
- Nipagin (8.1g)
- Sorbic acid (2.5g)
- Dried yeast extract (81 g)
- Ascorbic acid (8 g)
- Chloramphenicol (1g)
- Formaldehyde in 40 ml water (10 ml)

Autoclaved fourty grams agar in 1 liter water were added manually to the previous mixture for reaching a final volume of about 2.5 liter. The mixture was then poured into plastic container, and covered with aluminum foil. The diet can be kept in refrigerator for about one month, when needed, the diet can be cut into pieces for feeding.

The adults after emergence were fed with 10% sucrose solution in saturated cottons. Within three to five days, adults start laying eggs on

cottons placed inside the plastic container. Cottons with the insect eggs were transferred to another plastic container containing artificial diet. Eggs were hatched within nearly five days at 25 ±1ºC. After that, the neonate larvae leave the cotton to start feeding on artificial diet provided and grow until they separate to be fed individually.

When larvae reach 5th stage and after fifth molt they were transferred to wood bran for pupation. After pupation, the pupae were placed in Petri dishes inside plastic containers covered with glass board for adult emergence. The insects were incubated at 25 ±1ºC during all life stages. Healthy larvae of the desired instars were used for various experiments.

2.4.2. Fungal preparation

The *N. rileyi* isolates were cultured on Saboraud's maltose agar yeast extract medium (SMAY). The conidia of *N. rileyi* used in the experiments were harvested from 12-27 day old cultures.

2.4.3. Bioagent methods of application

First Experiment

Four methods of application of *N. rileyi* isolates (ARSEF 539 and ARSEF 1972) were tested in this experiment. Dipping 4th stage larvae of *H. armigera* in 10^9 conidia/ml aqueous suspensions for 3 sec, rearing the larvae on soil inoculated with 500 micro liter of the same suspension, rearing larvae on filter paper inoculated with 500 micro liter of the same suspension and the dry formulation (inoculating the larvae with dry conidia). Two control treatments were used in this experiment, dipping larvae for 3 seconds in sterile distilled water and rearing larvae on filter paper inoculated with 500 micro liter of distilled water.

Fourteen day old cultures of *N. rileyi* were flooded with distilled water and conidia were collected and concentration set at 10^9 conidia/ml. The experiment was completely randomized with five replicates per treatment, and a single larva in each replicate. Mortality percentages were recorded 4, 8, and 11 days after treatment. The tested larvae were incubated at 25 ± 1ºC under natural light and 100% R.H.

Second experiment

Three methods of application were tested in this experiment, dipping 4^{th} stage larvae in 10^7 conidia /ml 0.1% tween 20 suspensions for 3 seconds, inoculating the larvae with dry conidia and dipping the 4^{th} stage larvae in 10^7 conidia /ml distilled water suspension in addition to the control treatment (dipping 4^{th} stage larvae in 0.1% tween 20 without fungal inoculum). All the above treatments were applied to both *N. rileyi* isolates (ARSEF 539 and ARSEF 1972).

Seventeen days old cultures of *N. rileyi* were flooded with 0.1% tween 20 and distilled water and conidia were collected and concentration set at 10^7 conidia/ml. The experiment was completely randomized with six replicates per treatment, and two larvae in each replicate. Mortality percentages were recorded 6, 11, and 15 days after treatment. The tested larvae were incubated at 25 ± 1ºC under natural light and 100% R.H.

2.4.4. Serial culturing of *N. rileyi* inoculum

This experiment was conducted to test the difference in virulence of the two isolates, (ARSEF 539, ARSEF 1972), after 16 successive culturing. For comparison, virulence was evaluated as well after artificially inoculating *H. armigera* with the two same isolates which has been reisolated from dead inoculated larvae and called in the results (ARSEF 1972A and ARSEF 539A). Two inoculation methods were used in this experiment, the first one involving dipping 4^{th} stage larval instars in

10^8 conidia /ml of 0.05% tween 20 for 3 seconds. The second method, however involved rearing 4th stage larval instars on filter papers inoculated with 500 micro liter of the same suspension. Two control treatments were incorporated, dipping larvae in 0.05% tween 20 for 3 seconds, and rearing larvae on filter papers inoculated with 500 micro liter of 0.05% tween 20 for ten second. The inoculated larvae were then incubated at 25 ± 1 ºC under natural light. Conidia used in the experiment were harvested from 15 day old cultures of both isolates.

The experiment was completely randomized with 12 replicates for each treatment and single larvae per each replicate. Mortality percentages were recorded at 6, 11 and 15 days after inoculation.

2.4.5. Site of entry

The purpose of this experiment was to test which site of entry by the fungus *N. rileyi* that may result in highest larval mortality. Fourth larval instars were dipped in spores suspension of $4x10^7$ conidia /ml in 0.05% Tween 80. Conidia used as inoculum were harvested from 17 days old cultures of the isolates (ARSEF 539, ARSEF 1972, ARSEF539A, ARSEF 1972A). The inoculated larvae were then incubated at 25 ± 1ºC, under natural light and 100% R.H.

Two inoculation methods were used, the first one involving dipping 4th stage larval instars in 4 x 10^7 conidia /ml of 0.05% tween 80 for 3 seconds, and the second method involved feeding 4th stage larval instars on tomato leaves dipped in the same suspension for 72 hours. Two control treatments were incorporated, dipping 4th stage larval instars in 0.05% Tween 80, and the second involved feeding 4th stage larval instars on tomato leaves dipped in 0.05% Tween 80.

The experiment was completely randomized (CRD) with six replicates for each treatment, and single larvae per each replicate. Mortality percentages were recorded daily.

2.5. Virulence of Isolates

2.5.1. Virulence of the isolates (ARSEF 539 and ARSEF 1972)

This experiment was conducted to determine the LC_{50} (conidial concentration required to kill 50 % of tested pest population) and LT_{50} (time required to kill 50 % of tested pest population) for the isolates (ARSEF 539 and ARSEF 1972). Conidia of 17-day old cultures of both isolates were harvested in 0.02% Tween 80 solution. The numbers of conidia were determined by using Haemocytometer. Further concentrations were made to reach dilutions of 10^5, 10^6, 10^7, 10^8 and zero conidia/ml (control). Spore suspensions were then stored in the refrigerator at 5 ºC for three days.

Fourth larval instars of *H. armigera* were fed on tomato leaves dipped in both isolates fungal suspensions for 72 hr before they were fed on regular artificial diet. The larvae in the control treatment were fed on tomato leaves dipped in 0.02% Tween 80 without fungal conidia.
The treated larvae were then incubated at 25 ± 1 ºC under natural light, and 100% R.H.

The experiment was completely randomized (CRD) with eight replicates in each treatment and single larvae in each replicate. The larval mortality was recorded daily. The LT_{50} and LC_{50} were calculated by regression equations derived from time vs. mortality curves and dose vs. mortality curves, respectively (Vimala, 1994).

2.5.2. Virulence of native *Nomuraea* isolates

In preliminary study to test the virulence of native isolates, 4th stage larval instars of *H. armigera* were inoculated by dipping in 10^7 conidia / ml distilled water harvested from 21-day old cultures of the four native isolates. In additions, virulence was evaluated by dipping the artificial diet of the larvae in the same conidial suspension; control treatment involved using only distilled water.

The experiment was completely randomized with various replications. Larval mortalities were recorded at 10, 12, 13, and 14 days after inoculation.

In addition, the virulence of the four native *Nomuraea* isolates to *H. armigera* was studied. Seventeen day old cultures of the four native *Nomuraea* isolates were harvested in sterile distilled water. The numbers of conidia were determined using haemocytometer. The conidial concentration was calibrated to reach the dilutions of 10^5, 10^6, 10^7 and 10^8 conidia / ml. Fourth larval instars of *H. armigera* were fed on tomato leaves dipped in the prepared conidial concentrations, of the four native *Nomuraea* isolates, for 48 h before they were fed on regular artificial diet. Larvae of the control treatment were fed on tomato leaves dipped in sterile distilled water. The treated larvae were then incubated at 25 ± 1 ºC under natural light, and 100 % R.H. Larval mortalities were recorded daily until day thirteen.

The experiment was completely randomized with nine replicates for each treatment.

2.6. Pots bioassay

This experiment was conducted to test the virulence of *N. rileyi* to *H. armigera*, when the fungus was sprayed on 30-day old tomato seedlings under lab conditions during the summer of 2004. Seventeen-day old

cultures of both isolates (ARSEF 539 and ARSEF 1972) were harvested in 0.02% Tween 80 solution and the number of conidia was determined by haemocytometer. Sixty ml of 10^8 conidia /ml of 0.02% Tween 80 suspension were sprayed on each tomato seedling. The control treatment was sprayed by 0.02% Tween 80 solution without conidia. After inoculation, tomato seedlings were air dried. After that five 3rd instars larvae were placed on the plant in each pot, and the pots were then covered by transparent plastic bags to prevent larvae from leaving the pots and to provide humidity which is important for fungal infectivity. Larval mortalities were recorded after 10 days of inoculation.

The experiment was completely randomized, with three replicates; each replicates was single tomato seedling.

2.7. The Effect of *N. rileyi* metabolites

This experiment was conducted to evaluate the effect of fungal metabolites produced by both isolates of *N. rileyi*, (ARSEF 539 and ARSEF 1972) in SMAY broth media on 3rd larval instars of *H. armigera.* To obtain the filtrate, *N. rileyi* was cultivated aerobically in Saboraud's maltose yeast extract broth (4% Maltose, 1% peptone and 0.5% yeast extract, and pH was adjusted to 6.0 with phosphoric acid).

One hundred ml of the mentioned media was autoclaved and cooled in flasks. Flasks were inoculated with $1cm^2$ of 17-day old culture of both isolates of *N. rileyi*, and then incubated at 25 ± 1 ^{0}C with continuous shaking under constant light for 12 days. The media were filtered with whatman number 1 filter paper six times. The filtrate was then stored in refrigerator at 5 ^{0}C till experiment was started. In one treatment 3rd larval instars were dipped in culture filtrates of both isolates for 3 seconds. In second treatment, 3rd larval instars were fed on tomato leaves dipped in

culture filtrates of fungal isolates. The mortality percentages were recorded at 4, 8, 12 and 16 days after inoculation.

The experiment was completely randomized with ten replicates per each treatment and single 3rd instar larvae in each replicate.

2.8. Statistical analysis

The results of all experiments were analyzed statistically using analysis of variance (ANOVA) and LSD test. All analysis were conducted using Sigmastat software program.

Chapter 3

Results

3.1. Selective media

3.1.1. Effects of different concentration of dodine on germination of *N. rileyi*

First and second experiments

Conidial germinations of *N. rileyi* (ARSEF 539 and ARSEF 1972) in the preliminary first experiment were completely inhibited when both isolates were grown on SMAY amended with dodine (65% n-dodecyl-guanidine acetate) concentrations of more than 100 mg/ l. However, germinations started at dodine concentrations less than 100 mg / l (Table 3.1 & 3.2). When dodine concentrations below 100 mg/ l were used percentages of conidial germination of both isolates of *N. rileyi*, were inversely correlated with increasing dodine concentrations (r^2 = 0.77 and 0.74 for isolates 1972 and 539, respectively) (appendix 1 A). Germination of conidia started to appear in plates with 90mg /l dodine, for the isolate ARSEF 539; however, there was no significant difference between dodine concentration treatments at 90 mg /l or below except with the control treatment (zero concentration) (Table 3.1).

Concerning the effect of dodine on conidial germinations of the isolate ARSEF 1972, data showed that germination was less effected by dodine concentrations below 90 mg/l than the isolate ARSEF 539 (Table 3.1).

Table 3.1: The effect of dodine on conidial germination of *N. rileyi* isolates ARSEF 539 &ARSEF 1972.

Dodine Conc. mg/l	Percentage of conidial germination at (16)days	
	ARSEF 539*	ARSEF 1972
100	0 ± 0 c**	0 ± 0 e
90	27.5±17 b	30 ± 3 cd
80	40 ± 5 b	36 ±14 cd
70	40 ± 4 b	38 ± 18 bc
60	38 ±3 b	51 ± 25 abc
50	40± 10 b	57 ± 16 ab
40	42 ± 10 b	47 ± 5 b
30	44 ± 10 b	50 ± 0 ab
20	50 ± 0 ab	73 ± 22 a
0	70 ± 25 a	70 ± 21 a

*Values of data represent mean ± SD.
**Means followed by the same letter within the same column are not significantly different according to Fisher LSD method, with P≤ 0.05.

Third Experiment

In this experiment, conidial germination data were recorded after 8 days and percentages of germination were inversely correlated with increasing dodine concentrations (r^2 = 0.9) (Appendix 1B). Germination was almost completely inhibited at dodine concentration higher than 90 mg /l; the percentages of conidial germination were statistically different from the control treatment when dodine concentrations used were between 60 and 90 mg/l

On the contrary, statistical analysis showed that there were no significant differences in conidial germination when dodine was used at the concentration range of 0-50 mg/l (Table 3.2).

Table 3.2: The effect of dodine on conidial germination of *N. rileyi* isolates ARSEF 539 &ARSEF 1972.

Dodine Conc. mg/l	Percentage of conidial germination at (8)days	
	ARSEF 539*	ARSEF 1972
100	1.75 ± 1.5 d**	0 ± 0 e
90	37 ± 22 c	20 ± 15 d
80	---	23 ± 12 d
70	38 ± 13 c	39 ± 12 c
60	60 ± 6 b	45 ± 7 bc
50	74 ± 12 ab	49 ± 5 abc
40	77 ± 9 a	56 ± 6 ab
30	80 ± 5 a	57 ± 5 ab
20	86 ± 10 a	60 ± 9 a
0	88 ± 9 a	61 ± 4 a

*Values of data represent mean ± SD.

**Means followed by the same letter within the same column are not significantly different according to Fisher LSD method, with $P \leq 0.05$.

3.1.2. Effects of soil microbiota on growth of *N. rileyi*

Recovery percentages of *N. rileyi* from autoclaved soil ranged from 37 to 46 % with no effect of soil dilution (Table 3.3). However, in the unautoclaved soil treatment, recovery percentages were much lower under low dilution (10^{-3}) and almost undetectable at higher soil dilution (10^{-2}). Recovery percentages of the isolate ARSEF 1972 from soil was lower in general than that of the isolate ARSEF 539 (Table 3.3).

Table 3.3: Effects of soil microbiota, on recovery percentage of two Isolates of *N. rileyi* (ARSEF 539 and ARESF 1972) from soil on SMAY media amended with dodine.

	ARSEF 539		Other Fungi	ARSEF 1972		Other Fungi
Treatments	CFU/gm Soil x 10^3	*Recovery %	CFU/gm Soil x 10^3	CFU/gm Soil x 10^3	*Recovery %	CFU/gm Soil x
Autoclaved soil (10^{-3})	55	46±1.1a**	0	45	38±1.9a**	0
Autoclaved soil (10^{-2})	49	41±3.2a	0	44.5	37±4.8a	0
Unautoclaved soil (10^{-3})	37	31 ± 6 b	3	42.5	36±6 a	2
Unautoclaved soil (10^{-2})	1.13	0.95±0.91c	5.3	0.9	0.75±0.48b	7.8

* Values of data represent mean of four replicates ± SEM.

** Means followed by the same letter within the same column are not significantly different according to Fisher LSD method with $P \leq 0.05$.

3.1.3. The use of dodine-Benlate combinations in SMAYTD medium to enhance their selectivity to *N. rileyi*

Preliminary experiment

Conidial germination of *N. rileyi* (isolate ARSEF 539) was completely inhibited when Benlate was added at the concentration 2 mg /l and above (Table 3.4). However, results showed that Benlate can be safely incorporated into SMAYTD at the concentration of 1 mg /l or below; conidial germinations were 35 and 47.8 % when Benlate was added at the concentrations of 1.0 and 0.8 mg /l, respectively.

Table 3.4: Effects of Dodine-Benlate combination on germination of *N. rileyi* Isolate (ARSEF 539) on SMAYTD.

Benlate Conc.	Spore germination (%) *
0.8 mg / l	47.8 ± 12 a **
1 mg / l	35 ± 12 a
2 mg / l	0 ± 0 b
4 mg / l	0 ± 0 b

* Values represent mean of four replicates ± SD

** Means followed by the same letter within the same column are not significantly different according to Fisher LSD method with P≤ 0.05

Second experiment

The isolate ARSEF 539 was recovered successfully from soil when dodine (38%) or Dodine / Benlate combination (35%) were used in the selective media; other fungi populations were very much inhibited (Table 3.5). On the other hand, the isolate ARSEF 1972 was more affected by dodine and Dodine / Benlate combinations giving lower percentages of recovery (7.5 and 1.2 %), respectively. However, ARSEF 1972 was less affected by the Benlate treatment giving higher recovery percentages (15%).

Table 3.5: Effect of Dodine - Benlate combinations on recovery % of *N. rileyi* isolates (ARSEF 539 and ARSEF 1972) from soil.

Treatments	ARSEF 539		Other Fungi	ARSEF 1972		Other Fungi
	*** CFU/gm soil x 10^3	* % Recovery	Number of CFU/gm Soil x 10^3	*** CFU/gm soil x 10^3	* % Recovery	Number of CFU/gm Soil x 10^3
Control	0	0 ± 0c**	100	0	0 ± 0b**	100
Dodine	378	38 ± 6a	1.5	75	7.5 ± 4b	0.5
Dodine + Benlate	347	35 ± 6a	0.75	12	1.2 ± 0.4b	0
Benlate	183	19 ± 3b	17	150	15 ± 2a	8.8

* Values of data represent Mean of four replicates ± SEM

**Means followed by the same letter within the same column are not significantly different according to Fisher LSD method with $P \leq 0.05$

*** Number of recovered CFU / gram soil

3.2. Isolation and native isolates

3.2.1. Isolation of *N. rileyi* from local field soils

In this experiment, two hundred and ten soil samples collected from various regions in the West Bank were screened for presence of *N. rileyi* by using SMAYTD medium

Four isolates of the fungus *Nomuraea* was isolated only from four soil samples from the Jenin area (NsP18, NsP17, NsP5 and NsP3).

3.2.2. Growth parameters of *Nomuraea* native isolate (Influence of temperature & light)

3.2.2.1. Mycelial growth

This experiment was conducted to check the optimum temperature for mycelial growth of the four local isolates of *Nomuraea* spp. Temperature range for hyphal growth of all isolates was 15-30 ºC (Table 3.6). Hyphae of all isolates failed to grow at 35 ºC.

The optimum temperatures for mycelial growth of the isolate NsP18 were 20 ºC and 30 ºC under light and dark conditions, respectively. However, the optimum temperature for the isolate NsP17 was 25 ºC under both conditions. The isolate NsP5 grew best at both 20 and 25 ºC under light, whereas the isolate NsP3 grew best at 20 ºC under both conditions (Table 3.6 and Fig. 3.1).

It is worth mentioning however, that there were no significant differences in general between temperatures for the same isolates with very slight exceptions.

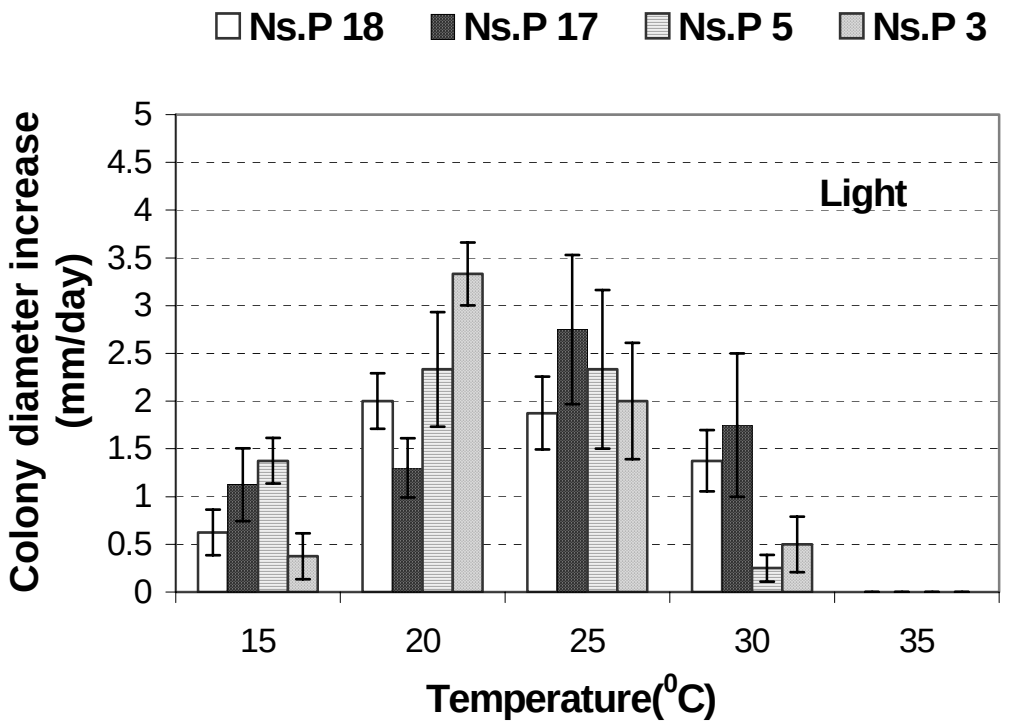
Table 3.6: Effect of temperature on hyphal growth of four native isolates of *Nomuraea* incubated under light and dark conditions.

	Isolates growth rate (mm/day)							
Temp.(°C)	**NsP18**		**NsP 17**		**NsP 5**		**NsP 3**	
	Light*	Dark**	Light	Dark	Light	Dark	Light	Dark
15	*** 0.63 ± 0.24 bc	1.25 ± 0.32 ab	1.13 ± 0.38 bc	2.33 ± 0.83 ab	1.38 ± 0.24 a	1.38 ± 0.32 a	0.38 ± 0.24 c	0.5 ± 0.20 ce
20	2 ± 0.29 a	1.38 ± 0.13 ab	1.3 ± 0.31 bc	2.88 ± 0.66 ab	2.33 ± 0.6 a	2.17 ± 0.67 a	3.33 ± 0.33 a	2.5 ± 0.29 ad
25	1.88 ± 0.38a	1.38 ± 0.13 ab	2.75 ± 0.78 ab	3.63 ± 1.20 a	2.33 ± 0.83 a	1.5 ± 0.5a	2 ± 0.61 bd	1.25 ± 0.25be
30	1.38 ± 0.32 ab	2.25 ± 1.05 a	1.75 ± 0.75 bc	0.25 ± 0.25 c	0.25 ± 0.14 b	0.13 ± 0.13 b	0.5 ± 0.29 ce	0.25 ± 0.25 c
35	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 b	0 ± 0 b	0 ± 0 c	0 ± 0 c

* Values of data represent mean of four replicates ± SEM.

** Values of data represent mean of three replicates ± SEM.

*** Means followed by the same letter within the same isolate are not significantly different according to Fisher LSD method with $P \leq 0.05$.



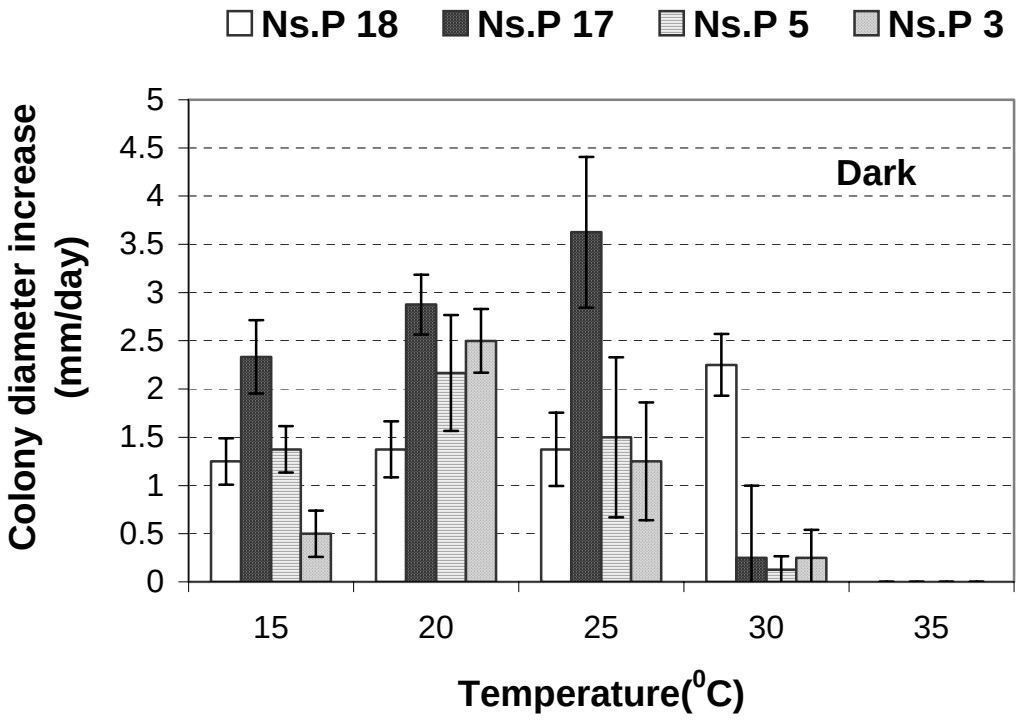


Fig. 3.1: Effect of temperature on hyphal growth of four native isolates of *Nomuraea* incubated under light and dark condition.

3.2.2.2. Spores germination

Conidia of all isolates germinated at temperatures ranged between15-30 ºC, but failed to germinate at 35 ºC under both light and dark conditions (Table 3.7 and Fig. 3.2). The isolate NsP18 conidia germinated well at the range of 15-30 ºC under dark and at the range of 20-25 ºC under light condition with no significant difference between the treatments in general. The isolates NsP5 and NsP3 recorded the highest germination rates at almost all temperatures tested (15-30 ºC) with no significant difference between treatments with few exceptions.

Table 3.7: Effect of temperature on spore's germination of native isolates *Nomuraea* grown on SMAY media incubated under light and dark conditions.

Temperature (ºC)	Isolates (percentage of germinated spores)							
	Ns P 18		Ns P 17		Ns P 5		Ns P 3	
	Light*	Dark**	Light	Dark	Light	Dark	Light	Dark
15	*** 40 ± 7 b	64 ±17 a	65 ± 10 b	90 ± 4 a	58 ± 11 b	84 ± 10 a	63 ± 5 b	85 ± 12 a
20	60 ± 6 a	63 ± 7 a	69 ± 3 b	66 ± 5 b	76 ± 5 ab	84 ± 1 a	84 ± 7 ab	82 ± 3 ab
25	63 ± 4 a	67 ± 4 a	70 ± 4 ab	67 ± 3 b	98 ± 1 a	95 ± 1 a	86 ± 8 a	88 ± 5 a
30	15 ± 7 c	69 ± 8 a	37 ± 13 d	65 ± 2 b	60 ± 14 b	88 ± 3 a	53 ± 8 b	89 ± 8 a
35	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 c	0 ± 0 c

* Values of data represent mean of four replicates ± SEM.

** Values of data represent mean of three replicates ± SEM.

*** Means followed by the same letter within the same isolate are not significantly different according to Fisher LSD method with $P \leq 0.05$.

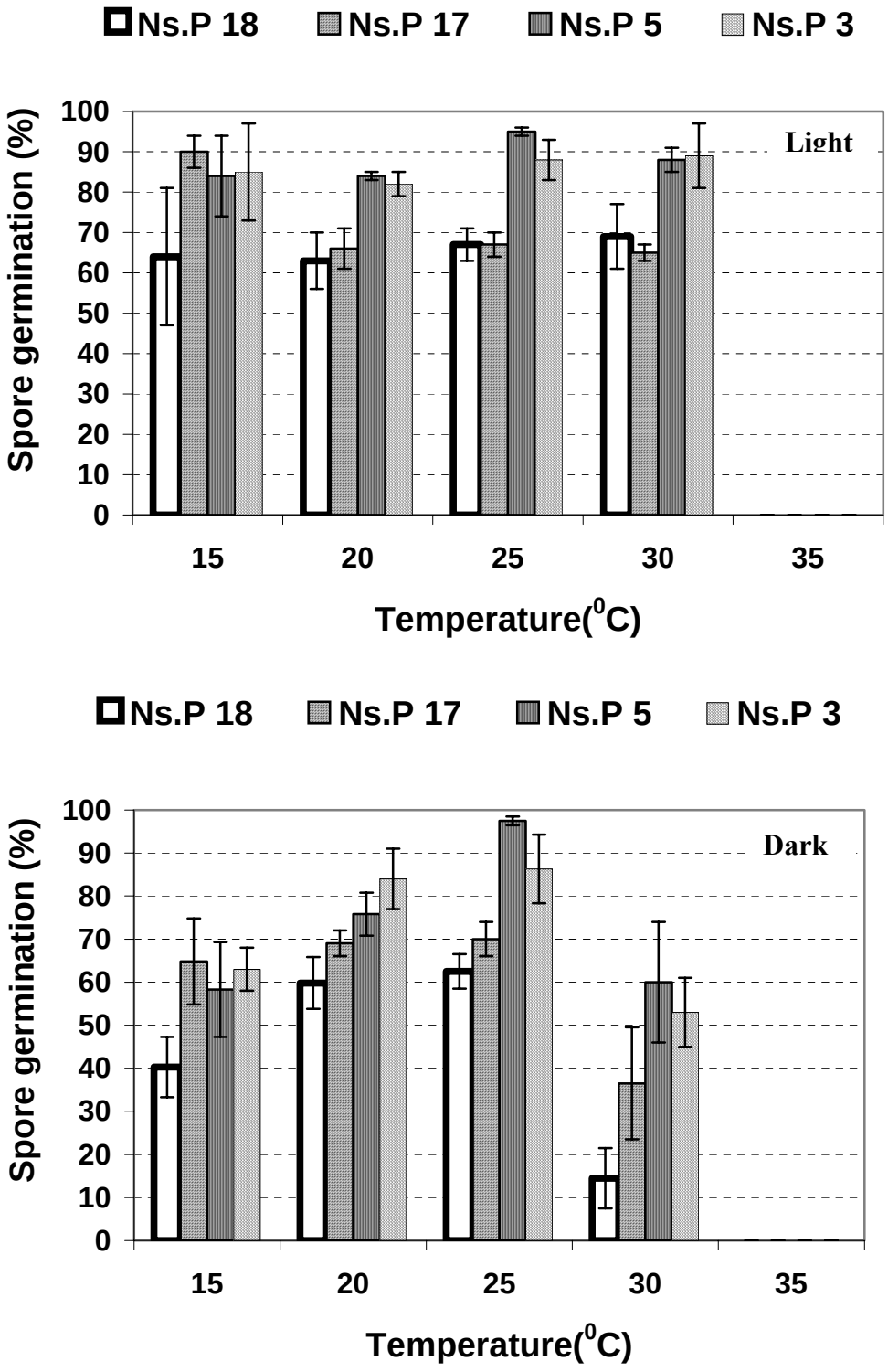


Fig. 3.2: Effect of temperature on spore's germination of native isolates of *Nomuraea* grown on SMAY medium incubated under light and dark conditions.

3.2.2.3. Sporulation

All isolates were able to produce spores at the range of 15-30 ºC (Table 3.8). Sporulation ranged from $1x10^6$ to $6.8x10^7$ spores/cm^2 of mycelial colony under light and from $3x10^6$ to $5.8x10^7$ spores/cm^2 under dark (Table 3.8). Sporulation was low at 30 ºC for all isolates under both conditions and completely stopped at 35 ºC. The highest sporulation rates were recorded by the isolates NsP3 at the temperature range of 20-25 ºC with one exception under dark. However, the lowest sporulation rates at almost all temperatures were recoded by isolate NsP17 under both conditions (Fig.3.3).

Table 3.8: Effect of temperature on sporulation of native isolates of *Nomuraea* grown on SMAY media and incubated under Light and dark conditions.

Temperature (°C)	Isolates (Means x 10^6)							
	NsP18		Ns P17		NsP5		Ns P3	
	Light*	Dark**	Light	Dark	Light	Dark	Light	Dark
15	*** 58 ± 10 a	50 ± 4 a	4 ± 2 bc	37 ± 6 a	16 ± 7 b	42 ± 7 a	48 ± 9 ab	45 ± 5 ab
20	50 ± 6 a	56 ± 3 a	6 ± 1 bc	6 ± 2 bc	13 ± 5 b	22 ± 20 ab	68 ± 29 a	51 ± 2 ab
25	50 ± 5 a	41 ± 19 a	12 ± 7 b	5 ± 3 bc	4 ± 4 b	20 ± 12 ab	60 ± 6 a	56 ± 7 a
30	5 ± 2 b	12 ± 6 b	1 ± 1 c	3 ± 3 bc	2 ± 2 b	4 ± 2 b	20 ± 4 bc	8 ± 2 c
35	0 ± 0 b	0 ± 0 b	0 ± 0 c	0 ± 0 c	0 ± 0 b	0 ± 0 b	0 ± 0 c	0 ± 0 c

* Values of data represent mean of four replicates ± SEM

** Values of data represent mean of three replicates ± SEM

*** Means followed by the same letter within the same isolate are not significantly different according to Fisher LSD method with $P \leq 0.05$.

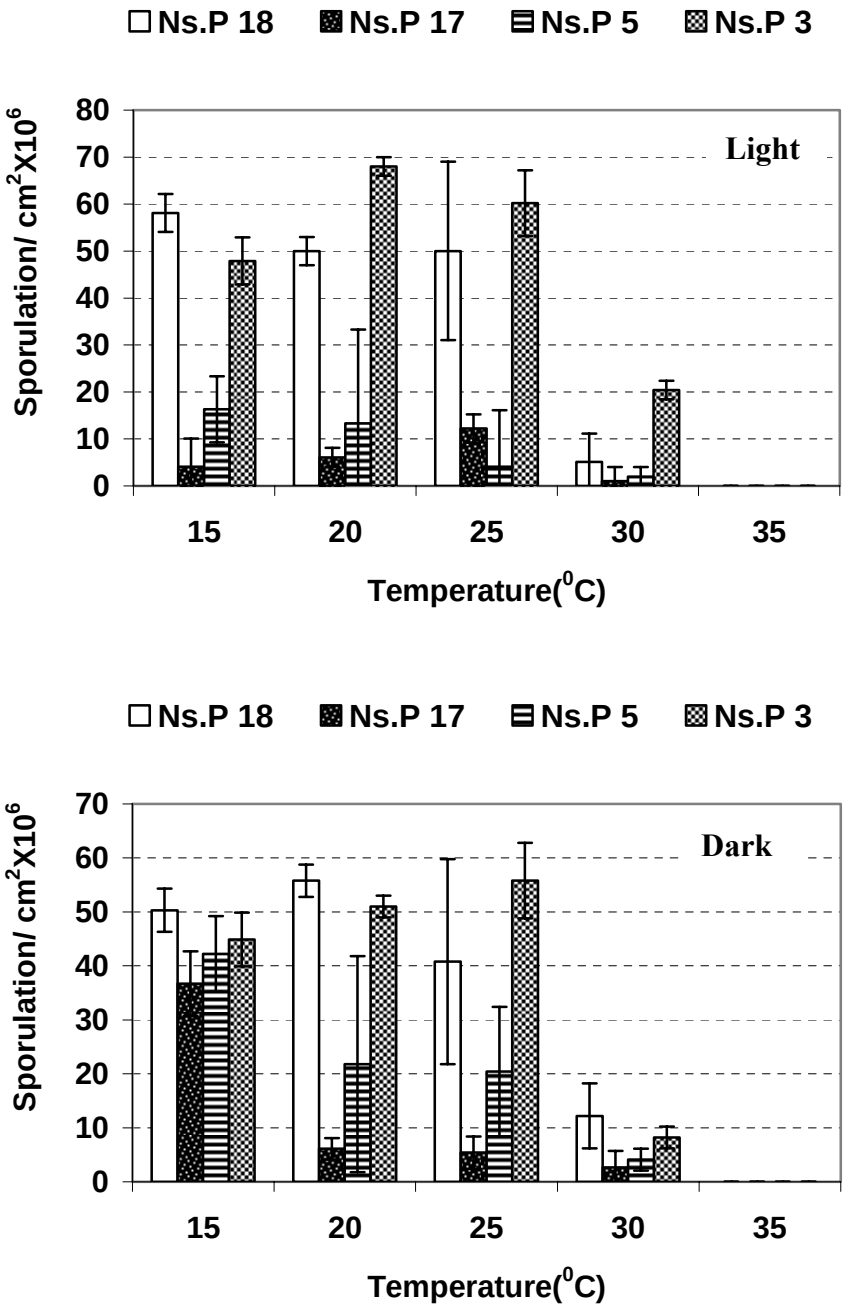


Fig. 3.3: Effect of temperature on sporulation of native isolates of *Nomuraea* grown on SMAY medium and incubated under light and dark conditions.

3.3. Bioassays

3.3.1. Bioagent methods of application

Preliminary experiment

This experiment was conducted to explore which bioagent methods of application are most efficient for biocontrol.

Mortality percentage rates ranged from 40% to 83% for the isolate ARSEF 539 and from 20% to 100% for the isolate ARSEF 1972 (Table 3.9). Mortality percentage was highest (83.3%) when 4th stage larvae of *H. armigera* were exposed to dry conidia of *N. rileyi* isolates ARSEF 539. However a very high mortality (100%) was induced when the larvae were exposed to spores suspension of the isolate ARSEF 1972 on filter paper. The lowest mortality percentages (40 and 20%) were recorded when larvae were exposed to soil formulated with spores of isolates ARSEF 539 and ARSEF 1972, respectively. These percentages were not statistically different from the control treatment. Furthermore, there were no significant differences between the dry treatment and filter paper and dipping treatments when the isolate ARSEF 539 was used (Table 3.9).

Table 3.9: Mortality percentages of *H. armigera* due to *N. rileyi under* different methods of application.

Application Methods	Mortality (%)	
	ARSEF 539*	**ARSEF 1972**
Dry	83.3 ± 16.7 ab **	50 ± 22.3 ae
Soil	40 ± 24.5 bcde	20 ± 20 de
Filter paper	80 ± 20 ac	100 ± 0 a
Dipping (aqueous)	60 ± 24.5 ad	40 ± 24.5 bcde
Control / Dipping	0 ± 0 e	0 ± 0 e
Control / Filter paper	0 ± 0 e	0 ± 0 e

* Values of data represent mean of five replicates ± SEM.

** Means followed by the same letter within the same column and/or row are not significantly different according to Fisher LSD method with $P \leq 0.05$.

Second experiment

This experiment was conducted to compare the efficiency of different conidial application methods (Tween, dry and distilled water), when applied on insect's external cuticle**.** Mortality percentage of *H. armigera* ranged from 25% to 83% when the isolate ARSEF 539 was used and from 33% to 58% when the isolate ARSEF 1972 was used (Table 3.10). Mortality percentage of *H. armigera* was highest (83%) when 4th stage larvae of *H. armigera* were treated with the aqueous (distilled water) formulation of *N. rileyi* isolates ARSEF 539. However, the highest mortality percentage (58%) was induced when the larvae were treated with the conidia of *N. rileyi* (isolate 1972) as dry and aqueous (distilled water) formulations (Table 3.10).

The lowest mortality percentages (25 and 33) were recorded when larvae were treated with spores in Tween 0.1% of both isolates (ARSEF

539, ARSEF 1972), respectively. These percentages were not significantly different from the control treatments (Table 3.10).

Table 3.10: The mortality percentages of *H. armigera* due to *N. rileyi under* different methods of appication.

Application methods	Mortality (%)	
	ARSEF 539*	**ARSEF 1972**
Tween (0.1%)	25 ± 17 bc **	33.3 ± 10.5 bc
Dry	50 ± 13 ab	58.3 ± 15.4 ab
Aqueous (distilled water)	83.3 ± 10.5a	58.3 ± 15.4ab
Control	0 ± 0 c	0 ± 0 c

* Values of data represent mean of six replicates ± SEM

** Means followed by the same letter within the same column and/or row are not significantly different according to Fisher LSD method with $P \leq 0.05$.

3.3.2. Serial culturing of *N. rileyi* inoculum.

This experiment was conducted to test if the virulence of *N. rileyi* is influenced by long term culturing on artificial media, and to check if virulence of the fungus is enhanced by reisolating the fungus from infected larvae.

Mortality percentages induced by *N. rileyi* isolates decreased when these isolates were used for inoculation obtained from previously infected dead larvae, with no significant difference between treatments except with the control treatment (Table 3.11). Accordingly long term culturing of the fungus seems not to influence virulence.

Table 3.11: Mortality percentages induced by *N. rileyi* isolates subjected to serial culturing (ARSEF 539 & 1972) and those isolated from infected larvae (ARSEF 539 A & ARSEF 1972 A).

	Mortality (%)				
Treatments	**ARSEF 539** *	**ARSEF 539A**	**ARSEF 1972**	**ARSEF 1972 A**	**Control**
Dipping	58.3 ±14** ab	50±15 ab	50±15 ab	25±13 bc	0 ± 0 c
Filter paper	67 ±14 a	33.3±14 abc	58.3±15 ab	33.3 ±14 abc	8.3±8.3 c

* Values of data represent mean of twelve replicates ± SEM

** Means followed by the same letter within the same column and/or row are not significantly different according to Fisher LSD method with P≤ 0.05.

3.3.3. Site of entry

The fungus site of entry to the insect body was evaluated in this experiment. The highest mortality percentages (100 %) were recorded when the 4th stage larvae of *H. armigera* fed on tomato leaves inoculated by spore suspension of the fungus with obvious significant differences recorded between treatments of both isolates (ARSEF 539 and ARSEF1972) (Table 3.12). On the contrary, dipping larvae in spore suspensions of the two fungal isolates induced at least 50 % less mortality than the feeding treatments (Table 3.12). This experiment as well further confirmed that long term culturing does not influence *N. rileyi* virulence.

Table 3.12: Effect of site of entry on virulence.

	Mortality (%)				
Sites of entry	**ARSEF * 539**	**ARSEF 539A**	**ARSEF 1972**	**ARSEF 1972A**	**Control**
Feeding	100 ± 0** a	100 ± 0 a	100 ± 0 a	100 ± 0 a	17 ± 17 bc
Cuticle	50 ± 22 b	50 ± 22 b	50 ± 22 b	33± 21 bc	0 ± 0 c

* Values of data represent mean of six replicates ± SEM.

** Means followed by the same letter within the same column and/or row are not significantly different according to Fisher LSD method with $P \leq 0.05$.

3.4. Virulence of isolates

3.4.1. Virulence of the isolates (ARSEF539 & ARSEF 1972)

This experiment was conducted to estimate the lethal time (LT_{50}) and the lethal concentration (LC_{50}) that is required for the fungus to kill 50% of the tested pest population.

The results shown that time (LT_{50}) required for killing the 4^{th} stage larvae decreased as the concentrations of fungal spore suspensions applied increased (Table 3.13) for the two isolates down to certain limit. However, the spore concentration (10^5) of the isolate ARSEF 1972 was ineffective and similar to the control treatment; LT_{50} for the isolate ARSEF 539 was 11 days at the same concentration (Table 3.13).

The isolate ARSEF 1972 induced 100% mortality after 13 days at the concentrations 10^7 spore/ml and after 12 days at the concentration 10^8 spore/ml; however, the isolate ARSEF 539 induced that mortality after 9 and 11 days at the concentrations (10^8 and 10^7 spore/ml), respectively (Fig. 3.4 a, b).

The lethal concentration (LC_{50}) of *N. rileyi* required to kill 50 % of tested 4th stage larvae of *H. armigera* was 10^5 and 10^6 for the isolates ARSEF 539 and ARSEF 1972 respectively (Fig. 3.5).

Table 3.13: Lethal time required to kill 50% of tested population of the 4th stage larvae of *H. armigera* (LT_{50}) at various conidial concentrations.

Concentrations (Conidia /ml)	LT_{50} (day)	
	ARSEF 539	ARSEF 1972
0	_	_
10^5	11	_
10^6	8	12.6
10^7	6.3	9
10^8	6.3	8.22

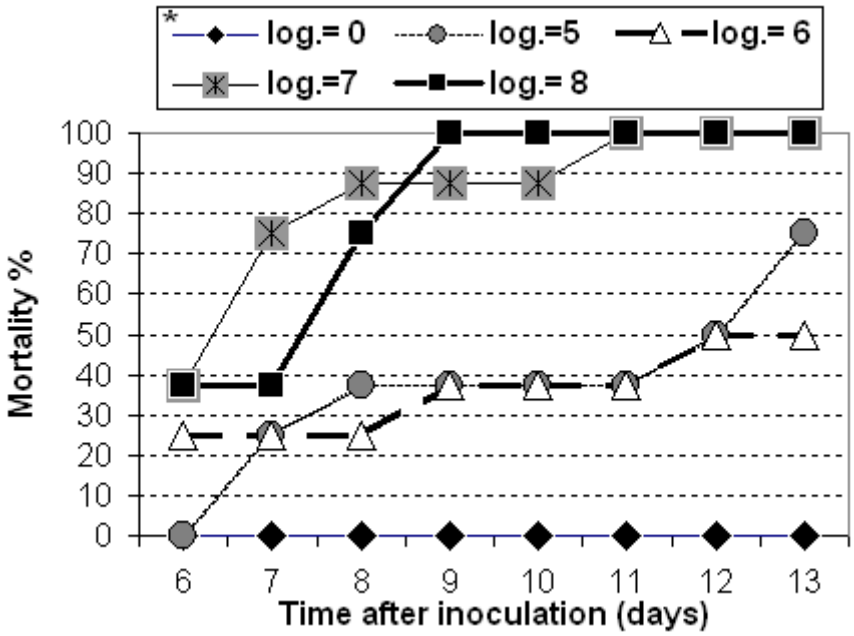
Table 3.14: The mortality percentages of 4th instar larvae of *H. armigera* due to different conidial concentrations of *N. rileyi* isolates ARSEF 539 & ARSEF 1972 at 12 days after inoculation.

Conidial Concentration/ ml	Mortality %	
	ARSEF 539*	ARSEF 1972
10^5	50 ± 19 b**	0 ± 0 c
10^6	50 ± 19 b	50 ± 19 b
10^7	100 ± 0 a	87± 13 a
10^8	100 ± 0 a	100 ± 0 a
Control (zero)	0 ± 0 c	0 ± 0 c

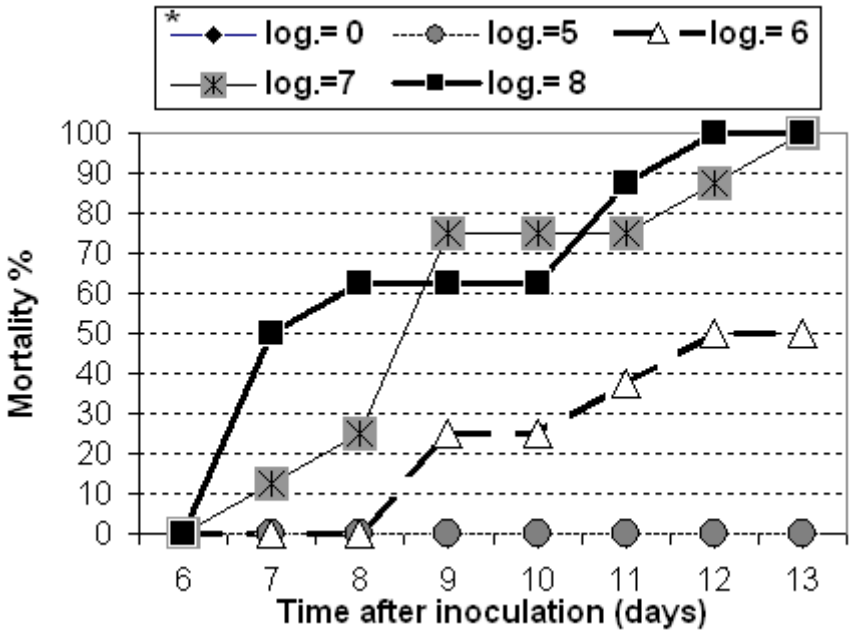
* Values of data represent mean of eight replicates ± SEM

** Means followed by the same letter within the same column and/or row are not significantly different according to Fisher LSD method with $P \leq 0.05$

A (ARSEF 539)



B (ARSEF 1972)



* Log. of conidial concentrations

Fig. 3.4: (A): Effect of time and spores concentrations of *N. rileyi* (ARSEF 539) on mortality percentage of 4th instars larvae of *H. armigera,* (B): Effect of time and spores concentrations of *N. rileyi* (ARSEF 1972) on mortality percentage of 4th instar larvae of *H. armigera*.

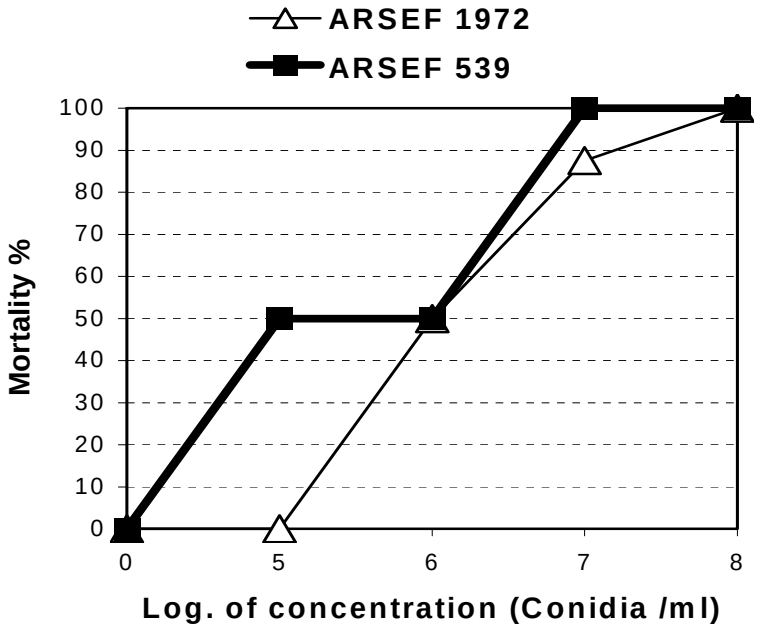


Fig. 3.5: The mortality percentages of 4th instar larvae of *H. armigera* due to different conidial concentrations of *N. rileyi* isolates ARSEF 539 & ARSEF 1972 at 12 days after inoculation.

3.4.2. Virulence of native isolates

3.4.2.1. *In vitro* studies

In this experiment, 4th stage larvae were dipped in conidial suspension and fed on artificial media inoculated with the same conidial suspension of the four native isolates of *Nomuraea* (NsP18, NsP17, NsP5 and NsP3). The isolate NsP18 recorded the highest mortality followed by NsP3. Lowest mortality was recorded by the isolate NsP17 with no significant difference from the control treatment (Fig. 3.6).

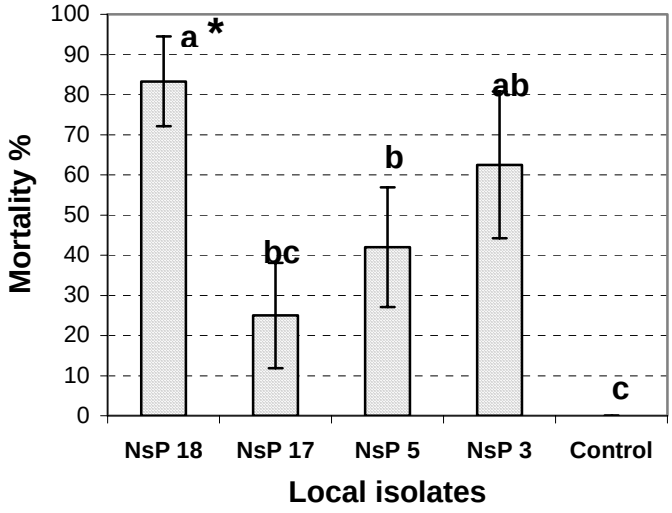


Fig. 3.6: Mortality % of 4th stage larvae inoculated with spore suspension of four native isolates of *Nomuraea*

* Columns labeled by the same letter are not significantly different according to Fisher LSD method, With $P \leq 0.05$.

3.4.2.2. *In vivo* studies

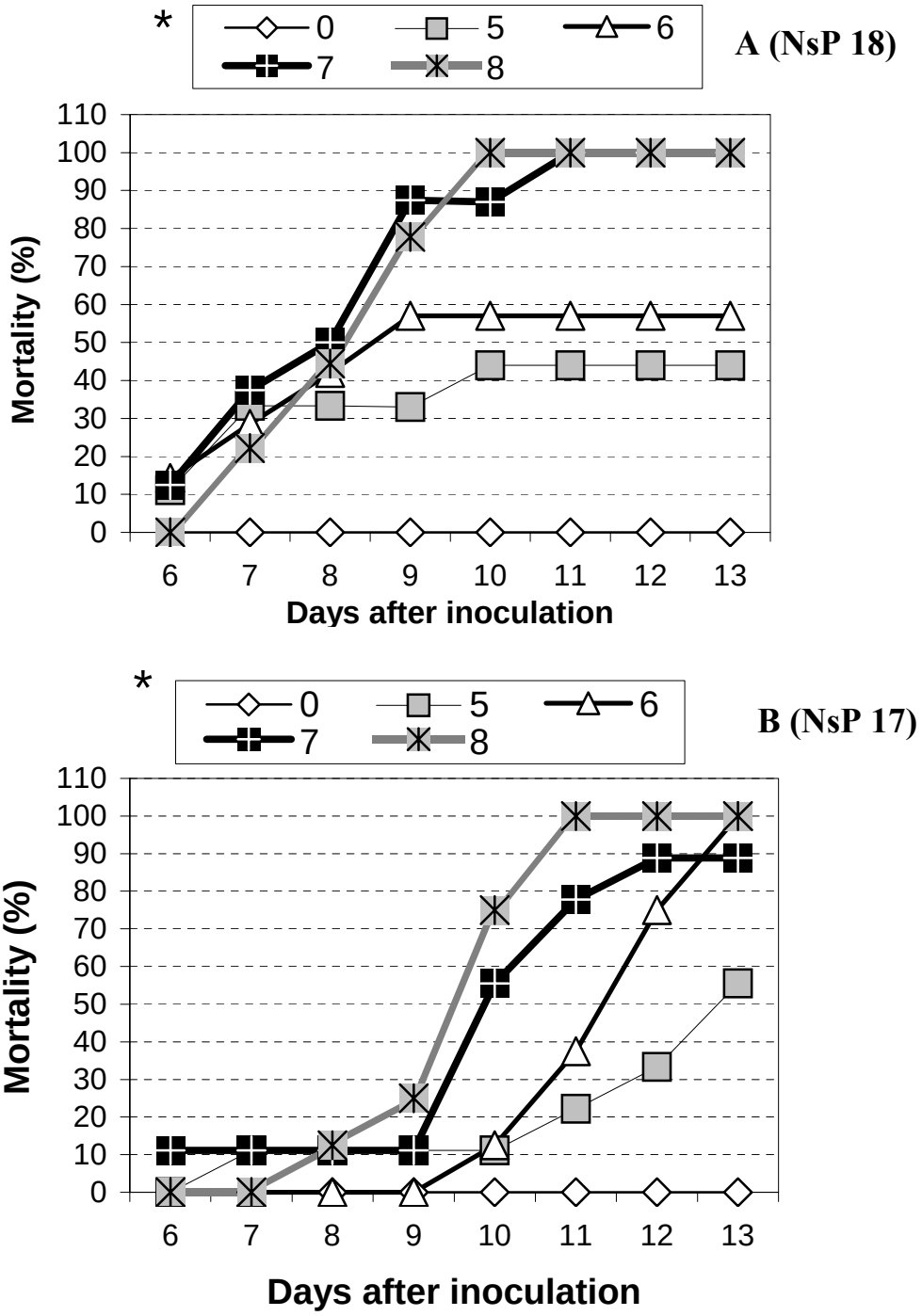
In this experiment, 4th stage larvae were fed on tomato leaves dipped in different concentrations of conidial suspension of the four local *Nomuraea* isolates.

Mortality of 4th stage larvae started at day 6 when concentrations of conidia were used (10^7 and 10^8); mortality percentages ranged from 10% to 100% depending on inoculum concentration and the bioagent isolate (Fig. 3.7 & 3.8).

Lethal time required to kill 50% of larval population (LT_{50}) decreased as the concentration of conidial inoculum increased (Table 3.15), with minimum LT_{50} of 7.8 days at high concentration (10^8), and more than 13 days at low inoculum concentration (10^5) (Table 3.15).

The maximum mortality (100%) was recorded at 10 days after inoculation of tomato leaves with 10^8 spore/ml inoculum of NsP18 and NsP3, while mortality reached maximum (100%) at 11 days when isolates NsP17 and NsP5 were used at the same concentration (Fig 3.7 & 3.8).

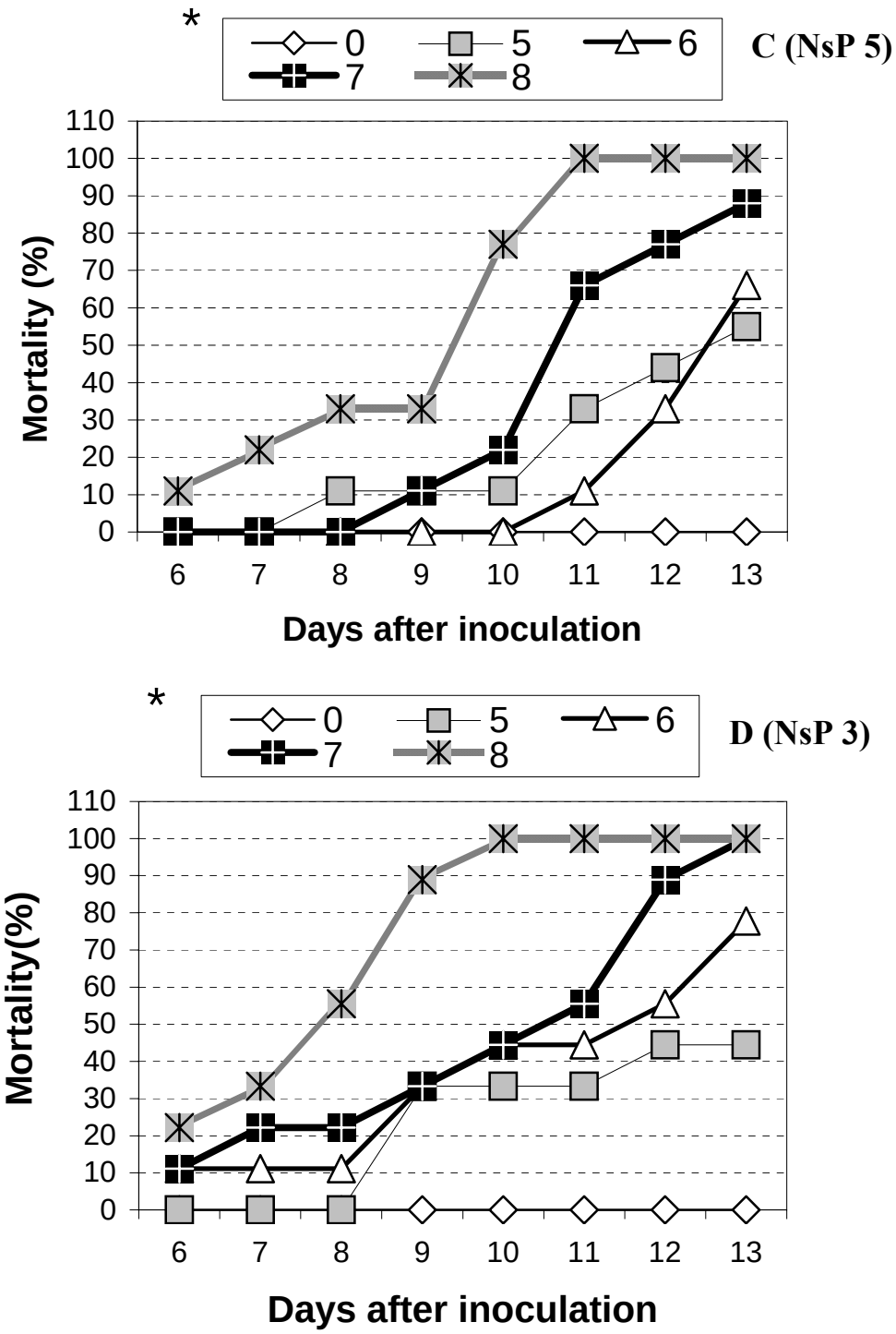
To evaluate the conidial concentrations that kill 50% of tested populations (LC_{50}) for the four local isolates, graphs that represent mortality vs. Log of conidial concentrations were plotted; LC_{50} was calculated from regression equations of curves in (Fig 3.9), which was between 10^5 and 10^6 conidia/ ml.



* Log of conidial concentrations.

Fig. 3.7: The mortality % of 4th stage larvae of *H. armigera* caused by native isolates of *Nomuraea* at indicated days after inoculation

A: NsP18 isolate, B: NsP17 isolate.



* Log of conidial concentrations.

Fig. 3.8: The mortality % of 4th stage larvae of *H. armigera* caused by native isolates of *Nomuraea* at indicated days after inoculation

C: NsP5 isolate, D: NsP3 isolate.

Table 3.15: The LT_{50} of 4th instar larvae of *H. armigera* at different conidial concentrations of *Nomuraea* native isolates.

	LT_{50} (day)			
Concentration Conidia / ml	**NsP 18**	**NsP 17**	**NsP 5**	**NsP 3**
0 (Control)	_	_	_	_
10^5	_	_	_	_
10^6	10.2	11	_	10.9
10^7	7.8	9.9	10.7	9.7
10^8	8.3	9.4	8.9	7.9

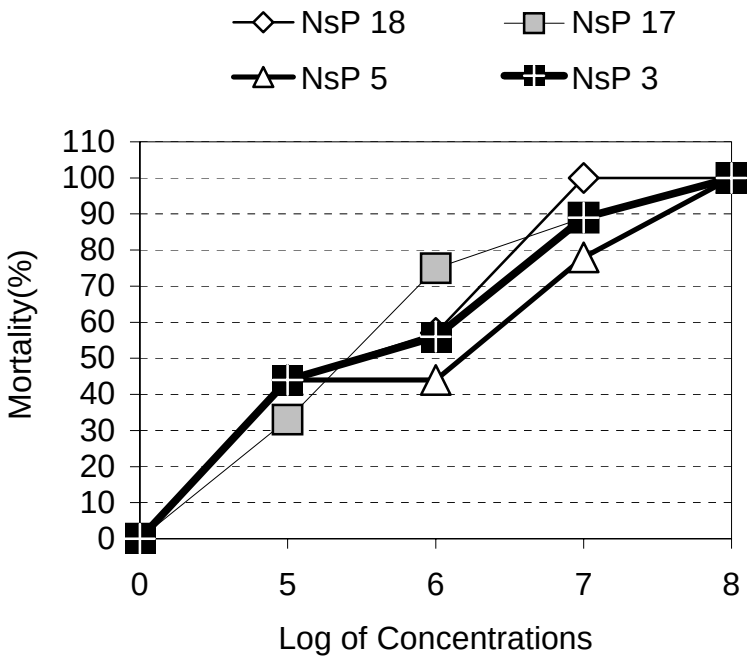


Fig. 3.9: Mortality percentages of 4th stage larvae of *H. armigera* due to four native *Nomuraea* isolates at different conidial concentration.

3.5. Pots bioassay

This experiment was conducted to test the virulence of *N. rileyi* isolates when sprayed on tomato plants before infestation. Data showed that the isolate ARSEF 539 induced 100% mortality to 3rd instars larvae of *H. armigera* when fed on inoculated tomato plants (Fig. 3.10). The isolates ARSEF 1972 induced 80% mortality. Both isolates were significantly different from the control treatment.

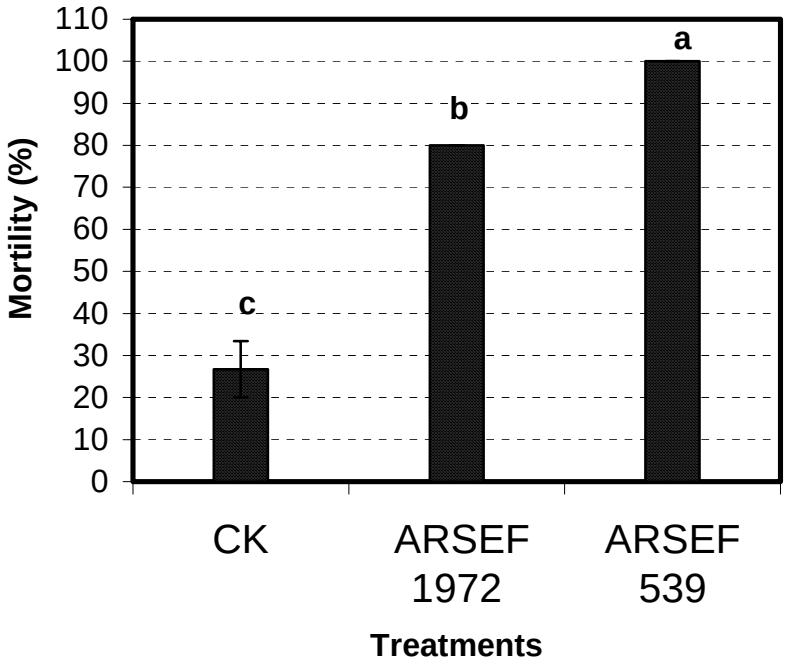


Fig. 3.10: Mortality percentages of 3rd instar larvae of *H. armigera* fed on tomato plants sprayed with conidial suspension of *N. rileyi* isolates ARSEF 539 and ARSEF 1972.

3.6. Effect of *N. rileyi* metabolites.

This experiment showed that the metabolites of *N. rileyi* two isolates (ARSEF 539 and ARSEF 1972) have no influence on mortality of the 3rd larval instars of *H. armigera* (Fig. 3.11). Mortalities due to the two concentrations used were not statistically different from the control treatment for the two isolates. However, the mortality of 3rd larval instars of *H. armigera* was zero when the two concentrations of crude culture filtrate suspensions applied directly on the larval cuticle.

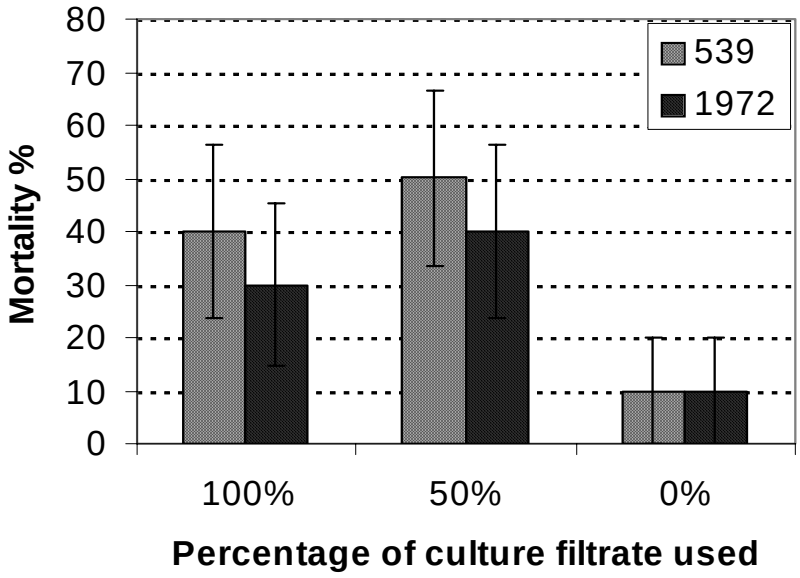


Fig. 3.11: Mortality percentage of *H. armigera* 3rd larval instars due to crude culture filtrate of *N. rileyi* isolates (ARSEF 539 and ARSEF 1972).

Chapter 4

Discussion

4.1. Selective media

Soil is the natural reservoir of conidia of the entomopathogenic fungus *Nomuraea rileyi* (Ignoffo *et al*., 1978). The isolation of the fungus *Nomuraea rileyi* from the soil is a difficult task and the information available on this is very scarce. Most selective media contain either a fungicide and / or antibiotic which encourage growth of entomogenous fungi and discourage the growth of saprophytic fungi and bacteria.

The standard media used for multiplication of *N.rileyi* is Saboraud's maltose agar yeast extract medium (SMAY) (Bell, 1975; Bell *et al*., 1982). The present study modified this medium to be selective for the isolation of *N. rileyi* from soil. When the effect of different concentrations of dodine on germination was tested, it was found that the conidia of *N. rileyi* germinated effectively on modified standard medium (SMAYTD) when the concentration of dodine added was less than 100mg/l. Germination was completely inhibited when dodine concentration was 100mg/l and above.

Furthermore, it was found that the best concentration of dodine to be used in the standard SMAY medium was 60–90 mg / l for isolation of *N. rileyi* from soil. Beilhatz *et al*., 1982 introduced dodine to oat meal agar selective medium for isolation of *Beauveria bassiana*. Another modification on the oat meal dodine media was the addition of Benlate to the medium; this composition increased the recovery of both *M. anisopliae* and *Beauveria bassiana* (Beilhatz *et al*., 1982). Furthermore,

SMAYTD selective medium was further modified by adding Benlate at the concentration of 0.8 mg / l with no improvement in the recovery percentage of *N. rileyi* (isolate ARSEF 539) from the soil. On the contrary, the recovery percentage of the isolate ARSEF 1972 was highly improved when Benlate was added to SMAY medium, but the growth of other fungi was also high when Benlate added to SMAY. This means that both Dodine and Benlate could be added to SMAY medium to become selective for isolation of *N. rileyi* from soil, but the selectivity of both fungicides (Dodine and Benlate) depends on the isolate itself.

In addition, Benlate was totally inhibitory for *N. rileyi* at the concentration of 2 mg / l. In this respect Sneh (1991) described wheat germ selective agar medium (WGSA) adding to it 0.8 mg Benlate (50% benomyl) and 0.3 g dodine for the isolation of *Beauveria bassiana* from soil. In this modification, Sneh (1991) has found that the growth and the recovery of *M. anisopliae* colonies on medium containing 0.8mg/l Benlate and 460mg/l Dodine was satisfactory. In addition, Sneh (1991) also indicated that the growth of *M. anisopliae* colonies and recovery was considerably inhibited at higher Benlate concentration (1.8mg/l) and was completely stopped at Benlate concentration more than 2.8 mg/l. He also described that the reduction in dodine concentration from 460 mg/l to 300 mg/l resulted in earlier and better growth of *M. anisopliae*.

Furthermore, it was found that the best soil dilution to be used in dilution plate technique to isolate the fungus *N. rileyi* from the soil using SMAYTD was 10^{-3}. Sneh (1991) has found that high CFU recovery of *M. anisopliae* was calculated from counts made on plates on which lower propagule concentration were spread than on those from higher concentrations.

The selective media designed in this study using dodine (SMAYTD) was used to isolate native *Nomuraea* isolates from soil which will be discussed in the coming sections.

4.2. Soil samples collection and isolation

The occurrence of number of entomopathogenic fungi in Palestine was studied by Shtayeh *et al*., (2002). *Nomuraea rileyi* however, was not isolated or studied by the investigator and this possibly due to its scarcity and lack of appropriate selective media for isolation.

The fungus *N. rileyi* is known to be overwintering in the soil mostly as conidia (Bell, 1975 and Ignoffo *et al*., 1978). Four local isolates of *Nomuraea* were isolated from Jenin area by using SMAYTD selective medium and dilution plate technique.

4.3. Local isolates (Growth parameter)

Infection, sporulation, spore germination and growth rate of entomopathogenic fungi are influenced by environmental factors, especially temperature and humidity (Tanada and Kaya, 1993). Virulence of entomopathogenic fungi is always affected by environmental factors such as temperature, humidity, soil moisture, and light. Spore survival, persistence, and sporulation are influenced drastically by temperature (Walstad *et al*., 1970; Hajek *et al*., 1990).

In this study, it was found that the isolates of *Nomuraea spp* isolated from the Jenin area favored relatively moderate temperatures from (15 – 25 ºC) for germination, sporulation and mycelial growth. Growth, sporulation and germinations of the fungus were completely inhibited at 35 ºC.

Tang and Hou, (2001) found that the conidial germination of *N. rileyi* were best at 20 and 25 ºC when it was tested on SMAY.

Furthermore, most stages of development of the isolates ARSEF 539 and ARSEF 1972 were favored by the temperature range 25–30 ºC (Barakat, unpublished data).

Mohamed *et al*., (1977) found that the infection of *N. rileyi* was best at 20 - 25 ºC, when tested against *Heliothis zea*. Tang and Hou, (2001) found that *N. rileyi* fungus favors moderate temperature particularly at 20 ºC for conidial germination and infection. The high temperature of 35 ºC was unsuitable for conidial germination of *N. rileyi* (Boucias & Pendland, 1984). Accordingly, it seems that the optimum temperature for *Nomuraea spp* is an isolate dependent. The same conclusion was documented by (Boucias *et al*., 1984).

4.4. Fungal methods of application

In larval bioassay, the mortality of the larvae is highly related with type of fungal formulation, and type of application methods (Ignoffo *et al*., 1975, 1978). The soil could be contaminated either by natural infestation that leads to production of conidia on dead larvae or by applying conidia to the soil artificially which could initiate new epizootics to lepidopterous larvae. Further more, any plant parts (cotyledons and leaflets) that could be in contact with soil are contaminated by surface soilborne conidia of the fungus *N. rileyi* and this contamination could be transmitted to caterpillars (Ignoffo *et al*., 1977).

In this study, the mortality of 4th stage larvae of *H. armigera* due to applying aqueous conidial suspension to soil in which the larvae were reared on was 40 % and 20 % for the isolates, ARSEF 539 and ARSEF 1972, respectively. Larval mortalities in the soil are due to direct contact between conidia of the fungus and the external cuticle. The low level of mortality obtained by this application method however might be due to either poor attachment of conidia that incorporated in the soil to the

external cuticle of the larvae or due to insufficient conidial concentration per mm^2 of soil.

The same results has been found by (Ignoffo *et al*., 1977) in which larvae of *Trichoplusia ni* fed on soybean cotyledons grown in contaminated soil showed low mortalities. they reported that the mortality of *Trichoplusia ni* fed on cotyledons of soybean plants germinated in soil treated with *N. rileyi* was 68.8% and 11.6% when conidial concentrations of 896x 10^3 and 89.6x10^3 conidia / mm^2 of soil, were used respectively. However, they indicated that the percentage of mortality of *T. ni* increased as the conidial concentration inoculum in the soil increased.

Furthermore, low level of mortality percentages obtained when applying the fungus *N. rileyi* to the soil might be due to the low level of nutrients available to the fungus in soil. In this respect, Vimala (1995) described that the application of *N. rileyi* fungus to the soil surface with its substrate (crushed sorghum with 1% yeast extract) in which groundnut plants were grown, resulted in 100% mortality of *S. litura* larvae. So it is believed that applying the fungus *N. rileyi* with low cost substrate or in sporulated dead larvae to the soil surface in the microclimate of the plants could be an efficient application method which could also be sufficient to initiate epizootic for the whole coming season.

Since application of the fungus to the filter paper gave the highest percentages of mortalities to *H. armigera* and there was no nutrients additives provided, then it is most probable that low mortalities recorded in the soil treatment was due to the soil physical properties, insufficient inoculum and poor inoculum attachment to the host cuticle as mentioned earlier.

One of the major factors in successful use of entomopathogenic fungi for biological control is the infectivity, which is a factor of conidial

adhesion to larval cuticle; the conidial adhesion would be useful to bypass the first barrier to pathogenicity (Moore & Prior, 1993).

In this respect three formulations were used as external application methods. The highest larval mortalities were recorded when fungal inoculum was carried by distilled water. However, mortality percentages were considerably reduced when inoculum was applied with 0.1 % Tween 80 formulation. This suggests that Tween 80 might have some slight toxicity to the fungus *N. rileyi*; this is supported by the fact that the Tween 80 control treatment without the bioagent has not influenced *H. armigera* larvae. In this respect, Butt and Gaettel (2000) reported that Tween added to water can be toxic to conidia if the concentration used was above 0.1 % in conidial suspension.

Ignoffo *et al*, (1967) described that treatment of soybean leaves with 0.05% tween 80 formulation of *N. rileyi* resulted in 97% mortality of *T. ni*, at 12 days after inoculation and at application rate of 307 conidia / mm^2 of leaflet side. This suggests that Tween formulation could be suitable when the fungus applied to host larvae through feeding.

Boucias *et al*., (1984) described that the leaf surface treatment with *N. rileyi* resulted in highest mortality to *Anticarsia gemmatalis* larvae caused by the fungus. They also suggested however, that the virulence of insect pathogens could be reduced by leaf texture, plant developmental stage, or antimicrobial substances produced by plants. Ignoffo (1981) suggested that the entomopathogenic fungus *N. rileyi* have to be applied to control insect pests as insecticidal methods of application. Since noctuid larvae are known to hide in the soil in day time and come out to feed on leaves at night, it is most effective to spray conidia on either soil surface or on leaf surface.

In this study, the mortality of *H. armigera* due to *N. rileyi* (ARSEF 539 and ARSEF 1972) was 100 % when 4^{th} stage larvae were fed on

tomato leaves dipped in conidial suspension. On the contrary, the mortality did not exceed 50 % when fungal conidia were applied to the larval cuticle. The same result has been found by (Tang and Hou, 1998) in which the mortality of *H. armigera* 4^{th} stage larvae due *N. rileyi* was (90.1- 100%), when the larvae fed on treated different kinds of plants with 10^8 conidia /ml suspension. In addition, Tang and Hou (2001) found the mortality percentage of 4^{th} stage larvae of *H. armigera* due to the external application of *N. rileyi* at the rate of $5x10^6$ conidia/ml with 0.05% tween 40 was 62% at 25 ºC.

Not all areas of the insect cuticle are equally vulnerable to penetration by propagules of entomopathogenic fungi. Butt *et al*., (1995), and Schabel (1976) stated that the buccal cavity can be preferential sites of infection. Therefore, the location where the inoculum lands can influence the probability of infection and the speed of kill.

The difference in mortality between applying conidia to elementary canal through feeding and applying conidia to the external cuticle could be due to the strength of external cuticle more than the inner walls of elementary canal, so external cuticle may be protected against the mechanical action applied by the spores of *N. rileyi*, or it may be resistant to be analyzed by chitinases secreted by the fungus, during penetration processes.

4.5. Serial *in vitro* culturing of *N. rileyi* inoculum

Attenuation in virulence of certain isolates of *Beauveria bassiana*, *Metarhizium anisopliae* and *Verticillium lecanii* fungi when these fungi have been serially transferred on artificial media have been reported by Nagaich (1973). This study was designed to test the influence of serial culturing on virulence of *N. rileyi* isolates. No significant difference was detected between those isolates serially cultured for 16 times and those

passed through inoculation to living larvae. Hall (1980) reported that serial *in vitro* transfer didn't result in the loss of virulence of these fungi to host insects. Morrow *et al*., (1989) have concluded that the effect of repeated *in vitro* subculture of entomopathogenic fungi could vary among species and pathotypes.

Early works by Kawakami (1960) indicated that serial passage reduced the virulence of *N. rileyi* to silkworms. Ignoffo *et al*., (1982) demonstrated that serial passage (12-18 transfers) of *N. rileyi* didn't result in attenuation of virulence when assayed against *T. ni*.

On the other hand, Morrow *et al.*, (1989) reported that continuous serial conidial transfer (more than 10) of certain pathotypes on SMAY resulted in conidia with reduced virulence against neonate larvae; by 16^{th} conidial transfer, conidia become nonvirulent to larvae.

Morrow *et al.*, (1989) further reported that there was no decrease in virulence of *N. rileyi* after multiple vegetative transfers (more than 80) of the hyphal body stage on SMAY plates. Virulence of attenuated isolates of *N. rileyi* can be regained with passage through an appropriate host (Morrow *et al.*, 1989). He also reported that the attenuation of *N. rileyi* virulence was related to the sporulation process.

4.6. Virulence of *Nomuraea* isolates

Virulence against the target insect's pest is the most important criterion for commercial exploitation of the fungal pathogens (Vimala *et al*., 2003). Several parameters for virulence of entomopathogenic fungi have been documented (Butt, and Goettel, 2000); of these are mortality percentage, LT_{50}, and LC_{50}.

In testing the two isolates ARSEF 539 and ARSEF 1972, the results shown that LT_{50} decreased as the concentrations of *N. rileyi* spore suspensions applied increased. The same result has been found by Tang

and Hou, (1998). In this study the mortality of the two isolates ARSEF 539 and ARSEF 1972 was 100% at 9 and 12 days after inoculation respectively, at conidial concentration of 10^8 conidia/ ml

The LT_{50} for both isolates were 6.3 and 9 days, respectively at conidial concentration of 10^8 conidia/ ml.

In this study, four local isolates were studied for their virulence. The isolates NsP18 and NsP3 were capable of causing significant larval mortalities (78 % and 89 %) of *H. armigera* at 9 days, whereas isolates NsP17 and NsP5 were capable of causing this mortality (75 % and 78 %) at 10 days. The LT_{50} for all local isolates were more than 13 days at conidial concentration 10^5 and was reduced to be in the range of 7.9 to 9.4 days at conidial concentration of 10^8. These variations in LT_{50} values within isolate could be due to genetic variations in virulence.

The difference in larval mortality percentage and LT_{50} due to *N. rileyi* infection depends on environmental factors especially temperature and humidity (Tanada and Kaya, 1993). It also depends on virulence of the isolate itself; however geographical isolates of entomopathogenic fungi vary in their virulence to target insects (Ignoffo and Garcia, 1985). Tang and Hou (2001) found that the infection of *H. armigera* by *N. rileyi* resulted in approximately 95 % mortality of 4^{th} stage larvae, and LT_{50} of only 5.8 days, at inoculation concentration of 5 x 10^6. They further found that the *N. rileyi* infection resulted in 94.3% of 3^{rd} instars mortality and LT_{50} of 4.59 days, at inoculation concentration 5 x 10^8. However, 100% 4^{th} instars larval mortality with LT_{50} of 7.2 days occurred at incubation temperature of 20 ºC, and LT_{50} of 6.8 days at incubation temperature of 25 ºC.

Lethal concentrations LC_{50} for the isolates ARSEF 539 and ARSEF 1972 were 10^5 and 10^6 respectively, while LC_{100} were 10^7 and 10^8, respectively. The lethal concentrations (LC_{50}), for the native isolates

were between 10^5 and 10^6. However, the LC_{100}, were between, 10^7 and 10^8.

In this respect, it has been found that the LC_{50} of *N. rileyi*, the lethal dose that kill 50 % of tested 2nd instars of *S. litura* populations at 6th, 7th, and 8th days were $2.23x10^8$, $2.89x10^7$, and $2.2x10^7$ conidia/ml (Vimala, 1994). In this study the results indicates that the recommended dose could be between 10^7 and 10^8 conidia /ml for *N. rileyi* isolates.
This difference in lethal concentration could be due to isolate virulence characters, such as conidial germination, efficacy in laboratory bioassay and chitinolytic activity (El- Sayed *et al*., 1989).

In pots bioassay experiment which was conducted to test the efficiency of *N. rileyi* to be used in insecticidal approach against *H. armigera*, the mortality of *H. armigera* due to the two isolates of *N. rileyi* (ARSEF 539 and ARSEF 1972) were 100% and 80%, respectively. This difference could be due virulence characteristics of both isolates. Whereas the isolate ARSEF 539 was more virulent than ARSEF 1972.

In net house study of the effects of *N. rileyi* on 2nd instars of *S. litura*, Vimala (1994) found that the mortality percentage was 84% at the conidial concentration of $5x10^8$ and was 66 % at the conidial concentration $1x10^8$.

4.7. Fungal metabolites

Many fungi produce secondary metabolites, which acts on other organisms, causing inhibition of growth, and death. The crude metabolites of the isolates ARSEF 539 and ARSEF 1972, has no effects on the 3rd stage larvae of *H. armigera*. This could be due to either the genetic nature of the isolates in production of toxins, since the amount of toxin produced by certain isolates may be more than other isolates, or due to the dilution of toxins in broth medium.

Mohamed and Nelson (1984) reported that *N. rileyi* crude extracts caused mortality percentage of 23.3%, 44.5% and 68.9% for *H. virensens* larvae, and 28.7%, 53.8% and 78.3% for *H. zea* larvae at 42, 48 and 72 hours after treatment, respectively.

Conclusions

The fungicide Dodine can be safely added at the concentration of 90 mg / l, to modify the standard SMAY media to become selective for the isolation of the *Nomuraea rileyi* from soil. Four native isolates of *Nomuraea* were isolated from soil samples collected from the Jenin area by using selective SMAY (SMAYTD).

The optimum temperatures for growth parameters of native isolates were in the range of 20 – 25ºC; optimum growth temperature was an isolate dependent.

Sterile distilled water was the best conidial carrier for pathogenicity. *Nomuraea rileyi* induced 100 % larval mortalities when the 4^{th} stage larvae of *H. armigera* fed on tomato leaves sprayed with conidial suspension of the fungus. Larval mortalities have not exceeded 50 % when conidia were applied to the external cuticle of the larvae.

Virulence of *N. rileyi* has not been attenuated after 16 successive culturing, and was not enhanced by isolating the fungus from infected *H. armigera* larvae.

The lethal time needed to kill 50 % of 4^{th} stage larvae of studied population (LT_{50}) was 6.3 and 9 days for the isolates ARSEF 539 and ARSEF 1972, respectively. LT_{50} of the native *Nomuraea* isolates ranged between 7.9 and 9.4 days at the inoculum concentration of 10^8 conidia / ml. LC_{50} for all *Nomuraea* isolates was in the range of 10^5 to 10^6 conidia / ml. The conidial concentration 10^8 conidia / ml induced 100% larval mortality.

Finally, and in light this study, future investigations are needed in the following fields:

- Developing new methods for mass production of *N. rileyi* in cost effective manner.
- Developing new fungal formulations that are relatively stable to environmental factors, and have relatively long shelf life.
- Testing the biocontrol efficiency of *N. rileyi* isolates under field conditions.
- Using the fungus in the context of IPM.

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المراجع العربية.

غـازي الحريـري، الحـشرات الاقتـصادية فـي سـوريا والبلادالمجـاورة، منـشورات جامعة حلب ، 1984

نعـيم شـر ف واخـرون، الحـشرات العامـة ، دار زهـران للنـشر والتوزيـع، عمان،الاردن، 1993

Appendix A:

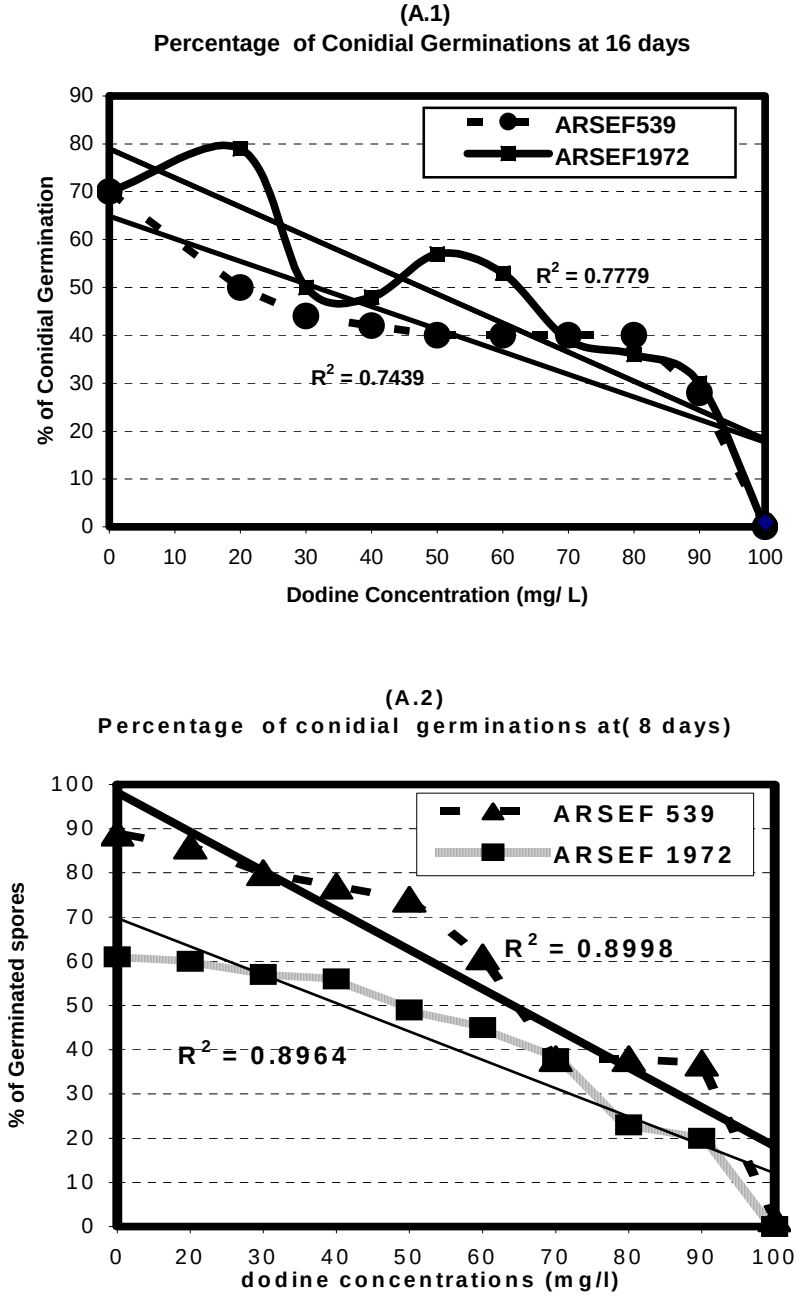


Fig. A.1: Effect of dodine concentrations on percentage of conidial germinations of the fungus *Nomuraea rileyi* at 16 days after inoculation; A.2: Effect of Dodine concentrations on percentage of conidial germinations of the fungus *Nomuraea rileyi* at 8 days after inoculation.

Appendix B: Mortality of *Helicoverpa armiger* 4th instars induced by native *Nomuraea* isolates at spore concentration (10^8 spore / ml)

Isolate	Mortality % at indicated days after inoculation*					
	6 days	7 days	8 days	9 days	10 days	11 days
NsP18	0 ± 0cd**	22 ± 15bc	44 ±18b	78 ±15a	100 ±0a	100 ±0a
NsP17	0 ± 0bc	0 ± 0b	13 ±13b	25 ±16b	75 ±16a	100 ±0a
NsP5	11 ±11bc	22 ±15b	33 ±17b	33 ±17b	78 ±15a	100 ±0a
NsP3	22 ±15bd	33 ±17bd	56 ±18bcd	89 ±11ac	100 ±0a	100 ±0a

* Values of data represent mean of nine replicates ± SEM.

** Means followed by the same letter within the same column and / or row are not significantly different by Fisher LSD method with $P \leq 0.05$.

Appendix C: Mortality of *Helicoverpa armiger* 4th instars induced by native *Nomuraea* isolates at spore concentration (10^7 spore / ml)

Isolate	Mortality % at indicated days after inoculation*							
	6 days	7 days	8 days	9 days	10 days	11 days	12 days	13 days
NsP18	12±12 c **	37±18 bc	50±19 b	87±12 a	87±12 a	100±0 a	100±0 a	100 ±0 a
NsP17	11±11 b	11±11 b	11±11 b	11±11 b	55.5±17.5 a	78±15 a	89±11 a	89 ±11 a
NsP5	0±0 b	0±0 b	0±0 b	11±11 b	22±14 b	67±17 a	78±15 a	89±11 a
NsP3	11±11 d	22±15 cd	22±15 cd	33±17 cd	45±18 cd	56±18 bc	89±11 ba	100 ±0 a

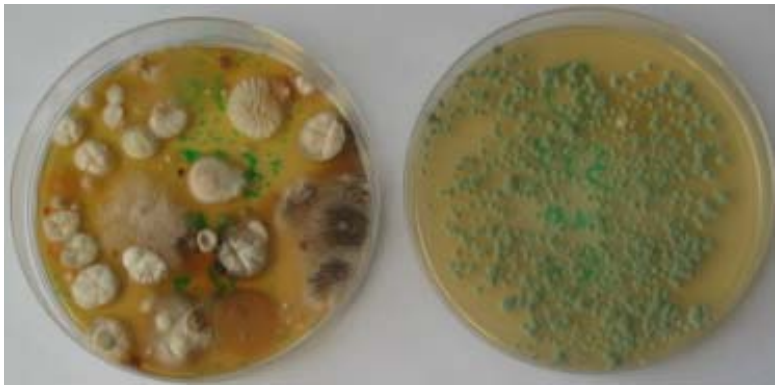
* Values of data represent mean of nine replicates ± SEM.

** Means followed by the same letter within the same row are not significantly different by Fisher LSD method with $P \leq 0.05$.

Appendix D: ANOVA Tables

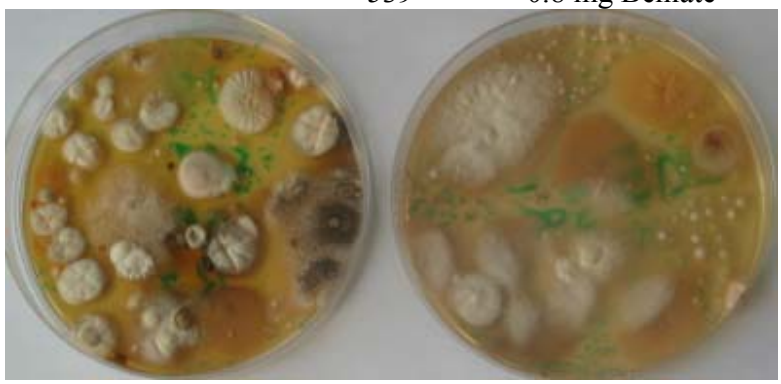
Experiment	D.F treat	D.F residual	D.F total	SS treat	SS residual	SS total	MS treat	MS residual	F
Effect of dodine on germinations (16 days)	19	47	66	26505.6	8885.4	35391	1395	189	7.4
Effect of dodine on germinations (8 days)	18	63	81	51631.9	6453.5	58085.4	2868.4	102.4	28
Effect of field soil and soil dilutions on % of germinations	7	23	30	9202	1157.4	10359.4	1314.6	50	26
Effect of dodine benlate combination on % of germinations.	3	12	15	7201.5	8595	8061	2400.5	71.6	33
Effect of dodine benlate combination on % of recovery	7	23	30	6233	1050	7283	890.4	45.7	19.5
Effect of application methods on mortality %	9	42	51	$5.447x10^4$	$7.533x10^4$	$1.3x10^5$	$6.05x10^3$	$1.79x10^3$	3.38
Effect of formulations on mortality %	7	40	47	$3.66x10^4$	$3.46x10^4$	$7.1x10^4$	$5.2x10^3$	$8.65x10^2$	6.1
Effect of serial culturing of *N. rileyi* on virulence of the fungus	9	110	119	$5.367x10^4$	$2.3x10^5$	$2.8x10^5$	$5.96x10^3$	$2.09x10^3$	2.85
Effect of site of entry	9	49	58	$7.6x10^4$	$6.7x10^4$	$1.4x10^5$	$8.4x10^3$	$1.4x10^3$	6.2
Conidial concentration vs mortality	8	63	71	$1x10^5$	$6.9x10^4$	$1.7x10^5$	$1.31x10^4$	$1.1x10^3$	11.96
Mortality % due to external application of the local isolates	4	50	54	$4.7x10^4$	$8.7x10^4$	$1.3x10^5$	$1.2x10^4$	$1.7x10^3$	6.7
Effect of time on mortality % due to NsP18	5	48	53	78703	53333	132037	15740	1111	14.2
Effect of time on mortality % due to NsP17	5	42	47	71041	38750	109791	14208	922	15.4
Effect of time on mortality % due to NsP5	5	48	53	54259	80000	134259	10851	1666	6.5
Effect of time on mortality % due to NsP3	5	48	53	53333	66666	120000	10666	1388	7.6
N. rileyi sprayed on tomato seedling vs mortality	2	6	8	$8.6x10^3$	$2.7x10^2$	$8.9x10^3$	$4.3x10^3$	44	97
Culture filtrate vs mortality	4	45	49	$9.2x10^3$	$1.03x10^5$	$1.1x10^5$	$2.3x10^3$	$2.3x10^3$	1
Effect of temp. on growth rate of isolate NsP18	9	29	38	21.4	18.9	40.3	2.4	0.65	3.6
Effect of temp. on growth rate of isolate NsP17	9	29	38	58.9	44	103	6.5	1.52	4.3
Effect of temp. on growth rate of isolate NsP5	9	25	34	29.7	11.8	41.5	3.3	0.47	6.97
Effect of temp. on growth rate of isolate NsP3	9	28	37	41	9.3	50.4	4.569	0.33	13.7
Effect of temp. on germination% of NsP18	9	25	34	25012	4257.5	29269	2779	170	16.3
Effect of temp. on germination% of NsP17	9	24	33	28897	3928	32826	3210.8	163.7	19.6
Effect of temp. on germination% of NsP5	9	23	32	38329	5031	43360	4258	218	19.5
Effect of temp. on germination% of NsP3	9	22	31	37600	3396.7	40997	4177.8	154.4	27
Effect of temp. on sporulation of isolate NsP18	9	25	34	19453	4666	24149	2164	186	11.6
Effect of temp. on sporulation of isolate NsP17	9	24	33	3346	1058	4404	371	44	8.4
Effect of temp. on sporulation of isolate NsP5	9	23	32	5025	4445	9471	558	193	2.9
Effect of temp. on sporulation of isolate NsP3	9	23	32	20157	7180	27337	2239	312	7.2

Appendix E:



Control ARSEF 539 90 mg Dodine

Control ARSEF 539 90 mg Dodine 0.8 mg Benlate



Control ARSEF 539 0.8 mg Benlate

Germination of *N. rileyi* (Isolate ARSEF 539) on selective media compared to control

Appendix F:

Control ARSEF 1972 90 mg Dodine

Control ARSEF 1972 90 mg dodine 0.8 mg Benlate

Control ARSEF 1972 0.8 mg Benlate

Germination of *N. rileyi* (Isolate ARSEF 1972) on selective media compared to control