

**IMPACT AND BIOLOGICAL CONTROL OF *OMMATISSUS LYBICUS*
DE BERGEVIN (HEMIPTERA: TROPIDUCHIDAE) ON DATE PALM
(*PHOENIX DACTYLIFERA* L.)**

NASSER AL-ABRI, M.Phil

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Division of Plant and Crop Sciences, University of Nottingham, Sutton
Bonington, LE12 5RD, UK

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List of Abbreviations

ANOVA	Analysis of variance
Ap	Appressoria
°C	Degree Celsius
BLAST	Basic local alignment search tool
cm	Centimetre
CV	Co-efficient of variation
cv.	Cultivar
DNA	Deoxyribonucleic acid
dpi	Days post inoculation
EDTA	Ethylenediaminetetraacetic acid
EPF	Entomopathogenic fungi
g	Grams
GPS	Global positioning system
hpi	Hours post inoculation
Hy	Hypha
ICP-MS	Inductively coupled plasma mass spectrometry
ITS	Internal transcribed spacer region
kg	Kilograms
LC-MS	Liquid chromatography mass spectrometry
LSD	Least significant difference
m	Meter
min	minutes
mm	Millimetre

NCBI	National Center for Biotechnology Information
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
pH	Potential of Hydrogen
ppm	Parts per million
R _{c1}	Hydrometer blank reading after 40 seconds
R _{c2}	Hydrometer blank reading after 6 hours
R _{clay}	Hydrometer reading of clay after 6 hours
R _{sand}	Hydrometer reading of sand after 40 seconds
SDA	Sabouraud Dextrose Agar
SE	Standard error
SEM	Scanning electron microscopy
Sp	Spores
V	Volume of EDTA titrated for the sample (ml)
V1	Volume of soil extract used for titration (ml)

Abstract:

The dubas bug, *Ommatissus lybicus* De Bergevin (Hemiptera: Tropiduchidae), is a piercing-sucking insect species of significant economic importance. The insect attacks date palms (*Phoenix dactylifera* L.), consuming phloem sap and producing large volumes of honeydew on crop leaves. The deposited honeydew on leaves results in dust accumulation and secondary growth of sooty moulds, which are likely to reduce photosynthesis. Both adults and nymphs cause damage to the palms, with the females also causing necrosis to the palm tissue through oviposition. Major efforts are made every year to control this pest in Oman using repeated applications of insecticides on more than twenty thousand acres infested with *O. lybicus*. Despite these applications, control has been inconsistent and more research are needed to develop more sustainable and environmentally safe control strategies. The aim of this study was to quantify the effects of *O. lybicus* on date palm growth and to identify and develop biological control measures using native and commercial entomopathogenic fungi.

The effect of different infestation levels of *O. lybicus* on chlorophyll, date palm biomass, and date palm nutritional composition were investigated. Initial infestations of *O. lybicus* nymphs exceeding 300 nymphs per palm seedling caused a reduction in palm chlorophyll of up to 40% and plant biomass of 29-34%. *Ommatissus lybicus* populations of more than 600 insects per palm reduced oviposition by females, possibly due to intraspecific competition over resources. Honeydew droplets showed significant association with chlorophyll content or eggs oviposited on the rachis, which suggests that control can be performed at 3-6 nymphs/leaflet. The pest activities affected the palm nutrients Mg, Ca, P,

and K. Magnesium content in palms was associated with chlorophyll reduction as *O. lybicus* density increased.

To investigate the abundance, diversity, and distribution of local entomopathogenic fungi (EPF) in Omani soils, a survey was conducted using the Galleria bait method. The obtained EPF with high sporulation and growth rate were further characterized for their optimal temperature, and infection potential against *O. lybicus* adults was assessed using scanning electron microscopy (SEM). Fungal species known to be biological agents against insects were identified from 46 isolates collected through this survey and belonged to five genera, *Metarhizium* spp., *Beauveria bassiana*, *Clonostachys* spp., *Fusarium* spp., and *Aspergillus* spp. along with five entomopathogenic nematode species that were identified as *Acrobelloides saeedi*, *Oscheius tipulae*, *Pratylenchus goodeyi*, *Heterorhabditis indica*, and *Mesorhabditis* sp. In vitro, *B. bassiana* and *M. quizhouense* were able to grow and sporulate at temperatures between 25°C and 35°C. *Clonostachys rosea* had rapid growth on *O. lybicus* adult cuticle and was first seen after 2 hours post inoculation (hpi). *Beauveria bassiana* was the most effective fungi against *O. lybicus* adults, with a 93.4% mortality rate within 3 days post inoculation (dpi).

To evaluate the efficacy of native and commercial entomopathogenic fungi control of *O. lybicus*, four native fungal pathogen isolates, *Clonostachys rosea*, *Metarhizium quizhouense* and *Beauveria bassiana* and *Metarhizium anisopliae* together with three commercial products, Bio-Power *B. bassiana*, Bio-Catch *Verticillium lecanii*, and Botanigard 22WP *B. bassiana* strain GHA were evaluated for their pathogenicity against *O. lybicus* eggs, nymphs, and adults. The insects were hosted in Petri dishes and palm seedlings. Furthermore, two

fungi *M. anisopliae* and *B. bassiana*, were evaluated against the *O. lybicus* adults under different relative humidity treatments. The highest death rate was achieved by *B. bassiana* with 76.7% mortality at the adult stage of *O. lybicus* after 2 weeks of inoculation. The best formulated fungi against *O. lybicus* nymphs and adults was Botanigard 22WP with a 90.8% and 84.2% mortality rate at the 1st and 2nd instars, and 100% mortality rate at the 3rd, 4th, 5th, and adult stages after 7 dpi. *Ommatissus lybicus* egg hatching was significantly reduced by Botanigard 22WP and *C. rosea* with 75.8% and 74.7% unhatched eggs, respectively. *Ommatissus lybicus* adults inoculated with two native fungi *B. bassiana* and *M. anisopliae* had a significantly higher mortality rate of 100% when incubated at a relative humidity of 50% and above. This study showed a promising biological control alternative against *O. lybicus* as part of Integrated Pest Management (IPM).

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Chapter 1:

Chapter 1: Literature review

1.1 Date palm (*Phoenix dactylifera* L.)

Date palm (*Phoenix dactylifera* L.) is an important ancient crop for many arid-region countries around the world. Date palm is a monocotyledonous, perennial and dioecious species belonging to Arecaceae. Male and female date palms grow separately; males produce pollen and females bear fruits (Figure 1.1a & 1.1b). The production of date palm fruits has increased sharply from 1.5 million tons in 1950 to 359 million tons in 2018, and expected an increase of approximately 500 million tons by 2025 due to high demand, at a growth rate of 5% per annum (4). Palms also offer a suitable microclimate for other vegetation, especially in the arid and semi-arid areas, which in turn increases the income of the farmers (5). Date palm fruits are rich in natural fibre and a suitable replacement for refined sugar. In addition, dates contain minerals and vitamins in sufficient quantities to prevent deficiency and have been shown to reduce heart disease, and to have anti-mutagenic and antioxidant properties (6). Date palm fruits contain carbohydrates characterised by 14 different fatty acids, proteins containing 23 amino acids, 15 diverse minerals and salts, high dietary fibre, and six vitamins (7). Assirey, (8) determined that date fruits contain high sugar content (71.2–81.4%) and low lipids (0.12–0.72%), protein concentration (1.7–4.7%).

The natural distribution of date palms has been altered substantially by human activities moving from Spain to India, Australia, Peru, and California and are also present in sub-Saharan Africa (9). The plantation areas in North African and Middle East countries comprise 60% of date palms grown in the world with

nearly 60 million date palms (10). Egypt, Iran, Algeria and Saudi Arabia are the leading world date palm producers (2) (Figure 1.2). Iraq harvested 242 632 ha of the world date palm area in 2014, followed by Algeria, Iran, and Saudi Arabia with a sum of 422 866 ha (2) (Figure 1.3). However, Egypt has high yields per unit area (Figures 1.2 & 1.3).



Figure 1.1 Date palms (*Phoenix dactylifera* L.), a) female date palm bearing fruits, b) Male date palm, source: (www.commonswikimedia.org)

In Oman, date palms have cultural, economic and religious significance as the major crop planted in the country. Date palms represent 82% of the total fruit crops cultivated in the Sultanate and constitute 49% of the total cultivated area. Oman is the eighth date palm producing country, with an annual production of more than 320000 metric tons (4.2% of the total world production) in 2014 (2).

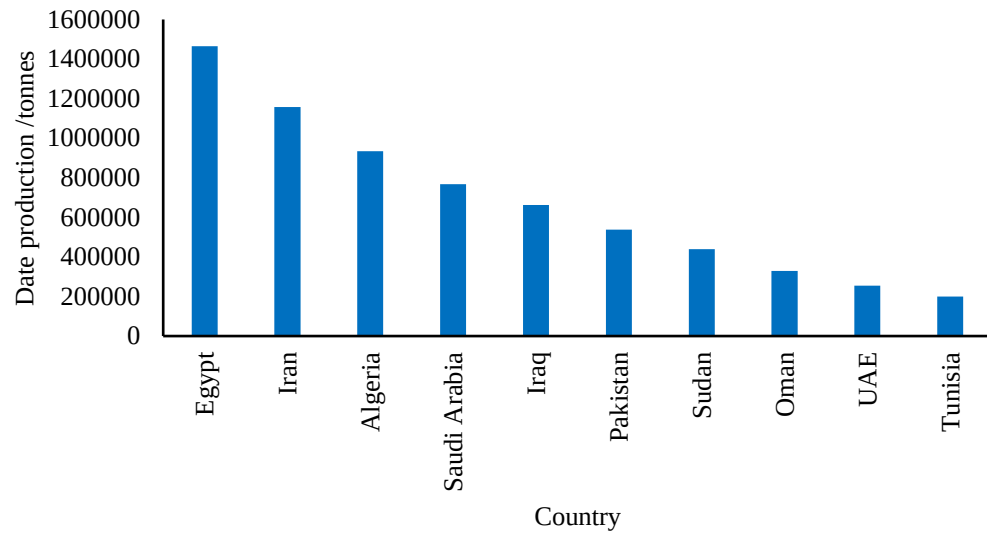


Figure 1.2 Date palm production in the first 10 leading countries (2).

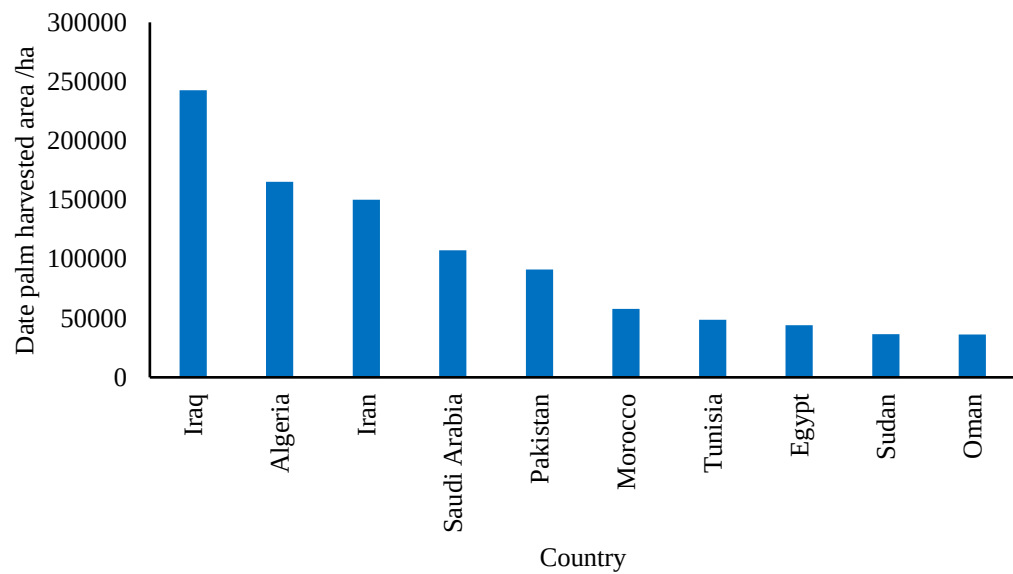


Figure 1.3 Date palm harvested area in the first 10 leading countries (2).

1.1.1 Genetic diversity in date palms

Date palm genetic improvement is essential to improve the resistance to increase tolerance to insects, diseases, abiotic stresses, and improve yield and fruit quality (11). In Iran, date palm cultivars Shahani and Khasi were reported to have resistance against *O. lybicus* by reducing the female fecundity up to 41 - 45% in

comparison to Zahdi cultivar (12). This finding could provide a genetic resource for breeding programs to obtain resistance varieties against *O. lybicus* infestation.

A huge genetic diversity is found in date palm (13), with around 3000 date palm cultivars found worldwide (5,14). Date palms fruits are drupe type of fruits exhibiting high variation in shape, colour, texture, and chemical composition (5).

Oman has a long season of date palm production, extends for seven months from May to November. This variability in climatic conditions, together with topography, and cultivation practices resulted in cultivar adaptation to different geographic regions in Oman. There are currently about 250 date palm cultivars grown in Oman (15).

1.1.2 Biotic and abiotic stress

Date palms are able to withstand severe abiotic stresses, including salinity, water stress, and hot and dry climatic conditions (5). In Oman, date production declined steadily due to an increased soil salinity in major date palm-growing regions (Al-Batinah and Al-Sharqia). Nutrient deficiency and water stress are other major limiting factors in date fruit production (16).

Biotic stress is another major challenge facing date palms. Many major pathogenic fungi are associated with date, such as, *Fusarium oxysporum* f. sp. *albedinis* (Foa) (17), *Alternaria arborescens*, *A. jacinthicola*, *A. slovacica*, *A. alternata*, *A. tomato*, and *A. burnsii* (18,19). Also, a number of destructive insect pests are associated with date palm, in which they are attacking fruits, fronds, and the main trunk. A comprehensive list of 16 major and 15 minor insect pests on the arthropod pest complex of date palm and their management was reported by Blumberg, (20). Worldwide, 112 species of mites and insects are associated

with date palm, including 22 species attacking stored date fruits (5,21). The major insect pest attacking the main trunk is red palm weevil *Rhynchophorus ferrugineus* Olivier, while the major pests attacking date fruits are lesser date moth *Batrachedra amydraula* Meyer and old-world date mite *Oligonychus afrasiaticus* McGregor, and date palm fronds are attacked by Dubas bug *Ommatissus lybicus* de Bergevin (22).

1.2 The Dubas bug *Ommatissus lybicus*

1.2.1 Palm damage and economic importance

The dubas bug, *Ommatissus lybicus* De Bergevin (Hemiptera: Tropiduchidae), is an invasive phloem-feeding insect that attacks date palms (*Phoenix dactylifera* L.) producing large volumes of honeydew. The substantial secretion of honeydew leads to the growth of the black sooty mould, *Meliola camellia* (23,24), and the accumulation of dust on the palm fronds reducing photosynthesis and reducing yield both qualitatively and quantitatively. Thus *O. lybicus* infestation results first in chlorotic frond surfaces (3,25). *Ommatissus lybicus* is not known to be a vector of pathogens (24,26,27). The pest is monophagous and limited to *P. dactylifera*. The females of *O. lybicus* cause the most physiological damage to the palm tissue by digging 0.4 to 0.5 mm deep tunnels into the green or soft tissue using a powerful chitinous saw-like tool laying one egg per tunnel (3,28,29). The oviposition causes scar necrosis on the fronds surface (3,26). Nymphs and adults directly and indirectly reduce palm productivity through sap draining and honeydew production, respectively. In Oman, (30) losses of up to 28% are estimated due to *O. lybicus* infestation on date palms. However, an economic loss of up to 50% due to *O. lybicus*

infestation has been recorded on date palms in Iraq (31), which could be one of the reasons in having lower yields per unit area (Figures 1.2 & 1.3). Five hundred and twenty-three tons of insecticides were used by The Ministry of Agriculture and Fisheries in Oman to combat *O. lybicus* infestation in date palm plantations through an aerial application from 1993 to 2010. The estimated cost of these applications was £18.5 million (3). Such inefficient insecticide control is challenging to estimate in cost because of negative effects on human health, non-targeted organisms, and the environment (3,32,33).

1.2.2 Taxonomy and distribution

Ommatissus lybicus De Bergevin belongs to the family Tropiduchidae, order Hemiptera and suborder Auchenorrhyncha commonly referred to as “Planthoppers”. Tropiduchidae distributed mainly in tropical regions and comprising of 330 species. Tropiduchidae is common in tropical forests and the genus *Ommatissus* is distributed in the eastern hemisphere. Most species of the family are greenish or yellowish to a brownish in colour and 5-7 mm long. Tropiduchidae have clear forewings, which are longer than their body. Unlike other palmivorous species of Auchenorrhyncha, which are associated with multiple palm species, the genus *Ommatissus* seems to be host-specific (26). *Ommatissus lybicus* is monophagous insect of *P. dactylifera* found in almost all date palm plantations grown in Oman (3,22,34), Pakistan (35), Iraq (36), Iran (37,38), Yemen (39), Tunisia, Algeria, Morocco, Egypt, Libya (40), United Arab Emirates, Saudi Arabia, Kuwait and Bahrain (3) (Figure 1.4).

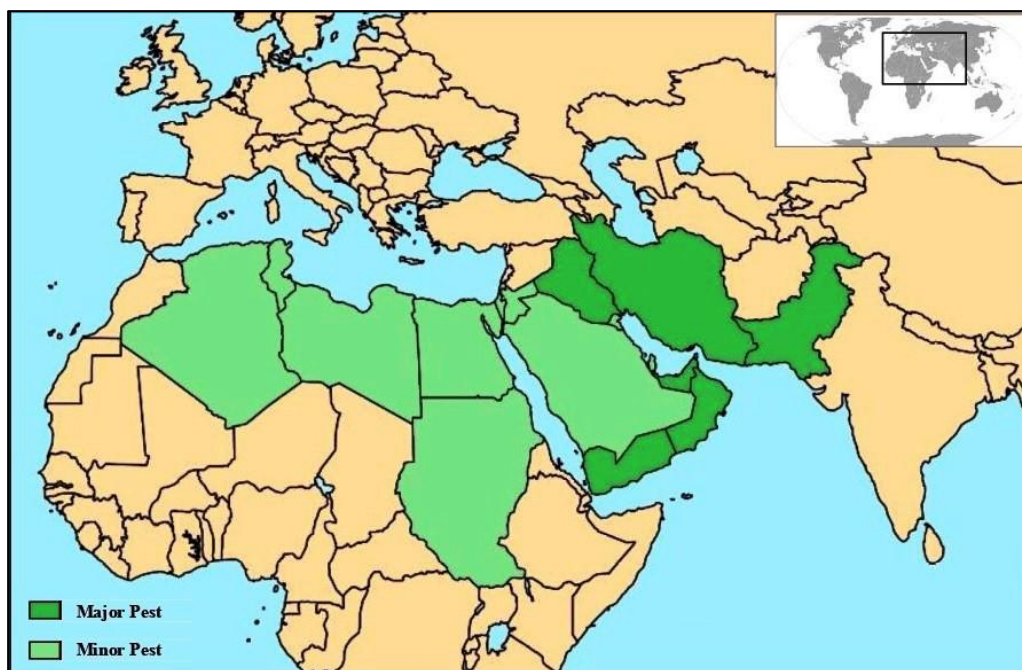


Figure 1.4 *Ommatissus lybicus* current global distribution (3).

1.2.3 Ecology and life cycle

Ommatissus lybicus undergoes hemimetabolous metamorphosis and passes three life stages in its life cycle: egg, nymph, and adult (Figure 1.5). *Ommatissus lybicus* develops from nymphs that resemble adults and undergoes five moults before metamorphosis. Following the fifth moult it obtains wings and genitalia (3). The insect is bivoltine (has two generations a year) with a generation in the spring (February - May) and in the autumn (August - November) (1,3,29,31,39,41–44).

Elwan and Al-Tamimi, (1999) studied the development of *O. lybicus* on date palm seedlings for two years (1995 -1996) under laboratory conditions (Table 1.1). The spring generation remained on the host for 161.9 and 155.6 days in 1995 and 1996, respectively. The nymphal stage lasted for 48.6 and 43 days in 1995 and 1996, respectively.

In the autumn generation, the insect lasted for 155.1 and 149.4 days in both years 1995 and 1996. The nymphal stage lasted for 32.7 and 34.8 days in 1995 and 1996, respectively (1).

Table 1.1 *Ommatissus lybicus* development on date palm seedlings for two years (1995 -1996) under laboratory conditions (1)

Stage	Generation	
	Spring	Autumn
	Duration/Days	
1	9.4	5.6
2	9.6	6.0
3	7.9	5.5
4	8.7	7.2
5	10.3	9.5
Adult male	51.2	64.8
Adult female	45.1	56.1
Pre-oviposition period	13.1	11.9
Oviposition period	27.8	37.1
Post-oviposition period	4.2	7.2
Egg incubation Period	99.9	106.7

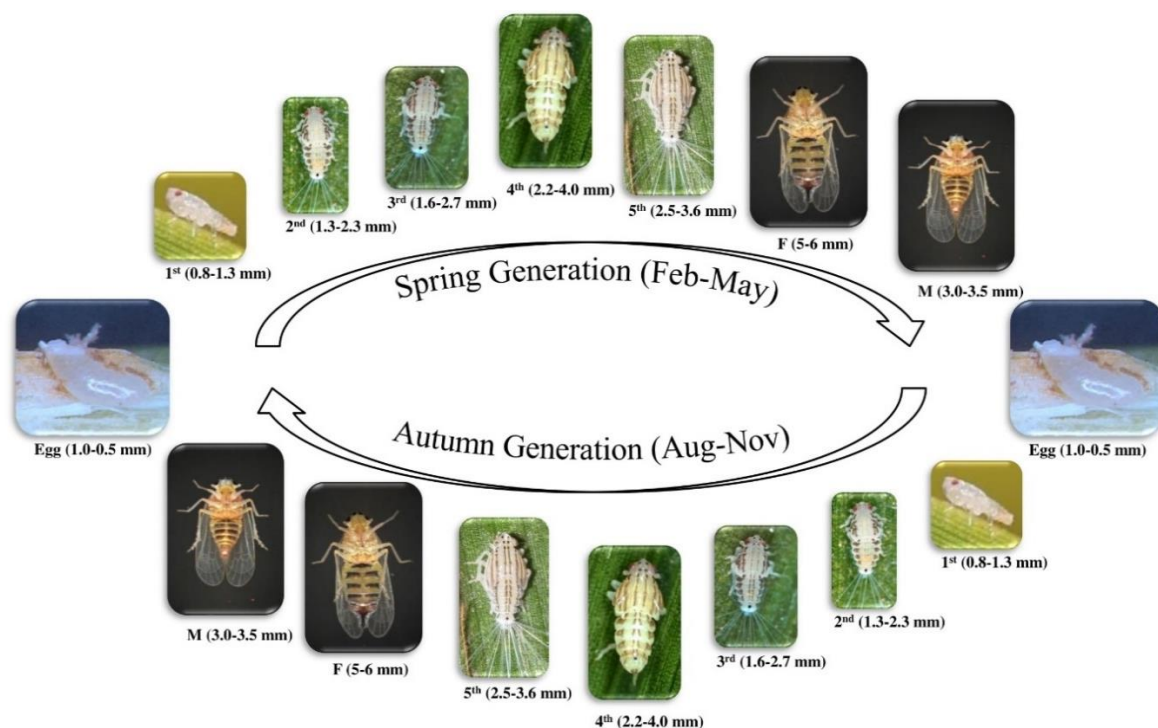


Figure 1.5 *Ommatissus lybicus* life-cycle from egg to adult stage, two generations per year (Bivoltine). In brackets (showing each stage size), F = Female, M = Male.

1.2.4 Morphology

Ommatissus lybicus adults are yellowish brown in colour and the wings are clear with concentrated venation at the apices. The female is longer (5-6 mm) than the male (3.0-3.5 mm) (Figure 1.5). The male wings are larger in proportion to the body size. Females are characterized by pairs of black spots at the frons base, head vertex, laterally on the pronotum and on the seventh and eighth abdominal segments (3,29,45,46). The adults can fly for a short distance when disturbed and jump covering a distance of about 12 times their length (47). The *O. lybicus* sex-ratio is 1:1 (3,29).

Ommatissus lybicus nymphs undergo five instars before reaching adult stage. The number of the waxy caudal filaments clusters are used to distinguish

between the five nymphal instars (3,29,42,47,48). Caudal filaments are used to initiate takeoff, and their numbers in each nymphal instar are consistent and increase after each nymphal moult (42). In addition, nymphs can jump for a short distance when disturbed (47). First nymphal instars (0.8 - 1.25 mm) are pale gray in colour (Figure 1.5). They are characterized by obvious dark patches on both sides of the thorax and abdomen segments. The first instars have no lines on the thorax and on the abdomen dorsal surface. Second nymphal instars (1.3 – 2.25 mm) have two lines of dark brown spots formed on the thorax and abdominal dorsal surface. Third nymphal instars (1.6 - 2.68 mm) are dorsal surface of the thorax and abdomen are characterized by two longitudinal lines. Fourth nymphal instars (2.18 - 4 mm) are characterized with three lines on both thorax sides (3). Fifth nymphal instars (Figure 1.6) (2.5 - 3.64 mm) are yellowish brown in colour. This instar is characterized by the coverage of the first wing bud on the second wing and the third abdomen segment (3). Eggs are bright green in colour when first oviposited and change to yellow-white and to bright yellow just before hatching (3,29,49).



Figure 1.6 The fifth instar of *Ommatissus lybicus* nymph

1.2.5 Methods of control for *O. lybicus*

1.2.5.1 Cultural control

Date palm plantation density plays an important role in pest reduction (42). Previous studies have indicated that dense plantation of date palms results in high humidity and reduced air flow that subsequently increase *O. lybicus* infestation (23,42). The recommended date palm density is 165 palms/ha (50), which provide good spacing between palms and provide good airflow between date palms and subsequently would provide unsuitable environment for *O. lybicus* infestation. *Ommatissus lybicus* oviposit 64% of their eggs on the 1st and 2nd frond rows (51). Pruning the lower old fronds of date palms can also help to reduce the pest population in the next season (42).

1.2.5.2 Chemical control

Chemical control remains the basic measure taken to control *O. lybicus* infestation on date palm plantations. In Oman, the chemical applications against *O. lybicus* are carried out using ground and aerial spraying (Figure 1.7 & 1.8). Ground-based application achieves optimum coverage on young trees and offshoots grown under date palms and is used in spraying infested date palm grown in harsh mountainous areas where aerial application is not possible. However, ground-based applications have many disadvantages, including increased time to spray the targeted area. The chemical application should be achieved before emergence of *O. lybicus* adult stage to avoid egg oviposition (52,53). Furthermore, most of the ground sprayers cannot reach tall palms of more than 15 m.

Chemical control of *O. lybicus* through aerial spraying is faster than the ground-based application with greater area covered. However, this type of application is not efficient in dense date palm plantations, in which fewer insecticide droplets can penetrate underneath the foliage. Aerial spraying is more difficult to perform in harsh mountainous areas, and has high pesticide drift (22). Several insecticides have been evaluated for their effectiveness to control *O. lybicus* in Oman. Insecticides from different chemical groups such as pyrethroids and organophosphates can be applied using aerial application for the control of *O. lybicus*. This application is typically performed once a year by air for autumn generation control, whilst the ground application can be carried out twice a year to control the autumn and spring pest generations. Insecticides used include deltamethrin (Decis® 12.5% ULV), malathion (Malathion® 96% ULV), dichlorvos (Nogos® 50 EC), fenitrothion 1% and esfenvalerate 49%

(Somicomdi Alfa® 50% ULV), fenitrothion (Somithion 99% ULV), and etofenprox (Treon® 30 ULV) (3,53,54). Insecticides including deltamethrin (Decis® 25 EC), phenthoate (Elsan 50 EC), dichlorvos (Nogos 50 EC), esfenvalerate (Somi Alfa® 5% EC), and etofenprox (Treon® 20 EC) (54) have also been used in ground-based applications with high pressure sprayers.



Figure 1.7 Ground chemical spraying against *O. lybicus* using high pressure sprayers



Figure 1.8 Aerial chemical spraying against *O. lybicus* using helicopter

1.2.5.3 Biological control

Natural enemies are capable of balancing natural ecosystems through efficient regulation of insect pest populations (55). Biological control utilizes living organism to reduce the fitness and density of another organism on a population level for the control of diseases, animals, and weeds (56). It is considered as the sustainable element of pest management in contrast to chemical control (57). Biological control is an important component of integrated pest management (IPM) that has been implemented successfully to suppress several key insect pests.

In Oman, several natural enemies have been identified as preying or parasitizing *O. lybicus*. Beg et al. (58) carried a field survey for natural enemies of *O. lybicus* in Oman in which five invertebrate species were identified. The identified species obtained during the survey were two predatory insects *Cheilomenes sexmaculata* (Fab.) (Coccinellidae, Coleoptera) (Figure 1.9) and *Chrysoperla carnea* (Stephens) (Chrysopidae:Neuroptera), one predatory spider (*Runcinia* sp. (Thomisidae, Araneae)), and one parasitoid (*Aprostocetus* sp. (Hymenoptera: Eulophidae)) (58). Other natural enemies were also recorded *Bocchus hyalinus* (Hymenoptera: Dryinidae) (59,60) and the egg parasitoid *Pseudoligosita babylonica* (Figure 1.10) (54). The *O. lybicus* egg parasitoid *P. babylonica* is considered a potential biological control agent showing more than 70% egg parasitism in some locations in Oman (3,61,62) This parasitoid was first identified by The Natural History Museum as *Oligosita* sp., however, it was then confirmed as *P. babylonica* by G. Viggiani (3). Al-Khatiri. et al. (61) studied the distribution of the *P. babylonica* in Oman. The survey revealed a diverse distribution of the parasitoid from one village to another in Oman. The highest

parasitism was recorded in Ghyadhah village in A'Sharqiyyah Governorate. A mean parasitism rate of 20.9% *P. babylonica* against *O. lybicus* eggs was recorded in Oman (3).



Figure 1.9 *Coccinellida* sp. preying on *O. lybicus*



Figure 1.10 *Pseudoligosita babylonica* Viggiani, an egg parasitoid of *O. lybicus* (3).

1.2.5.4 Entomopathogenic Fungi

Biological control of crop and forestry pests using entomopathogenic fungi has been widely investigated (63–68). Government regulators classify entomopathogenic fungi as posing a low risk to beneficial organisms including parasitoid wasps, earthworms, and bees and they also present low human safety concerns (69,70). Fungal species of *Metarhizium* (Figure 1.11a), *Beauveria*, *Isaria*, and *Lecanicillium* are most commercially utilised because they are relatively easy to mass produce (71).

Entomopathogenic fungi (EPF) specialize in direct penetration of the insect cuticle followed by development in the hemocoel. EPF play an important role in insect pest population control (66,72). Out of approximately 100,000 described fungal species in the world (73), about 750-1000 are entomopathogenic fungi placed in over 100 genera (67,74–77). These fungi have the ability to infect and kill arthropods and belong to the phyla Ascomycota, Basidiomycota, Chytridiomycota, Entomophthoromycota, and Microsporidia (78).

Entomopathogenic fungi differ in infection strategy to viruses and bacteria as they do not need to be ingested to cause disease (79–81). The infection takes place when a fungal spore adheres to the insect cuticle and germinates under favourable conditions. The conidial germ tube penetrates the host cuticle through a combination of mechanical force and cuticle-degrading enzymes (82,83), including amino peptidases and metalloproteases (84). The fungus produces new spores within and onto the infected host followed by dispersal to new hosts (85,86). Bassi, (87) was the first to recognize a fungal pathogen causing disease on the silkworm *Bombyx mori*, named later *Beauveria bassiana* (Figure 1.11b).

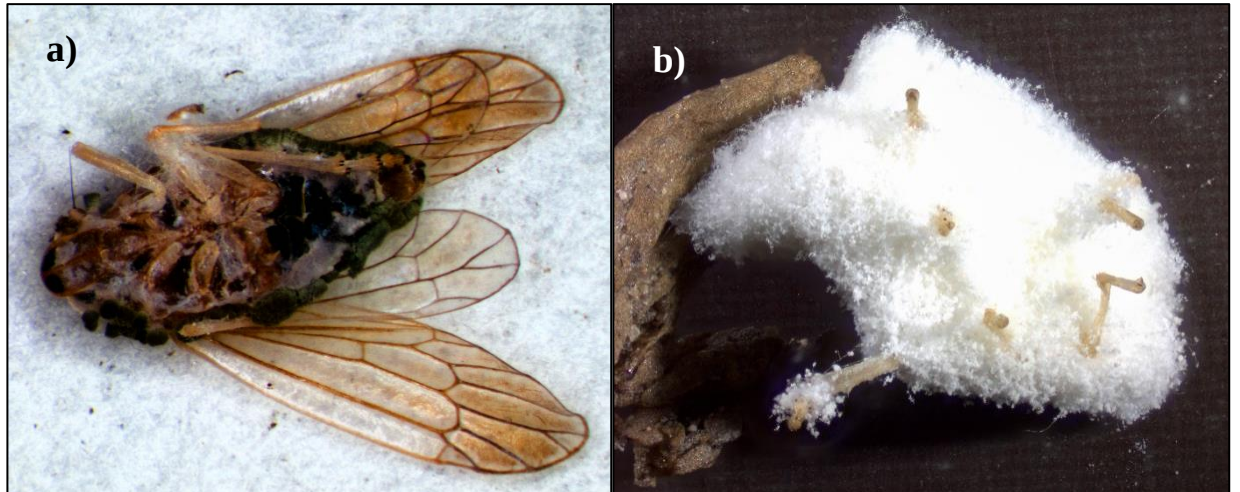


Figure 1.11 *Ommatissus lybicus* infected with **a)** *M. anisopliae*, **b)** *B. bassiana*

Aim of the study:

The overall aim of this study was to determine the effects of *O. lybicus* infestation on date palm growth and identify potential biological control agents to use against this pest.

The main objectives of this study were to:

Chapter 2:

- a) Determine the impact of different population densities of *O. lybicus* on chlorophyll, nutritional content, and final biomass of date palms.
- b) Define the relationships between different populations of *O. lybicus* and number of oviposited eggs or honeydew droplets on the palm host.

Chapter 3:

- c) Isolate native EPF from local environments, define their phylogenetic relatedness and determine their optimum conditions for growth and sporulation.
- d) Evaluate and demonstrate the infection patterns of entomopathogenic candidate strains against adults of *O. lybicus* using scanning electron microscopy (SEM).

Chapter 4:

- e) Determine the pathogenicity and aggressiveness of native and commercially available EPF against *O. lybicus* eggs, nymphs and adults.
- f) Determine the effect of relative humidity on the performance of native isolates against *O. lybicus* adults.

Hypothesis:

- a) There is a strong correlation between the *O. lybicus* population densities feeding on date palms and chlorophyll, nutritional content, and final biomass of date palms.
- b) There is a negative relationship between the different populations of *O. lybicus* and the number of oviposited eggs or honeydew droplets on the palm host.
- c) EPF isolates will be obtained from the *O. lybicus* environment.
- d) Temperature does affect spore germination, vegetative growth and spore production of native EPF isolates.
- e) The infection patterns of entomopathogenic candidate strains against adults of *O. lybicus* will be evaluated and demonstrated using scanning electron microscopy (SEM).
- f) The native commercially available EPF isolates will negatively affect the population growth of *O. lybicus* eggs, nymphs and adults.
- g) The relative humidity will positively increase the performance of the native EPF isolates against *O. lybicus* adults.

Chapter 2:

Chapter 2: Impact of initial population density of the Dubas bug, *Ommatissus lybicus* (Hemiptera: Tropiduchidae), on oviposition behaviour, chlorophyll, biomass and nutritional response of Date Palm (*Phoenix dactylifera*)

Abstract

The Dubas bug (*Ommatissus lybicus*) is an economically significant pest of date palm. In this study, the effect of population density of *O. lybicus* on chlorophyll, measured by the soil plant analysis development (SPAD) chlorophyll meter, palm biomass, and the nutritional composition of date palms was investigated. Another objective was to determine if there was any significant relationships between population density of *O. lybicus*, number of honeydew droplets and number of oviposited eggs. Reductions of up to 40% and 29-34% in chlorophyll content and plant biomass, respectively were caused by infestations exceeding 300 nymphs per palm seedling. Increasing population density of *O. lybicus* to 600 insects per palm decreased oviposition by females suggesting intraspecific competition for resources. There were significant relationships between honeydew droplets, produced by the pest population and chlorophyll content or eggs oviposited in the rachis suggesting that treatment can be triggered at 3-6 nymphs/leaflet. Egg oviposition was preferentially on the rachis and was predicted from honeydew produced by the adults. Ca, Mg, K, and P were the main nutrients significantly reduced by the activity of the pest. Mg content was associated with reduced chlorophyll content under increasing pest density

suggesting that supplemental nutrition can be potentially utilized to sustain chlorophyll and increase palm tolerance to pest infestation.

2.1 Introduction

Date palms (*Phoenix dactylifera* L.) are an economically important crop in the arid and semi-arid regions of the Middle East and North Africa (3,88) as dates are a rich source of sugars, containing 72% - 88% sugar content (89), and natural fibre (90) for human nutrition. The area under date palm plantations in North African and Middle East countries comprises 60% of the global cultivation with nearly 60 million date palms (5).

The Dubas bug (*Ommatissus lybicus* de Bergevin) (Hemiptera: Tropiduchidae) (Figure 2.1) is a phloem-feeding insect-pest characterised by modified mouthparts containing needle-like stylets to allow feeding through piercing date palm leaf tissue (91–93).



Figure 2.1 Dubas bug (*Ommatissus lybicus* de Bergevin) nymphs and adults on date palm.

Feeding disrupts photoassimilate flow and hinders phloem transport by interfering with sugar and nitrogen delivery to cells, as in the case of aphids (91,94). Endophytic oviposition, probing and salivation during feeding result in anatomical changes and mechanical injury to palm tissues (95,96). Severe insect infestations can cause up to 50% date palm yield loss (25,97,98) and are associated with significant reductions in fruit size, sweetness and delayed date ripening (29,52,99,100). As a bivoltine insect, *O. lybicus* has two generations a year, a spring generation (February to May) and an autumn generation (August to November) (3,44). The insect has five nymphal instars and can live after hatching for up to 155 days in the autumn and 162 days in the spring (1). The economic threshold of at least one nymph of *O. lybicus* per leaflet, determined by Arbabtafti et al. (101), has been shown inconsistent as was significantly influenced by geographical location, date palm variety and environmental conditions.

Ommatissus lybicus ingests large amounts of sugar-rich palm sap to obtain amino acids required for growth and reproduction and excretes the surplus sugars (102). The substantial secretion of honeydew results in the growth of the black sooty mould, *Meliola camellia* (23,44,103–105) on fronds contributing to the accumulation of dust on palm fronds and the intercropped plantation below (3). Honeydew is a water-soluble complex comprising of carbohydrates (mainly sucrose, glucose, fructose and others), amides, amino acids, alcohol, salts, and auxins (106–109). Honeydew droplets secreted by *O. lybicus* can be positively related to insect numbers on the host, and potentially utilised to estimate size of populations without the need to manually count insects on fronds or leaflets (25).

Insect population growth can be significantly impacted by intraspecific competition (110,111) whereby a population density that cannot be nutritionally supported by the host is regulated by a proportional decrease in reproduction rate and/or an increase in mortality or emigration (111,112). The effect of intraspecific competition is reported to affect fecundity in many species (113–118). For example, Luft et al. (119) demonstrated that the average number of oviposited eggs by eugenia psyllid *Trioza eugeniae* Froggatt females on Magenta Lilly Pilly *Syzygium paniculatum* Gaertn leaves was significantly decreased when insect population increased beyond three insects per leaf (119). Shah et al. (35) reported that morphological and physiological characteristics of date palm fronds (leaflets thickness, chlorophyll, and frond position) failed to affect the oviposition behaviour of *O. lybicus* females, however, no previous studies have been conducted to address the effect of different population densities of *O. lybicus* on fecundity of the pest.

Phloem-sap feeding insects reduce plant chlorophyll (120–122) and Shah (24) previously associated significant chlorophyll reduction of date palm leaflets with 30 insects/leaflet. Numerous studies have indicated a significant impact of plant phloem-feeders on plant biomass (123–126), nutritional response (127,128), and photosynthetic rate of their hosts (129). However, no studies have investigated the feeding effects of *O. lybicus* on the nutritional status of date palms. The objectives of this study were thus to: i) define the relationship between different populations of *O. lybicus* and number of eggs oviposited on the palm host, ii) define the relationship between different populations of *O. lybicus* and number of honeydew droplets deposited on the palm host, and iii) determine the impact

of different population densities of *O. lybicus* on chlorophyll, nutritional content, and final biomass of date palms.

2.2 Materials and Methods

Culturing *O. lybicus* and all experiments were performed in the Entomology Laboratory of the Plant Protection Research Centre, Directorate General of Agricultural and Livestock Research, Al-Rumais, the Sultanate of Oman.

2.2.1 2.1. Insects and experimental procedures

The experiments were conducted in an insect growth room with artificial climate control and pre-set conditions of $25\pm 2^{\circ}\text{C}$ with 12:12 hours photoperiod (3). Fluorescent lighting tubes (GE Starcoat T5 f28w/865 (6500K)) with automated timers were used in the room for photoperiod maintenance. Two-years-old, tissue culture-derived date palms, cultivar Khalas, were used as host plants. Khalas was selected due to being the most widely grown variety in Oman (130). The palms were planted in pots (8 L) containing peat moss as a growing medium and were confined inside well-ventilated insect-proof net cages (W 45.5 cm x L 60 cm x H 90 cm). The experiment was designed as randomized complete block design and consisted of five treatments: control (no insects), 100, 300, 600 or 1000 *O. lybicus* individuals per palm. Treatments were chosen to achieve low (approx. 1 insect/leaflet), medium (3 insects/leaflet), high (6 insects/leaflet) or extremely high (10 insects/leaflet) population densities based on infestation categorization used previously by Al-Khatiri (3) calculated per palm with an average of 5 fronds and 20 leaflets as used in our experiments.

Treatments had four replicates, and each caged pot was considered a single experimental unit. Immature *O. lybicus* (1st and 2nd instars) were collected from

an infested date palm orchard and used for the experiments (23°21'20.9"N 57°38'09.0"E). The palms received 20:20:20+TE NPK fertilizer two weeks before releasing the insects and were irrigated evenly twice a week. All experiments were repeated twice.

2.2.2 Chlorophyll measurement

The chlorophyll of palm seedlings was measured using a SPAD-502 plus chlorophyll meter (Konica Minolta Sensing, Inc. Japan) (131–135). Three SPAD readings were taken on ten leaflets of each frond per palm for eleven weeks following insect release. The mean SPAD reading per experimental unit (palm) was used for chlorophyll analysis.

2.2.3 Honeydew droplets count

Yellow, water-sensitive papers, (26 mm x 76 mm) were used to mark the honeydew droplets secreted by *O. lybicus*. Four papers were placed in four different directions under each palm for two hours (from 8 am to 10 am) (100). Honeydew droplet visual counts were carried out once a week over eleven weeks following insect release in the cages.

2.2.4 *Ommatissus lybicus* oviposition density

After obtaining the last set of chlorophyll and honeydew data, *O. lybicus* egg numbers oviposited on all leaflets and rachises on each date palm were counted to assess the oviposition density in different treatments. Palm tissues were examined with a stereoscope ZEISS SteREO Discovery. V20, (Carl Zeiss Microscopy GmbH, Germany). Oviposition density was determined by dividing the number of oviposited eggs by the palm surface area.

2.2.5 Plant area and biomass measurements

After procuring the last data set at eleven weeks following insect release, date palms were removed from the growing containers and washed with water to remove the peat moss from the roots and left to dry for one hour. Date palms were then separated into three parts (leaflets, roots, and rachis) to measure their fresh weight. Leaflets were scanned using CANON, Canoscan LIDE120 Flatbed Scanner, and a known reference scale (10 cm ruler) was added for further image analysis. Image J (<https://imagej.nih.gov/ij/>) was used to process the scanned images and obtain the leaflets area. Rachis surface area was calculated as a cylinder by measuring the length of the rachis and the average radius of both ends. Measured individual rachises were then summed to obtain surface area estimates (136–138).

$$A=2\pi rh$$

Where A, r, h and π denote area, radius, height and Pi (3.141592), respectively.

Each biomass partition was placed into pre-heated (70°C for one hour to avoid moisture intrusion) paper bags and weighed individually using a digital sensitive balance Kern EG 220-3NM (Kern and Sohn, Germany). The palm tissues were dried using an air oven (Binder GmbH, Tuttlingen, Germany) set at 70°C for three days and were immediately weighed again. The biomass data are presented as dry weight and fresh weight.

2.2.6 Plant nutritional measurements

Palm tissues were used to investigate nutritional variation between infested and non-infested plants. An initial screen of samples of date palms infested with 1000

O. lybicus individuals and the control palms (no infestation) was first performed to determine if any nutrients were affected by extremely high infestation with *O. lybicus*. Nitrogen (N), phosphorus (P), potassium (K), boron (B), calcium (Ca), copper (Cu), iron (Fe), manganese (Mn), molybdenum (Mo), sodium (Na), sulphur (S), zinc (Zn), and magnesium (Mg) were measured as described by Estefan et al. (139). The analyses were carried out in Global Laboratories and Testing LLC., Sultanate of Oman. Rachises, leaflets, and roots were separated, oven-dried at 60°C overnight and mechanically ground into a fine powder using a mortar grinder (RM200, Retsch, Germany). Nitrogen and phosphorous were analysed by digesting 0.5 g of the dried material in a 100ml digestion tube containing 3-4 anti-bumping granules, 3 g catalyst mixtures, and 10 ml concentrated H₂SO₄ mixed well using vortex tube stirrer. The mixture was first heated at 100°C for 20 minutes in a block-digester, agitated thoroughly and then heated at 380°C for two hours. Following digestion, the mixture was cooled down, made to 100 ml volume by adding distilled water and filtered using Whatman filter no. 42 (Whatman Ltd, UK). Nitrogen was analysed using Kjeltac 2400 distillation unit (Foss Analytical, Denmark). Ammonium-vanadomolybdate (10 ml) was added to 10 ml of the fluid into a 100 ml flask and diluted with distilled water to 100 ml. P concentration was then determined using a photo spectrometer (Shimadzu UV-1800, Japan) at 410 nm wavelength. A set of external standards diluted from 1000 ppm standard (Romil, UK) was used to determine phosphorus linear calibration curve (139). The other elements (K, Na, B, Ca, Cu, Fe, Mn, Mo, S, Zn, and Mg) were analysed using the dry ash method. Ground plant materials were weighed (1 g) into 50 ml porcelain crucibles and heated in the furnace to 550°C for 5 hours. After heating, the

crucible was allowed to cool and 5 mL portions of 2 N HCl were added to the ash before mixing thoroughly. After 20 minutes, distilled water was added to make 50 ml before filtering the mixture after 30 minutes using Whatman filter no. 42. K and Na were analysed using a flame photometer (Jenway PFP7, UK). The remaining elements (B, Ca, Cu, Fe, Mn, Mo, S, Zn, and Mg) were analysed using inductively coupled plasma (ICP) spectroscopy (ICPE-9000, Shimadzu, Japan) (139). Nutrient analysis as described above was subsequently performed on all samples from the first and the second experiments to confirm the main nutrients affected by different levels of insect infestation.

2.2.7 Statistical analysis

Statistical analysis was performed using Genstat 22nd edition. Experiments were repeated twice, and the experiment was included as a factor in the analysis to test for significant interactions with time of measurements or treatments. Chlorophyll, honeydew droplets, and oviposited eggs were analysed using generalized linear mixed model for binomial distribution with link function of logit transformation that included treatment (insect density) and time of measurement (weeks following infestation) for the first two variables. Poisson distribution with link function of logarithm was used to model honeydew and egg count data. Fisher's least significant difference at $p < 0.05$ was used to compare means for egg oviposition sites in experiments. Plant nutritional values for the initial screen comparing the control (no infestation) and infestation of 1000 insects were analysed using a two-tailed t-test at 95% confidence level. Plant nutritional values and biomass for the full experimental treatments were analysed using analysis of variance (ANOVA) and the least significant difference at $p < 0.05$ was used to compare means between treatments. Single or

multiple linear regression with groups for insect density were used to predict chlorophyll, rachis fresh weight and egg oviposition on the rachis. Regression models tested if data fitted on common, parallel, or separate lines for treatment groups before final model selection at $p < 0.05$. Explanatory variables (that were not correlated) were included for multiple linear regression analysis.

2.3 Results

2.3.1 Loss of chlorophyll

Generalized linear mixed model analysis revealed significant interaction ($p < 0.001$) between time of measurement (weeks) and treatment (population density) for chlorophyll measurements (Figure 2.2).

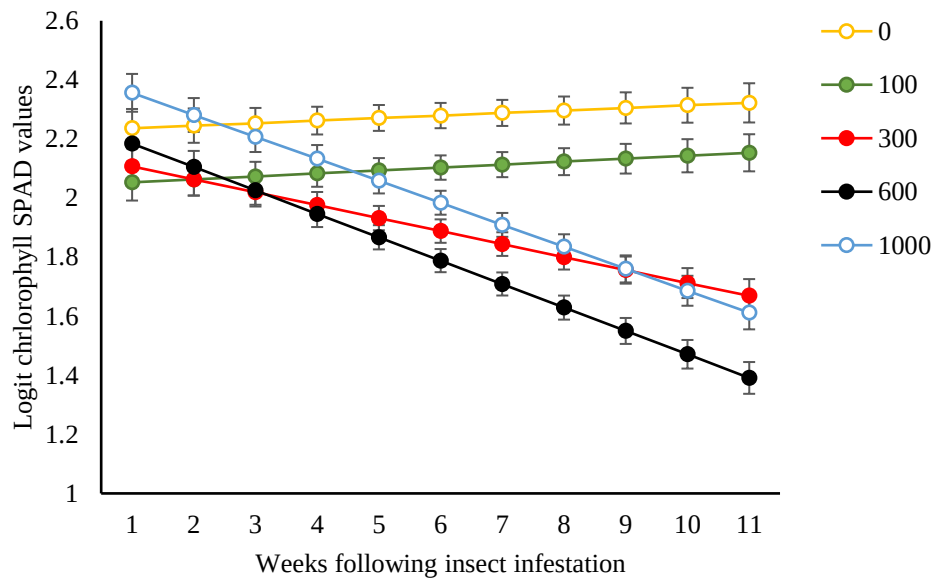


Figure 2.2 Effect of initial density of *O. lybicus* on chlorophyll measured by SPAD meter on date palm leaflets in the period of eleven weeks following insect release. Predicted means with standard error bars were derived by Generalized linear mixed model analysis for Binomial distribution with link function of logit transformation ($p < 0.001$).

A significant chlorophyll loss occurred in response to an increasing population beyond 300 *O. lybicus* per palm from week five onwards. The population of 600 caused the highest SPAD loss of 40% followed by 31% and 28% for densities of 1000 and 300 insects, respectively (Figure 2.2).

2.3.2 Honeydew droplets

Significant interaction between time and population density was observed for honeydew droplet secretion ($p < 0.001$, Figure 2.3).

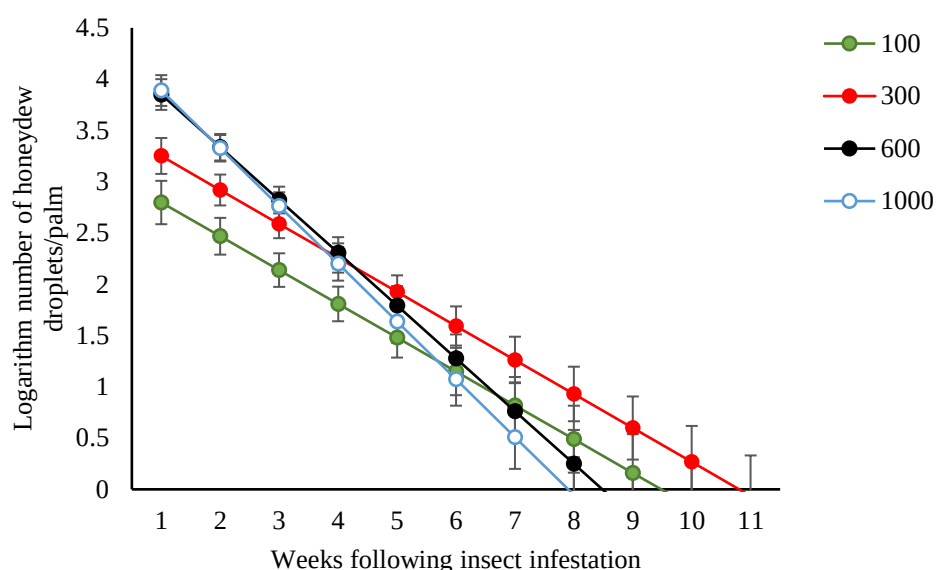


Figure 2.3 Honeydew secretion (square root transformed number of droplets/palm) by different initial populations of *O. lybicus* in the period of eleven weeks following insect release. Predicted means with standard error bars are shown from Generalized linear mixed model analysis for Poisson distribution with link function of logarithm ($p < 0.001$).

Honeydew secretion increased with population density reaching a plateau at 600 insects in the first two weeks of insect infestation (Figure 2.3). Honeydew production at a density of 300 *O. lybicus* remained significantly higher than any

other population at weeks 5-11 following insect release. Increasing insect density to 600-1000 individuals per palm resulted in the a significant reduction in honeydew being produced compared to lower population densities following week 6 (Figure 2.3).

2.3.3 *Ommatissus lybicus* oviposition

There was a significant interaction between oviposition site and experiment for numbers of oviposited eggs ($p < 0.001$, Figure 2.4). Irrespective of population density, *O. lybicus* oviposited preferentially on the rachis rather than on palm leaflets. However, there were 18% more eggs being oviposited in the rachis but less eggs in the leaflets in the first experiment compared to the second experiment.

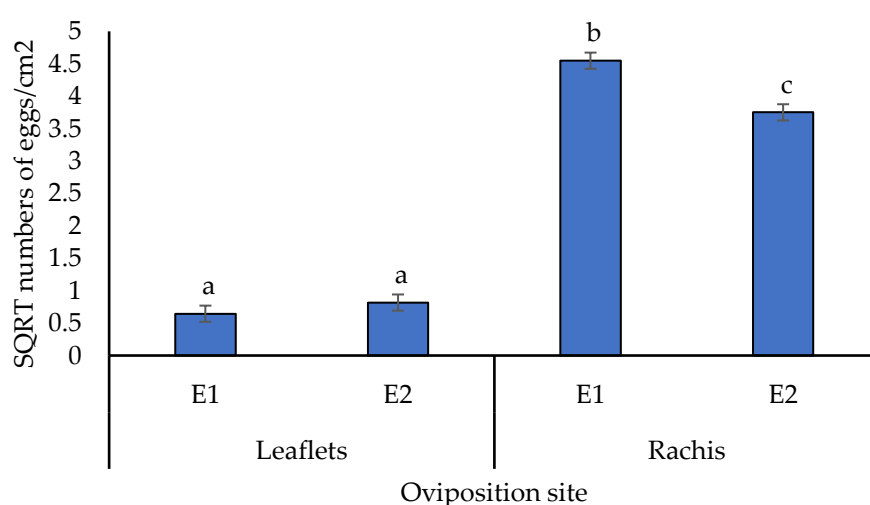


Figure 2.4 Oviposition site preference (square root transformed number of eggs cm⁻² of rachis or leaflet) of *O. lybicus* in experiments (E). Predicted means with standard error bars were derived by general linear regression analysis for Poisson distribution with link function of square root ($p < 0.001$). Fisher's least significant difference test was used to compare between means.

A significant interaction between experiment and population density was also observed for number of oviposited eggs in the rachis ($p < 0.001$, Figure 2.5). The highest egg oviposition on the rachis at the end of both experiments was made by 300 insects (mean of 5.2 and 4.4 eggs/cm² for the 1st and 2nd experiment respectively), which declined as *O. lybicus* population increased to 600 and 1000 insects (Figure 2.5). Although trends were similar for both experiments, number of oviposited eggs by 100 and 300 insects were not significantly different in the first experiment.

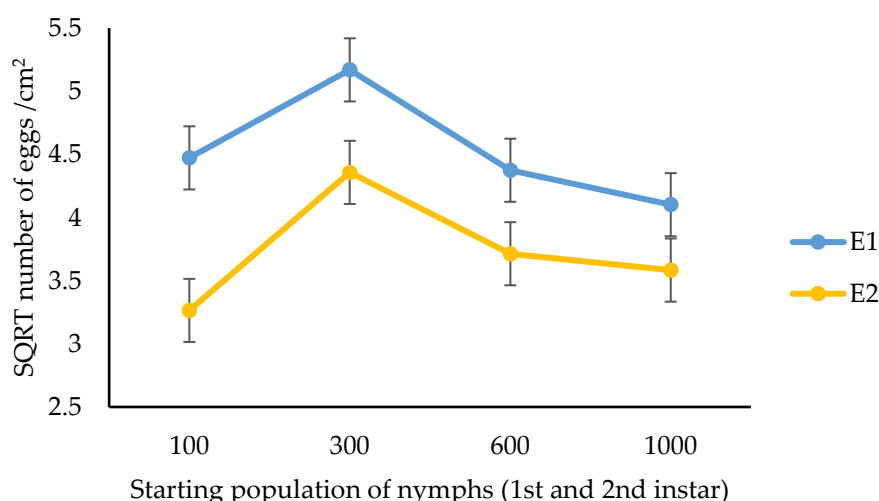


Figure 2.5 Oviposition (square root transformed number of eggs cm⁻²) on the rachis of palms by different populations of *O. lybicus* in the two experiments (E). Predicted means with standard error bars were derived by general linear regression analysis for Poisson distribution with link function of square root ($p < 0.001$).

2.3.4 Plant biomass

Fresh weight of leaflets was significantly reduced by 29% and 22% by insect infestations of 300 or 600 *O. lybicus*, respectively (Table 2.1). Infestations beyond 300 insects also caused up to 34% reduction in the fresh biomass of the

rachis compared with the control. Roots fresh weight was reduced by insect populations exceeding 100 individuals with 600 insects per palm reducing root weight by 34%. There were no significant differences detected for dry weight of leaflets or rachis. The highest root dry weight reduction of 32% was caused by the population density of 300 insects in the first experiment, however in the second experiment the population density of 600 insects reduced root dry weight by 28% compared to the control (no insects). There were no significant interactions between experiment and treatment for plant biomass of the rachis, leaflets and roots, however fresh and dry biomass of roots was significantly higher in the second experiments compared to the first (Table 2.1).

Table 2.1 Fresh weight and dry weight of the rachis, leaflets, and roots of date palms. ANOVA and the least significant difference at $p < 0.05$ were used to compare means between treatments. T = Treatment, E = Experiment.

N nymphs	Fresh weight (g)				Dry weight (g)			
	Leaflets	Rachis	Roots		Leaflets	Rachis	Roots	
			E1	E2			E1	E2
0	53.4	87.1	51.4	91.8	16.4	16.1	9.6	22.3
100	50.2	73.0	38.1	104.7	15.7	15.8	11.7	28.6
300	37.9	57.2	33.7	69.2	13.7	13.2	6.5	16.8
600	41.6	60.7	36.3	57.6	15.4	14.5	10.8	16.0
1000	48.8	69.1	33.1	66.4	15.7	15.7	9.4	17.5
<i>p</i> -value (LSD)								
T	0.02 (9.9)	0.002 (14.2)	0.008 (17.2)		0.62 (3.5)	0.49 (3.8)	0.03 (5.2)	
E	0.2 (6.2)	0.27 (9.0)	<0.001 (10.9)		0.18 (2.2)	0.25 (2.4)	<0.001 (3.3)	
T x E	0.58 (14.0)	0.55 (20.0)	0.123 (24.3)		0.69 (5.0)	0.55 (5.4)	0.22 (7.4)	
CV%	20.7	19.9	28.8		22.3	24.5	34.2	

2.3.5 Nutritional measurements

The nutrition screen with 13 elements of N, P, K, B, Ca, Cu, Fe, Mn, Mo, Na, S, Zn, and Mg on material from palms exposed to 1000 insects or the control revealed that *O. lybicus* affected only Mg, P and K content in leaflets, rachises, or roots (Table 2.2).

Table 2.2 Effect of infestation of 1000 *O. lybicus* /palm on nutrient concentration of leaflets, rachises and roots of date palm (Khalas cv.). Only significantly different ($p < 0.05$) elements between populations are shown.

	Nutrient content (mg/kg of dry weight)											
	Leaflets				Rachises				Roots			
	0	1000	<i>P</i> -value	SE±	0	1000	<i>P</i> -value	SE±	0	1000	<i>P</i> -value	SE±
N nymphs												
Magnesium (Mg)	3.85	3.48	0.01	0.099	5.65	5.78	0.88	0.759	5.73	4.53	0.02	0.371
Phosphorus (P)	1.38	1.00	0.01	0.095	2.78	2.08	0.02	0.207	1.18	0.98	0.29	0.172
Potassium (K)	9.73	7.53	0.02	0.696	18.33	16.50	0.53	2.734	12.25	9.73	0.01	0.689

Nutrient analysis of all samples in experiments 1 and 2 confirmed that the concentration of the same elements as shown in the screen, in addition to Ca, was affected by insect infestation. Significant interactions between experiment and treatment were observed for K and Mg in the leaflets, and P in both the leaflets and the rachis (Figure 2.6).

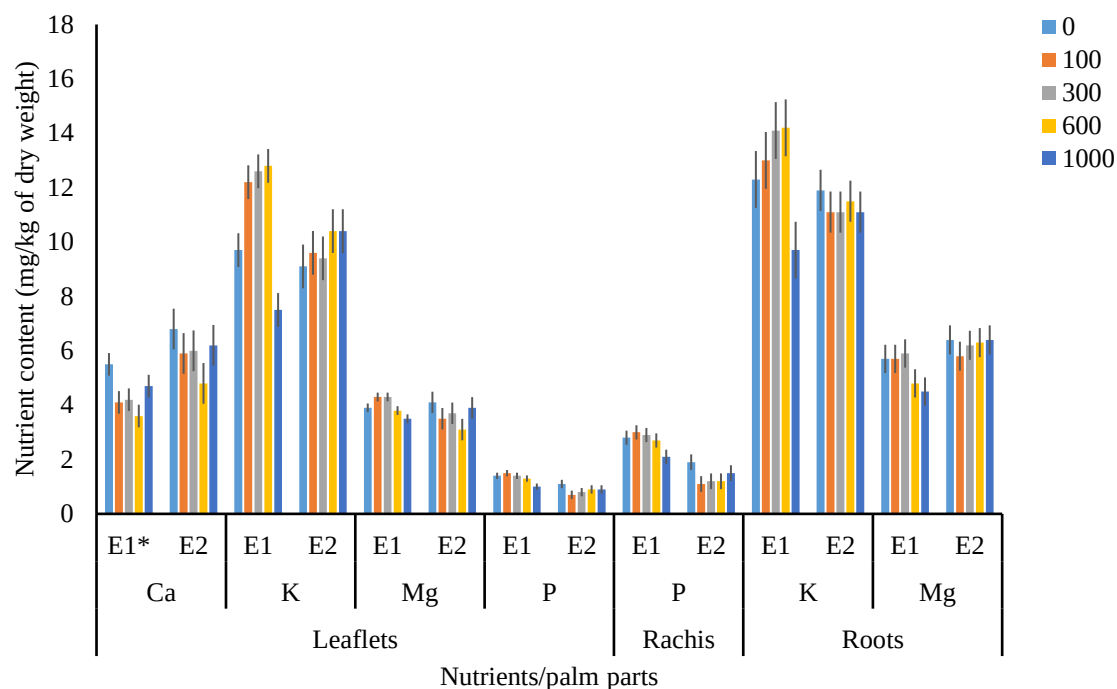


Figure 2.6 Effect of population density of *O. lybicus* (1st and 2nd instar of nymphs) on the concentration of nutrients in leaflets, rachis and roots of date palms (Khalas cv.). ANOVA and the least significant difference at $p < 0.05$ were used to compare means between treatments. E = Experiment. Only significantly different ($p < 0.05$) elements between populations are shown.

Ca content in leaflets was lower in the first experiment compared to experiment two with any infestation causing significant reduction compared to the control. The lowest Ca concentration of 3.6 mg/kg was quantified in palms with the starting population of 600 insects. K concentration in leaflets increased in both experiments with insect infestation of up to 600 individuals but in experiment two only 1000 *O. lybicus* reduced K compared to the control. In experiment one Mg in leaflets was not affected by insect infestation but was reduced by 100 and 600 insects in experiment two. The effect on P in leaflets by insect density was inconsistent in the two experiments, being reduced in experiment one by 1000

individuals whilst in experiment two reductions were observed at lower infestations by 100-300 insects. P in the rachis was reduced by any infestation in experiment two but only by 1000 individuals in experiment one. K in roots was significantly higher in experiment one and was reduced only by infestation with 1000 individuals. Mg in roots was significantly reduced by infestations, with greatest reductions observed when population increased beyond 600 *O. lybicus*.

2.3.6 Regression analyses

Simple linear regression analysis with insect populations as groups revealed that data for each population density fitted parallel lines for honeydew droplets in Week 1 and Mg content in leaflets explaining 48% and 62%, respectively, of the variance associated with chlorophyll content in week 11 ($p < 0.001$, Table 2.3).

Rachis fresh weight was related to eggs oviposition on the rachis accounting for 38% of the variance, with data fitting common line for *O. lybicus* population density ($p < 0.001$, Table 2.3). Variation in eggs oviposition on the rachis was related to honeydew droplets secretion in the ninth- and tenth weeks following infestation accounting for 36% of the variance with the data fitting common line for population density ($p < 0.001$, Table 2.3).

Table 2.3 Linear regression analysis of chlorophyll content after eleven weeks of *O. lybicus* infestation, rachis fresh weight and oviposited eggs in the rachis with honeydew secretions in the first week, magnesium (Mg) content in leaflets, egg oviposition and honeydew droplets in weeks 9 and 10, respectively. (HD1, HD9, and HD10) = (Honeydew 1st, ninth, and tenth week, respectively), (ER) = (Eggs oviposited in rachis).

Response variate (Y)	Equation	R ²	p-value
Chlorophyll content at 11 weeks	Y (100) = 66.38 - 0.305 (HD1)	0.48	<.001
	Y (300) = 62.28 - 0.305 (HD1)		
	Y (600) = 59.64 - 0.305 (HD1)		
	Y (1000) = 62.39 - 0.305 (HD1)		
Chlorophyll content at 11 weeks	Y (0) = 1.47 (Mg) + 60.36	0.62	<.001
	Y (100) = 1.47 (Mg) + 59.49		
	Y (300) = 1.47 (Mg) + 54.78		
	Y (600) = 1.47 (Mg) + 52.28		
	Y (1000) = 1.47 (Mg) + 54.55		
Eggs in rachis	Y = 2.91 + 0.487 (HD9) + 0.713 (HD10)	0.36	<.001
Rachis fresh weight	Y = 87.57 - 5.62 (ER)	0.38	<.001

2.4 Discussion

This study investigated the impact of different population levels of *O. lybicus* on honeydew excretion and egg oviposition and on chlorophyll, plant nutrients and biomass of date palm. The results revealed that the infestations by populations greater than 300 nymphs of *O. lybicus* caused significant chlorophyll reduction in leaflets associated with feeding and sugar drain over the 11 weeks of insect infestation. Several studies have previously reported negative effect of phloem feeding insects on the chlorophyll of different host plants. Rafi et al. (116) showed that probing by the Russian wheat aphid *Diuraphis noxia* Mordvilko caused a reduction in the total chlorophyll content by 18.2% at a density of 80 insects/plant on *Triticum aestivum* L. Similarly, Huang et al. (122) reported that initial population of five nymphs of the invasive mealybug *Phenacoccus solenopsis* Tinsley per plant caused 57% reduction in chlorophyll content after 38 days of feeding on leaves of *Solanum lycopersicum* L. Previously, Shah, (24)

exposed three date palm cultivars (Kehraba, Jan Sore, and Mozavati) to different initial *O. lybicus* population densities of 5, 10, 15, 20, 25 and 30 first instar nymphs per leaflet confined to micro cages of 1.9 cm diameter. His results revealed that the chlorophyll of palm leaflets was reduced significantly by 30 insects/leaflet in comparison with the control treatment and there were no differences at lower insect populations. In contrast to using individual leaflets by Shah(24), we studied the effects of insect populations confined to whole young palms. The effects of *O. lybicus* feeding on date palm seedlings were not immediate, and chlorophyll loss increased significantly beyond the first five weeks of sustained feeding causing a maximum loss of 40% with an optimum infestation of 600 insects, roughly equating to starting population of 6 insects per leaflet. This is in agreement with other authors (120,121,140) showing that adequate exposure time at an optimum infestation population was needed to cause significant chlorophyll content reductions by phloem-feeding insect pests. The time taken for metamorphosis of nymphs to adults is approximately 5 weeks for *O. lybicus* and it is clear from our results that chlorophyll loss of leaflets increased once nymphs morphed into adults on the palms. Increased loss in chlorophyll over time was associated with reductions in honeydew secretion by populations greater than 300 insects suggesting nutrient resource exhaustion caused by feeding of the nymphs and diminished ability of the host to recover. Our studies also indicated that date palms have the potential to tolerate the lower infestations of 100 nymphs for up to 11 weeks because at this population density losses remained lowest for the period of the studies.

Mechanical probing and salivation by *O. lybicus* are likely contributing to chlorophyll loss through feeding as shown with other insect-host systems (95).

Indeed, in our studies honeydew secretion in week one following insect infestation was negatively related to chlorophyll content on leaflets at the end of experimentation. However, endophytic oviposition by *O. lybicus* females utilizing a saw-like robust chitinous tool around its ovipositor to tear date palm tissue to deposit eggs at 0.4 to 0.5 mm deep in tunnels (3,29) causing acute tissue necrosis can potentially increase chlorophyll loss in time significantly. Shah et al. (35) showed a negative relationship between measured chlorophyll and the oviposited eggs in different frond rows in spring ($r=-0.85$) or autumn ($r=-0.77$). In the current study, *O. lybicus* females exhibited a preference to oviposit their eggs in rachises rather than in leaflets with a ratio of 2:1. This mechanism avoids rapid exhaustion of host assimilates as photosynthetically active leaflets are not being destructively used by females for oviposition but rather to support feeding of the developing nymphs of the next generation.

A decline in reproductive output due to direct intraspecific competition for food resources is often observed with increasing population density of insects and animals (113–115,117,118). Egg oviposition by *O. lybicus* on the rachis reached optimum with 300 individuals before decreasing at higher infestations and was significantly related to honeydew droplets at the end of experimentation (weeks 8 and 9) by the adults rather than to the initial nymph population density. This is likely to be associated with decreased availability and/or quality of nutrition from the host. Indeed, palms with infestation beyond 300 individuals sustained the greatest reductions of chlorophyll and biomass. Similarly, Rafi et al. (116) reported that high initial population density of the wheat aphid *D. noxia* caused a reduced reproduction rate in its wheat host, whilst Mamai et al. (115) showed

an adverse effect of increased adult density of *Anopheles arabiensis* Patton on egg production.

Previous studies by Mokhtar & Al-Mjeni (25) and Shah et al. (100) have reported associations between the density of *O. lybicus* and the amounts of honeydew droplets. However, our results suggested that honeydew deposits increased with initial population density of the nymphs only at the very first few weeks of infestation and subsequently honeydew deposition was best related to final egg production by the adults. Since there was a significant negative relationship between egg oviposition and rachis biomass, honeydew droplets can be utilized to aid predictions for likely pest damage in the following season. We note that the observed relationships with honeydew deposits in our studies were of moderate strength ($R^2=0.36-0.48$) suggesting that other factors, such as environmental conditions, for example, not accounted for here are also likely to be important. A significant reduction in fresh palm biomass of leaflets was observed in our studies when *O. lybicus* density increased between 300-600 individuals per palm. Similarly, rachis fresh biomass was reduced when populations increased beyond 100 individuals. We also found significant reductions in root fresh and dry biomass being greater in the first experiment than in the second and in response to infestations by 300-600 individuals. The observed differences in fresh biomass but not for dry biomass of leaflets and rachises suggested that insect infestation affected water content of palm tissues. This suggests that optimum insect feeding, and egg oviposition cause reductions in sugar flow to the roots via the phloem decreasing root biomass and thus affecting the overall water use of the palms.

Four nutrients involved in essential plant growth and photosynthesis, Mg, K, Ca, and P, were affected by infestation with *O. lybicus* on date palm leaflets. Calcium is an element used for plant tissue development, promoting cell elongation and strengthening of plant cell walls (141,142) in addition to being involved in signaling during defense against pests and pathogens (141). In our experiments Ca was consistently reduced in both experiments by insect infestation with the greatest reductions caused by 600 insects which also caused significant decrease in chlorophyll of the leaflets. Phosphorous and magnesium play essential roles in plant energy transfer and biochemical processes including chlorophyll synthesis, respiration and photosynthesis (143,144). Potassium is also needed for photosynthesis, phloem translocation of assimilates, and metabolism (145,146)-(147). However, in our studies we observed interactions between experiments and treatments for K, Mg and P in plant tissues suggesting that the effects of insect populations on nutrient content were inconsistent under our experimental conditions. The greater reduction of Mg and P in leaflets by increased insect density in the second experiment maybe the result of the increased oviposition of eggs in leaflets rather than in rachises in the second experiment compared to the first. However, of all analyzed elements only Mg concentration was strongly ($R^2=0.62$) related to chlorophyll content in leaflets of palms with different *O. lybicus* densities at the end of experimentation suggesting that Mg can significantly contribute to chlorophyll maintenance of date palms. Previous studies have indicated that Mg can contribute to disease resistance and plant yield (148,149). To maintain optimum growth (150,151) a date palm requires 1.5-3 kg, 0.5 kg, and 2-3 kg of NPK, respectively, in addition to organic manure of 20 - 44 kg per year (151,152). The finding that Mg contributes to chlorophyll

content under *O. lybicus* infestation suggests that supplementing Mg to conventional fertilizers can be exploited to reduce losses. Further understanding of the effects of targeted applications of Ca, K, and P to supplement nutrition for increased tolerance may also be useful to improve pest management in the field.

2.5 Conclusions

The present study reported for the first time the impact of *O. lybicus* on date palm nutrition and biomass of single young date palms. *Ommatissus lybicus* infestation beyond 300 nymphs per palm significantly decreased chlorophyll, plant biomass and palm nutrients. The effect of the feeding process and oviposition was not immediate and intraspecific competition for space/food resources between *O. lybicus* individuals adversely affected the number of oviposited eggs as the population density of *O. lybicus* reached 600 individuals. This study showed a significant relationship between honeydew droplets produced by different densities of the nymphs and chlorophyll content suggesting that starting nymph populations of 3-6 nymphs/leaflet equating to medium to high initial infestation is likely to result in chlorophyll loss of 28-40% and can be used to identify potential risk and trigger treatment. High to extremely high infestations of 6-10 nymphs/leaflet (600 or 1000 insects per palm seedling as our treatments) resulted in intraspecific competition and decline in egg oviposition. Egg oviposition was preferentially on the rachis and was predicted from honeydew produced by the adults. Thus honeydew droplets at the end of the season could indicate potential egg oviposition associated with reductions in fresh weight biomass of the rachis. Magnesium content in leaflets together with population density of the nymphs were significantly related to chlorophyll content suggesting Mg as a potential supplement for improved palm

nutrition under pest attack. Our study was conducted on date palm seedlings in a controlled setting, therefore, validation of these results in a commercial date palm plantation is required. Further in field validation of the relationship between the *O. lybicus* infestation and nutritional losses of date palms would provide information to improve crop and pest management via rational application of targeted fertilizers to increase host tolerance to the pest.

Chapter 3:

Chapter 3: Identification and Characterization of Entomopathogenic Fungal Candidates for Biological Control of the Dubas Bug (*Ommatissus lybicus*) in Oman

Abstract

A survey was conducted to investigate the abundance, diversity, and distribution of native entomopathogenic fungi (EPF) in Omani soils using the *Galleria* bait method. Sixty-six soil samples were collected from environments with high infestation by *Ommatissus lybicus*. EPF candidates with high growth and sporulation rate were characterized for their optimal temperature and evaluated for infection potential against *O. lybicus* adults using scanning electron microscopy (SEM). Thirteen fungal species belonging to five genera, *Beauveria bassiana*, *Clonostachys* spp., *Metarhizium* spp., *Aspergillus* spp., and *Fusarium* spp., known to be biological control agents for use against insects, were identified from 46 isolates collected through our survey. In addition, five entomopathogenic nematode species were identified as *Pratylenchus goodeyi*, *Acrobeloides saeedi*, *Oscheius tipulae*, *Mesorhabditis* sp., and *Heterorhabditis indica*. Four fungal isolates of *Beauveria bassiana*, *Clonostachys rosea*, *Metarhizium anisopliae*, and *Metarhizium quizhouense*, were selected as EPF candidates on their ability to grow and sporulate rapidly. *Beauveria bassiana* and *M. quizhouense* were able to grow and sporulate in vitro at temperatures between 25°C and 35°C. Rapid spore germination on *O. lybicus* adult cuticle was first seen with *C. rosea* after 2 hours post inoculation (hpi), 6 hpi with *M. anisopliae*, and *M. quizhouense*. and 12 hpi with *Beauveria bassiana*. *Beauveria*

bassiana achieved the highest mortality rate against *O. lybicus* adults with 93.4% within 3 days post inoculation (dpi). This study showed for the first time the abundance and diversity of indigenous entomopathogenic fungi in Omani soils and identified potential biological control agents against *O. lybicus*. The adhesion and penetration of the native fungi into the *O. lybicus* adults cuticle were proven using a scanning electron microscope (SEM). *Beauveria bassiana* was the most promising EPF that could kill *O. lybicus* adults within three days.

3.1 Introduction

Entomopathogenic fungi (EPF) are potent insect natural enemies and are strong candidates for developing bioinsecticides for controlling pest populations on a large scale (77,153). EPF as biological control agents can possess narrow host range and are thus considered safer for biodiversity and the environment than broad-spectrum synthetic insecticides (154–157). In contrast to other entomopathogens such as bacteria, protozoans and viruses, that require passive entry into the insect midgut, EPF exerts mechanical force and produces degrading enzymes such as lipases, chitinases and proteases (158) to penetrate the insect cuticle (81,82,159–161). Temperature and relative humidity fluctuations are two of the most important environmental factors that influence the efficacy of EPF in field environments (162,163). Therefore, native EPF that are adapted to local environmental conditions are considered first as potential candidates. The most suitable fungal isolates are characterized by high virulence, fast germination and hyphal growth rate and profuse sporulation to spread (82,164–167).

Clonostachys rosea, *Beauveria bassiana* and *Metarhizium* spp. have been evaluated for their pathogenicity against many sucking insects, including *Peregrinus maidis* (168), *Aphis glycines* (169), *Bemisia argentifolii* (170) and *Bemisia tabaci* (171). *Clonostachys rosea* is an ubiquitous soil-dwelling mycoparasite shown to have a potential as a biological control agent on both insects and plant pathogens of economic importance, including *Botrytis cinerea* (172), *Bipolaris sorokiniana* (173), *Didymella rabiei* (174), *Fusarium verticillioides*, *Fusarium graminearum*, *Fusarium culmorum*, and *Fusarium proliferatum* (175). *Beauveria bassiana* was first reported by Agostino Bassi in

1834 as a pathogen of the silkworm *Bombyx mori* L. (176,177) and is widely distributed in temperate and tropical areas (70) infecting a variety of insects belonging to different orders (178,179). *Metarhizium* species have been used for 130 years as biological control agents (180) against insect species of the orders of Orthoptera (181–184), Diptera (185,186), Hemiptera (187), Hymenoptera (188), and Homoptera (189). Several commercial products containing formulations of EPF, including *B. bassiana* and *M. anisopliae* have already contributed to pest management programs (190). For example, the biopesticide product “Green Muscle” by CABI Bioscience/NPP containing *M. anisopliae* var. *acridum* was shown to kill locusts within 14 to 20 days with a mortality rate of 70% to 90% (182). The demand for these mycoinsecticides is growing at 10% per year in the global crop protection business (66).

In the Middle East, very few studies have investigated the potential use of local entomopathogenic fungi for biological control against important pests such as the Dubas bug (*Ommatissus lybicus* de Bergevin) (Hemiptera: Tropiduchidae). *Ommatissus lybicus* is a major pest on date palm *Phoenix dactylifera* Linnaeus; Areaceae causing palm yield loss of up to 50% (25,97,98). *Ommatissus lybicus* is a bivoltine insect with a spring generation (February to May), aestivation as egg diapause, followed by autumn generation (August to November) and eggs diapause to overwinter in date palms (44,191,192). Damage caused by *O. lybicus* nymphs and adults is through feeding on palm sap and excreting copious amounts of a viscous, sugary honeydew on leaves leading to the accumulation of dust and the growth of saprophytic fungi black sooty mould, *Meliola camellia* (23,104,105,193).

Chemical control using synthetic insecticides is the primary control method against *O. lybicus* in Oman, with about 523 tons applied in the period between 1993 - 2010 to infested palms via aerial application with an estimated cost of £18.5 million (3). Subsequently, the increased use of the insecticides deltamethrin and fenitrothion has led to the evolution of resistance in populations of *O. lybicus* in date palm plantations in Oman (98). Therefore, developing alternative control methods, such as the use of EPF for biological control of *O. lybicus* infestations is of high priority for palm protection (194). Isolates of *Cladosporium* sp., *B. bassiana*, *Acremonium alternata* and *Paecilomyces* sp have been shown previously to be pathogenic against *O. lybicus* in Iraq and Iran (195,196). Although some candidates have been identified in bordering states of Oman, native insect pathogenic fungi that are already adapted to local environmental conditions and present in the environment would be more desirable as biocontrol agents (156,178).

There is no previously published information on the existence of naturally occurring Omani EPF against *O. lybicus*, although biological control agents for other pests, for example, the coconut mite *Aceria guerreronis* have been isolated in Dhofar (197). This study aimed to identify and characterise potential entomopathogenic fungal candidates against *O. lybicus*, isolated in areas of high infestations with *O. lybicus* on date palms. The objectives were to: i) isolate native EPF from local environments and define their phylogenetic relatedness; ii) identify any relationships between species, distribution and frequency of isolation of native EPF and soil texture, nutrients, pH, and salinity; iii) determine the optimal temperature conditions for growth and sporulation of candidates; and iv) evaluate and demonstrate the infection patterns of entomopathogenic

candidate strains against adults of *O. lybicus* using scanning electron microscopy (SEM).

3.2 Materials and methods

3.2.1 Soil samples collection

Soil samples were obtained from sixty-six date palm orchards in 2017 in Oman (Figure 3.1). Coordinates of each plot were recorded using a handheld Global positioning system (GPS) device (Garmin Ltd, Schaffhausen, Switzerland). Two soil cores were collected from each plot (20 cm deep, 10 cm diameter and 25 cm distant from each other) using a trowel. The trowel was sterilized with NaOCl 5.2% after each plot sampling, followed rinsing with distilled water. Collected samples were placed in one pre-labelled plastic bag and treated as subsamples. Subsamples were sieved using a 5 mm sieve to remove any collected stones or roots, homogenized evenly, and stored at 4°C until inspection (198).

water for 15 s to prevent excessive silk webbing in the soil (198,200,205,206). A sub-sample of soil (220g) from each plot was placed in a round plastic container (12 cm diameter), and sterilized distilled water was added to moisten the soil. Ten wax moth larvae (fourth and fifth instar) were placed in each container (one container for each soil plot) with a closed lid with holes to allow air movement. The containers were incubated in the dark in a growth chamber (Panasonic: MLR-352H) at 22°C and 50% relative humidity (207–209). The larvae were inspected every 3 days. The soil was agitated on each inspection day to increase the probability of spores contacting the larvae (198). Dead larvae were collected and washed in NaOCl 1% for 3 minutes before being rinsed three times with distilled water and placed in a Petri dish on filter paper (70 mm). Petri dishes were sealed using Parafilm and incubated at 25°C in Sanyo incubator MIR-154 (Sanyo Electric, Osaka, Japan) until fungal growth was visible.

3.2.3 Single spore isolation and maintenance

Galleria mellonella showing fungal colonisation were placed in a petri dish containing Sabouraud Dextrose Agar (SDA, Oxoid) containing the bacterial antibiotics chloramphenicol 0.1 g/l and streptomycin sulphate 0.05 g/l and the selective fungicide *N*-dodecylguanidine monoacetate (Dodine) at 0.1 g/l to restrict the growth of common bacterial and fungal soil contaminants (208,210–214). A tip from a growing colony was transferred to a potato dextrose agar (PDA, Oxoid) and left at 25°C for 7 days. A modified protocol used by Choi et al. (215) was used to isolate a single spore isolate in order to get a pure culture of the targeted fungi. A small piece (3 mm) was taken from the newly grown colony from the PDA and suspended on 100 µl sterilized water containing 0.05% Tween 80 (Sigma-Aldrich) and vortexed to homogenize the spore suspension.

The spore suspension was then plated onto 1.5% Agar Bacteriological (Oxoid) and incubated at 25°C for 18 hours. Spore germination was inspected under a stereo microscope. A sterilised hypodermic needle was used to transfer single germinated spores on a new PDA agar and incubated at 25°C for 2 weeks.

3.2.4 Genomic DNA extraction

Two weeks old fungal cultures grown on PDA were harvested to obtain ≤ 100 mg mycelium using sterilized scalpel and placed into a 2 ml microcentrifuge tube. Steel grinding beads (5 mm diameter) were used to ground the mycelia in the tubes using a mixer mill (MM 200, Retsch, Haan, Germany) set at maximum frequency for 5 minutes. DNA was extracted using the DNeasy Plant Mini Kit (QIAGEN, Hilden, Germany) following the protocol by the manufacturer (216). The extracted DNA was purified with DNeasy PowerClean Pro CleanUp Kit (Qiagen) to eliminate PCR inhibitors as per the protocol by the manufacturer (216).

PCR amplification was carried out on the internal transcribed spacers (ITS) region. The universal primers ITS1 (50-TCCGTAGGTGAACCTGCGG-30) and ITS4 (50- TCCTCCGCTTATTGATATGC-30) were used to amplify the (ITS) region of the ITS1-5.8S-ITS2 rRNA gene (217–223). The PCR reaction mixture of 25 μ l contained 12.5 μ l Platinum™ SuperFi™ PCR Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA), 1.0 μ l ITS1 primer, 1.0 μ l ITS4 primer, 2.0 μ l DNA template, and 8.5 μ l nuclease-free water. The cycling conditions were 98°C for 30 sec, followed by 35 Cycles of 98°C for 10 sec, 55°C for 10 sec, and 72°C for 30 sec. The extension was at 72°C for 5 min and finally held at 4°C. The amplified PCR products were visualised on 2% agarose gel and

stained with ethidium bromide. PCR products were purified using a QIAquick PCR Purification kit (Qiagen, Germany) following the protocol of the manufacturer (224). The purified PCR products for all isolates were sent sequenced by Macrogen Inc. (Korea). The resulting sequences were aligned using MEGA X software and blasted in National Center for Biotechnology Information (NCBI) GenBank database (Table 3.1).

Nematodes were harvested from the infested larva (20 nematodes), placed in a 1.5 ml microcentrifuge tube and stored at -80°C till use. Nematode DNA was extracted using DNeasy Blood and Tissue Kit (Qiagen, Germany) following the manufacturer protocol (225). DNeasy PowerClean Pro CleanUp Kit (Qiagen) was used to eliminate PCR inhibitors (216). The ITS region primers TW81 (5'-GTTTCCGTAGGTGAACCTGC-3') and AB28 (5'-ATATGCTTAAGTTCAGCGGGT-3') were used in the PCR amplification process (226–228), and the same primers were used in sequencing.

The PCR protocol and sequencing facility used for the amplification of the nematode ITS region was identical to the above used for the EPF, except that the annealing temperature was reduced to 50°C.

The obtained sequence data were edited and assembled with MegaX. GenBank database of the National Center for Biotechnology Information (NCBI) was used to compare our sequences of ITS region by Basic Local alignment search Tool (BLAST). Selected accessions were aligned with our sequences in Mega X using Clustal W. Phylogenetic tree of aligned sequences was performed using a Maximum Likelihood method, and Bootstrap based on 1000 replications was used to generate the dendrogram for each region.

3.2.5 Soil analysis

Soil texture analyses were carried out at the soil laboratory in Rumais, Oman, following a procedure described by Gavlak et al. (229). Soil, clay, and silt were calculated using the following formulas:

$$\text{Sand \%} = \frac{(\text{oven-dry soil}) - (R_{\text{sand}} - R_{\text{c1}})}{(\text{oven-dry soil})} \times 100$$

$$\text{Clay \%} = \frac{(R_{\text{clay}} - R_{\text{c2}})}{(\text{oven-dry soil})} \times 100$$

$$\text{Silt} = 100 - (\text{Sand \%} + \text{Clay \%})$$

Where:

R_{sand} = hydrometer reading of sand after 40 seconds

R_{clay} = hydrometer reading of clay after 6 hours

R_{c1} = hydrometer blank reading after 40 seconds

R_{c2} = hydrometer blank reading after 6 hours

Soil pH, soil salinity, water-soluble sodium (Na) and potassium (K) concentrations were determined by making a water paste 1:5 (soil: water) ratio suspension (139,230). The extracted fluid was used to measure pH using a WTW Inolab pH-meter (WTW GmbH, Weilheim, Germany) and salinity using a conductivity meter (inoLab® Cond 7310, WTW, Germany). The soil extract was injected into a flame photometer (Sherwood M410, Sherwood Scientific Ltd, Cambridge, UK) to measure Na and P concentrations at 589-nm and 767-nm wavelengths, respectively (139).

Calcium (Ca) and magnesium (Mg) were titrated with disodium salt -ethylene diamine tetra acetic acid (EDTA), and calculations were done using the following formulas (139):

$$\text{EDTA normality: } N_{\text{EDTA}} = \frac{10 \times N_{\text{CaCl}_2}}{V_{\text{EDTA}}}$$

$$\text{Extractable Ca or Ca + Mg (Meq/L)} = \frac{(V-B) \times N \times V_1 \times 1000}{W_t \times N_2}$$

$$\text{Extractable Mg (Meq/L)} = \text{Extractable (Ca + Mg)} - \text{Extractable Ca}$$

Where:

V = Volume of EDTA titrated for the sample (ml)

B = Blank titration volume (ml)

V1 = Volume of soil extract used for titration (ml)

N = Normality of EDTA solution

Phosphorous (P) concentration was analyzed by preparing fluid extract following the procedure described by Estefan et al. (139). The phosphorous concentration (ppm) was measured using a spectrophotometer (Biochrom Libra S22, Cambridge, England) at 882nm wavelength (139). Total Nitrogen was analyzed following Kjeldahl method described by Estefan et al. (139) and measured using a nitrogen analyzer (VELP scientifica, Italy). Heavy metals were analyzed at the United Integrated Laboratories LLC following the procedure illustrated by Estefan et al. (139) and analyzed using inductively coupled plasma mass spectrometry (ICP-MS) (Thermo fisher scientific). Soil Organic Matter was analyzed using the ash-free massing method (231).

3.2.6 Isolate characterization

Two isolates of *B. bassiana*, six *Clonostachys* spp., and fifteen *Metarhizium* spp. were first characterized for their sporulation and growth rate at 26°C to select the best candidates for subsequent studies. The criteria for selection was rapid, abundant sporulation and growth. For each isolate, a small piece (3 mm) was cut from 2 weeks old non-sporulating mycelia on Petri dishes using a sterilized blade and placed in the center of new SDA medium plates. The plates were sealed using parafilm and incubated in the dark at 26°C (Sanyo incubator MIR-154). Surface radial growth was measured every 4, 7, 10, and 14 days using 2 cardinal diameters previously drawn underneath each plate. Sporulation of all isolates was measured by harvesting the conidia after 14 days. The conidia were scraped by a scalpel after flooding the plates with 10 ml sterilized distilled water containing 0.05% Tween 80 (Sigma-Aldrich) as a surfactant additive, and mycelia were filtered using double layer medical mesh. The filtered spore concentration was then calculated by adding 10 µl of spore suspension into a hemocytometer (Brand, Wertheim, Germany) and observed under a microscope (Primo Star; Carl Zeiss Microscopy, GmbH, Germany). Spores were imaged with a Zeiss AxioCam HRc (Carl Zeiss, Germany) and the free software ImageJ (<https://imagej.nih.gov/ij/>) was used for counting. There were 4 replicates for each isolate.

3.2.7 Optimum temperature for entomopathogenic fungal growth and sporulation

Four entomopathogenic fungal strains of *B. bassiana* (BeHa02), *C. rosea* (ClNa1), *M. anisopliae* (MeFa05), and *M. guizhouense* (MeBo17) with high

sporulation potential selected in the previous characterization studies were assessed for their optimum growth temperature. Radial growth rate and sporulation, as described above, were carried out on isolates grown on SDA agar at 9 different temperatures (15°C, 17.5°C, 20°C, 22.5°C, 25°C, 27.5°C, 30°C, 32.5°C, 35°C, and 37.5°C). The temperature in the incubators was monitored using data loggers (Watchdog Model A150; Spectrum Technologies Inc.) to avoid inaccuracy.

3.2.8 Scanning electron microscope (SEM)

Ommatissus lybicus adults were obtained from an existing laboratory colony in the Entomology laboratory in Rumais, Oman. Three male and female adults of *O. lybicus* were enclosed separately in a petri-dish containing double white filter papers. The insects were inoculated with the candidate native entomopathogenic fungal strains of *B. bassiana*, *M. anisopliae*, *C. rosea*, and *M. guizhouense* obtained from the survey. The insects were sprayed using a spore suspension adjusted to 1×10^8 spore/ml for each strain using a hand sprayer. The insects were then incubated in a growth chamber set at $26^\circ\text{C} \pm 2$ and 80% relative humidity. The length of exposure to EPF was set at 2 hours, 6 hours, 12 hours, 36 hours, 48 hours, 5 days, and 7 days. Following each time interval, insects were removed and preserved in 75% ethanol in a refrigerator overnight and before being washed in 100% ethanol 3 times, 5 minutes at a time. Specimens were dried using a Tousimis Autosamdri-815 critical point drier. Subsequently, insects were mounted on aluminium stubs with carbon-coated double-sided sticky tape and metalized with gold-palladium using a sputter coater (Bio-Rad, UK). Microbial development and infection were observed by SEM (JEOL JSM-

5600LV, Japanese Electronic and Optical Ltd., Tokyo, Japan), operated at accelerating voltages of 20 kV.

3.2.9 Statistical analyses

Statistical analyses were performed using Genstat 22nd edition (VSN International, Hemel Hempstead, UK). The relationship between the soil positive isolates and soil nutrients, soil texture, organic matter, soil Ph, and soil salinity were assessed using Spearman's rank correlation coefficient test. Differences between the screened isolates were subject to General Analyses of variance ANOVA at 95% confidence level. The predictive model of fungal radial growth at different temperatures was described by nonlinear curve fitting line plus exponential (addition of linear trend) regression, whilst sporulation rate was described by Gaussian regression at $p < 0.05$.

3.3 Results

3.3.1 Occurrence and distribution of EPF

Entomopathogenic fungi were found occurring in 40% (26 out of 66) of the sampled soils, yielding 46 fungal isolates. Thirteen fungal species were isolated from infected larvae belonging to five genera known for their pathogenicity against insects (153,219,232–241) (Table 3.1). *Metarhizium* species were most abundant, present on 43.5% of the infected larvae, with *Metarhizium anisopliae* as the most frequently isolated with thirteen isolates (37.7%). *Clonostachys* species were found in 20.8% of the infected larvae, followed by 13.2% containing *Beauveria bassiana*. Five *Fusarium* species were present in 9.4% of the infected larvae. The least occurring genera were *Aspergillus* spp. found in 5.7% of the infected larvae. However, seven larvae (13.2%) were parasitized by

entomopathogenic nematodes identified as *Acrobelloides saeedi*, *Heterorhabditis indica*, *Pratylenchus goodeyi*, *Mesorhabditis* sp., and *Oscieus tipulae* (Table 3.1).

Correlation analysis showed that the number of positive isolates had significant, but weak ($r=0.3$) positive associations with the pH, sand%, silt% and Pb content and negative associations with Na content (Supplementary Table 3.2). There were also weak, positive associations between the presence of nematode and Cu and C:N ratio.

Table 3.1 Isolation frequency and number of fungal and nematode species from Omani soils in 2017.

No.	Species	No. of Isolates	Isolation frequency %
1	<i>Aspergillus cristatus</i>	1	1.9
2	<i>Aspergillus flavus</i>	2	3.8
3	<i>Beauveria bassiana</i>	7	13.2
4	<i>Clonostachys buxi</i>	1	1.9
5	<i>Clonostachys rosea</i>	3	5.7
6	<i>Clonostachys</i> sp.	1	1.9
7	<i>Clonostachys swieteniae</i>	6	11.3
8	<i>Fusarium falciforme</i>	1	1.9
9	<i>Fusarium solani</i>	2	3.8
10	<i>Fusarium</i> sp.	2	3.8
11	<i>Metarhizium anisopliae</i>	13	24.5
12	<i>Metarhizium robertsii</i>	2	3.8
13	<i>Metarhizium quizhouense</i>	5	9.4
14	<i>Acrobelloides saeedi</i>	1	1.9
15	<i>Heterorhabditis indica</i>	2	3.8

16	<i>Mesorhabditis</i> sp.	1	1.9
17	<i>Oscheius tipulae</i>	2	3.8
18	<i>Pratylenchus goodeyi</i>	1	1.9

3.3.2 Phylogenetic analysis

Isolated fungi (Figure 3.2) and nematodes (Figure 3.3) were analyzed for their relatedness visualized as phylogenetic trees. The EPF phylogenetic tree showed two main clusters with 78% and 79% identity of Hypocreales and Eurotiales, respectively (Figure 3.2). The majority of the isolated EPF (92%) belonged to the Hypocreales, and the remaining 8% of isolated *Aspergillus* spp. belonged to the Eurotiales. Hypocreales showed two sub-clusters with 80% analogy at the sub-cluster *Clonostachys* spp., which gathered the isolates between ClSe07 (*C. swieteniae*) to ClBi10 (*C. buxi*). The second sub-cluster divided into two sub-clusters with an identity of 86%. The first sub-cluster, *Fusarium* spp. had an identity of 80%. The second sub-cluster divided into *Metarhizium* spp. and *Beauveria bassiana* with an identity of 86%. *Metarhizium* spp. had an identity of 83%, while *Beauveria bassiana* had an identity of 88% (Figure 3.2).

The entomopathogenic nematodes phylogenetic tree had two main clusters. The first cluster contained *Heterorhabditis indica*, *Pratylenchus goodeyi*, and *Acrobeloides saeedi* with an identity of 88%, and the second cluster consisted of *Oscheius tipulae* and *Mesorhabditis* sp. with an identity of 78% (Figure 3.3).

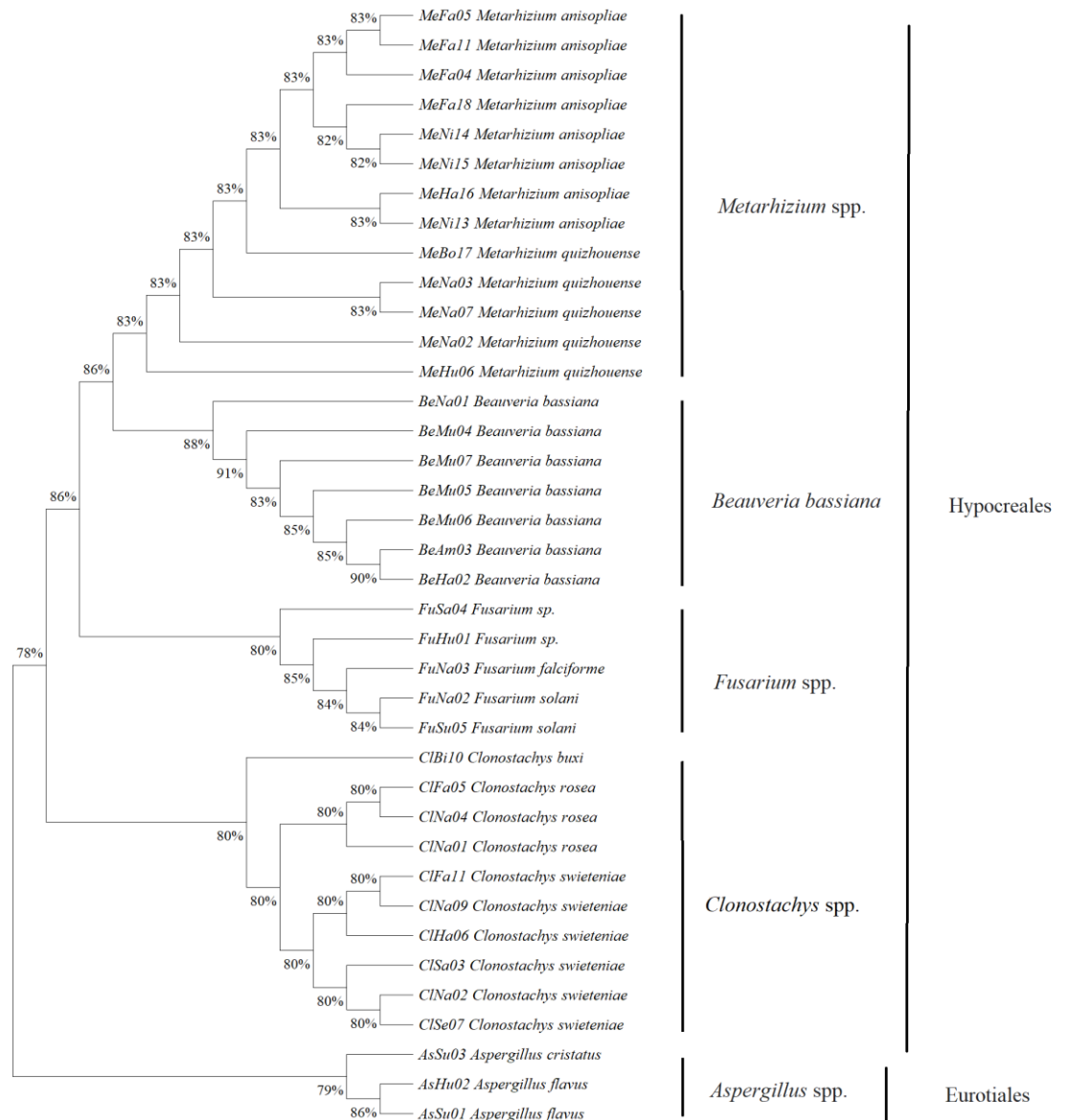


Figure 3.2 Phylogenetic tree of fungal isolates on Internal Transcribed Spacer (ITS) region. The tree was constructed using maximum likelihood by megaX. Bootstrap procedure of 1000 replicates was used. Isolate codes are followed by species name.

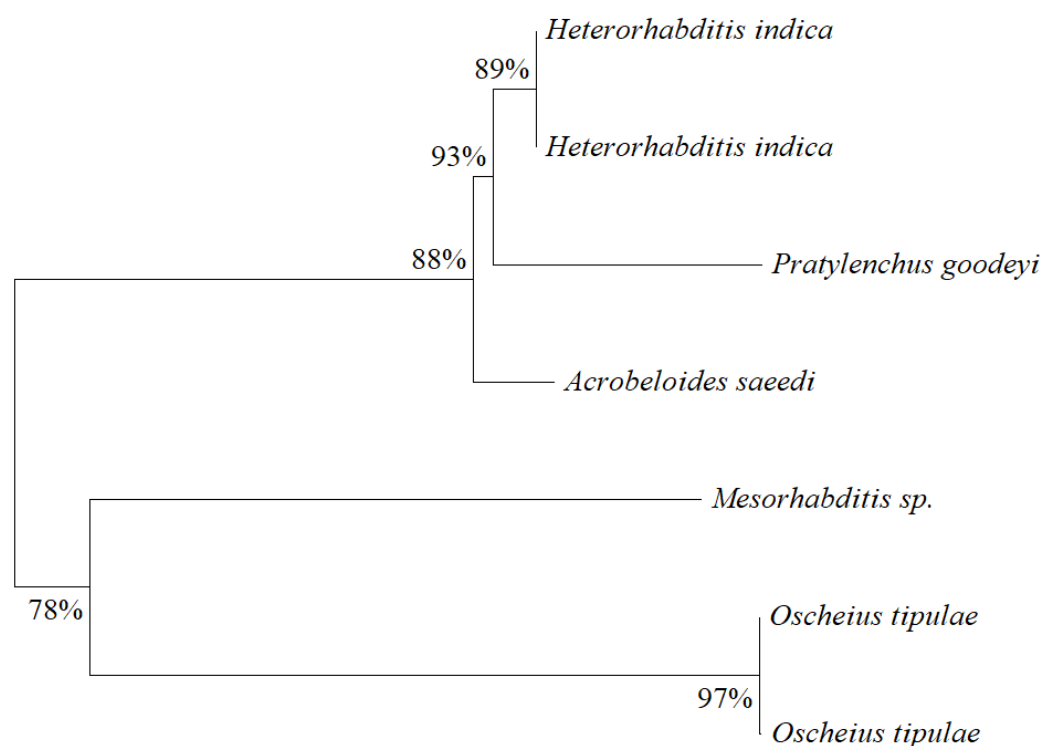


Figure 3.3 Phylogenetic tree of isolated nematodes based on ITS. The tree was constructed using maximum likelihood by megaX. Bootstrap procedure of 1000 replicates was used.

3.3.3 Optimum temperature for fungal growth and sporulation

The characterization of 23 fungal isolates revealed significant differences in sporulation rate at 26°C between the species of the genera *Clonostachys*, *Metarhizium* ($p < .001$), and *Beauveria* ($p = 0.02$). Four isolates *Beauveria bassiana* (BeHa02), *Clonostachys rosea* (ClNa01), *Metarhizium anisopliae* (MeFa05), and *Metarhizium quizhouense* (MeBo17), exhibiting the most abundant sporulation were selected from an initial screen of all fungal isolates within species (Supplementary Table 3.3) and were further characterized for their optimal temperature conditions for radial growth and sporulation by measuring radial growth rate per day (mm/day) and spore production,

respectively. Temperature had a significant effect on the radial growth of EPF strains ($p < .001$, Figure 3.4). *Metarhizium guizhouense* had the highest optimum growth temperature of 30°C (Figure 3.4), followed by *M. anisoplia* with and *C. rosea* with optimum growth at 27.5°C and 25°C, respectively (Figure 3.4). The predicted optimum growth temperature for *B. bassiana* was 25°C. The growth rate of *M. guizhouense* *M. anisoplia* under a temperature of 35°C was estimated to be 1.8 mm.day⁻¹ and 1.6 mm.day⁻¹, respectively. *Metarhizium anisoplia* and *C. rosea* exhibited higher growth rates of 4.5 mm.day⁻¹ and 4.4 mm.day⁻¹, respectively and grew at temperatures below 27.5°C. Furthermore, the lowest temperature in our study of 15°C was tolerated by all isolates; however, only the isolates of *B. bassiana* and *M. guizhouense* grew at higher temperatures between 35°C and 37.5°C (Figure 3.4).

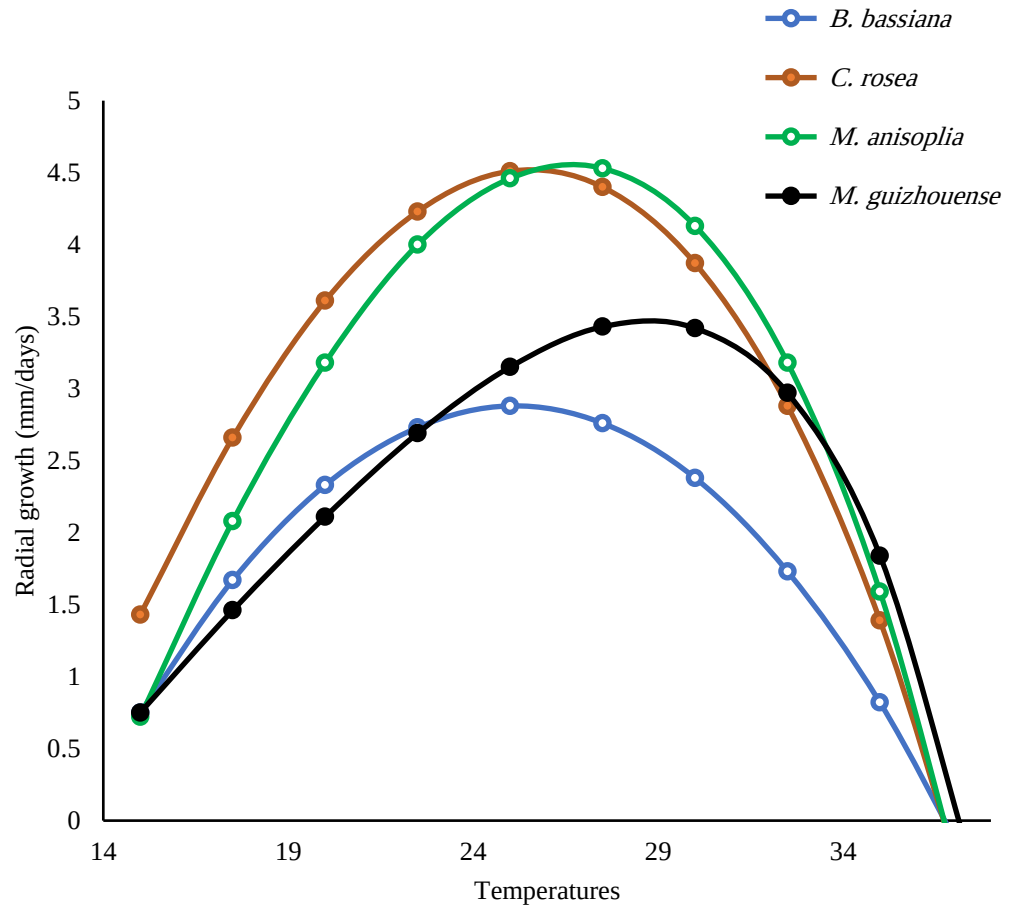


Figure 3.4 Radial mycelial growth rate (mm/day) of *C. rosea* (ClNa01), *M. anisoplia* (MeFa05), *B. bassiana* (BeHa02), and *M. guizhouense* (MeBo17) (followed by isolate code) at a temperature ranged between 15°C and 37.5°C. The predicted model of radial growth was described by line plus exponential (addition of linear trend) regression analysis ($p < 0.001$).

Temperature significantly affected the sporulation rate of EPF strains ($p < .001$, Figure 3.5). *Clonostachys rosea* sporulated most abundantly within a temperature range of 22.5°C and 32.5°C with an optimum temperature for sporulation at 27.5°C achieving 1×10^9 spores/ml after 14 days post inoculation (dpi). *Beauveria bassiana* sporulated at temperatures between 15°C and 35°C

with an optimum temperature of 22.5-27.5°C (3×10^8 spores/ml). *Metarhizium* spp. sporulated between 17.5°C and 35°C. *Metarhizium guizhouense* produced 3×10^8 spores/ml at an optimum of 30°C. *Metarhizium anisoplia* sporulated less and had an optimum sporulation of 1×10^8 spores/ml at a temperature between 25°C-27.5°C (Figure 3.5).

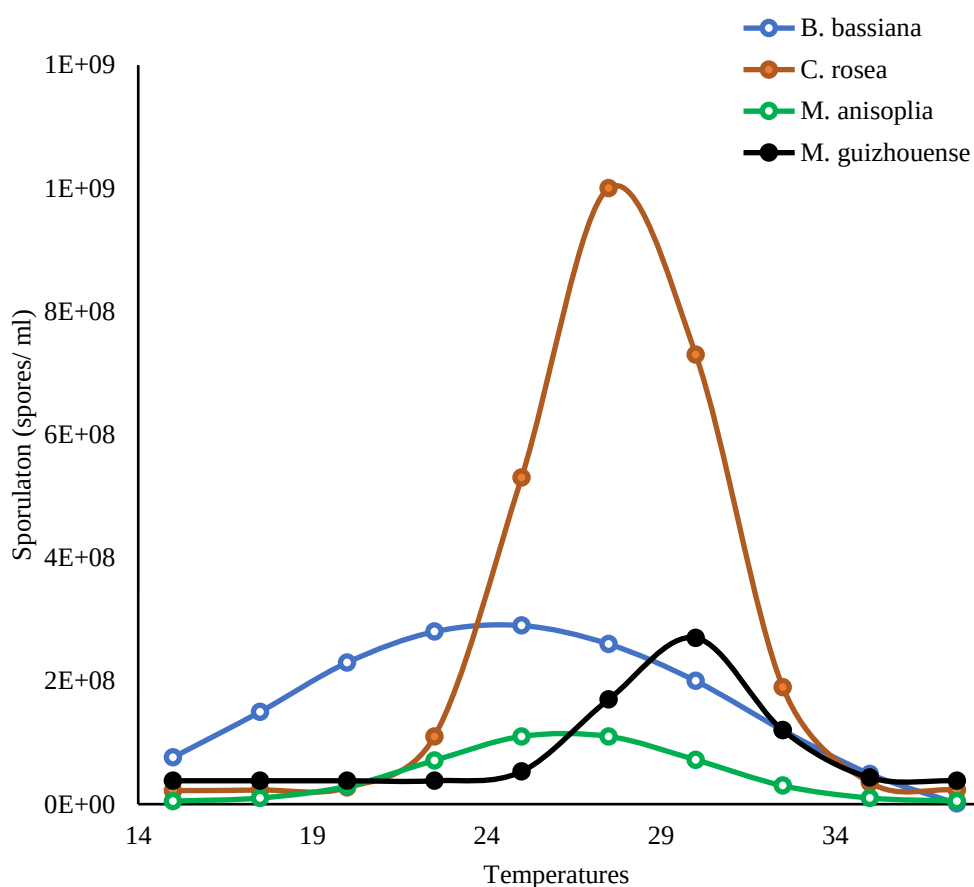


Figure 3.5 Sporulation rate (spores/ml) of *B. bassiana* (BeHa02), *M. anisoplia* (MeFa05), *C. rosea* (ClNa01), and *M. guizhouense* (MeBo17) (followed by isolate code) at different temperatures ranging between 15°C and 37.5°C. Predicted means were derived by Gaussian regression analysis ($p < 0.001$).

3.3.4 Infection of *O. lybicus* adults by EPF

Insect mortality at 3 dpi with the isolates of *B. bassiana*, *C. rosea*, *M. anisopliae* and *M. quizhouense* was 93.4%, 27.2%, 49.6%, and 59.8%, respectively. By 7 dpi, mortality increased to 96.8%, 89.2%, 93.5%, and 94.7% for insects infected with *B. bassiana*, *C. rosea*, *M. anisopliae* and *M. quizhouense*, respectively.

SEM allowed the observation of adhesion and penetration structures of the EPF on the cuticle of *O. lybicus*. Spore germination, germ tube extension and appressorial formation by *B. bassiana* (Figure 3.6a) were observed on the head, antenna, and abdomen of *O. lybicus* adults at 12 hours post infection (hpi). Hyphal networks forming conidiophores of *B. bassiana* (Figure 3.6b) were seen between the ommatidia of the compound eye, legs, and head frons of *O. lybicus* adults between 36 and 48 hpi. The mycelium thoroughly covered the insect 5 dpi (Figure 3.6c) with abundant sporulation (Figure 3.6d) at 7 dpi.

Good spore adherence (Figures 3.7a & 3.8a) with hyphal extension of *M. anisoplia* and *M. quizhouense* between 6-12 hpi were observed on the head and ommatidia of the compound eye of *O. lybicus*, respectively, (Figure 3.8b). Appressoria formation of *M. anisoplia* was observed on the head of *O. lybicus* 12 hpi (Figure 3.7b). Hyphal networks formed by the two strains (Figures 3.7c & 3.8c) were observed between the ommatidia of the compound eye, legs, labium, and head frons of *O. lybicus* adults from 36 to 48 hpi. Dense mycelium by *M. anisoplia* and *M. quizhouense* thoroughly covered the insects at 5 and 7dpi, respectively. Spore formation on *O. lybicus* adults by *M. anisoplia* and *M. quizhouense* was observed at 5 dpi (Figures 3.7d & 3.8d).

Clonostachys rosea spores were seen attached onto the head of *O. lybicus* adults 2 hpi (Figure 3.9a). Rapid germination was observed at 2-6 hpi leading to the extension of fungal hypha on the head and abdomen of *O. lybicus* adults (Figure 3.9b). Hyphal growth extended onto the whole adult body of *O. lybicus* by 12-36 hpi (Figure 3.9c). *Clonostachys rosea* fully colonized insect bodies with mycelium by 48 hpi with spore formation observed at 7 dpi (Figure 3.9d).

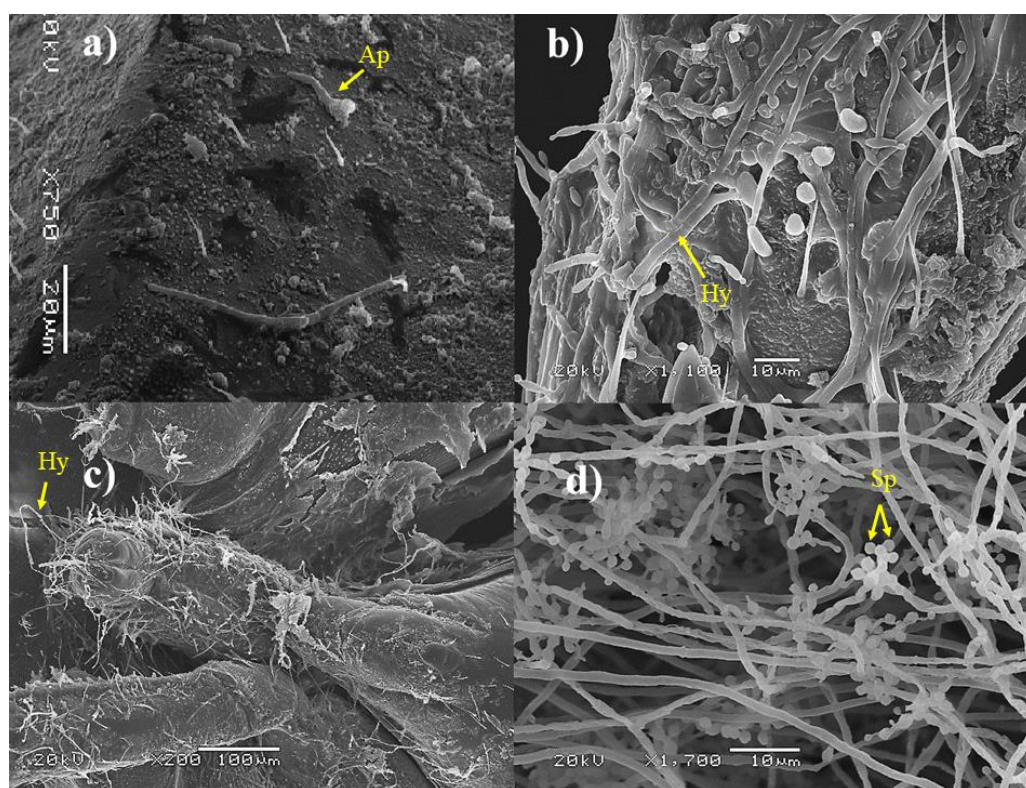


Figure 3.6 Scanning electron microscopy SEM of *O. lybicus* adult infected with *B. bassiana*; a) spore germination, germ tube extension and appressorial (Ap) formation on the head of *O. lybicus* adult female, 12 hpi, b) Hyphae (Hy) of *B. bassiana* on the leg of *O. lybicus* adult female at 36 hpi, c) Hyphal networks of *B. bassiana* forming on labium and legs of *O. lybicus* female 5 dpi, d) Spore (Sp) formation on the abdomen of *O. lybicus* adult male at 7 dpi. Days post inoculation (dpi), hours post inoculation (hpi).

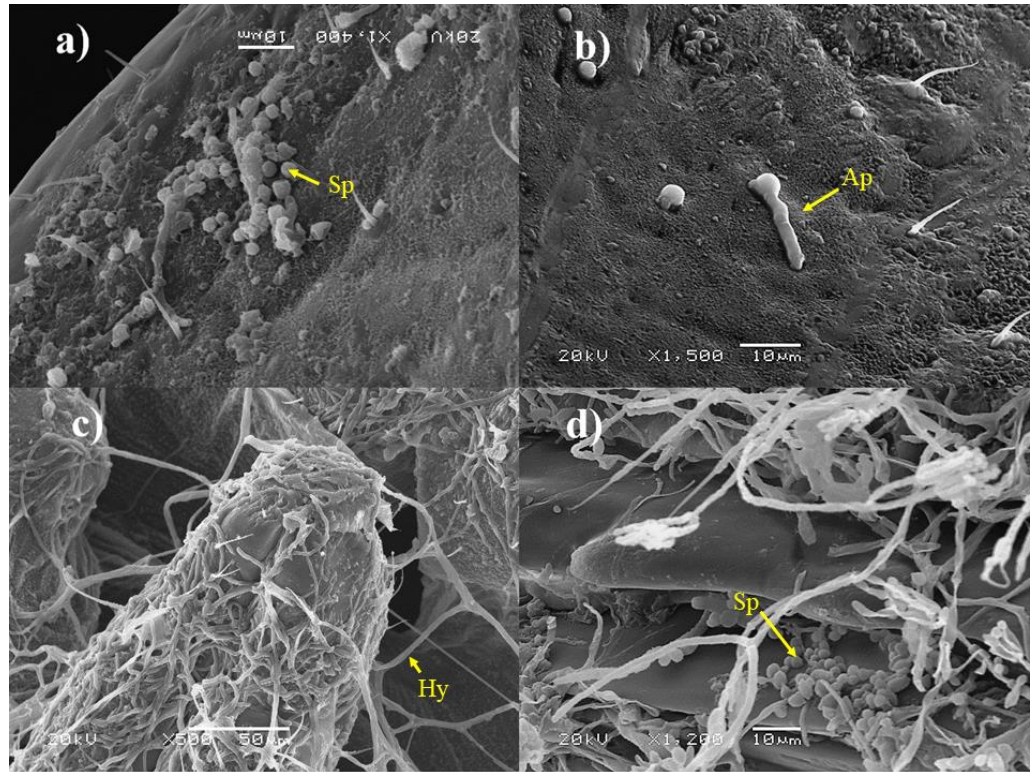


Figure 3.7 SEM of *O. lybicus* adult infected with *M. anisoplia*; a) Spores (Sp) attached to the head of *O. lybicus* adult female at 12 hpi, b) Appressoria (Ap) formation on the head of *O. lybicus* adult female at 12 hpi, c) Hyphal (Hy) networks on labium of *O. lybicus* adult male at 48 hpi, d) Spore (Sp) formation on *O. lybicus* adult female at 7 dpi. Days post inoculation (dpi), hours post inoculation (hpi).

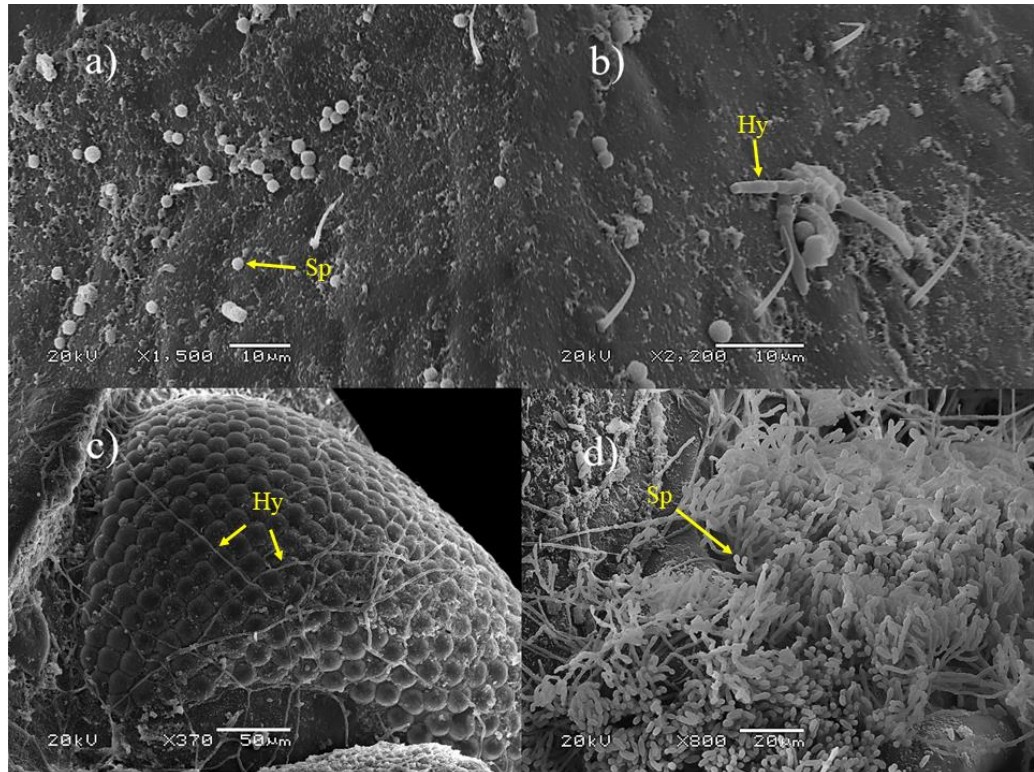


Figure 3.8 SEM of *O. lybicus* adult inoculated with *M. guizhouense*; a) Spores (Sp) attached to the head of *O. lybicus* adult male at 12 hpi, b) Hyphal (Hy) extension between the ommatidia of the compound eye of *O. lybicus* adult male at 12 hpi, c) Hyphal (Hy) networks on the eye of *O. lybicus* adult female at 48 hpi, d) Spore (Sp) formation on *O. lybicus* adult female at 5 dpi. Days post inoculation (dpi), hours post inoculation (hpi).

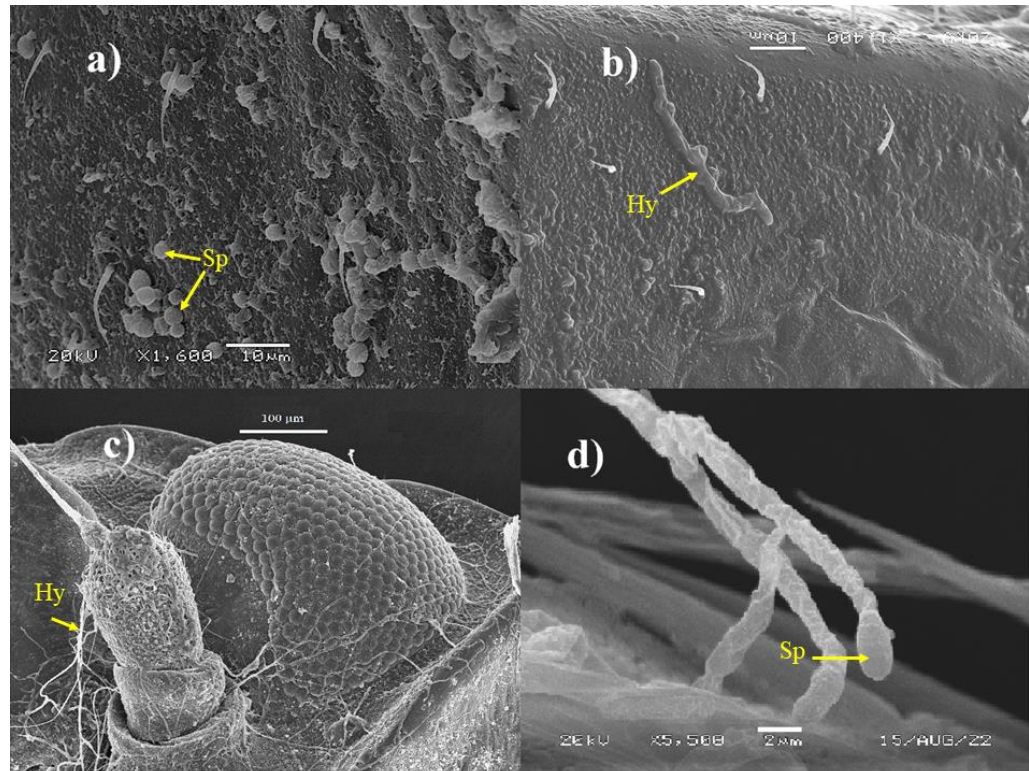


Figure 3.9 SEM of *O. lybicus* adult infected with *C. rosea*; a) Spores (Sp) attached on the head of *O. lybicus* adult male at 2 hpi, b) Hyphal (Hy) extension on the head of *O. lybicus* adult female at 6 hpi, c) Hyphal (Hy) growth extended onto the head, eye, and antenna of *O. lybicus* adult female, at 36 hpi, d) Spore (Sp) formation on the abdomen of *O. lybicus* adult female at 7 dpi. Days post inoculation (dpi), hours post inoculation (dpi).

3.4 Discussion

This study revealed new information about the abundance and diversity of EPF that attack *O. lybicus* in the environment in Oman. Forty-six EPF (40%) of the genera of *Clonostachys*, *Metarhizium*, *Beauveria*, *Fusarium*, and *Aspergillus* were isolated from different locations with high *O. lybicus* infestation. The insect baiting method used in this study was effective in obtaining candidate EPF from

the soil. This method was first introduced by G Zimmermann (1986) and is still widely used for capturing EPF from soil (200,205,242–245). Soil physical and chemical properties are known to influence EPF abundance (199,246,247). Many species of Ascomycota, including *Metarhizium*, *Beauveria*, and *Paecilomyces* can survive in the soil environment (248,249). For example, *M. anisopliae* forms chlamydospores to resist the harmful effects of soil biodegradation and abiotic stress, allowing persistence and long-term survival in soil (250). However, in our study, the associations between soil nutrients/traits and EPF abundance were weak, suggesting that environmental conditions and/or other agronomy factors not included here may be involved.

Metarhizium fungi are considered one of the most abundant genera in soil (182,249,251,252) and were the most frequently isolated species in our survey (37.7%). *Beauveria bassiana* occurred at a frequency of 13.2%, whilst only 3 isolates of *C. rosea* were obtained from soil samples. *Clonostachys rosea* is a mycoparasitic fungus that is pathogenic to both insect pests, such as *O. tucumana*, *S. grossa* Signoret (240), *Bemisia tabaci* (253), and to plant pathogens, including *Verticillium dahliae* Kleb. (254) and *Sclerotinia sclerotiorum* (240,255). Our study also revealed the existence of *Aspergillus* and *Fusarium* species which were capable of causing infection to *G. mellonella* larvae. The latter fungal species have been reported to be pathogenic against insects (153,256,257) but are also known to negatively impact human health (258–260) and were thus excluded from further investigations in this work. Our studies allowed the isolation of insect-parasitic nematodes (261–263) recovered from 13.2% of the infected larvae. The only previous study mentioning entomopathogenic nematodes in Oman was by Elawad et al. (264), who isolated

and named new species from alfalfa fields known as *Steinernema abbasi* sp. n. The diversity and phylogenetic relationships of the lineage of *Aspergillus* spp., *Clonostachys* spp., *Beauveria bassiana*, *Fusarium* spp., and *Metarhizium* spp, based on the sequence analysis of the ITS region of 38 isolates, were visualized showing two main clusters, Hypocreales and Eurotiales, with the majority of the identified isolated in Hypocreales. This is not surprising as the most abundant and diverse EPF have been previously found within this order (265).

Variations in different temperature requirements of EPF are due to genetic adaptations associated with geographical origin (266–269). The weather in Oman is characterized by a high annual temperature of an average of 27°C, with minimum and maximum daily average temperatures of 18.2°C and 33.4°C, respectively (270). Our study showed significant differences in the sporulation rate and radial growth of *Clonostachys* spp., *Beauveria bassiana*, and *Metarhizium* spp. isolates in vitro. Further characterisation of thermal tolerance and optimum temperatures for the growth and sporulation of candidate strains of *B. bassiana*, *C. rosea*, *M. anisopliae*, and *M. quizhouense* revealed that the selected EPF were able to grow and sporulate beyond the average annual temperature of Oman (27°C) with *M. quizhouense* and *B. bassiana* being capable of growing and sporulating at the very high temperature of 35°C in vitro. The isolate of *M. anisoplia* had an optimum growth temperature of 27.5°C whilst *C. rosea* was found to have an optimum sporulation temperature of 22.5°C to 32.5°C.

Successful fungal infection begins with rapid spore germination and hyphal growth to infect and colonise the insect host (82,164–167). SEM of *O. lybicus* adults treated with the *B. bassiana*, *M. anisopliae*, *C. rosea*, and *M. quizhouense*

revealed detailed information about the spore adhesion potential and hyphal penetration structures on the cuticle of *O. lybicus* adults. Spore adhesion, and germination as rapid as 2 hpi, was first seen with *C. rosea*, followed by *M. anisopliae* and *M. guizhouense* at 6-12 hpi, and lastly by *B. bassiana*. *Beauveria bassiana* and *M. anisopliae* are known to produce asexual spores with hydrophobic surface allowing the attachment to the insect cuticle to maintain close contact for germination and subsequent invasion (271). Appressorium is the specialized infectious structure that penetrates the cuticle and produces hyphae in the insects hemolymph (81,272). Here was clearly seen with *B. bassiana* and *M. anisopliae*; however, no appressoria formation was seen with *C. rosea*, and *M. guizhouense*. Penetration is further aided by digestive enzymes such as lipases, proteases, and chitinases to facilitate nutrition from the insect integument (158). In our study, external hyphal networks by *C. rosea* was visible at 12-36 hpi, and slower at 36-48 hpi with the other fungi. All tested fungi formed dense mycelial growth and sporulated on the insect cuticle at 5-7 dpi. Pathogenic fungal hyphae of *B. bassiana* can spread into the internal tissues and evade the host immune system when they reach the insects nutrient-rich hemolymph (273,274). *Beauveria bassiana* and *M. anisopliae* are also known to produce secondary metabolites, including toxins such as beauvericin, bassianolide, bassianin, beauverolides, tenellin, beauveriolides, oosporein, oxalic acid, destruxin and bassiacridin, during the colonization to aid virulence, causing nutrient depletion and leading to host death (70,180,275,276).

In the present study, the *B. bassiana* was most effective in achieving a rapid mortality rate against *O. lybicus* adults, with 93.4% within 3 dpi, in contrast to *C. rosea* causing 27.2% mortality after 3 dpi. *Metarhizium. guizhouense* and *M.*

anisopliae ranked in the middle with a mortality rate of 59.8% and 49.6% after 3 dpi and 94.7% and 93.5% after 7 dpi, respectively. In another study, a 100% mortality rate of third instar nymphs of *O. lybicus* was obtained by Aminaee et al. (195) using a native *B. bassiana* isolated from Iran. The study was performed in an environmental chamber set at 26°C and 80% relative humidity at a conidial concentration of 1×10^8 conidia/ml (195). *Clonostachys rosea*, and *M. guizhouense* were able to spread to the whole *O. lybicus* adult parts within 36 hpi and start producing spores after 5 dpi. A few studies have reported the virulence of *C. rosea*, and *M. guizhouense* against insects (277,278) and to best of our knowledge, there are no studies that addressed their mode of action on insect hosts. Therefore, further studies are required on the mode of action of these two fungi on insect hosts.

3.5 Conclusion

This study showed the natural distribution and abundance of native EPF in Omani soils for the first time, providing a guide for future isolate selection programs. A total of thirteen EPF species from the genera *Metarhizium*, *Clonostachys*, *Fusarium*, *Aspergillus*, and *B. bassiana*, were obtained from this study. These fungi were known for their pathogenicity against many insects. Insect-parasitic nematodes were identified as *H. indica*, *P. goodeyi*, *A. saeedi*, *Mesorhabditis* sp., and *O. tipulae*. The in vitro characterization of the obtained fungi showed that *B. bassiana* and *M. quizhouense* were able to grow and sporulate at a wide range of temperatures between 25°C to 35°C. Therefore, those EPFs will be tested for their pathogenicity against the different life stages of *O. lybicus* at different spore concentrations and formulations to test for control under various conditions. This study confirmed the adhesion patterns of native

EPF on *O. lybicus* adult cuticle through SEM. Rapid spore germination and hyphal growth of the native EPF were seen after 2 hpi with *C. rosea*, at 6 hpi with *M. guizhouense* and *M. anisopliae*, and 12 hpi with *B. bassiana*. The most promising fungus in this study was *B. bassiana* which could grow and sporulate at an extremely high temperature of 35°C in vitro and was the most effective killing fungus with a 93.4% mortality rate within 3 dpi. Studying the mechanisms of infection and disease by those EPF will be useful to direct their selection as biological control agents as part of Integrated Pest Management (IPM). This study was conducted under laboratory conditions; therefore, further studies on the relationship between the EPF and their surrounding environment would provide information about the influence of temperature, humidity, and sunlight on adhesion and penetration on insect cuticle.

3.6 Supplementary data

Supplementary Table 3.2 Spearman's rank correlation matrix of associations between the number of positive isolates of soil samples and soil variables considered in our study.

All Fungi	1																		
<i>Metarhizium</i> spp.	0.6	1																	
<i>Clonostachys</i> spp.	0.5	0.1	1																
<i>Beauveria</i> spp.	0.3	-0.1	-0.1	1															
<i>Fusarium</i> spp.	0.4	0.1	0.1	-0.1	1														
<i>Aspergillus</i> spp.	0.3	0.1	-0.1	-0.1	-0.1	1													
Nematodes	0.1	0.0	0.2	-0.1	-0.1	-0.1	1												
C:N	0.0	-0.1	0.0	0.1	-0.2	0.1	0.3*	1											
Ca	0.1	0.3*	0.0	0.0	0.0	0.0	0.0	0.1	1										
Cu	0.1	0.0	0.3*	0.0	-0.1	-0.1	0.3*	0.2	0.3	1									
Gravel%	-0.2	-0.1	-0.1	0.2	0.3*	0.3*	0.2	0.1	0.0	0.1	1								
Mg	0.0	-0.1	0.1	0.3*	0.0	0.0	0.0	-0.3	-0.3	-0.1	-0.1	1							
Na	-0.3*	-0.2	0.0	-0.1	-0.1	0.3*	-0.1	-0.1	-0.1	0.0	0.1	0.1	1						
Pb	0.3*	0.2	0.2	0.0	0.2	0.0	0.2	0.2	0.3	0.5	0.1	-0.2	-0.1	1					
PH	0.3*	0.0	0.4***	-0.1	0.2	0.0	0.1	0.1	-0.1	0.3	-0.2	0.2	0.0	0.3	1				
Sand%	-0.3**	-0.1	-0.2	-0.1	-0.1	-0.2	-0.1	-0.2	-0.2	-0.3	0.3	0.0	0.3	-0.4	-0.5	1			
Silt%	0.3**	0.1	0.1	0.1	0.2	0.2	0.1	0.0	0.0	0.1	-0.3	0.2	-0.4	0.3	0.4	-0.9	1		

* $p < .05$, ** $p < .01$, *** $p < .001$

Supplementary Table 3.3 Characterization of entomopathogenic fungi isolates for mycelial radial growth and sporulation at 26°C. Differences between isolates was subject to General Analyses of variance ANOVA at 95% confidence level followed by standard error.

Entomopathogenic fungi	Species Code	Spores/ml	<i>p</i> -value ± <i>SE</i>	Fungal growth rate cm/days							
				4	7	10	14				
				<i>p</i> -value ± <i>SE</i>	<i>p</i> -value ± <i>SE</i>	<i>p</i> -value ± <i>SE</i>	<i>p</i> -value ± <i>SE</i>	<i>p</i> -value ± <i>SE</i>			
<i>Beauveria bassiana</i>	BeAm03	7E+09	<.001 ±7E08	1.275	<.001 ±0.1	2.25	<.001 ±0.1	3.113	<.001 ±0.3	3.875	<.001 ±0.3
<i>Beauveria bassiana</i>	BeHa02	5E+09		1.562		2.262		3.038		3.663	
<i>Clonostachys buxi</i>	ClBi10	3E+09		1.887		3.012		4.013		5.905	
<i>Clonostachys swieteniae</i>	ClFa11	2E+08		2		3.575		4.85		6.838	
<i>Clonostachys rosea</i>	ClNa01	6E+09		2.188		3.775		5.2		6.475	
<i>Clonostachys rosea</i>	ClNa04	5E+07		2.012		3.487		4.725		5.95	
<i>Clonostachys swieteniae</i>	ClNa09	1E+07		1.7		3.025		3.888		5.525	
<i>Clonostachys swieteniae</i>	ClSa03	7E+07		1.85		3.475		4.638		6.313	
<i>Metarhizium quizhouense</i>	MeBo17	1E+09		1.662		2.7		3.825		5.313	
<i>Metarhizium anisopliae</i>	MeFa04	2E+09		1.862		3.35		4.363		6.138	
<i>Metarhizium anisopliae</i>	MeFa05	7E+09		1.912		3.525		4.825		6.4	
<i>Metarhizium anisopliae</i>	MeFa11	2E+09		1.587		2.95		3.9		5.413	
<i>Metarhizium anisopliae</i>	MeFa18	3E+09		1.875		3.325		4.663		6.125	
<i>Metarhizium anisopliae</i>	MeHa16	3E+09		1.65		3.025		4.238		5.913	
<i>Metarhizium robertsii</i>	MeHu01	4E+09		2.012		3.575		4.813		6.125	
<i>Metarhizium quizhouense</i>	MeHu06	-6E+07		2.462		4.35		5.963		7.923	
<i>Metarhizium robertsii</i>	MeHu10	2E+09		1.975		3.037		3.738		4.663	
<i>Metarhizium quizhouense</i>	MeNa02	8E+08		1.387		2.125		3.1		4.025	
<i>Metarhizium quizhouense</i>	MeNa03	8E+08		1.5		2.325		3.013		3.975	
<i>Metarhizium quizhouense</i>	MeNa07	1E+09		1.462		2.325		3.275		4.163	
<i>Metarhizium anisopliae</i>	MeNi13	5E+09		1.825		3.3		4.838		6.625	
<i>Metarhizium anisopliae</i>	MeNi14	4E+09		1.65		3.05		4.4		5.675	
<i>Metarhizium anisopliae</i>	MeNi15	2E+09		1.837		3.275		4.55		5.988	

Chapter 4:

Chapter 4: Effectiveness of Native and Commercial Entomopathogenic Fungi Against The Dubas Bug (*Ommatissus lybicus* De Bergevin) on Date Palm in Oman

Abstract

The current study was conducted to evaluate the efficacy of native and commercial entomopathogenic fungi (EPF) as an alternative strategy for the sustainable control of *Ommatissus lybicus* and determine the influence of the native fungi on their virulence against *O. lybicus* adults. Four native EPF *Metarhizium guizhouense*, *Metarhizium anisopliae*, *Clonostachys rosea*, and *Beauveria bassiana* isolated from Omani soil and 3 commercial products Botanigard 22WP *B. bassiana* strain GHA, Bio-Power *B. bassiana*, and Bio-Catch *Verticillium lecanii* were evaluated for their efficacy against *O. lybicus* eggs, nymphs, and adults. The pathogenicity test was performed for the native fungi against *O. lybicus* contained in Petri dishes and inoculated using two concentrations of 1×10^6 and 1×10^8 spores/ml. The same native fungi were then tested as spray treatments against *O. lybicus* on palm seedlings. Furthermore, the native fungi *C. rosea* and the commercial strain of *B. bassiana* GHA were Tested against *O. lybicus* eggs. Also, the impact of the relative humidity on the pathogenicity of the native fungi *M. anisopliae* and *B. bassiana* against the *O. lybicus* adults was evaluated. The results indicated that the native EPF efficacy against *O. lybicus* hosted in palm seedlings varied significantly. The effect of the fungal pathogens against *O. lybicus* increased as the insects grew up and the fungal spore concentration increased. The highest mortality rate of insects hosted

on palm seedlings was obtained with *B. bassiana* at the *O. lybicus* adult stage with 76.7%, followed by *M. guizhouense* and *M. anisopliae* with mortality rates of 65%, after 2 weeks of inoculation. Botanigard 22WP was the best commercial formulation used against *O. lybicus* eggs, nymphs, and adults with a mortality rate of 90.8% and 84.2% at the 1st and 2nd instars, and 100% at the 3rd, 4th, 5th, and adult stages after 7 days of inoculation. *Clonostachys rosea* along with Botanigard 22WP significantly affected the *O. lybicus* egg hatching with 74.7% and 75.8% unhatched eggs, respectively. The mortality rate of the two native pathogenic fungi, *B. bassiana* and *M. anisopliae*, against *O. lybicus* adults was significantly high when incubated at a relative humidity of 50% and above. The native and commercial EPF showed their capability to infect *O. lybicus* eggs, nymphs, and adults. The commercial strain Botanigard had a high potential in infecting all the *O. lybicus* stages. The native pathogenic fungi could successfully infect *O. lybicus* adults with 100% mortality rate at a relative humidity ranged between 50% to 90%. The high efficacy of the pathogenic fungi in the current study indicates that they can be effective biological control agents against *O. lybicus*.

4.1 Introduction:

Date palm (*Phoenix dactylifera* L.) is a crop of economic significance in arid and semi-arid regions of the world (279). Around the world there are more than 100 million date palms (280,281) consisting of more than 3000 different cultivars (5) grown as cash crops (282). Date palm fruit is considered an essential component of the diet and a staple food in North Africa and the Middle East (283,284). The Dubas bug (*Ommatissus lybicus* De Bergevin) (Hemiptera: Tropiduchidae) is one of the most important insect pests of date palm capable of causing 50% yield loss ((97). The Dubas bug is a bivoltine, monophagous insect with both nymphs and adults feeding on the host and producing large amounts of honeydew (285–287). The honeydew accumulates on leaves leading to the growth of the saprophytic fungi sooty mould *Meliola camellia* (23,24). *Ommatissus lybicus* infestations on date palms have been reported in Oman, Saudi Arabia, United Arab Emirates, Iraq, Egypt, Yemen, Israel, Pakistan, and Qatar (40,52,105).

Insecticides are the primary means of controlling *O. lybicus* in Oman (3,42) and field populations of *O. lybicus* have been confirmed to have developed resistance to the synthetic insecticides fenitrothion and deltamethrin in Oman Khan et al. (98). The development of pesticide resistance has prompted the research and adoption of alternative control methods including the use of fungal entomopathogens (288,289). Usage of entomopathogenic fungi (EPF) as a biological control agents is considered to be environmentally friendly and a safer alternative to synthetic insecticides (290). EPF invade insects once spores adhere to the cuticle and germinate when conditions are suitable. Following germination, emerging germ tubes penetrate the insect cuticle using a

combination of mechanical force and digestive enzymes to enter the body (82,291). Relative humidity is an essential factor for fungal development (85), and can be an important environmental constraint to fungal infectivity (292). Different fungal species differ in their humidity requirements; for example, *Entomophthora muscae* can infect adult Diptera at relative humidity of 40-60% (85,293), while *Aschersonia aleyrodalis* Webber requires 100% humidity for 16 hours to invade whiteflies at leaf level (294).

Species of EPF including *Beauveria bassiana*, *Isaria fumosorosea* and *Metarhizium brunneum* are commercially available as biopesticides against arthropod pests in various cropping systems (295). (296) tested four isolates of *B. bassiana* (two isolates obtained from Iraq and two from Jordan) and one isolate of *Lecanicillium* (synom. *Verticillium*) *lecanii* (obtained from Jordan) against *O. lybicus* eggs, nymphs, and adults. Isolates of both species applied at a concentration of 8×10^7 spores/ml to eggs achieved more than 93% mortality of adults and the first, third, and fifth nymphal instars of the pest. Although more than 700 arthropod host species have been recorded to be naturally infected with EPF (219,297,298), there are no studies on the isolation and use of native EPF in Oman against *O. lybicus*. In this study, we determined the effectiveness of native to Oman EPF and compared these native agents to commercial EPF against *O. lybicus* to determine the most effective products available for pest management programs to reduce the use of synthetic insecticides. The objectives of this study were i) to determine the pathogenicity and aggressiveness of native and commercially available EPF against *O. lybicus* eggs, nymphs and adults, ii) to determine the impact of relative humidity on the performance of native isolates against *O. lybicus* adults.

4.2 Materials and Methods

4.2.1 Entomopathogenic fungal species source and preparation

Four EPF *Beauveria bassiana* (BeHa02), *Metarhizium anisopliae* (MeFa05), *Clonostachys rosea* (ClNa1), and *Metarhizium guizhouense* (MeBo17) were obtained from Omani soils from a previous survey (Chapter 3). Each single spore isolate was maintained for short term storage on Potato Dextrose Agar (PDA, Oxoid) slants and was sub-cultured onto sterilized Petri dishes plates with PDA to raise cultures for inoculation. Cultures were incubated at 26°C for 14 days. Spores were harvested by adding 10 ml sterilized water with tween-80 (0.05%) solution to the growth medium. Spores were released using a flat spatula, and the resulting mycelia and spore suspension was sieved through muslin. Spore suspension concentrations for each fungus were adjusted to two concentrations 1×10^6 spores/mL and 1×10^8 spores/mL in 0.05% Tween-80 using a hemocytometer (Brand, Wertheim, Germany).

All *O. lybicus* nymphs and adults used in this study were obtained from infested date palm orchards (23°21'19.7"N 57°38'07.7"E).

4.2.2 Pathogenicity of commercial and native EPF against nymphs and adults of *O. lybicus*

The four native entomopathogenic fungal strains of *B. bassiana*, *C. rosea*, *M. anisopliae*, and *M. guizhouense*, previously characterized in Chapter 3, were assessed for pathogenicity against *O. lybicus* nymphs and adults at two spore concentrations of 1×10^6 or 1×10^8 spores/ml. The first study was designed to host the insects in Petri dishes, whilst a second study was performed by hosting the insects on palm seedlings.

The commercial products, Botanigard 22WP, with *Beauveria bassiana* strain GHA produced by BioWorks, Inc., USA, Bio-Power with *B. bassiana*, and Bio-Catch with *Verticillium lecanii* produced by the Indian company T. Stanes & Company Limited were evaluated for the control of the Dubas bug. Commercial formulations were diluted in distilled water and used at the recommended application rate by the manufacturing company (Table 4.1).

Table 4.1 Commercial entomopathogenic fungi and their application rate used against *O. lybicus*

Trade Name	Fungi Name	Application rate	Producing Company
Botanigard 22WP	<i>B. bassiana</i>	2.4g/L	BioWorks, Inc.
Bio-Power	<i>B. bassiana</i>	8g/L	T. Stanes
Bio-Catch	<i>V. lecanii</i>	8g/L	T. Stanes

In the first study ten insects were enclosed in Petri dishes (90 mm) containing double filter papers. Two ml of each fungal treatment were pipetted onto the Petri dishes, and a 2 ml sterilized water tween-80 (0.05%) solution was used as control treatment. The insects were incubated at 25°C and 75% relative humidity in a growth chamber (Panasonic: MLR-352H). In the second study ten insects were hosted on six months old palm seedlings enclosed in a ventilated transparent plastic cup. The insects were sprayed with the four native EPF strains using a 1 L hand sprayer, and the control treatment was sprayed with sterilized water tween-80 (0.05%) solution. Insect mortality was inspected after 7 dpi in the first study and at 7 and 14 dpi in the second study. Dead insects were collected with a sterilised fine brush and rinsed with distilled water to remove any attached spores, and then placed on Petri dishes containing moistened filter

papers to detect and stimulate the growth of fungi. Random dead *O. lybicus* were placed on PDA agar for further confirmation of the presence of the fungi. The presence of fungi was confirmed using a light microscope (195). The experiments contained ten insects for each treatment, replicated six times, and the experiments were repeated twice.

4.2.3 Effectiveness of EPF against eggs of *O. lybicus*

A native isolate of *C. rosea* and a commercial product of *B. bassiana* (Botanigard 22wp), were tested for their efficacy against *O. lybicus* eggs. *Clonostachys rosea* was chosen as is known to effectively reduce insect fecundity (299) whilst *B. bassiana* in the commercial formulation was used as the control. Twenty *O. lybicus* adults (10 males and 10 females) were confined in two years palm seedlings using a ventilated transparent container for egg oviposition. The seedlings were kept outdoor in a shade house for one month and were removed after two months. The oviposited eggs were sprayed using a 1L hand sprayer with a dose of 1×10^8 spores/ml for *C. rosea* and 1×10^{13} spores/ml for Botanigard 22wp as recommended dose. The hatched and unhatched eggs were calculated after most of the nymphs of *O. lybicus* reached the fifth instar to avoid the oviposition of new eggs. Each treatment was replicated ten times in a randomized block design, and the experiment was repeated twice.

4.2.4 Effect of humidity on the virulence of *B. bassiana* and *M. anisopliae*

The effect of humidity on the virulence of two native EPF, *B. bassiana* and *M. anisopliae*, was tested in this study against *O. lybicus* adults. These two fungi are the most common commercially available fungi (300). Ten *O. lybicus* were placed in seedling palms planted in small pots (0.5 L) and covered with a

ventilated transparent plastic cover. The *B. bassiana* and *M. anisopliae* spores suspended in sterilized water tween-80 (0.05%) solution at a concentration of 1×10^6 spores/ml were applied to the insects using a 1L hand sprayer, and the control was sprayed using sterilized water tween-80 (0.05%) solution. The insects were then placed into growth chambers set to constant temperature (26°C) and five different humidity (50%, 60%, 70, 80%, and 90%) and 12:12 light to dark cycle. Each treatment contained four replicates. The *O. lybicus* mortality rate was inspected after 7 days post inoculation (dpi). The experiment was repeated twice.

4.2.5 Data analysis

Statistical analysis was performed using Genstat 20th edition. *Ommatissus lybicus* mortality rate was corrected by Abbot's formulae and presented as a percentage reduction of *O. lybicus*. General ANOVA and the least significant difference at $p < 0.05$ were used to compare means between treatments.

4.3 Results

4.3.1 Effect of native EPF and commercial products on adults and nymphs of *O. lybicus* hosted in Petri dishes

Four native EPF were tested for their effectiveness in controlling *O. lybicus* adults and nymphs in Petri dishes using two concentrations of 1×10^6 and 1×10^8 spores/ml. At the concentration of 1×10^6 spores/ml, the results showed that there were significant differences ($p < 0.001$, Table 4.2) in the mortality of the first instars after 3 dpi, and no significant differences were observed after 7 dpi ($p > 0.05$, Table 4.2). The highest mortality rate of 47.8% was recorded for *M. guizhouense* whilst the lowest mortality rate of 16.9% was observed with *C.*

rosea after 7 dpi. The mortality rate of the second and third instars was significantly different after 3 ($p < 0.05$) and 7 dpi ($p < 0.01$, Table 4.2). *Metarhizium guizhouense* achieved the highest mortality rates of 82.3% and 93.5% of second and third instars, respectively. *Metarhizium anisopliae* was observed to have a significantly higher mortality rate of 67.7% and 90.1% of the fourth instar after 3 and 7 dpi, respectively ($p < 0.001$, Table 4.2), whilst the *O. lybicus* mortality of the fifth instar and adults after 7 dpi with different fungal species was not significantly different between them ($p > 0.05$, Table 4.2). The *O. lybicus* mortality rate of the adult stage was significantly high between the fungi 3 dpi, with 59.6% achieved by *B. bassiana* (93.7%), whilst the *C. rosea* was the least effective fungi with 37.8% ($p = 0.01$, Table 4.2).

Table 4.2 Mortality of *O. lybicus* inoculated with native entomopathogenic fungi at the concentrations of (1×10^8) and (1×10^6) hosted in Petri dishes. General ANOVA and the least significant difference at $p < 0.05$. Abbott's correction was applied to mortality data prior to analysis.

Dose (spores /ml)	Instar	Mortality % (days following inoculation)											
		<i>B.</i> <i>bassiana</i>	<i>C.</i> <i>rosea</i>	<i>M.</i> <i>anisoplia</i> <i>e</i>	<i>M.</i> <i>guizhou</i> <i>ense</i>	<i>p</i> value	LSD	<i>B.</i> <i>bassiana</i>	<i>C.</i> <i>rosea</i>	<i>M.</i> <i>anisoplia</i> <i>e</i>	<i>M.</i> <i>guizho</i> <i>uense</i>	<i>p</i> value	LSD
		3 days						7 days					
10^6	1	46.5	16.9	33.4	47.8	<.001	15.2	65.2	72.7	66.3	71.1	0.89	23.2
	2	26.4	11.8	33.6	43.6	0.04	21.6	80.5	50.3	72.6	82.3	0.004	18.3
	3	54.0	37.2	55.8	64.8	0.02	17.1	75.0	52.2	90.2	93.5	<.001	14.9
	4	46.8	20.0	67.7	43.0	<.001	16.1	81.4	55.0	90.1	72.6	<.001	12.0
	5	55.6	41.7	53.7	39.8	0.15	17.0	77.3	73.9	80.7	83.0	0.66	15.6
	Adults	59.6	37.8	53.1	43.3	0.01	13.4	93.7	88.4	91.4	89.4	0.58	8.4
10^8	1	25.7	32.1	32.1	23.2	0.649	17.5	77.6	80.9	76.3	85.4	0.63	15.2
	2	65.2	38.6	54.0	67.1	0.002	15.4	84.0	69.2	74.5	90.4	0.004	11.7
	3	35.9	15.6	46.6	39.3	0.03	20.6	94.6	88.4	94.6	98.9	0.10	8.3
	4	83.2	41.1	69.1	59.7	<.001	17.1	97.9	96.8	91.4	92.5	0.17	6.8
	5	84.6	31.2	79.2	55.5	<.001	19.8	97.7	76.9	92.9	100.0	<.001	6.0
	Adults	93.4	27.2	49.6	59.8	<.001	15.3	96.8	89.2	93.5	94.7	0.101	6.1

The mortality rate of the *O. lybicus* first instar treated with the four native EPF at the concentration of 1×10^8 spores/ml hosted in Petri dishes had no significant differences after the third and the seventh dpi ($p > 0.05$, Table 4.2). A significant difference between the treatments was observed for the second *O. lybicus* instar after the third and the seventh dpi ($p < 0.01$, Table 4.2). *Metarhizium guizhouense* and *M. anisopliae* showed significantly higher mortality rate of 67.1% and 46.6% of second and third *O. lybicus* instars, respectively, 3 dpi, whilst *B. bassiana* achieved significantly higher *O. lybicus* mortality rate of 83.2%, 84.6%, and 93.4% of fourth, fifth, and adult stages, respectively, 7 dpi ($p < 0.001$, Table 4.2). *Clonostachys rosea* had the lowest efficacy against *O. lybicus*

nymphs and adults with 32.1%, 38.6%, 15.6%, 41.1%, 31.2%, 27.2% at the 1st, 2nd, 3rd, 4th, 5th, and adult stages, respectively, 3 dpi. *Metarhizium guizhouense* achieved the highest mortality rate of 85.4%, 90.4%, 98.9%, and 100.0% of the 1st, 2nd, 3rd, 5th *O. lybicus* instars, respectively, 7 dpi, whilst *B. bassiana* showed a mortality rate of 97.9% and 96.8% of the 4th and adult stages, respectively, 7 dpi ($p > 0.05$, Table 4.2). The death rate of *O. lybicus* instars inoculated with the native EPF hosted in Petri dishes increased with increasing concentration from 1×10^6 to 1×10^8 spores/ml. *Clonostachys rosea* exhibited the highest increase in the mortality rate of *O. lybicus* stages with up to 42%, due to dosage increase up to 1×10^8 spores/ml, whilst the lowest increase of up to 12% on the mortality rate of *O. lybicus* stages was obtained *M. anisopliae*. *Ommatissus lybicus* instars inoculated with a higher dose of *M. guizhouense* and *B. bassiana* achieved a higher mortality rate of up to 20%.

Three commercial fungal products were tested for their efficacy against *O. lybicus* hosted in Petri dishes. Botanigard 22wp demonstrated the highest effectiveness against *O. lybicus* stages, with a mortality rate between 42.2% to 95.8% 3 dpi ($p < 0.001$, and $p < 0.05$ at the adult stage, Table 4.3) and 100% mortality rate after 7 dpi ($p < 0.001$, and $p < 0.01$ at the third instar, Table 4.3) except at the fourth stage, where the mortality rate was 98.9%. Bio-power was the least effective product, with mortality rate at the 1st, 2nd, 3rd, 5th, and adult stages with 41.9%, 37.9%, 77.3%, 47.9%, and 80.3% mortality, respectively, after 7 dpi (Table 4.3).

Table 4.3 Mortality of *O. lybicus* inoculated with commercial entomopathogenic fungi hosted in Petri dishes at 3 and 7 days post inoculation. General ANOVA and the least significant difference at $p < 0.05$. Abbott's correction was applied to mortality data prior to analysis.

Instar	Mortality % (days following inoculation)									
	Bio-Catch	Bio-power	Botanigard 22wp	<i>p</i> value	LSD	Bio-Catch	Bio-power	Botanigard 22wp	<i>p</i> value	LSD
	3 days					7 days				
1	-1.7	-1.7	95.8	<.001	3.6	58.1	41.9	100.0	<.001	7.2
2	2.5	14.4	91.5	<.001	9.4	47.7	37.9	100.0	<.001	10.3
3	55.8	37.4	85.3	<.001	12.7	87.5	77.3	100.0	0.004	12.7
4	47.1	19.6	62.7	<.001	14.8	64.9	69.2	98.9	<.001	11.6
5	8.4	1.9	42.2	<.001	11.0	52.2	47.9	100.0	<.001	8.0
Adults	76.0	61.5	92.2	0.013	19.7	92.8	80.3	100.0	<.001	9.5

4.3.2 Effectiveness of native and commercial EPF against *O. lybicus* hosted on palm seedlings

Native EPF were evaluated for their efficacy against *O. lybicus* nymphs and adults hosted in palm seedlings. The *O. lybicus* mortality of the first instar was not significant between the different treatments after 7 or 14 dpi ($p > 0.05$, Table 4.4), although *M. anisopliae* and *B. bassiana* showed 54.2% and 52.5% mortality 14 dpi. *Metarhizium guizhouense* achieved a significantly higher mortality of the second and third instars of 55.0% and 53.3%, respectively 14 dpi ($p < 0.001$, Table 4.4). There were no differences between treatments for fourth instar ($p > 0.05$, Table 4.4); however, *B. bassiana* caused the highest mortality of 57.5% after 14 dpi. *Metarhizium anisopliae* achieved significantly higher mortality of 75.0% 14 dpi of the fifth stage compared to comparing to the other treatments. *Beauveria bassiana* had the highest mortality rate at the adult stage with 76.7%

($p < 0.01$, Table 4.4) after 14 dpi. *Clonostachys rosea* was the least effective fungus against *O. lybicus* nymphs and adults comparing to the other fungi used in this study, except with the fourth *O. lybicus* stage where *M. guizhouense* had the the lowest mortality rate, with 43.3% after 14 dpi.

Table 4.4 The weekly death rate of *O. lybicus* inoculated with native entomopathogenic fungi (1×10^6) hosted in seedlings. General ANOVA and the least significant difference at $p < 0.05$. Abbott's correction was used to mortality data prior to analysis.

Instar	Mortality % (days following inoculation)											
	<i>B. bassiana</i> <i>a</i>	<i>C. rosea</i>	<i>M. anisopliae</i>	<i>M. guizhouense</i> <i>e</i>	<i>p</i> value	LS D	<i>B. bassiana</i>	<i>C. rosea</i>	<i>M. anisopliae</i> <i>e</i>	<i>M. guizhouense</i> <i>e</i>	<i>p</i> value	LSD
	7 days						14 days					
1	31.7	27.5	30.0	30.8	0.649	9.82	52.5	44.2	54.2	51.7	0.103	12.07
2	32.5	25.8	25.0	30.0	0.263	12.2	49.2	30.0	43.3	55.0	<.001	16.1
3	28.3	20.8	24.2	29.2	0.255	13.2	42.5	29.2	42.5	53.3	0.001	15.9
4	30.8	29.2	25.0	30.8	0.523	12.8	57.5	50.8	49.2	43.3	0.197	18.5
5	40.8	46.7	44.2	41.7	0.679	15.0	57.5	55.0	75.0	56.7	<.001	13.4
Adults	65.8	32.5	33.3	44.2	<.001	15.8	76.7	55.8	65.0	65.0	0.002	14.3

Two commercial EPF products (Bio-power and Botanigard 22wp) were tested for their efficacy against *O. lybicus* hosted in palm seedlings. Botanigard 22wp was superior to Bio-power and showed significantly higher mortality rate against *O. lybicus* nymphs and adults, with 90.8% and 84.2% mortality at the 1st and 2nd stages after 7 dpi, and with 93.3% and 86.7% mortality after 14 dpi, respectively. Botanigard 22wp showed 100% mortality rate the third *O. lybicus* instar onward after 7 and 14 dpi (Table 4.5).

Table 4.5 The death rate of *O. lybicus* inoculated with commercial entomopathogenic fungi hosted in seedlings. General ANOVA and the least significant difference at $p < 0.05$. Abbott's correction was used to mortality data prior to analysis.

Instar	Mortality % (days following inoculation)							
	Bio-power	Botanigard 22wp	<i>p</i> value	LSD	Bio-power	Botanigard 22wp	<i>p</i> value	LSD
	7 days				14 days			
1	66.7	90.8	0.008	23.2	67.5	93.3	0.007	24.1
2	55.0	84.2	<.001	17.0	59.2	86.7	<.001	18.5
3	40.8	100.0	<.001	13.1	53.3	100.0	<.001	14.8
4	31.7	100.0	<.001	19.0	40.8	100.0	<.001	21.6
5	39.2	100.0	<.001	10.1	51.7	100.0	<.001	16.9
Adults	58.3	100.0	<.001	9.7	71.7	100.0	<.001	6.9

4.3.3 The effect of native and commercial EPF against *O. lybicus* eggs

The effect of the commercial fungi Botanigard 22wp (*B. bassiana*) and the native fungi *C. rosea* was tested for their efficacy against *O. lybicus* eggs. The results indicated highly significant differences in *O. lybicus* unhatched eggs between the treatments in comparison with the control ($p < 0.001$, Figure 4.1). The highest unhatched egg rate of 75.8% and 74.7% was observed with Botanigard 22wp and *C. rosea*, respectively (Figure 4.1).

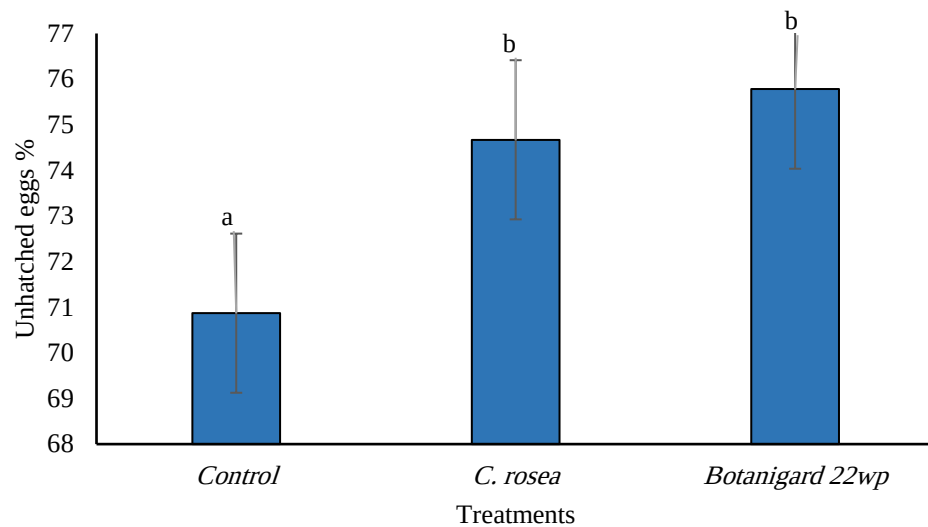


Figure 4.1 The *O. lybicus* unhatched eggs rate of the native fungi *C. rosea* and the commercial fungi Botanigard 22wp (*B. bassiana*) compared to the controlled treatment (no inoculation). General ANOVA test with standard error bars ($p < 0.001$).

4.3.4 The effect of humidity on efficacy of control by *B. bassiana* and *M. anisopliae* against *O. lybicus* adults

Two native fungi were used in this study, *B. bassiana* and *M. anisopliae*, to evaluate their humidity requirement in *O. lybicus* infection. Mortality rates with all *Beauveria bassiana* and *M. anisopliae* treatments were significantly higher than control treatments ($p > 0.001$, Table 4.6), achieving 100% mortality at humidity ranging between 50% to 90% after 7 dpi (Table 4.6). Higher relative humidity above 50% appeared to have lethal effect against *O. lybicus* infection (Table 4.6).

Table 4.6 The death rate of *O. lybicus* inoculated with native entomopathogenic fungi *B. bassiana* and *M. anisopliae* at different humidity. General ANOVA and the least significant difference at $p < 0.05$.

Humidity	Treatments			<i>p</i> value	LSD
	<i>B. bassiana</i>	<i>M. anisopliae</i>	Control		
50	100	100	6.25	<.001	4.423
60	100	100	7.5	<.001	6.55
70	100	100	8.75	<.001	4.961
80	100	100	5	<.001	4.203
90	100	100	10	<.001	4.766

4.4 Discussion:

The study aimed to determine the pathogenicity and aggressiveness of native and commercially available EPF against *O. lybicus* eggs, nymphs, and adults through contact. The results showed that both commercial and native EPF have the potential to control *O. lybicus* eggs, nymphs, and adults. Native EPF used in this experiment were obtained from a survey carried out in date palm orchards infested with *O. lybicus*. The highest *O. lybicus* mortality rate was obtained by *M. anisopliae* against the first and fifth instars hosted in seedlings. The second and third instars were best treated by *M. guizhouense*, and *B. bassiana* gave the best control against the fourth and adult stage. The commercial strain of *B. bassiana* and the local *C. rosea* isolate showed a potential effect on *O. lybicus* eggs. Overall, *B. bassiana* showed the best results against *O. lybicus* late instars hosted in seedlings, when applied at a dose of 1×10^6 spores/ml, whilst *M. guizhouense* was best against the early instars.

Infection of *O. lybicus* adults by EPF was more successful than infection of nymphs. Interaction between the insect integument infected with fungus and nymph stage ecdysis could be responsible for the differential susceptibility at various life stages (301). Ecdysis is an important factor affecting fungal infection, mainly when the time interval amid successive molting is short (302,303). Molting in *O. lybicus* take from 5 to 10 days, depending on the insect instars and the atmospheric conditions (1). However, the performance of all native fungi was best when inoculation took place in Petri dishes at 75% relative humidity achieving a mortality rate of more than 90% at the concentration of 1×10^8 of the isolates of *B. bassiana*, *M. anisopliae*, and *M. guizhouense*. Fungal performance was reduced when sprayed on seedlings. This could be due to lower dose 1×10^6 and humidity, as room humidity fluctuated between 25% to 40%. Aminaee et al. (195) obtained *B. bassiana* from naturally infected *O. lybicus* collected from infested date palms in Iran. The *B. bassiana* strain used in their study achieved 100% mortality of third instar nymphs of *O. lybicus* at a conidial concentration of 1×10^8 conidia/ml within 6.5 days in an environmental chamber set at 25°C and 80% relative humidity (195). Jassim et al. (296) studied four *B. bassiana* isolates and one isolate of *L. lecanii* obtained from Iraq and Jordan against *O. lybicus* nymphs and adults under lab conditions. The highest mortality rate was achieved after applying the four isolates of *B. bassiana* and *L. lecanii* isolates against *O. lybicus* adult and the first, third, and fifth nymphal instars at the high spore concentration of 8×10^8 (296).

The commercial formulation of *B. bassiana* (Botanigard 22wp) gave the best mortality rate against *O. lybicus* nymphs and adults, with a mortality rate of 100% after 7 dpi. Botanigard 22wp was found to be effective against *Bactericera*

cockerelli (Sulc), a key pest of solanaceous crops with an average nymph mortality rate of 82% in laboratory trials (304). Parker et al. (305) tested a lower concentration of 1×10^7 against second instar *Halyomorpha halys* (Stål) under controlled laboratory conditions scoring 100% mortality after 12 dpi. An approach by using the endophytic *B. bassiana* extracted from date palm tissues was developed by Khudhair et al. (306). They tested two strains, *B. bassiana* strains isolated from date palms and one strain isolated from soil. The three isolates were injected into palm trunks using a concentration of 1×10^9 conidia/mL, and the *O. lybicus* nymph mortality rates reached 92%, 96% and 100% after 15 dpi (306).

The results of the mortality rate of the commercial strain of *B. bassiana* and the native one *C. rosea* against *O. lybicus* eggs showed some effect. *Ommatissus lybicus* egg is a legitimate target for biological control associated mainly with control by the egg parasitoids such as *Pseudoligosita babylonica* Viggiani (3,307). This study indicates for the first time the pathogenicity of *C. rosea* and the commercial product Botanigard 22wp against *O. lybicus* eggs. Also, Jassim et al. (296) obtained promising results after using four *B. bassiana* isolates and one isolate of *L. lecanii* against *O. lybicus* eggs. A high mortality rate was obtained after applying both EPF on eggs at a concentration of 8×10^7 , in which the mortality rate at the *B. bassiana* isolates ranged between 93.7% and 97.9% after 18 dpi. The highest egg infection rate at *L. lecanii* was at the concentration of 8×10^7 with 97.9% after 18 dpi (296). A study was carried out by Samuels et al. (308) on the virulence of *B. bassiana* and *M. anisopliae* isolates on the eggs of the chinch bug *Blissus antillus* using two conidial concentrations of 1×10^4 and 5×10^8 conidia/ml. They found that *B. bassiana* strains had less or no effect

on the *B. antillus* eggs, while *M. anisopliae* isolates had a high infection rate at both concentrations reaching an infection rate of 96.7%. Furthermore, Permadi et al. (309) tested the pathogenicity of nine isolates of *B. bassiana* and *Metarhizium sp.* strains against Green ladybug *Nezara viridula* L. eggs. They found that all the isolates were pathogenic to *N. viridula*, and the lowest egg hatching rate was found at the *Metarhizium sp.* (MetTrP), and *B. bassiana* isolates with a reduction in egg hatching rate of 0% and 10%, respectively (309). These results indicate a possible new approach to the control of *O. lybicus* by targeting their eggs which are available all the year. More studies on the pathogenicity of EPF against *O. lybicus* eggs are needed, and pathogenicity needs to be confirmed through transmission and scanning electron microscopes. Also, fungi penetrations through the egg chorion need to be confirmed as well.

High relative humidity is required in all developmental stages of fungi on most occasions (85). Previous research indicated that the *B. bassiana* pathogenic activity, conidial germination, and its sporulation on fungus-killed cadavers highly depend on moisture conditions (292,310,311). Mishra et al. (312) found that the 100% mortality of housefly adult *Musca domestica* L. was achieved at 90 and 100 % relative humidity at 30°C when inoculated with *B. bassiana* HQ917687 and only achieved 50% mortality at 50% relative humidity. However, our study indicated that *B. bassiana* and *M. anisopliae* strains used in our study could have 100% mortality after *O. lybicus* adult inoculation at a low humidity of 50%. This could be due to the characteristics that the fungi obtained from, where extremely high temperature and extremely low humidity do exist.

4.5 Conclusion

Our study showed for the first time the efficacy of four native pathogenic fungi *C. rosea*, *M. guizhouense*, *M. anisopliae*, and *B. bassiana* obtained from Omani soil against *O. lybicus* eggs, nymphs, and adults in Oman. Data from the study showed that the commercial strain GHA was shown to be a promising controlling agent against *O. lybicus* infestation that could be adopted for controlling this pest. *Clonostachys rosea* and Botanigard 22WP showed a significant effect in controlling *O. lybicus* eggs. *Beauveria bassiana* and *M. guizhouense* achieved the best results against *O. lybicus* nymphs and adults hosted in seedlings at a dose of 1×10^6 spores/ml. Furthermore, our native fungal isolates of *M. anisopliae* and *B. bassiana* showed promising results when applied to the insects at a relative humidity above 50% in growth chambers. Also, they showed lethal effect against *O. lybicus* adults at higher humidity above 50%. This could be used in date palm plantations at coastal areas and at areas with condense plantation (high density of palms per unit area), where relative humidity relatively is high. Formulating these native fungi would be recommended to enhance their viability and pathogenicity in field, and to avoid the introduction of exotic organisms to a new environment which might affect non-target organisms like honeybees or *O. lybicus* arthropods natural enemies. Entomopathogens are considered safer organisms with minimal risks to humans, invertebrates and the environment. These organisms could be part of an integrated program in *O. lybicus* management. In addition, studies on the effect of entomopathogens on non-targeted organisms such as *O. lybicus* parasitoids and predators and other beneficial insects like honeybees are vital. The current study was conducted in laboratory conditions, therefore, field studies on the

pathogenicity of the native and exotic pathogenic fungi against *O. lybicus* are needed.

Chapter 5:

Chapter 5: General discussion

Ommatissus lybicus is an important insect pest of economic importance infesting date palm trees. The insect produces large amounts of honeydew, which accumulates on leaves and results in dust accumulation and the growth of sooty mould. The pest is capable of causing 50% yield loss. This study showed a significant relationship between different *O. lybicus* population densities and honeydew droplet production and chlorophyll content (Chapter 2). This suggests that medium to high initial infestation of *O. lybicus* of starting nymph populations equating to 3-6 nymphs/leaflet is likely to cause chlorophyll loss of 8-13% which can be implemented to identify potential risk and trigger treatment. In the current study, honeydew secretion was affected by the increase in population density. The *O. lybicus* population density of 600 insects per date palm had a higher level of honeydew secretion in the first two weeks. The removal of large quantities of phloem sap when feeding and producing honeydew can affect the nutritional state of the host palm. (313). The study found clear evidence that *O. lybicus* reduced palm biomass and that this is partly because of the large amounts of honeydew produced.. Roots fresh weight was subject to a reduction of 34% at the population density of 300 insects in the first experiment.

This study found evidence for intraspecific competition in *O. lybicus* because oviposition levels was reduced at higher population densities. *Ommatissus lybicus* females preferentially oviposited their eggs in the rachis and could be predicted from honeydew produced by the adult insects. This finding would allow the prediction of the pest intensity in the following season which in turn

would help decision-makers to determine the best treatment timing and improve pest management in the field. Furthermore, honeydew droplets could indicate potential egg oviposition associated with reductions in fresh weight biomass of the rachis at the end of the season.

The results of this study cast a new light on the effect of *O. lybicus* infestation on the nutritional status of date palms. Date palms exposed to 1000 insects had a significant effect on Mg, P and K content in leaflets, rachises, or roots. The population density of the *O. lybicus* infestation nymphs together with Mg content in leaflets were significantly associated with chlorophyll content suggesting Mg fertilisation under pest attack is vital for improved palm nutrition. Data on the relationship between the infestation level of *O. lybicus* and nutritional losses of date palms would provide information on the *O. lybicus* pest management via rational application of targeted fertilizers to increase host tolerance to the pest in field validation.

The main objective of chapter 3 was to identify and isolate native EPF from Omani soils to be used against *O. lybicus*. The survey yielded 46 EPF fungal species belonging to two orders Hypocreales and Eurotiales, and divided into five genera of *Metarhizium*, *Clonostachys*, *Aspergillus*, *Beauveria*, and *Fusarium*. The majority of the isolates were Hypocreales, which is in line with other EPF surveys (314–316). *Metarhizium* was the most abundant genus with three species of *M. anisopliae*, *M. robertsii*, and *M. quizhouense*, and *Clonostachys* with four species of *C. buxi*, *C. rosea*, *C. swieteniae*, and one unknown species *Clonostachys* sp.. In contrast, *Aspergillus* was the least abundant fungi with three isolates of two species of *Aspergillus cristatus* and *Aspergillus flavus*. In the current study, the relationship between soil

nutrients/traits and EPF abundance was weak, suggesting that other environmental and agronomy factors not included in this study may be involved. Furthermore, five entomopathogenic nematodes were identified through this study as *A. saeedi*, *Mesorhabditis* sp., *O. tipulae*, *H. indica*, and *P. goodeyi*.

EPF obtained through this survey were screened to identify candidates with high sporulation potential, and four fungal isolates: *B. bassiana* (BeHa02), *C. rosea* (ClNa1), *M. anisopliae* (MeFa05), and *M. guizhouense* (MeBo17) were further characterized for their optimum growth temperature. The thermal characterization was carried out to select EFF candidates suitable for Omani conditions of annual average temperature of 27°C (270). The selected EPF strains were able to grow and sporulate in vitro beyond the average annual temperature of Oman (27°C). *Beauveria bassiana* and *M. guizhouense* were capable of growing and sporulating at 35°C in vitro. The isolates of *M. anisopliae* and *B. bassiana* had a wide range of growth temperature between 20-30°C in vitro.

Inoculation of *O. lybicus* using native EPF of *B. bassiana* (BeHa02), *guizhouense* (MeBo17), *M. anisopliae* (MeFa05), and *C. rosea* (ClNa1) allowed the examination by SEM of the pattern and speed of infection. SEM revealed detailed information about spore adhesion and hyphal penetration structures of *C. rosea*, *M. anisopliae*, *M. guizhouense*, and *B. bassiana* on the adult cuticle of *O. lybicus*. Spore adhesion and germination were observed with *C. rosea* as rapidly as 2 hpi, followed by *M. guizhouense* and *M. anisopliae* at 6-12 hpi, and lastly by *B. bassiana*. Appressoria formation was clearly seen with *B. bassiana* and *M. anisopliae* whilst not observed with *C. rosea* and *M. guizhouense*. Hyphal networks formation on *O. lybicus* adults were visible at 12-36 hpi with *C. rosea*,

and with the other fungi slower at 36-48 hpi, and all tested fungi showed dense mycelial growth and sporulation on the insect cuticle at 5-7 dpi.

Despite having rapid spore germination on *O. lybicus* adult cuticle, *C. rosea* was least effective against *O. lybicus* adults causing 27.2% mortality after 3 dpi, in contrast to *B. bassiana* which achieved rapid mortality of 93.4% within 3 dpi. Further studies are required to address the mode of action of the EPF identified in this study on the insect host. This study provides for the first time a pool of diverse pathogenic fungi with shown potential as biocontrol agents against *O. lybicus* for pest management programs in Oman.

The fourth chapter of this study aimed to determine the effectiveness of native (obtained from chapter 3) and commercial EPF against *O. lybicus*. In this study, adult insects were more susceptible to EPF infection than nymphs, which could be due to the molting speed of the nymphs (302,303). When insects were hosted in Petri dishes and on palm seedlings, and all native fungi performed better when inoculation took place in Petri dishes, which could be due to the high humidity rate. A mortality rate of more than 90% was achieved by the isolates of *M. guizhouense*, *B. bassiana*, and *M. anisopliae* against *O. lybicus* hosted in Petri dishes at a concentration of 1×10^8 .

Botanigard 22wp composed of *B. bassiana* was the most effective commercial fungal formulation with a 100% mortality rate against *O. lybicus* nymphs and adults after 7 dpi. This product could undergo field trial evaluation and be used as part of *O. lybicus* pest management, however, enhancing the effectiveness of the native EPF would be best to avoid any disastrous shift to the biological balance from exotic fungal isolates.

The current study showed some effect caused by the commercial product Botanigard 22wp and the native fungi *C. rosea* against *O. lybicus* eggs. *Ommatissus lybicus* eggs are present throughout the year therefore, targeting this susceptible life stage of the pest them would provide a new approach to the control of *O. lybicus*. However, fungi penetrations through the egg chorion need to be demonstrated using scanning electron microscopes.

In this study (Chapter 4), native strains of *M. anisopliae* and *B. bassiana* were evaluated for their optimal humidity against *O. lybicus* adult. The inoculated fungi could achieve a 100% mortality rate against *O. lybicus* adults, which could be due to the high temperature and low humidity conditions in the places where the fungi were obtained from. On most occasions, the developmental stages of fungi require high relative humidity (85). Knowledge of the minimum relative humidity requirements through further studies will establish suitable native fungal strains against the different *O. lybicus* stages under changing environmental conditions.

This study suggests that supplementing Mg with conventional fertilizers could improve pest management to increase palm tolerance and reduce chlorophyll losses under *O. lybicus* infestation. Furthermore, (Chapter 3) a pool of native EPF obtained from Omani soils guides future isolate selection programs for *O. lybicus* management. Finally, this study (Chapter 4) showed the potential of the local isolates *B. bassiana* and *M. anisopliae* strains in controlling 100% of *O. lybicus* adults at a low humidity of 50%, which could be due to the characteristics of the fungi origin where the extremely high temperature and low humidity do exist.

Future work

- Validate any relationships between the *O. lybicus* infestation in lab conditions and nutritional losses of date palms to provide information that would improve crop and pest management via rational application of targeted fertilizers to increase host tolerance to the pest.
- Determine the influence of temperature, humidity, and sunlight on the adhesion and penetration of the cuticle *O. lybicus* by the native fungal candidates obtained from this study.
- Investigate *O. lybicus* metabolic profiles after infection with the native entomopathogenic fungi to identify compounds with insecticidal action.
- Study the influence of different doses of UV light on the sporulation rate of native EPF to *O. lybicus* in vitro.
- Explore the efficacy of the native and commercial EPF against *O. lybicus* under field conditions.

Chapter 6:

Chapter 6: References

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