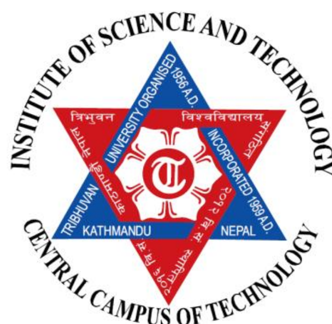
EFFICACY OF ENTOMOPATHOGENIC FUNGUS (*Metarhizium anisopliae*) AGAINST WHITE GRUBS UNDER LABORATORY CONDITION



A

Dissertation Submitted to the **Department of Microbiology**

Central Campus of Technology

Tribhuvan University, Dharan, Nepal

In Partial Fulfillment of the Requirements for the Award of Degree

of Masters of Science in Microbiology

(Agriculture)

By:

Sushila Khadka

Department of Microbiology

Central Campus of Technology, Dharan, Nepal

T.U.Regd.No.5-2-459-9-2011

2019

Tribhuvan University

TRIBHUVAN UNIVERSITY
INSTITUTE OF SCIENCE & TECHNOLOGY
Central Campus of Technology

Phone No.: 025-520228, 025-526530
Post Box No. 4

Department of ....

DHARAN-14, HATTISAR
SUNSARI, NEPAL

Ref. .... Date : ....

RECOMMENDATION

This is to certify that **Ms. Sushila Khadka** has completed this dissertation work entitled **"Efficacy of Entomopathogenic fungus (*Metarhizium anisopliae*) against White Grubs under Laboratory Condition"** as a partial fulfillment of the requirements of M.Sc. degree in Microbiology (Agriculture) under my supervision. To my knowledge, this work has not been submitted for any other degree.

…………………………..

Prince Subba
Teaching Assistant
Department of Microbiology
Central Campus of Technology
Hattisar, Dharan, Nepal

Date: ..../........../............

TRIBHUVAN UNIVERSITY
INSTITUTE OF SCIENCE & TECHNOLOGY
Central Campus of Technology
Phone No.: 025-520228, 025-526530
Post Box No. 4
DHARAN-14, HATTISAR
SUNSARI, NEPAL
Department of ....

Ref. ....   Date : ....

CERTIFICATE OF APPROVAL

On the recommendation of **Mr. Prince Subba** this dissertation work of **Sushila Khadka** entitled **"Efficacy of Entomopathogenic fungus (*Metarhizium anisopliae*) against White Grubs under Laboratory Condition"** has been approved for the examination and is submitted for the Tribhuvan University in partial fulfillment of the requirements for M.Sc. degree in Microbiology (Agriculture).

…………………………	………………………..
Shiv Nandan Sah	Hemanta Khanal
Head of Department	Asst. Prof /Program Co-Ordinator
Department of Microbiology	Department of Microbiology
Central Campus of Technology	Central Campus of Technology
Hattisar, Dharan Nepal	Hattisar, Dharan Nepal

Date: ..../........./................

ACKNOWLEDGEMENTS

Among the wide panorama of people who provided me help and motivation to prepare this dissertation work, I must thank my inspiring and co-operative supervisor, **Mr. Prince Subba,** without his continuous encouragements, inquisitive minds and interchange of ideas, I would not have been able to prepare this dissertation work. His friendly guidance and adroit advice have been invaluable throughout all stages of this work.

I would wish to express my gratitude to my Campus Chief **Professor Dr. Dhan Bahadur Karki**, **Asst. Professor Mr. Hemanta Khanal, Asst. Professor Shiv Nandan Shah**; Head of Department of Microbiology, Central Campus of Technology, and **Mr. Amar Bahadur Pun** ; Director of ARS, Pakhribas, for providing me with the required facilities and instructions for the dissertation work.

I humbly remember all my teachers especially thankful to **Asst. Professor Mr. Suman Rai** and laboratory staff **Ain Bahadur Karki**, **Prajwal Bhandari**, librarian **Mr. Om Khatiwada** and library staff for their great cooperation and help.

I am also thankful to **Mr. Narayan Bhandari** and **Mrs. Bhagawati Shrestha** for providing their field for sample collection during my thesis work.

I wish to express my sincere thanks to my colleagues **Bidhya Dhungana, Kabiraj Khadka, Romika Shrestha, Bijay Shrestha and Jenish Shakya** for their constant encouragements, supports and immense assistances.

Finally, I would like to express my profound gratitude to my dear husband and my beloved family members for their love and continuous supports both spiritually and materially.

………………….

Sushila Khadka

Date: ..../........./................

ABSTRACT

White grub is a polyphagous and nefarious larvae of chafer beetles (Scarabaeidae: Coleoptera) cause damage to wide range of crops of agricultural field. An integrated pest management (IPM) is required to control the white grub using this pest effectively, within which biological control is now beginning to play a key role. Hence the main objective of this study was to isolate entomopathogenic fungi: *Metarhizium anisopliae* and study the insecticidal effect of it against white grubs under maintained laboratory conditions. Overall 100 white grubs were collected from different agricultural field of Paripatle, Dhankuta, Nepal and maintained viable in lab by standard method fedding carrot pieces. Among them 2 white grubs were noticed to be infected with *M. anisopliae* and from each cadavers (died white grub) was isolated and identified by conventional microbiological methods from and indicated as (Ma 1 and Ma 2). Selective media with Streptomycin and Tetracycline antibiotics were used for the isolation of entomopathogens. For insecticidal assay 30 larvae (for each of three replication) were dipped individually into the conidial suspension (10^7 /ml) for five seconds. Efficacy % of Ma1 (54.05) was more than Ma2 (40.52) which was calculated from bioassay record. One way ANOVA shows significant results for FD and SR with treatments (control, Ma1 and Ma2).The result concluded that, there is great possibility that the entomopathogenic fungi could be safe microbial control agents in managing white grubs.

Key words: Bioassay, efficacy, entomopathogenic fungi, White Grubs

TABLE OF CONTENTS

Contents	Page No
Title	I
Recommendation	II
Certificate of Approval	III
Board of examiners	IV
Acknowledgements	V
Abstract	VI
Table of contents	VII-IX
List of abbreviations	X
List of tables	XI
List of figures	XII
List of photographs	XIII
List of appendices	XIV
CHAPTER I : INTRODUCTION AND OBJECTIVES	**1-6**
1.1 Background	1
1.2 Objectives	6
1.2.1 General objectives	6
1.2.2 Specific objectives	6
CHAPTER II: LITERATURE REVIEW	**7- 20**
2.1 Fungi attacking white grubs	7
2.1.1 Entomopathogenic fungi	7
2.1.2 Major characteristics of hyphomycetes	8
2.1.3 Major hosts of insect pathogenic fungi	8
2.1.4 Taxonomy of *Metarhizium anisopliae*	9
2.1.5 Morphology of *Metarhizium anisopliae*	11
2.1.6 Mycotoxins of *Metarhizium anisopliae*	11
2.1.7 Biology of *Metarhizium anisopliae*	12
2.1.7.1 Adhesion	13
2.1.7.2 Pre penetration and germination	13
2.1.7.3 Penitration into hosts	13
2.1.7.4 Mode of infection	13

2.1.7.5 Signs and symptoms 15
2.1.7.6 Saprophytic development of the fungus 15
2.1.8 Environment influence on *Metarhizium anisopliae* 15
2.1.9 Isolation of entomopathogenic fungi 16
2.1.10 Production of fungal spores 16
2.1.11 Enumeration of the spores 17
2.1.12 Storage of the fungal spores 17
2.1.13 Formulation of entomopathogenic fungi 17
2.1.14 Storage of fungus material 17
2.2 White Grubs 18
2.2.1 Morphology 18
2.2.2 Life cycle 19
2.2.3 White grub management 19
2.2.4 Efficacy of *Metarhizium anisopliae* against White Grubs 19
CHAPTER III : MATERIALS AND METHODS 21-26
3.1 Materials 21
3.2 Methods 21
3.2.1 Study site 21
3.2.2 Sample size and types 21
3.3 Sample collection 21
3.4 Rearing of white grubs 21
3.5 Screening of entomopathogens 22
3.5.1 Isolation of entomopathogens 22
3.5.2 Identification of fungus 22
3.5.3 Maintance of culture 23
3.5.4 Preparation of spore suspension 23
3.6 Bioassay of fungal pathogen 23
3.6.1 Mortality of white grubs 24
3.6.2 Survival rate of white grubs 24
3.6.3 Efficacy of *M. anisopliae* (Ma1 and Ma2) 24
3.7 Data analysis 25
CHAPTER IV: RESULTS 27-35
4.1 Screening of fungus (*M. anisopliae*) on white grubs 27

4.2 Identification of *M. anisopliae* 28
4.3 Dead white grubs record of bioassay 29
4.4 Mortality of white grubs within three treatments 30
4.5 Fungal mortality(FD%),other reason mortality (OD%) and survival (S%) record of bioassay 31
4.6 Fungal death of White grubs during bioassay 32
4.7 Other reason death of White Grubs during bioassay 33
4.8 Survival of White Grubs during bioassay 34
4.9 Efficacy of *M. anisopliae* 35
CHAPTER V: DISCUSSIONS **36-39**
CHAPTER-VI: CONCLUSION AND RECOMMENDATIONS **40-41**
6.1 Conclusion 40
6.2 Recommendations 41
REFERENCES **42-50**
APPENDICES **i-vi**
Appendix A **I**
Appendix B **Iii**
Appendix C **Iv**
Appendix D **V**
Appendix E **Vi**

LIST OF ABBREVIATIONS

CFC	: Conidia Forming Cell
C	: Conidia
Cp	: Conidiophores
DAI	: Days After Inoculating
EMP	: Entomopathogen
EPF	: Entomopathogenic Fungi
EPSF	: Entomopathogenic Soil fungi
FD	: Fungal Death
FE	: Fungal Emergence
H	: Haemocoel
IPM	:Integrated Pest Management
Ma1	: Isolated Metarhizium anisopliae 1
Ma2	: Isolated Metarhizium anisopliae 2
Ma	: Metarhizium anisopliae
ODP	: Other Reason Death
PDA	: Potato Dextrose Agar
SIP	: Soil Insect Pest
SM	: Selective media
TDP	: Thermal Death Point
UV	: Ultra Violet

LIST OF TABLES

Table No	Title of Table	Page No
Table 2.1 :	Common entomopathogenous fungi and their major hosts	9
Table 2.2 :	Classification of *Metarhizium anisopliae*	10
Table 4.1 :	Morphological and cultural characteristics of *M. anisopliae*	28
Table 4.2 :	Dead Record of Bioassay	29
Table 4.3 :	Mortality (M%) of white grubs within three treatments	30
Table 4.4 :	FD %, OD % and S % Record of Bioassay	31
Table 4.5 :	Fungal Death During Bioassay	32
Table 4.6 :	Other Reason Death of White Grubs During Bioassay	33
Table 4.7 :	Survival of White Grubs During Bioassay	34
Table 4.8 :	Efficacy Percent of Fungal Isolates	35

LIST OF FIGURES

Figure No.	Name of figure	Page No.
Figure 2.1:	Life cycle of *Beauveria bassiana* comparing with *M.* anisopliae	14
Figure 4.1:	Screening of fungus (Metarhizium anisopliae) on white grubs	27

LIST OF PHOTOGRAPHS

Photograph 1: White grub sccumbed with *Metarhizium anisopliae*

Photograph 2: Pure culture of *Metarhizium anisopliae*

Photograph 3: Microscopic view of *M. anisopliae*

Photograph 4: Researcher inoculating organism into the media

LIST OF APPENDICES

APPENDIX A: Materials and equipments, reagents/strains, media/ antibiotics

APPENDIX B : Preparation of soil for White Grubs rearing

APPENDIX C : Enumeration of spores using haemocytometer

APPENDIX D : Calculation of efficacy

APPENDIX E : Data analysis

CHAPTER - I

INTRODUCTION AND OBJECTIVES

1.1 Background

Insect pests are major limiting factors in the agricultural commodity to crop production system, which cause 12-15% crop losses worldwide and 15-20% in Nepal (Upadhyaya 2014). Among them soil insect pests are becoming major biological constraints to the productivity of different crops. White grubs are the larvae of chafer beetles (Family*: Scarabaeidae,* Order: *Coleoptera)* cause damage to wide range of crops (polyphagous). This pest is reported from all eleven districts of the eastern hills of Nepal in different crops such as maize, cabbage, cauliflower, potato, tea and ginger etc (Shrestha 2000).

Moreover field reports from various sources indicate that white grubs species are the most important insect pest of cash crops like vegetables, ginger, tea and even large cardamom and food crops like maize and millet during summer and rainy season. In hills of eastern Nepal the extent of damage by the grubs varies with the crops (Timsina 2003).The real damage is done by the beetles larvae i.e. white grubs which feed on roots, young larvae feed on fine roots. Second and especially third instar larvae are able to injure even trees by peeling the cambium from the roots (GC 2013). Beetles larvae commonly known as white grubs are found feeding on roots of many plants or decaying organic matter (Mishra and Chandel 2003).

White grubs are larvae of chafer beetles and are defoliating pests. They damage a large number of fruit crops and forest trees as a result of feeding on apical buds and tender leaves, whereas, the grubs feed on plant roots, causing yellowing. They cause wilting which is characterized by an initial purpling of the leaves, followed by death of small plants and reduced lodging of larger ones (GC 2006). The world fauna of white grub exceeds 30,000 species (Mittal 2000). Various types of grubs in terms of morphology, occurrences, species etc are observed in different agro-environment. Among those

commonly available are the masked chafers, *Cyclocephala* spp (annual grubs); May/June beetles, *Phyllophaga* spp (three-year grubs) and most recently the Japanese beetle, *Popillia japonica* (Mittal 2000). (Yadava and Vijayavergia 1994) reported that the extent of damage caused by white grubs solely depends upon the species involved, the numbers present and host crop. In India, white grub is one of the five pests of national importance. In many crops, white grubs cause losses to the extent of 40-80 % (Prasad and Thakur 1959).

Wegner and Niemczyk (1981) had found that historically used chemical insecticides were found ineffective in controlling white grubs as the larvae present in the soil do not come into direct contact with the insecticides. During off season of their host crops, being subterranean, the white grubs go deep into the soil and are difficult to control by soil application of insecticides (Khagta 2006). Baker and Gyawali (1994) had reported that the use of chemical insecticides applied to the white grubs in the soil also have hazardous effects on some non-target soil organisms. Thus, crop protection emphasis has shifted from the dominant chemical pesticides to integrated pest management (IPM), where the focus is on biological control and other natural resources with reduced reliance on chemicals. Guppy and Harcourt (1970) had reported that white grubs have become serious pests of most agricultural crops, fruits, vegetables, ornamental plants, plantation crops, pastures, turf and meadow grasses, lawns, golf courses and forest trees in different part of the world.

Schweigkofler and Zelger (2002) had found that Scheduled chemical application on pest control is ineffective. Chemical pesticides help to protect crops and to kill pests (Schweigkofler and Zelger 2002). However there are many drawbacks including pest resistance, resurgence of pest, emergence of secondary pest, effect on non-target organisms, contamination of environment etc. Due to this harmful effect, the necessity for sustainable crop production through eco-friendly pest management technique is being largely felt in the recent times. Microbial control is an aspect of biological insect control and consists of the rational use of pathogens to maintain pest balances in agricultural environments, with increases in the numbers of other natural

enemies often being observed in fields where microbial control has been used (Maria 2014).

Nepal has always been an agricultural country with rich biodiversity of entomopathogens and exploitation of these natural and renewable resources are essential in a successful bio control strategy. A careful evaluation of beneficial pathogens can lead to gainful exploitation in microbial control programs (Burges 1998).Microbial control strategies are valuable components in IPM and have advantages over chemical pesticides (Rosset and Moore 1997). Mean while, a number of fungal species have also been investigated,undergone commercial development. Products based on *Beauveria bassiana* (Balsamo) Vuillemin*, Beauveria brongniartii* (Sacc.) Petch*, Metarhizium anisopliae* (Metschnikoff) Sorokin*, Paecilomyces fumusoroseus* (Wize) Brown and Smith and *Verticillium lecanii* (Zimm) Viegas are applied in a number of countries (Keller 2000; Rath et al 1995).

Entomopathogenic fungi (EPF) are a polyphyletic group comprising approximately 1000 species reported from many taxonomical divisions of the fungal kingdom (Kaya and Vega 2012). Among entomopathogens, fungi are important as they are virulent, infect insect by contact, persist in environment for long time and have one of the largest host lists (Santharam 2001). Important genera among EPF are *Beauveria*, Metarhizium and *Lecanicillium*. (Roy et al 2006) had found that the efficacy of EPF as biocontrol agents is affected by many biotic and abiotic factors in their environment. Entomogenous fungi comprise a heterogenous group of cover 100 genera with approximately 750 species, reported from different insects and living in diverse habitats including fresh water, soil surfaces and aerospaces, many of which offer greater potential in pest management (Hajek and Ledger 1994; Maddox 1994).

Metarhizium anisopliae a microbial bio-control agent commonly known as green muscardine fungus, can be a suitable alternative as an eco-friendly pest management tool to develop an intelligent pest management system (Keller and Zimmermann 1989). Ferron (1978) reported that Microbial control strategy is valuable component in IPM and has advantages over chemicals due

to improved performance, cost effectiveness and increasing resistance of insects to the chemical insecticides. However, Pokhrel (2004) reported that accurate dose of the virulent strain application is to be known for proper recommendation to the farmers as variable mortality is observed in different concentration of fungus.

The sustainability and economics of production of microbial insecticidal agents is very important which also have broad efficacy to the target organisms and at the same time to the non-target organisms (Burges and Hussey 1971). Robertson (1993) reported that the host range of *M. anisopliae* (Metchnikoff) Sorokin is wide exceeding two hundred species of seven orders of the insects. The pathogenicity however varies with strains or isolates (Aizawa 1987). Therefore, the selection of effective or more virulent strains of entomopathogens is essential for the development of microbial insecticides as a biocontrol by eco friendly way. Environmental factors like temperature, humidity and sunlight play a profound role on the field persistence of entomopathogenic fungi. One of the critical factors in the effective use of microbial agents as insecticides is their relatively short persistence on leaf surfaces. The realization of the economic potential of mycoinsecticides would benefit from advances in biotechnology (Miranpuri and Khachatourians 1995).

M. anisopliae has been reported to be effective in the suppression of soil borne pests like termites, crickets, locusts, brown plant hopper in rice, pyrilla, spittle bug in sugarcane and root grubs. The fungus as commercial product "metaquino" has been in use in Brazil. It was also used against coffee berry borer in Brazil and coconut leaf beetle in Taiwan. Usage of entomopathogenic fungi in IPM of rhinoceros beetle paid good dividend in Samoa (Ferron et al 1975).

Entomopathogenic fungi are being used worldwide for the control of many pest of agricultural importance. Entomogenous fungi are potentially the most versatile biological control agent, which helps to suppress pest population by eco – friendly way. An attractive feature of these fungi is that infectivity is by contact and the action is through penetration (Nadeau et al 1996). *Metarhizium anisopliae* is the most widely exploited entomogenous fungus in bio-control

attempts. It is known to attack over 200 species and insects belonging to orders Coleopteran, Dermoptera, Homoptera, Lepidoptera and Orthoptera (Moore et al 1996). It is categorized as a green muscardine fungus due to the green color of the sporulation colonies. Therefore, entomopathogenous fungi are interesting biocontrol agents, due to their epizootics and pathogenicity (Devi et al 2001). Hence in this study *M. anisopliae* had used as a biological control agent to minimize the use and ill effects of the chemical pesticide for possible integration in the IPM program.

1.2. Objectives

1.2.1 General objectives

- To assess efficacy of entomopathogenic fungus (*Metarhizium anisopliae)* against white grubs under laboratory conditions.

1.2.2 Specific objectives

1. To isolate and identify entomopathogenic fungi from different agricultural field of Dhankuta, district.

2. To assess about the insecticidal property of entomopathogenic fungi against white grub.

CHAPTER – II

LITERATURE REVIEW

2.1 Fungi Attacking White Grubs

Biological control or bio- control, is defined as "the use of living organisms to suppress the population density or impact of specific pest organisms, making it less abundant or less damaging than it would otherwise be" (Eilenberg et al 2001). Microbial control to various pest insects has successfully been applied in other countries like Switzerland, Austria, New Zealand and Australia (Keller 2000). They were either based on the insect pathogenic bacteria *Serratia entomophila* (Jackson et al 1992) or the insect pathogenic fungi, *Metarhizium anisopliae* and *Beauveria brongniartii* against wide range of soil insects (Zimmermann 1993; Zimmermann 1992).

2.1.1 Entomopathogenic Fungi

Entomopathogenic fungi have played an important role in the history of microbial control of insects as a biological control agent. Entomopathogenic fungi can be used as a component of integrated pest management of many insect pests. Entomopathogenic fungi are distributed in a wide range of habitats including aquatic forest, agricultural, pasture, desert and urban habitats (Lacey et al 1997) many of which offer greater potential in pest management and known by greater value in the environment as a biological control agent (Maddox 1994). Soil is the main source and natural habitat for entomopathogenic fungi (Zare 2014). Entomopathogenic soil fungi (EPSF) are a polyphyletic group comprising approximately 1000 species (Rahman 2016). Most entomopathogenic fungi are mesophilic, with growth between 10 and 40˚C and optimal temperature between 25 and 35˚C (Miller 2009). Entomopathogenic fungi (EPF) have emerged as bio pesticide of considerable potential because of their multiple modes of action for insect killing, broad host range, etc (Malik et al 2015). The EPF are found in the division Eumycota and in the following subdivisions: Mastigomycotina, Zygomycotina, Ascomycotina, Basidiomycotina. They are known to infect all

life stages of insects and are commonly found in aquatic, terrestrial, and subterranean habitats (Ferron 1978). Entomopathogenic fungi, *M. anisopliae* has ubiquitous in nature, with extremely wide host range, more often affecting hosts in soil than on aerial plant parts (Humber 1997). *Metarhizium anisopliae* is a fungus that kills the host insect by physically invading its body and consuming the insect's nutritional reserves and also producing toxins (Kannan et al 2008).

2.1.2 Major Characteristics of Hyphomycetes

The Hyphomycetes are a large, rather heterogeneous group of fungi which include *Aschersonia, Beauveria, Culicinomyces, Hirsutella, Metarhizium, Nomouraea, Paecilomyces, Tolypocladium* and *Verticillium* (Inglis et al 2001).They are characterized by mycelial forms that bear asexual spores, termed "conidia" borne on specialized conidiogenous cells, lack of a teleomorph state (Samson et al 1988; Humber 1997). The conidia are microscopic in nature and susceptible to adverse environmental conditions, ultraviolet radiation and desiccation. All entomopathogenic species with exception of a several Entomothorales can be cultured on artificial media (GC 2013).

2.1.3 Major Hosts of Insect Pathogenic Fungi

More than 800 fungal species comprising of 125 genera have been reported to infect insects (GC 2013). David (1967) reported that Fungi infect individuals in all orders of insects; most common are Hemiptera, Diptera, Coleoptera, Lepidoptera, Orthoptera, and Hymenoptera.The major host insects infected with *M. anisopliae* is presented in **Table2.1**. *M. anisopliae* is being tested as a natural enemy of white grubs (scarabs), corn rootworm, and some root weevils. It has a very broad host range and is extensively used in different parts of the world.

Table 2.1: Common entomopathogenous fungi and their major hosts (Source: Butt and Goettel 2000)

Entomogenous fungus, Division Deuteromycotina	**Invertebrate Host**
Beauveria bassiana	Cockchafers and borers
Beauveria brongniartii	Cockchafers
Culicinomyces spp.	Spider mites, citrus mites
Hirsutella thompsonii	Mosquitoes
Metarhizium album	Mosquitoes
Metarhizium anisopliae	Orthopteran insects
Metarhizium flavoviride	Wide host range
Nomuraea rileyi	Lepidoptera
Paecilomycesf arinosus	Coleoptera, Lepidoptera
Paecilomyces fumosoroseus	Wide host range
Tolypocladiu mcylindrosporum	Mosquitoes
Verticillium lecanii	Wide host range, Aphid

2.1.4 Taxonomy of *Metarhizium anisopliae*

Metarhizium is one of the best known genera of entomopathogenic fungi, commonly known as “green muscardine fungus” due to the green colour of the sporulation colonies and is applied as spores or mycelia in various formulations (GC 2013). The fungus was first isolated from the wheat chafer *Anisoplia austriaca* by Metschnikoff in 1879 and named *Entomophthora anisopliae*. He suggested its use as microbial agents against insect pests

(Steinhaus 1949). The genus *Metarhizium,* was established by Sorokin (1883) retaining the scientific ethics. It is known to attack over 200 species and insects belonging to orders Coleopteran, Dermoptera, Homoptera, Lepidoptera and Orthoptera. It is categorized as a green muscardine fungus due to the green color of the sporulating colonies (Moore et al 1996).

Zimmermann (1993) reported that Four groups of insect pests (termites, locusts, spittlebugs and beetles) are targeted for control by *M. anisopliae* Several other species of *Metarhizium* have been described from insects, including *M. flavoviride* from weevils (Gams and Rozsypal 1973), however, only *M. anisopliae* has been found attacking scarabs.

The current classification of *Metarhizium* is based on morphological characters and was reviewed by Tulloch (1976), several species of *Metarhizium* were described prior to 1976, but Tulloch (1976) accepted only *M. anisopliae* and *M. flavoviride;* all other species were synonymized or treated as varieties (Robert and Leger 2004). The separation of species strictly on morphology and sometimes colour proved unsatisfactory for the wide number of isolates held in fungus collections world-wide since 1976. There have been efforts to add a molecular level to the taxonomic studies, and identification methods based on physiology and/ or nucleic acid have been attempted in recent years (Bridge et al 1993). The general classification of *M. anisopliae* is presented in **Table 2.2.**

Table2.2: Classification of *Metarhizium anisopliae* (after Ainsworth, 1971).

Kingdom	Fungi
Division	Eumycota
Subdivision	Deuteromycotina
Class	Hyphomycetes
Order	Hyphomycetales
Family	Moniliaceae
Genus	*Metarhizium*
Species	*anisopliae*(Metsch)

2.1.5 Morphology of *Metarhizium anisopliae*

When studied on the genus *Metarhizium,* Tulloch (1976) reported that *M. anisopliae* appears white when young but as the conidia mature turn to dark green. Similarly, *M. album* produces white colonies and *M. brunneum* produces yellow or brown colonies. *M. anisopliae* has two types, the short spored form *M. anisopliae* var *anisopliae* (conidia 3.5-9.0 μm) and long spored *M. anisopliae var* majus (conidia 9.0-18 μm). Tulloch (1976) also reported that, the morphological characteristics of mycelia, conidia and conidiophores depend on different factors such as temperature, pH, nutrition, light humidity and age of isolate. *M. anisopliae* forms a loose or tough mycelial mat with cushions or areas of conidial structures. The shape of phialide is cylindrical. The conidiophores of *M. anisopliae* are arranged in compact to nearly stomatic patches, mostly, mononematous. Conidiogenous are, arranged in a candle like fashion, clavate to cylindrical, conidia are single celled, smooth walled hyaline to slightly coloured, forming long chain often aggregated into prismatic columns. The cadavers show a dark green mycelia growth on the insect surface however difference in conidial colour was observed. Hence microscopic observation is necessary (Talwar 2005). *M. anisopliae* shows mycelium often wholly covering affected hosts; conidiophores in compact patches; individual conidiophores broadly branched (candelabrum-like), densely intertwined; conidiogenous cells with rounded to conical apices, arranged in dense hymenium; conidia aseptate, cylindrical or ovoid, 9 μm long, forming chains usually aggregated into prismatic or cylindrical columns or a solid mass of parallel chains, pale to bright green to yellow-green, olivaceous, sepia or white in mass (Humber 1997).

2.1.6 Mycotoxins of *Metarhizium anisopliae*

Strasser et al (2000), reported that toxins produced by *Metarhizium is* potentially present in bio-control formulations and or in fungus-killed insects were examined as to safety to non-target organisms and also concluded that *M. anisopliae* will not secrete copious metabolites into the environment and that the toxins do not pose a health risk. Ferron (1978) found that fungi usually

cause insect mortality either due to nutritional deficiency, invasion and destruction of tissues, and release of toxins.

The infective unit in most of the entomopathogenic fungi is a conidium or spores which when land on a susceptible host, put forth germ tubes or infection pegs from aspersoria. These structures secrete a complex of cuticle degrading enzymes *viz.*, chitinases, proteases and lipases, which are capable of hydrolyzing corresponding cuticular constituent's *viz.*, chitin, protein and lipids (Leger et al 1992). This facilitated the germ tube to invade haemocoel and fat bodies. The invading vegetative hyphae consume the contents of haemolymph for its growth and metamorphosis. On exhaustion of the haemolymph content the host insect become moribund and the fungi sporulate after death of the host.

The cuticle is the first barrier to infection by fungi. Hence, rapid and direct penetration of the cuticle is important for virulence pathogenic fungi. Cultures of *M. anisopliae* contain the cyclo depsipeptides, destruxins A, B, C, D, and E, and desmethyldestruxin B (Suzuki et al 1966). Destruxins B has been considered as new generation insecticides. They cause titanic paralysis when inoculated into larvae of *Galleria mellonella* (Roberts 1966) and cause death. Cytopathology occurs in the mid-gut cells with changes in the mitochondria and endoplasmic reticulum causing strongly pycnotic nuclei (Tanada and Kaya, 1993).

2.1.7 Biology of *Metarhizium anisopliae*

The life-cycle of *M. anisopliae* comprises both a parasitic as well as a saprophytic phase. The parasitic phase begins after the contact with a potential host (adhesion and germination of the spore on the insect's cuticle), penetration into the haemocoel and development of the fungus (germination of the host's cuticle), followed by a rapid proliferation of fungal cell which ultimately results in the death of the host (Aidross and Roberts 1978). The parasitic phase is also divided into the two steps such as events before penetration and after penetration. Steps involved in life cycle of *Metarhizium anisopliae* are as fallows;

2.1.7.1 Adhesion

Adhesive processes have not yet been intensively studied in entomogenous fungi; however, both physical and chemical interactions are probably important. Fargues (1984), had reported that, electrostatic forces and molecular interactions may be involved in adhesion. Conidia strongly adhere to insect cuticles, and their attachment to cuticles is thought to involve non-specific adhesion mechanisms mediated by the hydrophobicity of the conidial cell wall (Boucias et al 1991).

2.1.7.2 Pre penetration and Germination

Spore germination is highly dependent on moisture and probably requires free water (Kramer 1980). After the pathogen reaches and adheres to the host surface, it proceeds with a sequence of activities such as spore germination, hyphal growth and formation of infection structures that may be stimulated or inhibited by the potential host.

2.1.7.3 Penetration into hosts

Penetration is both a mechanical and an enzymatic process (McCoy et al 1988). With the help of lipases, proteases and chitinases, the germination tube penetrates through the cuticle and epidermises of the insect towards the haemocoel which have been observed by (Leger 1993). Penetration of the cuticle is accomplished by the germ tube itself or by the formation of an appressorium which attaches to the cuticle and gives rise to a narrow penetration peg (Boucias and Pendland, 1982). However, in some cases, the fungus may not be able to penetrate the cuticle. Inglis et al 2001 reported number of factors, such as inappropriate moisture and inhibitory factors, such as fatty acids or melanin, within the cuticle are responsible.

2.1.7.4 Mode of infection

Fungi have a unique mode of infection in contrast to bacteria, protozoa and viruses. They reach the haemocoel through the cuticle or possibly through the mouth parts (Inglis et al 2001). The mode of penetration mainly depends on

the property of the cuticle, its thickness, sclerotization, and the presence of antifungal and nutritional substances (Charnley 1984) The newly molted larva and the newly formed pupa are more susceptible to infection than those in which the cuticle has fully hardened (Fox 1961).

After the germinating hypha has penetrated the insect's integument and entered the haemocoel, it produces yeast like hyphal bodies, essentially blastospores that multiply by budding. In addition to hyphal bodies, hyphal strands and wall-less protoplasts may develop in the haemocoel. The dispersal throughout the haemocoel and tissue invasion varies with the fungal species. Some fungal species form both hyphal bodies and protoplasts depending on the nutritional environment (Tanada and Kaya 1993). The life cycle of *M. anisopliae* is similar with that of *Beauveria brongniartii* as shown in the below **Figure 2.1**, however, *M. anisopliae* produces no aerial mycellium and has very short and arranged conidia.

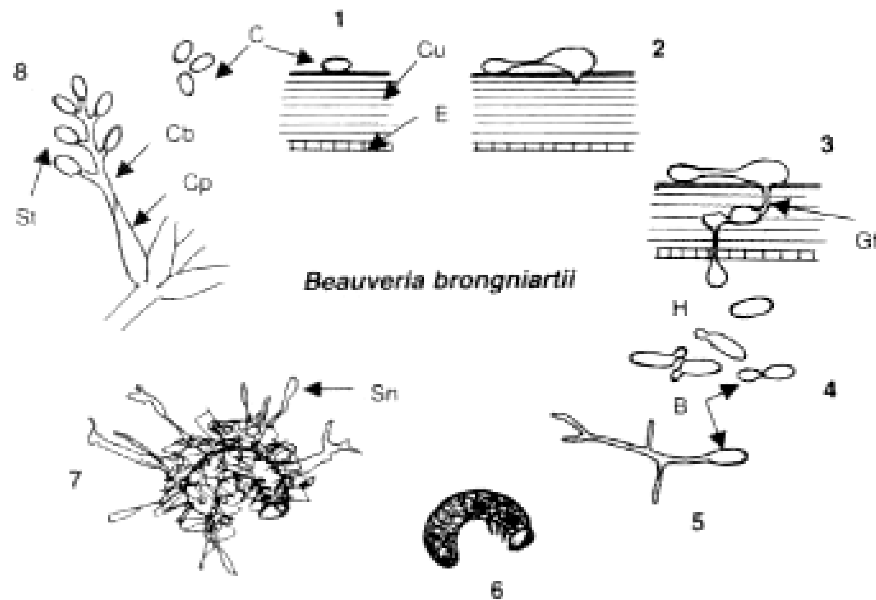


Figure 2.1: Life cycle of *Beauveria bassiana* comparing with *Metarhizium anisopliae* (1-5) parasitic and (6-8) saprophytic phase: (1) Adhesion of conidia on the insect cuticle (2) Germination of the conidia on the surface of the cuticle (3) Penetration of the germination tube through cuticle and epidermis (E) into the haemocoel (H) of the insect (4) Production and multiplication of blastospores (B) in the haemocoel (5) Germination of blastospres and

colonisation of the insect body (6) Mummified insect cadaver filled with mycelium of *B. bassiana* (7) Outgrowth of hyphal strands into the surrounding soil (8) Growth of conidiophores with conidia forming cells (CFC) (GC and keller 2013).

2.1.7.5 Signs and Symptoms

At an early stage of fungal infection, the insect shows little or no signs and symptoms except for a few necrotic spots which may develop at the invasion sites. In advance stage of infection, the insects generally become less active, their appetites are reduced, and they lose coordination. Infected insects often move to high places or if subterranean, rise to the soil surface (McCoy et al 1988). The fungal hyphae continue to grow usually resulting in mummification, and the dead insects retain their form and shape. Shortly prior to or at death, the insect may have a characteristic color.

2.1.7.6 Saprophytic Development of the Fungus

The saprophytic phase commences when the infected insect dies (end of parasitic phase) and generally ends with the formation of reproductive organs. The saprophytic phase is essential for the completion of the developmental cycle of entomogenous fungi. Reproductive spores are produced within the sclerotium or on sporophores (sporangiophore and conidiophore) (Ferron et al 1991).

2.1.8 Environment Influence on *Metarhizium anisopliae*

Environmental factors have dramatic effects on the efficacy of entomopathogenic fungi on insect pests. The most important abiotic factors are temperature, water availability, precipitation, and oxygen in the soil, pH, soil texture and wind (Zimmermann 1982).

Conidia may be very sensitive to solar radiation (Ignoffo et al 1977). Goettel and Inglis 1997 had reviewed the effect of temperature extensively. Most of the entomopathogenic fungi have a wide range of temperature tolerances (0-40ºC), however, temperature optima for infection, growth and sporulation are

usually much more restricted (generally 20-30ºC). Inglis et al 2001 reported that moisture can have very significant effects on the persistence of fungal inoculum. For the most part, fungal conidia usually exhibit greatest stability under cool and dry conditions (Roberts and Campbell 1977). In contrast, conidia of *M. anisopliae* survive better at moderate temperatures when relative humidity is high (Daoust and Roberts 1983).

In vitro germination of *M. anisopliae* occurs at 25- 30ºCwith a germination range of 15-35ºC. Sporulation occurs between 10ºC and 35ºC. The thermal death point (TDP) for fungi is approximately 50ºC. Conidia are reported to survive more than a year at 8ºC, but at 21ºC, *B. bassiana* for 0.5 months and *M. anisopliae* for 2.5 months (Walstad et al 1970). Agricultural practices such as ploughing, crop rotation and application of pesticides and fertilizers may radically alter the population of soil organisms. This may be due to desiccation, a change in aeration of soil, a direct effect of pesticides on target or non-target organisms, or by the addition of un-decomposed or partially decomposed organic materials. Soil water not only affects the growth and survival of microorganisms and insects, but also profoundly affects their movement (Keller and Zimmermann, 1989).

2.1.9 Isolation of Entomopathogenic Fungi

Selective media rich with antibiotics and growth substances are frequently used for the isolation of entomopathogens (Goettel and Inglis 1997). Entomopathogenic Hyphomycetes may be isolated directly from insect cadavers on which the fungus has already sporulated or from soils. Most common methods include the soil dilution plating (Beilharz et al 1982) and insect baiting (Zimmermann 1986).

2.1.10 Production of Fungal Spores

After pure culture isolation, the isolates can be stored at the suitable temperature usually at 22-25ºC. Individual colonies free of bacteria can then be harvested using Tween 80 (0.2%). Continued sub-culturing can result in changes in virulence and other characteristics (Glare 1992). Submerged

fermentation can be used for production of blastosproes and conidia (Ignoffo 1981).

2.1.11 Enumeration of the Spores

A wetting agent such as Tween 80 is necessary to make *M. anisopliae* conidia hydrophilic because of their hydrophobic nature. To quantify number of propagules per unit volume of haemocytometer is commonly used. When the concentration of propagules in the original suspension is too high to get an accurate count on the haemocytometer (e.g.> 300/cell), it is necessary first to dilute the suspension prior to enumeration (Goettel and Inglis 1997).

2.1.12 Storage of the Fungal Spores

Once the fungus is cultured, it must be stored unless it is used immediately. Conidia and mycelium should be stored in cryovials under nitrogen, or freeze-dried and stored in sterile glass ampoules (Humber 1997) for short or long term storage. Freshly harvested conidia can also be air dried and stored in desiccators at 4ºC or room temperature. Several hyphomycetes fungi such as *V. lecanii or M. anisopliae* are stored as conidia bound to silica gel at -40ºC (Hedgecoc et al 1995).

2.1.13 Formulation of Entomopathogenic Fungi

To maintain or improve the efficacy of the spores, different components can be added such as carrier, diluents, binder, dispersant, UV protectants and virulence-enhancing factors (Moore and Caudwell 1997). The most widely used carriers are oil and water. Oils are reasonably effective in sticking spores to insect and plant surfaces and as protectant against desiccation (Inglis et al 1996).

2.1.14 Storage of Fungus Material

Once the fungus is cultured, it must be stored unless it is used immediately. Many fungi, especially from the Hyphomycetes, can be maintained *in vitro* on several media. Several hyphomycetes fungi such as *V. lecanii, M. anisopliae* can be stored as conidia bound to silica gel at -40ºC (Humber 1997). It is well documented, however, that storage at refrigerator temperatures (approximately

4ºC) affords much longer survival than higher temperatures, and that temperatures above normal room temperature can be severely debilitating (Robert and Leger 2004).

2.2 White Grubs

White grubs are the larval stage of beetles of the family Scarabaeidae. Some important species are also called “June beetles” or “May beetles” referring to the flight period of the adults. The family consists of about 30,000 species worldwide. They mostly live underground or under debris, not exposed to sunlight (GC 2013).The grubs are subterranean and attack a wide range wild plants and crops such as potato, chilies, tomato, okra, brinjal, ginger, and Cole crops are the major vegetables severely damaged (Singh and Mishra 2003). The larvae prefer to feed upon the plant roots of corn, groundnut, potatoes, strawberries and several other hosts, however, they dislike legumes (Matherson 1985), the sweet clover (Metcalf and Flint 1975).The adults of the green beetle (*Anomala dimidiate*) feed the whole leaves and flowers whereas, adults of European chafer, *Melolontha melolontha* feed from the margins without leaving the midribs or stout veins causing host plants completely denuded (Keller 2000).White grub is placed in the order Coleoptera, Sub- order-Polyphaga and in the family Scarabaeidae (Arrow 1917).

2.2.1 Morphology

The scarabs are heavy bodied, oval or elongated usually more or less convex, varying enormously in size and chiefly distinguished by having the lamellae at the tips of the antennae. Their organs of smell are located on the movable lamellae or leaves on the thickened end of the antennae (Linsenmair 1972). White grubs are C– shaped, called grubs and are pale yellow or white. The legs are well developed and often hairy. The head is large, hypognathous and heavily sclerotized, yellow brown or red brown in color, and equipped with powerful exposed mandibles.

2.2.2 Life Cycle

Different species of white grubs have similar patterns of life cycle but may vary according to the climatic factors at the time of emergence, egg lying, active larval period, time of pupation and other stages (Sharma 1989). Few species may complete their life cycle in one year e.g. all the known species of *Holotrichia* (Yadav and Mathur, 1987) the others like European cock chafer *Melolontaha melolontha* has at least of three years cycle and many others have biannual cycle. White grubs completed their life cycle within four steps they are Adult emergence, Oviposition, Larvae, and Pupation (Keller 2000).

2.2.3 White Grub Management

White grubs can be controlled by different ways like cultural practices, Mechanical control, Biological control, chemical control and integrated pest management. The insect pathogenic fungi *M. anisopliae* and *B. brongniartii* (Keller 2000) have been reported throughout the world. Fungus based natural enemies have successfully been applied in countries like Switzerland, Austria, New Zealand and Australia (Rath et al 1995). After application, the fungi persisted in the soil due to their capacity to multiply in the host (Fox 1949). They are also easily isolated from the soils (Zimmermann 1993) and insects (Madelin 1963).

2.2.4 Efficacy of *Metarhizium anisopliae* Against White Grubs

Bioassay is a one method through which efficacy of *Metarhizium anisopliae* can be measured by making spore suspension. Virulence may be measured in a bioassay by exposing a known number of hosts to a known number of pathogen and observing the number of dead over time (Reichelderfer 1993). In case of white grubs, they are kept under controlled conditions and the mortality is assessed for periods of more than two months depending on the life span (Glare and Milner, 1991). The dipping method (DP) is the most common bioassay method for the evaluation of fungus in white grubs (Keller 2000). Susceptibility is often a quantitative phenomenon (Zimmermann 1993). The high dosage of 10^8 to 10^9 conidia per ml causes mortality of 90% (Glare and Milner 1991). A sharp increase in mortality was observed 3 days after

treatment with 10 mg of *M. anisopliae* per 100 adults (Lacey et al 1997). Insects infected with certain fungal pathogens show stimulation of feeding, followed by a reduction in feeding and finally reduced mobility, behavioral response and changes in migration patterns (Khachatourians 1996).

CHAPTER - III

MATERIALS AND METHODS

3.1 Materials

A complete list of materials, equipment’s, chemicals, reagent, antibiotics and Media used for this study are listed in Appendix A.

3.2 Methods

3.2.1 Study site

The study was carried out in ARS, Pakhribas, Dhankuta, and Central campus of Technology, Dharan, Nepal from May to November 2018.

3.2.2 Sample Size and Types

During the study 400 white grubs were analyzed (100, for identification of the entomopathogenic fungi and 300 for its effect). All the work concerning this research was carried out in ARS, Pakhribas Laboratory and CCT, Dharan. The samples analyzed were from different crop field of Dhankuta.

3.3 Sample Collection

The sample for study were collected randomly from the different crop field within soil and placed individually in cap perforated polyvial (about 6cm length and 2.5 diameters). 100 white grubs (all size) were collected from maize field of Dhankuta (Pari Patle-3) on May and 300 white grubs (same size, large) were collected from Sidhuwa, Dhankuta on August. The entire collected sample was brought to the ARS, laboratory for further study.

3.4 Rearing of White Grubs

100 white grubs were brought to the laboratory and were kept in quarantine for one week to observe the mechanical injury. The grubs after the quarantine were reared individually in each container of 100ml capacity (Leanth- 6cm

and Breath- 4.5cm) with half-filled sterilized soil supplying pieces of carrot as diet on weekly. The rearing room was protected from direct sun-light and UV-light by making it dark. The rearing temperature was within the range of 22-23ºC during the rearing period. While rearing of the grubs, the following biological parameters such as, date of collection, date and causes of grub mortality were recorded.

3.5 Screening of Entomopathogens

Observations were taken for the live and dead grubs. All the larvae kept for rearing were checked daily until the emergence of adults or their death whether they are attacked by the insect pathogenic fungi. Dead larvae were carefully separated and transferred into another vial with cotton in order to assess the causes of mortality either due to fungus or any other reasons. The dead grubs during rearing suspected to be affected by fungi were kept at humid and damp condition at 25ºC for pathogen proliferation on the body cadaver. Before isolation, confirmation of the attack by *Metarhizium anisopliae* was checked under the compound microscope as shown in photograph.

3.5.1 Isolation of Entomopathogens

Selective media rich with antibiotics (Strasser et al 1997) were used for the isolation of entomopathogens. Isolation of the fungus was carried out following the loop dilution method from such cadavers that were fully covered by the sporulating fungus (GC 2006).

3.5.2 Identification of Fungus

Pure culture of fungi on selective medium was used for morphological identification.Identification of the entomopathogenic fungus was done by preparing the slides of the fungus. For slide preparation, a drop of water was put in the centre of the glass slide. Now some water was added in selective media culture slants and a drop of fungus culture was transferred to the glass slide using sterile bacteriological loop. Then cover slip was put carefully avoiding the formation of air bubbles. The slide was examined under

compound microscope. The morphological and colonial characteristics were recorded on10th days after inoculation, Conidiophore structures were observed under 40X magnification using compound microscope and identified according to the key described by Humber (1977) and Samson et al (1998).

3.5.3 Maintenance of Culture

The actively growing mycelia colonies were subcultured to obtain pure cultures through streak plate method. A loop full of fungal spores was streaked on the surface of the PDA and selective medium in Petri plates under aseptic conditions. These plates were incubated at 26±1°C for 15 days. Again the sporulated fungus was transferred to sterilized PDA and SM slants and plates and incubated. After 15 days, the slants and plates showing full growth were stored at 4˚C in refrigerator.

3.5.4 Preparation of Spore Suspension

The fungal conidia from pure SM plates and slants were harvested after 13 days incubation by scrapping and mixed with distilled water with 0.2 %Tween-80 to get the spores suspension. The number of conidia was determined using a haemocytometer. Finally, the spore suspension containing (1x10^7, conidia/ml were obtained for the two isolates (Ma1 and Ma2) of *Metarhizium anisopliae.* The conidial suspension (10^7 conidia/ml) was prepared in a glass Petri dish.That concentration was found to be most effective against the common white grubs (GC et al 2008).

3.6 Bioassay of Fungal Pathogen

Two isolates (Ma1 and Ma2) having 10^7 conida /ml concentration and one control (distilled water) were assessed for their efficacy against white grubs (Large size) in the laboratory. The experiment was conducted from August 22, 2018. The methodology followed during bioassay experiment was 30 larvae per replication for each treatment were dipped individually into the conidial suspension of isolates (Ma1 and Ma2) and control (distilled water) for five seconds (Goettel and Inglis 1997) by holding them loosely at the leg with forceps. Three replications were done and the experiment was carried out at

25-27℃ on the population of white grubs from potato field of Sidhuwa, Dhankuta. Excess liquid was allowed to drop off and the larvae were returned individually to the rearing vials containing sterile soil, and were incubated at a temperature of 22-24˚C. The lid was perforated for air circulation. The larvae were fed with slices of potato and carrot and checked for mortality every third day for four weeks. Dead larvae were observed under the light microscope for causes of mortality.

3.6.1 Mortality of White Grubs

Mortality of white grubs was determined from bioassay record and was calculated by using following formula;

$$\text{Mortality \%} = \frac{\text{No. of dead larvae}}{\text{Total number of larvae}} \times 100$$

(GC 2006)

3.6.2 Survival Rate of White Grubs

During bioassay some white grubs were not affected by inoculating treatments (i.e. Ma1 Ma2 and distilled water) and were found to be alived as normal. The survival rate was calculated from bioassay record by using following formula;

$$\text{Survival rate} = \frac{\text{No. of live larvae}}{\text{Total number of larvae}} \times 100$$

3.6.3 Efficacy of *M. anisopliae* (Ma1 and Ma2)

In this experiment laboratory test (ioassay) was conducted to assess the efficacy of isolated *M. anisopliae* (Ma1 and Ma2) against white grubs with 10^7conidia/ml. For the determination of efficacy, live and dead number of white grubs inoculating two fungal treatments (Ma1, Ma2) and distilled water

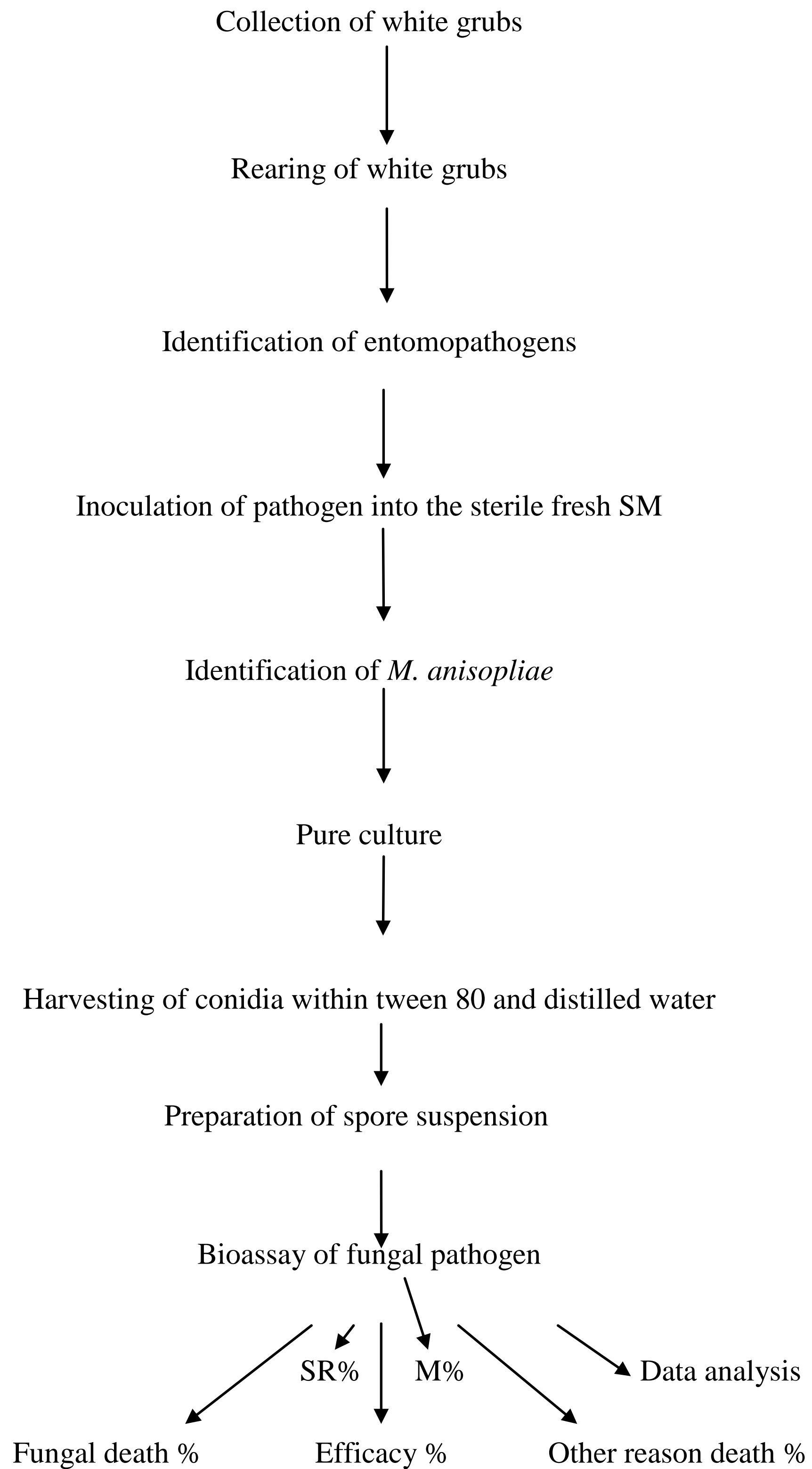
(control) during bioassay were recorded. The corrected mortality (efficacy %) was calculated by using modified Abbotto's formula (Abbott 1925) as given below.

$$\text{Efficacy\%} = 100 \times \frac{\text{Survival untreated\%} - \text{Survival treated\%}}{\text{Survival untreated\%}}$$

3.7 Data Analysis

The data recorded from bioassay was documented and tabulated. The data (FD, OD and SR) within three treatments (control, Ma1and Ma2) were statistically analyzed using ANOVA at 5% confidence level by SPSS. The test was considered statistically significant if p value obtained was less than 0.05.

Flow chart for the study



CHAPTER - IV

RESULTS

4.1 Screening of Fungus (*Metarhizium anisopliae*) on White Grubs

The study was done in ARS, Pakhribas, Dhankuta and in microbiology lab of central campus of technology. Study period was from May to July 2018.

One hundred white grubs (all size) were collected from Dhankuta and the white grubs after the quarantine were reared individually in each container (polyvial) with half filled sterilized soil supplying pieces of carrot as diet on weekly. Observations were taken for the live and dead white grubs upto two months. The dead white grubs during rearing were kept at 25˚C for pathogen proliferation on the body cadaver. Out of 100 samples analyzed three larvae were found to be dead due to other reason (may be bacteria, nematode, mechanical injuries, etc) and only two larvae were found to be infected with suspected fungi (*M. anisopliae)* which is illustrated in **Figure 4.1.**

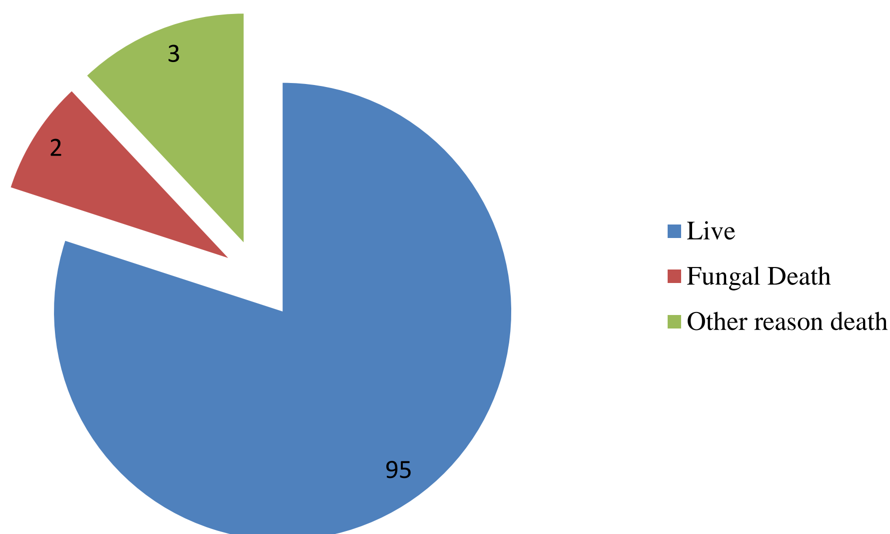


Figure 4.1: Screening of fungus (*M. anisopliae*) on White grubs

4.2 Identification of *M Anisopliae*

The cadavers showed a dark green mycelial growth on the insects (white grubs) surface. Conidiogenous cells (phialides) occurred in a dense layer (hymenium).*M. anisopliae* formed branched conidiophores. The shape of the phialides was clavate or cylindrical with a rounded to conical apex. The conidia of *M. anisopliae* produced in long, aggregated into prismatic columns. *M. anisopliae* appeared white when young but as the conidia matured turned to green and dark green. Some morphological and cultural characteristics of isolated *M. anisopliae* (Ma1 and Ma2) is as shown in **Table4.1** and the microscopic view of *M.anisopliae* is shown in **Photograph number 3**.

Table 4.1: Morphological and cultural characteristics of *M. anisopliae*

Fungal isolates	No. of days for sporulation	Colour of conidia	Time taken to cover the cadaver (Days)	Colony diameter on 10th DAI (mm)
Ma1	5	Dark green	15	41.10
Ma2	8	Green with yellowish pigmentation	19	30.28

4.3: Dead white Grubs Record of Bioassay

Dead larvae record of bioassay was recorded from day after inoculation (DAI) as weekly for four weeks within three replication using three treatments (control, Ma1and Ma2). In control sterile distilled water is used but isolated *metarhizium anisopliae1* (Ma1) and isolated *Metarhizium anisoliae* 2 (Ma2) were used by making their spore concentration (i.e 10^7 spore/ml) in10ml sterile water. In bioassay 30 larvae were kept in per replication for each treatment and were reared individually in cap perforated polyvial, half filled with sterile soil by supplying carrot slices for their food. During bioassay many white grubs were found to be dead with different reason like some were death by fungal infection where as some were found to be other reason(may be bacteria, nematodes, mechanical injury, etc) death. In this study,no fungal death was observed in control where as 39 larvae were found to be death by fungus in Ma1 and 31 larvae were found to be death due to fungal infection in Ma2.The detailed record for this study is illustrated in **Table 4.2,** where **T-** total death, **F-** fungal death and **O-** other reason death.

Table 4.2: Dead record of bioassay

Week		1st			2nd			3rd			4th			Total death	Total fungal death	Due to other reason death
Treatments	Rep	T	F	O	T	F	O	T	F	O	T	F	O			
Control	1	1	0	1	2	0	2	0	0	0	1	0	1	4	0	4
	2	2	0	2	1	0	1	0	0	0	2	0	2	5	0	5
	3	3	0	3	2	0	2	1	0	1	1	0	1	7	0	7
													Total	16		16
Ma1	1	1	0	1	3	2	1	5	3	2	7	5	2	16	10	6
	2	2	1	1	6	4	2	8	5	3	7	6	1	23	16	7
	3	2	2	0	4	3	1	5	4	1	6	4	2	17	13	4
													Total	56	39	17
Ma2	1	3	2	1	5	3	2	4	3	1	7	4	3	19	12	7
	2	1	0	1	4	2	2	3	2	1	3	3	0	11	7	4
	3	2	2	0	3	2	1	5	4	1	6	4	2	16	12	4
													Total	46	31	15
													Grand Total	118	70	48

4.4 Mortality of White Grubs within Three Treatments

When our bioassay experiment was completed, mortality of white grubs was calculated from bioassay record and was found to be greater in isolated *M. anisopliae* 1(Ma1=62.22 %) as compared to isolated *M. anisopliae* 2 (Ma2= 51.11%) and control(17.77%) within three replication.The detail information of this study is given in **Table 4.3**.

Table 4.3: Mortality (M %) of white grubs within three treatments

Treatments	Total no. of white grubs tested	No of dead white grubs	Mortality (%)
Control	90	16	17.77
Ma1	90	56	62.22
Ma2	90	46	51.11

4.5 Fungal Mortality (FD %), Other Reason Mortality (OD %) and Survival (S %) Record of Bioassay

The FD%, OD % and S % were calculated from bioassay record. In control survival rate was found to be greater (SR%= 82.22) as compared to Ma1 (37.78%) and Ma2 (48.9%). In this study, no fungal death was observed in control (FD%= 0) and was found greater in Ma1 (43.34%). Other reason death was found to be also greater in Ma1 (OD%= 18.89). Detailed information for this study is as shown in **Table 4.4.**

Table 4.4: FD %, OD % and S % record of bioassay

Treatment	Death % by fungus	% other reason death	% survival
	0	13.34	86.68
Control	0	16.68	83.34
	0	23.34	76.68
Total(mean)	0	17.78	82.22
	33.34	20	46.68
Ma1	53.34	23.34	23.34
	43.34	13.34	43.34
Total(mean)	43.34	18.89	37.78
	40	23.34	36.68
Ma2	23.34	13.34	63.34
	40	13.34	46.68
Total(mean)	34.44	16.67	48.9

4.6 Fungal death (FD) of White Grubs during Bioassay

In this study, during bioassay the Ma1 was found to be more virulent as compared to other treatment. The detailed relationship between treatment and fungal death on White Grubs is shown in **Table 4.5**.

Table 4.5: Fungal death during bioassay

Treatment	Rep	FD	P value
Control	1	0	0.001
	2	0	
	3	0	
Total		0	
Ma1	1	10	
	2	16	
	3	13	
Total		39	
Ma2	1	12	
	2	7	
	3	12	
Total		31	

4.7 Other Reason Death (OD) of White Grubs during Bioassay

On our laboratory experiment (bioassay), some white grubs are dead with other reason like due to bacteria, nematodes or mechanical injuries but not due to entomopathogenic fungi .The detailed relationship between other reason death and treatment is shown in **Table 4.6**.

Table 4.6: Other reason death of white grubs during Bioassay

Treats	Rep	OD	P value
Control	1	4	
	2	5	
	3	7	
Total		16	
Ma1	1	6	
	2	7	0.880
	3	4	
Total		17	
Ma2	1	7	
	2	4	
	3	4	
Total		15	

4.8 Survival (S) of White Grubs during Bioassay

In this experiment, some white grubs are not affected by our treatment and they are live as normal during bioassay within three replication. The detailed relationship between survival rate and treatment is as shown in **Table 4.7**.

Table 4.7: Survival of white grubs during bioassay

Treats	Rep	SR	P value
Control	1	26	0.006
	2	25	
	3	23	
Total		74	
Ma1	1	14	
	2	7	
	3	13	
Total		34	
Ma2	1	11	
	2	19	
	3	14	
Total		31	

4.9 Efficacy of *M. anisopliae*

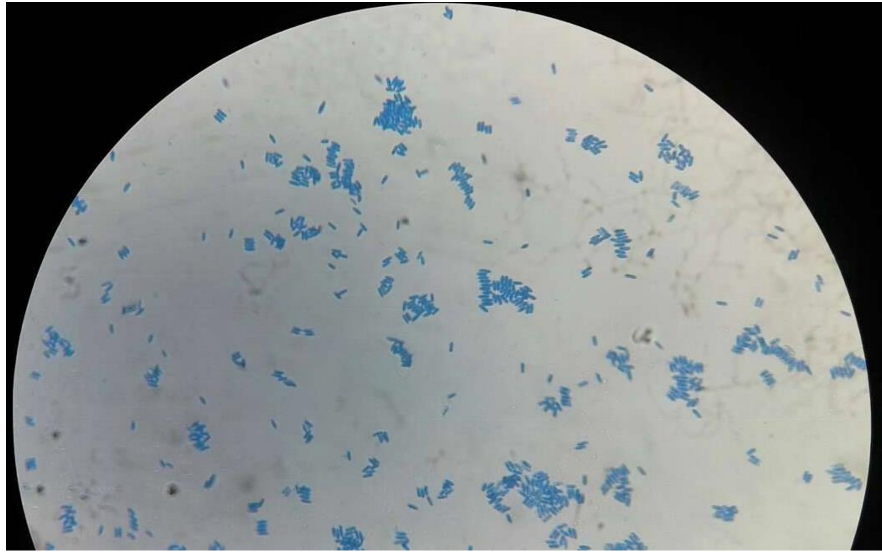
The efficacy % was calculated from bioassay data record by using modified Abbotto's (Abbott 1925). In this study efficacy of isolated *M. anisopliae*1 is found to be greater (Ma1=54.05 %) on white grubs as compared to isolated *M. anisopliae*2 (Ma2= 40.52 %).The detail of this study is as shown in **Table 4.8,** where sUtrt= Survival untreated and strt = survival treated.

Table 4.8: Efficacy Percent of Fungal Isolates

Treatment	Death % by fungus	other reason death %	survival%	sUtrt-strt	Efficacy %
Control	0.00	17.78	82.22	-	-
Ma1	43.34	18.89	37.78	44.44	54.05
Ma2	34.44	16.67	48.89	33.34	40.52

Photograph1: White Grub sccumbed with *M. anisopliae*

Photograph 2: Pure culture of *Metarhizium anisopliae*



Photograph 3: Microscopic view of *Metarhizium anisopliae*

Photograph 4: Researcher inoculating organism into the media

CHAPTERV

DISCUSSIONS

Soil is considered an excellent environmental shelter for entomopathogenic fungi since it is protected from UV radiation and other adverse abiotic and biotic influences. Many fungal groups contain fungi that attack insects, either specific developmental stage like eggs of all stages. Entomogenous fungi are potentially the most versatile biological control agent, which helps to suppress pest population by eco–friendly way. An attractive feature of these fungi is that infectivity is by contact and the action is through penetration (Nadeau et al 1996).This study is carried out to know the efficacy of isolated entomopathogenic fungi against white grubs under maintained lab condition.

In this study, Out of 100 white grubs collected from Paripatle, Dhankuta two white grubs were found to be infected with fungus on different days during rearing period at laboratory of ARS, Dhankuta. The cadavers showed a dark green mycelial growth on the insect surface which is similar to the study carried out in Nepal which found four fungal infected white grubs showing dark green mycelial growth on their surface, from observation of 200 white grubs of Parbat, Mid hill, Western region, Nepal (GC 2006).

Entomopathogenic fungi may be isolated directly from insect cadavers on which the fungus has already sporulated or from soils. Most common methods include the soil dilution plating and insect baiting (Beilharz et al 1982, Zimmermann 1986). In this study, cadavers, having white mycelial growth and green sporulated fungus on insects (white grubs) bodies were used for isolation of fungi(*M. anisopliae*) using selective medium containing peptone, glucose, agar and some antibiotics like streptomycin tetracycline, etc (Strasser et al 1996). Our study is similar to the study where selective medium adapted from Strasser et al (1996) was used for the isolation of *M. anisopliae* from such cadavers having white mycelial growth and green sporulated fungus (GC 2006, GC et al 2008). However our study is not relatable to the study carried out in Germany which used Potato Dextrose Agar (PDA) for isolation of isolation of entomopathogenic fungi from insect cadavers (Ashraf et al 2017).

In this study, two fungal isolates from white grubs of Paripatle, Dhankuta, were successfully grown and maintained on selective medium and named them as isolated *M. anisopliae*1 (Ma1) and isolated *M. anisopliae*2 (Ma2), Similar result was obtained in Nepal where out of 70 isolates of *M. anisopliae* 24 isolates were isolated from white grubs of different geographic origin (Chitwan= 10, Parbat= 7,Tanahun= 4,Nawalparasi= 3) of Nepal where as 41isolates were isolated from soil/ *Galleria* bait method and remaining 5 isolates were isolated from soil/ selective medium (Keller et al 2008).

In this study, some morphological and colonial characteristics of *M. anisopliae* are: the cadavers showed a dark green mycelial growth on the insects (white grubs) surface, conidiogenous cells (phialides) occurred in a dense layer (hymenium), *M. anisopliae* formed branched conidiophores, the shape of the phialides was cylindrical with a rounded to conical apex, the conidia produced in long, aggregated into prismatic columns, *M. anisopliae* appeared white when young but as the conidia matured turned to green and dark green. Those characteristics of *M. anisopliae* are similar to the morphological characteristics of *M. anisopliae* which were described by Humber (1997).

In our study, the color of conidia after 5 days of sporulation was found to be dark green in Ma1where as after 8 days of sporulation the color of conidia was found to be dark green with yellow pigmentation in Ma2. The colony diameter at 10th days after inoculation (DAI) was found to be 41.10mm in Ma1 and 30.28mm in Ma2.This result is somewhat similar to the study done in Dharwad, India where color of conidia was green with yellowish pigmentation and the colony diameter after 10th DAI was 38.60mm in Ma1 where as the conidial color was dark green and the colony diameter after 10th DAI was 42.30mm (Talwar 2005).

Laboratory bioassay was performed to assess the efficacy of two fungal isolates isolates (Ma1 and Ma2) on white grubs population which were collected from potato field of Sidhuwa, Dhankuta. For bioassay freshly grown and sporulated plates and slants of *M. anisopliae* were used and the conidia were collected by scraping off those sporulated plates and slants using sterile bacteriological loop and the conidial suspension was adjusted to 10^7

conidia/ml with the help of haemocytometer in 10 ml distilled water with the addition of two drops of Tween80 solution for both Ma1 and Ma fungal isolates. In this study, three treatments were used to conduct bioassay i.e Ma1 Ma2 and control. In control sterile water (D/W) was used and three replication were done for each treatment. The bioassay was performed using the dipping method (Goettel & Inglis 1997).The dead and live record was reported up to four week for each replication.

In bioassay mortality was found to be greater in Ma1 (62.22 %) followed by Ma2 (51.11%) and 17.77% mortality was observed in control. Here, the cause of mortality is due to fungi and other reason (bacteria, nematodes, mechanical injury etc).Similar results were reported by GC et al (2008) where greater mortality (67.5%) was observed in white grubs origin fungus, *M. anisopliae* (Ma1) within 10^7 conidia/ml and in control 24.7% mortality was observed against white grubs.

During bioassay, the cause of mortality was found to be different. In this study, after four week of days after inoculation (DAI) of bioassay, fungal death was found to be greater in isolated *M. anisopliae*1 (Ma1 = 56) fallowed by isolated *M. anisopliae*2 (Ma2 =46) , whereas no any white grubs were found to be death by fungus in control (C= 0). This result reveals that, for the control of white grubs, Ma1 has greater fungal mortality/ mycosis (FD% of Ma1= 43.34%) and shows more virulent as compared to Ma2 (FD% of Ma2 = 34.44%). This result is ratable to the study where the mycosis/ fungal mortality of *M. anisopliae* (M1) was 52.0% after 70 days of inoculations against white grubs (GC et al 2008).There was significant difference between treatment and fungal death ($p<0.05$)

For the determination of efficacy of an organism, all dead and live record of bioassay is required. During bioassay, some white grubs were found to be death without fungal mortality and were may be due to other reason like (bacteria, nematodes or mechanical injury). In this study, greater other reason death mortality was observed in Ma1 (OD% = 18.89%) followed by 17.78% in control and 16.67% in Ma2. There was no significance difference between treatment and other reason death ($p> 0.05$). Our bioassay data shows some

white grubs were found to be live as normal and were not affected by any treatment. The greater survival percent was found in control (S% of control = 82.22%) as compared to Ma2 (S% of Ma2=48.89%) and Ma1 (S% of Ma1= 37.78). The lower survival % reveals that, it's have greater capacity to control insect pest. In our study Ma1 shows the lower survival % of white grubs so this one can be called as more effective to control white grubs as compared to Ma2. The relationship between survival and treatment was statistically significant ($p<0.05$). When an actual count of the living and dead insects in both the treated (Ma1 and Ma2) and untreated (distilled water) plats or checks is available, we can use Abbott formula to determined the actual effectiveness of insecticides against tested insects.The difference between the percentage of living scales in the untreated check and the percentage of living scales in the treated plat gives the percentage of the original actually killed by the treatment. Finally efficacy of two fungal isolates (Ma1 and Ma2) against white grubs were determined from bioassay results by using Abbotto's formula (Abbotts 1925).

In this study, corrected mortality (efficacy %) of white grubs by isolated entomopathogenic fungi, Ma1 and Ma2 within10^7 conidia/ml were found to be 54.05% and 40.52% respectively. This result is somewhat relatable with one of the study where 41.16% corrected mortality of white grub was noted after eight weeks of inoculations within 10^7 conidia/ml followed by 34.88% within 10^2 conidia/ml (Kafle et al 2018).

CHAPTER – VI

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

White grub is a polyphagous and nefarious larvae of chafer beetles (Scarabaeidae: Coleoptera) cause damage to wide range of crops of agricultural field. In hills of eastern Nepal the extent of damage by the grubs varies with the crops, the white grubs population of maize field of Dhankuta Paripatle was found in lesser number as compared to potato field of Sidhuwa, Dhankuta. Thus, there is urgent need of environment friendly alternative to chemical management practices against white grubs. Entomogenous fungi have great promise for use as biological control agents against different insects. *Metarhizium anisopliae*, is a fungus that grows naturally in soils throughout the world and causes disease in various insects by acting as a parasitoid through ecofriendly way. The disease caused by the fungus (*M. anisopliae*) is sometimes called green muscardine disease because of the green colour of its spores. Our study was aimed in evaluating efficacy of two fungal isolates Ma1 and Ma2 against white grubs at maintained lab condition and was found to be greater in Ma1(54.05%) as compared to Ma2(40.52%). From these results, it is concluded that the entomopathogenic fungi (*M. anisopliae*) could be a safe microbial control agents in managing white grubs and due to greater efficacy Ma1 could be better for mass production for the management of white grubs in fields as compared to Ma2.

6.2 Recommendations

1. Insect Pests are major limiting factors in the agricultural commdity to crop production system; those nefarious grubs should be controlled by *M. anisopliae* as bio-pesticides.
2. Entomopathogenic fungi should be isolated from White grubs cadaver using selective medium.
3. Metarhizium anisopliae should be grown within 15-25˚C on its selective medium.
4. Mass production of Entomopathogenic fungus (*Metarhizium anisopliae*) should be done on different substrates by selecting more virulent isolates of *Metarhizium anisopliae*.
5. The fungus material should be preserved at refrigerator (4˚C) and handled with care for maintaining its virulence.
6. Detection of EMF must be done through PCR based experiment for correct identification of fungal species.

REFERENCES

Abbott, W.S. (1925)."A method of computing the effectiveness of an insecticide." Journal of Economic Entomology 18:265-267.

AI-Aidroos, K. and D. W. Roberts (1978)."Mutants of *Metarhizium anisopliae* with increased virulence toward mosquito larvae." Canadian Journal of Genetic Cytology 20: 2-11.

Ainsworth, GC. (1971)."Ainsworth and Bisby Dictionary of the fungi." Commonwealth Mycological Institute, Kew, U.K.

Aizawa, K. (1987)."Recent development in the utilization of microbial insecticides in Japan.Food and Fertilizer technology Center." Republic of China on Taiwan 6-10.

Arrow, G.R. (1917)."Coleoptera Lamellicornia the fauna of British India including Ceylon and Burma."London, Taylor and Francis: 387.

Ashraf, M., M. Farooq, M. Shakeel, N. Din, S. Hussain, N. saeed, Q. Shakeel and N. A. Rajput (2017)."Influence of entomopathogenic fungus, *Metarhizium anisopliae*, alone and in combination with diatomaceous earth and thiamethoxam on mortality, progeny production, mycosis,and sporulation of the stored grain insect pests."

Baker, SL.and BK. Gyawali (1994)."Promoting proper pesticide use: Obstacles and opportunities for an integrated pest management programme in Nepal." HMGN/MOA/Winrock International, Kathmandu, Nepal.

Beilharz,V. C., D. G. Parberry and H. J. S. Wart (1982). "Dodine: A selective agent for certain soil fungi."Transactions of the British Mycological Society 79: 507-511.

Boucias, D.G. and Pendland, JC. (1982)."Ultrastructural studies on the fungus, *Nomuraea rileyi*, infecting the velvet bean caterpillar, *Anticarsiagemmatalis*." Journal of Invertebrate Pathology 39: 321-338.

Boucias, D.G., JC. Pendland, and J.P. Latge (1991). "Attachment of mycopathogens to cuticle: the initial event of mycoses in arthropod hosts." The Fungal Spore

and Disease Initiation in Plants and Animals. Plenum Press, New York, 101-128.

Bridge, P.D., M. Williams, C. Prior and R.R.M. Paterson (1993)."Morphological biochemical and molecular characteristics of *Metarhiziuman isopliae* and *Metarhizium flavoviridae."* Journal of General Microbiology 139: 1163-1169.

Burges, H.D.(1998)."Formulation of mycoinsecticides." Kluwer Academic Publishers Dordrecht 131-185.

Burges, H.D. and N.W. Hussey (1971)."Microbial control of insect and mites." .Development in insect pathology, Academic Press London 1-11.

Butt, T.M. and M.S. Goettel (2000)." Bioassays of entomopathogenous fungi." CABI, Wallingford, U. K 141-194.

David, H., K.,K. C. Ananthanarayan, and A. S. Ethirajan (1967)."Integrated control of white grubs, *Holotrichia serrata* on sugar cane." Madras Agricultural Journal 63: 637-41.

Devi, KU., J. Padmavathi, H.C. Sharma and N.Seetharama (2001)."Laboratory evaluation of the virulence of *Beauveria bassiana* isolates to the sorghum shoot borer *Chilo partellus* Swinhoe (Lepidoptera: Pyralidae) and their characterization by RAPD-PCR."World Journal of Microbiology and Biotechnology 17: 131-137.

Eilenberg,J., A. Hajek and C. Lomer (2001)."Suggestions for the unifying the terminology in biological control." Biocontrol 46: 387-400.

Fargues, J. (1984). "Adhesion of the fungal spore to the insect cuticle in relation to pathogenicity."

Ferron, P., P.H. Roberts and A. Deotte (1975)."Susceptibility of *Oryctes rhinoceros* adults to *Metarhizium anisopliae*." Journal of Invertebrate Pathology, 25: 313-319.

Ferron, P. (1978). "Biological control of insect pests by entomogenous fungi."Annu ; 23:409-442.

Fox, C. J. S. (1961). "The incidence of green muscardine in the European wireworm, *Agriotesobscurus*(Linnaeus), Nova Scotia." Journal of Insect Pathology 3: 94-95.

Gams, W.and J. Rozsypal (1973)."*Metarhizium flavoviride* n. sp. isolated from insects and from soil." *Acta Botanica Neerlandica* 22: 518-521.

GC, Y.D. (2006)."White grubs (Coleoptera: Scarabaeidae) associated with Nepalese agriculture and their control with the indigenous entomopathogenic fungus *Metarhizium anisopliae* (Metsch.) Sorokin." Basel, Switzerland, 2.

GC, Y. D. and S. Keller (2013)."Crop pest of Nepal and their management." Kathmandu 44-45.

GC, Y. D., and S. Keller (2013). "Crop Pests in Nepal and their Management." Kathmandu 148.

GC, Y. D. and S. Keller (2013)."Crop Pests of Nepal and their Management." Kathmandu150.

GC, Y.D., S.Keller and P. Nagel (2004)."Microbial control of white grubs with entomopathogenic fungi and its value in Integrated Pest Management (IPM) in Nepal." Tribhuvan University Journal 24: 37-47

GC, Y. D., S. Keller, P. Nagel,and L.N. Kafle. (2008)."Virulence of *Metarhizium anisopliae* and *Beauveria bassiana* against common white grubs in Nepal." Formosan Entomol. 28: 11-20.

Glare, T. R. and R. J. Milner (1991). "Ecology of entomopathogenic fungi." Handbook of Marcel Dekker, New York, 2: 547-612.

Goettel, M. S.and D. G. Inglis (1997). "Fungi: Hyphomycetes Manual of Techniques in Insect Pathology." CRC Press, Boca Raton, FL 209-232.

Goettel, MS and AE.Hajek (2000)."Evaluation of non target effects of pathogens used for management of arthropods." CABI , Wallingford. 81-97.

Guppy, JC. And GD. Harcourt (1970)."Spatial pattern of the immature stages and general adults of Phyllophaga spp. (Coleoptera: Scarabaeidae) in permanent meadow." Canadian Entomologist. 102:1345-1359.

Hedgecoc, S., D. Moore, P. M. Higgins and C. Prior (1995). "Influence of moisture content on temperature tolerance and storage of *Metarhizium flavoviride* conidia in an oil formulation." Biocontrol Science and Technology 5: 371-377.

Humber, R. A. (1997)."Fungi: Preservation of cultures Manual of Techniques in Insect Pathology." Academic Press, London 269-279.

Humber, RA. (1997). “Fungi Identification." Manual of techniques in insect pathology. San Diego, CA: Academic Press 153-185.

Ignoffo, C. M. (1981)."The fungus *Nomuraea rileyi* as a microbial insecticide." Microbial Control of Pests and Plant Diseases, 1970-1980. Academic Press, London 513-538.

Inglis, G. D., M. S. Goettel, M. T. Butt and H. Strasser (2001)."Use of Hyphomyceteous Fungi for Managing Insect Pests." CABI Press, Wallingford, U. K: 23-69, 409-442.

Inglis, G. D., D. L. Johnson and M. S. Goettel (1996)."Effect of bait substrate and formulation on infection of grasshopper nymphs by *Beauveria bassiana*." Biocontrol Science and Technology 6: 35-50.

Jackson,T. A., J. F. Pearson and M. Callaghan (1992)."Pathogen to Product – Development of *Serratiaentomophila*(Enterobacteriaceae) as a commercial biological control agent for the New Zealand grass grub (*Costelytrazealandica*)."Andover U. K: 191-198.

Khagta R (2006). "Evaluation of some entomopathogenic fungi for the suppression of *Brahmina coriacea."* UHF, Nauni, Solan, H.P. India.

Kafle, L., S. Marahatta, R. Regmi, V. Bista, K. Karki, A. poudel, S. Maharjan and J. R Bohora (2018)."Efficacy of different concentration of Metarhizium anisopliae (Metsch.) Sorokin against white grub at lab condition in Chitwan, Nepal."Journal of Pharmacognosy and Phytochemistry 149-153.

Kannan, S. K., K. Murugan, A. N. Kumar,N. Ramasubramanian, And P. Mathiyazhagan (2008). "Adulticidal effect of fungal pathogen, *Metarhizium anisopliae* on malarial vector Anopheles *stephensi* (*Diptera: Culicidae*)." African Journal of Biotechnology, 7 (6), 838-841.

Kaya H.K. and FE. Vega 2012)."Scope and basic principles of insect pathology." Insect pathology,London, Academic Press; 1:12.

Keller, S. (2000). "Use of *Beuveriabrongniartii*in Switzerland and its acceptance by farmers." Switzerland, 23: 67-72.

Keller, S.,Y.D. GC, P. Nagel, and L.N. Kafle. (2008)."Virulence of *Metarhizium anisopliae* and *Beauveria bassiana* against common white grubs in Nepal." Formosan Entomol. 28: 11-20.

Keller, S. and G. Zimmermann (1989)."Mycopathogens of soil insects."239-270.

Khachatourians, GG. (1996)."Biochemistry and molecular biology of entomopathogenic fungi." Springer, Heidelberg. 331-363.

Kramer, J. P. (1980)."The house-fly mycosis caused by *Entomophthora muscae*: Influence of relative humidity on infectivity and conidial germination." New York Entomological Society of Journal 88: 221-236.

Lacey, L. A. and W. M. Brooks (1997)."Initial handling and diagnosis of diseased insect." Academic Press, London:1-15.

Linsenmaier, W. (1972). "Insects of the world: drawing, photographs and text." New Delhi: McGraw-Hill Book Company: 137-143.

Maddox, J.V. (1994)."Insect pathogens as biological control agents."New York. : 199-244.

Madelin, M. F. (1963)."Diseases caused by Hypomycetous fungi." An advanced treatise. London: Academic Press, 2: 233-271.

Maria, S. (2014)."Biological insect control using *Metarhizium anisopliae*: Morphological, molecular and ecological aspects." ISSNO103-8478,44,46:45-651.

McCoy, C. W., R. F. Brooks, J. C. Allen and A. G. Selthime (1988)."Proc. Tall Timbers Conf. Ecol. Anim. Control Habitat Man." 6: 1-17.

Miranpuri, GS and GG. Khachatourians (1995)."Application of *Beauveria bassiana* and *Verticillium lecanii* against Saskatoon berry leaf aphid *Acyrthosiphon macrosiphum*."Journal of Insect Science, 8: 93-95.

Misra, SS. And RS. Chandel (2003). "Potato white grubs in India." Central Potato Research Institute, Shimla, H.P. 60: 47.

Mittal, I.C. (2000). "Survey of Scarabaeid (Coleoptera) fauna of Himanchal Pradesh (India)." Entomological Research ; 24:133-141.

Moore, M., P.M. Higgins And C. J.Lomer (1996)."The effects of simulated and natural sunlight on the viability of conidia of *Metarhizium flavoviride."* Biocontrol Science Technology, 7: 87-94.

Nadeaue, M. P., G.B. Dumphy and J. L. Boisvert (1996). "Development of Eryniaconica (Zygomycetes: Entomopthorales) on the cuticle of the adult black flies *Simuliamro stratum* and *Simuliam decorum* (Diptera: Simullidae)."Journal of invertebrate pathology, 68: 50-58.

Pell JK, J. Elienberg,AE. Hajek and DC. Steinkraus (2001)."Biology, ecology and pest management potential of Entomophthorales."CAB International, Wallingford 71-153

Pokhrel, M.R.(2004)."Field survey of white grubs and laboratory evaluation of Metarhiziumanisopliae (Metsch.) Sorokin for its control with side effects on Bombyx."

Prasad, S. K. and C. Thakur (1959)."White grub *Lachnosterna consanguinea* Blanch: a new menace to sugarcane." Indian Journal of Entomology 21: 184-189.

Rath, AC., D. Woreledge, T.B. Koen and B.A. Rowe (1995)."Long-term field efficacy of the entomogenous fungus *Metarhizium anisopliae* against the subterranean scarab, Adoryphorus couloni." Biocontrol Science and Technology 5: 439-451

Rath, A. C., G. C. Anderson, D. Worledge and T. B. Koen (1995)."The effect of low temperatures on the virulence of *Metarhizium anisopliae* (DATF-001) to the subterranean Scarab, *Adoryphoruscouloni."* Journal of Invertebrate Pathology 65: 186-192.

Reichelder, C. F. (1993). "Biological assays with insect pathogens." New Delhi. CBS: 489-515.

Roberts, D. W. and R. J. St.Leger (2004)."*Metarhizium* spp., cosmopolitan insect-pathogenic fungi: Mycological aspects."Elsevier Inc., London, U. K, 54: 400.

Robertson, L. N. (1993). "Population dynamics of false wireworms (*Gonocephalum macleayi, Pterohelaeus alternatus*, *P. darlingensis*) and development of an integrated pest management program in Central Queensland field crops." Australian Journal of Environmental Agriculture 33: 953-962.

Rosset, P. and M. Moore (1997)."Food production and local production of biopesticides in Cuba."LEISA Newsletter13 (4): 18-19.

Roy, H.E., D.C. Steinkraus, J. Elienberg, A.E. Hajek, and J.K. Pell (2006). "entomopathogenic fungi and their arthropod." Bizarre interactions and endgames: hosts 51:331–357.

Santharam G. (2001)."Prospectus of using *Metarhizium anisopliae*, *Beauveria bassiana* and *Paecilomyces fumosoroseus* for pest management." Department of Agricultural Entomology Tamil Nadu Agricultural University, Coimbatore. 95-103

Samson, R. A., H. C. Evans and J. P. Latge (1988)."Atlas of Entomopathogenic Fungi." Springer-Verlag, Berlin: 187.

Schweigkofler,W. and R. Zelger (2002)."Control measures responsible for the decline of Melolontha populations in South Tyrol." IOBC/WPRS 25:65-72.

Sharma, M. L. (1989)."White grub and its management in Rajasthan." Indian Farming, 2:33-36.

Shrestha, K. P. (2000)."Situation analysis and problem identification through PRA tools." Agricultural Research Station, Pakhribas, Dhankuta. August 2000.

Singh, M. P. and P. N. Mishra (2003)."White grubs in hilly and mountain agro ecosystem of Uttaranchal." Indian Farming: 20-23.

Steinhaus, E. A. (1949). "Principles of insect pathology." Hafner Publishing Company, New York and London: 757.

Strasser,H., A.Vey and T. M. Butt (2000)."Are there any risks in using entomopathogenic fungi for pest control, with particular reference to the

bioactive metabolites of *Metarhizium, Tolypocladium* and *Beauveria* species?" Bio. Sci. and Technol. 10, 717-735.

Strasser, H., A. Forer and F. Schinner (1997)."Development of media for the selective isolation and maintenance of virulence of *Beauveria brongniartii*." Ag Research Lincoln, New Zealand :125-130.

Suzuki, A., S. Kuyama , Y. Kodaira and S. Tamura (1966)."Structural elucidation of seed corn maggot." J. Econ. Entomol. 53: 517-518.

Talwar, B.(2005). "Isolation and characterization of entomopathogenic fungi and their effectiveness." Doctors of Philosophy,Department of Agricultural microbiology , college of agriculture,University of agricultural sciences, Dharwad.

Tanada,Y. and H. K. Kaya (1993)."Fungal Infections. In: Insect Pathology." Academic Press, San Diego :319-366.

Teja, K.N.P.and S.J. Rahman (2016)."Characterisation and evaluation of Metarhiziumanisopliae (Metsch.) Sorokin strains for their temperature tolerance." Mycology, 7:4, 171-179.

Timsina, G.P. (2003)."Exploring biological control of white grub using entomopathogenic fungi in Nepal."Annual technical report presented to Sustainable Soil management Programme (SSMP).

Tulloch, M. (1976)."The genus *Metarhizium.*Trans." Br. Mycol. Soc.66: 407-411.

Upadhyaya, N. S. (2003)."Status of community integrated pest management in Nepal." Himalayan Resource Institute, Kathmandu, Nepal :209- 216.

Vago, C. (1963)."Predispositions and interrelations in insect diseases." Academic Press, New York: 339-379.

Wegner, G.S. and H.D. Niemczyk (1991)."Bionomics and phenology of Ataeniusspretulus." Annals of Entomological Society of America; 74:374-384.

Yadava, C. P. S. and Y. K. Mathur (1987)."White grub: a national pest and strategy of its management." Kanpur, India.

Zare,H. (2014)."Isolation and characterization of entomopathogenic fungi from hibernating sites of sunn pest (*Eurygaster integriceps*) on Ilam Mountains, Iran." International Journal of current microbiology and applied sciences,12: 314-325.

Zimmermann,G. (1982)."Effects of high temperature and sunlight on the viability of conidia of *Metarhizium anisopliae* (Metsch.) Sorokin." Journal of Invertebrate Pathology 40: 36-40.

Zimmermann,G. (1992). "Use of the fungus, *Beauveria brongniartii*, for the control of European Cockchafers, *Melolontha* spp., in Europe." Use of pathogens in Scarab Pest Management. Intercept, Andover, U. K 199-208.

Zimmermann, G. (1986)."The Galleria baits method for detection of entomopathogenic fungi in soil." Journal of applied Entomology. 102: 213-215.

Zimmermann, G. (1993). "The entomopathogenic fungus *Metarhizium anisopliae* and its potential as a biocontrol agent.Pesticide."Science37: 375-379.

APPENDIX A

Materials and Equipments:

List of Materials

Glass wares

Beaker

Conical flask

Polyvials

Glass rod

Slides

Pipettes

Test tubes

Measuring cylinder

Micropipette

Micropipette tips

Petridishes cork borer

Miscellaneous

Bacteriological loop

parafilm

Bunsen burner

test tube rack

Sterile cotton swabs

Spirit lamp

Forceps

Gloves

Marker

Soaps

Tissue paper

Labeling stickers

Tube holder

Equipments

Autoclave

Haemocytometer

Incubator

Refrigerator

Hot air oven

Compound Microscope

Reagents/ Strains

Tween 80 0.2 %

Lysol Lactophenol cotton blue Blue

Media / Antibiotics

Media -

Potato dextrose agar

Selective media for *Metarhizium anisopliae*

Antibiotics -

Streptomycin sulphate , Tetracycline

Direction

(Preparation of SM for *M. anisopliae*):

10g peptone from meat pancreatically digested, 20 g glucose, 18g agar agar, were suspended in 1000ml distilled water, autoclaved the solution at 120 ^{0}C for 20 minutes and cool up to 60^{0}C and following mixture were mixed in it.

Streptomycin 0.6g, Tetracycline0.05g + 20ml sterilized distilled water.

APPENDIX B

Preparation of Soil for White Grubs Rearing:

Required soil i.e 2 kg for 100 larvae was collected from dry crop field and brought to the laboratory. Collected soil was sprayed on tray and Soil aggregates were breaked by hand or using hammer, sieved out, and Sterilized by autoclaved at 121 ˚ C for 15 minutes ,then leave to cool down , finally ready for used in sterile polyvials for White grubs larvae rearing.

APPENDIX C

Enumeration of Spores Using Haemocytometer :

Number of conidia on big square chamber of Haemocytometer :

55, 48, 29, 28

$$\text{Average number of conidia on Big square Chamber} = \frac{160}{4}$$

$$= 40$$

$$\text{The average number of conidia in the smallest unit} = \frac{40}{16}$$

= 2.5 Spores/ unit area

The area of the smallest unit of the haemocytometer (L× B) =0.0025 mm^2(This value is given on haemocytometer)

Height of all units of the Haemocytometer (H) = 0.100 m

Thus the volume of smallest Unit = (L× B × H) = 0.00025 mm^3

Here, 2.5 spores / smallest unit of haemocytometer means 0.00025mm^3 of suspension

Or, 0.00025 mm^3 contains, 2.5 conidial spores

Or, 1 mm^3 contains, 2.5/0.00025 conidial spores

Or, 1000 mm^3contains , 2.5/ 0.00025 × 1000 conidial spores

$=1\times 10^7$ spores /ml

(Note: 1000 mm^3 contains 1 ml water)

APPENDIX D

CALCULATION OF EFFICACY:

The efficacy % was calculated by using modified Abbotto's formula given below.

$$\text{Efficacy\%} = 100\text{X} \ \frac{\text{Survival untreated\% -Survival treated\%.}}{\text{Survival untreated\%}}$$

We have , survival untreated (control) = 82.22 %

Survival treated (Ma1) = 37.78 %

Survival treated (Ma2) = 48.9 %

So ,

$$\text{Efficacy of Ma1} = 100 \times \frac{82.22 - 37.78}{82.22}$$

$$= 54.05\ \%$$

$$\text{Efficacy of Ma2} = 100 \times \frac{82.22 - 48.9}{82.22}$$

$$= 40.52\ \%$$

APPENDIX E

Statistical Analysis:

ANOVA

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	282.889	2	141.444	24.481	.001
Within Groups	34.667	6	5.778		
Total	317.556	8			

Here, The test is statistically significant difference in both between groups and within groups ($p < 0.05$).Where, Dependent variable = Fungal Death (FD) and factor = treatment

ANOVA

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	.667	2	.333	.130	.880
Within Groups	15.333	6	2.556		
Total	16.000	8			

Here, The test is not statistically significant in both between groups and within groups (i.e $p > 0.05$) .Where Dependent variable = Other Reason Death (OD)

Factor = Treatment

ANOVA

	Sum of Squares	Df	Mean Square	F	Sig.
Between Groups	288.889	2	144.444	13.131	.006
Within Groups	66.000	6	11.000		
Total	354.889	8			

Here, the test is statistically significant in both between groups and within groups ($p < 0.05$).Where, Dependent = Survival Rate (SR) and factor = treatment