PATHOGENICITY OF ENTOMOPATHOGENIC FUNGI TO WHITE GRUBS OF MAIZE BASED CROPPING SYSTEM IN NEPAL

Yubak Dhoj G. C.[1] Siegfried Keller[2] and Peter Nagel[3]

[1]Tribhuvan University, Institute of Agriculture and Animal Sciences (IAAS), Rampur, Chitwan, Nepal
[2]Swiss Federal Research Station for Agroecology and Agricultrue, Reckenholzstrasse, 191, CH-8946 Zurich
[3] Basel University, Institute for Environmental Sciences (NLU)/Biogeography, St. Johanns Vorstadt 10, CH 4056 Basel

Abstract

White grubs are increasingly important pests of cereal and cash crops in Nepal. The situation is also aggravated by the fact that they are one of the most difficult insect pests to manage due to high levels of insecticide resistance. An alternative method with insect pathogenic fungal based antagonists was sought. Series of activities were conducted in this regard since the mid of 2002 at Tribhuvan Unversity, Institute of Agriculture and Animal Sciences (IAAS), Rampur and in farmers' fields in Nepal. Several dozens of isolates of the insect pathogenic fungi, *Metarhizium anisopliae* (green muscardine fungus) and more than half a dozen of *Beauveria bassiana* (white muscardine fungus) were recovered from infested soil and diseased insects using a selective medium and the *Galleria* bait method (GBM). Isolation, maintenance, mass production and efficacy tests with *M. anisopliae* were conducted under captivity. A three-tired screening strategies; exploration of the indigenous fungal based bioagents, screening of the virulence under captivity and field tests was initiated with *M. anisopliae.* With the preliminary screening, four isolates namely, M1, M6, M18 and M48 being identified as pathogenic for pests larvae (*Anomola* sp and *Xylotrupes gidion)* at concentrations of 10^5 and 10^7 spores/ml. Mass production in barley kernels was achieved into autoclavable polybags and however, field tests are under way in different locations of farmers field in Nepal.

Key words: Entomopathogens, white grubs, bioassays, *Metarhizium anisopliae, and Beauveria bassiana*

01. Introduction

White grubs are economically important pest insects in Nepal, however, its strategic management is lacking in the country (G. C. and Keller, 2002). Chemical pesticides have been the practical method used by growers for many decades, but their effects on non-target organisms, groundwater contamination, residues on food crops and the development of insect resistance to chemicals have forced the industry and scientists to focus on the development of alternative control measures. Alternatives, including biological control with reference to entomopathogenic fungi in the genera of *Metarhizium* have been sought.

Biological control has well recognized success story and was initiated since 1762 with the introduction of Mynab bird (*Acridotheres tristis)* from India into Mauritius for the control of sugar cane red locust, *Nomadacris septemfasciata.* Control of insect pests, particularly by their natural enemies comprising parasitoids, predators and pathogens in agro-ecosystems is a continuous process. In the search for the new avenues in biological control, the importance of entomopathogens has been high lighted as an environmentally friendly pest control method. Fungi constitute a large group of more than 500 species which can parasitize insects. Most of the taxonomic groups contain entomopathogenic genera, such as *Metarhizium, Beauveria,*

Verticillium, Nomuraea, Entomophthora, Neozygites, to name a few (Desphande, 1999). Fungal biocontrol agents are promising because they act by contact and do not need ingestation, can be mass produced very easily and are quite host specific.

Metarhizium anisopliae is an imperfect, entomopathogenic fungus found in soils throughout the world. It was first recognized as a biocontrol agent in the 1880s. Four groups of insect pests (termites, locusts, spittlebugs and beetles) are currently being targeted for control by *M. anisopliae* (Zimmermann, 1993). The Government of Canada, Netherlands, Switzerland, UK and USA have supported the LUBISOSA research programme for the control of Sahelian locusts and grasshoppers using *M. flavoviride* (Prior *et al.,* 1992). *M. anisopliae* is applied as spores or mycelia in various formulations. Control is achieved through the induction of a fungal epizootic where new spores and vegetative cells produced in infected insects are spread to healthy members of the population. . *M. anisopliae* is a ubiquitous species but strain selection is vital, since a high level of variation exists among isolates in relation to pathogenicity, optimal temperature and viability (Moutia, 1936).

In Nepal, *Phyllophaga* sp, *Anomala, Xylotrupus gidion* etc. are the main white grub species involved in various up land crops (G. C. *et al.,* 2003). Larval stages of these insect live inside the soil and causes poorly quantifiable losses in the crops. Their infestation has been reported across the country and incidence is increasing every year. Control is mainly targeted with the use of chemical pesticides; however, none of them are effective in lowering down their population.

The biological factors that influence populations of white grubs' complex are relevant to the potentiality of the biological control with soil fungi. There has been much speculation on the role played by antagonistic micro-organisms and pathogens acting on the larvae in the soil. Pathogens are in general more effective than entomophagous insects in the biological control of white grubs. The present study deal with isolation and identification of entomopathogenic hyphomyceteous strains, study of their virulence under captivity, mass production and field tests on maize based cropping areas so as to reduce the dependence on chemical pesticides and, therefore, lower const of production in the long run.

02. Materials and methods

2.1 Collection and rearing of white grubs larvae

All stages of white grubs' complex from *Phyllophaga, Anomala, Xylotrupes* and other un-identified species were collected over six localities of farmers' field of Nepal (Table 1). Live specimens were maintained in the laboratory upon feeding slices of carrots and potatoes inside the polypots where moisture and aeration were maintained. Rearing was continued until either their death or adult emergence. Dead and diseased specimens for which mycosis was suspected were retained for diagnosis.

Table 1 Sites representing the collection of white grubs species and natural emergence of entomopathogenic fungi during 2003 in Nepal

Origin	Site characteristics	Number of total grubs collected	Number and species of EPF emergence	EPF emergence (%)

Pang, Parbat	High mid hill (maize-millet)	300	13 *M. a.*	4.33
Jamune, Tanahun	Mid hill (maize-pulses)	300	1 *B. bass.*+ 6 *M. a.*	0.33 (*B. bass.*) + 2 (*M. a.*)
Nahala, Tanahun	Mid hill (maize-pulses)	300	11 *M. a.*	3.66
Gaindakot, Nawalparasi	Low hill (maize millet) forest site	300	5 *M. a.* + 1 *B. bass.*	1.66 (*M. a.*) + 0.33 (*B. bass.*)
Mangalpur, Chitwan	Low hill (maize-potato)	300	8 *M. a.*	2.66
Gunganagar, Chitwan	Low hill (maize-millet)	300	10 *M. a.*	3.33

M. a. = Metarhizium anisopliae *B. bass. = Beauveria bassiana*

2.2 Presence of entomopathogenic fungi in soils and insects

Strains were isolated from soil, either by soil dilution plating or *Galleria* bait method, as described by Goettel and Inglis (1997), or from naturally infected insects. Presence of mycosis or any observable disease symptoms was recorded. Entomopathogens from local insect pest species were isolated and identified using Koch's Postulates. Forty six soil samples were taken with a soil borer from fields in Chitwan, Syangja, Tanahun and Parbat districts (Fig 1). They originated from arable land and from meadows in Chitwan (Rampur, National Maize Research Programme – NMRP), from Syangja (Dhanubanse),

Figure 1: Study sites: 1: Durlung and Pang/Parbat; 2: Syangja; 3: Rishing Patan/Tanahun; 4: IAAS and NMRP Rampur/Chitwan. White grubs were collected from 1 and 2; and soil samples were collected from 1, 3 and 4

from Tanahun (Rising Patan) and Parbat (Pang, Durlung) and from fruit plantations, arable land and meadows from Chitwan (Rampur, Tribhuvan University, Institute of Agricultural and Animal Sciences – IAAS). All samples were checked with the *Galleria* bait method (Zimmermann, 1986) (Table 2). Thirty samples, ten each from Rising Patan, Pang, and NMRP were analyzed by plating soil suspension on a selective medium (Keller *et al*., 2000).

Table 2 Presence and density of entomopathogenic fungi in soil samples of Nepal as pointed out with the *Galleria* bait method in 2003

Origin	Crop	Number of samples	Soil samples with EPF (%)	Species of EPF
Parbat, Durlung	Arable land	4	3 (75)	3 *M.a.*
	Grassland	4	2 (50)	2 *M.a.*
Parbat, Pang	Arable land	5	3 (60)	3 *M.a.*
	Grassland	5	3 (60)	3 *M.a.* + 1 *B. bass.*
Tanahun, Rishing Patan	Arable land	5	1 (20)	1 *B. bass*.
	Grassland	5	4 (80)	1 *M.a.* + 4 *B. bass*. + 2 *P*. spp.
IAAS, Rampur	Arable land Fruit plantations	4	2 (50)	2 *M.a.*
	Grassland	4	1 (25)	1 *M.a.*
NMRP, Rampur	Arable land	5	2 (40)	2 *M.a.* + 1 *B. bass*.
	Grassland	5	3 (60)	3 *M.a.*
All	Arable land	23	11 (48)	10 *M.a*. + 2 *B. bass*.
	Grassland	23	13 (57)	10 *M.a*. + 5 *B. bass*.

EPF = Entomopathogenic fungi. *M. a. = Metarhizium anisopliae; B. bass. = Beauveria bassiana*; *P.* ssp = *Paeciolomyc; NMRP* = Samples taken in fields of the National Maize Research Programme

The soil plating method was adapted from Fornallaz (1992). 10 g soil/sample of fresh soil are shaken for 3 h at 140 rpm on a rotary shaker in 250 ml Erlenmeyer flasks with 50 ml tap water containing 1.8 g/l tetra-Sodiumdiphosphate-Decahydrat ($Na_4P_2O_7$*$10H_2O$) to favour disaggregation of the soil. After 15 seconds of sedimentation 0.1 ml the suspension is distributed with a Trigalsky spatula on a Petri dish with selective media and intensively rubbed. Three replicates/soil samples were prepared. After 8-10 days at 20^0C and darkness the colonies of *M. anisopliae* were counted and a selection of colonies was isolated in tubes with Sabouraud-glucose-agar with 0.6 g/l Streptomycine.

The selective medium adapted from Strasser *et al.* (1997) with the following composition and preparation was used: 10 g peptone from meat pancreatically digested, 20 g glucose, and 18 g agar, all dissolved in 1 litre distilled water and autoclaved at 120^0C for 20 minutes. At a temperature of 60^0C 0.6 g streptomycin, 0.05 g tetracycline and 0.05 g cyclohexamide previously dissolved in distilled, sterile water and 0.1 ml Dodine were added.

The *Galleria* bait method (GBM) was adapted from Zimmermann (1986). About 60 ml of soil/sample was filled in a cylindrical plastic tube with a diameter of 40 mm and a height of 65 mm and 4 larger *Galleria* larvae added. The samples were kept in darkness at a temperature of 22^0C. During the first five days the tubes were turned daily to keep the larvae moving in the soil. After 16-18 days the larvae were examined, fungus infections were recorded and the fungus from infected larvae was isolated.

2.3 Isolation of insect pathogenic fungi

Single spore isolation i. e. loop dilution method (Fig 2) was followed for the isolation of

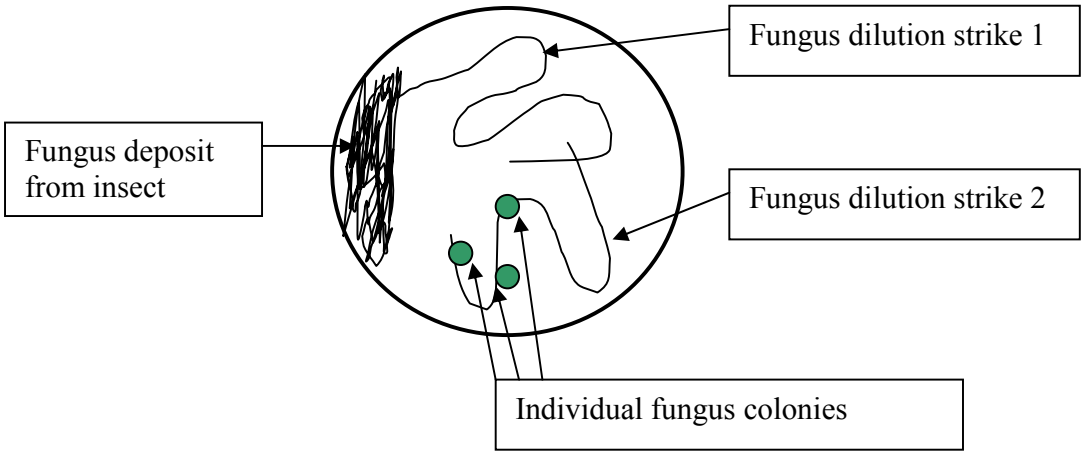


Fig 2 Loop dilution method for obtaining single spores of fungus

entomopathogenic fungi. Fungal inoculums form specimens showing mycelial growth and/ or sporulation, was transferred onto plates of semi-selective medium composed with peptone, agar-agar, glucose. Antibiotics such as streptomycin, tetracycline, cyclohexamide and dodine were added to avoid the contamination. Pure cultures were obtained by subsequent reinoculation and reisolation.

2.4 Laboratory infectivity trails

2.4.1 Maintenance of core isolates: Insect pathogenic fungi based on *M. anisopliae* (green muscardine fungus) and *B. bassiana* (white muscardine fungus) were isolated from white grubs' cadaver, Galleria bait method (GBM) and selective medium through soil dilution plating. The fungus pathogens are maintained in the selective medium adapted from Strasser *et al.* (1997). The list of master isolates with their detailed information is presented in table 3. Isolation and re-isolation while maintaining their virulence in a host passage sequence has been done as routine operation. Security copies of the isolates are kept at FAL, Switzerland in order to minimize the risks of loosing the work.

Table 3 Entomopathogenic fungi isolated from various sources and maintained at IAAS, Rampur, Chitwan, Nepal until the end of 2003

Fungus species	Origin		Date of isolation	Name of Isolator	Remarks	FAL Number	IAAS Number
	Host	Locality					
M. anisopliae	White grub	Parbat/Nepal	31.5.02	S. Keller	Cropland	800	M1
M. anisopliae	White grub	Parbat/Nepal	12.6.02	Y. GC	Cropland		M2
M. anisopliae	White grubs	Parbat/Nepal	12.6.02	Y. GC	crop land		M3
M. anisopliae	Soil/SM	Parbat/Nepal	31.5.02	S. Keller	grassland	801	M4
M. anisopliae	Soil/SM	Parbat/Nepal	31.5.02	S. Keller	cropland	802	M5
M. anisopliae	Soil/SM	Parbat/Nepal	31.5.02	S. Keller	cropland	803	M6
M. anisopliae	Soil/Galleria	Rampur/Nepal	03.7.02	S. Keller	cropland	804	M7
M. anisopliae	Soil/Galleria	Rampur/Nepal	03.7.02	S. Keller	cropland	805	M8
M. anisopliae	Soil/Galleria	Rampur/Nepal	03.7.02	S. Keller	grassland	806	M9

M. anisopliae	Soil/Galleria	Parbat/Durlong	03.7.02	S. Keller	cropland	807	M10
M. anisopliae	Soil/Galleria	Parbat/Durlong	03.7.02	S. Keller	cropland	808	M11
M. anisopliae	Soil/Galleria	Parbat/Durlong	03.7.02	S. Keller	cropland	809	M12
M. anisopliae	Soil/Galleria	Parbat/Durlong	03.7.02	S. Keller	grassland	810	M13
M. anisopliae	Soil/Galleria	Parbat/Durlong	03.7.02	S. Keller	grassland	811	M14
M. anisopliae	Soil/Galleria	Parbat/Pang	12.9.02	Y.GC	crop land		M15
M. anisopliae	Soil/Galleria	Parbat/Pang	12.9.02	Y.GC	crop land		M16
M. anisopliae	Soil/Galleria	Parbat/Pang	12.9.02	Y.GC	Cropland		M17
M. anisopliae	White grub	Parbat/Pang	01.2.03	Y.GC	Cropland	885	M18
M. anisopliae	GBM	NMRP/Rampur	16.4.03	SK/YGC	Cropland	886	M19
M. anisopliae	GBM	NMRP/Rampur	16.4.03	SK/YGC	Cropland	887	M20
M. anisopliae	GBM	NMRP/Rampur	16.4.03	SK/YGC	Cropland	888	M21
M. anisopliae	GBM	NMRP/Rampur	16.4.03	SK/YGC	Cropland	889	M22
M. anisopliae	GBM	NMRP/Rampur	16.4.03	SK/YGC	Cropland	890	M23
M. anisopliae	GBM	NMRP/Rampur	16.4.03	SK/YGC	Cropland	891	M24
M. anisopliae	GBM	NMRP/Rampur	16.4.03	SK/YGC	Cropland	892	M25
M. anisopliae	GBM	NMRP/Rampur	16.4.03	SK/YGC	Grassland	893	M26
M. anisopliae	GBM	NMRP/Rampur	16.4.03	SK/YGC	Grassland	894	M27
M. anisopliae	GBM	NMRP/Rampur	16.4.03	SK/YGC	Cropland	895	M28
M. anisopliae	GBM	NMRP/Rampur	16.4.03	SK/YGC	Cropland	896	M29
B. bassiana	GBM	R Patan/Tanahun	16.4.03	SK/YGC	Cropland	897	B1
M. anisopliae	GBM	RPatan/Tanahun	16.4.03	SK/YGC	Cropland	898	M30
M. anisopliae	GBM	R Patan/Tanahun	16.4.03	SK/YGC	Cropland	899	M31
M. anisopliae	GBM	R Patan/Tanahun	16.4.03	SK/YGC	Cropland	900	M32
B. bassiana	GBM	R Patan/Tanahun	16.4.03	SK/YGC	Grassland	901	B2
B. bassiana	GBM	R Patan/Tanahun	16.4.03	SK/YGC	Grassland	902	B3
B. bassiana	GBM	R Patan/Tanahun	16.4.03	SK/YGC	Grassland	903	B4
B. bassiana	GBM	R Patan/Tanahun	16.4.03	SK/YGC	Grassland	904	B5
M. anisopliae	GBM	Pang/Parbat	16.4.03	SK/YGC	Cropland	905	M33
M. anisopliae	GBM	Pang/Parbat	16.4.03	SK/YGC	Cropland	906	M34
M. anisopliae	GBM	Pang/Parbat	16.4.03	SK/YGC	Cropland	907	M35
M. anisopliae	GBM	Pang/Parbat	16.4.03	SK/YGC	Cropland	908	M36
M. anisopliae	GBM	Pang/Parbat	16.4.03	SK/YGC	Cropland	909	M37
M. anisopliae	GBM	Pang/Parbat	16.4.03	SK/YGC	Grassland	910	M38
M. anisopliae	GBM	Pang/Parbat	16.4.03	SK/YGC	Grassland	911	M39
M. anisopliae	GBM	Pang/Parbat	16.4.03	SK/YGC	Cropland	912	M40
M. anisopliae	GBM	Pang/Parbat	23.4.03	SK/YGC	Cropland	913	M41
M. anisopliae	GBM	Pang/Parbat	23.4.03	SK/YGC	Grassland	914	M42
M. anisopliae	GBM	Pang/Parbat	28.4.03	SK/YGC	Grassland	915	M43
M. anisopliae	GBM	Pang/Parbat	28.4.03	SK/YGC	Grassland	916	M44
M. anisopliae	GBM	Pang/Parbat	23.4.03	SK/YGC	Grassland	917	M45
B. bassiana	GBM	Pang/Parbat	15.4.03	SK/YGC	Cropland	918	B6
M. anisopliae	SM/soil	Pang/Parbat	10.4.03	SK/YGC	Grassland	919	M46
M. anisopliae	SM/soil	Pang/Parbat	7.5.03	SK/YGC	Grassland	920	M47
M. anisopliae	White grubs	Pang/Parbat	12.4.03	Y.GC	Cropland		M48
M. anisopliae	White grubs	Pang/Parbat	17.5.03	Y.GC	Cropland		M49
M. anisopliae	White grubs	Pang/Parbat	9.6.03	Y.GC	Cropland		M50

B. bassiana	White grubs	Chitwan	17.9.03	Y.GC	Cropland		B7
B. bassiana	White grubs	Chitwan	17.9.03	Y.GC	Cropland		B8

Note: GBM = *Galleria* Bait Method; SM = Selective medium M1-M52 = *Metarhizium anisopliae* isolates; FAL = Federal Research Station for Agroecolgy and Agriculture, Zurich, Switzerland; IAAS = Institute of Agriculture and Animal Sciences

2.4.2 Bioassay with fungus strains in first tier experiment

Laboratory bioassays were performed to assess the infective potential of various strains of pathogens on common species of white grubs prevalent in the study sites. The insect bioassay was performed using the dipping method (Goettel & Inglis, 1997), with third instars larvae of white grubs as targets. Infectivity of the total of 50 isolates of *M. anisopliae* was carried out against third instars larvae of white grubs (*Anomala* spp and *Xylotrupes gidion)*. The fungus culture was used as fresh. The observation units, treated white grubs were replicated twice each with 15 white grubs per replication and single larvae in each poly pots were treated with a single dose (10^7spores/ml) for 50 isolates and positive check with water treatment. The experiment was arranged in a completely randomized design. The experimental unit consisted poly pots and sterile soils. The soil used in the experiment was sterilized in an autoclave at 120 ^{0} C and 15 lbs pressure.

The white grubs larvae used in the experiment were collected from the damaging sites of Parbat, Tanahun and Chitwan districts. Their identification was followed as per the instruction of Prof. Dr. Peter Nagel, University of Basel, Switzerland. The larvae were quarantined into laboratory for 6 weeks before they were experimented. The fungus isolates were grown on selective medium (Strasser *et al*., 1997) and the spores washed off with 0.1% Tween 80. Two types of bioassays, the time and dose mortality bioassay were performed under laboratory conditions of the Tribhuvan University (TU) of Institute of Agriculture and Animal Sciences (IAAS), Rampur, Chitwan Nepal in 2003. Dipping (5 seconds) method was followed upon holding the grubs into its leg with loose forceps to the individual concentration of the fungi. Excess liquid was dropped off the grubs and they were placed individually in rearing containers of 250 ml, where the larvae were fed with the slices of potatoes. Poly-pots were made air tight with its lid and soil was moisten to prevent desiccation, and incubated at 25^0C until recording time. The lid was perforated for air circulation. The experiment was conducted until 12 weeks and the number of fungus invading each insect larva was determined under the stereomicroscopic microscope where fungal infection was checked daily.

In dose mortality experiment, four different doses of M1 strain of *M. anisopliae* such as 10^3 spores/ ml, 10^5 spores/ ml, 10^7 spores/ ml and un-treated treatments were tested in the screening experiments. In both the experiment the treated insects were returned into the same environment of rearing with the supplement of artificial diet i.e. slices of carrots, where ad-libitum moisture and aeration was also provided. Mortality and mycoses insects due to the original fungus were recorded in both the experiments. Following formulae were used to determine the virulent strains on lethal time (LT 50).

Following parameters were calculated using following formulae;

1. Mortality % = No. of dead insects

---------- X 100
30

2. Infection % = No. of infected insects
---------- X 100
30
3. LT 50 = Lethal time of 50% of the population due to *Metarhizium anisopliae* expressed in weeks.

4. LD 50 = Lethal dose of 50% of the population due to the infection of *Metarhizium anisopliae* and is presented in weeks.

2.4.2 Mass production of the insect pathogenic fungi

A mass production system for the development of an entomopathogen as a microbial control agent was set up for rapid multiplication of promising isolates for field application as and when required. Peeled grains of barley were chosen as the solid substrates for this purpose. A di-phasic method consisting of a submerged fermentation for mycelia growth followed by incubation on a solid substrate was adopted. A liquid culture containing bacteriological peptone and sucrose was inoculated with a conidial suspension of the mycopathogen in distilled water. The liquid culture is then transferred to partially cooked and sterilized barley in autoclavable bags which were incubated for about 10 days for sporulation inside the incubator at a temperature of 22-23^{0}C. Upon colonization of the grains they were transferred into the cool, dark room temperature until field application. Before application the colonized grains were tested into water agar and sporulation tests as part of quality control.

03. Results and discussion

3.1 Natural incidence of the entomopathogens

Low level of incidence was found in field-collected white grubs' complex (Table 1). In majority of the cases, heavy mortality was recorded; however, sporulation of the fungus was recorded very low in all the sites. Table 1 showed that *M. anisopliae* presence is comparatively higher in case of Pang, Parbat and low in Jamune of Tanahun. This fungus is available more or less in all the study sites whereas, *B. bassiana* is present almost negligible extent in two sites and it is nil in most of the study area. The possible reason of low natural occurrence of *B. bassiana* might be the low infective potentials in the natural situation. Similarly, virulence of the fungus and congenial environment may also be other attributing factors for this reason.

Isolation of the fungus was proved to be difficult due to bacterial contamination of the plates. Identification of the fungus was performed as instructed by Swiss scientist, Dr. Siegfried Keller, Federal Research Station for Agriculture and Agro-ecology (FAL), Reckenholz, Switzerland based on the conidia sizes and arrangement patterns of the fungi.

3.2 Density of entomopathogenic fungi in soil samples

Two methods were used to study the presence of entomopathogenic fungi in soils; the Galleria bait method (GBM) and spreading soil suspensions on selective media. Using the GBM 52% of the soil samples proved to contain entomopathogenic fungi. The difference between soils from arable land (48% positive samples) and soils from grassland (57% positive samples) were only

small and statistically not significant (P = 0.73). *M. anisopliae* was found at all locations and in all types of crop except in arable land at Tanahun. *B. bassiana* was found in grassland at Parbat (Pang), Tanahun (Rishing Patan), and in arable land at Tanahun (Rishing Patan), and Chitwan (NMRP, Rampur) (Table 2). Two fungus specimens attributed to *Paecilomyces* spp. were found in grassland at Tanahun district.

3.3 Isolation and culturing of entomopathogenic hyphomycetes

A total of fifty entomopathogenic hyphomycetous strains of *M. anisopliae* and eight strains of *B. bassiana* were isolated from various sources (Table 3). Among them seven strains of *M. anisopliae* were originated from white grub's cadavers, five from the selective medium and rests are from the *Galleria* bating from the soil. Whereas in another hand, only two strains of *B. bassiana* were recovered from white grubs and rests from the *Galleria* baiting. This result has conveniencingly showed that, *Galleria* bait method is very common one for obtaining the entomopathogens from soil environment in a reasonably shorter period of time. In addition, it demands low sophisticated techniques and knowledge compared to other methods. These master isolates so far recovered are preserved at IAAS, Rampur, Chitwan, Nepal and FAL, Zurich, Switzerland as a foundation materials for further works in this regard.

3.4 Time mortality response under captivity

Susceptibility study is summarized in figures 2 and 3. This study has revealed that higher the infections better the isolates and vice-versa. It means the isolates that killed the insects in shortest time are considered as better compared to slow killer. Based on the infection rate, the isolate M1 stood as better sporulator (93.33%) followed by the isolates M6, M18 and M48 with 86.67%. Interestingly, isolate M6 was able to cause mortality (2 weeks) in a shorter period as compared to M1 (3 weeks) however, it resulted a comparatively low infection (86.67%) as compared to M1 (93.33%). Isolates having shorter LT 50 such as four weeks period are considered as effective isolates and they need to be further tested and promoted in the development of mycoinsecticides. In majority of the cases the infection percentage is not corresponding with that of percent mortality and LT50 of the test insects.

Figure 2 Mortality and infection (percentage) with respect to different fungus isolates

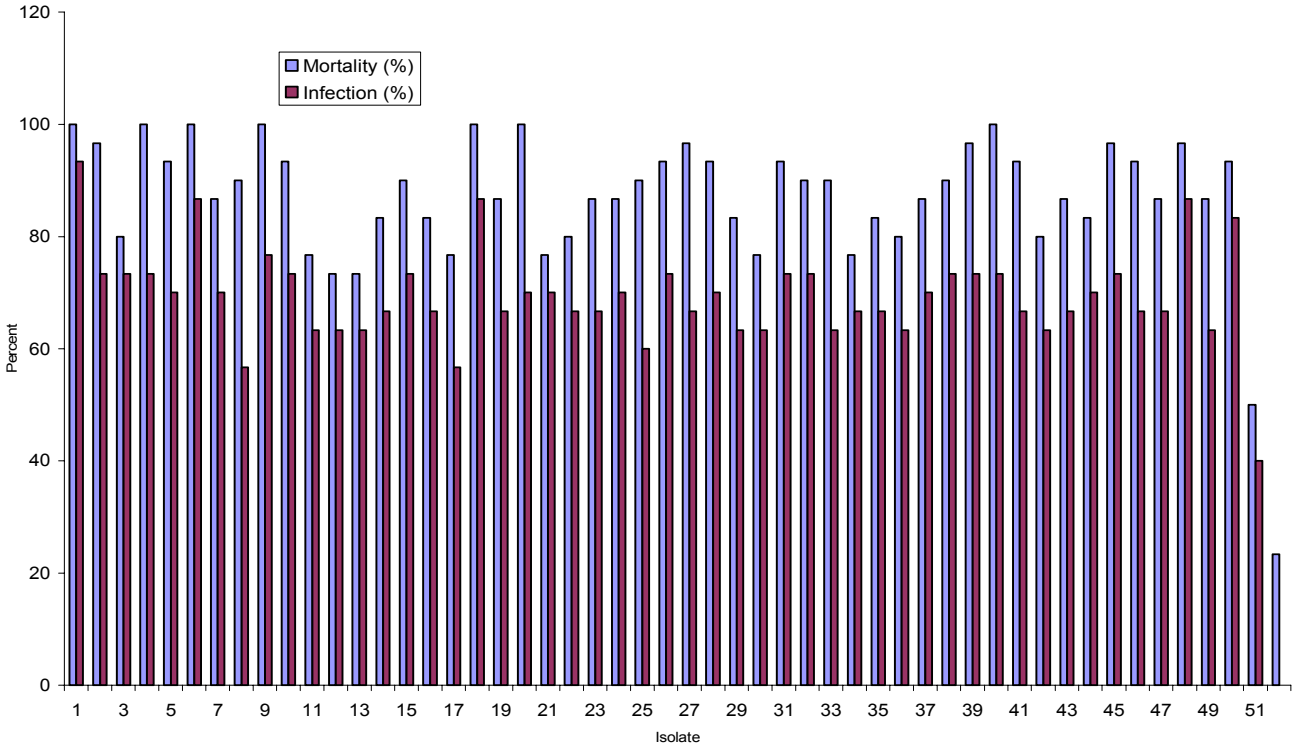
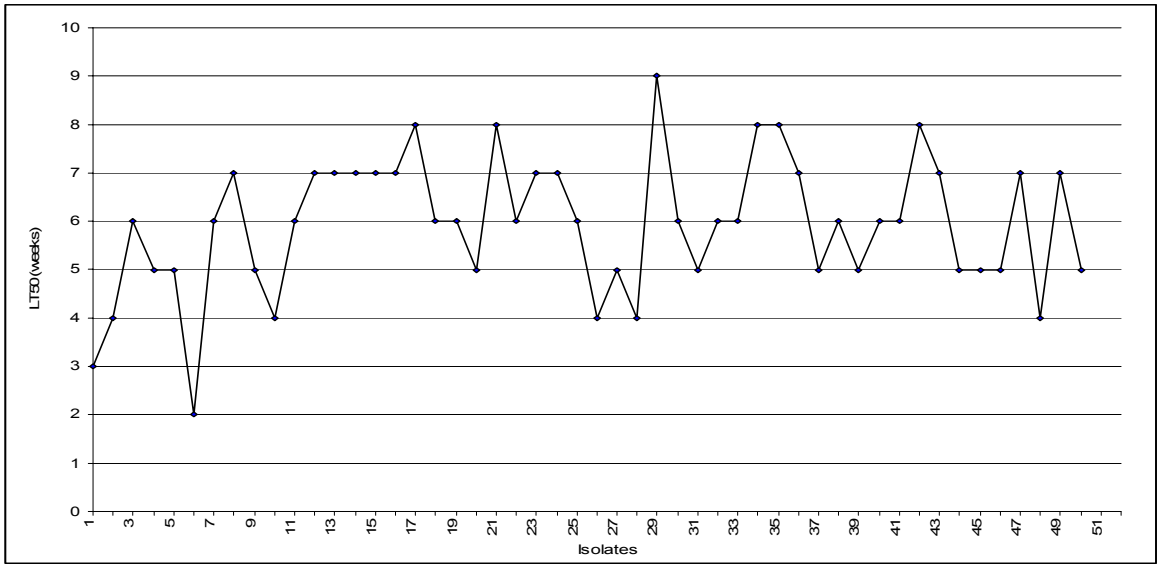


Figure 3 Fungal isolates and their LT50 recorded over the weeks



The pathogenicity however, varies with strains or isolates (Aizawa, 1987). Therefore, the selection of effective strains of entomopathogens is essential for the development of microbial insecticides. The bioassay experiment convincingly showed that, some of the strains isolated from the white grubs are found to be relatively virulent compared to soil isolates (Table 3; figures 2 and 3). Hence, further experiments are suggested with such isolates to reduce the use of chemical pesticide and in contributing eco-friendly pest control measures. They are regenerative in the natural environment and resistance build up by the insect pest is less likely. In addition,

most of the solely chemical based technologies are likely to be worth for a few years before the insect builds resistance. In this sense, microbial control will be more sustainable approach for the increased hill farm production. The population of these agents should be encouraged in the endemic localities either by inoculative or inundative release.

3.5 Dose mortality response under captivity

The effect of different doses of insect fungus to third instars white grubs species is summarized in table 4 to 5 and figure 4. The result showed that higher doses of the fungus inoculums achieving greater killing and vice versa. It is clear that, the mortality and infection percentage is significantly different (P= 0.010) while comparing with control, however, the mean separation revealed that, it is only different between the higher and lower dose i. e. 10^7 and 10^3 and not between the middle dose (10^5 spores / ml solution). It means there is no significant difference between 10^7 and 10^5 spores /ml. Therefore, the effective dose in laboratory killing of the grubs could be targeted from 10^5 spores/ml solution.

Table 4 Effect of different doses of insect fungus to white grubs in percent infection

S.N.	Fugus dose	Percent infection
1	10^7spores/ml	52.00 a
2	10^5spores/ml	45.33 a
3	10^3spores/ml	35.33 b
4	Control	0.00 c
LSD (p=0.010)		9.764
SEM		2.427
CV%		23.14

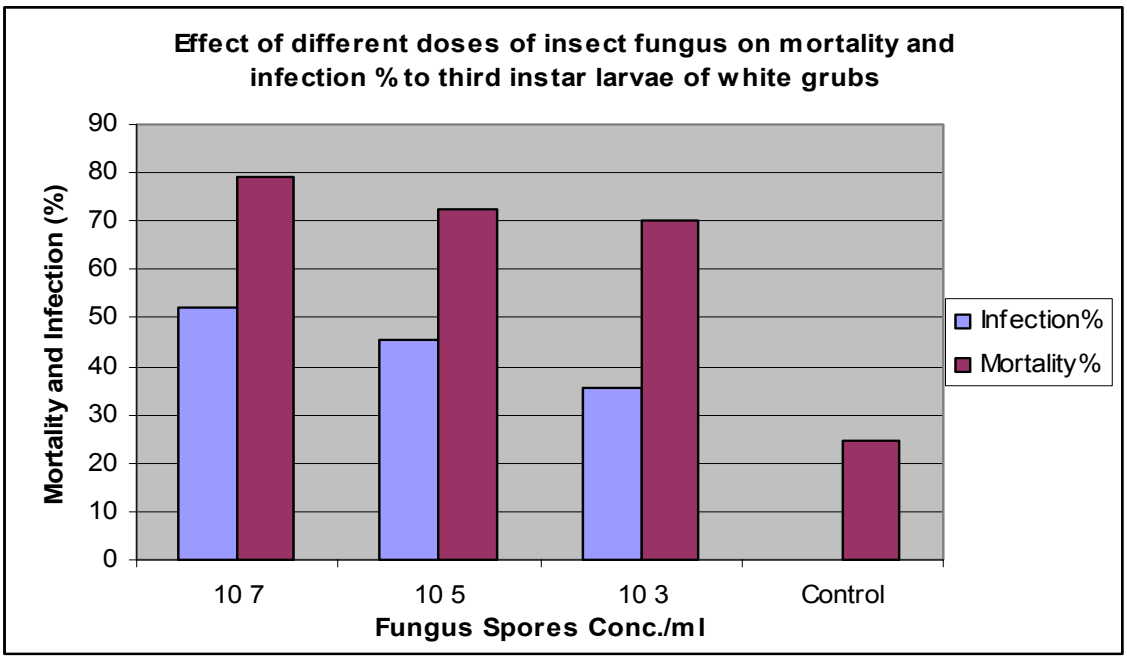
Figures in column followed by same letter are not significantly different at p<0.010 by DMRT.

Table 5 Effect of different doses of insect fungus to white grubs in percent infection

S.N.	Fungus dose	Percent mortality
1	10^7spores/ml	79.33 a
2	10^5spores/ml	72.67 ab
3	10^3spores/ml	70.00 b
4	Control	24.67 c
LSD (p=0.010)		8.483
SEM		2.108
CV%		10.81

Figures in column followed by same letter are not significantly different at p<0.010 by DMRT.

Figure 4 Effect of different doses of *Metarhizium anisopliae to* third instar larvae of white grubs under bioassay experiment



04. Overall conclusion

Entomogenous fungus have great promise for use as biological control agents against different insects however, their infectivity is quite different depending on fungus species and developmental stage of the insects (Samson, 1981). Therefore, when a particular insect pest control programme is considered using these fungus, the particular species or strains which are most suitable have to be taken into account. Similarly, dose and time of exposure of the host to insect fungus and time taken to kill the host are also important parameters for evaluating virulence of insect fungus. Funguses which need shorter exposure period and kill the host quickly are very important in the practical application. It is therefore, important that the activity of selected fungus isolates should be screened against the particular target host at the initial stage. Such pathogenic relationship, may give ideas related to virulence of the species and the number of fungus necessary to kill at least 50% or more insect pests.

They are regenerative in the natural environment and resistance build up by the insect pest is less likely. In addition, most of the solely chemical based technologies are likely to be worth for a few years before the insect builds resistance. In this sense, microbial control will be more sustainable approach for the increased hill farm production. The population of these agents should be encouraged in the endemic localities either by inoculative or inundative release. The development of commercial products based on entomopathogenic fungi for the use in integrated pest management programme needs several steps. Fungal species and isolates must first be obtained from diseased insects or from the environment, and identified. The most promising candidates are evaluated under controlled condition and produced in large scale as mycopesticides for field tests. Working with fungus based mycopesticides in Nepal is very favorable since the Government and donor organizations has given due priority. The preliminary studies has indicated ample opportunity of proceeding ahead with the fungal based mycopesticides in Nepal targeting white grubs in particular and soil-dwelling insect pests in general.

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