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Janak Raj Bohara
B. Sc. Agriculture, Institute of Agriculture and Animal Science, Rampur, Chitwan, Nepal

Sarju Maharjan
B. Sc. Agriculture, Institute of Agriculture and Animal Science, Rampur, Chitwan, Nepal

Asmita Poudel
B. Sc. Agriculture, Institute of Agriculture and Animal Science, Rampur, Chitwan, Nepal

Kasmita Karki
B. Sc. Agriculture, Institute of Agriculture and Animal Science, Rampur, Chitwan, Nepal

Vivek Bist
B. Sc. Agriculture, Agriculture and Forestry University, Rampur, Chitwan, Nepal

Rajendra Regmi
Assistant Professor, Department of Entomology, Agriculture and Forestry University, Rampur, Chitwan, Nepal

Santosh Marahatta
Assistant Professor, Department of Agronomy, Agriculture and Forestry University, Rampur, Chitwan, Nepal

Lekhnath Kafle
Visiting Professor, Department of Entomology, Agriculture and Forestry University, Rampur, Chitwan, Nepal

Correspondence
Janak Raj Bohara
B. Sc. Agriculture, Institute of Agriculture and Animal Science, Rampur, Chitwan, Nepal

Efficacy of different concentration of Metarhizium anisopliae (Metsch.) Sorokin against white grub at lab condition in Chitwan, Nepal

Janak Raj Bohara, Sarju Maharjan, Asmita Poudel, Kasmita Karki, Vivek Bist, Rajendra Regmi, Santosh Marahatta and Lekhnath Kafle

Abstract
An experiment was conducted to evaluate the effect of different concentration of entomopathogenic fungus, *Metarhizium anisopliae* (Metsch.) Sorokin against white grubs in the Entomology Laboratory, at Institute of Agriculture and Animal Science, Rampur, Chitwan in 2015. For the experiment, 6 treatments were tested against white grubs at the rate of 10^7 commercial conidial spores per ml and indigenous 10^7, 10^5, 10^4, 10^2 of conidial spores per ml including control and each treatment were replicated thrice. Total 270 grubs were treated, with 15 grubs in each replication of each treatment. They were screened for relative pathogenicity, and the mean lethal time eliciting 50% mortality (LT_{50}) was calculated. Indigenous 10^4 spores/ml concentration of fungus had the lowest LT_{50} value of 61.45 days followed by 10^2 spores/ml with 65.35, 10^7 spores/ml with 68.98, 10^5 spores/ml with 76.04 and commercial 10^7 spores/ml with 152.71 days. Indigenous isolate showed higher mortality of white grubs in comparison to commercial isolate.

Keywords: white grub, entomopathogenic fungi, *Metarhizium anisopliae,* isolate

Introduction
White grubs (Coleoptera: Scarabaeidae) are soil inhabiting and root feeding immature stages of scarab beetles. 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).

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 (Guppy and Harcourt, 1970).White grubs are polyphagous pests which tend to injure the roots throughout the crop period because of their overlapping life cycle. Beetles are defoliating pests and 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 vigour or lodging of larger ones(G. C., 2006). They are attributed to have caused an average of about 25% yield loss on groundnut (*Arachis hypogea*) in western Nepal (Thapa and G. C., 2000). White grub causes losses to the extent of 12 to 60 percent in many crops. It is emerging as a serious pest of maize and vegetables in Nepal (Pokhrel, 2004).

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 (Wegner and Niemczyk, 1981).Chemical method for white grub control using persistent organochlorines such as chloradane, aldrin, dieldrin, heptachlor (Niemczyk and Lawrence, 1973); DDT, BHC, carbaryl, malathion, endrin, phorate, carbofuran and quinolphos (Misra, 1995) were found ineffective in controlling white grubs as the larvae present in the soil do not come into direct contact with the insecticides (Wegner and Niemczyk, 1981) and have even shown resistance to organo-chlorine (Pokhrel, 2004). The uses 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 (Baker and Gyawali, 1994).

Metarhizium anisopliae (Metsch.) Sorokin, 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 (Zimmermann, 1992; Keller and Zimmermann, 1989). 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 (Ferron, 1978). However, 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 (Pokhrel, 2004).

Materials and methods

An experiment was conducting in laboratory of Department of Entomology, Institute of Agriculture and Animal Science (IAAS), Rampur, Chitwan, Nepal during September - November, 2015. Statistical data analysis was carried out using Excel and Genstat software. LT_{50} was calculated by using SAS 2016.

Experimental design

The experiment was conducted on CRD design. There were six treatments (1 commercial strain, 4 indigenous concentrations and 1 control)and three replications for each treatment. Altogether there were 270 larvae, 45 per treatment and 90 per replication. The details of treatments are given in the table below:

Table 1: Treatment details of the experiment

Treatments	Strains	Concentration (spores/ml)
T1	Commercial	10^7
T2	M9	10^7
T3	M9	10^5
T4	M9	10^4
T5	M9	10^2
T6	Control	Distilled water

Collection of white grubs

White grubs (larvae) were collected from agricultural fields of Rampur, Chitwan. Fields with relatively high population of grubs like mango orchard, maize farm, etc. were selected. The field was dug beneath the crop root zone, grubs were picked up and placed individually in perforated poly pots filled with humid, cool, loose soil up to its two third level. They were then brought to laboratory and classified based on the instars and species with the help of larval keys. Small and injured ones were discarded. Emphasis was given in collection of similar sized larva of same species.

Preparation of larvae for bioassay

The selected larvae were reared in individual perforated poly pots of 4.5 cm diameter and 6 cm height. Soil collected from the sampling farms was poured in the containers upto two third level to provide natural habitat to the grubs. Potato slices were fed and optimum moisture and humidity was maintained by adding distilled water. Larvae were placed in quarantine for about 4 weeks with regular inspection. The rearing room was kept dark and protected by UV light. The temperature of the room was around 29ºCand relative humidity 79.2%. Grubs were checked time to time (every third day).

Preparation of media for bioassay

To prepare media for growth and *in vitro* analysis of the fungus *Metarhizium anisopliae*, 800 ml of water was boiled in 1000 ml conical flask. Agar agar (17 g) was added in the flask followed by peptone (8 g) and glucose (16 g). The conical flask was made air tight using cotton plug and aluminium foil. The flask and petri plates were than autoclaved for 25 minutes at 121ºC in 15 psi pressure for sterilization. Flask containing media and petri plates were than allowed to cool in laminar air flow bench. About 3 to 4 drops of iodine was added in the media and shaken slowly to control bacterial growth. The working bench was completely sterilised to prevent contamination. The prepared media was then poured in 28 petri plates at the rate of 19 – 20 ml per plate.

Isolation and culture

Multiplication of fungal conidia in the selective medium (SM) was adopted from Strasser *et al*. (1996). The fungal isolates (strain M_9) preserved in lab were used for experimentation. Petriplates with media were inoculated with the M_9 strain of fungus and incubated at 22ºC for 2 weeks.

Preparation of different concentration of spores of *Metarhizium anisopliae*

Spores were counted under microscope using Thoma Haemocytometer and different concentrations were prepared as:

Concentrations	Stock volume(ml)	Water(ml)
10^7	15.5	34.5
10^5	3.1	96.9
10^4	10	90
10^2	1	99

Assessment of bioassay experiment

Bioassay was carried out using the dipping method (Goettel and Inglis, 1997) against third instar larva. Bioassay was carried out with different concentration of spores like 10^7 spores/ml, 10^5spores/ml, 10^4 spores/ml and 10^2spores/ml for indigenous *Metarhizium* and 10^7 ml for commercial *Metarhizium* while one group of white grub was maintained as control. Larvae were dipped in spore suspension individually for 3 – 5 seconds and returned into rearing pot. The treated larvae were checked at every third day for 75 days. The temperature and relative humidity was maintained at 25ºC and 79% respectively.

Statistical Data Analysis

The recorded data was entered and tabulated in Microsoft Excel Worksheet and analyzed by Analysis of Variance (ANOVA) andDuncan's Multiple Rage Test (DMRT) at 5% level of significance in GENSTAT. LT_{50} was calculated by SAS 2016. A correction was made for those grubs that died in untreated control with Abbott's formulae (Abbott, 1925).

$$\text{Corrected percent mortality} = \frac{\text{Percent test mortality} - \text{Percent control mortality}}{100 - \text{Percent control mortality}} \times 100$$

Parameters used

i. Mortality %= No. of dead insects/15 X 100
ii. Infection %= No. of infected insects/15 X 100
iii. LT_{50}

Results and Discussion

Pathogenicity

All the concentrations tested were observed to be pathogenic against white grubs (Table 8, Figure 3). The percentage of mortality corrected by Abbott's formula varied from 35.90 to

97.44 (Table 8). The indigenous isolate with concentration of 10^4 spores/ml and 10^2 spores/ml showed highest mortality of white grubs while lowest mortality of larva was seen in commercial isolate of 10^7 spores/ml at 108 days after treatment. Although, concentration of 10^4 spores/ml and 10^2 spores/ml showed same mortality at 108 days after treatment but from figure 1 it is clear that mortality caused by 10^4 spores/ml is higher than that of 10^2 spores/ml concentration during most of the time.

Table 8: Corrected percentage mortality of white grub larvae by different concentration of *Metarhizium anisopliae* in the laboratory, IAAS, Rampur, 2015

	Days after treatment																	
Concentration	1-6	6-12	12-18	18-24	24-30	30-36	36-42	42-48	48-54	54-60	60-66	66-72	72-78	78-84	84-90	90-96	96-102	102-108
Commercial (10^7spores/ml)	0.04	4.44	6.67	8.89	11.11	11.11	11.11	13.33	13.64	15.91	18.60	23.26	24.39	22.50	23.08	28.21	33.33	35.90
Indigenous (10^7spores/ml)	0.02	2.22	4.44	4.44	8.89	8.89	15.56	17.78	20.45	25.00	41.86	51.16	53.66	62.50	71.79	89.74	89.74	92.31
Indigenous (10^5spores/ml)	0.02	2.22	4.44	4.44	4.44	4.44	6.67	6.67	4.55	25.00	25.58	32.56	53.66	60.00	69.23	84.62	84.62	84.62
Indigenous (10^4spores/ml)	0.02	4.44	4.44	11.11	17.78	17.78	20.00	24.44	29.55	31.82	48.84	58.14	68.29	77.50	89.74	97.44	97.44	97.44
Indigenous (10^2spores/ml)	0.02	6.67	8.89	8.89	8.89	8.89	15.56	15.56	15.91	22.73	34.88	48.84	68.29	80.00	87.18	94.87	94.87	97.44

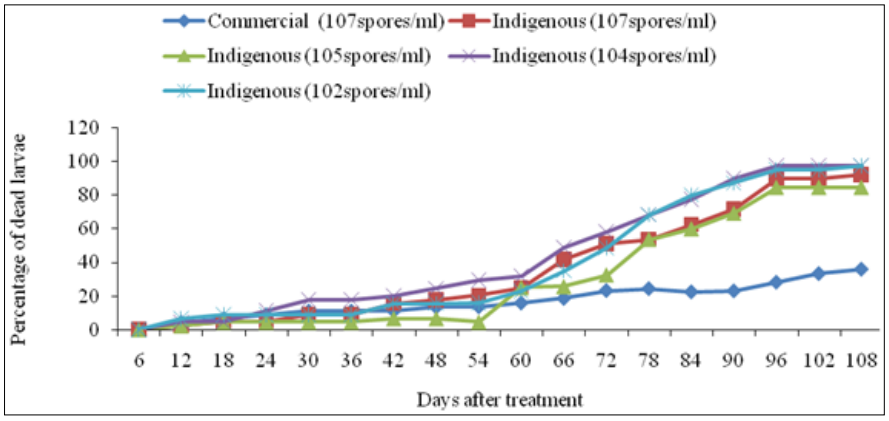

Fig 3: Mortality of white grub by different concentration of *Metarhizium anisopliae* in the laboratory, IAAS, Rampur, 2015

Comparison between cumulative mortality of indigenous and commercial isolates

Both indigenous and commercial isolates showed the similar effects on the mortality of white grub until 54 days after treatment. Although, there was no significant difference between the isolates, mortality was found slightly higher in commercial isolate at 6 DAT, 12 DAT, 18 DAT, 24 DAT, 30 DAT and 36 DAT. However, from 42 DAT to 54 DAT, mortality percentage of grub was found higher for indigenous isolate but non-significant. After 54 to 108 days of treatment, the mortality percentage of white grub by indigenous isolate was found significantly higher than commercial isolates. The comparison between cumulative mortality of indigenous and commercial isolates is shown in figure 1.

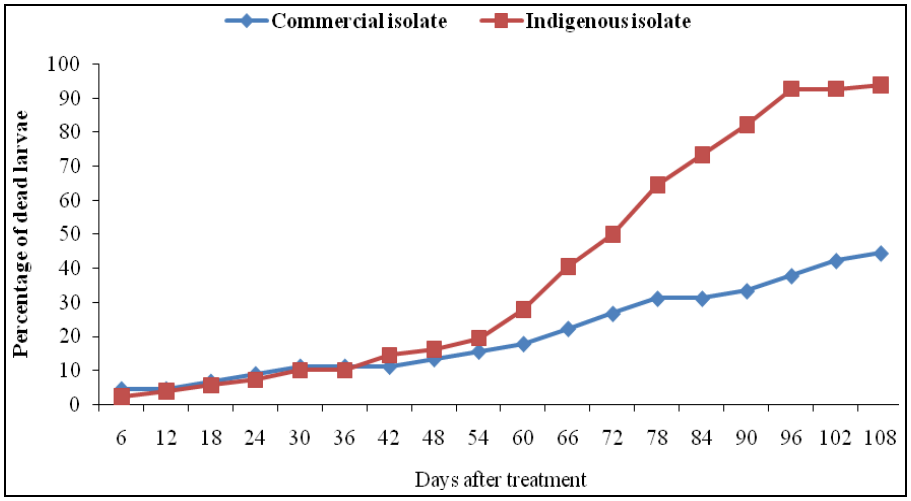

Fig 1: Grand mean of cumulative mortality of white grubs caused by different concentrations of commercial and indigenous isolate of *Metarhizium anisopliae* in the laboratory, IAAS, Rampur, 2015

Cumulative mortality of white grubs

The line graph of cumulative mortality of grubs at different days of treatment showed that the death of larvae occurred slowly during first 30 DAT, remained constant from 30 DAT to 36 DAT, increased steeply from 36 DAT to 96 DAT and increased at very slow rate from 96 DAT to 108 DAT as shown in figure 2.

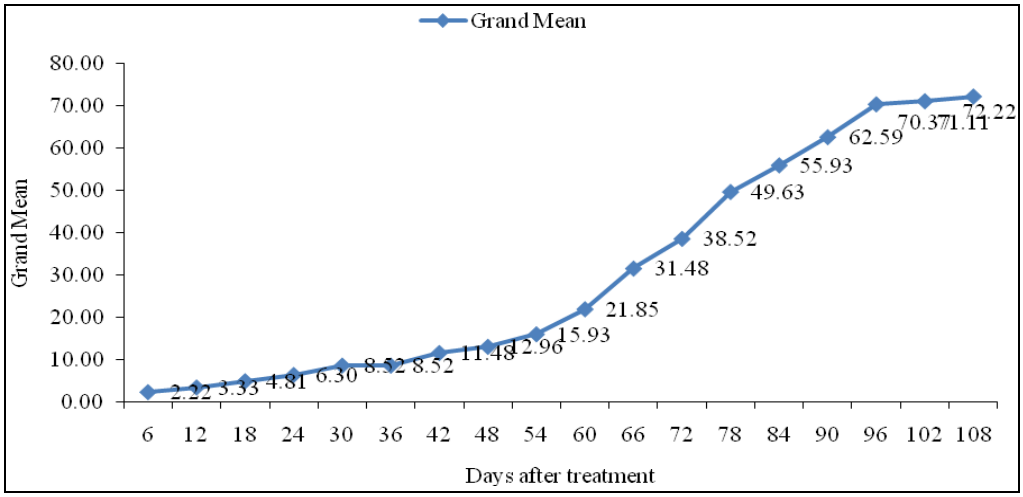

Fig 2: Grand mean of cumulative mortality of white grubs caused by different concentration of *Metarhizium anisopliae* in the laboratory, IAAS, Rampur, 2015

Mean Lethal Time (LT_{50})

At the end of the study (108 DAT), when the total number of test insects killed by the test items (commercial 10^7 spores/ml, indigenous 10^7 spores/ml, indigenous 10^5 spores/ml, indigenous 10^4 spores/ml, indigenous 10^2 spores/ml, and control) were compared, significantly higher number of test insects were killed by the indigenous 10^4 spores/ml and 10^2 spores/ml concentration of fungus. However, number of test insects killed by indigenous 10^7 spores/ml, indigenous 10^4 spores/ml and indigenous 10^2 spores/ml or indigenous 10^7 spores/ml indigenous and 10^5 spores/ml concentration were not significantly different (p = 0.001, F = 251.1). Based upon the LT_{50} values, indigenous isolate with concentration 10^4 spores/ml has the highest test insect killing speed than indigenous 10^2 spores/ml, indigenous 10^7 spores/ml, indigenous 10^5 spores/ml and commercial 10^7 spores/ml concentration of fungus (Table 9).

Table 9: Efficacy of different concentration of test item on test insect under laboratory conditions

Concentrations of fungus (spores/ml)	No. of dead grubs (mean ± SE)[a]	LT_{50} (Days)[b]
Commercial 10^7	6.67±0.33c	152.71
Indigenous 10^7	14±00ab	68.98
Indigenous 10^5	13±0.58b	76.04
Indigenous 10^4	14.67±0.33a	61.45
Indigenous 10^2	14.67±0.33a	65.35
Control	2±0d	-

[a]Means within the same column followed by the same letter are not significantly different ($p \leq 0.05$) using SNK test of SAS (2016).
[b]LT_{50} values (in days) were determined by probit analysis (StatPlus, 2015).

4. Conclusion

The infectivity of indigenous strain of *M. anisopliae* to white grubs in laboratory condition indicates good potential for further use of the fungus. The selection of virulent strains, there mass production, extensive field evaluation have to be done before commercial application and recommendation to the farmers. This experiment has further suggested for the use of virulent strains while conducting mass production into a suitable substrates as a means of white grub control. Retention of the fungus virulence throughout production, storage and application of the fungus is very important. The fungi are highly sensitive to higher temperature; therefore appropriate temperature should be maintained during storage. The fungus material should be preserved at refrigerator temperature and handled with care for maintaining its virulence. Mass production, formulation, storage and field application are important factors which should be considered into full account for the larger scale production of fungus based bio-pesticides.

5. Recommendation for further research

From the findings of this study, following recommendations are made for further study:

- Extensive field evaluation of the virulent indigenous fungus.
- Research on seasonal variation on virulence of the fungus.
- Similar research is suggested for other agro-climatic zones.
- Comparative study of different bio-control agents.
- Development of integrated eco-friendly management practices for white grubs.
- Research on intra-specific competition in M. anisopliae

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