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Evaluation of effects of indigenous entomopathogenic fungus, *Metarhizium anisopliae* (Metsch.) Sorokin on on-target organism, the *Bombyx mori* Lin.

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ABSTRACT

A laboratory experiment was conducted to evaluate the level of effects by different concentrations of indigenous entomopathogenic fungus, *Metarhizium anisopliae* (Metsch.) Sorokin spores by dipping the non-target organisms, the silkworm, *Bombyx mori* L. in the fungus suspension at laboratory condition in Rampur, Chiwan, Nepal. The selected most virulent fungus isolate, among 26 was tested at different concentrations of 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 number of spores per ml including a control each replicated thrice to each of 30 fourth instar silkworm larvae. The mortality of silkworm larvae due to fungus started only after five days of the treatment and finally caused 70, 44.44, 28.88, 26.66, 23.33 and 3.33 percent death of silkworm larvae with 10^7, 10^6, 10^5, 10^4, 10^3 and 10^2 spores per ml of fungal conidial concentrations respectively. The larval body weight was significantly lower even one day after treatment in the lots treated with 10^7 spores as compared to other concentrations. On the third and fourth day of treatment, there was no significant difference in the body weight of silkworm larvae in all the treatments. There was significantly lower weight in the lots treated with 10^7 spores after fifth, sixth and seven days of treatment but after eighth days, there was no significant difference in the body weight with the treatments. The laboratory result indicated that even a small number of conidia of *M. anisopliae* (Metsch.) Sorokin caused mortality and was found hazardous to silkworms.

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INTRODUCTION

Soil insects like white grubs and red ants have become serious pests of most cereals, fruits, vegetables, ornamental plants, plantation crops, pastures, turf and meadow grasses, lawns, golf courses and forest trees in different part of the world (Arita *et al.*, 1993). In many crops, white grub as a soil pest caused losses to the extent of 40 to 80 percent (Raodeo *et al.*, 1974).

Many chemicals like chlordane, aldrin, dieldrin and heptachlor (Niemczyk and Lawrence, 1973); DDT, BHC, carbaryl, malathion, endrin (Kaunsale *et al.*, 1978); phorate, carbofuran, quinolphos, fenithion and diazinon (Fujiie and Yokoyama, 1996) were found ineffective in controlling the soil pests. The active stage of soil insects present in the soil do not come into direct contact with the insecticides (Wegner and Niemczyk, 1981). The insects have also shown resistance with many chemicals (Niemczyk and Lawrence, 1973). The uses of chemical insecticides to control the pests have hazardous effect to the non-target organisms due to cryptic nature of the soil pests. Therefore, alternative methods to control the pest are essential and various control agents have received considerations in different part of the world (Alm *et al.*, 1994; Benyakir *et al.*, 1995; Bednarek *et al.*, 2000). Microbial control strategy is valuable component in integrated pest management and has advantages over chemicals due to its improved performance, cost effectiveness and increasing resistance of insects to the chemical insecticides (Roberts *et al.*, 1991; Rosset and Moore, 1997). The microbial control is compatible with biological, toxicological, environmental and social requirements (Inglis *et al.*, 2001).

The sustainability and economics of microbial agents rest on the efficacy to the target pests and at the same time safety to the non-target organisms (Burges and Hussey, 1971). The host range of *Metarhizium anisopliae* (Metsch) Sorokin is wide exceeding 200 species of 7 orders of the insects (Samuels *et al.*, 1989; Robertson, 1993). The pathogenicity, however, varies with isolates and the insects (Aizawa, 1987). The fungus applied to control the soil pests may be carried to the silkworm

feed plant: the mulberry. The white grub adults carrying fungal spores can feed on newly developed twigs of the mulberry and there is a chance of contamination to the rearing rooms. Therefore, the evaluation of potential fungal isolate in causing infection to non-target organism is essential for the development of microbial insecticides (Moorhouse *et al.,* 1993). So, keeping these points in view, a laboratory research was undertaken to evaluate the effects of different concentrations of the fungus on mulberry silkworm, *Bombyx mori* Lin. as non-target organism. The general objective of the study was to explore the potentiality of *Metarhizium anisopliae* (Metsch.) Sorokin by assessing the effects on potential non target organism: the silkworms. The following were the specific objectives of the study: to estimate the rate of infection and death by different concentrations of *Metarhizium* isolates in silkworm larvae by bioassay method and to evaluate the effect of virulent fungus isolate on growth of non-target organism, the mulberry silkworm.

Metarhizium is a fungus of form family Moniliaceae, form order Hyphomycetes and form class Deuteromycetes (Tulloch, 1976). The fungus was first isolated from the wheat chafer *Anisopliae austriaca* Hbst. by Metschnikoff in 1879 and suggested its use as microbial agents against insect pests (Steinhaus, 1949). Tulloch (1976) studied a number of species in this genus and concluded that two species *M. anisopliae* (Metsch.) Sorokin and *M. flavoviridae* Gams and Rozsyopal were most promising in causing disease in insects. There are many studies on this fungus to control different pests and is getting dimension to be used as a commercial product in different part of the world. The non-target organism indicates all living things except the pests being treated. Generally, entomopathogens and particularly *Metarhizium anisopliae* parasitise insects and there are rare cases that the fungus also attack outside of Insecta. The direct and indirect effect on all members of ecosystem is equally important which makes the evaluation difficult (Hajek and Goettel, 2000). The entomopathogenic fungus is generally perceived to be ecologically preferable to chemical treatment, if found safe to the non-target organisms. However, biological control agent carries its own risks (Simberloff and Stiling, 1996), including their potential for damage to the non- target organisms. At least, it is more important to study the effects on commercially important organisms like the honeybees and silkworms.

Non-target effects may be of less concern when native organisms are used for biological control (Goettel *et al*., 2001). *M. anisopliae* occurs naturally in soils (Humber, 1992) and has a broad host range of more than 200 insect species from different orders (Zimmermann, 1993). Though the wide host range is desirable from the commercial view point of production, it is a risk from an environmental perspective as it may include beneficial organisms in the host range (Vestergaard *et al.,* 2003). Therefore, further studies of the possible non-target effects are needed to weight the ecological risks against the expected benefits in pest control (Hajek and Goettel, 2000).

No evidence has been found for this fungus being pathogenic applied by different methods to birds, mice, rats, guinea pigs and rabbits (Austwick, 1980; Shadduck *et al*., 1982; El-Kadi *et al*. 1983; Zimmermann, 1993) and by inference for humans but lung lesions have been detected in crocodiles and these has been produced artificially in lizards (Shadduck *et al*., 1982). Subcutaneous and intravenous injection of 0.5 ml of 2 percent suspension of *M. anisopliae* to rats did not show any morbid changes (Heimpel, 1971) and showed the absence of fungal mycelium in the blood after two months period. Similarly, found no pathogenic or toxic effects or allergic manifestations in the rats by inhalation (Heimpel, 1971). On feeding also, all rats survived, were normal, exhibited no losses in weight and had unreduced appetites. It is reported that physically stressed animals are susceptible to this fungus (Shadduck *et al*., 1982).

Beneficial insects are generally not reported as being susceptible. *M. anisopliae* has been detected in nests of bumblebees but has not been reported causing disease in any bees including honeybee *Apis mellifera* L. (Macfarlane, 1976). *M. anisopliae* is recognized as a natural enemy of silkworms in Japan and India (Aoki, 1958; Ganga and Chetty, 1999). Silkworms can be reared in isolation from pathogens used against pest insects in the field (Krieg, 1971; Bailey, 1971). Though silkworms are more susceptible, it is not necessary that the pathogens safe to silkworms are safe to other beneficial field insects (Bailey, 1971). Insect pathogens that have failed to infect silkworms in the laboratory include *Bacillus popillae* Dutky *and B. lentimorbus* Dutky which cause milky disease in *Popilliae japonica* Newman (Kriger *et al.*, 2000).There are many reports that elaborate the

restricted use of the microbial control agents. Like, *Rickettsia* is restricted in white grub as is not completely safe to warm-blooded animals (Krieg, 1971). The others like the members of *Aspergillus* are pathogenic to vertebrates (Heimpel, 1971). There are many hosts that are infected in the laboratory are never found infected in the field (Goettel *et al*., 2001). Therefore, the evaluation of agents of microbial control is crucial.

The biological assay or bioassay is a form of experiment for the estimation of the potency of a substance or comparing the efficacy of two or more substances by means of reaction that follows their application to living matter (Rangaswamy, 1995). The bioassay involves a stimulus applied to a subject and the response of the subject to the stimulus (Reichelderfer, 1993). The pathogenicity or the virulence of microbial insecticide is a function of many interacting factors. Virulence may be measured in a bioassay by exposing a known number of hosts to a known number of pathogens and observing the number of dead hosts over time (Reichelderfer, 1993). The amount of microbial agent that is required to produce a desired mortality is a function of susceptibility of the host and virulence of pathogen (Steinhaus, 1949). The bioassay method should expose the pathogen to the host by the natural route (Reichelderfer, 1993). Insect cuticle is the site of infection of silkworms (Inglis *et al.*, 2001). The dipping method is the most common bioassay method for the evaluation of fungus in white grubs and termites (Keller, 2000). The bioassay and rearing methods are unique and differ with test insects and the pathogen (Grace, 1994).

MATERIALS AND METHODS

The study encompasses bioassay test to evaluate the effects of *M. anisopliae* on body weigth and mortality of *Bombyx mori* L.

Fungus source and its preparation

The fungal isolate for bioassay was obtained from the soil of midhill condition of Nepal and was maintained on Saboraud Dextrose Agar (SDA) supplemented with antibiotics. The isolate used in the bioassay was maintained on its natural host, *Galleria mellonella.* The isolate *M. anisopliae* isolate used in the bioassay was obtained from the cropland soils of Durlung, Parbat, Nepal. The fungus was multiplied on selective medium SDA adopted from Strasser *et al.* (1997) with the following composition: 20 gm dextrose, 10 gm peptone pancreatically digested, 18 gm agar-agar, 0.05 gm of Cyclohexamide and Tetracycline each and 0.6 gm Streptomycin all dissolved in 1000 ml of distilled water and transferred into sterilized petriplates and test tubes. That was incubated at 27 $\pm$ 2^0C and 80$\pm$5 percent RH to induce the growth and sporulation of the fungus. After 15 days, the conidia were harvested by scrapping off the contents of each petridishes and test tube with sterile bacteriological loop.

Enumeration of fungal conidia

The concentration of conidia per ml was calculated by using a Thoma Haemocytometer. The conidia were first harvested by scrapping off the contents of each petridish with a sterile bacteriological loop. The conidial mass was dispersed in water by using a drop of Tween 80. From the original solution, 1 ml was taken and observed under microscope in Haemocytometer. This dense original solution was diluted to make the counting easy and possible, and later it was diluted to different concentrations.

Statistical analysis

Data were tabulated and analyzed using Excel 2000 to see the effect of the isolate to calculate LT_{50}. T-test was used to test whether the variances and mean among the isolates were significantly different. A correction was made for those individuals that died in untreated control with Abbott's formulae (Abbott, 1925).

Description of experiment

Completely Randomized Design (CRD) was used to study the effects of different concentrations of the fungus M. anisopliae. Thirty III- instar larvae of mulberry silkworm were taken as an experimental unit and replicated three times. The entire experimental material was divided into 21 experimental units and numbered serially starting with one and proceeding in serpentine manner. The different concentrations of 10^7 (T_1), 10^6 (T_2), 10^5 (T_3), 10^4 (T_4), 10^3 (T_5), 10^2 (T_6) conidial spores per ml of suspension and a control (T_7- distilled water without conidia) were used as the treatments (T1 to T7). The treatments were allotted randomly using

three-digit random numbers selected from random number table (Gomez and Gomez, 1984).

Preparation of silkworm larvae and rearing

The eggs of bi-voltine mulberry silkworm, Japanese and Chinese (J_{12} X C_{12}) cross were obtained from Sericulture Development Section, Khopasi, Chitwan, Nepal. The silkworm eggs were incubated and were brushed and reared after hatching larvae on Kanva-2 mulberry. The young and grown-up larvae were reared on mulberry following the standard practice (Jolly, 1987). After third moult, the newly moulted out IV- instar larvae were treated with fungus material and were reared on polyethylene trays of 11 X 9 X 3 inches with side and bases netted.

Bioassay experimentation

The virulent isolate (M_6) of the *M. anisopliae* was taken for the side effect study. The fourth instar silkworm larvae were submerged for 5 seconds in 50 ml of conidial suspension of different concentrations. The treated larvae were transferred to petri dishes containing sterile filter paper and then placed in the rearing tray. The daily record was taken to note mortality. The dead larvae were preserved in 60 ml transparent vials on cotton to facilitate the fungal growth and to confirm the cause of death. The larval body weight was taken just before the fungus treatment and followed daily up to the end of the trial i.e. before cocooning. The daily maximum and minimum experimental room temperature and humidity was recorded for the entire bioassay period.

RESULTS AND DISCUSSION

Mortality of silkworm larvae

The fungus *M. anisopliae* caused infection and death to silkworms. The mortality was recorded highest in the silkworm lots with increased spore concentration. The concentration of 10^7 spores per ml caused the highest mortality was significantly different from other lower concentrations. The larval death due to fungus was not recorded within control group. Within the first four days of treatment, there was no death of silkworm larvae due to the fungus in either of the treatments. The higher concentrations caused earlier death and the lower on later period. All the dead silkworms were covered with fungal growth in the treated lots. After 10 days of treatment, the mortality due to concentrations of 10^6, 10^5, 10^4, 10^3, 10^2 *M. anisopliae* spores per ml was 43±5%, 30±5%, 26±3%, 23±12% and 3% respectively. The mortality was higher in lots treated with higher concentration of fungus (Figure 3).

Table 1. Mortality of silkworm ($J_{12}XC_{12}$) larvae by different conidial concentrations of *M. anisopliae* after tenth day of treatment in the laboratory

Concentrations	Mean no of dead silkworm larvae *
10^7 spores/ml	$21±4.00^a$
10^6 spores/ml	$13±1.53^b$
10^5 spores/ml	$9±1.53^c$
10^4 spores/ml	$8±1.00^c$
10^3 spores/ml	$7±3.79^c$
10^2 spores/ml	$1±0.00^d$
Control (Water)	$0±0.00^d$

* Means followed by same letter in a column are not significantly different at 5% level

There was no significant difference between control and 10^2 spores per ml (Table 2, 3 & 4). The concentrations 10^3, 10^4 and 10^5 did not significantly differ from each other on the mortality.

Table 2. Mortality of silkworm ($J_{12}XC_{12}$) larvae over time by different conidial concentrations of *M. anisopliae* in the laboratory

Conidia per ml	Days after treatment					
	5^{th}	6^{th}	7^{th}	8^{th}	9^{th}	10^{th}
10^7	1.67^a	2.67^a	4.67^a	5.33^a	2.33^a	4.33
10^6	0.00^b	0.00^b	1.33^b	2.67^b	2.67^a	3.33
10^5	0.00^b	0.00^b	0.33^b	2.00^b	1.67^a	2.67
10^4	0.00^b	0.33^b	2.67^a	2.00^b	1.33^a	1.67
10^3	0.00^b	0.00^b	0.33^b	0.00^c	0.67^a	0.00
10^2	0.00^b	0.00^b	2.00^a	1.67^{bc}	1.33^a	1.33
Control	0.00^b	0.00^b	0.00^b	0.00^c	0.00^b	0.00
P (at 0.05)	*	***	*	***	*	ns
$LSD._{05}$	1.011	1.081	2.702	1.986	1.986	2.702
Mean	0.24	0.43	1.62	1.95	1.43	1.90

Average of 30 larvae/replication; values followed by the same letter in each column are not significantly different by LSD and ns means none significant and *, ** , *** means significant at P< 0.05, P<0.01 and P <0.001, respectively.

Table 3. Mean square values of mortality of silkworm ($J_{12}XC_{12}$) larvae after days of *M. anisopliae* treatment in the laboratory

Source of variation	Days after treatments						
	df	5th	6th	7th	8th	9th	10th
Concentration	6	1.190	2.968	8.27	9.82	2.52	8.07
Error	14	0.3333	0.3810	2.381	1.286	1.286	2.381
CV%		22.50	14.00	9.30	8.00	9.40	8.00

df means degrees of freedom and CV means coefficient of variation

Larval body weight

There was no significant difference in the body weight of silkworm on the day of treatment. The following day, the larvae treated with 10^7 spores had significantly lower weight than the others. On the third and fourth day, there was no significant difference in the body weight of silkworm larvae among the treatments. Then afterwards, there was significantly lower weight in the lots treated with 10^7 spores. However, again after the eighth day of treatment, there was no significant difference in the body weight (Table 5, 6) of the lots treated with different concentrations.

Infection by *M. anisopliae* in silkworms

The healthy IV-instar silkworm larvae became sluggish just after a day of treatment with *M. anisopliae* conidial suspension, yellow and brown spots occurred on the cuticle, spread over other parts of the body on third days of treatment, on five to six days of the fungus treatment, the larvae became extremely feeble, stiff, after which they became rigid in form and brownish-yellow in color, mummified and died, after seven days of treatment or three days after death, the hyphae broke through the integument everywhere. Thereafter, the spores covered the body of the dead silkworms. In later days of death the spores became olive green in color, cohering in masses, which made large flakes The fungus *M. anisopliae* caused infection and death to silkworms. The mortality was higher with increased concentration of fungus as suggested by Steinhaus (1949), McCauley *et al.* (1968), Glare and Milner (1991). The silkworm larvae began to die after four to five days of infection. Similar results have been reported in different insects (Steinhaus, 1949). The mortality result demonstrated that *M. anisopliae* was highly pathogenic to silkworm larvae. Mortality of fungus treated silkworms and survival of worms on control confirmed that *M. anisopliae* was the cause of death.

Table 4. Live body weight (gm) of silkworm ($J_{12}XC_{12}$) larvae in treatments with different concentrations of *M. anisopliae* in the laboratory

Conidia per ml	Days of treatment▲									
	1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
10^7	0.62	0.65^{c}	0.72	0.89	1.05^{c}	1.27^{d}	1.58^{c}	2.08	2.19	2.13
10^6	0.59	0.68^{bc}	0.74	0.89	1.14^{b}	1.38^{cd}	1.71^{bc}	2.09	2.19	2.17
10^5	0.68	0.75^{ab}	0.79	1.02	1.32^{a}	1.59^{bc}	1.94^{abc}	2.29	2.32	2.35
10^4	0.67	0.79^{a}	0.8	1.02	1.32^{a}	1.88^{a}	2.05^{a}	2.19	2.32	2.28
10^3	0.65	0.75^{ab}	0.77	1.01	1.32^{a}	1.66^{ab}	2.01^{a}	2.25	2.28	2.39
10^2	0.72	0.78^{a}	0.82	0.93	1.32^{a}	1.53^{bc}	1.92^{ab}	2.25	2.26	2.21
Control	0.67	0.78^{a}	0.79	0.91	1.33^{a}	1.97^{a}	2.10^{a}	2.08	2.18	2.23
P (at 0.05)	ns	*	ns	ns	*	**	*	ns	ns	ns
SE $m_{\pm}$	0.02	0.01	0.01	0.02	0.04	0.05	0.05	0.04	0.03	0.07
Grand mean	0.65	0.73	0.77	0.95	1.23	1.52	1.84	2.17	2.24	2.08

▲Average of 30 larvae/replication; means followed by the same letter in each column are not significantly different by DMRT and ns means none significant and *, * *, * * * means significant at P< 0.05, P<0.01 and P< 0.001 respectively.

Table 5. Mean square values of body weight (gm) of silkworm ($J_{12}XC_{12}$) larvae after days of treatment in the laboratory.

Source of variation	df	Days of treatment									
		1st	2nd	3rd	4th	5th	6th	7th	8th	9th	10th
Treatments	6	0.005	0.007	0.004	0.010	0.063	0.099	0.083	0.023	0.01	0.133
Error	14	0.005	0.002	0.004	0.009	0.017	0.015	0.023	0.031	0.014	0.091
CV %		11.37	5.55	7.92	9.76	10.60	8.08	8.31	8.16	5.24	14.47

df means degrees of freedom and CV means coefficient of variation

The gain in body weight of silkworm larvae was lower in lots treated with higher concentration. Hajek and Goettel (2000), Simberloff and Stiling (1996), Goettel *et al*. (2001) and Vestergaad *et al.* (2003) similarly reported the results in different insects. No significant body weight difference among silkworms was recorded in the lots treated with different concentrations after eighth day of treatment. The cause might be because the left over worms were free from *M. anisopliae* spores.

The fungus *M. anisopliae* was found pathogenic to silkworm larvae at 10^7 spores per ml of concentration and caused 70% mortality. The lowest concentration even with 100 spores per ml also caused three per cent mortality to the silkworm larvae. So the fungus should be handled with care during production and application in the area where the silkworm is a promising business. The larvae were treated by directly dipping the silkworm larvae in the fungus suspension. So, it seems not so risky to seri-business if handled with care as the worms are reared in captivity and there is less chance of direct contact and contamination with higher doses of the fungus. The infection and death percent of silkworm by the fungus still opens the opportunity of using silkworm larvae as the bait insect to trap this entomopathogenic fungus from the soil, which can easily be re-isolated in the laboratory. Other studies on the effect of fungus to silkworm by ingestion, contaminated leaf feeding and its effects to different stages are suggested.

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