Efficacy of *Metarhizium anisopliae* (Metsch.) Sorokin against the Potato Tuber Moth, *Phthorimaea operculella* (Zeller) in Consumable Potato, under Laboratory Conditions

Sunita Pandey[1], Marc Sporleder[2] , Yubak Dhoj Gharty Chhetry[1]., Yagya Prasad Giri[3] and Jürgen Kroschel[2]

ABSTRACT

The potato tuber moth Phthorimaea operculella (Zeller) (Lepidoptera: Gelechiidae) is major pest of potato (Solanum tuberosum L.) in tropical and subtropical regions of the world causing serious economic damage especially in non-refrigerated potato storerooms. Chemical pesticide use for post-harvest pest management in farmers' rustic storerooms causes health risks to farmers and consumers, triggered a search for safer pest management alternatives, such as use of entomopathogens. Metarhizium anisopliae (Metsch.) Sorokin culture originally isolated from white grub larvae native from Nepal was grown on artificial media and its biological activity assessed against the potato tuber moth in the laboratory, using potato tuber surface contamination bioassay. A total of 5 bioassays were conducted. In four bioassays fresh fungus stock suspensions were tested while in one bioassay the fungus stock suspension of the first bioassay was reused after 8-month storage period at ambient temperature. All probit lines revealed a common slope of 0.57. The first stock suspension revealed an LC50-value of 6.9×106 conidia/ml while after 8-month storage a significant activity loss of about 90% (7.2 ×107 conidia/ml) was observed. The other three bioassays revealed LC50-values of mean 4.2×105 (2.9 to 6.1×105) conidia/ml without significant differences in their potencies. According to the probit lines (3-5) > 7.5x10^7 conidia/ml would be required to kill >90% PTM. Hence it can be concluded that the M. anisopliae strain showed high biological activity against PTM larvae and has potential as bio-control agent for controlling the pest; however, activity can be significantly reduced if stored inappropriately or for longer periods.

Key words: biological control, entomopathogens, potato pests, probit analysis

INTRODUCTION

The potato tuber moth *Phthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae), which is cosmopolitan pest of potato (*Solanum tuberosum* L.) and other solanaceous crops, was reported for the first time in 1966 from the Kathmandu valley, Nepal (NARC, 1967). Today, it is distributed along mid-hills where it produces economic losses especially in the summer crop starting from late May or early June to October-November (Pradhan, 1984) and after harvest in traditional, non-refrigerated storages. The damaging life stage of the pest is the larva, which feeds on potato foliage as well as tubers in the field and tubers in storage (Haines, 1977; Raman, 1980; Sileshi, 2001; Povolny, 2004). In Nepal, among 40 phytophagous species associated with potato, the potato tuber moth is the most important pest (NPRP, 2004/05). Post harvest losses might reach 30-85% (Joshi, 1989) and in some cases 100% (NPRP, 2004/05) in rustic potato

[1] Department of Agriculture, Hariharbhawan, Lalitpur, Nepal
[2] International Potato Center, Apartado 1558, Lima 12, Peru
[3] Nepal Agricultural Research Council
Corresponding Email: pandey.sunita2009@gmail.com

storages. Such losses are similar to the losses reported from other tropical-subtropical regions (CIP, 1988; Palacios and Cisneros, 1996; Chandel and Chandla, 2005; Lagnaoui and El-Bedewy, 1997) as infested tubers are subject to secondary infestation.

Tiwari et al. (2006) reported that 80% of the farmers in Kathmandu valley rely on chemical pesticides to manage potato tuber moth in storage. Such kind of control is not only environmentally hazardous; it also jeopardizes farmers and consumers health, and helps to induce resistance in potato tuber moth against diverse insecticides (Saour, 2004; Dogramaci and Tingey, 2008) and outbreaks of secondary pests (Lagnaoui and El-Bedewy, 1997). These facts trigger a search for safer alternatives, such as the use of entomopathogens that can be included as a component in integrated pest management (IPM) strategies.

The ubiquitous fungi, *Metarhizium anisopliae* (Metsch.) Sorokin (Ascomycota: Hypocreales) is an important agent for biological control of several insect pests (Ekesi et al, 2003; Marannino et al, 2006). It produces mycotoxins and destruxins, i.e. a group of secondary metabolites, which are considered as an important new generation of insecticides (Tanada and Kaya, 1993). It is a recognized pathogen of more than 200 insect species, including several major pests (Roberts and Hajek, 1992). The pathogen penetrates its hosts directly through the insect exoskeleton and proliferates throughout the insect's body, producing toxins and draining the insect nutrients, eventually killing it. Once the fungus has killed its host, it grows through the softer portions of the cuticle, covering the insect with a layer of green mold. Sabbour (2002) assayed *M. anisopliae* against *P. operculella* neonate larvae by dipping potato tubers in suspensions of the fungus prior moth attack and determined a LC_{50} of 8.61×10^7 conidia/ml seven days after inoculation of moths.

The objective of the study was to determine the biological activity (LC_{50}) of *Metarhizium anisopliae* through laboratory bioassays for evaluating the entomopathogen's potential use as an IPM component targeting the potato tuber moth in rustic farmers' potato storerooms.

MATERIALS AND METHODS

Mass rearing of potato tuber moths

Potato tuber moth was mass reared at the laboratory of the Entomology Division Khumaltar, Lalitpur, of the Nepal Agriculture Research Council (NARC). The rearing was initiated with potato tuber moth-infested potato tubers collected from different farmers' storerooms in Sankhu, Nepal. The tubers were placed in a plastic box (30×23×14.5 cm), which were partially filled with fine sterilized sand, and incubated at ambient temperature until pupae developed. Dry sand helped to absorb the moisture from infested potato and served as pupation medium. When the larvae had completed its larval stage, the pupae (with their cocoons) were harvested through sieving (2.5 mm mesh width). Cocoons were removed and pupae were surface-sterilized by washing them in a sodium hypochlorite solution (0.3%). The collected pupae were air-dried and placed in a cylindrical plastic container (∅ 13 cm, 12 cm depth), which was covered with mesh cloth (15 cm). After adult emergence, a filter paper was placed on the mesh cloth providing oviposition site. Adults were fed with a diluted honey solution (1:5 honey: water) which was dropped on the edges beside the filter paper. The filter papers were changed daily and the papers containing the eggs collected in a box stored at 10 ºC until use in bioassays or for further rearing. For rearing, the papers containing the eggs were placed into a plastic container with potato tubers as food source. For bioassays, filter papers with eggs were placed individually in petri dishes (Ø 15 cm × 3 cm depth), the dishes sealed with parafilm and incubated at room temperature (between 25 to 27 ºC) until larvae emergence. Fresh eggs generally hatched after 4 days of incubation and neonates were used immediately in bioassays.

Preparation of *M. anisopliae* culture

The fungus *M. anisopliae* was grown on artificial media in insect pathology laboratory Department of Entomology, Institute of Agriculture and Animal Science (IAAS), Rampur, Chitwan. The materials used for preparing selective medium were composed of 20 g dextrose, 10 g peptone, 18 g agar-agar and 1000 ml of distilled water. These components were mixed thoroughly and dissolved in the water in a conical flask. The prepared medium was sterilized in autoclave at 121 ºC with a pressure of 15 lb for 15 min. When the medium was cooled down to 60 ºC, 0.6 g streptomycin, 0.05 g tetracycline, and 0.05 g cyclohexamide dissolved in 20 ml sterilized distilled water was poured in conical flask that contained the medium and thoroughly mixed. Sterilized liquid medium was than poured into sterilized petri plates (116 ºC for 4 hours in oven) and kept to solidify for streaking fungus culture. The culture was incubated at 24 ºC and 75% RH to induce growth and sporulation of fungus in an aseptic condition in the laboratory. After 16 days, the conidia were harvested by scrapping off the contents of each Petri dish using a sterile metal spatula. The culture was stored in cool temperature at 4 ºC.

Preparation of *M. anisopliae* concentrations

For the preparation of *M. anisopliae* concentrations, the conidia scrapped off from the Petri dish were mixed homogenously in water together with 2 drops of Tween 20 (0.1%, dispersing agent). Conidia in the stock solution were quantified by counting under microscope using the hemocytometer (Thoma) and the stock solution was adjusted to ~2×10^7 conidia/ml by adding water. Five bioassays were conducted. For each bioassay a new stock solution (No I, III-V) was prepared with the exception of bioassay II, for which purpose the stock solution No I was stored over an 8-month period at ambient temperature. Since exact adjustment to 2×10^7 conidia/ml was not always possible, the quantity (titer) assessed through counting (average of three counts) of conidia was taken as the most exact estimate of the highest concentration used in each bioassay. The stock suspension "Ma-stock" was further diluted using different dilution factors to obtain different concentration levels (see Table 1).

Bioassay procedure

In bioassays, neonate larvae were exposed to surface contaminated potato tubers. Each suspension of different *M. anisopliae* concentrations (see Table A 1 for concentration level used in each bioassay) was filled in a plastic container (0.5 liter) and 12 washed and air-dried potato tubers per suspension (3 tubers with a total weight of about 100 g were used in each replication) were posed in a net and dipped into its respective suspension for a period of five seconds to obtain an even coating of the pathogen on the tuber surface.

Table A 5. *Metarhizium anisopliae* concentrations used in five bioassays for assessing the concentration–mortality regression lines of the fungus-host system.

Date	Concentration levels (conidia/ml)						
	1st	2nd	3rd	4th	5th	6th	7th
2008/9/7	1.9×10^7	3.8×10^6	7.5×10^5	1.5×10^5	3×10^4	6×10^3	1.2×10^3
2009/5/13	1.6×10^7	3.1×10^6	6.3×10^5	1.3×10^5	2.5×10^4	5×10^3	10^3
2009/6/24	2.9×10^7	5.8×10^6	1.2×10^6	2.3×10^5	4.6×10^4	9.3×10^3	1.9×10^3
2009/7/7	2×10^7	4.1×10^6	8.2×10^5	1.6×10^5	3.3×10^4	6.6×10^3	1.3×10^3
2009/8/7	2×10^6	4.1×10^5	8.2×10^4	1.6×10^4	3.3×10^3	-	-

Each bioassay included one control (water with Tween-20 only).

Each bioassay included a control without the fungus; i.e. tubers were dipped into water with Tween only. After surface contamination of the tuber (tuber dipping), treated tubers were air-dried for few minutes and placed in bioassay containers (12 cm depth and 13 cm in diameter).

Fifty neonate larvae, not older then 4-h after hatching, were inoculated on the treated potatoes per container using a camel hair paint brush. Containers were closed with a lid, which contained a mesh window for ventilation, and incubated at ambient room temperature (25-28 °C). Larval survival was recorded after sixteen and twenty-one days (second evaluation) of incubation. During evaluation, developed pupae and adults were considered as survivors. After the second evaluation no further development to pupae of test insects was expected.

Statistical analysis

Probit regression lines for each bioassay replication were calculated according to Finney (1971). The mortality responses (scored as failure to pupate, repetitions were pooled) were adjusted for the bioassay-specific natural mortality by using Abbott's formula (Abbott, 1925).

$$Adjusted\ mortality\ rate = \frac{mortality\ rate\ observed - control\ mortality\ rate}{1 - control\ mortality\ rate}$$

Natural mortalities were estimated as a parameter in the probit models using the control responses (background mortality) observed in each assay as the provisional estimate. The data were analyzed in a parallel line assay. A variance ratio test (G-test; Chi^2_{total} - $Chi^2_{heterogeneity}$) was applied to test the parallelism of all five probit regression lines. If heterogeneity was significant in the data a heterogeneity factor (= Chi^2/df) was included in the calculation of variances, co-variances and confidence limits. The obtained Probit regression lines were compared by their LC_{50}-values and relative potencies. 95% confidence limits for potencies were calculated according to Fillers theorem (Finney, 1971). The analysis was conducted using R-statistics (version 2.2) (R Development Core Team, 2013).

RESULTS

Control mortality varied between the five assays ranging from 21.1% (±1.46%) (bioassay 2) and 52.3% (±1.19%) (bioassay1); however, the background mortality was almost similar (around 35%) in bioassays 3 to 5 (Figure A 1). Mortalities in the total set of data revealed significant heterogeneity (χ^2= 28.9, *df* = 18, *P* = 0.042), and therefore a heterogeneity factor was included for calculating variances and 95% confidence limits for LC_{50}-values and potencies. The Likelihood-Chi^2 test confirmed parallelism between all five regression lines (χ^2= 9.488, df = 4, P = 0.73) and the common slope for all concentration-response lines was found to be 0.57 (SE ± 0.00087). The LC_{50} values ranged from 2.94×10^5 (bioassay 3) to 7.16×10^7 (bioassay 2) conidia per ml (Table A 2).

Potencies of the LC_{50}-values revealed significant differences between some of the 5 bioassays. Lowest activity was observed in bioassay 1 and 2 (i.e. from the first Ma-stock suspension); bioassay 2 revealed a significantly reduced activity (about 10-times) compared to bioassay 1, which can be attributed to loss of viability during the 8-month storage period of the fungus. Bioassays 3-5 showed no significant variability in their potencies but revealed significantly increased potencies compared to bioassay 1 (Table A 2). This might indicate that the Ma-culture used in the first stock suspension was of lower viability, while the bioassays 3-5 showed the full potential of the fungus against neonate *P. operculella* larvae.

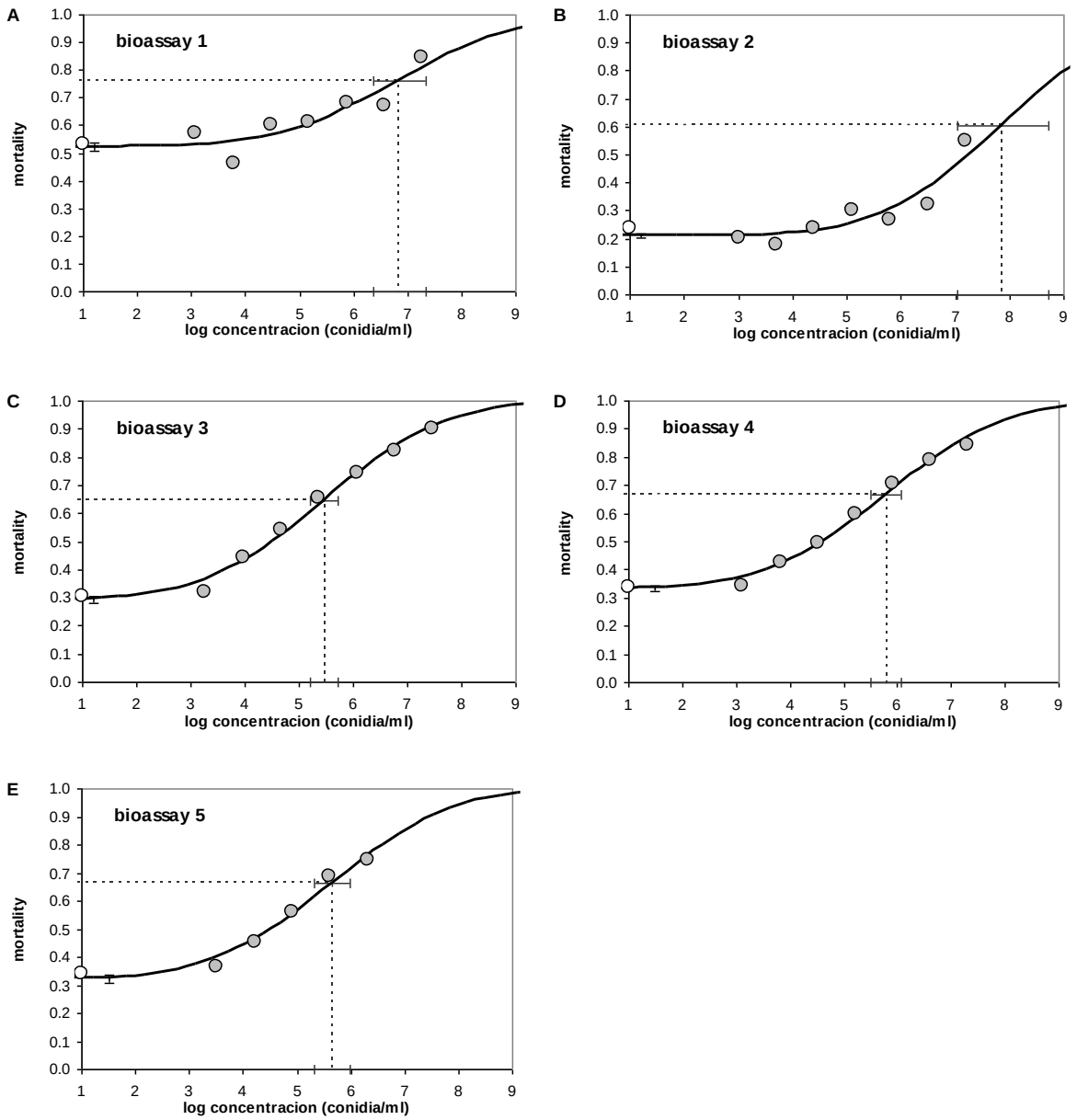

Figure A 1. Probit regression lines retransformed into percentage mortalities plotted against log concentration of *M. anisopliae* (conidia/ml) treated to neonate larvae of *P. operculella* during a series of 5 assays. Dots are observed mortalities, open dots are control mortalities observed, lines are model predictions, x-bars are 95% confidence

Table A 6. Probit statistics for *M. anisopliae* in *P. operculella* larvae

Date	Equation a + b	*Chi* ^{2}	*P*	LC_{50} [c] (conidia/ml)		Relative potencies [c]	
2008/9/7	-3.9 + 0.57	13.1	0.02	6.92×10^6	(2.36×10^6 - 2.11×10^7)	1	
2009/5/13	-4.5 + 0.57	11.7	0.04	7.16×10^7	(1.12×10^7 - 5.17×10^8)	0.095	(0.03 - 0.27)
2009/6/24	-3.1 + 0.57	3.4	0.64	2.94×10^5	(1.56×10^5 - 5.28×10^5)	23.9	(9.4 - 65.9)
2009/7/7	-3.3 + 0.57	2.8	0.73	6.14×10^5	(3.24×10^5 - 1.15×10^6)	11.4	(4.46 - 31.1)

2009/7/8	-3.3 + 0.57	2.3	0.50	4.38×10^5 (2.06×10^5 - 9.67×10^5)		15.8	(5.92 - 42.3)

a = intercept, and b = slope of the Probit model; SE(b) ± 0.00087.

[c]number in parenthesis are 95% confidence limits. Due to significant heterogeneity, a heterogeneity factor was included to calculate confidence limits for LC_{50} values and potencies.

DISCUSSION

The slope of the concentration–mortality regression line for the fungi *M. anisopliae* and the host *P. operculella* larvae was constant throughout the series of assays; however the value observed in this study of 0.57 is much smaller than the slope of 1.9 reported by Sabbour (2002) who used almost the same treatment procedure as used in this study for the same pathogen-host system but mortality was evaluated after seven days of treatment. The difference between the slopes obtained in this and Sabbour's study might be due to different experimental conditions like variation of isolates used, environmental condition, insect culture, and differences in experimental procedures (differences in pathogen concentrations, incubation periods). Sabbor evaluated mortality after 7 days while this study determined mortality after >14 days. This study revealed LC_{50}-values ranging from 2.94×10^5 to 7.16×10^7 conidia per ml also according to the probit lines (3-5) > 7.5x10^7 conidia/ml would be required to kill >90% PTM, which was still lower then the LC_{50}-value of 8.61×10^7 conidia/ml obtained by Sabbour (2002). The maximum value 7.16×10^7 was obtained from *M. anisopliae* after storing the stock solution I for an 8-month period.

This study revealed significant differences in potencies between different *M. anisopliae* preparations, with lowest activities of the pathogen during the first two bioassays. The reason for lower fungal activity in the first bioassay might be partly due to experimental differences like a reduced fitness of test insects (assay revealed an natural background mortality of >50% while in other assays background mortality was around 30%), whereas in the second assay the reduction in fungal activity can be attributed to a loss of activity during an 8-month storage period of the *M. anisopliae* preparation at ambient temperature (varied between 15 and 24 ºC). Clerk and Madelin (1965) reported that the viability of the conidia of *M. anisopliae* decreased as the storage temperature increased from 8 to 25 ºC. In this experiment the preparation had lost about 90% of its activity within 8 month of storage. Therefore, the fungus should be stored best at about 4 ºC, in dark and at very low humidity. The longevity of conidia is generally more stable at cool and dry condition (Hong et al., 1997). The last three bioassays, which were conducted within a short period of time and hence at more or less similar temperatures using always fresh fungus culture, resulted in quite similar LC50-values. This shows that the results on low LC50-values are repeatable if the fungi preparations are fresh (viable) and experimental conditions are held constant. The latter three bioassays reveal probably the full potential of the fungus against neonate *P. operculella* larvae.

CONCLUSIONS

The *M. anisopliae* strain showed high biological activity against potato tuber moth neonate larvae and hence has potential as biocontrol agent for controlling the pest in potato storerooms; however, activity can be significantly reduced if stored inappropriately or for longer periods. Optimal storage conditions and the products shelf life need to be assessed before the fungus can be used as a biopesticide. In addition, further experiments needs to be carried out under farmers' or simulated storage conditions before final recommendations can be made.

ACKNOWLEDGEMENTS

This study was part of a collaborative project between the National Agriculture Research Council (NARC), Lalitpur, Nepal, and the International Potato Center (CIP), Lima, Peru, funded by the German Federal Ministry for Economic Cooperation and Development (BMZ), Germany. We thank Mr. Tika Ram Bagale for lab assistance.

LITERATURES CITED

Abbott, W. S. (1925). A method for computing the effectiveness of an insecticide. *Journal of Economical Entomology*. **18**, 265-267.

Burges, H. D. (1971). Possibilities of Pest Resistance to Microbial Control Agents. In: H. D. Burges and N. W. Hussey. (Eds.), *Microbial Control of Insects and Mites*. London, Academic Press. pp 445 - 456.

Chandel, R.S., Chandla, V. K. (2005). Integrated control of potato tuber moth (*Phthorimaea operculella*) in Himachal Pradesh. *Indian Journal of Agricultural Science*. **75**: 837-839.

CIP. (1988). Annual report. International Potato Centre. Lima, Peru. pp 99-101.

Clerk, G. C., Madelin, M. F. (1965). The longevity of conidia of three insect-parasitizing hyphomycetes. *Transactions of the British Mycological Society*. **48**(2): 193-209.

Dogramaci, M., Tingey, W. M. (2008). Comparison of insecticides resistance in a North American field population and a laboratory colony of Potato Tuber Worm (Lepidoptera: Gelechiidae). *Journal of Pest Science*. **81**: 17-22.

Ekesi, S., Maniania, N. K., Lux, S. A. (2003). Effect of soil temperature and moisture on survival and infectivity of *Metarhizium anisopliae* to four tephritid fruit fly puparia. *Journal of Invertebrate Pathology*, **83**:157–167.

Finney, J. R. (1971). Probit analysis. 3rd edn. Cambridge University Press, Cambridge, UK.

Haines, C.P. (1977). The potato tuber moth *Phthorimaea operculella* (Zeller): a bibliography of recent literature and review of its biology and control on potatoes in the field and in stores. Report of the Tropical Production Institute 112: p15.

Hong, T. D., Ellis, R. H., Moore, D. (1997). Development of a model to predict the effect of temperature and moisture on fungal spore longevity. *Annual Botany*, **79**:121–128.

Joshi, S. L. (1989). Comparative life cycle of the potato tuber moth, *Pthorimaea operculella* (Zeller) (Lepidoptera: Gelechiidae) on potato tubers and foliages and its economic loss in yield. *Journal of Entomological Society of Nepal*. **1**: 59-69.

Lagnaoui, A., El-Bedewy, R. (1997). An integrated pest management strategy for controlling potato tuber moth in Egypt. *CIP Circular* 22(3): 6-7.

Marannino, P., Alvarez, C. S., Lillo, E. de., Moraga, E. Q. (2006). A new bioassay method reveals pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana* against early stages of *Capnodis tenebrionis* (Coleoptera; Buprestidae). *Journal of Invertebrate Pathology*. **93**: 210–213

NARC. (1967). Annual technical report of 1966/67. Entomology Division. Nepal Agriculture Research Council (NARC), Khumaltar, Nepal.

NPRP. (2004/05). Annual Report. National Potato Research Program. NARC, Khumaltar, Lalitpur, Nepal. p 70.

Palacios, M., Cisneros, F. (1996). Integrated management of potato tuber moth in pilot units in the Andean Region and the Dominican Republic. Program report 1995-96. International Potato Center, Lima, Peru. pp 162-168.

Povolny, D and V. Hula. (2004). A new potato pest invading Southwestern Europe, the Guatemala potato tuber moth Scrobipalpopsis solanivora (Lepidoptera: Gelechiidae). *Entomologia Generalis*. 27: 155–168.

Pradhan, R. B. (1984). Control of potato tuber moth by weeds. A report. National Potato Development Program HMG/NISATA, Lalitpur, Nepal. p 1

R Development Core Team, (2013). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria. (http://www.R-project.org).

Raman, K.V. (1980). Potato tuber moth. Technical Information Bulletin **3**. International Potato Center (CIP), Lima, Peru. pp 14.

Roberts, D. W., Hajek, A. E. (1992). Entomopathogenic fungi as bioinsecticides, pp. 144-159. *In*: G. F. Leatham (Ed.), Frontiers of industrial mycology. Chapman & Hall, New York

Sabbour, M. M. (2002). The Role of Chemical Additives in Enhancing the Efficacy of *Beauveria bassiana* and *Metarhizium anisopliae* Against the Potato Tuber Moth *Phthorimaea operculella* (Zeller) (Lepidoptera:Gelechiidae). *Pakistan Journal of Biological Sciences*. **5**(11): 1155-1159.

Saour, G. (2004). Efficacy assessment of some *Trichogramma* species (Hymenoptera: Trichogrammatidae) in controlling the potato tuber moth *Phthorimaea operculella* Zell. (Lepidoptera: Gelechiidae). *Journal of Pest Science*. **77**: 229–234.

Sileshi, G and J. Teriessa. (2001). Tuber damage by potato tuber moth, Phthorimaea operculella (Zeller) (Lepidoptera: Gelechiidae) in the field in eastern Ethiopia. *International Journal of Pest Management*. **47**: 109–113.

Tanada, Y., Kaya, H. K. (1993). Insect Pathology. Academic Press, Inc. Harcout Bace Jovanovich Publishers San Diego New York Boston, London Sydney Tokyo Toronto. pp 359-360.

Tiwari, D. B., Thapa, R. B., Shrestha, S. M., Joshi, S. L. (2006). Field Survey and Monitoring of Potato Tuber Moth (*Phthorimaea operculella* (Zeller)) (Lepidoptera: Gelechiidae). *Journal of the Institute of Agriculture and Animal Science*. **27**: 157-160.