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Full Length Article

Pathogenicity of *Paecilomyces tenuipes* to Diamond Back Moth, *Plutella xylostella* at Three Temperatures in Trinidad

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ABSTRACT

The aim of this study was to conduct laboratory and field evaluations to determine the potential of *Paecilomyces tenuipes* as a biological control agent of *Plutella xylostella*. *P. tenuipes* radial diameter increased linearly for cultures at 20, 25 and 30°C with no growth at 35°C. Colony growth rate as measured by the time for 50% conidial germination (GT_{50}) was fastest at 25°C (1.409 ± 0.058 μm h^{-1}) and approximately double that at either 20 or 30°C. A LC_{50} of 1.09×10^6 spores/mL at 25°C caused the highest mortality to 3rd instar *P. xylostella* larvae. The LT_{50} at this concentration and temperature was 3.28 days. Percentage mycosis for both larvae (66.7%) and pupae (57.0%) of *P. xylostella* under field conditions was highest 7 days post first fungal application. *P. tenuipes* caused mean mortalities of 17.8% and 21.7% to larvae and pupae, respectively over a 25 day period.

Key Words: Conidial germination; *Paecilomyces tenuipes;* Mortality; *Plutella xylostella*

INTRODUCTION

Diamondback moth, *Plutella xylostella* Linnaeus (Lepidoptera:Plutellidae) is a major pest of cruciferous vegetable crops in both temperate and tropical countries, attacking a wide range of wild and cultivated cruciferous crops (FAO, 2000; Lohr *et al.,* 2007). Chemical control is most frequently practiced and a wide spectrum of insecticides (primarily organophosphates, pyrethroids & neonicotinoids) is currently used locally for *P. xylostella* control. The benefit of chemical control of *P. xylostella* is its rapid killing to avoid economic losses to high priced commodities including cabbage (*Brassica oleracea* L. var. *capitata*), cauliflower (*B. oleracea* L. var. *botrytis*) and broccoli (*B. oleracea* L. var. *italica*), which fetch premium prices and have niche markets in Trinidad and Tobago and the Caribbean. However, *P. xylostella* has a short life cycle with several generations in one growing season and hence can develop resistance to insecticides very rapidly (FAO, 2000; Sharma *et al.,* 2008; LiJuan *et al.,* 2011). Biological control of *P. xylostella* offers long term management of this pest compared to chemical control. Additionally, it is cost effective, host specific, environmentally friendly and self-perpetuating. Towards the end of 2002, a fungus identified by CAB International, United Kingdom as *Paecilomyces tenuipes* (Peck) Samson (IMI 189/02) was found infecting *P. xylostella* larvae inside cabbage heads for the first time in Caura Valley, Trinidad (Lopez *et al*., 2003). The present study seeks to determine (1) the effect of temperature on *in vitro* radial growth rate and germination of *P. tenuipes,* (2) the pathogenicity of *P. tenuipes* against third instar *P. xylostella* larvae and (3) *P. tenuipes* for its potential as a biological control agent of *P. xylostella* under field conditions.

MATERIALS AND METHODS

Fungus: An isolate of *P. tenuipes* (IMI 189/02) from *P. xylostella* larvae on cabbage in Aranguez, Trinidad was identified by and pure cultures obtained from CAB International, London. The isolate was obtained as 6-day old first subculture on Potato Dextrose Agar (PDA).

Effect of temperature on *in vitro* germination of fungal conidia: *P. tenuipes* conidia were extracted by lightly scraping the original culture with a sterile loop and were used to inoculate thirty-two 90 mm petri dishes of PDA. Eight Petri dishes of *P. tenuipes* were placed in each of four clear, sterile polythene bags (20 cm x 35 cm) and then placed in four incubators maintained at 20, 25, 30 and 35°C. At four intervals during the 96 h period following inoculation, some of the conidia in each of two petri dishes from each temperature were placed on glass slides and fixed with cotton blue in lactophenol (100 g/L). One hundred randomly chosen conidia were assessed microscopically for germination and the times at which germination was recorded were used for analysis. A conidium was considered to have germinated if it had a germ tube at least as long as the smallest diameter of the conidium. Random

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fields of view were examined using an eyepiece graticule with a grid, which allowed the field of view to be counted in the same pattern each time until 100 conidia had been examined (Yeo *et al.,* 2003; Lawrence & Khan, 2009). For each replicate of each temperature, the percentage germination over time (n=100) was analyzed. The time taken in hours for 50% of conidia to germinate (GT_{50}) was calculated using probit analysis (SAS Institute Inc., 1989).

Effect of temperature on *in vitro* radial colony growth of *P. tenuipes*: Circular plugs (5 mm diameter) were cut from 14-days old culture dishes incubated at 27°C. A single plug was placed in the centre of a new dish of medium (20 mL of PDA in a 90 mm petri dish). Dishes were sealed with Parafilm® and incubated in darkness in separate incubators at 20, 25, 30 and 35°C. Eight replicate dishes were prepared for each temperature. Radial growth was recorded at 2-day intervals using previously drawn cardinal lines on the Petri dishes. The experiment was conducted for 26 days or until the fungal colony had covered the entire petri dish at any of the temperatures. For each *P. tenuipes* replicate at each temperature, the colony radial growth rate, K_r ($\mu m\ h^{-1}$), was estimated from the slope of the linear regression of colony radius on time. The values of the slopes were used to determine differences in growth rates at different temperatures (Yeo *et al*., 2003; Lawrence & Khan, 2009).

Effect of temperature on pathogenicity of *P. tenuipes* on *P. xylostella* third instar larvae: Third instar *P. xylostella* larvae collected from cultures maintained under field conditions were used for this experiment. *P. xylostella* larvae were reared on Chinese cabbage (*Brassica chinensis* L var. *gracious*). Cultures of *P. tenuipes* maintained for 25 days at three temperatures of 20, 25 and 30°C (35°C was not used since larvae died at this temperature) were used for this bioassay. Conidial suspensions were prepared for cultures maintained at each of the three temperatures by flooding the mature fungal colony with sterile distilled water and 0.2% Tween 80®. The conidia were extracted by gently scraping the surface of the fungal colony with a sterile, bent glass rod. A 1:10 dilution of the original conidial suspension was made and used for conidial counts on an improved Neubauer Haemocytometer. Four serial dilutions of 10^8, 10^7, 10^6 and 10^5 conidia mL^{-1} were prepared from the original conidial suspensions from cultures at each of the three temperatures.

Petri dishes (90 mm) were prepared by placing filter paper, lightly moistened with sterile distilled water at the bottom of the dish. Leaves of Chinese cabbage were cut into 60 mm^2 pieces, sterilized in a Clorox® bleach solution (0.01%), rinsed thoroughly in distilled water and left to air dry on sterile paper towels in an Envair® laminar flow cabinet (Model HLF/4/B). One piece of sterilized leaf was immersed for 5 sec in a particular conidial concentration and placed on moistened sterile Whatman No. 4 filter paper in a Petri dish. Each conidial concentration experiment was replicated 5 times. Five 3rd instar *P. xylostella* larvae were placed in each of the petri dishes and mortality was recorded daily. The lethal time causing 50% mortality (LT_{50}) was calculated for *P. tenuipes* at each temperature using probit analysis (SAS Institute Inc., 1989).

Field evaluation of *P. tenuipes*: A field evaluation at Caura Valley, Trinidad was conducted using *P. tenuipes* and a control. A completely randomized design was used comprising four replicates/treatment with each replicate being on a plot 7 x 2 m with 70 cauliflower plants spaced 30 x 30 cm. Sample plants were taken from within each plot such that there were at least 2 plants around the sample plant and a distance of at least 1 m between treatment plots. Grain rice was used as the substrate for the solid culture stage and a physical support for *P. tenuipes* to produce aerial conidia. The rice was autoclaved in plastic bags at 121°C and 1.05 kg cm^{-2} for 40 min and, after cooling to room temperature (25–27°C), inoculated with *P. tenuipes* and left for 10 days during which sporulation occurred. Conidial extraction was achieved by pouring 3 L of sterile distilled water (with 1mL of Cropspray® paraffinic adjuvant) into the bags and gently massaging the rice. A concentration of 10^8 conidia mL^{-1} was applied in the field using a Jacto® 18.9L knapsack sprayer fitted with a hollow cone nozzle at a flow rate of 1 L 14 m^{-2} for each treatment plot. Water and adjuvant (1.25 mL Cropspray® paraffinic adjuvant/4 L of water) was used as the control. Treatments were applied on 3-weeks old plants once per week for 4 weeks. For each treatment, 4 plants (randomly taken one from each of the replicate plots) were removed. Data were recorded for the number of each stage of *P. xylostella* present on sample plants.

Statistical analysis: Mean GT_{50} and LT_{50} values were compared among themselves using Tukey's Multiple Comparisons Test at P=0.05. Colony growth rates were compared at three temperatures using the slopes of their regression equations (SAS Institute Inc., 1989).

RESULTS AND DISCUSSION

The time taken for 50% conidial germination (GT_{50}) was not significantly different ($p>0.05$) for *P. tenuipes* cultures maintained at 20°C and 25°C, but was significantly different ($p<0.05$) at 30°C compared to that at 20 and 25°C. *P. tenuipes* achieved 50% conidial germination the slowest and fastest at 20 and 30°C, respectively (Table I).

The radial diameter of *P. tenuipes* colonies increased linearly for cultures maintained at 20, 25 and 30°C. However, no growth was observed at 35°C for the duration of this experiment. Colony growth rate was fastest at 25°C ($1.409 \pm 0.058\ \mu m\ h^{-1}$) and significantly different ($p<0.05$) from that at either 20 or 30°C. Growth rate was approximately doubled at 25°C when compared to 20 and 30°C as evidenced by the slope. There was no significant difference ($p>0.05$) between the slopes at 20 and 30°C (Table II). Wu *et al.* (2008) also obtained maximum colony growth rate of both *Sclerotinia minor* and *S. sclerotiorum* at 25°C.

Table I: Regression equations and time (hours) taken for 50% germination (GT_{50}) of *Paecilomyces tenuipes* at three temperatures

Temperature	Regression equation	GT_{50} ± S.E. (95% confidence interval)* (hours)
20	Y = 4.21x – 1.92	43.99 ± 1.04 (40.79, 47.45)[a]
25	Y = 2.41x + 0.84	52.82 ± 1.09 (44.74, 62.37)[a]
30	Y = 3.84x – 2.07	69.27 ± 1.04 (64.21, 74.73)[b]

Table II: Summary of regression analysis of colony radius against time for *Paecilomyces tenuipes* at three temperatures

Temperature	Regression equation	Slope±S.E. (95% confidence interval) (=Growth rate, K_r μm h^{-1})
20	Y=0.714x + 50.794	0.714 ± 0.034 (0.6296, 0.7981)[a]
25	Y=1.409x + 5.476	1.409 ± 0.058 (1.2663, 1.5524)[b]
30	Y=0.604x + 68.099	0.604 ± 0.014 (0.5705, 0.6373)[a]

Table III: Lethal time (days) for 50% mortality (LT_{50}) of 3rd instar *Plutella xylostella* larvae exposed to different concentrations of *Paecilomyces tenuipes* at three temperatures

Temperature	Concentration (conidia mL^{-1})	LT_{50} ± SE (days) (95% confidence interval)
	10^8	2.11 ± 1.05 (1.92, 2.31)[a]
	10^7	3.55 ± 1.03 (3.34, 3.78)[b]
20	10^6	3.99 ± 1.04 (3.73, 4.27)[b]
	10^5	5.29 ± 1.08 (4.54, 6.17)[c]
	10^8	2.33 ± 1.08 (2.01, 2.70)[a]
	10^7	2.49 ± 1.07 (2.19, 2.84)[a]
25	10^6	3.28 ± 1.10 (2.74, 3.93)[b]
	10^5	3.78 ± 1.06 (3.36, 4.24)[b]
	10^8	1.93 ± 1.07 (1.68, 2.21)[a]
	10^7	2.02 ± 1.05 (1.84, 2.21)[a]
30	10^6	9.14 ± 1.20 (6.37, 13.12)[d]
	10^5	64.31 ± 2.17 (14.08, 293.83)[e]

*Values followed by the same letter along a column are not significantly different from each other (P=0.05) based on Tukey's Multiple Comparisons Test

The percent corrected mortality for *P. xylostella* 3rd instar larvae exposed to *P. tenuipes* over time was calculated for each of the temperatures: 20, 25 and 30°C (Fig. 2). LT_{50} values were calculated for cultures of *P. tenuipes* separately for each concentration at each of the three temperatures. LT_{50} increased with decreasing spore concentration at all temperatures examined; however the most dramatic increase in LT_{50} was noted at 30°C and at the lowest concentration (Table III). At 20°C a concentration of 1 x 10^8 conidia mL^{-1} had an LT_{50} of 2.11 days, which was the lowest and significantly different ($p<0.05$) when compared to the other three concentrations (Table III). These results are similar to Vega-Aquino *et al.* (2010) who investigated the activity of oil formulations of *Nomuraea rileyi* and *P. tenuipes* against four lepidopterous species and also obtained low LT_{50} values (2.5–5.6 days) as observed in this experiment.

The LC_{50} of 3rd instar *P. xylostella* larvae was calculated for each of the temperatures and the results indicated that the LC_{50} value for cultures at 25°C

Fig. 1: Effect of three temperatures on colony growth of *Paecilomyces tenuipes* on Potato Dextrose Agar

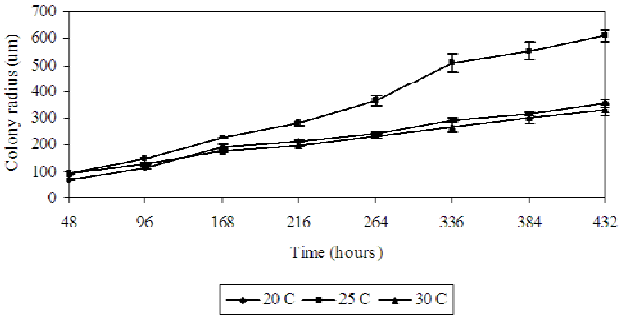
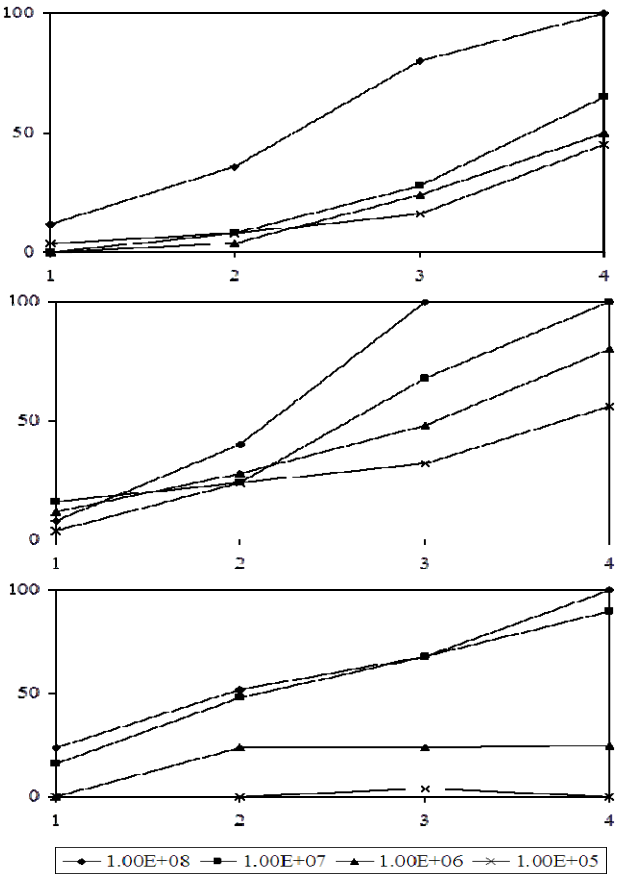


Fig. 2: Variation of corrected mortality (%) of *Plutella xylostella* third instar larvae with time when exposed to four concentrations of *Paecilomyces tenuipes* at three temperatures



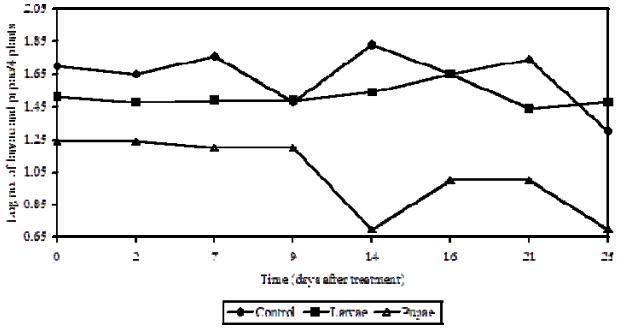
(LC_{50}=1.09 × 10^6 conidia mL^{-1}) was the lowest and significantly different ($p<0.05$) from the values at either 20 or 30°C. There was no significant difference ($p>0.05$) between LC_{50} values at 20 and 30°C (Table IV). Under field conditions the percentage mycosis for both *P. xylostella* larvae and pupae was highest at 7 days post first treatment (66.7% & 57.0%, respectively). The mean mycosis over the data collection period for larvae and pupae was 17.8% and 21.7%, respectively (Fig. 3).

Table IV: Lethal concentration for 50% mortality (LC_{50}) of 3rd instar *Plutella xylostella* larvae exposed to *Paecilomyces tenuipes* at three temperatures

Temp.	Probit equation	LC_{50} spores/ml (95% confidence interval)	S.E. of LC_{50}	χ^2
20	Y = 0.61x + 0.60	1.58×10^7 (5.53×10^6, 4.55×10^7) [a]	1.71	4.28
25	Y = 0.47x + 2.18	1.09×10^6 (2.60×10^5, 4.58×10^6) [b]	2.08	0.03
30	Y = 0.69x + 0.21	9.11×10^6 (3.55×10^6, 2.34×10^7) [a]	1.62	4.26

*Values followed by the same letter along a column are not significantly different from each other (P=0.05) based on Tukey's Multiple Comparisons Test

Fig. 3: Effect of *Paecilomyces tenuipes* application on larval and pupal density of *Plutella xylostella* on cabbage



At the four test temperatures, GT_{50} increased with increasing temperature with the GT_{50} value at 30°C being the highest and significantly different ($p < 0.05$) to values at 20 and 25°C. In this study *P. tenuipes* germinated fastest at 20°C but was not significantly different ($p>0.05$) from values at 25°C. Morley-Davies *et al.* (1996) obtained similar results for *Metarhizium flavoviridae* isolates, where temperature had a limiting effect on conidial viability between 30 and 40°C with a slight decrease occurring between 20 and 30°C. A fast germination rate of conidia *in vitro* has been related to virulence in other hypomycetous fungi (Yeo *et al.,* 2003). Hall (1989) also stated that fungal spore pathogenicity can decline disproportionately faster compared to spore viability and target spore viability should always be near 100%. For *P. tenuipes*, growth rate increased *in vitro* from 20 to 25°C (growth rate doubled at 25°C compared to 20 or 30°C) but at temperatures higher than 25°C growth rate decreased until 30°C with no growth at 35°C. Butt *et al.* (2001) stated that optimum temperature for most entomopathogenic fungi was between 20 and 25°C, while above 30°C, the negative growth of most taxa is inhibited and growth effectively ceases at approximately 37°C.

It is well documented that ambient temperature influences the rate of infection and time to death of insects treated with entomopathogenic hypomycetous fungi (Wraight *et al*., 2000; Butt *et al.,* 2001; Ugine, 2011). Generally, as temperatures decrease or increase above the optimum for *in vivo* infection, the time taken for insects to succumb to fungal infection increases; while the total number of hosts infected may be related to the ability to germinate rapidly, the speed of killing is more closely related to the ability to colonize the host rapidly (Yeo *et al*., 2003).

The GT_{50} at 25°C (52.82±1.09 h) was low compared to that at 30°C (69.27±1.04 h). At 25°C the LT_{50} for 1 x 10^5 conidia mL^{-1} was lowest and significantly different to LT_{50} (at 1 x 10^5 conidia mL^{-1}) values at 20°C and 30°C. Radial growth was also slowest at 30°C although this was not significantly different ($p > 0.05$) to that at 20°C. At 20°C, GT_{50} and LT_{50} values were significantly lower than at 30°C. The low LC_{50} value at 25°C suggests that cultures of the isolate at this temperature produce more infective propagules and hence requires a lower concentration of spores to cause mortality. This can therefore be considered as the ideal temperature for mass production of *P. tenuipes*.

A delay in death can be critical to a pest manager, especially in the management of pests with explosive population growth rates and/or pests of high value crops (Butt *et al.,* 2001). This situation can ideally describe *P. xylostella's* relationship with crucifers. Based on GT_{50}, LT_{50}, growth rate and LC_{50} values, it appears that 25°C is the ideal temperature for culture of *P. tenuipes*, however the data suggest that there is no difference between the LT_{50} values at a spore concentration of 1 x 10^8 conidia mL^{-1} and 1 x 10^7 conidia mL^{-1}. At higher concentrations mortality appears to be least dependant on temperature. Consequently for large scale production (provided that the production of infective propagules can be done easily & cost effectively) concentrations of 1 x 10^7 conidia mL^{-1} and above should be produced at room temperature. An economic analysis may be necessary to determine the cost of producing conidia for high dose application versus that for lower dose applications at a temperature of 25°C.

Host-fungus interactions are often assessed in bioassays done under optimal conditions for fungal development. If these are not representative of the conditions that the fungus will be exposed to in the field, predictions from laboratory experiments will be inaccurate (Yeo *et al.,* 2003). *P. tenuipes* was found in the same country as the field evaluation and hence should possess inherent characteristics for survival under these conditions. This however, does not mean that its performance as a biological control agent would be the same throughout the country, as localities would have different microclimates. Laboratory experiments conducted also demonstrated the influence of temperature on GT_{50}, LT_{50}, LC_{50} and growth rate of *P. tenuipes*. The field trial conducted was preliminary in nature and was intended to determine if *P. tenuipes* would cause mycosis to a population of *P. xylostella* when applied as a mycoinsecticide. Bateman (1997) stated that induced mortality from a pathogen will

always be much slower than 'knock down' chemicals. However, from the field experiment conducted, the effect of *P. tenuipes* on *P. xylostella* can be equated with that for chemical control. Throughout the period for the field trial both larval and pupal population densities were not significantly different between the biological (*P. tenuipes*) and chemical (Pirate® (chlorfenapyr) treatments. With the exception of the data collected seven days after first application of the treatments, both larval and pupal population densities were not significantly different ($p>0.05$) among all treatments. Additionally, larval population densities were lower for *P. tenuipes* treatment (64% over the trial period) compared to the control treatment. On the 7th day after first treatment both larval and pupal population densities had the same trend and populations densities were not significantly different ($p>0.05$) between *P. tenuipes* and Pirate® treatments, but significantly different between control and *P. tenuipes* treatments and between control and Pirate® treatments. On the 7th day after first treatment there was 66.7% and 57.1% larval and pupal mycosis, respectively.

Field studies have indicated that three distinct routes for fungal infection occur: direct impaction with spray droplets; secondary pick up of spray residues on vegetation and horizontal transmission (or secondary cycling) of the pathogen from individuals infected *via* the first two routes, which are governed by density-dependant relationships (Bateman, 1997). Butt *et al.* (2001) also noted that insect density was of particular importance in the epizootiology of disease and as insect density increased; there is a higher probability of an insect coming into contact with a pathogen. Density-dependant relationships were observed between *P. xylostella* larval population densities and levels of mycosis. The highest levels of mycosis in the *P. tenuipes* treated plots were associated with peak larval population densities in the control plots (Fig. 3). Mycosis also appears to follow cycles with alternating high and low levels, which are associated with high and low larval and pupal densities in the control plots. However, although a density-dependant relationship exists, there is no evidence to suggest, which of the methods outlined by Bateman (1997) contributed to mycosis. Average mycosis for *P. xylostella* larvae and pupae during the trial period was 17.8% and 21.7%, respectively and could have contributed to fluctuations in population levels.

Data (unpublished author's data) collected from the field trial suggest that *P. tenuipes* may not affect the cabbage budworm (*Hellula phidealis* Walker (Lepidoptera: Pyralidae). Budworm damage observed was 36%, 38% and 0% in the control, *P. tenuipes* and Pirate® treated plots, respectively. Analysis of the data suggested that there was no significant difference between *P. tenuipes* treated and control plots. Further assessment may however be required to determine the host range of *P. tenuipes* under field conditions.

CONCLUSION

Based on the present study, *P. tenuipes* conidia had fastest growth rate at 25^{o}C with a LC_{50} of 1.09×10^{6} spores/mL and a LT_{50} of 3.28 days at this temperature. We conclude that this entomopathogenic fungus caused mortality to both larvae and pupae of *P. xylostella* under field conditions and has potential as a biological control agent of this insect particularly in organic and ecologically sustainable agriculture.

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