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Original article**Susceptibility of the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera: Chrysomelidae) to microsporidian parasites of the genus *Nosema* from lepidopteran hosts**

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Abstract

Microsporidia are intracellular parasites of animals and some protists. They are promising agents of biological protection of plants against insect pests in which they cause chronic diseases, ultimately leading to death. In this study, five species of microsporidia of the genus *Nosema* from different Lepidoptera (the silkworm, cotton bollworm, cinnabar moth, beet webworm, and European corn borer) were tested for their ability to infect larvae of the Colorado potato beetle *Leptinotarsa decemlineata* (Say). Infection was orally induced by feeding fresh potato leaves treated with microsporidian spores. Only the microsporidium *Nosema* sp. NspHA from the cotton bollworm *Helicoverpa armigera* (Hübner) had a negative impact on the growth of the Colorado potato beetle, resulting in delayed development and abnormalities during metamorphosis. Insect mortality was also increased after treatment with spores of *Nosema polyvora* (Blunck) from the cabbage white *Pieris brassicae* (Linnaeus). Further studies will include testing low doses of these isolates on beetle larvae and assessing their impact on the viability and fertility of the offspring of infected beetles.

Keywords: biological control, laboratory culture, microsporidia, mortality rate, violation of metamorphosis

Introduction

Microsporidia are widespread intracellular parasites of animals and some protists (Issi 2020). At least 1,300 species in 220 genera have been described (Park and Poulin 2021) so far. Most microsporidian species are described in arthropods and fish (Bojko *et al.* 2022). Microsporidia may cause systemic infections affecting almost all tissues or infect specific tissues of all developmental stages of their hosts. In arthropods, they most often infect the adipose tissue and the midgut epithelium, causing severe dysfunction of these organs and disrupting vital processes (Pan *et al.* 2018). Chronic infections caused by microsporidia, as well as delayed host development, are used to control insect abundance in natural ecosystems and in agriculture (Issi 2020). For example, the formulation "Nolo bait", made from wheat bran coated with *Paranosema* (*Nosema*) *locustae* (Canning) spores, was applied to control locusts, grasshoppers and crickets (Zhang and Lecoq 2021). It showed compatibility with other insect pathogens (Dakhel *et al.* 2019). Microsporidia *Amblyospora* spp. and *Edhazardia aedis* (Kudo) are considered as promising pathogens to combat mosquitoes (Andreadis *et al.* 2018; Bukhari *et al.* 2022). *Kneallhazia solenopsae* Sokolova and Fuxa and *Vairimorpha invictae* (Jouvenaz and Ellis) were effectively used against fire ants (Oi *et al.* 2019; Willis and Reinke 2022);

Vairimorpha (*Nosema*) *lymantriae* (Weiser) and *Vairimorpha disparis* (Timofejeva) were used on gypsy moth *Lymantria dispar* (Linnaeus) (Boukouvala *et al.* 2022). The microsporidium *Myrmecomorba nylanderiae* (Plowes *et al.*) was tested for several years under field conditions against the tawny crazy ant *Nylanderia fulva* (Mayr) in North America, resulting in a steady decline of the pest populations (LeBrun *et al.* 2022).

In insects, as in other animals, microsporidia are transmitted either horizontally, between individuals of the same generation (Nattoh *et al.* 2021), or vertically, from parents to offspring (Haag *et al.* 2020). The variety of transmission routes contributes to the preservation and spread of pathogens in insect populations.

Horizontal transmission of infection is mainly carried out by alimentary means as a result of cannibalism (within the population of the same host species) or predation (in the case of heterospecific transmission), as well as necrophagy or consumption of food, contaminated with spores that are excreted by infected individuals with feces (Nattoh *et al.* 2021).

In addition to infecting their typical hosts, some microsporidia are able to infect other taxa that are considered non-specific. The expansion of the host range can involve insects of different genera, families and orders (Malysh *et al.* 2018; Lipa *et al.* 2020; Tokarev *et al.* 2022), as well as hosts of different classes and phyla (Simakova *et al.* 2016; Hinney *et al.* 2016; Tokarev *et al.* 2016).

Different microsporidian species vary greatly in geographical range, frequency of occurrence, and ability to infect non-specific hosts (Murareanu *et al.* 2021; Willis and Reinke 2022). This may even apply to closely related species. Lepidoptera and Coleoptera are among the most numerous and widespread taxa of arthropods (Stork 2018). Because many of them occupy the same ecological niches (e.g., larvae of different taxa feeding on plant leaves, or adults feeding on flowers), this increases the chances of pathogen transmission between the host orders. In this regard, the aim of this study was to assess the potential of microsporidia of the genus *Nosema*, which infect lepidopteran insects, as a biological agent against the Colorado potato beetle *Leptinotarsa decemlineata* (Say) (Coleoptera, Chrysomelidae). To assess this, the intensity of microsporidian development and its impact on the pest's viability under experimental conditions were examined.

Materials and Methods

Insects

Adult females of the Colorado potato beetle were collected in the Krasnodar Area on a potato field in mid-June 2025. They were kept in ventilated plastic buckets with a volume of 30 L (height 27.5 cm, diameter 22.5 cm), 20 per bucket, at ambient temperatures (+ 25-28°C). The beetles were fed freshly cut potato shoots, placed in 50 ml test tubes filled with water to preserve the freshness of the leaves on which the beetles fed and laid eggs. Larvae hatched from the eggs were routinely maintained in a similar way for 4-5 days and then exploited in the experiments.

Microsporidia

The following five microsporidia of the genus *Nosema* were used in the experiments: *Nosema bombycis* (Naegeli), *Nosema* sp. NspHA which is closely related to *N. bombycis* (Kononchuk *et al.* 2022; Malysh *et al.* 2023), *Nosema tyriae* (Canning *et al.*), *Nosema pyrausta* (Paillot), and *Nosema polyvora* (Blunck) (Table 1). All microsporidia were cultivated in larvae of the mulberry pyralid *Glyphodes pyloalis* (Walker) (Lepidoptera, Crambidae) using a temporary laboratory culture of insects collected in Uzbekistan. The spores were isolated from the homogenized tissues of infected pupae. To purify the spores from insect tissues, 1.5 ml tubes were used, to which 1 ml of distilled water was added. The tubes with the contents were centrifuged at 4000 rcf for 5 minutes, after which the supernatant was carefully removed and distilled water was added again to wash the pellet. This procedure was repeated 10 times. Subsequently, the tubes with spores were stored at +4°C (Malysh *et al.* 2023).

Table 1. Microsporidia used for this experiment.

Microsporidian species/isolate	Insect host in which the microsporidia/isolate was obtained	Collection area for infected insects	Year of collection
<i>Nosema bombycis</i>	The larvae of the silkworm <i>Bombyx mori</i>	The Institute of Sericulture (Tashkent, Uzbekistan)	2024
<i>Nosema</i> sp. NspHA	The pupae of the cotton bollworm <i>Helicoverpa armigera</i>	The Krasnodar Area	2023
<i>Nosema tyriae</i>	The pupae of the cinnabar moth <i>Tyria jacobaeae</i>	The Karasuk district of the Novosibirsk Province	2020
<i>Nosema pyrausta</i>	The European corn borer <i>Ostrinia nubilalis</i>	All-Russian Institute of Plant Protection (Saint Petersburg, Russia)	2025
<i>Nosema polyvora</i>	The pupae of the cabbage white <i>Pieris brassicae</i>	The Nevelsky district of the Pskov Province	2022

Infecting beetles with microsporidia

A water suspension of fresh spores (< 1 month of storage, 10^6 spores per larva) was applied to both sides of the upper leaves of the potato foliage measuring 40 x 20 mm, slightly scratched with a needle for better absorption by the surface. The excess liquid was allowed to evaporate for 30 minutes. In the control group, distilled water without spores was applied. Each treatment had three replicates, with 10 larvae per replicate. One leaf was used for treatment of each replicate. For all infection experiments beetles of one replicate were kept in a separate plastic Petri dish measuring 90 x 15 mm for the second instars and as the larvae of the Colorado potato beetle increased in dimensions, the Petri dishes were replaced with larger ones (145 x 20 mm), keeping the same number of insects per dish (or fewer due to mortality). On the next day post

treatment, the contaminated feed was consumed by 95% (only the streaks of leaves remained), and fresh uncontaminated feed was added. The insects were transferred daily to clean Petri dishes with fresh feed. Mortality records and feed replacement were carried out daily.

Light microscopy

All individuals of the Colorado potato beetle were examined for the presence of spores using a “Micromed” light microscope (LOMO) equipped with a ToupCam video camera (ToupTek) and ToupView software at a magnification of 680 X (without immersion) and 1700 X (with oil immersion).

Statistical analysis

Statistical data processing of the mortality and infection rate was performed by the single-factor analysis using Past v3 software (Hammer *et al.* 2001) with the built-in Kruskal-Wallis model and the posteriori Dunn's post hoc test.

Molecular analysis

DNA was extracted using a simplified protocol of Sambrook *et al.* (1989) with modifications (Malysh *et al.* 2019). Beetle tissues which remained after microscopy, and contained a notable number of spores were used for molecular analysis. DNA amplification was performed with primers targeting the ribosomal RNA small subunit locus (SSU rRNA) 18f:1047r at an annealing temperature of 54°C, specific product sized ca 900 bp (Weiss and Vosbrinck 1999). For PCR analysis, a 5X Screen Mix (Evrogen, Russia) mixture was used with the following cycling conditions: initial denaturation at 95°C for 3 min, 40 denaturation cycles at 95°C for 0.5 min, annealing for 0.5 min at 54°C, elongation at 72°C for 1 min and the final elongation at 72°C for 9 minutes. Amplicons were visualized by electrophoresis in 1% agarose gels with a molecular weight marker GeneRuler Ladder Mix, 75-20000 bp (Thermo Fisher Scientific, USA).

Results

Among untreated insects, the mortality rate on the 10th and 20th days after the start of the experiment was 3.33 ± 0.34 and $6.67 \pm 0.05\%$, respectively. In the treatment with *Nosema* sp. NspHA spores, the mortality rate of larvae was $3.33 \pm 0.34\%$ (number of perished larvae n=1 out of 30 larvae) on day 10 and $26.67 \pm 0.07\%$ (n=8) on day 20. In the treatment of *N. bombycis*, the mortality rate of host larvae was 3.33 ± 0.03 (n=1) and $16.67 \pm 0.08\%$ (n=5), respectively.

Larval mortality in the *N. tyriae* treatment was $3.33 \pm 0.03\%$ (n=1) on the 10th day and $26.67 \pm 0.08\%$ (n=8) on the 20th day after infection. In the *N. pyrausta* treatment, larval mortality was $16.67 \pm 0.07\%$ (n=5) and $26.67 \pm 0.08\%$ (n=8), respectively. The highest larval mortality was observed in the case of *N. polyvora*, where it reached $13.33 \pm 0.06\%$ (n=4) on day 10 and $46.67 \pm 0.09\%$ (n=14) on day 20 after treatment (Table 2).

Table 2. Average mortality and number of spores in the Colorado potato beetle after feeding microsporidian spores (10^6 spores per individual) to the second instars.

Microsporidian species/isolate (million spores per individual)	Insect death on the 10th and 20th days after feeding the spores (out of 30 individuals), % $\pm$ SE		Presence of spores in cadavers	Proportion of individuals with developmental disorders, % $\pm$ SE
	10	20		
Control (without spores)	3.33 ± 0.03 (n=1) a*	6.67 ± 0.05 (n=2) ab	-	0
<i>Nosema</i> sp. NspHA	3.33 ± 0.03 (n=1) a*	26.67 ± 0.08 (n=8) abc	5.29 ± 0.78 million spores per individual (n=7)	66.66 ± 8.61
<i>Nosema bombycis</i>	3.33 ± 0.03 (n=1) a*	16.67 ± 0.07 (n=5) b	Single spores, up to 3 spores per field of view	0
<i>Nosema tyriae</i>	3.33 ± 0.03 (n=1) a*	26.67 ± 0.08 (n=8) abc	Single spores, up to 5 spores per field of view	0
<i>Nosema polyvora</i>	13.33 ± 0.06 (n=4) a*	46.67 ± 0.09 (n=14) c	Single spores, up to 5 spores per field of view	0

<i>Nosema pyrausta</i>	16.67 ± 0.07 (n=5) b*	26.67 ± 0.08 (n=8) abc	Single spores, up to 5 spores per field of view	0
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Values significantly different from control are indicated by different letters – $p < 0.01$; * – $p < 0.05$.

When the pathogens' effect of different isolates was compared, *N. polyvora* caused the highest mortality on the 10th day after treatment, which significantly ($p < 0.05$) differed from the mortality in all other variants. On day 20, the mortality in this treatment was significantly ($p < 0.01$) higher than that in the control and the *N. bombycis* application (Table 2).

It is noteworthy that insects treated with NspHA showed marked deviations from the normal course of metamorphosis. In comparison to the controls the larvae turned black, acquired a wrinkled appearance, and had decreased sizes, while remaining alive (Fig. 1, B, C) for another 6 days. No visible pathologies were observed in other variants (Fig. 1, A, F).

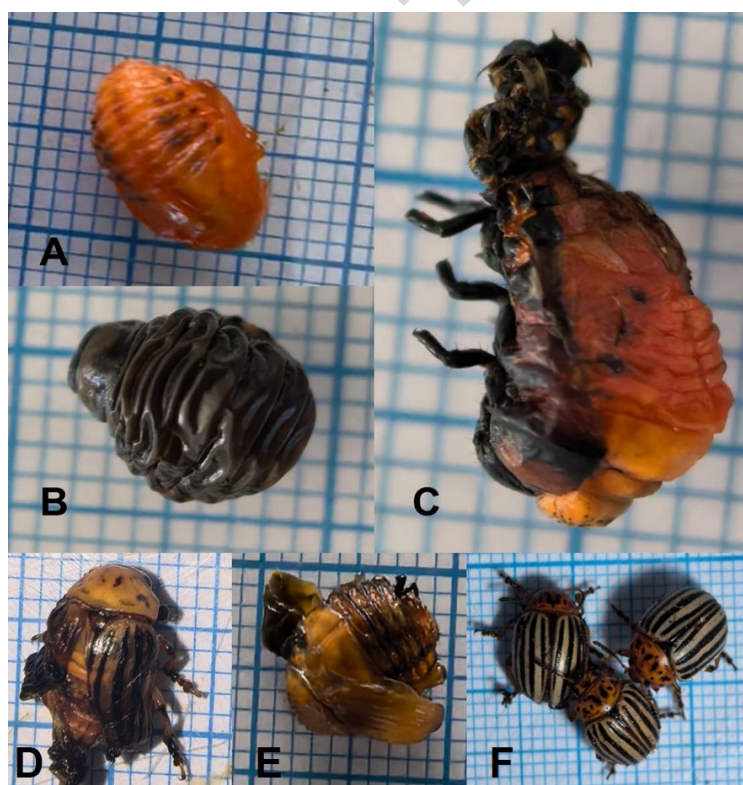


Fig. 1. Infection of the Colorado potato beetle with microsporidian isolate *Nosema* sp. NspHA: deformed larvae, pupae, and adults of the infected host (B, C, D, E) compared with uninfected control (A, F).

Thirty days post treatment with NspHA, 9 out of 22 individuals which survived ceased to pupate while another 9 resulted in adults with deformed wings (Fig. 1, D, E). Only four adults were normal. Interestingly, adult specimens with deformities lived for 3-4 days, while normal specimens were viable for 5-7 days.

Microscopy of the homogenized cadavers demonstrated spores in all treatments, but with differing intensities of microsporidian development. Out of 30 analyzed individuals in the treatment of *Nosema* sp. NspHA, there were 25 (84%) microsporidia-positive specimens, containing at least five spores of the pathogen per microscopic field (at $\times 400$ magnification). Among these, as many as seven insects (28% of the whole treatment sample $n=30$) had 10 to 50 spores per field. In this group, the average number of spores per individual was $5.29 \pm 0.78 \times 10^6$ ($n=7$). In the treatments of *N. polyvora* and *N. tyriae*, one to five spores per field of view were seen in 26 and 24 insects, corresponding to 87% and 81% ($n=30$), respectively. In the case of *N. pyrausta*, there were only two positive samples, i.e., 7% ($n=30$). The lowest intensity was observed in the treatment of *N. bombycis*, where 16 individuals (54%, $n=30$) contained only one to 10 spores per 20 microscopic fields of view examined per smear prepared from the whole body homogenate (Table 2).

PCR analysis showed only faint amplicons with a length of about 900 bp in samples of *Nosema* sp. NspHA, *N. polyvora*, *N. pyrausta*, and *N. tyriae*. At the same time, the NspHA amplicon was brighter than the other three, yet weaker than the positive control. In the case of *N. bombycis*, no luminescence was detected in the region of 900 bp (Fig. 2). An attempt to purify a sufficient amount of PCR products for further sequencing was not successful.

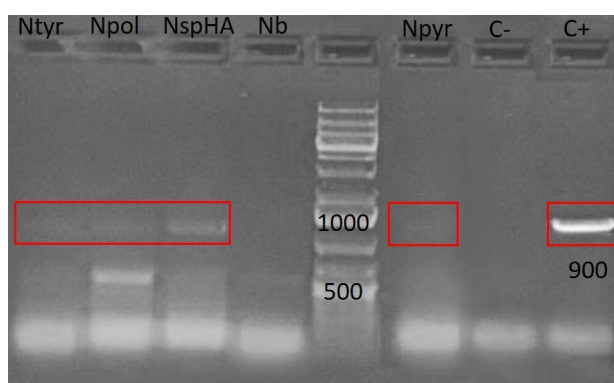


Fig. 2. Electrophoretic profile of amplification products obtained with 18f:1047r SSU rRNA primers. C+ - positive control, *Nosema bombycis* from the silkworm; C- – negative control, no DNA; Npyr – *N. pyrausta* from the European corn borer; Nb – *N. bombycis* from the silkworm; NspHA – *Nosema* sp. from the cotton bollworm; Npol – *N. polyvora* from the cabbage white; Ntyr – *N. tyriae* from the cinnabar moth. The red border indicates the presence of the specific amplicon.

Discussion

Microsporidia have been repeatedly found in nature in beetles of the Chrysomelidae family, including *L. decemlineata* (Hostounsky and Weiser 1973; Brooks *et al.* 1988; Bauer and Pankratz 1993; Yaman *et al.* 2011; Bekircan 2020). However, in the present study, microsporidian spores were not detected in untreated insects, which were the progeny of field-collected adults. According to previous observations, microsporidia were not found in populations of the Colorado potato beetle native to southwestern (Krasnodar Area) and northwestern Russia (St. Petersburg), as well as western Siberia (Novosibirsk Province). On the other hand, it is important to test the possibility of infecting the Colorado potato beetle with microsporidia from another order, namely Lepidoptera, which is one of the most common and known for a high abundance of microsporidia. For example, *N. pyrausta*, found in the European corn borer *Ostrinia nubilalis* (Hübner) (Lepidoptera, Crambidae), successfully infected the beet webworm *Loxostege sticticalis* Linnaeus (Lepidoptera, Crambidae), the garden pebble moth *Evergestis forficalis* Linnaeus (Lepidoptera, Crambidae), the small tortoiseshell *Aglais urticae* Linnaeus (Lepidoptera, Nymphalidae), and the Siberian moth *Dendrolimus sibiricus* Chetverikov (Lepidoptera, Lasiocampidae) (Malysh *et al.* 2021; Tokarev *et al.* 2022). In addition, cases of microsporidia transmission from insect hosts of one order to those of another order have been documented. For example, the microsporidium *Vairimorpha* (*Nosema*) *ceranae* Fries *et al.*, parasitizing honey bees *Apis mellifera* Linnaeus (Hymenoptera, Apidae) was found in the larvae of *L. sticticalis* (Malysh *et al.* 2018). Similarly, the microsporidium *Anncaliia* (*Nosema*) *meligethi* Issi and Radishcheva from the pollen beetle *Brassicogethes* (*Meligethes*) *aeneus* Fabricius (Coleoptera, Nitidulidae) could infect the lepidopteran host, namely the cabbage white *Pieris brassicae* Linnaeus (Lepidoptera, Pieridae), under laboratory conditions (Sokolova *et al.* 2018). It was also found that *N. pyrausta* and *N. bombycis* were able to infect the spined soldier bug *Podisus maculiventris* (Say) (Hemiptera, Pentatomidae), whose nymphs had been feeding on infected lepidopteran larvae (Rumiantseva *et al.* 2024).

Microsporidia such as *Nosema gastroideae* Hostounsky and Weiser, *Nosema polygrammae* Hostounsky and Weiser, *Endoreticulatus* (*Plistophora*) *fidelis* Hostounsky and Weiser, and *Pleistophora grossa* Hostounsky and Weiser, caused acute infections in the Colorado potato beetle, and “larvae died before the parasite could produce large number of spores”. That prompted the authors to search for alternative laboratory hosts within the family Chrysomelidae (Hostounsky 1984). On the other hand, the microsporidium *Nosema scripta* Bauer and Pankratz from the cottonwood leaf beetle *Chrysomela scripta* Fabricius (Coleoptera, Chrysomelidae) infected the Colorado potato beetle at low levels, and the infected individuals

could transmit the infection vertically to their progeny (Bauer and Pankratz 1993). In spite of these studies, there is no published data describing transfer of these parasites from Lepidoptera to Coleoptera. Microsporidian infections have been reported in predatory beetles that feed on lepidopteran insects in greenhouses, yet there is no evidence indicating that the infection originated from the lepidopteran hosts (Yaman *et al.* 2016, 2022). As shown in the present study, among five microsporidia from Lepidoptera, notable effects were exerted by two isolates: *Nosema* sp. NspHA from the cotton bollworm *Helicoverpa armigera* (Hübner) (Lepidoptera, Noctuidae), which impaired the development of 67% of beetles, and *N. polyvora* from *P. brassicae*, which caused 40% additional mortality (given the baseline 6% mortality in the untreated group). The number of spores in the insect homogenates was negligible, with the exception of only NspHA, in which the spores could accumulate at a remarkable level, alongside impaired host metamorphosis, serving as fairly common symptoms of microsporidian infection in holometabolic insects (Pan *et al.* 2018). Microsporidia often cause developmental pathologies in their hosts. *Vairimorpha* sp. caused symptoms of an abnormal abdomen with a malformation of the second pair of wings in *Gonipterus platensis* Marelli (Coleoptera, Curculionidae) (Jordan *et al.* 2021). Transovarial transmission of *N. pyrausta* during several laboratory generations of *L. sticticalis* caused a gradual decrease of insect fitness and zero fertility in the third generation (Malysh *et al.* 2021). The silkworm treated with *N. bombycis* had an increased life cycle, decreased reproduction, and decreased silk thread secretion (Bhat *et al.* 2017).

The life cycle of microsporidia of the genus *Nosema* takes 3-5 days at a temperature range of +24-35°C (Odnosum and Yefimenko 2021; Pei *et al.* 2021), and may extend up to 14 days at suboptimal thermal regimes (Odnosum and Yefimenko 2021). As the temperature conditions in the present study were within the optimal range and the experiment lasted for 20 days the pathogens had enough time for at least one round of multiplication. However, only in NspHA treatment the number of accumulated spores exceeded that of the initial parasite's load by an order of magnitude, which warrants the infective process. As for the other species, the observation of low numbers of spores observed at the end of the experiment (especially in *N. bombycis*) does not allow for reliable conclusions.

Meanwhile, the developmental abnormalities induced in nearly 70% of the Colorado potato beetles by *Nosema* sp. NspHA suggest that the beetles which survived would not be able to reproduce normally. This indicated the possible adverse effect on pest population density and invokes practical interest in this microsporidian isolate. It will be necessary to study the prospects of its use in microbial control, but this will require testing with low spore doses to

make it economically feasible. In addition, it will be important to assess the impact of these microsporidia on the viability and fertility of the offspring of infected beetles.

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