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ORIGINAL RESEARCH ARTICLE

Assessing the efficacy of un and gamma-irradiated entomopathogenic nematode, *Steinernema carpocapsae* as an eco-friendly approach to control *Agrotis ipsilon* larvae

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Abstract
The black cutworm, *Agrotis ipsilon* (Hufnagel) is one of the most devastating agricultural pests that attack different crops worldwide. Entomopathogenic nematodes (EPNs) have been utilized in classical, conservation, and augmentative biological control programs. Consequently, this study was designed to estimate the efficacy of an unirradiated and gamma-irradiated entomopathogenic nematode, *Steinernema carpocapsae* (all), against *A. ipsilon* larvae as a safe control tool to reduce chemical environmental hazards. The virulence of different concentrations (10, 20, 30 and 40 IJs/ml) of unirradiated and gamma-irradiated (with 2 Gy) of *S. carpocapsae* was evaluated. Changes in peroxidase (Px) and polyphenyl oxidase (PPO) zymographic patterns were also investigated after larvae were treated with LC_{50} values for *S. carpocapsae*. The results revealed that larval mortality increased with increasing *S. carpocapsae* concentration and time after treatment. The 2 Gy gamma-irradiated *S. carocapsae* caused a greater pathogenic effect than the unirradiated *S. carocapsae*, where the LC_{50} values of 2 Gy gamma-irradiated *S. carocapsae* were 31.19 and 17.78 IJs/ml after 1 and 2 days, respectively, compared to 113.23 and 65.83 IJs/ml for unirradiated *S. carocapsae*. For the toxicity index, gamma irradiation at a dose of 2 Gy was selected and given an arbitrary 100 units and considered a standard treatment. Analysis of gel electrophoresis revealed 3 bands of Px and PPO in control larvae; treatment with unirradiated and irradiated *S. carpocapsae* caused the disappearance of the first band of both enzymes and lowered the intensity of the other 2 bands. The laboratory studies revealed that 2 Gy gamma-irradiated *S. carpocapsae* could be used as an eco-safe management tool against *A. ipsilon* larvae under field conditions.

Keywords Black cutworm · Entomopathogenic nematode · Gamma rays · Biological control · Isoenzyme

Introduction

The black cutworm, *Agrotis ipsilon* (Hufnagel), the black cutworm, is a destructive pest not only in Egypt but also throughout the world; it is most common in places that are temperate or subtropical (Kononenko 2003). This noctuid insect is polyphagous and infects numerous crops (Abo El-Ghar et al. 1996). Vegetables, cucurbitaceous plants, and agricultural crops are all infested by the greasy cutworm. The most damaged crops are cotton, essential-oil cultures, maize, tobacco, sunflower, tomatoes, sugar beet, and potato. The larval stage is the most devastating; it prefers to live in the ground and feed on the stem, cutting off the stem and sometimes dragging the plants into their burrows. The larvae can consume over 400 cm^2 of foliage during their development. According to the larval habitat, it is difficult to control it (Amin et al. 2019).

The traditional control tool for this pest is using chemical insecticides, but the widespread use of these pesticides has resulted in a residual effect in the plant that produces new resistant insects and negative impacts on nontarget organisms.

Entomopathogenic nematodes (EPNs) of the "families Steinernematidae and Heterorhabditidae", along with the symbiotic bacteria *Xenorhabdus* and *Photorhabdus*, have been employed as biocontrol agents of pest insects that are harmful to agriculture (Gaugler 2002). When these bacteria are discharged from the nematode gut, they quickly grow in the host haemolymph, and within 24 to 48 h, they produce

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deadly bacteraemia. Deadly endoparasites of insects known as Steinernematidae and Heterorhabditidae have the ability to secrete poisons, proteases, and other insecticidal active ingredients that can have a lethal effect on infected host insects (Toubarro et al. 2009). The deadly actions of insecticidal active ingredients frequently involve altered enzyme activity in host insects (Grewal et al. 2005).

Nematodes, like other living things, can be activated by low levels of gamma radiation (Marples and Collis 2008). Earlier research suggested that 2 Gy gamma-irradiated EPNs could be used to control certain insect infections (Yussef 2006; Sayed 2011; Salem et al. 2014; Sayed et al. 2015, 2018; Sayed and Shairra 2017).

Insects have an extremely effective cellular and humoral immune system (Gillespie et al. 1997). Phagocytosis, encapsulation, and nodule formation are all aspects of cellular immunity (Lavine and Strand 2002). Humoral defenses refer to soluble effector molecules, including antimicrobial peptides (AMPs), complement-like proteins, enzymes, and products generated by complex proteolytic cascades (Blandin and Levashina 2004).

Biochemically, enzymes are present in many alternative forms with the same enzyme activity, called isoenzymes, such as peroxidases and polyphenole oxidase. Peroxidases (Px) play a significant role in a variety of metabolic processes (Cheng et al. 2008) and contribute to xenobiotic detoxification, innate immunity, the pathogenesis of inflammatory diseases, and hormone biosynthesis (Lubos et al. 2011). Moreover, Prakasha and Umesha (2016) reported that peroxidases play a role in enhancing the plant's defense against pathogens and start working within minutes after a bacterial attack. Polyphenole oxidase (PPO) catalyzes the oxidation of phenols to quinones (Vanitha et al. 2009) and was reported at high levels after plant infection with bacterial diseases (Ngadze et al. 2012).

The current study aimed to evaluate the vulnerability of *A. ipsilon's* 5th larval instar to unirradiated and gamma-irradiated *S. carpocapsae*. Beyond furthering its impact on some isoenzyme activity (Px and PPO) of the *A. ipsilon* larvae.

Materials and methods

Rearing of *A. ipsilon*

The black cutworm larvae were reared on castor leaves away from any insecticidal contamination in the Natural Control lab., Natural Products Research Department, National Centre for Radiation Research and Technology, Egyptian Atomic Energy Authority, Cairo, Egypt, under laboratory constant conditions of 24 ± 2 °C and 65 ± 5% relative humidity and a photoperiod of 16 L:8 D. The newly hatched larvae were kept in a clean glass jar and provided with castor oil leaves daily until the 3rd instar larvae emerged. Then, they were transferred to larger, clean glass jars to prevent larval cannibalism. The bottom of each jar was covered with a thick layer of fine sawdust, and the usual rearing techniques were performed along with the developing larvae until pupation occurred. The developed pupae were transferred to a clean jar for adult emergence after pupation. The adult moths (males and females in a 7:5 ratio) were transferred to a glass jar and given a dietary supplement of a hanged piece of cotton wool soaked in 10% sugar solution (El-Shershaby 2010).

Nematode

Stinernema carpocapsae (all) was obtained from the Biological Control Department, Plant Protection Research Institute; Agricultural Research Centre (ARC), Giza, Egypt. *S. carpocapsae* was cultivated on the last larval instar of *Galleria mellonella* L. (Bedding and Akhurst 1975) at the Natural Control lab., Natural Products Research Department, National Centre for Radiation Research and Technology, Egyptian Atomic Energy Authority, Cairo, Egypt, under controlled conditions of 25 ± 2 °C. Twenty larvae were placed in a 15 cm petri dish filled with filter paper, and then 2 ml of the infective juvenile suspension was sprayed. After the larvae were dead, they were placed in the collecting dishes, which consisted of filter paper on the glass watch placed in a larger and deeper Petri dish in which distilled water was added to reach the edge of the filter paper. The cadavers were placed in the middle of moist filter paper to migrate down into water. The juvenile suspension was collected daily by a special pipette and stored in glass jars containing distilled water in a refrigerator at 5 °C until it was used in the experiments.

Irradiation technique

The 3rd infective juveniles (IJs) of the EPN species were gamma irradiated with 2 Gy (Sayed 2011) using the Gamma Cell Irradiation Unit located in the National Centre for Radiation Research and Technology, Egyptian Atomic Energy Authority. The dose rate used for the cesium unit (Cs^{137}) was 0.613 Rad/sec at the time of irradiation.

Virulence of unirradiated and irradiated *S. carpocapsae* on *A. ipsilon* larvae

The bioassay was carried out according to Woodering and Kaya (1988). Different concentrations of unirradiated and irradiated IJ nematode suspensions were prepared: 10, 20, 30 and 40 IJs/ml; the control consisted of the same volume of sterilized distilled water. Six larvae were placed in 100 cm^3 plastic cups, and 1 ml of each nematode concentration

was sprayed. Each concentration and the control were replicated 5 times under controlled conditions of 25 ± 2 °C. The mortalities were counted daily, and the cumulative mortalities were calculated. The LC_{50} values were calculated using Ldp-line software (copyrighted by Ehab Bakr, Plant Protection Research Institute, Agricultural Research Center, Giza, Egypt).

Isoenzyme electrophoresis

For isoenzyme extraction, 0.5 g of normal and treated larvae (with LC_{50} values of unirradiated and irradiated *S. carpocapsae*) were homogenized in 1 ml of extraction buffer (10% glycerol). The extract was then transferred in to clean Eppendorf tubes and centrifuged at 10,000 rpm for 5 min. The supernatant was transferred to new, clean Eppendorf tubes and kept at –20 °C until used for electrophoretic analysis. Native-polyacrylamide gel electrophoresis (Native-PAGE) was conducted to identify isozyme variations among the studied larvae using two isozyme systems, according to Stegemann et al. (1985). The utilized isoezymes were peroxidase (Px) and polyphenol oxidase (PPO). A volume of 40 µl extract of each larvae group was mixed with 20 µl sucrose and 10 µ bromophenol blue, and then a volume of 50 µl from this mixture was applied to each well. The run was performed at 150 V until the bromophenol blue dye reached the separating gel, and then the voltage was increased to 200 V. The electrophoresis apparatus was placed inside a refrigerator during the running duration. After electrophoresis, the gels were stained according to their enzyme systems with the appropriate substrate and chemical solutions and then incubated at room temperature in the dark for complete staining (in most cases, incubation for approximately 1 to 2 h is enough).

For peroxidase, the gel was incubated at room temperature with 0.125 g of benzidine dihydrochloride, 2 ml of glacial acetic acid, 50 ml of dilute water, and 5 drops of hydrogen peroxide until bands appeared.

For polyphenyl oxidase, the staining substrate solution was prepared by 0.1 M phosphate buffer pH 6.5, 100 mg sulphonillic acid, and 200 mg cathecol in 2 ml acetone. The gel was placed into this solution and incubated at 30 °C for 30 min until bands appeared. Gels were photographed, scanned, and analyzed using the Gel Doc VILBER LOURMAT system.

Statistical analysis

The Minitab program was used to adjust and analyze the obtained results using ANOVA, followed by Tukey's pairwise comparisons test to examine the significant differences ($P < 0.05$) across the means of the treatments.

Results

The mortality rates of 5th instar larvae of *A. ipsilon* by unirradiated *S. carpocapsae* at various time intervals were showed in Fig. 1. The data indicated a positive relationship between increased *S. carpocapsae* concentration and larval mortality, as well as exposure time (Fig. 1). The mortalities were 3.33, 6.67, 10, and 23.33% after 1 day and increased to 10, 16, 30, and 36.67% after 2 days of treatment with 10, 20, 30 and 40 IJs/ml, respectively. On the third day of the treatment with 10, 20, 30 and 40 IJs/ml, the percentages of mortality recorded the highest rate, being 16.67, 30, 40, and 56.67%, respectively.

The overall death rate showed a parallel correlation with increasing nematode concentration and exposure time (Fig. 2). The 5th larval instar of *A. ipsilon* was highly susceptible to irradiated *S. carpocapsae*; larval mortalities were 100% after 2 and 3 days of treatment with irradiated 40 and 30 IJs/ml, respectively. After 1 day of treatment with 10,

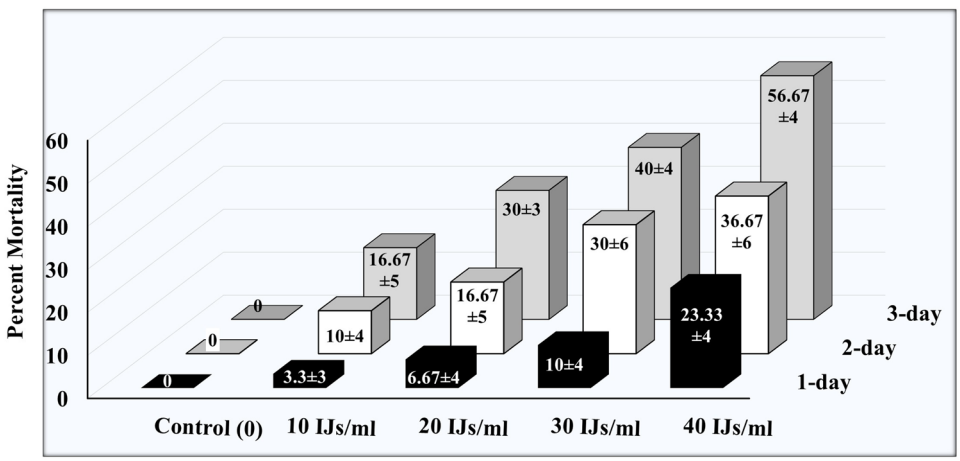

Fig. 1 Cumulative percentage mortality (mean±SE) of 5th instar larvae of *A. ipsilon* by unirradiated *S. carpocapsae* at different time intervals

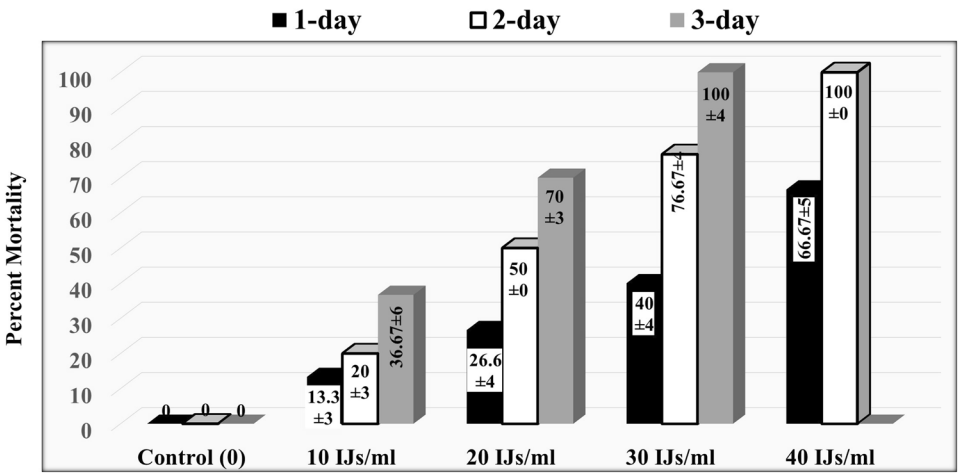

Fig. 2 Cumulative percentage mortality (mean±SE) of 5th instar larvae of *A. ipsilon* by irradiated *S. carpocapsae* at different time intervals

20, 30 and 40 IJs/ml, the mortality percentages were 13.33, 26.67, 40, and 66.67, respectively.

The data in Table 1 display the calculated LC_{50} and LC_{90} values of unirradiated and irradiated *S. carpocapsae* after 1 day and 2 days of treatment. The LC_{50} values of unirradiated *S. carpocapsae* were 113.23 and 65.83 IJs/ml after 1 day and 2 days of treatment, respectively. For the irradiated *S. carpocapsae*, the corresponding values of 32.19 and 17.78 IJs/ml after 1 day and 2 days of treatment, respectively (Fig. 3). Generally, the results implied that *A. ipsilon* larvae were more susceptible to irradiated *S. carpocapsae,* with a toxicity index of 100, than unirradiated larvae**.**

The peroxidase (Px) zymogram of 5th instar larvae of *A. ipsilon* either normal or treated with the LC_{50} of unirradiated and irradiated *S. carpocapsae* presents in Table 2 and Fig. 4a show The analysis revealed 3 isomers, Px1, Px2, and Px3, with relative mobilities of 0.1, 0.3, and 0.45, respectively. Px1 appeared with moderate density only in control larvae and disappeared in the analysis of treated larvae. Px2 was detected in all samples, but in the control larvae, the density was high and lowered to moderate density in larvae treated with the LC_{50} of unirradiated and irradiated *S. carpocapsae*. The density of the 3rd band (Px3) was moderate in control larvae or larvae treated with the LC_{50} of unirradiated *S. carpocapsae*. Px3 density was low in larvae treated with the LC_{50} of irradiated *S. carpocapsae*.

Polyphenyl oxidase (PPO) isozyme patterns are presented in Table 3 and Fig. 4b. Three PPO isoezymes, PPO1, PPO2, and PPO3, were discovered in the 5th instar larvae of *A. ipsilon* either normal or treated with the LC_{50} of unirradiated and irradiated *S. carpocapsae,* and their relative mobility values were 0.1, 0.4, and 0.45, respectively. The PPO1 band was detected in control larvae only at a moderate density. Moreover, PPO2 and PPO3 showed high and moderate densities, respectively, in control larvae and decreased to low densities in larvae treated with the LC_{50} of unirradiated and irradiated IJs.

Discussion

Stinernema carpocapsae has demonstrated its effectiveness in controlling different insect pests (Bhairavi et al. 2021). The infectivity of the nematode depends on the species, stage, size, and habitat of the pest, and no one nematode species was the most infective for all insect species (Bedding et al. 1983).

Our results showed that there was a positive correlation between 5th instar larvae of *A. ipsilon* mortality and *S. carpocapsae* concentration increases (unirradiated or irradiated) as well as the time of treatment increase. This agrees with Hassan et al. (2016), who stated that increasing the

Table 1 Lethal concentrations of *S. carpocapsae* against 5th instar larvae of *A. ipsilon*

Treatment	LC_{50}	LC_{90}	Toxicity Index	Resistance Ratio	Slope
After 1-day					
Unirradiated *S. carpocapsae*	113.23	538.78	28.43	3.51	1.89
Irradiated *S. carpocapsae*	32.19	106.57	100	1.00	2.46
After 2-day					
Unirradiated *S. carpocapsae*	65.83	395.99	27.01	3.70	1.64
Irradiated *S. carpocapsae*	17.78	36.45	100	1.00	4.11

Toxicity index and resistance ratio compared with irradiated *S. carpocapsae*

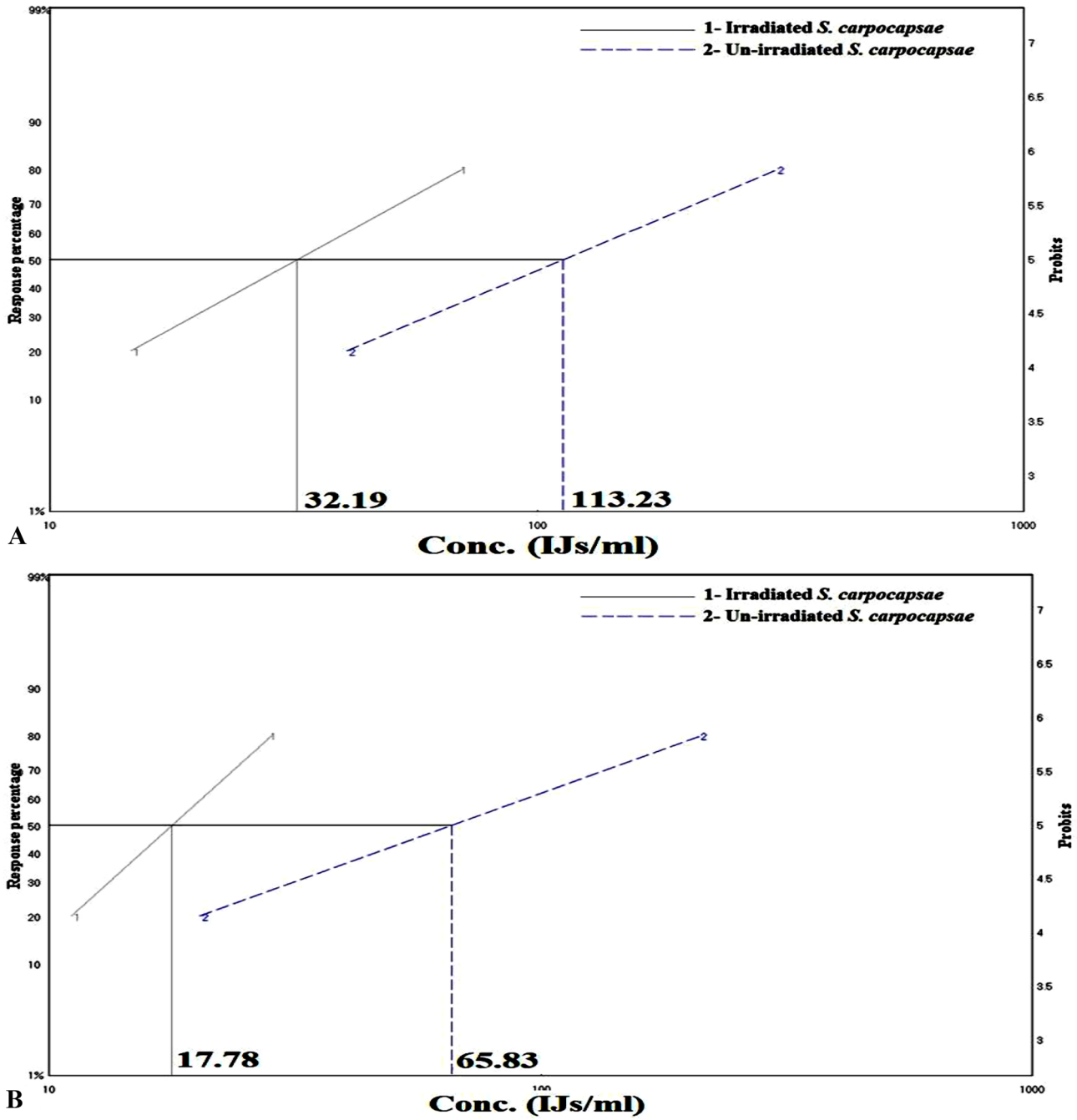

Fig. 3 Median lethal concentration (LC_{50}) after 1 and 2 days of treatment with *S. carpocapsae* on the 5th larval instar of *A. ipsilon.* A: LC_{50} after 1 day, B: LC_{50} after 2 days

Table 2 Distribution of peroxidase isozyme groups of 5th instar larvae of *A. ipsilon* normal or treated with LC_{50} of unirradiated and irradiated *S. carpocapsae*

Groups	Relative Mobility	Control	larvae of *A. ipsilon* normal or treated with LC_{50} of unirradiated *S. carpocapsae*	larvae of *A. ipsilon* normal or treated with LC_{50} of irradiated *S. carpocapsae*
Px1	0.1	1^{+}	0	0
Px2	0.3	1^{++}	1^{+}	1^{+}
Px3	0.45	1^{+}	1^{+}	1^{+}

1: present band, 0: Absent band, + +: High density, +: Moderate density

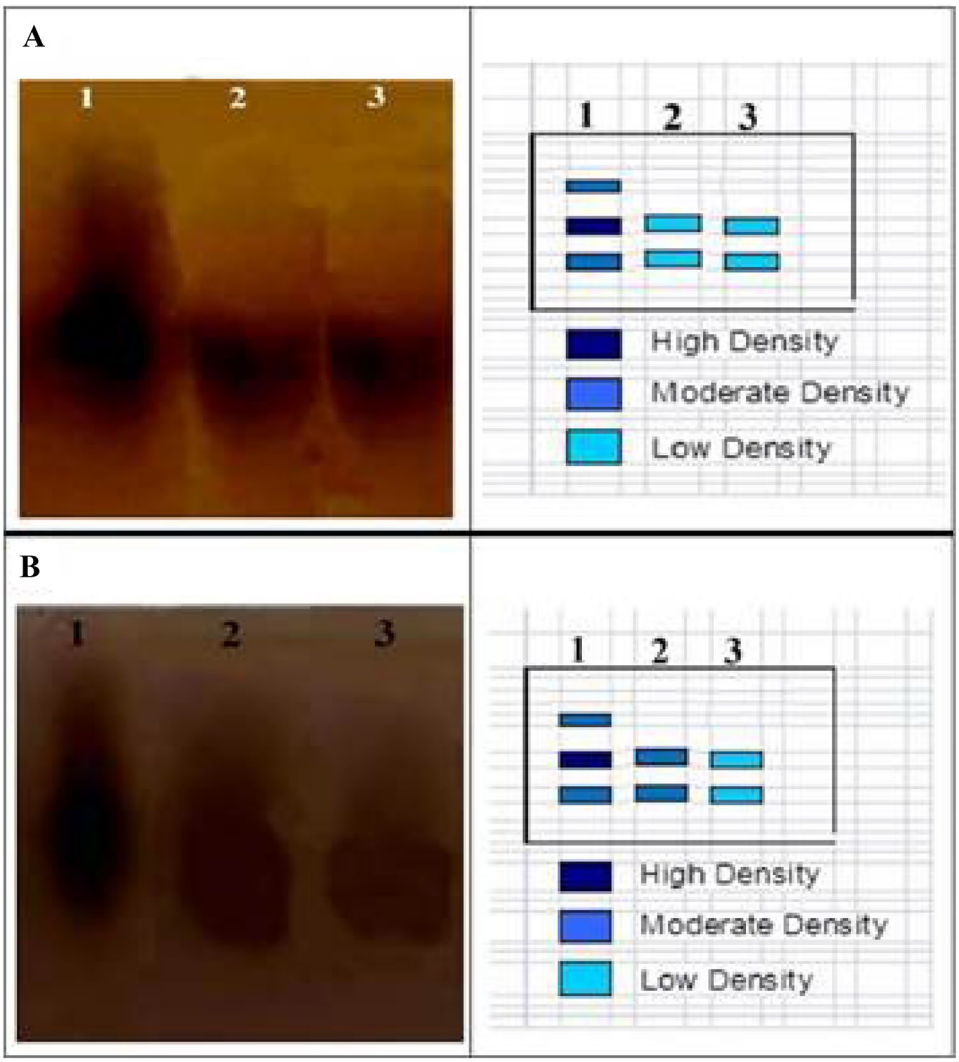

Fig. 4 Zymogram analysis of (**A**) peroxidase and (**B**) polyphenyl oxidase of 5th instar larvae of *A. ipsilon* normal or treated with LC_{50} of unirradiated and irradiated *S. carpocapsae*. 1: Control, 2: Larvae of *A. ipsilon* normal or treated with the LC_{50} of unirradiated *S. carpocapsae* and 3: Larvae of *A. ipsilon* normal or treated with the LC_{50} of irradiated *S. carpocapsae*

Table 3 Distribution of polyphenyl oxidase isozyme groups of 5th instar larvae of *A. ipsilon* normal or treated with LC_{50} of unirradiated and irradiated *S. carpocapsae*

Groups	Relative Mobility	Control	larvae of *A. ipsilon* normal or treated with LC_{50} of unirradiated *S. carpocapsae*	larvae of *A. ipsilon* normal or treated with LC_{50} of irradiated *S. carpocapsae*
Px1	0.1	1^{+}	0	0
Px2	0.4	1^{++}	1^{+}	1^{-}
Px3	0.45	1^{+}	1^{+}	1^{-}

1: present band, 0: Absent band, + +: High density, +: Moderate density, -: Low density

concentrations of *S. glaseri* and *Heterorhabditis bacteriophora* and the time of exposure raised the mortality of *A. ipsilon* larvae. Moreover, Shairra et al. (2016) declared that there was a positive correlation between *S. carpocapsae* or *S. scapterisci* concentrations and larval mortality of *A. Ipsilon*. Additionally, Sobhy et al. (2020) reported that *A. ipsilon* larvae were susceptible to *S. monticolum* and *H. bacteriophora* concentrations and exposure time increases.

According to the prior findings on LC_{50} values, it was clear that the 2 Gy gamma-irradiated *S. carpocapsae* was more virulent against the 5th instar larvae of *A. ipsilon*. The same finding of the increased pathogenicity of gamma-irradiated EPN compared to unirradiated EPN was previously reported by Yussef (2006), who stated that irradiated *S. carpocapsae* was more virulent against *Callosobruchus maculatus* (F.). Similarly, irradiated *S. carpocapsae* killed

G. mellonella, *Corcera cephalonica* (Stainton), and *Ephestia kuehniella* (Zeller) larvae faster than unirradiated larvae (Sayed 2011). Moreover, Sayed and Shairra (2017) evaluated the effectiveness of 2 Gy γ-irradiated *S. scapterisci* among *Spodoptera littoralis* (Boisd.), and they obtained the same result. Sayed et al. (2018) confirmed that the LC_{50} of 2 Gy gamma-irradiated *S. scapterisci* was lower than that of unirradiated *S. scapterisci* when used against *Bactrocera zonata* (Saunders) larvae and pupae. This increase in pathogenicity could be explained by the fact that low doses of gamma radiation cause the symbiotic bacteria to grow more, increasing their toxin production. This was in accordance with Bashandy and El-sinary (2002), who stated that insect mortality rose from 20 to 66% when using gamma-irradiated *Bacillus thuringiensis*.

The gel electrophoresis analysis of peroxidase and polyphenyl oxidase patterns of 5th instar larvae of *A. ipsilon* (control and treated with LC_{50} of unirradiated and irradiated *S. carpocapsae*) revealed the absence of bands and lowered the density of the obtained bands in the larvae treated with *S. carpocapsae*. There were no reports on the activity of EPN toxins on zymographic analysis of enzymes. Although, the changes in band analysis might indicate a change in the enzymes' activities due to the immune-stimulating function of peroxidase in protecting organisms from any stress (Zamocky et al. 2008) or bacteria and parasites infection (Acharya and Ackerman 2014), and the host defence systems of polyphenol oxidases (including, pigmentation and sclerotization) (Malviya et al. 2011).

Conclusions

The above mentioned results revealed that the 5th instar larvae of *A. ipsilon* were susceptible to *S. carpocapsae*, which was more obvious when using 2 Gy gamma-irradiated juveniles. So, it is possible to conclude that gamma-irradiated *S. carpocapsae* may serve as a safe environmentally safe method for *A. ipsilon* management under field conditions.

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Data availability All data and materials are available if requested.

Declarations We confirm that this work is original and has not been published elsewhere and is not currently under consideration for publication elsewhere.

We confirm that the results presented clearly, honestly, and without fabrication, falsification or inappropriate data manipulation. Additionally, no data, text, or theories by others are presented.

Ethics approval and consent to participate Not applicable.

Consent for publication All authors declare that there are no issues relating to journal policies.

Competing interests The authors declare that they have no conflicts of interest.

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