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PLANT PROTECTION
Article

Effects of entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* on the fitness of a Vip3A resistant subpopulation of *Heliothis virescens* (Noctuidae: Lepidoptera)

Asim Gulzar[1,2,*], Tariq Mukhtar[3], Denis John Wright[1]

1. Imperial College London - Silwood Park Campus - Department of Life Sciences - Ascot, UK.
2. Pir Mehr Ali Shah Arid Agriculture University - Department of Entomology - Rawalpindi, Pakistan.
3. Pir Mehr Ali Shah Arid Agriculture University - Department of Plant Pathology - Rawalpindi, Pakistan.

ABSTRACT: The widespread use of transgenic plants imposes selection pressure on insect pest populations to develop insecticide resistance. Evaluation of effectiveness of resistance management strategies is very important in resistance management programs. Resistance management to insecticides is widely believed to depend in part on associated fitness costs. Fitness costs can delay the development of resistance. In the present study, the effects of two entomopathogenic nematode species, *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* were studied on the fitness of first insect population of *Heliothis virescens* selected with Vip3A in the laboratory. It was found that both nematodes species increased the fitness cost of Vip3A selected insects. The mortality of the Unsel subpopulation after exposure to either nematode species was significantly lower than that of the Vip3A-Sel subpopulation. Likewise, the reproduction of both nematode species was significantly greater in cadavers of the Unsel compared with the Vip3A-Sel subpopulation of *H. virescens*. There was positive correlation between nematode reproduction and the larval instar infected with nematodes. The penetration of infective nematode juveniles (IJ) was greater in the Vip3A-Sel subpopulation than in the Unsel subpopulation of *H. virescens*. It is concluded that entomopathogenic nematodes could increase the fitness costs and subsequently delay the resistance.

Key words: fitness costs, entomopathogenic nematodes, *Heliothis virescens*, mortality.

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*Corresponding author:
asim@uaar.edu.pk

INTRODUCTION

Genetically modified plants expressing insecticidal crystal (Cry) proteins derived from *Bacillus thuringiensis* (Bt) have been one of the major successes of applying genetic engineering technologies to agriculture (Gatehouse 2008; Gulzar et al. 2012; Gulzar and Wright 2014). These crops are effective against many lepidopteran pest species and with the introduction of Bollgard I (expressing Cry1Ac), Bollgard II (Cry1Ac and Cry 2Ab) (Monsanto, St Louis, USA) and WideStrike (Cry1Ac and Cry1F) (Dow Agro Sciences, Indianapolis, USA) cotton, key pests such as *Heliothis virescens* and *Pectinophora gossypiella* have been effectively controlled (Greenplate et al. 1999; Jackson et al. 2007; Llewellyn et al. 2007). Although these Bt crops and commercially available Bt spray formulations provided an important alternative to chemical insecticides to control the pests, their widespread use could increase the risk of the development of Bt resistance in insects (Ferré and Van Rie 2002; Gassmann et al. 2009). Lepidopteran species, *Plutella xylostella*, *Helicoverpa zea*, *Spodoptera frugiperda* and *Bussiola fusca*

have developed resistance to Bt Cry toxins in the field, while *Trichoplusia ni* has developed resistance to Cry1Ac in some greenhouse populations (Janmaat and Myers 2005).

Genetically modified crops expressing toxins, which are likely to have no cross-resistance with Cry toxins are therefore of interest. One such group of toxins are the vegetative insecticidal proteins (Vip) produced during vegetative growth of Bt (Estruch et al. 1996; Selvapandiyan et al. 2001), which show broad-spectrum insecticidal activity against many lepidopteran (Vip3A) (Lee et al. 2003; Hernández-Martínez et al. 2013; Chakroun et al. 2016) and coleopteran pest species (Vip1 and Vip2) (Warren 1997). Vip toxins have a different mode of action (Lee et al. 2003) and no sequence homology compared with Cry toxins (Estruch et al. 1996; Lee et al. 2003; Chakroun et al. 2016).

Heliothis virescens is an important, highly polyphagous pest (Neunzig 1969; Sudbrink and Grant 1995; Gulzar and Wright 2015). Its distribution extends through North and South America, with a permanent population between 40° N and 40° S (Neunzig 1969; Fitt 1989; King 1994; McCaffery 1998). It attacks a wide range of important food, fiber, oil crops and is one of the key pests of cotton in America (Fitt 1989; Terán-Vargas et al. 2005; Blanco et al. 2008).The first insect population reported with resistance to a Vip toxin is a subpopulation of *H. virescens* collected from cotton fields in the USA in 2006 and selected with Vip3A in the laboratory (Pickett 2009; Gulzar et al. 2012).

Resistance management to insecticides is widely believed to depend in part on associated fitness costs; with the frequency of resistance alleles within a population declining in the absence of selection pressure (Tabashnik 1994), although some researchers consider that even strong fitness costs have only a minimal impact on the evolution of resistance (Roush 1998). Fitness costs can vary between different ecological and environmental conditions (Carrière and Tabashnik 2001; Bird and Akhrust 2007; Raymond et al. 2005; 2010; Gassmann et al. 2009).

Entomopathogenic nematodes (EPN) of the families *Steinernematidae* and *Heterorhabditidae* are used to control many insect pests (Ehlers 1996; Gouge et al. 1999; Liu and Yue 2000; Fitters et al. 2001; Susurluk et al. 2009; Rahoo et al. 2011; 2017;2018 a; b;2019 a; b; Javed et al. 2019 a; b). Infective nematode juveniles (IJ) may enter in the insect's haemocoel directly via thin parts of the cuticle, through the midgut epithelium via mouth or anus, or through the tracheae via spiracles (Koppenhöfer et al. 2000). Infective nematode juveniles release symbiotic bacteria into the insect haemocoel; the bacteria start to grow and release toxins that kill the host insect, usually within 24 to 48 h (Burnell and Stock 2000).

Entomopathogenic nematodes are being used as biological control agents against different insect pests. In laboratory experiment, *Steinernema riobrave* reduced survival of larvae, pupae and adults of red flour beetle, *Tribolium castaneum* (Ramos-Rodriguez et al. 2007). Larvae and adults of Indian meal moth, *Plodia interpunctella* (Hübner) (Lepidoptera: Pyralidae) were found to be susceptible to different heterorhabditid species in laboratory (Mbata and Shapiro-Ilan, 2005). Studies on the effects of entomopathogenic nematodes and baculoviruses on the fitness of Cry1Ac-selected *P. gossypiella* and *P. xylostella* have shown that both types of entomopathogens increase the fitness cost of resistance (Gassmann et al. 2006; 2009; Raymond et al. 2007; Hannon et al. 2010) and it has been suggested that the use of entomopathogens in Bt resistance management could increase the effectiveness of the use of refuges (Gassmann et al. 2008). In the present studies, fitness costs associated with the response to two entomopathogenic nematodes *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* in Vip3A-Sel insects were investigated.

MATERIALS AND METHODS

Insect culture

Population of *H. virescens* used in the studies was collected from velvetleaf, *Abutilon theophrasti*, on Wildy Farms, Leachville, Mississippi County, Arkansas and designated as WF06. The WF06 population was divided into two subpopulations; one subpopulation (Vip-Sel) was selected with Vip3A toxin (Syngenta, Research Triangle, North Carolina, USA), the other subpopulation was left unselected (Vip-Unsel) (Pickett 2009; Gulzar et al. 2012). At the time of experiments the Vip-Sel resistance ratio was > 200.

Nematodes

Commercially-produced entomopathogenic nematodes, *Steinernema carpocapsae* (Weiser) (Nematoda: Rhaditida) and *Heterorhabditis bacteriophora* (Poinar) (Nematoda: Rhabditida) were obtained from Becker Underwood Ltd (Littlehampton, West Sussex, UK) and stored at 4°C prior to use. The nematodes were used within one week of storage.

Nematode bioassays

Mortality

Nematode bioassays were conducted on 2nd, 3rd, 4th, 5th and 6th instar larvae of Vip3A-Sel and Unsel subpopulations of *H. virescens*. A preliminary experiment was conducted to check the effect of starvation on the insect larvae. The results indicated that there was no mortality for any instar of either subpopulation after 24 and 48 h periods of starvation. Subsequent bioassays were conducted in Petri dishes (5 cm dia.) containing a Whatman No. 1 filter paper (4.5 cm dia.). Four hundred µl of water was applied to the filter paper to moisten it. A single insect larva was put in each Petri dish. Five nematode concentrations, i.e. 0, 10, 20, 50, 100 and 150 IJ in distilled water, were applied directly to the individual larvae by pipette. Insect mortality was noted after 24 and 48 h. Surviving insect larvae were transferred to individual plastic cups (1 oz plastic cups, No. 9051, Bio-Serv, Frenchtown, NJ, USA) containing diet. Larvae that pupated were transferred to new containers and the emergence of adults was recorded. Twelve larvae per treatment were used in bioassays and the bioassay was replicated four times. Experiments were conducted at 25 ± 5°C and 70% ± 5% RH under a 16-h-light/8-h-dark cycle.

Nematode reproduction

Reproduction of nematodes was determined in 2nd, 3rd, 4th, 5th and 6thinstar larvae of both subpopulations using the White Trap method (Kaya and Stock 1997). Five dead larvae per treatment from the above bioassay were placed in individual round plastic cups (250 ml) and kept at 25 °C. After 2 weeks, the number of IJs that emerged from the insect cadavers in the water were counted under a stereomicroscope.

Statistical analyses

All data were analyzed using statistical program R version 2.9.0 (R Development Core Team 2009). Mortality data for both nematode species were corrected for control mortality using Abbott's correction (Abbott 1925) and subjected to analysis of covariance (ANCOVA). Nematode reproduction data for both nematode species were also analyzed by ANCOVA. Penetration data for both nematode species were log transformed and then analyzed by ANOVA.

RESULTS

Effect of entomopathogenic nematodes on mortality of Vip3A-Sel and Unsel subpopulations of *Heliothis virescens*

There was no survival in the Vip3A-Sel or Unsel subpopulations of *H. virescens* for 2nd, 3rd, and 4th instar larvae after 48 h in any of the nematode treatments. The data were therefore excluded from the analysis. For 5th instar larvae, mortality of the Unsel subpopulation after exposure to either nematode species for 48 h was significantly lower than the Vip3A-Sel subpopulation ($p < 0.05$) (Fig. 1). The overall mortality of both subpopulations increased with increasing nematode

concentration ($p < 0.001$). The overall mortality caused by *S. carpocapsae* was significantly higher than that by *H. bacteriophora* in both Unsel and Vip3A-Sel subpopulations of *H. virescens* ($p < 0.001$).

For 5th instar larvae, mortality of the Unsel subpopulation after exposure to either nematode species at eclosion was significantly lower than the Vip3A-Sel subpopulation ($p < 0.05$) as shown in Fig. 2.

Similarly, with the 6th instar larvae of *H. virescens*, mortality in the Unsel subpopulation at 48 h and eclosion following exposure to either nematode species was significantly lower than in the Vip3A-sel subpopulation ($p < 0.05$) and have been shown in Figs. 3 and 4. Mortality for both subpopulations and nematode species showed a general trend to increase with increasing nematode concentration ($p < 0.001$).

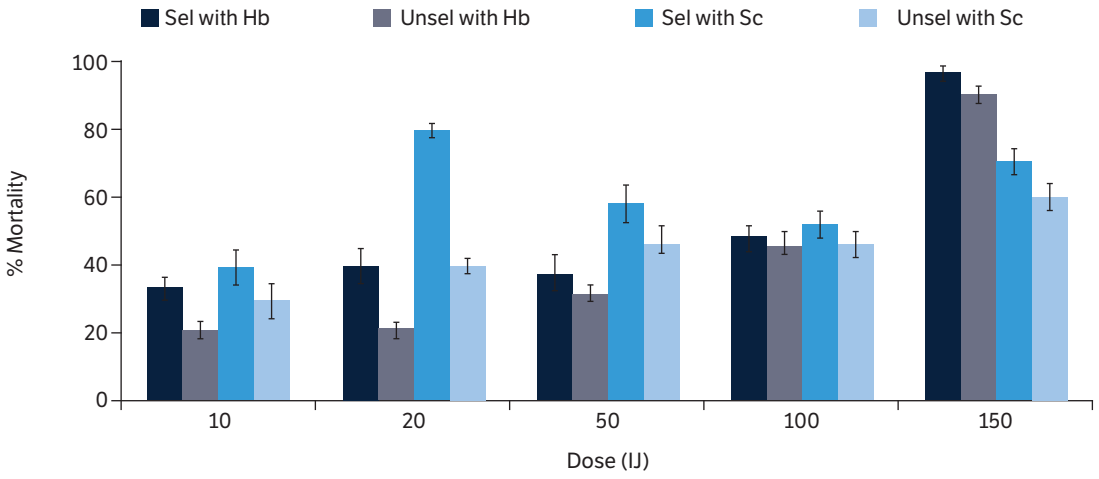

Figure 1. Mortality (% ± SE) of 5th instar larvae of Vip3A-Sel and Unsel subpopulations of *Heliothis virescens* at different concentrations of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* infective juveniles after 48h.

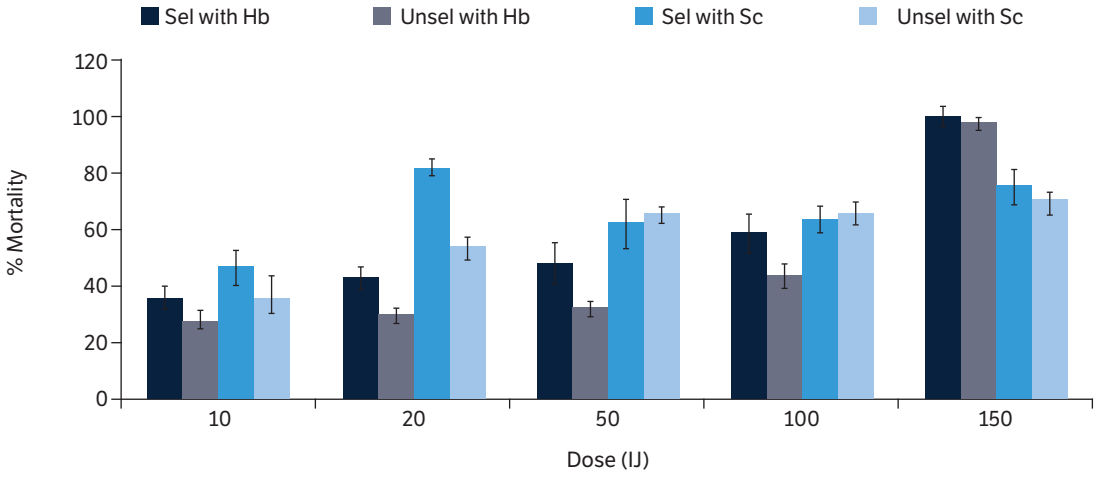

Figure 2. Mortality (% ± SE) at eclosion following exposure of 5th instar larvae of Vip3A-Sel and Unsel subpopulations of *Helithis virescens* to different concentrations of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* infective juveniles.

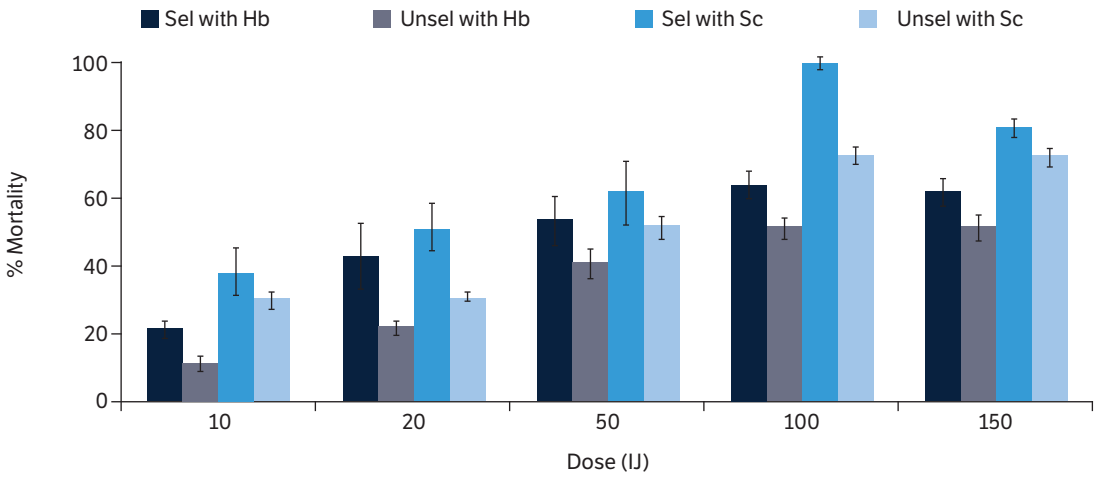

Figure 3. Mortality (%) ± SE at 48 h of 6th instar larvae of Vip3A-sel and Unsel subpopulations of *Heliothis virescens* at different concentrations of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* infective juveniles.

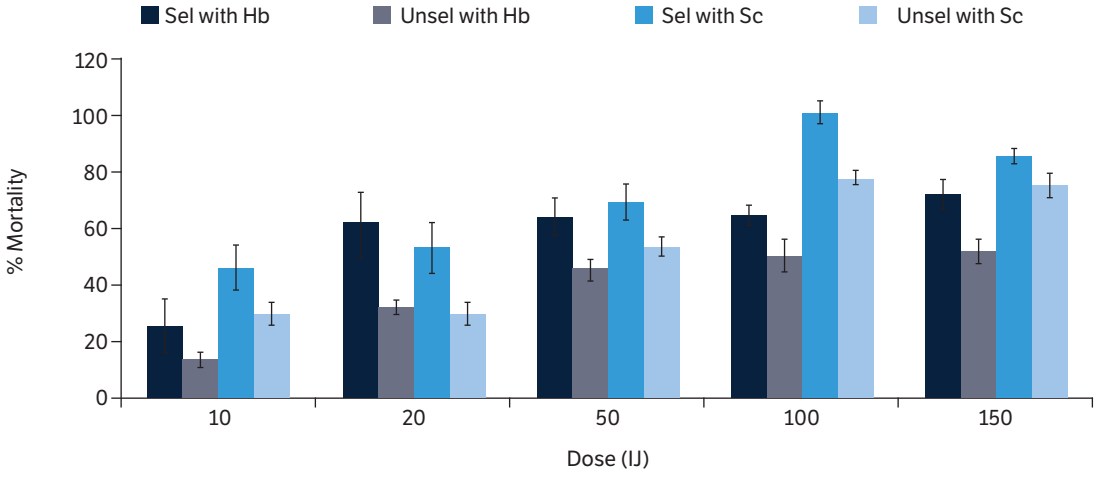

Figure 4. Mortality (%) ± SE at eclosion of 6th instar larvae of Vip3A-Sel and Unsel subpopulations of *Heliothis virescens* at different concentrations of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* infective juveniles.

Nematode reproduction

The overall reproduction of *S. carpocapsae* and *H. bacteriophora* was significantly greater in cadavers of the Unsel compared with the Vip3A-Sel subpopulation of *H. virescens* for all larval instars ($p < 0.001$). The individual productions of IJs in each instar at different concentrations have been shown in Fig. 5 (a) to (e). There was a positive correlation between nematode reproduction and the larval instar infected with nematodes ($p < 0.001$).

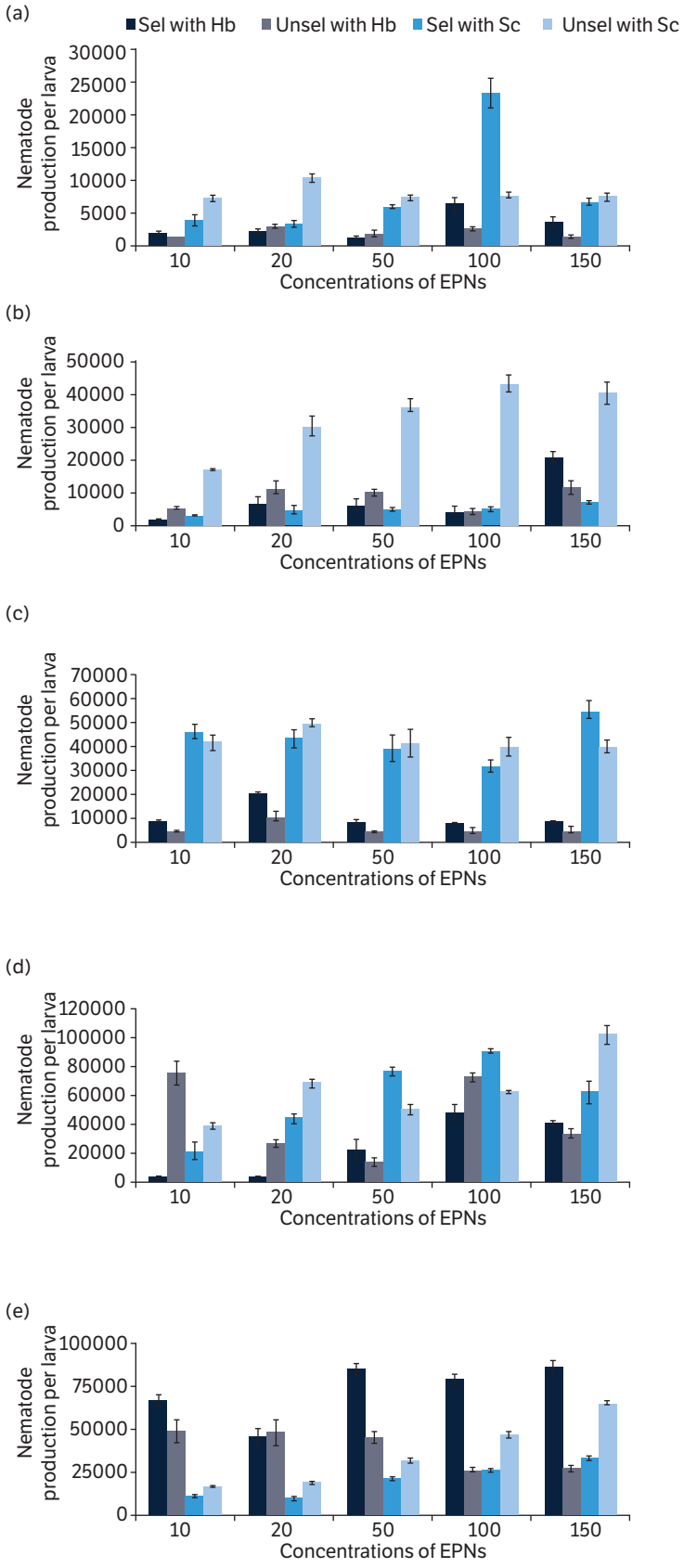

Figure 5. Reproduction ± SE of *Steinernema carpocapsae* and *Heterorhabditis bacteriophora* in cadavers of 2nd (a), 3rd (b), 4th (c), 5th (d) and 6th (e) instar larvae of Vip3A-Sel and Unsel subpopulations of *Heliothis virescens* at different nematode doses.

DISCUSSION

The present study showed that 5th and 6th instar larvae of the Vip3A-Sel subpopulation of *H. virescens* were significantly more susceptible to both *S. carpocapsae* and *H. bacteriophora* compared with the Unsel subpopulation. These results, which suggest a fitness cost, are in accordance with previous studies, where *S. carpocapsae* marginally increased the fitness costs of Bt resistance in *P. xylostella* (Baur et al.1998) and *S. riobrave* and *H. bacteriophora* increased the fitness costs of Cryl1Ac resistance in *P. gossypiella* (Gassmann et al. 2006; 2009).Raymond et al. (2007) have reported that baculoviruses (NPV) also increased the fitness costs of a Cryl1Ac resistant population of *P. xylostella*. Lopez et al. (2010) also reported the similar findings that *Nosema pyrausta* delayed larval development of partially and fully Cryl1Ab resistant *Ostrinia nubilalis*. Similarly, the nematode *S. riobrave* increased the fitness costs of resistance to Bt toxin Cry1Ac in pink bollworm (Hannon et al. 2010).

The mechanisms by which the entomopathogenic nematodes cause greater mortality in Bt resistant insect populations remain unclear. The studies have shown that CO_2 is involved in the long-distance attraction of plant parasitic and entomopathogenic nematodes (Klingler 1965; Lewis et al. 1993; Robinson 1995; Susurluk et al. 2009). One hypothesis is that the Bt toxin makes insects more debilitated and subsequently more susceptible to entomopathogens and these debilitated insects respire more and release more CO_2; ultimately the selected population attract more EPNs. The other hypothesis is that Bt resistant insects show a reduced capacity to fend off pathogen infections compared with susceptible insects (Gassmann et al. 2006). For example, organophosphate resistant mosquitoes have been reported to have higher levels of Wolbachia infections than susceptible mosquitoes and to show a clear interaction between the presence of resistance alleles and Wolbachia load (Berticat et al. 2002).

Stress can reduce the general immunocompetence in insects against natural enemies. The major immune defense against endoparasitoids are encapsulation and melanization (Karimzadeh and Wright 2008). Insects may fend off infection from nematodes through encapsulation or melanization of nematode infective juveniles before they release bacteria (Li et al. 2007). Melanization occurs by the action of the prophenoloxidase pathway and difference in phenoloxidase activity could contribute to differences in susceptibility to pathogens between Bt resistant and susceptible insects (Gassmann et al. 2009). *Bacillus thuringiensis* resistance has been associated with higher phenoloxidase activity in *Ephestia kuehniella* and *Helicoverpa armigera* (Rahman et al. 2004; Ma et al. 2005), but it is not known whether these insects are susceptible to the entomopathogenic nematodes (Gassmann et al. 2009).

The present results indicate that *S. carpocapsae* imposed greater fitness costs compared with *H. bacteriophora* by causing greater mortality in the Vip3A resistant than in the susceptible population of *H. virescens*. This difference could be due to differences in the nematodes` behavior (Gaugler and Campbell 1993; Lewis et al. 1993; Boff et al. 2001; Susurluk et al. 2009) or to the different bacterial symbionts found in *Steinernema* (*Xenorhabdus* spp.) and *Heterorhabitis* (*Photorhabdus* spp.) species (Kaya and Gaugler 1993). Similarly, in the present study, penetration of IJs of both nematode species was greater in the Vip3A-Sel subpopulation than in the Unsel subpopulation of *H. virescens.* While the reason for this is unknown, it could be due to differences in insect behavior in the Petri bioassay. The greater number of IJ in Vip3A-Sel insects is the simplest explanation for the greater mortality in resistant insects rather than a reduced immune response. Further studies are required to determine whether Vip3A-resistant insects are more susceptible to entomopathogenic nematodes under conditions more relevant to the field.

CONCLUSION

The significant findings of the present study showed that *Steinernema carpocapsae* imposed greater fitness costs compared with *Heterorhabditis bacteriophora* by allowing greater penetration of infective juveniles and causing greater mortality in the Vip3A-Sel subpopulation than in the Unsel subpopulation of *Heliothis virescens*. It is therefore, concluded that entomopathogenic nematodes could increase the fitness costs and subsequently delay the resistance.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR'S CONTRIBUTION

Conceptualization, Gulzar A. and Wright D. J.; Methodology, Gulzar A. and Mukhtar T.; Investigation, Gulzar A. and Mukhtar T; Writing – Original Draft, Gulzar A.; Writing – Review and Editing, Wright D. J. and Mukhtar T.; Funding Acquisition, Gulzar A.; Resources, Gulzar A. and Wright D. J.; Supervision, Wright D. J.

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