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Technological aspects of *Steinernema carpocapsae* spray application alone or mixed with *Bacillus thuringiensis aizawai* in spinach crop

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Abstract

Foliar application of entomopathogenic nematodes (EPN) is generally carried out with traditional boom sprayers. However there is a possibility that this operation may cause physical stress to nematodes due to the action of the mechanical components. Our aim was to evaluate the effects of a spray application, with a conventional hydraulic sprayer, on the viability of *Steinernema carpocapsae* (Weiser) in spinach crop. Laboratory tests preceded foliar application in the field to exclude possible effects on the nematodes due to the spray equipment. In a field trial, applications of EPN alone and in a mixture with *Bacillus thuringiensis* Berliner (Bt) subsp. *aizawai* were carried out evaluating their level of control against noctuid moths in spinach. The biopesticides action was also compared with conventional chemical control. In addition, the interaction from the combination of Bt and EPN was investigated. Results showed that a static pressure up to 14 bar causes no significant damage to *S. carpocapsae* and that the passage of the nematode through the flat fan nozzles do not affect their viability. Repeated recirculation of nematodes by hydroejector did not affect their viability even at high recirculation level (36.9 l/min). In the field trial, treatment with EPN alone and with the mixture EPN+Bt showed no significant differences with respect to the control. Nevertheless, in general these treatments were not significantly different from chemical treatment. The combined application of EPN and Bt showed an additive interaction compared to EPN alone.

Key words: application technology, biocontrol agents, entomopathogenic nematodes, *Steinernema carpocapsae*, *Bacillus thuringiensis*, joint action.

Introduction

Biopesticides are living organisms (plants, microscopic animals such as nematodes, and microorganisms, including bacteria, viruses, and fungi) or natural products derived from these organisms, that are used to suppress pest populations (Thakore, 2006). These biological agents need precautions during their application to avoid a reduction in their efficacy (Gan-Mor and Matthews, 2003). No equipment specially adapted for spray application of biopesticides is available on the market (Gan-Mor and Matthews, 2003; Brusselman *et al*., 2008). Application is therefore usually done with existing spray technologies but its efficacy could be affected by equipment characteristics and operating conditions (Fife *et al*., 2003; Shapiro-Ilan *et al*., 2006).

Spray application of entomopathogenic nematodes (EPN), utilizing the sprayers commonly employed for chemical pesticides, can induce a variety of physical stresses on the organisms due to variations in pressure inside the spraying machine and while passing through the pump and nozzles (Nilsson and Gripwall, 1999; Brusselman *et al*., 2010a). Further physical stress may be caused by the effect of agitation inside the tank (Nilsson and Gripwall, 1999; Łaczyński *et al*., 2004) and by the rise in temperature produced by the recirculation system (Łaczyński *et al*., 2006; 2007; Brusselman *et al*., 2010a). However, studies on the reduction in viability of the nematodes following mechanical application do not provide uniform results. Fife *et al*. (2003; 2004) reported that a single passage through different types of pump, at operating pressures up to 828 kPa, did not influence the viability of *Heterorhabditis bacteriophora* Poinar, *Heterorhabditis megidis* Poinar Jackson et Klein and *Steinernema carpocapsae* (Weiser). Nilsson and Gripwall (1999) found no significant influence on *Steinernema feltiae* (Filipjev) viability with a high-pressure sprayer. However, they also noted a reduced viability of the nematodes in all the high-pressure treatments and a significant decrease in viability, increasing the pumping duration. Grewal (2002) reported the negative effect of excessive hydraulic agitation on the viability of nematodes and Łaczyński *et al*. (2004) indicated a linear decrease in the viability of *H. bacteriophora* with respect to the duration of the hydraulic agitation. Brusselman *et al*. (2010a) comparing pneumatic, mechanical and hydraulic agitation, found that only the hydraulic agitation, using a centrifugal pump, caused a significant reduction in both viability and infectivity of nematodes. Brusselman *et al*. (2010a) attributed this effect to temperature rise, due to recirculation, and not to a direct mechanical effect. The type of nozzle, flow rates and pressure were also important factors in nematode delivery, the type of nozzle (fan nozzle, full cone and spinning disc) influences the mean number of *S. carpocapsae* infective juveniles (IJs) deposited (Lello *et al*., 1996). They showed that the full cone and flat fan nozzle, if used at low pressure and with high flow rate, increase the number of larger sized droplets that enable a higher number of nematodes to be carried. However spraying large droplets also confers the danger of causing more bouncing and rolling of droplets from the leaves, and thus of getting less EPN deposition (Beck *et al*., 2013). Brusselman *et al*. (2010b) found that the volumetric distribution pattern of EPN is influenced

by nozzle type and concluded that further research is needed to evaluate if the differences in coverage due to nozzle type will result in significant differences in pest control.

The efficacy of EPN can be boosted by the addition of surfactants to increase leaf coverage (Williams and Walters, 2000; Head *et al*., 2004; Schroer and Ehlers, 2005; Schroer *et al*., 2005a).

A possible interaction from the combination of Bt and EPN has been found (Koppenhöfer and Grewal, 2005). Two control agents applied together might act independently of one another against a given pest, and their effects would be additive. This type of response will be observed if the action sites of the two components differ, i.e. if each one has a completely different mode of action and these modes of action are totally independent. They also might interact synergistically or antagonistically, thus rendering the combination more or less effective in control than in the case of an additive effect (Robertson and Preisler, 1992). Koppenhöfer and Kaya (1997), for example, have demonstrated an additive or synergistic interaction between *B. thuringiensis* subsp. *japonensis* (Btj) and *H. bacteriophora* or *Steinernema glaseri* (Steiner) on white grubs, *Cyclocephala hirta* LeConte and *Cyclocephala pasadenae* Casey (Coleoptera Scarabaeidae). Salem *et al*. (2007) found that the combination of *S. carpocapsae* All and *B. thuringiensis* subsp. *aizawai* against 2nd and 5th instar larvae of *Spodoptera littoralis* (Boisduval) (Lepidoptera Noctuidae) exhibit an additive interaction.

In Europe, Italy is the largest producer of spinach (FAO, 2009). Among the major insect pests in this crop, noctuid moths are considered the most important (Lanzoni and Burgio, 2010). The protection of processing spinach presents many problems related to the very low economic threshold. Indeed the commercial damage is caused by the presence of larvae, which can make the product unmarketable (Lanzoni *et al*., 2012). This characteristic requires many treatments with conventional insecticides that may lead to adverse effects, such as the selection of resistant insect populations or the presence of residues at harvest exceeding the legal limits. New biopesticides are needed for pest control in spinach due to the reduction of the number of pesticide formulations available on the market and to the emergence of resistance to the active ingredients in these formulations. Unlike applications with conventional pesticides, application with biopesticides is not subject to a mandatory interval between treatment and harvest, which gives biopesticides a distinct advantage over conventional pesticides (Bailey *et al*., 2010). The use of EPN may therefore represent an effective solution for the control of noctuid moths in this crop. In Italy spinach is cultivated mainly on small farms (in field of an average surface of 10 ha) where, to contain costs, it is necessary to use conventional spraying equipment that is economical and versatile and able to deliver both chemicals and living organisms. Also, alternative equipment such as row application and localization systems (Brusselman *et al*., 2012) seems inappropriate in relation to the spinach crop configuration.

This paper reports the results of mechanical application of the nematode *S. carpocapsae* with conventional hydraulic equipment for the control of noctuid moths in processing spinach in the open field. In order to exclude a possible reduction of viability of the nematode due to physical stress induced by the passage through the equipment, laboratory tests preceded foliar application on the field. The passage through the nozzles, effects of recirculation and effect of pressure were considered as main causes of physical stress. These three mechanical aspects were investigated separately, simulating the conditions which nematodes are subjected to during field application. The efficacy of *S. carpocapsae* was also evaluated, either alone or in a mixture with *B. thuringiensis* (Bt) with the aim of verifying a possible additive, synergistic or antagonistic effect.

Materials and methods

Laboratory trials

A commercial formulation of *S. carpocapsae* was used, i.e. NemoPAK-SC® (Bioplanet, Italy), containing 50 million IJs in an inert carrier. Prior to each trial 2 g of the product was suspended in 1 l of water (22 °C). Then, 0.25 l of this initial suspension was further diluted in 5 l of water to obtain a final concentration of approximately 62,500 IJ/l.

Static pressure

The effect of static pressure, comparable to the pressure to which the organism is subjected during traditional application, was tested using a test bench composed of a 150 ml capacity metal container connected to a manually operated hydraulic jack. During this trial, nematode viability was evaluated after exposure to different pressure levels (0, 2, 8 and 14 bar), lasting 15 s. The effect of the intermediate pressure (8 bar) was tested after 5, 15, 25 and 35 s. After each pressure exposure, a sample of the suspension was taken from a valve situated at the lower part of the compression chamber. The experiment was repeated three times each with a different nematodes suspension. At the end of each replication the test bench was washed by passing water through the metal container. The relative viability in the samples was observed as described in subsection "sample collection and nematode counting".

Passage through the nozzles

A test bench was set up in the laboratory to examine the effect of passage through different sized Teejet XR flat fan nozzles on the viability of the nematodes (table 1). To avoid damage to the nematodes before passing through the nozzles, a flexible impeller pump (Liverani 131 mod. INV MIDEX 3/4) was used. It was assumed that this type of pump would not damage the nematodes due to absence of pressure peaks in the delivered flow. The electric motor of the pump was equipped with a frequency converter that enabled a continuous regulation of the rotational speed (180-1400 revs/min). The nematode suspension was aspirated from a manually agitated spray tank (140 l) and transmitted to a spray boom equipped with one nozzle. Just before spraying, a

Table 1. Characteristics and operational parameters of the test bench used to evaluate the effects of passage through different nozzles.

Nozzles Type	BCPC code	Flow rate (l/min)	Pressure (bar)	Pump speed (revs/min)
Control		0	0	0
XR11008	white	3.16	3	1400
XR11004	red	1.58	3	1150
XR11001	orange	0.39	3	900
Pump only		88	0	1400

Table 2. Recirculation time and tank content in the agitation-return circuit during the emptying cycle in the laboratory spray application trial.

Treatment[a]	Tank content (litres)	Recirculation time (seconds)
1 (control, full tank)	300	0
2	225	407
3	150	814
4	25	1491

[a] Treatments correspond to different volumes in the spray tank.

control sample was taken directly from the central part of the spray tank. A sample was also taken at the output of the pump in order to point out any difference in viability between the effect of the passage through pump and nozzle, and the passage only through the pump. The experiment was replicated three times each with a different nematodes suspension.

Laboratory spray application

A test bench was set up in the laboratory to check the combined effect of recirculation in the spray tank and the delivery through the nozzles on the viability of the nematodes. The spray system was composed of a 300-litre spray tank equipped with a hydraulic agitation system, a pressure regulator and a spray boom with 7 flat fan nozzles (XR11004). A low-pressure piston-diaphragm pump (Comet BP 75) was used for spraying and agitation. Agitation was created by returning a part of the spray liquid back into the spray tank through a hydro-ejector. The filter installed between the pump and the spray tank was removed.

Two agitation levels (L1 and L2) were considered, obtained with pump speeds of 252 and 380 revs/min that generated a flow rate of 23.9 and 36.9 l/min respectively.

Samples were taken at the outlet of the nozzles at successive intervals corresponding to decreasing volumes in the spray tank (table 2). As a control, a sample was taken directly from the spray tank. The temperature of the suspension in the spray tank was measured at each sampling time. The spray pressure was 3.0 ± 0.1 bar throughout the time of delivery. The experiment was replicated three times each with a different nematodes suspension.

Sample collection and nematode counting

In each test and for each treatment, a sample of 100 ml was taken for analysis. After each trial, until EPN counting, samples were stored in plastic test tubes in the dark at a temperature of 14-16 °C for a maximum of 18-20 hours to limit the reduction in nematode survival following conservation (Molyneux, 1985).

Approximately 30 minutes before the count, three sub-samples of nematodes (each 1 ml) were extracted from each of the 100 ml sample using a calibrated pipette and left at ambient temperature in the dark to encourage mobility and facilitate the counting of the individuals (Łaczyński *et al*., 2006). The sub-samples, diluted with 3 ml of distilled water, were placed in Petri dishes with a grid base, and the nematodes were counted using a binocular microscope. Only whole nematodes were counted; fragments were not considered as they had been present in the commercial formulation probably due to the production process and packing. Nematodes were considered dead if they did not respond to prodding (Grewal, 2002). Relative nematode viability V_r was calculated as the percentage of living nematodes.

Field trial

A field trial was conducted in September and October 2010 in autumnal spinach in Forlì-Cesena province (44°12'19"N 12°15'01"E 12 m a.s.l.) northern Italy. The aim of this trial was to confirm the results of the laboratory trials and to check for possible additive or synergistic interaction between EPN and *B. thuringiensis* in a combined treatment. The same commercial formulation of *S. carpocapsae* as that utilized in the laboratory experiments and *B. thuringiensis* subsp. *aizawai* as the commercially available formulation XenTari® (Sumitomo Chemical Italia, Italy) were used.

A 0.32 ha experimental area was delimited in a commercial 5-ha spinach field sown on 27 August 2010. After emergence, plots (8 × 20 m) were assigned to the following treatments: 1- entomopathogenic nematodes (EPN); 2- entomopathogenic nematodes combined with *B. thuringiensis* (EPN + Bt); 3- *B. thuringiensis* (Bt); 4- chemical insecticides; 5- untreated control (table 3). Treatments 1 and 2 were repeated twice, with a time span of 14 days. Treatment 3 was repeated weekly, while the insecticide application (treatment 4) was performed in the presence of noctuid larvae. Each treatment had four replicates in a randomized block design. The spray application was performed with a conventional boom sprayer, of the same type as the one used for the recirculation trials in the laboratory. The filter between the tank and the pump was removed. The spray boom was equipped with 16 ISO 06 flat fan nozzles at a pressure of 2 bar and with an ejector flow-rate of 23.9 l/min (agitation level L_1). All treatments were applied after 4:00 p.m. to minimize the influence from UV light. To favour the maintenance of an adequate moisture level on the leaf, *S. carpocapsae* and the nematode-Bt mixture were applied at an application rate of 1,650 l/ha. The chemicals and Bt were applied at 625 l/ha. The different application rates were achieved by changing the spray boom speed (table 3).

Table 3. Application parameters of treatments in the field trial using traditional boom sprayer.

Active ingredient	Trade name	Application rate (l/ha)	Flow rate (l/min)	Forward speed (m/s)	Pressure (bar)	Dose	Number of applications	Date
S. carpocapsae	NemoPAK-SC®	1650	1.93	0.39	2	30 IJs/cm^2	2	23/09-07/10
S. carpocapsae + *B. t. aizawai*	NemoPAK-SC® + XenTari®	1650	1.93	0.39	2	30 IJs/cm^2 1 kg/ha	2	23/09-07/10
B. t. aizawai	XenTari®	625	1.93	1.03	2	1 kg/ha 1.5 kg/ha	2 2	23/09-30/09 07/10-14/10
Delthametrin Indoxacarb Delthametrin	Decis® Steward® Decis®	625	1.93	1.03	2	0.5 kg/ha 0.15 kg/ha 0.5 kg/ha	1 1 1	22/09 07/10 09/10

To evaluate nematode viability and concentration during spraying, samples were taken from the spray tank immediately after suspension preparation and at the outlet of the nozzles during spray application. The latter were collected at successive time intervals corresponding to each of the four replicates of the treatments with nematodes. Each treatment was sampled three times for replication purposes. The relative viability in the samples was then measured according to the trial protocol. Noctuid larval populations were sampled within each treatment plot using a hand-held vacuum suction device (modified, reversed Stihl BG75 leaf blower), without damaging spinach plants. Each vacuum sample consisted of 25 one-second suctions taken while moving around a 2 × 2 m spinach area. The central part of each plot was sampled at a rate of 4 suction areas randomly selected, 4 and 13 days after each nematodes application (table 3). Collected larvae were returned to laboratory, stored in Plexiglas cylindrical cages (Ø 9 cm, h 11 cm) and reared on an artificial diet in a climatic chamber at 25 ± 1 °C, 80 ± 10% RH, and L:D 16:8 photoperiod, for 4 days. Afterwards each larva from EPN and EPN + Bt treatments was dissected to assess nematode penetration.

Data analysis

The STATISTICA software for Windows (StatSoft, 2011) was used for all analyses. *S. carpocapsae* concentration and percentage viability (arcsine transformed) data were submitted to analysis of variance. Differences between treatment means were estimated by Fisher's LSD test ($P < 0.05$). The comparison of noctuid larval infestation between treatments in the field experiment was performed using Kruskal-Wallis test ($P < 0.05$).

Analysis for additive, antagonistic, or synergistic interaction between *S. carpocapsae* and *B. thuringiensis* was based on a binomial test and the observed and expected population reduction as a result of spraying were compared as adapted from Robertson and Preisler (1992) and Negrisoli *et al*. (2010). Expected population reduction was obtained using the formula $P_e = P_o + (1 - P_o)(P_1) + (1 - P_o)(1 - P_1)(P_2)$, where P_e is the expected population reduction for the combination of EPN and Bt, P_o is the natural population reduction or increase in the control treatment, P_1 is population reduction after treatment with EPN alone, and P_2 is population reduction after treatment with only Bt. Population reduction has been calculated as the difference between the mean number of moth larvae sampled on the first (28/09/2010) and last (20/10/2010) sampling. Other causes might have affected the mortality in the field but we have considered that they act uniformly among treatments. The chi-square value was calculated using the formula $\chi^2 = (L_o - L_e)/L_e + (D_o - D_e)/D_e$, where L_o is the number of living larvae sampled (observed), L_e is the number of larvae expected, D_o is the observed magnitude of population reduction and D_e is the expected magnitude of population reduction. The χ^2 was used to test the hypothesis of independence with one degree of freedom and P = 0.05. The correspondent critical χ^2 value is 3.84 (Zar, 1999). Additivity was characterized by $\chi^2 < 3.84$, antagonism by $\chi^2 > 3.84$ and $P_c < P_e$, where P_c is the observed population reduction of the EPN and Bt combination and P_e the expected population reduction of the combination, and synergism by $\chi^2 > 3.84$ and $P_c > P_e$.

Results

Effect of the static pressure

No significant effect of static pressure was observed on the relative mean viability (table 4). The mean values of relative viability ranged between 58.1% for the sample subjected to a pressure level of 8 bar for 5 seconds and 67.8% for the control.

Table 4. Effect of four static pressure and different time of pressure exposure on the relative mean viability (V_r) of the organisms in the laboratory.

Pressure (bar)	Time (s)	V_r (% ± SE)
0	0	67.8 ± 5.2
2	15	59.9 ± 5.9
8	5	58.1 ± 3.7
8	15	58.9 ± 4.6
8	25	64.1 ± 5.8
8	35	61.0 ± 5.6
14	15	64.0 ± 5.0
		$F_{(6, 54)} = 0.96$; $P = 0.463$

Table 5. Suspension temperature and nematode viability and concentration for the two levels of agitation-return, L_1 (23.9 l/min) and L_2 (36.9 l/min), in the laboratory spray application trial.

Treatment[a]	Tank content (l)	Temperature (°C) (Mean ± SE)		V_r (%) (Mean ± SE)		Nematodes concentration (IJs/ml) (Mean ± SE)	
		Agitation level L_1	Agitation level L_2	Agitation level L_1	Agitation level L_2	Agitation level L_1	Agitation level L_2
1 (control)	300	17.3 ± 0.9	16.8 ± 0.7	89.2 ± 2.5	94.5 ± 1.0	64.2 ± 4.6	53.6 ± 4.5
2	225	17.4 ± 0.9	17.0 ± 0.8	88.4 ± 1.9	91.1 ± 1.2	68.6 ± 7.4	55.7 ± 4.1
3	150	17.6 ± 0.8	17.7 ± 0.6	85.1 ± 2.7	92.4 ± 1.2	65.3 ± 5.8	55.7 ± 11.2
4	25	18.6 ± 0.8	19.9 ± 0.6	87.0 ± 2.0	91.2 ± 2.2	77.1 ± 11.7	64.0 ± 6.6
				$F_{(3, 30)} = 1.39$; $P = 0.266$	$F_{(3, 30)} = 0.78$; $P = 0.516$	$F_{(3, 30)} = 1.39$; $P = 0.266$	$F_{(3, 30)} = 0.44$; $P = 0.729$

[a] Treatments correspond to different volumes in the spray tank.

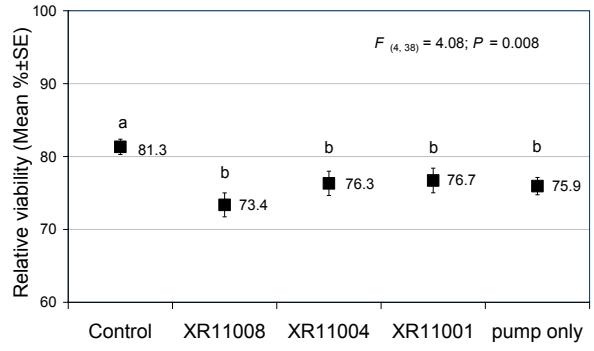

Figure 1. Viability of *S. carpocapsae* after spraying with different kind of nozzles. Means followed by the same letter are not significantly different (LSD test; P > 0.05).

Stress caused by passing through the nozzles

Nozzle size did not affect the relative viability of the nematodes (figure 1). Relative viability in all samples after treatment, even the passage through the pump at free discharge, was significantly lower than the viability of the nematodes in the control sample.

Laboratory spray application

The temperature of the suspension inside the tank increased by 1.3 and 3.1 °C after about 25 minutes of agitation for the L_1 and L_2 levels respectively (table 5). Neither nematode viability nor concentration were affected by the spray application at both agitation levels (table 5).

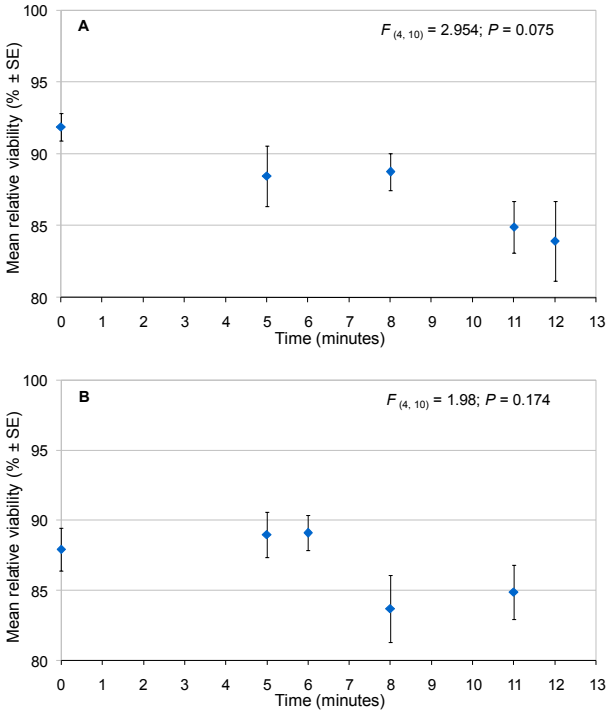

Figure 2. Viability (% ± SE) of *S. carpocapsae* measured inside the sprayer tank (time 0) in relation to application time in the treatments EPN (A) and EPN + Bt (B).

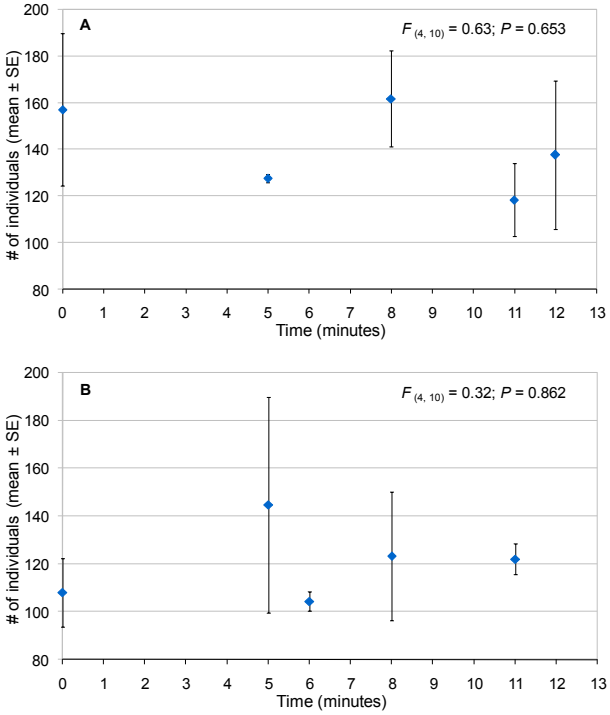

Figure 3. Concentration of infective juveniles of *S. carpocapsae* (IJs/0.1 ml) measured inside the sprayer tank (time 0) in relation to application time in the treatments EPN (A) and EPN + Bt (B).

Field trial

The survival of the nematodes was not affected by the spray application in either of the treatments EPN or EPN + Bt (figure 2). The concentration of nematodes also remained constant in relation to time (figure 3).

The population of noctuid larvae sampled was composed of *Spodoptera exigua* (Hubner) (51.0% of the total larvae sampled), *Autographa gamma* (L.) (38.9%) and *Chrysodeixis chalcites* (Esper), *Lacanobia oleracea* (L.), *Mamestra brassicae* (L.), *Helicoverpa armigera* (Hubner) (2.8% in total). The other 7.3% was represented by a species belonging to the Arctiidae family (Lepidoptera).

The number of larvae in the samples after EPN and EPN + Bt treatments was not different from the number of larvae in the control. Besides, with the exception of one larva sampled 4 days after the second nematode spraying in the EPN + Bt treatment, none of the noctuid larvae sampled were found to be infected with nematodes. Nevertheless, the treatments with EPN alone and with EPN + Bt showed, from the middle of the crop cycle to the harvest, a tendency to a reduction in the number of noctuid larvae (figure 4). Moreover the number of larvae sampled in these treatments was not significantly different from that sampled on chemical plots. Finally, the number of larvae sampled in the chemical treatment, excluding the first sampling date, was always significantly lower than the control.

When EPN were applied in association with Bt, an additive interaction was observed either considering all the larvae sampled ($\chi^2 = 0.09$) or only *S. exigua* ($\chi^2 = 0.04$) or *A. gamma* ($\chi^2 = 2.63$) (table 6). However this did not translate into a significant decrease in the number of larvae sampled respect to the treatment with only EPN (figure 4).

Discussion

The results of the trials demonstrate that a static pressure up to 14 bar, even protracted for 35 seconds, causes no significant damage to *S. carpocapsae*. These findings are consistent with other work (Fife *et al*., 2003) showing that the viability of exposed EPN remained above 85% at pressure less than or equal to 20 bar for *S. carpocapsae* and *H. bacteriophora* and 14 bar for *H. megidis*. Fife *et al*. (2003) tested much higher pressures than this to simulate extreme spraying conditions, whereas in our trials it was attempted to reproduce more realistic working conditions.

Results show that the flat fan nozzles do not affect *S. carpocapsae* viability during application. This is consistent with what was found by Łaczyński *et al*. (2006) for *H. bacteriophora.* The use of flat fan nozzles was chosen

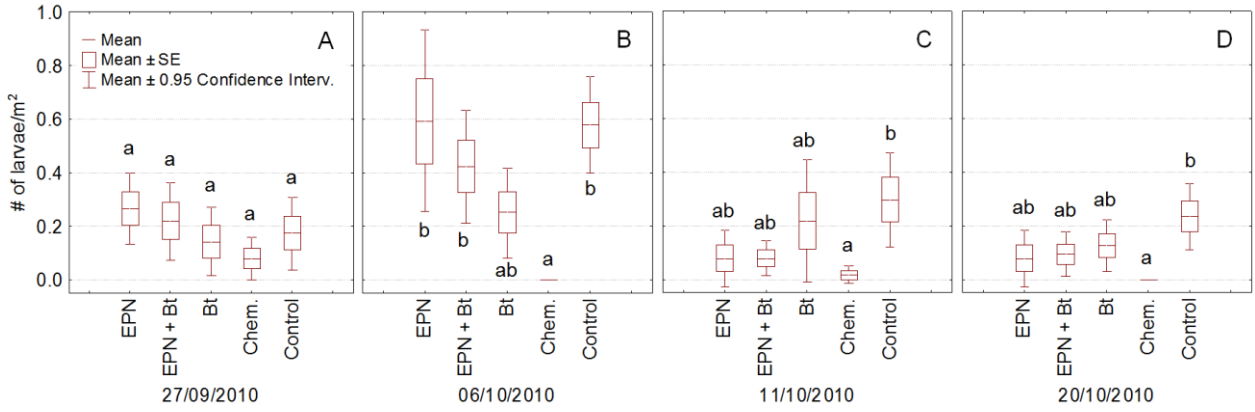

Figure 4. Number of noctuid larvae sampled in the field trial (A) 4 days and (B) 13 days after the first application of *S. carpocapsae* alone (EPN) or in association with *B. thuringiensis aizawai* (EPN+Bt), and (C) 4 days and (D) 13 days after the second application. Bt: *B. thuringiensis aizawai*; Chem.: delthametrin, indoxacarb; Control: untreated control. On each sampling date, different letters show significant differences among treatments (Kruskal-Wallis test, $P < 0.05$).

Table 6. Population reduction of noctuid larvae in the field after exposure to combined *S. carpocapsae* and *B. thuringiensis aizawai*.

Target	Population reduction (%)		χ^2	Interaction between treatments[b]
	Observed	Expected[a]		
All larvae	57.1	64.3	0.09	Additive
Spodoptera exigua	50.0	57.1	0.04	Additive
Autographa gamma	62.5	90.5	2.63	Additive

[a] Expected population reduction $P_e = P_o + (1\text{-}P_o)(P_1) + (1\text{-}P_o)(1\text{-}P_1)(P_2)$; P_e expected population reduction for the combination of EPN and Bt; P_o natural reduction or increase in the control treatment; P_1 population reduction after treatment with EPN alone; P_2 population reduction after treatment with only Bt.

[b] Additivity: $\chi^2 < 3.84$; antagonism: $\chi^2 > 3.84$ and $P_c < P_e$, P_c observed population reduction of the EPN and Bt combination, P_e expected population reduction of the combination; synergism: $\chi^2 > 3.84$ and $P_c > P_e$.

because they have the advantage of operating at very low pressures, producing a coarse spray that evaporates slowly and therefore avoids a rapid desiccation of the organisms. Indeed, nowadays the top manufacturers recommend pressures no higher than 4 bar for this type of nozzle, to obtain the best compromise between uniformity of distribution and droplet diameter. To isolate the effect of the nematodes passing through the nozzle from the other variables a flexible impeller pump was used. The choice of this pump was initially suggested based upon the hypothesis that the other alternative or centrifugal pumps would produce high peaks of pressure or intense shear effect. Instead, the results appear to demonstrate that the slight difference in the viability is to be ascribed more to the pump, than to the nozzle size.

In our trials no suspension temperature increase due to recirculation was observed. The high temperature increases reported by Grewal (2002), Fife (2003) and Brusselman *et al*. (2010a) which are likely to affect the viability of the nematodes, are mainly due to the use of centrifugal pumps and lengthy remixing times.

The tests on recirculation carried out with the traditional sprayer both in the laboratory and in the field, showed that repeated passages of the nematodes in the hydraulic system did not affect their viability even at the end of an application when a low amount of spray liquid remained in the tank. Actually the differences were never significant, even varying the intensity of the agitation. The concentration of nematodes did not alter in relation to the duration of the spray application, indicating that the level of remixing applied avoided the nematodes being deposited at the bottom of the sprayer tank. Lastly, from a physical-mechanical point of view, it can be attested that the traditional boom sprayers, even without modifications, if operating at low pressures, can be safely used for the application of *S. carpocapsae* for the levels of agitation tested.

In the field experiment chemicals proved to be effective in noctuid moth larval population control. Indeed only chemical treatment was significantly lower than the control. The low efficacy showed by *B. thuringiensis* when utilized alone may be related to the rainfall (14 mm in the four days after the second Bt spraying and 26 mm in the four days after the fourth Bt spraying) that could have washed away the product reducing the period of activity, since not even the increase of the dose resulted in a better pest control.

Abiotic factors such as temperature, desiccation and UV radiation could be responsible for the low performance recorded in the nematode treatments. Indeed desiccation is reported as the key factor influencing nematode efficacy on foliage (Glazer *et al*., 1992; Piggott *et al*., 2000; Arthurs *et al*., 2004). In our field experiment no nematodes infecting sampled larvae, even 4 days after EPN treatment, were detected. Considering that vacuuming normally does not collect dead larvae and that the symbiotic bacteria associated to *S. carpocapsae* usually kill the insect larva in about two days (Burnell and Stock, 2000), it could be possible that nematode activity was roughly limited to the first two days after spraying. The addition of chitosane, an organic biodegradable product with the active ingredient N-acetyl-glucosamine, to the nematode suspension could have improved nematode efficacy (Llácer *et al*., 2009). Chitosane forms a film which stabilizes and improves the adhesion of IJs acting as a protective agent against desiccation and UV light in foliar applications (Martinez Peña, 2002). However it cannot to be excluded that EPN did not fully reach the target host, in particular during the first treatment, when the foliage did not completely cover the ground. Nevertheless the nematodes that may have been leached into the soil are able to attack the noctuid prepupae and pupae. The key pest in our study, *S. exigua*, pupates in the soil and is particularly susceptible to *S. carpocapsae* (Kaya and Hara, 1980).

Previous studies have shown an additive interaction between *S. carpocapsae* and *B. thuringiensis* subsp. *aizawai* against *S. littoralis* in the laboratory (Salem *et al*., 2007) and between *S. carpocapsae* and *Heterorhabditis indica* Poinar, Karunakar et David and insecticides at half dose against *Spodoptera frugiperda* (Smith) (Lepidoptera Noctuidae) in controlled field conditions (Negrisoli *et al*., 2010). The present study is the first showing of an additive interaction between EPN and *B. thuringiensis aizawai* aiming to control noctuid moths, *S. exigua* and *A. gamma* in particular, in the open field. These findings are highly valuable in a crop such as spinach where, as in other vegetable crops, more than one pest has to be controlled, thus making the high cost of EPN formulations appear more tolerable. Moreover, the use of combined biocontrol agents could be a potential strategy to reduce pest resistance caused by intensive use of chemical insecticides and to manage restrictions of current insecticides. However the reason why the additive effect found in this study did not translate into a better pest control in the field needs to be investigated more thoroughly. Schroer *et al*. (2005b) observed promising results against *Plutella xylostella* (L.) (Lepidoptera Plutellidae) on cabbage either using a weekly rotation of EPN and Bt or both biological agents together.

The application technology and EPN formulations availability make nematode applications feasible against some foliar pests. However, the low pest control efficacy of EPN alone or mixed with *B. thuringiensis* found in this experiment, suggests new studies should be carried out on this subject. In particular it is necessary to investigate optimal biocontrol agent concentrations, water volume, actual reaching of the target, and treatment timing to coincide best with susceptible host stages and to maximize nematodes ability to rapidly locate and infect target hosts, along with the overall benefit of using EPN in chitosane formulation.

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References

ARTHURS S., HEINZ K. M., PRASIFKA J. R., 2004.- An analysis of using entomopathogenic nematodes against above-ground pests.- *Bulletin of Entomological Research*, 94: 297-306.

BAILEY A., CHANDLER D., GRANT W. P., GREAVES J., PRINCE G., TATCHELL M., 2010.- *Biopesticides: pest management and regulation*.- CABI Publishing, Wallingford, UK.

BECK B., BRUSSELMAN E., NUYTTENS D., MOENS M., POLLET S., TEMMERMAN F., SPANOGHE P., 2013.- Improving foliar applications of entomopathogenic nematodes by selecting adjuvants and spray nozzles.- *Biocontrol Science and Technology*, 23: 507-520.

BRUSSELMAN E., NUYTTENS D., DE SUTTER N., VIAENE N., STEURBAUT W., MOENS M., 2008.- Effect of several centrifugal pump passages on the viability and activity of *Steinernema carpocapsae*, a biopesticide.- *Communications in Agricultural and Applied Biological Sciences*, 73: 705-708.

BRUSSELMAN E., MOENS M., STEURBAUT W., NUYTTENS D., 2010a.- Evaluation of hydraulic, pneumatic and mechanical agitation for the spray application of *Steinernema carpocapsae* (Rhabditida: Steinernematidae).- *Biocontrol Science and Technology*, 20: 339-351.

BRUSSELMAN E., BECK B., TEMMERMAN F., POLLET S., STEURBAUT W., MOENS M., NUYTTENS D., 2010b.- The spray pattern of entomopathogenic nematodes. In: *Proceeding of ASABE Annual International Meeting. American Society of Agricultural and Biological Engineers*, Pittsburgh, PA, Paper Number: 1009541.

BRUSSELMAN E., BECK B., POLLET S., TEMMERMAN F., SPANOGHE P., MOENS M., NUYTTENS D., 2012.- Effect of the spray application technique on the deposition of entomopathogenic nematodes in vegetables.- *Pest Management Science*, 68: 444-453.

BURNELL A. M., STOCK S. P., 2000.- *Heterorhabditis*, *Steinernema* and their bacterial symbionts - lethal pathogens of insects.- *Nematology*, 2: 31-42.

FAO, 2009.- *FAOSTAT*.- [online] URL: http://faostat.fao.org/DesktopDefault.aspx?PageID=567#ancor

FIFE J. P., 2003.- Investigation of the effect of agricultural spray application equipment on damage to entomopathogenic nematodes - a biological pest control agent. *PhD thesis*, The Ohio State University, Columbus, OH, USA.

FIFE J. P., DERKSEN R. C., OZKAN H. E., GREWAL P. S., 2003.- Effect of pressure differential on the viability and infectivity of entomopathogenic nematodes.- *Biological Control*, 27: 65-72.

FIFE J. P., OZKAN H. E., DERKSEN R. C., 2004.- Physical effect of conventional spray equipment on a biological pesticide, pp. 495-502. In: *International Advances in Pesticide Application 2004. Aspects of Applied Biology 71* (BATEMAN R. P., COOPER S. E., CROSS J. V., GLASS C. R., ROBINSON T. H., STOCK D., TAYLOR W. A., THORNHILL E. W., WALKLATE P. J., Eds).- Association of Applied Biologists, Warwick, UK.

GAN-MOR S., MATTHEWS G. A., 2003.- Recent developments in sprayers for application of biopesticides - an overview.- *Biosystems Engineering*, 84: 119-125.

GLAZER I., KLEIN M., NAVON A., NAKACHE Y., 1992.- Comparison of efficacy of entomopathogenic nematodes combined with antidesiccants applied by canopy sprays against three cotton pests (Lepidoptera: Noctuidae).- *Journal of Economic Entomology*, 85: 1636-1641.

GREWAL S. G., 2002.- Formulation and application technology, pp. 265-287. In: *Entomopathogenic Nematology* (GAUGLER R., Ed.).- CAB International, Wallingford, UK.

HEAD J., LAWRENCE A. J., WALTERS K. F. A., 2004.- Efficacy of the entomopathogenic nematode, *Steinernema feltiae*, against *Bemisia tabaci* in relation to plant species.- *Journal of Applied Entomology*, 128: 543-547.

KAYA H. K., HARA A. H., 1980.- Differential susceptibility of lepidopterous pupae to infection by the nematode *Neoplectana carpocapsae*.- *Journal of Invertebrate Pathology*, 36: 389-393.

KOPPENHÖFER A. M., KAYA H. K., 1997.- Additive and synergistic interaction between entomopathogenic nematodes and *Bacillus thuringiensis* for scarab grub control.- *Biological Control*, 8: 131-137.

KOPPENHÖFER A. M., GREWAL P. S, 2005.- Compatibility and interactions with agrochemicals and other biocontrol agents, pp. 363-381. In: *Nematodes as biocontrol agents* (GREWAL P. S., EHLERS R.-U., SHAPIRO-ILAN D. I., Eds).- CABI Publishing, Wallingford, UK.

LLÁCER E., MARTÍNEZ DE ALTUBE M. M., JACAS J. A., 2009.- Evaluation of the efficacy of *Steinernema carpocapsae* in a chitosan formulation against the red palm weevil, *Rhynchophorus ferrugineus*, in *Phoenix canariensis*.- *BioControl*, 54: 559-565.

ŁACZYŃSKI A., DE MOOR A., MOENS M., SONCK B., RAMON H., 2004.- An application technique for biological plant protection products containing entomopathogenic nematodes, pp. 489-493. In: *International advances in pesticide application 2004. Aspects of applied biology 71* (BATEMAN R. P., COOPER S. E., CROSS J. V., GLASS C. R., ROBINSON T. H., STOCK D., TAYLOR W. A., THORNHILL E. W., WALKLATE P. J., Eds).- Association of Applied Biologists, Warwick, UK.

ŁACZYŃSKI A., DE MOOR A., DIERICKX W., MOENS M., DARIUS P., SONCK B., RAMON H., 2006.- The effect of hydraulic spraying on the viability of the nematode *Heterorhabditis bacteriophora*.- *Crop Protection*, 25: 1135-1141.

ŁACZYŃSKI A., DIERICKX W., DE MOOR A., 2007.- The effect of agitation system, temperature of the spray liquid, nematode concentration, and air injection on the viability of *Heterorhabditis bacteriophora*.- *Biocontrol Science and Technology*, 17: 841-851.

LANZONI A., BURGIO G., 2010.- Contro le nottue dello spinacio si parte dal monitoraggio.- *L'Informatore Agrario*, 66 (4): 57-60.

LANZONI A., BAZZOCCHI G. G., REGGIORI F., RAMA F., SANNINO L., MAINI S., BURGIO G., 2012.- *Spodoptera littoralis* male capture suppression in processing spinach using two kinds of synthetic sex-pheromone dispensers.- *Bulletin of Insectology*, 65: 311-318.

LELLO E. R., PATEL M. N., MATTHEWS G. A., WRIGHT D. J., 1996.- Application technology for entomopathogenic nematodes against foliar pests.- *Crop Protection*, 15: 567-574.

MARTINEZ PEÑA A., 2002.- Biological pesticide based on chitosan and entomopathogenic nematodes.- *WO Patent*, 037966.

MOLYNEUX A., 1985.- Survival of infective juveniles of *Heterorhabditis* spp. and *Steinernema* spp. (Nematoda: Rhabditida) at various temperatures and their subsequent infectivity for insects.- *Revue de Nématologie*, 8: 165-170.

NEGRISOLI A. S., GARCIA M. S., BARBOSA NEGRISOLI C. R. C., BERNARDI D., DA SILVA A., 2010.- Efficacy of entomopathogenic nematodes (Nematoda: Rhabditida) and insecticide mixtures to control *Spodoptera frugiperda* (Smith, 1797) (Lepidoptera: Noctuidae) in corn crops.- *Crop Protection*, 29: 677-683.

NILSSON U., GRIPWALL E., 1999.- Influence of application technique on the viability of the biological control agents *Verticillium lecanii* and *Steinernema feltiae*.- *Crop Protection*, 18: 53-59.

PIGGOTT S. J., WRIGHT D. J., MATTHEWS G. A., 2000.- Polymeric formulation for the application of entomopathogenic nematodes against foliar pests, pp. 1063-1068. In: *Proceeding of BCPC Conference on Pests and Diseases*, Brighton, UK.

ROBERTSON J. L., PREISLER H. K., 1992.- *Pesticide bioassay with arthropods*.- CRC Press, Boca Raton, USA.

SALEM S. A., ABDEL-RAHMAN H. A., ZEBITZ C. P. W., SALEH M. M. E., ALI-FAWKIA I., EL-KHOLY M. Y., 2007.- Interaction between entomopathogenic nematodes and *Bacillus thuringiensis* as a new approach for biological control of some lepidopterous pests.- *Journal of Applied Sciences Research*, 3: 333-342.

SCHROER S., EHLERS R.-U., 2005.- Foliar application of the entomopathogenic nematode *Steinernema carpocapsae* for biological control of diamondback moth larvae (*Plutella xylostella*).- *Biological Control*, 33: 81-86.

SCHROER S., ZIERMANN D., EHLERS R.-U., 2005a.- Mode of action of a surfactant-polymer formulation to support performance of the entomopathogenic nematode *Steinernema carpocapsae* for control of diamondback moth larvae (*Plutella xylostella*).- *Biocontrol Science and Technology*, 15: 601-613.

SCHROER S., SULISTYANTO D., EHLERS R. U., 2005b.- Control of *Plutella xylostella* using polymer-formulated *Steinernema carpocapsae* and *Bacillus thuringiensis* in cabbage fields.- *Journal of Applied Entomology*, 129: 198-204.

SHAPIRO-ILAN D. I., GOUGE D. H., PIGGOT S. J., FIFE J. P., 2006.- Application technology and environmental considerations for use of entomopathogenic nematodes in biological control.- *Biological Control*, 38: 124-133.

STATSOFT, 2011.- *Electronic Statistics Textbook*.- StatSoft Inc., Tulsa, OK, USA.

THAKORE Y., 2006.- Biocontrol: The biopesticide market for global agricultural use.- *Industrial Biotechnology*, 2: 194-208.

WILLIAMS E. C., WALTERS K. F. A., 2000.- Foliar application of the entomopathogenic nematode *Steinernema feltiae* against leafminers on vegetables.- *Biocontrol Science and Technology*, 10: 61-70.

ZAR J. H., 2010.- *Biostatistical analysis, 5th ed*.- Prentice Hall, Upper Saddle River, NJ, USA.

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