Effects of selected insecticides on osmotically treated entomopathogenic nematodes

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Abstract

Combining environmentally friendly insecticides with entomopathogenic nematodes may constitute an effective alternative to conventional chemical control of many insect pests. The objective of this laboratory study was to evaluate the effects of selected insecticides which are commonly used for pest control in vegetables in China on osmotically treated and untreated infective juveniles of *Steinernema carpocapsae* strain All. The insecticides azadirachtin, chlorpyrifos, cypermethrin, fipronil, imidacloprid, malathion, thiamethoxam and chlorantraniliprole-thiamethoxam had no adverse effects on nematode survival and infectivity. In contrast, bisultap, emamectin benzoate, phoxim and rotenone proved harmful to *S. carpocapsae* All mainly by reducing infectivity of the infective juveniles to larvae of the greater wax moth, *Galleria mellonella*. Osmotic induction is a promising way to induce entomopathogenic nematodes into partial anhydrobiosis and thus increase environmental stress tolerance of the nematodes as well as their shelf life after production. The present results showed that osmotic treatment did not adversely affect fitness of the nematode in terms of its susceptibility to insecticides and even increased its tolerance to chlorpyrifos and rotenone. The results of this laboratory study indicate that several of the tested insecticides can be safely combined with *S. carpocapsae* All within an integrated pest management approach.

Key words: Integrated pest management, osmotic treatment, *Steinernema carpocapsae*

Introduction

Chemical insecticides are the main method for many insect pests control in crops, vegetables and fruits in China. Heavy use of chemical insecticides leads to operator and consumer poisoning, hampers export of the products, and causes serious environmental pollution (Wang & Han 2008). With the development of insecticide resistance due to the high frequency of insecticide applications (Feng et al. 2000), environmentally friendly control methods are urgently needed.

Entomopathogenic nematodes (EPN) of the genera *Steinernema* and *Heterorhabditis* (Rhabditida: Steinernematidae and Heterorhabditidae) are natural enemies of various insect pests. They can actively search for their hosts in the soil (Grewal et al. 2005) and can be easily mass-produced using conventional fermentation technology (Grewal et al. 2002). They are increasingly used to control insect crop pests in different parts of the world (Kaya et al. 2006). In comparison with common chemical insecticides, however, the cost of EPN remains too high for the average Chinese grower. Combining EPN with insecticides has contributed to the suppression of a number of economically important crop pests (Koppenhöfer & Grewal 2005). Such an integrated pest management (IPM) approach could not only yield more effective control and slow down development of insecticide resistance, but could also lower EPN application rates and thus contribute to a reduction of control cost.

EPN can be induced into partial anhydrobiosis. Anhydrobiosis is a state of dormancy induced by dehydration (Barrett 1991). In an anhydrobiotic state, resistance of EPN to environmental extremes, hypoxia, radiation and metabolic poisons lethal to active organisms, is greatly increased (Grewal 2000). A previous study showed that exposure to (hyper)osmotic solutions (termed "osmotic induction") can improve storage stability of *Steinernema carpocapsae* All (Yan et al. 2011). The nematode can thus be stored after osmotic induction into partial anhydrobiosis and subsequently applied in the field after rehydration.

Improving insight in the response of EPN to insecticide exposure may aid the development of IPM programs against key pests. Therefore, the objective of the present laboratory study was to evaluate the susceptibility of both osmotically treated and untreated *S. carpocapsae* All to selected insecticides which are commonly used for the control of insect pests in vegetable production in China. Survival of infective juveniles (IJ) exposed to the insecticides at different concentrations and their infectivity to larvae of the greater wax moth *Galleria mellonella* (Lepidoptera: Pyralidae) were evaluated in the laboratory.

Materials and methods

Nematodes

Steinernema carpocapsae All was reared from an *in vitro* solid sponge culture in 1000 ml flasks according to the

method of Bedding (1981) with modifications (Han 1995). After 4 weeks of incubation at 25°C, the IJ were soaked out of the sponge and washed with sterile distilled water (6x), concentrated to 2×10^4 IJ ml^{-1} and then stored at 15°C. The stored IJ were tested within 2 weeks. Background mortality was below 5% throughout the study.

Osmotic induction of IJ was conducted as described by Yan et al. (2010). The IJ were treated with osmotic solution consisting of a mixture of fortified artificial seawater (1.692 M NaCl, 9.00 mM KCl, 9.27 mM CaCl$_2$, 22.94 mM MgCl$_2$·6H$_2$O, 25.50 mM MgSO$_4$ × 7H$_2$O, 2.14 mM NaHCO$_3$) and glycerol (6.4 M) at a proportion of 1:5 (v/v) in 250 ml flasks and incubated at 15°C for 24 h in a rotary shaker at 100 rpm. After that, 1 ml of osmotically treated or untreated nematode suspension was transferred to a 9 cm Petri dish containing 9 ml sterile distilled water. The dishes were incubated at 25°C for 24 h in order to rehydrate the IJ before exposure to the insecticides. The experiments were repeated twice using different batches of nematodes. Different batches of EPN were cultured at different times throughout the study. They were from the same ancestor but of different generations.

Insects

The greater wax moth, *Galleria mellonella*, is a model insect used for EPN bait trapping and for evaluation of EPN infectivity. *Galleria mellonella* was reared in an environmentally controlled room at 28 ± 2°C and 60% relative humidity (RH). The larvae were fed on an artificial diet modified from Vanhaecke & Degheele (1980).

Insecticides

Insecticides used in the present study are listed in Table 1; all were commercial formulations purchased on the Chinese market and commonly used in Chinese vegetable production.

Effects of insecticides on nematode survival

Effects of insecticides on nematode survival were tested using the method described by Zhang et al. (2006) with modifications. Insecticide dilutions were prepared in distilled water and added to each Petri dish to a final concentration corresponding with the recommended field concentration (RC), 1/10 RC or 1/50 RC. The insecticide-treated IJ were incubated at 25°C in the dark for 24 h. Each treatment had three replicates (dishes) with a density of 1000 IJ ml^{-1}. Infective juveniles treated with osmotic solution or distilled water only were used as controls. After that, three 100 µl samples were drawn from each dish and mortality of the IJ was calculated. Infective juveniles were considered dead when showing no response after probing with a needle. The infectivity of the exposed IJ to last instar larvae of *G. mellonella* was tested as described below when there were surviving IJ.

Effects of insecticides on nematode infectivity

A Petri dish bioassay was used to estimate the effects of the tested insecticides on nematode infectivity (Zhang et al.

Table 1: Insecticides used in the study

Insecticide	Main component	Manufacturer	Recommended field rate
Azadirachtin EC 0.3%	azadirachtin	Chengdu Green the High-Tech Co., Ltd, Chengdu	1200 ml ha^{-1}
Bisultap AS 18%	bisultap	Zhongshan Aestar Fine Chemical Inc., Ltd, Zhongshan	675 g ha^{-1}
Virtako WG 40%	chlorantraniliprole, thiamethoxam	Syngenta, Beijing	120 g ha^{-1}
Lorben EC 40%	chlorpyrifos	Zhejiang XinNong Chemical Co., Ltd, Hangzhou	1500 g ha^{-1}
Cypermethrin EC 4.5%	beta cypermethrin	Daguangming Nongyao Huagong Co., Ltd, Jiangmen	600 ml ha^{-1}
Emamectin benzoate EC 2%	emamectin benzoate	Zhejiang Qianjiang Biochemical Co., Ltd, Haining	125 ml ha^{-1}
Regent SC 80%	fipronil	Bayer CropScience, Hangzhou	20 g ha^{-1}
Admire WG 70%	imidacloprid	Bayer CropScience, Hangzhou	20 g ha^{-1}
Malathion EC 45%	malathion	Cangzhou Lekai Nongyao Huagong Co., Ltd, Cangzhou	3000 ml ha^{-1}
Phoxim EC 40%	phoxim	Lianyungang Liben Nongyao Huagong Co., Ltd, Lianyungang	750 ml ha^{-1}
Rotenone EC 2.5%	rotenone	Shenzhen Huanong Biological Engineering Co., Ltd, Shenzhen	2000 g ha^{-1}
Actara WG 25%	thiamethoxam	Syngenta, Beijing	300 g ha^{-1}

EC, emulsifiable concentrate; AS, aqueous solution; WG, water dispersible granules; SC, suspension concentrate.

2006). Two layers of 9 cm filter paper (Xinhua, China) were placed in a 9 cm Petri dish. Two hundred fifty live IJ were sampled from the Petri dishes in which they had been exposed to insecticide and insecticide dilutions at the respective concentrations (as described before) were added to a final volume of 1.5 ml. The IJ in different dilutions of the selected insecticides were then applied onto the filter paper. Subsequently, ten last instar *G. mellonella* were placed in each dish. Infective juveniles in distilled water were used as controls. The *G. mellonella* larvae were also exposed to all concentrations of the selected insecticides and to water only (control), without *S. carpocapsae*. Each treatment had three replicates (dishes). The dishes were sealed with Parafilm (SPI Supplies, West Chester, USA) and incubated at 25°C for 72 h. Mortality of the *G. mellonella* larvae was checked every 12 h and nematode infectivity was expressed as mortality of wax moth larvae 72 h after infection.

Statistical analysis

Nematode and insect mortality data were expressed in percentages. Mortality rates of the IJ were corrected for control mortality using Abbott's formula (Abbott 1925). All data were arcsine square root transformed prior to statistical analysis performed with SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). To assess effects of osmotic treatment, insecticide concentration and their interaction, a Univariate General Linear Model was used. Means were separated using Tukey's test. Paired sample *t*-tests were done to analyse differences between osmotically treated and untreated nematodes exposed to the same concentration of the same insecticide. Differences among means were considered significant at *P* < 0.05.

Results

Effect of insecticides on nematode survival

Corrected mortality rates of *S. carpocapsae* strain All exposed to different concentrations of the tested insecticides are shown in Fig. 1. Nine of these insecticides (azadirachtin, cholorantraniliprole-thiamethoxam, cypermethrin, malathion, emamectin benzoate, fipronil, phoxim, imidacloprid and thiamethoxam) had no or negligible effects on survival of both osmotically treated and untreated IJ at the different concentrations, with mortality rates below 4%.

Three insecticides, rotenone, bisultap, and chlorpyrifos, did affect survival of *S. carpocapsae* All to some extent with mortalities ranging from 10 to 58% for IJ that had not been exposed to the osmotic solution; the effect of osmotic treatment on nematode susceptibility also varied. The effects of osmotic treatment and insecticide concentration on mortality of *S. carpocapsae* All exposed to the latter three insecticides are shown in Table 2. Rotenone was harmful to the IJ at RC with mortality rates of 57.9 ± 2.2% (mean ± SE) for untreated IJ and 31.2 ± 2.3% for osmotically treated IJ. Bisultap at RC caused 21.1 ± 3.8% mortality in untreated IJ

Table 2: Two-way ANOVA results indicating the effect of osmotic treatment and insecticide concentration on infectivity of *Steinernema carpocapsae* All based on corrected mortality of *Galleria mellonella* 72 h post-infection

Factor	Insecticide		
	rotenone	bisultap	chlorpyrifos
Osmotic treatment (*df* = 1, 12)			
F-value	2.406	9.828	8.131
P-value	0.147	0.009	0.015
Insecticide concentration (*df* = 2, 12)			
F-value	128.787	53.754	19.469
P-value	< 0.001	< 0.001	< 0.001
Osmotic treatment × insecticide concentration (*df* = 2, 12)			
F-value	8.383	2.985	6.199
P-value	0.005	0.089	0.014

and 9.9 ± 1.1% in osmotically treated IJ. There were no effects of bisultap and rotenone on survival of both osmotically treated and untreated IJ at 1/10 and 1/50 RC. For bisultap and rotenone at RC, mortality of osmotically treated IJ was also higher than 10%. Chlorpyrifos was slightly harmful to the IJ without osmotic treatment at RC with a mortality rate of 10.0 ± 1.1%. There were no effects of chlorpyrifos on survival of IJ with osmotic treatment at all three concentrations and without treatment at 1/10 and 1/50 RC.

Osmotically treated IJ showed better survival than untreated IJ in insecticide solutions of chlorpyrifos, rotenone and emamectin benzoate. Mortality rates of osmotically treated IJ were significantly lower than those of the untreated IJ exposed to chlorpyrifos (*t* = 5.718; *df* = 2; *P* = 0.035), rotenone (*t* = 6.427; *df* = 2; *P* = 0.023) and emamectin benzoate (*t* = 5.810; *df* = 2; *P* = 0.028).

Effect of insecticides on nematode infectivity

Corrected mortality rates of *G. mellonella* 72 h after exposure to *S. carpocapsae* All in different dilutions of the selected insecticides are shown in Table 3. For the insecticides alone, only RC of emamectin benzoate caused significant mortality in *G. mellonella*, with an average of 43% after 72 h, indicating the low susceptibility of the exposed last instar larvae of this insect to the tested insecticide concentrations. For the nematodes in different dilutions of the tested insecticides, there were no effects on IJ infectivity for azadirachtin, chlorantraniliprole-thiamethoxam, chlorpyrifos, cypermethrin, fipronil, imidacloprid, malathion and thiamethoxam, which caused about 100% mortality of *G. mellonella* in 48−72 h, as did osmotically treated or untreated IJ alone.

Mortality rates of *G. mellonella* exposed to *S. carpocapsae* All in solutions of four insecticides (bisultap, emamectin benzoate, phoxim and rotenone) were significantly influenced by insecticide concentration but not by osmotic treat-
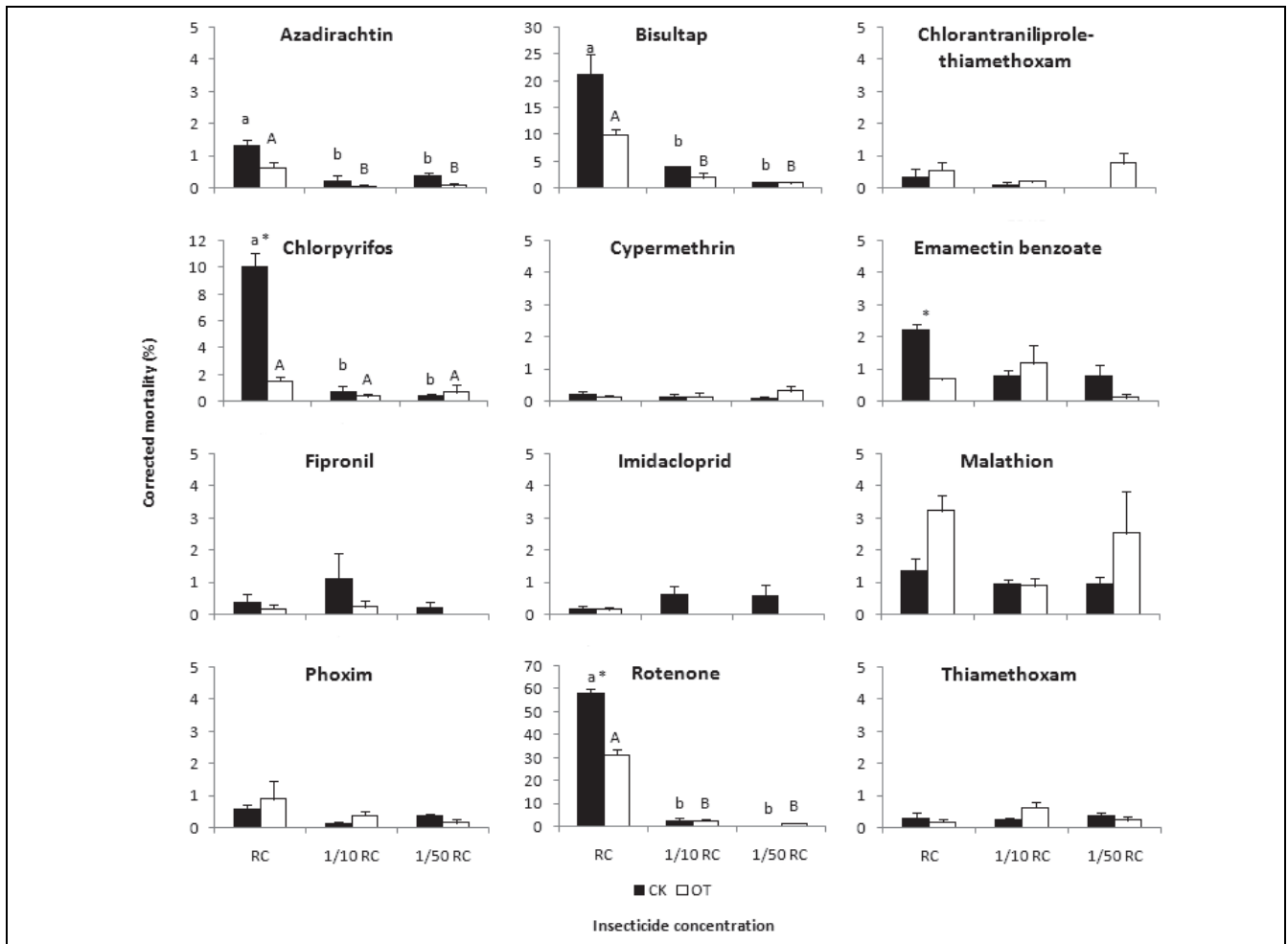
Fig. 1:   Corrected mortality rates (± SE) of osmotically treated and untreated *Steinernema carpocapsae* All after exposure to different insecticides at different concentrations.
CK, untreated *S. carpocapsae* All; OT, osmotically treated *S. carpocapsae* All. RC, recommended concentration; 1/10 RC, 10-time diluent of recommended concentration; 1/50 RC, 50-time diluent of recommended concentration. Asterisks (*) above bars indicate a significant difference of mortality rates between the osmotically treated and untreated nematodes treated with the same concentration of insecticide ($P < 0.05$). Bars with different uppercase or lowercase letters indicate significant differences in mortality rates among different concentrations of insecticides for osmotically treated or untreated nematodes, respectively ($P < 0.05$, Tukey's test).

ment and the interaction between the two factors was also not significant (Table 4). Infective juveniles treated with bisultap at the RC lost their infectivity to *G. mellonella*, but retained their infectivity when treated at 1/10 or 1/50 RC. Infective juveniles exposed to emamectin benzoate at RC and 1/10 RC showed weaker infectivity to *G. mellonella* when compared with IJ without insecticide treatments. At RC and 1/10 RC of latter compound, *G. mellonella* mortality caused by untreated and osmotically treated IJ at 72 h averaged 47–63%, but reached 100% at 1/50 RC. Exposure of IJ to phoxim and rotenone at the three different concentrations greatly reduced their infectivity to *G. mellonella*.

Discussion

Combining the use of biological control agents, like EPN, with insecticides is central to IPM programs against many

agricultural pests (Koppenhöfer & Grewal 2005). This strategy can not only facilitate the use of EPN, increase the control efficiency of programs including EPN and reduce the cost of EPN application, but it may also reduce the dependence on chemical insecticides and thus contribute to slowing down the development of insecticide resistance and preventing adverse effects on man and the environment. The present laboratory study explored the effects of several commonly used insecticides in China on osmotically treated and untreated IJ of *S. carpocapsae* All and indicated that some of these insecticides may be harmless to the nematode.

Commercial formulations of azadirachtin, chlorantraniliprole, chlorpyrifos, cypermethrin, fipronil, malathion, imidacloprid and thiamethoxam showed no negative effects on the survival and infectivity of both osmotically treated and untreated *S. carpocapsae* All. The results are consistent with those from former studies on the compatibility of *S. carpocapsae* with thiamethoxam, imidacloprid, azadirachitin,

Table 3: Corrected mortality rates of *Galleria mellonella* larvae 72 h after exposure to *Steinernema carpocapsae* All in different concentrations of selected insecticides

Insecticide	Treatment	Mortality (Mean ± SE, %)		
		RC	1/10 RC	1/50 RC
azadirachtin	CK	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	OT	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
bisultap	CK	0.00 ± 0.00 a	100.00 ± 0.00 b	100.00 ± 0.00 b
	OT	0.00 ± 0.00 a	96.67 ± 2.72 b	100.00 ± 0.00 b
chlorantraniliprole, thiamethoxam	CK	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	OT	96.67 ± 2.72	100.00 ± 0.00	100.00 ± 0.00
chlorpyrifos	CK	96.67 ± 2.72	100.00 ± 0.00	100.00 ± 0.00
	OT	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
cypermethrin	CK	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	OT	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
emamectin benzoate	CK	63.33 ± 2.27 a	53.33 ± 7.20 a	100.00 ± 0.00 b
	OT	63.33 ± 11.86 a	46.67 ± 7.20 a	100.00 ± 0.00 b
fipronil	CK	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	OT	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
imidacloprid	CK	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	OT	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
malathion	CK	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	OT	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
phoxim	CK	3.33 ± 2.72 a	3.33 ± 2.72 a	70.00 ± 9.43 b
	OT	3.33 ± 2.72 a	0.00 ± 0.00 a	73.33 ± 17.85 b
thiamethoxam	CK	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	OT	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
rotenone	CK	3.33 ± 2.72 a	16.67 ± 7.20 a	46.67 ± 7.20 b
	OT	26.67 ± 11.86 a	30.00 ± 4.71 a	50.00 ± 0.00 b

CK, untreated *S. carpocapsae* All; OT, osmotically treated *S. carpocapsae* All. RC, recommended concentration; 1/10 RC, 10-time diluent of the recommended concentration; 1/50 RC, 50-time diluent of the recommended concentration. Means within a row followed by different letters are significantly different ($P < 0.05$, Tukey's test).

fipronil and chlorpyrifos (Prakasa Rao et al. 1975, Rovesti et al. 1988, Koppenhöfer et al. 2003, Alumai & Grewal 2004, García del Pino & Jové 2005, Negrisoli Jr et al. 2010). Cypermethrin caused 12% mortality in *S. carpocapsae* in a study by Negrisoli Jr et al. (2010), whereas in our study mortality of *S. carpocapsae* caused by cypermethrin remained below 1%. Emamectin is fermented from *Streptomyces avermitilis* and is generally formulated as emamectin benzoate (Waddy et al. 2007). According to Fetoh et al. (2009) emamectin benzoate had no adverse effects on *S. carpocapsae* and a mixture of *S. carpocapsae* with formulated emamectin benzoate significantly increased mortality to greasy cutworm (*Agrotis ipsilon*) as compared with *S. carpocapsae* alone. In

our study, however, viability of *S. carpocapsae* IJ was not affected by emamectin benzoate, but their infectivity was impaired. Different findings among studies may be related to differences in formulation of the test compounds and in the experimental design (Koppenhöfer & Grewal 2005, Negrisoli Jr et al. 2010).

No studies have previously reported on the compatibility of *S. carpocapsae* with bisultap, malathion, phoxim and rotenone. The nereistoxin bisultap is a broad-spectrum insecticide with a strong stomach, contact, systemic and fumigant activity (Wang et al. 1995). Bisultap proved toxic to *S. carpocapsae* All only when applied at RC. At lower concentrations, however, adverse effects of bisultap in our

Table 4: Two-way ANOVA results indicating the effect of osmotic treatment and insecticide concentration on infectivity of *Steinernema carpocapsae* All based on corrected mortality of *Galleria mellonella* 72 h post-infection

Factor	Insecticide			
	bisultap	emamectin benzoate	phoxim	rotenone
Osmotic treatment (*df* = 1, 12)				
F-value	1.000	0.059	0.002	3.892
P-value	0.337	0.812	0.963	0.066
Insecticide concentration (*df* = 2, 12)				
F-value	829.740	61.808	64.477	167.695
P-value	< 0.001	< 0.001	< 0.001	< 0.001
Osmotic treatment × insecticide concentration (*df* = 2, 12)				
F-value	1.000	0.137	0.261	1.081
P-value	0.397	0.936	0.852	0.385

laboratory exposure tests were negligible. The organophosphate insecticide phoxim had no effects on the survival of *S. carpocapsae* All, but strongly reduced the infectivity of IJ, even at diluted concentrations. Rotenone has been widely used in organic farming in China to control leaf feeding pests, including flea beetles on brassicas. Rotenone kills fishes and other organisms by blocking the cellular uptake of oxygen (Singer & Ramsay 1994). However, it is rapidly bio-degraded under warm conditions and is poorly absorbed by the gastrointestinal tract of humans, rendering it relatively safe to man and the environment (Ling 2003, Robertson & Smith-Vaniz 2008). In our study, the compound proved lethal to *S. carpocapsae* All at RC, with 58% and 31% mortality of untreated and osmotically treated IJ, respectively, and it impaired the infectivity of the nematode even at very low concentrations (1/10 RC and 1/50 RC). The adverse effects of the insecticides tested in the present study on *S. carpocapsae* may be due to the direct toxicity of the active ingredient to the nematode or to the detrimental effects of the formulation on the symbiotic bacteria (Alumai & Grewal 2004, Hara & Kaya 1983).

Our results further indicate that osmotic treatment did not adversely affect fitness of the nematode in terms of its susceptibility to insecticides. Interestingly, an increase in tolerance of the IJ to chlorpyrifos and rotenone was observed when exposed to osmotic induction. Such an effect could not be demonstrated for the other insecticides, given the low mortality rates observed in our experiments. EPN can be induced into a state of partial anhydrobiosis using osmotic solutions, which may allow longer storage of the IJ (Feng et al. 2006). Molecular studies indicated that in response to desiccation and osmotic induction of anhydrobiosis, the expression of certain genes was up-regulated and some novel proteins were induced (Yan & Han 2010). After dehydration and the subsequent rehydration, these genes and proteins may still be in function, enabling the EPN to better resist environmental stresses including that from toxic substances. This may explain why the osmotically treated *S. carpocapsae* exposed to chlorpyrifos and particularly rotenone survived better than untreated ones.

In conclusion, the present laboratory study indicates that azadirachtin, chlorantraniliprole, chlorpyrifos, cypermethrin, fipronil, malathion, imidacloprid and thiamethoxam are harmless to IJ of *S. carpocapsae* All. However, laboratory and field studies using target insects are needed to confirm whether these compounds can be safely applied together with *S. carpocapsae* All for the control of insect pests in vegetable crops in China and beyond.

Acknowledgements

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