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Comparative Bioactivity of Emamectin Benzoate Formulations against the Pine Wood Nematode, *Bursaphelenchus xylophilus*

Jong-won Lee[1,2†], Abraham Okki Mwamula[2,3†], Jae-hyuk Choi[1,2], Ho-wook Lee[1,2], Yi Seul Kim[2,3], Jin-Hyo Kim[4], Yong-hwa Choi[5], and Dong Woon Lee [1,2,3*]

[1]*Department of Ecological Science, Kyungpook National University, Sangju 37224, Korea*
[2]*Research Institute of Invertebrate Vector, Kyungpook National University, Sangju 37224, Korea*
[3]*Department of Entomology, Kyungpook National University, Sangju 37224, Korea*
[4]*Department of Agricultural Chemistry, Gyeongsang National University, Jinju 52828, Korea*
[5]*School of Ecology and Environmental System, Kyungpook National University, Sangju 37224, Korea*

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The pine wood nematode (PWN), *Bursaphelenchus xylophilus* is a well-known devastating pathogen of economic importance in the Republic of Korea and other countries. In the Republic of Korea, trunk injection of nematicides is the preferred method of control. In this study, the efficacy of 16 locally produced formulations of emamectin benzoate against the PWN are compared through determining their sublethal toxicities and reproduction inhibition potentials. Nematodes were treated with varying concentrations of the tested chemicals in multi-well culture plates, and rates of paralysis and mortality were determined after 24 h. Reproduction inhibition potential was tested by inoculating pre-treated nematodes onto *Botrytis cinerea*, and in pine twig cuttings. Despite the uniformity in the concentration of the active ingredient, efficacy was contrastingly different among formulations. The formulations evidently conformed to three distinct groups based on similarities in sublethal activity (group 1: LC_{95} of 0.00768-0.01443 mg/ml; group 2: LC_{95} of 0.03202-0.07236 mg/ml, and group 3: LC_{95} of as high as 0.30643-0.40811 mg/ml). Nematode paralysis generally occurred at the application dose of 0.0134-0.1075 µg/ml, and there were significant differences in nematode paralysis rates among the products. Nematode reproduction was only evident at lower doses both on *B. cinerea* and pine twigs, albeit the variations among formulations. Group 1 formulations significantly reduced nematode reproduction even at a lower dose of 0.001075 µg/ml. The variations in efficacy might be attributed to differences in inert ingredients. Therefore, there is need to analyze the potential antagonistic effects of the large number of additives used in formulations.

Keywords **:** efficacy, nematicide, sublethal toxicity, trunk injection

The pine wood nematode (PWN), *Bursaphelenchus xylophilus*, is a well-documented devastating pathogen of economic importance causing pine wilt disease (PWD) on susceptible pine tree varieties. Significant pine tree damage and wood yield losses have been recorded in countries like China (Baojun and Qouli, 1989; Zhao, 2008), Japan (Kosaka et al., 2001; Mamiya, 1988), Portugal (Mota and Vieira, 2008), and the Republic of Korea (Choi and Moon, 1989; Shin, 2008). In the Republic of Korea, the disease was first recorded in Busan in 1988; and rigorous control efforts saw the major invasion and damage limited to areas within the southern region of the country until the early 2000s (Choi and Moon, 1989; Kwon et al., 2011).

†These authors contributed equally to this work.
*Corresponding author.
Phone) +82-54-530-1212, FAX) +82-54-530-1218
E-mail) whitegrub@knu.ac.kr
ORCID
Dong Woon Lee
https://orcid.org/0000-0001-9751-5390

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At present, the PWD is known to have gradually spread to several areas in the northern part of the country. According to Korean Forest Service, the total economic damage is estimated to have reached $6.5 million in the last decade (Kim et al., 2020).

In most affected countries, several control methods against the PWN and its insect vector (*Monochamus* spp.) have been applied in the past years. These include among others; physical removal of infected trees and subsequent fumigation of infected wood (e.g., with metam sodium), aerial insecticide application against the insect vector (e.g., with thiacloprid and acetamiprid); and trunk injection of nematicidal compounds against the nematodes proliferating in live pine trees (Bi et al., 2015; Kong et al., 2006; Liu et al., 2020). In the recent years, there have also been attempts towards testing naturally occurring nematicidal compounds isolated from plants, and other less toxic pesticides, with efforts to limit and combat environmental impacts, including effects on non-target organisms and health-related problems associated with the use of non-selective synthetic nematicides (Mwamula et al., 2022; Nunes da Silva et al., 2014; Park et al., 2007; Seo et al., 2014). However, among all the available and tested control approaches, aerial application of insecticides such as thiacloprid and acetamiprid, and trunk injection with synthetic pesticides such as fenitrothion, morantel tartrate, emamectin benzoate, abamectin, and abamectin + sulfoxaflor mixture have been widely used in the East Asian countries (Bi et al., 2015; Jung et al., 2021; Kishi, 1995; Liu et al., 2020). Trunk injection with especially the naturally occurring broad-spectrum avermectins (abamectin and emamectin benzoate) offers a direct nematicidal effect on the target nematode and is relatively seen as an environmentally friendly approach than direct aerial applications practiced in control of insect vector. These chemicals are known to offer a persistent nematicidal effect over successive number of years (Kwon et al., 2011; Lee et al., 2009; Shin, 2008; Takai et al., 2000, 2004).

Trunk injection has been in practice in the Republic of Korea since 2005 (Kwon et al., 2011). And, with the increasing demand for emamectin benzoate in nematode and other pathogen management, more chemical companies continue to roll out similar formulations of the chemical with the same active ingredient concentration (emamectin benzoate 2.15%) and recommended strength. The formulations are often produced as a single chemical compound or in combination with other insecticides aimed at controlling the vector insect as well. However, each chemical company possibly uses specific unique inert ingredients which may have an impact on both the efficacy of emamectin benzoate, and environmental safety in terms of toxicity levels. Determination of the toxic effect levels of a pesticide is normally based on the concentration of the pure active ingredient or technical grade (Beggel et al., 2010; Cox and Surgan, 2006). However, commercial pesticide formulations, including emamectin benzoate contain a significantly large proportion of more than 97% inert ingredients. Inert or other ingredients are not always mandatorily required by law to be listed on the chemical label, as they are normally considered to be trade secrets. But this also allows opportunity for production of pesticide formulation products which may possess substantially altered toxicity levels (Beggel et al., 2010; Schmuck et al., 1994). The current study was therefore designed to investigate, and compare the efficacy of emamectin benzoate formulations available on market against the PWN; by evaluating their sublethal toxicity levels, and subsequently comparing nematode activity (rate of paralyzed or immobilized nematodes) and nematode reproduction inhibition resulting from pre-exposure to selected concentrations of these chemical formulations.

Materials and Methods

Nematode population. *Bursaphelenchus xylophilus* isolate was isolated from infected pinewood sample (*Pinus densiflora*) taken from Gumi area, Gyeongsangbuk-do Province, Republic of Korea in 2021. The juvenile and adult stages were extracted from the chips of infected wood using the Baermann funnel method (Jenkins, 1964), and the extracted populations were maintained on *Botrytis cinerea*. Briefly, the extracted PWN isolates were maintained on a non-sporulating strain of *Botrytis cinerea* grown on potato dextrose agar at 25°C as described by Kishi (1995) and Takemoto (2008).

Chemical formulations. Sixteen emamectin benzoate formulations (14 EC, 1ME, and 1SL; 15 with a 2.15%, and one with a 2% emamectin benzoate active ingredient concentration) from different producing companies were sourced from the Korean local markets and (or) their respective local producing companies. The formulations were assigned blind-case codes from A to P to enable anonymous and impartial empirical analysis of chemical content, performance, and presentation of results (Table 1).

Quantification of actual emamectin in the formulations. Quantification was performed by a modified method of Rural Development Administration guidance for the pesticide quality inspection method (Rural Development

Table 1. List of emamectin benzoate compound formulations tested against *Bursaphelenchus xylophilus*

Code	Active ingredient[a]	Quantified concentration (%)
A	Emamectin 2.15 EC	2.68
B	Emamectin 2.15 EC	2.45
C	Emamectin 2.15 EC	2.79
D	Emamectin 2.15 EC	2.32
E	Emamectin 2.15 EC	2.57
F	Emamectin 2.15 EC	2.37
G	Emamectin 2.15 EC	2.88
H	Emamectin 2.15 EC	2.78
I	Emamectin 2.15 EC	2.7
J	Emamectin 2.15 EC	3.12
K	Emamectin 2.15 EC	2.5
L	Emamectin 2.15 EC	2.71
M	Emamectin 2.15 EC	2.85
N	Emamectin 2.15 EC	2.99
O	Emamectin 2.15 ME	2.63
P	Emamectin 2.0 SL	2.46

[a]EC, emulsifiable concentrate; ME, microemulsion concentrate; SL, soluble concentrate. Quantified concentration represents the actual quantity of emamectin measured in the current study. It is expressed to the sum of emamectin B1a and B1b ($n = 3$).

Administration, 2022). Briefly, for the quantitative analysis of emamectin benzoate, the pesticide samples were diluted with an equal volume of acetone and then 100-fold with acetonitrile. The diluted sample was then filtered with 0.20 μm of nylon syringe filter (BioFACT, BioFACT Co. Ltd., Seoul, Korea) for the instrumental analysis. The diluted sample was analyzed for emamectin B1a and B1b with UHPLC-UVD (Thermo Fisher Scientific Inc., Waltham, MA, USA). All the analyses were performed with three replications. The analytical column was C18 (4.6 × 250 mm, 5 μm, Agilent Technologies Inc., Santa Clara, CA, USA) and the detection wavelength was 245 nm for the emamectins. The analytical standard of emamectin benzoate was mixed with emamectin B1a and B1b (96.4%, Dr. Ehrenstorfer, LGC labor GmbH, Augsburg, Germany), and the working solution was prepared in the ranges of 10.0-500 μg/ml with acetonitrile. The linearity (R^2) was 1.0000 for the total emamectin. The quantity of emamectin was expressed to the sum of emamectin B1a and B1b (Table 1).

Sublethal toxicity test. Sublethal toxicity tests were conducted to evaluate the efficacy of the various formulations of emamectin benzoate. Preliminary tests at various dilutions were performed to ascertain the appropriate test range for each formulation or group of formulations. Depending on the preliminary test results, the formulations were grouped in four different concentration groups for proper screening (0.0025-0.008, 0.0025-0.0197, 0.0033-0.1003, and 0.0833-0.4606 mg/ml). Serial dilutions of the test formulations were prepared using distilled water to give seven different test concentrations within the given range. The lowest concentration being the amount of the active ingredient in the formulation capable of causing 1-10% mortality, and the highest value represented the concentration at which 90-100% mortality could be registered after a 24-h treatment of the formulation against the nematodes. A 0.5 ml nematode suspension containing 100 nematodes was prepared through homogenization. Briefly, nematode suspension was homogenized by adding distilled water to a subsample of the nematode population, before blowing air through the diluted suspension several times using a pipette (Van Bezooijen, 2006). A 0.5 ml nematode suspension containing 100 nematodes (a mixture of mainly 3rd, 4th stage juveniles and adults) was then filled in each well of a 12 multi-well culture plate (SPL Life Sciences Co., Ltd., Pocheon, Korea), and an equal volume of test chemical compound in selected varying dilutions was added. The multi-well culture plates were wrapped with aluminum foil and kept at 25°C in the growth chamber (HB 303 DH-0, Han Baek, Bucheon, Korea); and the number of both live and dead nematodes was counted under a Nikon SM2 1000 microscope (Tokyo, Japan) after 24 h. Nematodes were considered dead when no response was observed after several repeated touches with a nematode-picking needle. The test comprised of four replicates for each chemical compound concentration, and was repeated twice.

Paralysis test. Paralysis tests were also conducted to evaluate the effect of the various formulations of emamectin benzoate at lower concentration levels. Low concentration serial dilutions of the test formulations were prepared to give five different test concentrations as described above (0.01075-1.075 μg/ml). The lowest concentration being the amount of the active ingredient capable of causing paralysis of 1-10% of the test population, and the highest value represented the concentration at which 90-100% paralysis of the test population could be registered after a 24-h treatment. Experimental setup was conducted in the same way as described above in the sublethal toxicity test. The multi-well culture plates (SPL Life Sciences Co., Ltd.) were wrapped with aluminum foil and kept at 25ºC in the growth chamber (HB 303 DH-0, Han Baek); and the number of paralyzed nematodes was counted under a Nikon SM2

1000 microscope after 24 h. The experiments were set up with four replicates for each chemical compound concentration, and were repeated twice. Nematodes were considered paralyzed when no motion was observed but could respond after being prodded severally with a nematode-picking needle.

Reproduction inhibition test. Two bioassay studies were conducted to test the effect of the formulations on nematode reproduction potential. In the first bioassay, nematode populations (mixture of all stages and sexes) were exposed to the different concentrations of the chemical formulations (1.075, 0.1075, 0.01075, and 0.001075 μg/ml) for 24 h at 25°C, as described by Cheng et al. (2017). The nematodes were subsequently rinsed three times with sterilized water in a 10-ml centrifuge tube to get rid of the treated chemical, before being homogenized to the required nematode numbers for experimentation. One hundred nematodes were inoculated onto a uniform *B. cinerea* culture in a Petri dish. Fresh untreated nematodes were used in the control test. The experiment was arranged with four replicates for each chemical concentration and was repeated twice. The inoculated *B. cinerea* culture plates were kept for 10 days at 25°C in the growth chamber (HB 303 DH-0, Han Baek). Nematodes were extracted from all the Petri dish contents using the Baermann funnel method. Enumeration of the final populations was done under Nikon SM2 1000 microscope, and the nematode reproduction factor (Pf/Pi [Pf, final nematode population; Pi, initial nematode population]) was calculated for each formulation.

The second bioassay was conducted the same way as described above but, in pine tree twigs according to the method of Shin et al. (2015). Briefly, nematode populations were pre-exposed to the above-mentioned chemical concentration preparations for 24 h at 25°C, and were subsequently washed with sterilized water before being homogenized to the targeted nematode numbers. Twenty-centimeter-long fresh twigs were cut from *P. densiflora* tree stands in Gumi area, Gyeongsangbuk-do Province, Republic of Korea. The twigs were sealed at both ends with paraffin to minimize moisture loss and rapid drying. Small drilled holes (diameter × depth, 0.7 × 0.5 cm) were created in the middle of the twig and cotton wool was inserted to serve as a source of infection after nematode injection. One thousand nematodes were injected into the twigs through the cotton wool before carefully sealing off with parafilm. The treated areas were wrapped with aluminum foil before transferring the twigs into the growth chamber at 25ºC (HB 303 DH-0, Han Baek). The treatments were replicated and repeated the same way as noted above. The experiment was terminated after 30 days. Nematodes were extracted from all the twigs (twig portions of 5 cm from the treatment area in both directions were cut into small discs before extraction) using the Baermann funnel method. Nematode populations were counted under Nikon SM2 1000 microscope, and the nematode reproduction factor (Pf/Pi) (Pf, final nematode population; Pi, initial nematode population) was calculated for each formulation.

Data analysis. Data were tested for homogeneity of variance and subsequently subjected to analysis of variance using SAS statistical package version 9.4 (SAS Institute Inc., Cary, NC, USA). The targeted lethal concentration values ($LC_{10,\ 20,\ 50,\ 90,}$ and $_{95}$) were determined using probit analysis. There were no statistical differences between the two repetitions in nematode reproduction and paralysis data. Thus, all replications were used in analysis (n = 8 replications). Treatment means of nematode reproduction and paralysis data were subjected to analysis of variance according to the general linear model procedure and were compared using Tukey's honestly significant difference at $P \leq 0.05$, while the reproduction factors (Pf/Pi) of populations recovered from each chemical treatment were calculated in Microsoft Excel (Microsoft Corporation).

Results

Quantified emamectin concentration and sublethal toxicity. The analysis of the actual concentration of the emamectin benzoate in the tested formulations showed that the quantified concentrations were relatively higher than the indicated concentrations on the respective product labels (Table 1).

Generally, mortality of *B. xylophilus* consistently increased with increase in concentration in all the tested formulations. The lethal concentration values ($LC_{10,\ 20,\ 50,\ 90,}$ and $_{95}$) of the tested chemicals significantly differed among the formulations despite uniformity of original concentrations (Table 2). Emamectin benzoate formulations conformed to three groups based on relative similarity in sublethal efficacies (group 1: A, D, and E; group 2: B, F, G, I, K, L, M, and N; and group 3: C, H, J, O, and P) (Fig. 1). Formulations in group 1 were highly effective, with LC_{95} of as low as 0.00768-0.01443 mg/ml. Significant variations in sublethal toxicities were more evident in group 2 formulations (Table 2). For instance, LC_{95} values ranged between 0.03202 mg/ml in I and 0.07236 mg/ml in F. The LC_{10} and LC_{20} of group 1 and 2 were generally ≤ 0.00933 mg/ml, except for formulation K (0.01012 and 0.01339 mg/ml for LC_{10} and LC_{20}, respectively). Group 3 constituted the

Table 2. The toxicity of emamectin benzoate formulations against *Bursaphelenchus xylophilus*

Code	Lethal concentration (95% FL) (mg/ml)				
	LC_{10}	LC_{20}	LC_{50}	LC_{90}	LC_{95}
A	0.00221 (0.00191-0.00249)	0.00293 (0.00262-0.00322)	0.00503 (0.00469-0.00538)	0.01143 (0.01029-0.01302)	0.01443 (0.01271-0.01691)
B	0.00625 (0.00568-0.00682)	0.00891 (0.00825-0.00956)	0.01753 (0.01661-0.01848)	0.04915 (0.04575-0.05316)	0.06583 (0.06049-0.07229)
C	0.11998 (0.11204-0.12734)	0.14422 (0.13654-0.15129)	0.20509 (0.19895-0.21073)	0.35057 (0.34252-0.35966)	0.40811 (0.39569-0.4226)
D	0.00646 (0.0064-0.00652)	0.00668 (0.00662-0.00672)	0.0071 (0.00706-0.00714)	0.00781 (0.00774-0.00788)	0.00802 (0.00794-0.00811)
E	0.00411 (0.00395-0.00424)	0.00451 (0.00438-0.00463)	0.0054 (0.0053-0.0055)	0.00711 (0.00695-0.00729)	0.00768 (0.00747-0.00793)
F	0.00403 (0.00358-0.00449)	0.00623 (0.00566-0.0068)	0.01428 (0.01337-0.01523)	0.05056 (0.04633-0.05565)	0.07236 (0.06524-0.08116)
G	0.00342 (0.00305-0.00378)	0.00509 (0.00466-0.00551)	0.01091 (0.01027-0.01158)	0.03477 (0.03151-0.03887)	0.04831 (0.04294-0.05526)
H	0.11598 (0.1071-0.12418)	0.13941 (0.13071-0.14735)	0.19821 (0.19105-0.20472)	0.33875 (0.33093-0.34756)	0.39433 (0.38226-0.40852)
I	0.00148 (0.0006266-0.00254)	0.00234 (0.00114-0.00371)	0.00568 (0.00355-0.00768)	0.02185 (0.01933-0.0241)	0.03202 (0.02914-0.03576)
J	0.10781 (0.10206-0.11324)	0.13152 (0.12591-0.1368)	0.19236 (0.18731-0.19729)	0.34322 (0.33209-0.35581)	0.40444 (0.38848-0.42288)
K	0.01012 (0.00932-0.0109)	0.01339 (0.01252-0.01425)	0.02289 (0.02176-0.02405)	0.05175 (0.04845-0.05562)	0.06521 (0.06043-0.07096)
L	0.00661 (0.00576-0.00743)	0.00933 (0.00837-0.01024)	0.01804 (0.01686-0.01924)	0.04928 (0.04499-0.05471)	0.06552 (0.05871-0.07446)
M	0.00454 (0.00415-0.00493)	0.00645 (0.00601-0.00689)	0.01263 (0.01205-0.01323)	0.03514 (0.0329-0.03777)	0.04696 (0.04339-0.05125)
N	0.00587 (0.00532-0.00643)	0.00839 (0.00775-0.00902)	0.0166 (0.01573-0.01749)	0.0469 (0.04363-0.05077)	0.06295 (0.05777-0.06926)
O	0.13299 (0.12751-0.138)	0.15331 (0.14839-0.15785)	0.20124 (0.197-0.20551)	0.30451 (0.29425-0.31658)	0.34246 (0.32861-0.359)
P	0.21172 (0.20786-0.2151)	0.22382 (0.22065-0.2267)	0.24893 (0.24607-0.25198)	0.29268 (0.28672-0.2999)	0.30643 (0.29912-0.3154)

Lethal concentrations ($LC_{10, 20, 50, 90,}$ and $_{95}$ [mg/ml]) data were calculated after 24 h of treatment with emamectin benzoate formulations ($n = 8$). FL, fiducial limits.

least effective formulations, with LC_{95} values of as high as 0.30643-0.40811 mg/ml. Their LC_{10} and LC_{20} ranged between 0.10781 and 0.22382 mg/ml.

Paralysis test. Twenty-four h after treatment with varying concentrations of the listed emamectin benzoate formulations, variations in nematode paralysis were evident (Table 3). Generally, nematode paralysis occurred at concentrations lower than manufacturers' recommendation dose (1,000-fold lower than the recommendation dose). At the test dose of 1.075 μg/ml, rates of paralysis were highest in treatments A, B, C, D, E, F, G, and O, and these were significantly different from other treatments at the same rate of application ($F = 192.01$; $df = 16, 119$; $P < 0.0001$). Similarly, at a dose range of 0.0268-0.1075 μg/ml, significantly high rates of paralysis were observed in treatments with

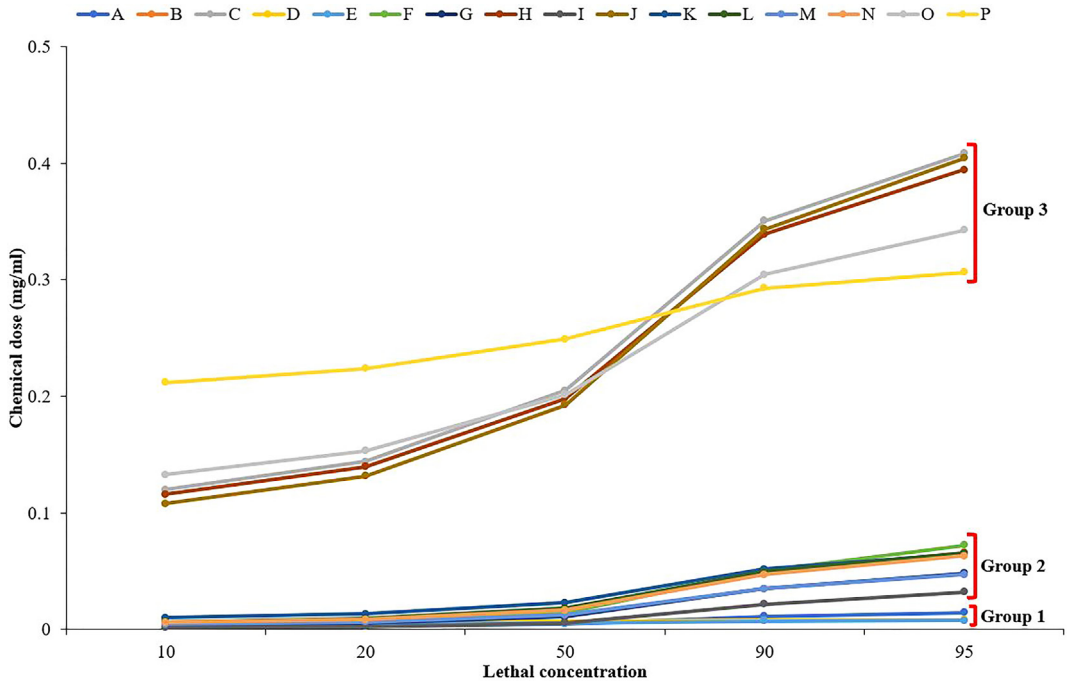

Fig. 1. Toxicity of emamectin benzoate formulations against *Bursaphelenchus xylophilus* after 24-h treatment.

formulations A, B, C, D, E, F, G, and O (paralysis rate of >90% except for formulation D, with paralysis rate of 87% at a dose of 0.0268 µg/ml) ($F = 366.23$; $df = 16, 119$; $P < 0.0001$ for 0.0268 µg/ml, and $F = 252.32$; $df = 16, 119$; $P < 0.0001$ for 0.1075 µg/ml). Nematode paralysis rates caused by formulations A, B, C, D, E, F, G, and O at the highest tested dose (1.075 µg/ml) were comparatively similar to the rates recorded at a lower dose of 0.1075 µg/ml (95.7-99.7% vs. 94.6-99.2%). Moderate paralysis rates were recorded in treatments with formulations H, I, J, K, L, M, N, and P at dose of 0.1075-1.075 µg/ml, albeit with significant variations (60-89%) (Table 3).

At the lower dose of 0.0134 µg/ml, only five formulations (A, C, D, G, and O) sustained paralysis effect of above 80%, with the highest effect (96%) recorded in treatment with formulation C ($F = 153.62$; $df = 16, 119$; $P < 0.0001$). Generally, the rate of paralysis was comparable to the pattern followed by sublethal efficacy test albeit with significant deviations. Most formulations with high sublethal efficacies (group 1 and 2) generally caused significant paralysis levels at the tested application doses. Disparities were however evident in some effective and less effective formulations. For instance, formulation E with a high sublethal toxicity was found to cause a lower paralysis rate of 35% at a 0.0134 µg/ml application dose compared to a less toxic formulation C that caused a high nematode paralysis rate of 96% at a similar application dose. At the lowest dose of 0.01075 µg/ml, formulations A and D showed nematode paralysis rates of 30%. All other formulations caused nematode paralysis rates of less than 20% (2-18%) ($F = 26.41$; $df = 16, 119$; $P < 0.0001$).

Reproduction inhibition test

Reproduction inhibition on *B. cinerea*. Treatment with varying concentrations of emamectin benzoate formulations inhibited the reproduction of *B. xylophilus* on *B. cinerea*. Nematode reproduction was completely inhibited in populations pre-exposed to high concentrations (1.075 and 0.1075 µg/ml) of all the formulations (reproduction factor = 0: data not shown). At a 0.01075 µg/ml application dose, reproduction inhibition was more pronounced in populations treated with formulations A, B, C, D, E, F, G, H, I, J, L, and O (reproduction factor = 0.11-2.30) (Fig. 2A). Formulations D and E showed the highest efficacy in inhibiting nematode production (reproduction factor = 0.11 and 0.14, respectively). Significant reproduction was also evident in populations treated with formulations K, M, and N (reproduction factor = 16.3-20.8), and the highest reproduction was recorded in a population treated with formulation P (reproduction factor = 89.7). There were no significant differences between nematode numbers recovered from treatment with formulation P and the control ($F = 1.33$; $df = 1.8$; $P = 0.2822$). The nematode numbers in all other emamectin benzoate treatments were significantly lower than that of

Table 3. Comparison of paralysis rates induced by emamectin benzoate formulations against *Bursaphelenchus xylophilus*

Code	Rate of paralysis (%, mean ± SD)				
	1.075 µg/ml	0.1075 µg/ml	0.0268 µg/ml	0.0134 µg/ml	0.01075 µg/ml
A	97.0 ± 1.2 ab	95.3 ± 1.7 a	94.7 ± 1.5 ab	93.3 ± 4.2 a	29.9 ± 6.3 a
B	98.8 ± 0.9 a	98.3 ± 1.6 a	97.7 ± 1.1 a	13.9 ± 2.5 efgh	9.6 ± 4.5 cdef
C	98.9 ± 1.3 a	98.2 ± 1.6 a	98.6 ± 0.8 a	96.4 ± 1.6 a	12.3 ± 4.7 bcde
D	95.6 ± 1.2 ab	95.6 ± 2.1 a	86.8 ± 3.7 b	82.4 ± 5.3 ab	29.7 ± 7.3 a
E	97.5 ± 0.8 ab	96.0 ± 1.2 a	93.7 ± 2.0 ab	34.9 ± 10.6 d	18.4 ± 6.9 b
F	95.7 ± 1.6 ab	94.6 ± 2.6 a	92.6 ± 7.0 ab	67.9 ± 10.5 bc	7.7 ± 1.8 defg
G	99.6 ± 0.6 a	98.4 ± 0.7 a	96.9 ± 1.5 a	94.5 ± 1.7 a	5.4 ± 1.4 efg
H	70.3 ± 11.7 ef	62.2 ± 6.6 de	46.3 ± 3.8 d	25.4 ± 3.7 de	15.9 ± 4.2 cbd
I	78.0 ± 3.4 de	70.2 ± 5.0 c	28.6 ± 8.1 ef	10.1 ± 2.2 fgh	5.7 ± 1.3 efg
J	88.9 ± 2.6 bc	79.8 ± 5.9 b	62.7 ± 5.7 c	53.5 ± 15.3 c	12.8 ± 1.5 bcde
K	75.7 ± 3.9 de	68.7 ± 6.4 cd	12.9 ± 3.8 g	2.2 ± 1.5 gh	1.5 ± 0.8 gf
L	81.5 ± 10.3 cd	67.2 ± 6.2 cde	55.9 ± 8.7 c	14.4 ± 8.9 efgh	6.9 ± 2.7 efg
M	70.3 ± 7.1 ef	67.0 ± 8.1 cde	22.9 ± 6.2 f	14.9 ± 13.9 efg	7.6 ± 9.5 de
N	71.9 ± 8.6 ef	69.5 ± 3 cd	32.7 ± 8.6 e	25.3 ± 7.6 de	17.0 ± 4.6 bc
O	99.7 ± 0.4 a	99.2 ± 0.6 a	98.6 ± 1.3 a	95.3 ± 2.9 a	6.2 ± 3.0 efg
P	66.0 ± 3.3 f	60.1 ± 7.6 e	26.6 ± 8.6 ef	20.3 ± 17.5 def	7.7 ± 6.4 defg
Control	0.0 ± 0.0 g	0.0 ± 0.0 f	0.0 ± 0.0 h	0.0 ± 0.0 h	0.0 ± 0.0 g

Paralysis rate data were analyzed after 24 h of treatment with emamectin benzoate formulations. Mean values followed by the same letters indicate similar groups (Tukey's honestly significant difference, $P < 0.05$) ($n = 8$).

the control (reproduction factor = 109.4) ($F = 57.63$; $df = 16, 68$; $P < 0.0001$).

At the least tested concentration of 0.001075 µg/ml, significant variations in nematode reproduction were more evident among the formulations (Fig. 2B). Formulations D and E were the most effective compounds (reproduction factor = 9.8 and 9.9, respectively). Relative reproduction was recorded in formulations A, C, G, H, I, J, L, and O, with a reproduction factor range of 15.2-27.9 (Fig. 2B). Similar to high concentration tests, the highest reproduction was recorded in a population treated with formulation P (reproduction factor = 98.6), and there were no significant differences when compared with the control ($F = 0.04$; $df = 1.9$; $P = 0.8476$). Nematode numbers in all other emamectin benzoate treatments were significantly lower than populations recovered in the control ($F = 22.74$; $df = 15, 67$; $P < 0.0001$).

Reproduction inhibition in twig cuttings. Generally, a similar trend of reproduction inhibition was evident in the pine twig treatments. Nematode reproduction was completely inhibited in treatments with high concentrations (1.075 and 0.1075 µg/ml) (reproduction factor = 0: data not shown). However, at a 0.01075 µg/ml application dose, differences in efficacy of the formulations were observed. All but C, I, and O formulations significantly inhibited nematode production in pine twigs (reproduction factor range of 0.01-0.52). Formulations D and E were the most effective in inhibiting nematode reproduction (reproduction factor = 0.05 and 0.01, respectively) (Fig. 3A). Significant reproduction was recorded in formulations C, I, and O, and the highest reproduction was recorded in formulation C, with a reproduction factor of 4.9. The nematode numbers recovered from all chemical-treated populations were significantly lower than in control (reproduction factor = 12.7) ($F = 19.51$; $df = 16, 34$; $P < 0.0001$). It is also important to note that low nematode numbers were recorded in all pine twig treatments than on *B. cinerea.*

Similar to the reproduction rates recorded at low concentration of 0.001075 µg/ml on *B. cinerea*, significant nematode numbers were recorded in pine twigs as well. Nematode numbers varied between formulations (Fig. 3B). Formulations A, B, D, E, G, L, and M were more effective in inhibiting nematode reproduction in pine twigs (reproduction factors 0.8-2.2). Consistent with the above findings, formulation D showed the highest negative effect on nematode reproduction (reproduction factor = 0.8). Formulations A, E, and L were also more effective when

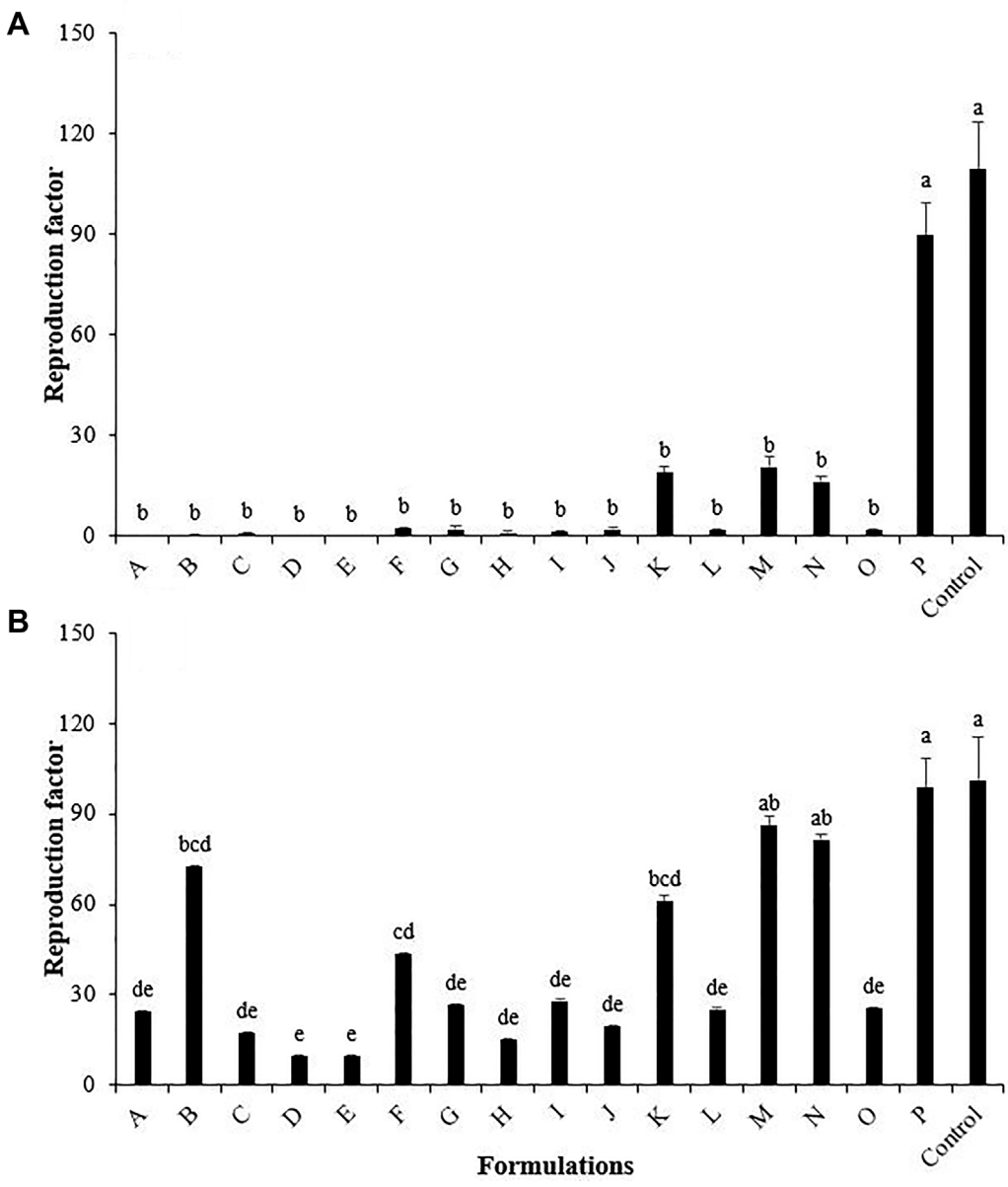

Fig. 2. Effect of emamectin benzoate formulations on reproduction of *Bursaphelenchus xylophilus* on *Botrytis cinerea* after 10 days treated with a 0.01075 µg/ml dose (A); treated with a 0.001075 µg/ml dose (B). Error bars indicate the standard error of the means. Bars with the same letter are not significantly different (Tukey's honestly significant difference, $P < 0.05$). Reproduction factor = Pf/Pi (Pf, final nematode population; Pi, initial nematode population).

compared with all the other formulations (reproduction factor of 1.1, 1.5, and 1.6, respectively). Contrastingly, relatively higher reproduction factors were recorded in treatments with formulations H, I, and P (reproduction factors: 10.2, 7.7, and 8.8, respectively). Highest reproduction was recorded in a population treated with formulation C (reproduction factor = 11.8), and no significant differences were recorded between nematode populations recovered from treatment with formulation C and the control ($F = 0.42$; $df = 1.4, 67$; $P = 0.5507$). Nematode populations recovered from all other emamectin benzoate treatments were significantly lower than the numbers recovered from the control ($F = 10.48$; $df = 15, 32$; $P < 0.0001$).

Discussion

Like in many published studies, our results underline the nematicidal efficacy, and the population suppressive effect

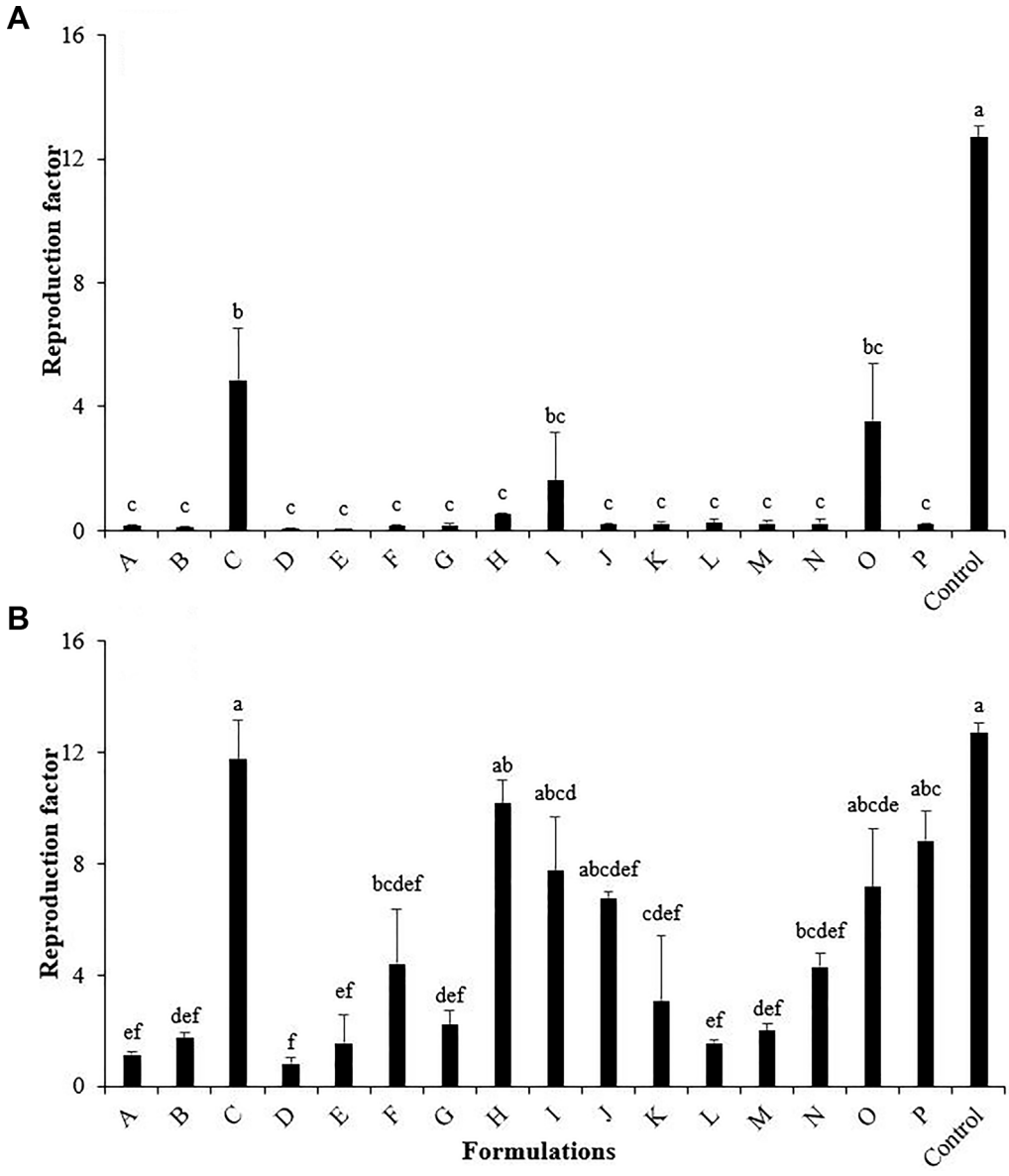

Fig. 3. Effect of emamectin benzoate formulations on reproduction of *Bursaphelenchus xylophilus* in pine twigs after 30 days treated with a 0.01075 μg/ml dose (A); treated with a 0.001075 μg/ml dose (B). Error bars indicate the standard error of the means. Bars with the same letter are not significantly different (Tukey's honestly significant difference, $P < 0.05$). Reproduction factor = Pf/Pi (Pf, final nematode population; Pi, initial nematode population).

of emamectin benzoate against *B. xylophilus* and many other pathogens and (or) pests (Bi et al., 2015; Khan et al., 2018; Sousa et al., 2013; Takai et al., 2000). Emamectin benzoate is an insecticidal, nematicidal macrocyclic lactone; a gamma-aminobutyric acid receptor agonist with proven extreme potency against *B. xylophilus* among several chemical substances (Sousa et al., 2013; Takai et al., 2000, 2001). The compound has been proven to be even more active in suppressing populations of *B. xylophilus* by lowering its fecundity and inhibiting egg hatchability, in addition to the lethal effect on the nematode (Bi et al., 2015). Importantly, compared to other available control agents, emamectin benzoate is recognized to be the most effective agent to control PWN, especially as a trunk injection compound. Its efficacy is touted to last more than 2 years in treated pine stands (Kwon et al., 2021; Sousa et al., 2013). However, our results highlight the fact that its performance is most likely dependent on the intricacies of its formulation. In our results, only formulations in group 1 (D, E, and A) attained nematicidal efficacy that

are comparable to those reported in tests performed using technical grade emamectin benzoate compound (99% and 95% mortality of *B. xylophilus* at 8.0 mg/l and 2 mg/l in Bi et al., 2015 and Wang et al., 2022, respectively; and LC_{90} of 18.20 mg/l against *Meloidogyne incognita* (Cheng et al., 2015). Reproduction inhibition induced by group 1 formulations was also comparable to the effective inhibition dose reported by Takai et al. (2000). However, formulations in group 2 and especially, group 3 demonstrated a much lower efficacy in terms of induced mortality and reproduction inhibition.

Emamectin benzoate is a derivative of avermectins (abamectin); sets of macrocyclic lactone isomers that are isolated from the fermentation of *Streptomyces avermitilis* (Guo et al., 2015). Avermectin production is regulated by complex mechanisms that involve intricate multistep biosynthetic framework. In the recent past, much attention has been given to the biotechnological overproduction, and structure diversification of avermectins to produce other related effective derivatives including emamectin benzoate (Zhuo et al., 2014). These avermectins undergo a purification process to enhance the content of B1a. However, impurities are still not easily separated during production; and these impurities might have an influence on the crystallization process and eventual performance of the final product (Liu et al., 2006; Zhang et al., 2020). For instance, in their analysis of generic products, Cheng et al. (2002) noted that whereas generic products of abamectin contained 2 to 6 times more total avermectin content that the registered 2% avermectins B1a/B1b, significant variations in the ratios of B1a/B1b were evident, in addition to the presence of other impurities. Emamectin benzoate is a mixture of no less than 90% (4″R)-4″-deoxy-4″-(methylamino)-avermectin B1a benzoate and no more than 10% (4″R)-4″-deoxy-4″-(methylamino) avermectin B1b benzoate salts (Wolterink et al., 2012). These ratios should technically be maintained in the final products for better performance. Biotechnological advances in the recent years have seen producers specialize in improved overproduction of avermectins and their derivatives using unique biotechnological processes, intricacies, and applications. The differences in technologies and intricacies may have an influence on the performance of the product formulations.

It is also important to note that toxicity or effectiveness of chemical compounds against the target species is normally determined using threshold lethal concentrations of the pure active ingredients of commercialized products (Cox and Surgan, 2006; U.S. Environmental Protection Agency, 2006). However, commercial formulations are an assortment of both the active ingredients mixed with non-nematicidal ingredients, often referred to as “inert” or “other” ingredients. These ingredients constitute more than 97% in volume of the currently studied formulations. Of recent, the assessment of health and environmental related hazards resulting from interactions between various chemical substances lanced in commercial pesticide formulations is increasingly being encouraged (Nagy et al., 2020). However, the approval of many pesticide products for agricultural use is mostly still reliant on determined toxicity of the individual active ingredients, thereby ignoring the possible interactive effects that may be definable in various formulations. These “inert” additives which act as solvents, synergists, surfactants, and solubilizers among other functions, are expected to improve the delivery, stability, and effectiveness of the nematicidal ingredient. However, numerous studies have already shown that inert ingredients may instead significantly enhance or lessen the toxicity of chemical formulations. And therefore, they may affect significant toxicologic endpoints, including neurotoxicity, genotoxicity, eventual disruption in hormone function in target- and even non-target organisms, and phytotoxicity (Cox and Surgan, 2006; Schmuck et al., 1994). Of interest, companies are not mandated to indicate the actual constituents of these mixtures on the product labels, unless the ingredients used are classified as highly toxic (Cox and Surgan, 2006). And yet, there is no uniformity in the choice of inert ingredients to be used in similar commercial formulations. The choice of these additives is dependent on the preference of the producer. In our results for instance, formulations C and O, that are among the least effective compounds in terms of sublethal toxicity, displayed a significantly higher paralysis activity on the PWN compared to some of the effective group 1 formulations, especially at a lower dose of 0.0134 µg/ml. Such disparities are most likely caused by the enhanced and sustained bioactivity of the preferred inert ingredients to the nematode.

Ideally, there should be no substantial differences between similar formulations of the same active ingredient concentration. However, as indicated in the current and many other studies, it is not always the case. For instance, Mayer and Ellersieck (1986) compared the toxicity of 161 technical grade pesticides to their formulations and showed that overall toxicity was not affected in 57%, decreased in 11%, and increased in 32% of the cases. Nagy et al. (2020), in their review identified another eight studies that demonstrated reduced toxicity of product formulations in relation to their active ingredient. The disparities were attributed to potential antagonistic effect between

the constituents. Li et al. (2015) demonstrated that the cytotoxicity of chlorfluazuron in Tn5B1-4 cells could be reduced by PEG6000, but could also be enhanced by Tween 80. Also, Azadieno, a product formulation of amitraz was shown to induce statistically significant genotoxic effect at lower concentrations than active ingredient amitraz alone (Padula et al., 2012). In the current study, it is likely that the preferred inert ingredients (or technical grade chemical) by the various producers translates into the significant differences in the bioactivity of emamectin benzoate against the PWN. It might also account for the specific grouping of the formulations based on the effect of the inert ingredients on the bioactivity of emamectin benzoate. An analysis of the actual concentration of the emamectin benzoate in all the tested formulations showed that the quantified concentrations (B1a and B1b) were relatively higher than the indicated quantities on the respective labels. It is therefore evident that emamectin concentration in all the formulations is sufficient to induce the intended effect of PWN control even though individual ratio of B1a/B1b were not determined. Thus, the plausible explanation is that the excipients of the formulated products that belong to the moderate, and least effective groups potentially interfered with the nematicidal activity of the active ingredient.

Essentially, emamectin benzoate is marketed as a trunk injection agent for control of the PWN. However, the transportation and bioavailability of the chemical-active ingredient in these trees largely depend on the water solubility of the chemical (Matsuura, 1984; Takai et al., 2001). The water solubility of emamectin benzoate is known to be very low (24 mg/l) (Tomlin, 2009). And thus, the formulations are normally prepared with improved water solubility. For instance, Matsuura (1984) reported that only compounds with a water solubility of more than 1000 mg/l were able to prevent wilting of pine trees that had been artificially inoculated with PWNs. Therefore, in addition to other inert ingredients, solubilizers are also deemed to be a crucial part of emamectin benzoate formulations (Takai et al., 2001). And thus, all these additives interact to bring about the final chemical complexity in the final product formulation. Therefore, there is a need to balance the trade offs in terms of potential antagonistic effects which may arise as result of the large number of constituents lanced in a single formulation. Based on our results and other published literature (Cox and Surgan, 2006; Li et al., 2015; Mayer and Ellersieck, 1986; Nagy et al., 2020; Padula et al., 2012), it is evident that there are inconsistencies in the potency of similar product formulations with the same active ingredient concentration against the target organism. Ignoring the possible drawbacks derived from the interaction between the active ingredient and other additives in various commercial product formulations of even similar active ingredient concentration culminates in misjudgment of the final toxicological effect on the target organism. Therefore, in order to avoid pesticide/nematicide failure, and the possibilities of potential resistance development in target organisms due to continued exposure to ineffective formulations, inert ingredients and the intricacies used in formulation preparations require rigorous testing complementary to the toxicity studies of the active ingredients alone. Product label disclosure of the main inert ingredients would facilitate these needed studies, especially by independent scientists. This would allow independent toxicity research, and proper environmental risk assessment studies to be carried out, and enable proper conclusions to be made on the potential effects of inert additives on the pesticide formulation performance and the environment in general.

Conflicts of Interest

No potential conflict of interest relevant to this article was reported.

Acknowledgments

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