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Sensitivity of *Meloidogyne incognita* second-stage juvenile hatch, motility and viability to pure cucurbitacins and cucurbitacin-containing phytonematicides

Zakheleni P Dube[1]*, Phatu W Mashela[1] and Dirk de Waele[1,2]

[1] Green Biotechnologies Research Centre, University of Limpopo, Sovenga, South Africa
[2] Laboratory of Tropical Crop Improvement, Department of Biosystems, Faculty of Bioscience Engineering, University of Leuven (KU Leuven), Heverlee, Belgium; and Unit for Environmental Sciences and Management, North-West University, Potchefstroom, South Africa
** Corresponding author, email: zakheleni_dube@yahoo.com*

Previous claims suggested that pure active ingredients from botanicals were less effective in pest management than their fermented crude-extracts. The objective of this study was to compare the toxicity of pure (98%) cucurbitacin A and B on nematode bioactivities with those of their fermented crude-extracts, the Nemarioc-AL and Nemafric-BL phytonematicides, respectively. Purified active ingredients were each diluted to 0.00, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25 and 2.50 μg ml^{-1} and their fermented crude-extracts to 0.0%, 0.5%, 1.0%, 1.5%, 2.0%, 2.5%, 3.0%, 3.5%, 4.0%, 4.5% and 5.0% phytonematicide. The exposure time for second-stage juveniles (J2) hatch, motility and viability of the J2 to each concentration was 24, 48 and 72 h. The overall sensitivities (Σk) of J2 hatch to Nemarioc-AL, cucurbitacin A, Nemafric-BL and cucurbitacin B were 1, 30, 5 and 2 units, respectively, for motility 7, 12, 2 and 12 units, respectively, and for viability 2, 4, 1 and 4 units, respectively. Generally, nematodes displayed high sensitivities to cucurbitacin-containing phytonematicides than to their respective purified active ingredients (cucurbitacins). In conclusion, the cucurbitacin-containing phytonematicides were more effective in nematode suppression than their purified active ingredients.

Keywords: botanicals, CARD, *Cucumis* species, *k*-value, plant extracts, triterpenoids

Introduction

During the past decade, efforts to develop botanicals as biopesticides have increased (Okwute 2012). As a result, a number of promising biopesticides have been identified (Manju and Sankari Meena 2015). Nonetheless, many issues remain to be solved before these biopesticides can be effectively applied. One of these issues is the claim that the purified active ingredients from botanicals are less effective compared with the fermented crude-extracted biopesticides (Javed et al. 2008; Okwute 2012; Chaudhary et al. 2013). For instance, the Aza formulation, which contains azadirachtin, a purified ingredient from neem seed, showed no effect on the motility and viability, and a lower effect on second-stage juveniles (J2) hatch of root-knot nematode (*Meloidogyne* species) compared with crude neem cake and leaf extracts (Javed et al. 2008). Grandison (1992 cited by Javed et al. 2008) also did not observe any direct effects of purified substances from neem seed kernels, such as aza, salanine and nimbin, on J2 of *Meloidogyne javanica*.

The first step in determining the bioactivity of plant extracts in most studies consists of *in vitro* bioassays of purified substances. The inactivity of some of the purified substances resulted in potential phytonematicides failing to move beyond *in vitro* bioassays, limiting progress in the development of phytonematicides. Okwute (2012) suggested two causes for the observed inactivity of purified substances: (1) less desirable solvents used during purification and (2) the bioactivity of crude extracts is based on synergism among some of their ingredients.

The Curve-fitting Allelochemical Response Data (CARD) computer-based model was developed to assess responses of organisms toward increasing concentration of allelochemicals (Liu et al. 2003). Responses of plant-parasitic nematodes to increasing concentration of phytonematicides have been characterised by density-dependent growth patterns, which were quantified by quadratic curves and seven biological indices (Liu et al. 2003). The sensitivity index (k) has been described as the number of $\ln(D + 1)$ transformations, where k-values serve as biological indicators of the degree of sensitivity of test organisms to increasing concentration of allelochemicals. The k index has been used to measure the degree of toxicity of phytonematicides to crops (Mafeo and Mashela 2010; Mafeo et al. 2011; Pelinganga et al. 2013; Mashela et al. 2015). Usually, k-values start from zero and increase as discrete numbers when the sensitivity of the test organism to the allelochemical decreased (Liu et al. 2003). Thus, the sensitivity of the test organism to the allelochemical is inversely proportional to the k-value. The overall sensitivity (Σk) is the sum of all the k-values associated with a specific test organism and, in the case of plants, it is the sum of all k-values of the tested organs (Mashela et al. 2015).

The sensitivity of *Meloidogyne incognita* to cucurbitacin-containing phytonematicides and how this sensitivity

compares with that to the purified active ingredients of these phytonematicides using the CARD-generated sensitivity index has not been examined. The objective of this study was to compare the bioactivity of cucurbitacin A and cucurbitacin B on J2 hatch, motility and viability of *M. incognita* J2 with the bioactivity of the crude-extracted phytonematicides Nemarioc-AL and Nemafric-BL, respectively, of which these cucurbitacins are one of the ingredients.

Materials and methods

Preparation of the pure extracts and crude-extracted phytonematicides

Purified (ca. 98% purity) cucurbitacin A and B were obtained from ChemFaces (Wuhan, China). Approximately 1 000 μg of each cucurbitacin was dissolved in 5 μL methanol to enhance solubility prior to adding 1 mL distilled water to make a stock solution. In two separate trials of cucurbitacins, different concentrations of cucurbitacin A and B made from diluting the stock solution with distilled water (0.00, 0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 1.75, 2.00, 2.25 and 2.50 μg cucurbitacin mL^{-1} distilled water) were each pipetted into a 9-cm-diameter petri dish. Nemarioc-AL and Nemafric-BL were prepared by Effective Microorganisms™ (EM™) fermentation of oven-dried ground fruits from *Cucumis myriocarpus* and *C. africanus*, respectively (Pelinganga et al. 2012). The two *Cucumis* species were propagated as described by Shadung (2016). Of each phytonematicide, 10 concentrations (0.50%, 1.00%, 1.50%, 2.00%, 2.50%, 3.00%, 3.50%, 4.00%, 4.50% and 5.00%) were made in distilled water. Distilled water and 0.005% methanol were included as controls in the bioassay with the pure extracts, while distilled water and EM™ (Microzone, Pretoria, South Africa) were included as controls in the bioassay with the crude-extracted phytonematicides.

Collection of Meloidogyne incognita *eggs and second-stage juveniles (J2)*

Dark-brown coloured egg masses of *M. incognita* were obtained from infected two-month-old tomato (*Solanum lycopersicum* L. 'Floradade') plants grown in soil in pots in a greenhouse. Egg masses were dislodged from the roots using a toothpick, placed in 1% NaOCl solution and shaken for 30 s to surface-sterilise the egg masses and remove the gelatin matrix, before thorough rinsing in distilled water. Surface-sterilised eggs were used for the J2 hatch bioassay. Freshly-hatched J2 were obtained by transferring surface-sterilised eggs in Petri dishes containing 10 mL distilled water and placed in an incubator at 25 ± 2 °C. J2 that hatched during the first 24 h were discarded. J2 that hatched during the subsequent 48 h were used in the bio-assay (Dube and Mashela 2016).

Effect on J2 hatch

The study was conducted at the Green Biotechnologies Research Centre, University of Limpopo, South Africa (23°53′10″ S, 29°44′15″ E) under *in vitro* conditions. The effect of the different concentrations of the pure extracts and the crude-extracted phytonematicides on the J2 hatch of the J2 was separately tested in 9-cm-diameter petri dishes. An average of 107 freshly collected eggs were transferred to each petri dish containing 10 mL concentration. The petri dishes were placed in a completely randomised design with three replications in an incubator at 25 ± 2 °C. The number of J2 that had hatched after 24, 48 and 72 h were counted using a stereomicroscope. The bioassay was repeated three times.

Effect on motility and viability of second-stage juveniles (J2)

The effect of the different concentrations of the pure extracts and crude-extracted phytonematicides on the motility and viability of J2 was examined following a modification of the method described by Wuyts et al. (2006). The assessments were carried out using 9-cm-diameter petri dishes containing 10 mL of each concentration. Approximately 450 freshly-hatched J2 were added to each concentration. The petri dishes were placed in a completely randomised design with three replications in an incubator at 25 ± 2 °C. After 24, 48 and 72 h, each petri dish was emptied into a counting chamber and the number of immobile J2 counted using a stereomicroscope. The J2 were considered immobile when no movement was observed during 2 s even after prodding with a needle. Concentrations were considered motile-inhibitive when significantly more J2 became immobilised compared with the controls (Wuyts et al. 2006). The immobile J2 were stained in 0.015% methylene blue for 1 h. All dark blue-stained J2 were considered dead (Saifullah 2002). The bioassay was repeated three times.

Statistical analysis

Number of hatched, immobile and dead J2 were $\log_{10}(x + 1)$ transformed to homogenise variances (Gomez and Gomez 1984) prior to analysis of variance using SAS 9.2 software (SAS Institute 2008). Significant treatment means for variables were further subjected to CARD modelling to generate sensitivity values (Mashela et al. 2015).

Results and discussion

The overall sensitivities (Σk) of J2 hatch to Nemarioc-AL, cucurbitacin A, Nemafric-BL and cucurbitacin B were 1, 30, 5 and 2 units, respectively (Table 1). In contrast, those for J2 motility were 7, 12, 2 and 12 units, respectively (Table 2), and for J2 viability were 2, 4, 1 and 4 units, respectively (Table 3). The bioactivity of the pure cucurbitacins and the fermented crude extract phytonematicides on J2 hatch, motility and viability of *M. incognita* J2 observed supports the results of other studies (Ibrahim et al. 2006; Lazzeri et al. 2004; Wuyts et al. 2006). Ibrahim et al. (2006) and Lazzeri et al. (2004) reported the nematostatic and nematicidal activity of pure chemical substances derived from plant extracts, whereas Javed et al. (2007) observed the same activities from crude extracts of other plants. Nevertheless, differences in sensitivities between the cucurbitacin-containing crude extracts and the pure cucurbitacins were observed.

The highest difference observed was the sensitivity of eggs to J2 hatch inhibition caused by Nemarioc-AL and cucurbitacin A, with Nemarioc-AL having a higher inhibitive effect compared with its active ingredient cucurbitacin A. In general, in terms of J2 hatch, motility and viability the eggs and J2 of *M. incognita* were more sensitive to the crude extracts than to their purified active

Table 1: Sensitivity of *Meloidogyne incognita* second-stage juvenile (J2) hatch to Nemarioc-AL phytonematicide, cucurbitacin A, Nemafric-BL phytonematicide and cucurbitacin B after 24, 48 and 72 h exposure

Biological index	Nemarioc-AL			Cucurbitacin A			Nemafric-BL			Cucurbitacin B		
	24	48	72	24	48	72	24	48	72	24	48	72
Threshold stimulation (D_m)	13.87	0.01	0.08	0.61	0.52	0.16	4.05	0.19	4.89	1.38	1.37	0.73
Saturation point (R_h)	12.70	0.02	0.16	0.41	0.64	1.12	0.70	0.09	0.37	1.06	0.68	0.26
0% inhibition (D_0)	13.29	0.04	0.25	0.82	1.28	2.24	3.08	0.60	3.00	2.28	1.71	0.76
50% inhibition (D_{50})	15.17	4.12	6.89	1.05	1.75	3.31	3.84	2.71	6.47	3.34	2.39	1.49
100% inhibition (D_{100})	20.77	9.92	23.09	1.12	1.92	4.28	4.94	10.90	12.37	4.40	3.07	3.02
R^2	0.96	0.96	0.95	0.86	0.86	0.81	0.97	0.96	0.98	0.90	0.88	0.95
Sensitivity index (k)	0	0	1	5	5	20	1	4	0	0	0	2
Overall sensitivity (Σk)		1			30			5			2	

Table 2: Sensitivity of *Meloidogyne incognita* second-stage juvenile (J2) motility inhibition to Nemarioc-AL phytonematicide, cucurbitacin A, Nemafric-BL phytonematicide and cucurbitacin B after 24, 48 and 72 h exposure

Biological index	Nemarioc-AL			Cucurbitacin A			Nemafric-BL			Cucurbitacin B		
	24	48	72	24	48	72	24	48	72	24	48	72
Threshold stimulation (D_m)	34.13	29.35	0.37	5.76	22.41	9.29	5.91	0.13	5.05	4.32	9.64	13.57
Saturation point (R_h)	291.67	587.00	22 462.14	1.64	1.68	1.71	285.84	4.13	263.53	1.70	0.30	1.68
0% inhibition (D_0)	454.57	880.50	22 462.51	5.34	13.73	7.21	297.66	6.26	273.63	4.71	5.27	9.31
50% inhibition (D_{50})	617.47	1 174.00	22 462.88	9.04	25.77	12.71	309.46	10.29	283.69	7.72	10.24	16.93
100% inhibition (D_{100})	780.37	1 467.50	22 463.28	12.74	37.82	18.21	321.26	14.22	293.69	10.73	15.21	24.56
R^2	0.97	0.97	0.97	0.99	0.99	0.99	0.96	0.96	0.98	0.99	0.99	0.99
Sensitivity index (k)	1	2	4	4	4	4	0	2	0	3	5	4
Overall sensitivity (Σk)		7			12			2			12	

Table 3: Sensitivity of *Meloidogyne incognita* second-stage juvenile (J2) viability inhibition to pure cucurbitacin A, Nemarioc-AL phytonematicide, cucurbitacin B and Nemafric-BL phytonematicide. R^2 = coefficient of determination

Biological index	Nemarioc-AL	Cucurbitacin A	Nemafric-BL	Cucurbitacin B
Threshold stimulation (D_m)	6.77	20.70	1.33	13.66
Saturation point (R_h)	1.56	1.69	1.53	1.67
0% inhibition (D_0)	5.73	12.89	27.43	9.34
50% inhibition (D_{50})	9.89	24.08	34.19	17.00
100% inhibition (D_{100})	14.06	35.28	41.53	24.67
R^2	0.99	0.99	0.99	0.99
Overall sensitivity (Σk)	2	4	1	4

ingredients. A similar trend was reported in several other studies (Javed et al. 2008; Okwute 2012). Okwute (2012) suggested two possible explanations to the different responses to plant crude extracts. The first explanation could be the undesirable solvents used during purification. For instance, the hexane extracts of neem (*Azadirachta indica*) seed were ineffective in causing losses of viability in *Heterodera glycines* J2 compared with water extracts, which were 240 times more effective (Silva et al. 2008). Alcohol extracts of *Chrysanthemum coronarium*, *Pistacia palestina*, *Matricaria discoidea* and *Tagetes patula* (Ibrahim et al. 2006), *Xanthium strumarium* and *Lantana camara* (Chaudhary et al. 2013) did not have any significant effect on *M. incognita* J2 hatch even though their aquous extracts were widely reported to be bioactive (Singh and Prasad 2014).

The second explanation could be that there are many active substances in plant crude extracts and that these may work in synergy to induce bioactivities on nematodes. Although the presence of multiple active substances in plant crude extracts is relatively well documented (Ibrahim et al. 2006; Nzanza and Mashela 2012), there is limited information on their synergistic interactions. Such synergistic effects were observed in NICOSAN, a drug used in the treatment of sickle cell anemia, when it was found to be less potent to sickle cells and more toxic to healthy cells when individual components were separately tested (Okwute 2012). Aza, a purified neem seed extract, affected motility and viability of *M. javanica* J2 less compared with neem cake and leaf crude extracts (Javed et al. 2008). Grandison (1992 cited by Javed et al. 2008) also reported the inactivity of salinine, Aza and nimbin, pure extracts of neem seed kernels, against the viability of J2 of *M. javanica*. These reports could explain the observed low sensitivities of *M. incognita* to pure active ingredients compared with their fermented crude extracts in the current study.

In this study, *M. incognita* response ranged from moderate to highly tolerant to the bioactivity of cucurbitacin A (k = 4–30 units), and similar trends were observed in cucurbitacin B (k = 2–12 units) tests. The difference in k-values between cucurbitacin A and B could be explained in terms of the polarity of cucurbitacin A, which readily breaks down to cucumin ($C_{27}H_{40}O_9$) and leptodermin ($C_{27}H_{38}O_8$). After 48 h, cucurbitacin A was still very bioactive as indicated by the

relatively higher sensitivities of the J2, but after 72 h the J2 became moderately sensitive, this being more evident in J2 hatch (Table 1). This was most probable due to accumulation of cucumin and leptodermin, which could also suggest that J2 hatch is less sensitive to these two substances compared with the parent compound, cucurbitacin A. Evidence of bioactivities of cucumin and leptodermin was reported in certain insects (Damalas 2011). In contrast, due to the stability of cucurbitacin B, *M. incognita* sensitivity remained high to this substance (Table 1).

Melodogyne incognita J2 hatch was highly sensitive to the two phytonematicides as shown by low *k*-values across all incubation periods, with a higher sensitivity to Nemarioc-AL compared with Nemafric-BL. Pelinganga (2013) observed similar trends with tomato plant variables being highly sensitive ($\Sigma k = 0$) to these two products, but with more plant organs having higher sensitivities to Nemarioc-AL compared with Nemafric-BL. Seed germination of various plants also had high sensitivities to crude extracts of *C. myriocarpus* fruit in granular formulation (Mafeo et al. 2011). The superior performance of traditionally produced crude-extracted phytonematicides over their purified ingredients reported in this study and in other studies does not conform to the registration requirements where purified materials are preferred.

Conclusion

The sensitivity index is used as an indicator of nematode sensitivity to cucurbitacin-containing phytonematicides and their purified active ingredients demonstrate that the latter were more than the former. Consequently, the products should be used in crude extract form. Given that crude plant extract preparation is easy to make, their high effectiveness makes them an ideal tool for the management of plant-parasitic nematodes in resource-poor farming communities.

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