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Compatibility of entomopathogenic nematodes with plant extracts and post-exposure virulence test under laboratory condition

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Abstract: The efficacy of both botanical pesticides and entomopathogenic nematodes (EPN) is largely dose-dependent and driven by environmental conditions. Combination of the EPNs and botanicals may enhance their efficacy; thus, we investigated the compatibility of the medicinal plants *Alepidea amatymbica* and *Elephantorrhiza elephantina* with five locally isolated EPN strains, three *Steinernema* (*S. khoisanae*, *S. biddulphi* and *S. innovationi*) and two *Heterorhabditis* (*H. bacteriophora* and *Heterorhabditis* sp. SGI 244). The experiments were designed to evaluate EPNs survival in plant extracts extracted using water and ethanol at 1%, 0.75%, 0.50%, 0.25% and 0.125% concentrations and nematode viability post-exposure. A concentration of 1000 IJs/mL in distilled water was used. Incubating extracts of *A. amatymbica* and *E. elephantina* with EPNs influenced the survival and virulence of the EPN species examined. The percentage survival of the EPNs post-exposure to the plant extracts was dependent on the plant extraction method and concentration in *A. amatymbica* but not in *E. elephantina.* The ethanol extraction method supported high percentage survival both at smaller and larger concentrations. The surviving infective juveniles (IJs) were virulent to *Tenebrio molitor* in both the aqueous and ethanol extracts of *A. amatymbica* and *E. elephantina*. However, virulence was observed to be strain-specific and not IJ concentration-dependent. Percentage mortality (against *T. molitor*) of strains with low percentage survival in aqueous extract of *A.* amatymbica compared effectively with those of higher percentage survival. The compatible relationship between extracts of the two plants and the EPN strains may lead to improved pest control in agricultural farming systems relative to either one of them applied individually.

Key words: *Alepidea amatymbica*, compatibility, entomopathogenic nematodes, *Elephanthorrhiza elephantina*, survival, virulence

1. Introduction

Alepidea amatymbica and *Elephantorrhiza elephantina* are medicinal plants possessing bioactive compounds such as terpenes, diterpenoids, tannins, flavonoids and anthocyanidins (Wintola and Afolayan, 2014; Mpofu et al., 2014). Despite the presence of these compounds in the two plants, their insecticidal potential is scarcely explored. The insecticidal potential of *A. amatymbica* have been implicated in the control of aphids and cutworms of cabbage (Skenjana and Poswal, 2018) and stalk borers of maize (Skenjana, 2018) among the smallholding farming communities in the Eastern Cape, South Africa.

Entomopathogenic nematodes are soil-inhabiting roundworms with proven biocidal properties against soil and above-ground pests (Laznik et al., 2010; Lacey and Georgis, 2012; Mahmoud, 2014a&b). The EPNs from the families of Steinernematidae and Heterorhabditidae are mutually associated with bacteria of the *Xenorhabdus* and *Photorhabdus* genera, respectively (Akhurst, 1982; Forst and Clarke 2002; Boemare et al., 2003; Ryssa et al., 2011). The steinernematid and heterorhabditid nematodes and their symbiont bacteria function as highly virulent insect pathogens (Gaugler, 2002). During host infection, the infective juvenile (IJ) carrying the symbiont bacteria finds and releases the bacterial symbionts into the haemocoel, causing septicemia and producing a multitude of insecticidal toxins and secondary metabolites killing the insect (Abdel-Razek, 2003; Ciche, 2007). The EPNs are eco-friendly, relatively inexpensive to culture, can be applied with most standard agricultural equipment, may persist for many years in the soil depending on the prevailing condition, can infect numerous insect species, and they are compatible with numerous biological and chemical pesticides (Akhurst and Smith, 2002; Askary et al., 2012; Shapiro-ilan et al., 2012; Ramakuwela et al., 2016).

The combination of two control agents against a given pest can result in antagonistic, additive or synergistic effects

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on speed of kill and mortality of the pest (Koppenhöfer and Grewal, 2005). Antagonistic effects of phenolic compounds in some plant extract, particularly on the developmental stages of *Heterorhabditis bacteriophora* (Poinar), have been reported (Santhi et al., 2017&2019). In order to optimize their potential, EPNs have been reported for their additive and synergistic interactions with other pest management tactics in integrated pest management (IPM) programs (Laznic et al., 2012; Shapiro-Illan et al., 2017). Guo et al. (2016) also reported a synergistic effect of the combination of *H. bacteriophora* (Poinar) with Chlorantraniliprole and Imidacloprid on the second instar larvae of *Holotrichia oblita* Faldermann (Coleoptera: Scarabaeidae). Some plant extracts alongside plant essential oils have also been compared with EPNs for compatibility and virulence against some insect pests. Shamseldean et al. (2013) investigated the compatibility of *H. indica* (Poinar) and *H. bacteriophora* with plant oils or plant extracts against grasshopper, *Heteracrir littoralis* (Orthoptera: Acrididae) at different inoculum levels. They reported that the grasshopper was susceptible to the tested EPN species, either combined with plant oils or plant extracts. In the same vein, EPN species, combined with neem pellets, were reported with additive effect on western flower thrips, *Frankliniella occidentalis* (Otieno et al., 2015).

Based on documented information on the interactions between EPNs and plant extracts, we investigated compatibility between two medicinal plants (*Alepidea amatymbica* and *Elephantorrhiza elephantina*) and EPNs isolated in South Africa. Furthermore, we tested virulence of nematode species post-exposure to different concentrations of the plant extracts against *T. molitor*.

2. Materials and methods

2.1. Plant collection and extraction

The corms and roots of *Alepida amatymbica* (Larger Tinsel Flower) and *Elephantorrhiza elephantina* (Elephant root) respectively were collected at Monontsha around Qwaqwa, Free State Province, South Africa. The identities of the two plants were confirmed and authenticated at the Department of Plant Sciences, University of the Free State, Qwaqwa campus. Voucher specimens OsoMed/01/2019/QwHB and OsoMed/02/2019/QwHB were prepared and deposited in Qwaqwa Herbarium. The corms and roots were adequately washed and sliced. They were aerated for three weeks and later ground to powder using an electric powered blender (Nanning Mainline Food Machinery Company Ltd, China). Fifty grams (50 g) of each powdered plant material was soaked individually in 500 mL of water and ethanol (99%) in separate conical flasks. This was then placed on a shaker at a speed of 120 rpm and kept for 24 h at room temperature (Handa et al. 2008). The mixture was filtered with Whatman No: 1 filter paper (Sigma-Aldrich Corp., South Africa). The filtrate from ethanol was concentrated at 40 °C using rotary evaporator, while that of water was evaporated in a water bath at 45 °C for 2 days. The extracts were later kept in the refrigerator at 4 °C until when needed for bioassays.

2.2. Source of EPN strains

Three native species of *Steinernema* [*Steinernema khoisanae* (Nguyen, Malan & Gozel), *Steinernema biddulphi* n. sp. and *Steinernema innovation* (Çimen, Lee, Hatting and Stock)] and two species of *Heterorhabditis* (*H. bacteriophora* and *Heterorhabditis* sp. SGI 244) from the EPN culture collection at the Agricultural Research Council –Small Grain (ARC-SG) used for the study were propagated in the larvae of *Galleria mellonella* (Linnaeus, Lepidoptera: Pyralidae) according to Kaya and Stock (1997). Larvae of *G. mellonella* were reared on a diet described by Mohamed and Coppel (1983) at ARC-SG (Bethlehem, South Africa). Infective juveniles were collected in modified White traps and stored (maximum for two weeks) in the cold room at 10 °C until they were needed for the experiment.

2.3. Source of *T. molitor* and Maintenance

Tenebrio molitor was reared in a colony at ARC-SG, Bethlehem. The insect culture was reared in a clear plastic container covered with a lid which allowed for air circulation through gauze. The container was filled to about 2–3 inches deep with bran and potato halves as a source of moisture for the larvae. Only last instars were collected and used in bioassays.

2.4. Compatibility of EPNs with plant extracts

The experimental dishes (35 mm Petri dishes) were arranged in completely randomized design (CRD) of 2×5×5 factors. The 2×5×5 factors were solvent (ethanol and water), extract concentration (1%, 0.75%, 0.50%, 0.25% and 0.125%) and EPN strains (*S. khoisanae, S. biddulphi*, *S. innovationi, H. bacteriophora* and *Heterorhabditis* sp. SGI 244). There were 12 treatments per strain (with two control, distilled water and ethanol) adding up to 60 treatment samples per replicate. The ethanol control was prepared at a concentration of 2% (Katiki et al., 2011). The plant extracts were reconstituted in their original solvent, and the stock solution was further diluted into five descending concentrations (1%, 0.75%, 0.50%, 0.25% and 0.125%) based on weight/volume. Nematode concentration of 1000 IJs/ mL in distilled water was prepared for each strain and 1mL of the nematode suspension was pipetted into 35 mm Petri dishes containing 2 mL of the extract. Infective juveniles in the distilled water served as control. The dishes were incubated in the dark inside a growth chamber at 25 ± 1 °C for 72 h. Nematode viability was assessed post incubation by examining a sample of 100 IJs loaded in a nematode counting slide (Kyron laboratory, Benrose, South Africa) (Josende et al., 2019) under a dissection

microscope, counting dead and alive IJs to calculate percentage survival (Kaya and Stock, 1997). To ensure that no live IJs were missed, non-moving juveniles were probed gently with a nylon brush bristle (Shapiro-Ilan et al., 2009). The experiment was repeated thrice at different times with a fresh batch of EPNs and plant extracts.

2.5. Virulence assay

The nematode suspension after the 72-h count was introduced into Eppendorf tubes and centrifuged at 3000 rpm for 4 min to allow the IJs to form a pellet. The supernatant was pipetted out, and the remaining nematode pellet was suspended in 1 mL distilled water. This was used to infect 10 *T. molitor* larvae in 90 mm Petri dishes lined with filter paper disc by pipetting the nematode suspension followed by introducing the larvae immediately (Ramakuwela et al., 2019). Control treatments received 1 mL distilled water only. The dishes were placed in zip-lock bags to conserve moisture and incubated at 25 ± 1 °C. There were 12 dishes per treatment and 60 dishes per replicate. Mortality against *T. molitor* was assessed by counting the number of dead larvae after 48 h post infection (Shapiro-Ilan et al., 2015). Potential differences in innate nematode virulence among the strains were corrected by applying a modified Schneider-Orelli's formula (Püntener, 1981) to larval mortality in the exposed treatments compared with the control treatment. The formula used was [(Mortality % in treatment – Mortality % in the control) / 100 – Mortality % in the control] *100. Cadavers were transferred to white traps to allow nematode emergence to confirm nematode recycling. The experiment was repeated three times, with fresh batches of EPNs and plant extracts.

2.6. Statistical analysis

Statistical analysis was performed using SAS 9.4 statistical software (SAS InstituteInc., Cary, NC, USA). All data were subjected to an analysis of variance (ANOVA). The standardized residuals showed an acceptable normal distribution (Shapiro and Wilk, 1965) with honestly significant difference value calculated as Tukey test at $p \leq 0.05$ (SAS 2015).

3. Results

3.1. Effect of extraction method and extracts concentrations on survival of the EPN strains

The percentage survival of the EPN strains in aqueous extract of *A. amatymbica* was observed to be extraction method dependent. The percentage survival of the EPNs in aqueous extract at higher concentrations (0.5%–1%) was lower than the survival in ethanol extract of the same concentrations ($F_{0.5} = 1.10_{a,}\ 1.39_{e}$; $df_{0.5} = 4_{a}, 4_{e}$; $P_{0.5} = 0.4090_{a}, 0.3052_{e}$) (Figure 1). At lower concentration levels of 0.25 and 0.125%, the EPNs in aqueous extract compared effectively with those in ethanol extract ($P_{0.25} = 0.9117_{a}, 0.0565_{e}$). However, the percentage survival of the EPNs in the ethanol extraction method was not dependent on concentration. At a lower concentration of 0.125%, percentage survival was 99.3% (*Heterorhabditis* sp. SGI 244), 89.7% (*H. bacteriophora*), 96.3% (*S. biddulphi*), 92.3% (*S. khoisanae*), and 81.3% (*S. innovationi*). Similarly, at a higher concentration of 1%, percentage survival was 93.5% (*Heterorhabditis* sp. SGI 244), 86% (*H. bacteriophora*), 91.3% (*S. biddulphi*), 90% (*S. khoisanae*), and 79.3% (*S. innovationi*).

In *E. elephantina*, there were no marked differences ($P_{0.5} = 0.5885_{a}, 0.6529_{e}$) in the percentage survival of the EPN strains subjected to either aqueous or ethanol extracts regardless of the concentrations (Figure 1).

The percentage survival of the individual strain at different concentrations was concentration-dependent for the EPNs subjected to aqueous extracts of *A. amatymbica*. For *S. khoisanae*, lowest survival of 51% was observed in 1% concentration with the highest survival of 83.3% in 0.25% concentration. *H. bacteriophora* recorded the lowest percentage survival of 17.5% at 1% concentration and the highest of 88.3% at 0.125% concentration. The lowest percentages of 16%, 60% and 28% and highest percentages of 95.7% 88% and 80% were recorded for *Heterorhabditis* sp. SGI 244, *S. biddulphi*, and *S. innovationi*, at 1% and 0.125% concentrations, respectively. However, for ethanol extracts of *A. amatymbica*, the percentage survival of the EPNs was not concentration dependent.

In the case of *E. elephantina* plant, individual EPN strain was observed to compare effectively ($P_{S.khoisane} = 0.7040_{a}, 0.5821_{e}$; $P_{H.bacteriophora} = 0.8274_{a}, 0.8591_{e}$; $P_{H.spSGT244} = 0.1129_{a}, 0.5808_{e}$; $P_{S.biddulphi} = 0.5575_{a}, 0.7897_{e}$; $P_{S.innovationi} = 0.9843_{a}, 0.9986_{e}$) at all the extracts doses as follows. For aqueous extract, *S. khoisanae* recorded highest survival (96%) at 0.25% with the lowest (88.7%) at 0.75%, *H. bacteriophora* and *S. biddulphi* had the highest (85.7% and 94.5%) at 0.125 and 0.50% with lowest (78.3% and 88.3%) at 0.25 and 1%, respectively, *Heterorhabditis sp. SGI 244* had the highest (89.3%) at the 0.25% and lowest (84%) at 0.75%, and *S. innovationi* had the highest (89.7%) at 1% and lowest (83.7%) at the 0.25%. For ethanol extracts, *S. khoisanae* had highest survival (91.7%) at 0.50% with the lowest (88.7%) at 0.125%, *H. bacteriophora* had highest (85.7%) at 0.125% with lowest (76%) at 0.75%, *Heterorhabditis sp. SGI 244* had highest (94%) at 1% with lowest (89.7%) at 0.125%, *S. biddulphi* had highest (94.7%) at 0.25% with lowest (89%) at 1%, and *S. innovationi* had highest (87.3%) at 1% with lowest survival (83.7%) at 0.5% concentration (Figure 2).

3.2. Virulence of EPN strains post incubation with plant extracts

The virulence of the entomopathogenic nematodes post 48 h incubation as indicated by corrected *T. molitor* mortality

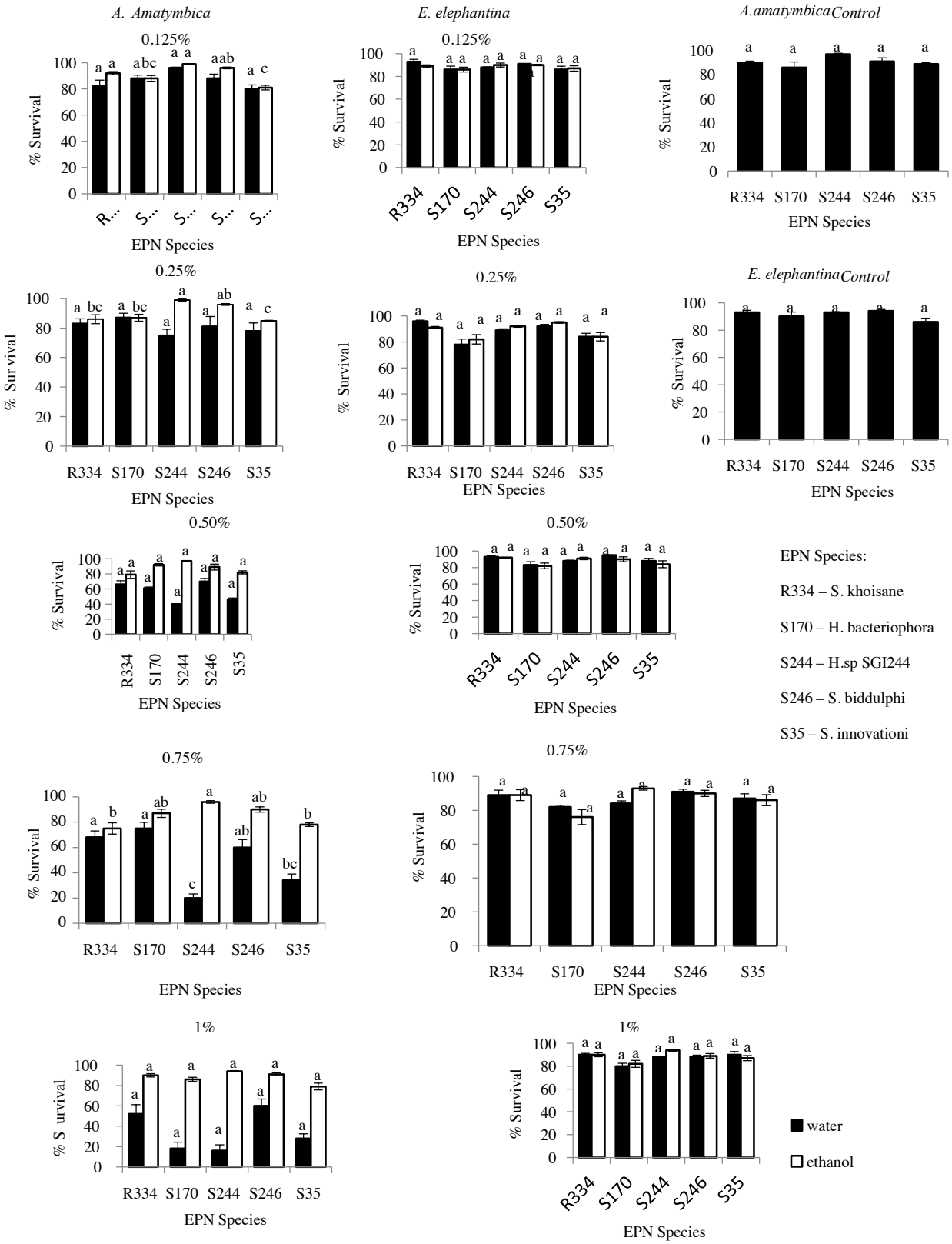

Figure 1. Percentage survival of the EPN Species in aqueous and ethanol extracts of *A. amatymbica* and *E. elephantina* after 72 h exposure. Different lower-case characters represent significant differences at $p < 0.05$.

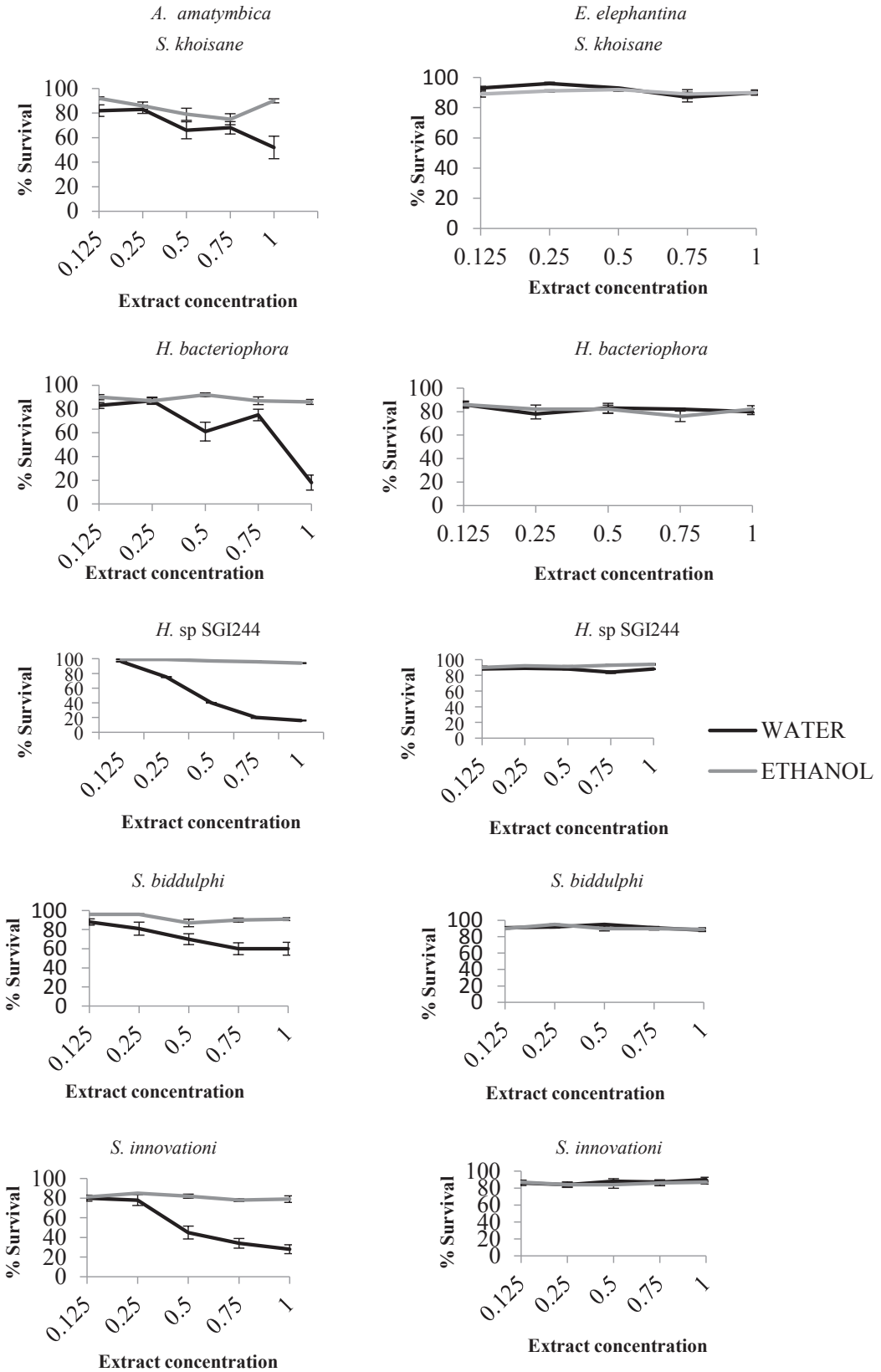

Figure 2. Percentage survival of the individual EPN species at different concentrations of aqueous and ethanol extracts of *A. amatymbica* and *E. elephantina.* Vertical bars illustrate the standard error of the mean (n = 3).

varied among the EPN strains and extract concentrations. It was interesting to note that mortality of *T. molitor* after exposure to IJs post incubation in different concentrations of aqueous extract of *A. amatymbica* showed that the IJs have low percentage survival compared effectively with those of higher percentage survival in some instances. *H. bacteriophora* at 0.125% plant extract concentration with 88% survival had 84% kill of *T. molitor*. The same strain at 1% plant extract concentration had a survival of 18% but killed 75% of *T. molitor* larvae. The same trend was observed among the inoculum of individual strains *Heterorhabditis* sp. SGI 244 and *S. innovationi* used to infect *T. molitor* at the same concentration. However, for the ethanol extract of the same plant which gave high survival rates across different concentrations, mortality of *T. molitor* varied among the strains and the different concentrations (Table 1).

For aqueous extract of *E. elephantina,* percentage survivals at different concentrations were high and subsequently their virulence except for *S. khoisanae* at 0.125 and 1% concentrations with <50% kill against *T. molitor* (Table 2). Most of the EPN strains had over 50% kill against *T. molitor* at the different concentrations of ethanol extract of *E. elephantina.* However, *Heterorhabditis* sp. SGI 244 (at 0.125%), *S. biddulpi* (at 0.125 &0.75%) and *S. khoisanae* (at 0.125 & 0.50%) had less than 50% kill against *T. molitor* (Table 2).

4. Discussion

This study has shown that the corm and root extracts of *A. amatymbica* and *E. elephantina* are compatible with EPN strains and exhibited virulence against *T.* molitor after 72 h post exposure. While they were impacted, some EPN strains can be tank-mixed with varying concentrations of these extracts as their survival and virulence were not affected. The percentage survival of the EPNs in *A. amatymbica* was dependent on extraction method and concentration of the extract used. *Alepidea amatymbica* may be reactive to the chemical constituents in aqueous solvent. Some plants are reported with ability to respond to physical and chemical stimuli, which are capable of triggering changes that could lead to a cascade of reactions (Sudha and Ravishankar, 2002). However, the EPNs survival in *E. elephantina*, was not dependent on extraction or concentration of the extract. The ethanol extraction method supported high percentage survival both at smaller and larger concentrations. Extraction solvent has been implicated with the efficiency of extraction protocol. This is because extraction solvents influence the extraction yield and the content of bioactive compounds; hence, they significantly affect the biological activity of the extract (Das et al., 2010; Lalitha and Jayanthi, 2012; Ngo et al., 2017).

In consistent with our results, Okwute (2012) noted that alcoholic extracts were found to be more active than aqueous extract as most of the active ingredients are lipophilic and are, therefore, more readily extracted into an organic medium. Moreover, aqueous extracts have been reported to be made up of non-active components such as carbohydrates, organic acids, proteins and minerals (Wigmore et al. 2016). Even though the survival of the EPNs in full dose of the plant extract was negatively affected in *A. amatymbica* aqueous extraction, sub-lethal doses of the extract may still be applied with EPNs for effective pest control as suggested by Elizabeth et al. (2003). Sub-lethal dose may not necessarily kill the pest; however, they are capable of weakening the pest such that they stop or feed less, translating to reduced plant damage. Furthermore, the weakened pest may be more susceptible to the EPN. The authors acknowledge the possibility of declined IJ survival in plant extracts post 72 h (The maximum exposure time that was tested in the current study). For example, application of the mixture in soil may result in prolonged contact of the extracts and the nematodes leading to poor survival and viability. A total of 72 h is long enough for nematodes to have infected the host due to the fast-killing nature of the EPNs (Shapiro-Ilan et al., 2012). Alternatively, application rates of the nematodes may be increased to make up for possible loss of IJs.

All tested nematode strains were capable of killing and recycling in *T. molitor* following exposure to plant extracts. *Heterorhabditis bacteriophora* presented low percentage survival below 20% at a full dose of *A. amatymbica* in aqueous extraction. However, those surviving IJs were virulent to *T. molitor* with percentage mortalities of over 60%. High virulence of this strain could be attributed to their ability to infect host by chewing through the cuticle with a buccal tooth-like structure (Bedding and Molyneux, 1982). Similarly, the survival of *S. feltiae* in the pesticide Gnatrol decreased to 17% after 72 h incubation, but infectivity to *Galleria mellonella* larvae was above 50% and 62% (Elizabeth et al. 2003). Contrary to our study under a different source of stress, Shapiro-Ilan et al. (2015) observed severe impacts on virulence beyond viability after exposure of some EPNs to ultraviolet rays. This suggests that, in many cases, viability may remain high but virulence can be compromised.

The virulence against *T. molitor* in both the aqueous and ethanol extracts of *A. amatymbica* and *E. elephantina* was observed to be strain-specific and not inoculum concentration-dependent with comparable mortalities 48 h post exposure. It has been established that different solvent extracts demonstrate differing inhibitory activities depending on the organism under study (Mpofu et al., 2014; Seleshe and Kang, 2019). Motility and foraging strategy of the microorganisms are virulence factors

Table 1. Virulence of entomopathogenic nematodes strains against *T. molitor* post 48 h incubation in *A.amatymbica.*

[Extract]	Strain	Aqueous		Ethanol	
		% Survival post incubation	Corrected mortality (%)	% Survival post incubation	Corrected mortality (%)
			48 h		48 h
0.125	*H. bacteriophora*	88	84	90	46
	H. sp SGI244	96	75	99	46
	S. biddulpi	88	55	96	83
	S. innovationi	80	38	81	59
	S. khoisanae	82	41	92	41
0.25	*H. bacteriophora*	87	75	87	46
	H. sp SGI244	75	63	99	46
	S. biddulpi	81	46	96	54
	S. innovationi	78	50	85	41
	S. khoisanae	83	21	86	38
0.50	*H. bacteriophora*	61	63	92	78
	H. sp SGI244	40	54	97	75
	S. biddulpi	70	71	89	75
	S. innovationi	46	54	82	46
	S. khoisanae	66	46	79	41
0.75	*H. bacteriophora*	75	59	87	59
	H. sp SGI244	20	34	96	41
	S. biddulpi	60	66	90	71
	S. innovationi	34	50	78	63
	S. khoisanae	68	34	75	41
1	*H. bacteriophora*	18	75	86	63
	H. sp SGI244	16	66	91	38
	S. biddulpi	60	25	94	75
	S. innovationi	28	63	79	55
	S. khoisanae	52	46	90	50

responsible for pathogenicity (Josenhans and Suerbaum, 2012; Griffin, 2012; Kao et al., 2014). Studies have implicated the *Heterorhabditis* spp. as cruise-foraging species best suited for finding immobile hosts, while *Steinernema* spp. (ambush-foragers) are considered suited for mobile hosts (Grewal et al.,1994; Gaugler et al., 1997; Kruitbos and Wilson, 2010).

5. Conclusion

In conclusion, the medicinal plant extracts, extraction method and concentration impacted survival of EPNs. The data are important for selection of plant extraction method and compatible strains. Ethanol extraction method supported IJ survival and virulence of the nematodes. However, ethanol availability to farmers

Table 2. Virulence of entomopathogenic nematode strains against *Tenebrio molitor* post 48 h incubation in *E elephantina.*

[Extract]	Strain	Aqueous		Ethanol	
		% Survival post incubation	Corrected mortality (%)	% Survival post incubation	Corrected mortality (%)
			48 h		48 h
0.125	*H. bacteriophora*	86	71	86	66
	H. sp SGI244	83	88	90	38
	S. biddulpi	91	66	90	41
	S. innovationi	86	54	87	59
	S. khoisanae	93	41	89	41
0.25	*H. bacteriophora*	78	50	82	71
	H. sp SGI244	89	84	92	84
	S. biddulpi	92	63	95	54
	S. innovationi	84	59	84	66
	S. khoisanae	96	50	91	66
0.50	*H. bacteriophora*	83	79	82	66
	H. sp SGI244	88	79	91	86
	S. biddulpi	95	59	90	59
	S. innovationi	88	54	84	66
	S. khoisanae	93	59	92	25
0.75	*H. bacteriophora*	82	91	76	54
	H. sp SGI244	84	84	93	84
	S. biddulpi	91	66	90	25
	S. innovationi	87	59	86	66
	S. khoisanae	89	63	89	54
1	*H. bacteriophora*	80	75	82	71
	H. sp SGI244	88	96	94	71
	S. biddulpi	88	75	89	50
	S. innovationi	90	59	87	88
	S. khoisanae	90	41	90	54

may be an issue as compared to water for plant extracts preparation. Moreover, the aqueous extraction was able to distinguish the EPN spp. survival in extracts. On that note, *S. khoisanae* and *S. biddulphi* would be selected for further compatibility studies (Efficacy of combined plant extracts and selected EPN strains) based on their appreciable survival of 50% at high concentration of 1% aqueous extractions of both plants. *Heterorhabditis bacteriophora* would also be selected based on its comparable mortalities from low versus high inoculum concentrations of the aqueous solutions. The combination of EPNs and other pest control agents has proved to be compatible and generates improved pest control than when either agent was applied alone (Shapiro-Ilan et al., 2010; Mahmoud, 2016). Future research will focus on virulence testing of combinations of these extracts and the three selected EPN strains for control of key agricultural pests and potential incorporation into IPM programs.

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