Sensitivity of stubby root nematodes (Trichodorus and Paratrichodorus spp.) to isothiocyanates associated with Brassicaceae in an in vitro assay

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Sensitivity of stubby root nematodes (*Trichodorus* and *Paratrichodorus* spp.) to isothiocyanates associated with Brassicaceae in an *In-vitro* assay

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Summary- Brassicas contain glucosinolates (GSLs), which are converted into different isothiocyanates (ITCs) that possess biocidal activity. These different ITCs result in a range of toxicities to various target species. Laboratory assays were conducted to evaluate the sensitivity of stubby root nematodes (SRN.)— *Trichodorus* and *Paratrichodorus* spp., to three pure commercially available ITCs i.e., 2-phenylethyl (PEITC), allyl (AITC) and sulforaphane (SITC). SRN were exposed to different concentrations of these three ITCs i.e., 1.625, 3.125, 6.25, 12.5, 25 and 50 µg ml^{-1}. Effect on nematode mobility was assessed after 24, 48 and 72h. Mortality of SRN was assessed after 48h incubation of the nematodes in distilled water post ITC treatment. Mortality for all ITCs at all tested doses was significantly higher ($P<0.05$) than the controls; distilled water and 1% DMSO. Concentration and type of ITC had a significant effect on SRN mobility and mortality, while increase in exposure time did not significantly increase the immobility of SRN. The average 24h ED_{50} (dose that resulted in immobility of 50%) for SRN were 7, 5 and 44 µg ml^{-1} while the average LD50 (dose that resulted in 50% mortality) after 48h recovery in distilled water was 7, 11 and 24.3 µg ml^{-1} for PEITC, AITC and SITC respectively. SITC was significantly less potent compared to PEITC and AITC which had LD50 values that were four times and two times lower respectively. These results indicate the potential use of brassica associated with the tested ITCs in the process of biofumigation for SRN suppression.

Keywords – Glucosinolates, mortality, brassica, toxicity, biofumigation

Members of the family Brassicaceae, contain a class of thioglucoside secondary metabolites known as glucosinolates (GSL) that are known to protect them from attack by pathogens (Ntalli & Caboni, 2017), and have been also successfully used in management of plant parasitic nematodes in the process of biofumigation (Aydınlı & Mennan, 2018; Dahlin & Hallmann, 2020; Ngala et al., 2014; Waisen et al., 2020; Yu et al., 2019). GSL are sulphur containing metabolites, stored in the cell vacuole. and are categorized based on the structure of their side chain (R).They occur in different quantities and have different profiles both quantitatively and qualitatively within the family Brassicaceae, in different cultivars and even species grown in the same environment (Bellostas et al., 2004). GSL distribution also varies with the age, where high concentrations have been recorded in the early growth stages in roots and then decreasing later in growth, while in the reproductive organs the concentration has been shown to increase during flowering where it is at peak (Bellostas et al., 2004). Physical damage of the tissue causes release of the glucosinolates from vacuoles into the cytoplasm where, they come into contact with endogenous thioglucosidases (myrosinases), leading to hydrolysis (Brown et al., 2003). The hydrolysis results in release of bioactive compounds such as nitriles, thiocyanates, and isothiocyanates depending on the R-group and prevailing chemical conditions in a process known as GLS-MYR system (Dutta et al., 2019; Ngala et al., 2015; Wathelet et al., 2004). The process of biofumigation relies on this system where cutting and incorporation of brassica residues leads to production of bioactive compounds including isothiocyanates (ITC) within the soil (Lord et al., 2011; Ntalli & Caboni, 2017). Additionally, other toxic sulphur containing hydrolysis products such as dimethyl sulphide, methyl sulphide, dimethyl disulphide, carbon disulphide, methaneiol, are also released ,and may contribute to the biofumigation process as they are present for a longer period of time compared to ITC which have a shorter half-life (Bellostas et al.,

2004) . Isothiocyanates are the most toxic glucosinolates catabolites and are recognised as the main cause of the biocidal activity of brassica green manures (Dutta et al., 2019). Various studies have attempted to explain the possible reactions that occur between ITC and nematodes. Some studies have suggested reaction of the active sites of the ITCs with the nucleophiles of the nematode, mainly thiols and amine groups of certain enzymes making them alkylated (Avato et al., 2013). In other cases, the ITC have been shown to induce oxidative DNA damage and also impairing nematode host finding ability (Murata et al., 2000). Another study observed that the dorsal pharyngeal gland nucleus in *Globodera rostochiensis* reduced upon exposure to ITC, ultimately reducing parasitism (Wu et al., 2011). The ITC derivatives differ in their toxicity among and within the different Brassica species (Zasada & Ferris, 2003). Their effectiveness differs depending on the type and structure which is partly explained by the different biosynthetic pathways involved (Matthiessen & Kirkegaard, 2006; Pinto et al., 1998). This study is one of the steps in a project that includes laboratory and field experiments to investigate suitability of brassicas as cover crops in the management of stubby root nematodes (SRN). As highlighted in previous studies, ITC derived from different brassicas and even varieties differ in their toxicity (Kruger et al., 2013; Lord et al., 2011; Melakeberhan et al., 2006; Scott & Antoon, 2014). The use of commercially available pure ITC makes it possible to evaluate the toxicity of the ITC by eliminating the conversion process from glucosinolates (Zasada & Ferris, 2003). The objectives of this study were to 1) compare the toxicity of different types of commercially available ITC on SRN mobility and mortality 2) determine the lethal dose (LD50) values of the different ITC, 3) determine the effect of exposure time on the mortality of SRN. This is the first study that investigates the effect of commercial isothiocyanates on the mobility and mortality of stubby root nematodes (SRN).

Materials and methods

Assay chemicals

Pure commercial isothiocyanates were obtained from Sigma-Aldrich, UK. The ITCs used in this study included: Allyl (AITC), sulforaphane (SITC) and 2-phenylethyl (PEITC). AITC and SITC are aliphatic ITCs while PEITC is an aromatic ITC and these ITC are derived from sinigrin, glucoraphanin and gluconasturtiin respectively as parent glucosinolates. Sinigrin is commonly found in brassicas such as *B. juncea* and *B. carinata*, glucoraphanin has been isolated from *B. rapa* and *Raphanus sativus*, while gluconasturtiin has been found in *B. juncea* and *B. campestris*. The criteria used in selecting ITCs used in this study was guided by previous studies that have reported their toxicity on a wide range of plant parasitic nematodes (Ntalli & Caboni, 2017; Wood et al., 2017; Wu et al., 2011; Zasada et al., 2009) as well as their association with brassica plants used in biofumigation (Aydınlı & Mennan, 2018; Lord et al., 2011; Ngala et al., 2015; Waisen et al., 2020).

Source of stubby root nematodes (SRN)

Mixed stages of SRN (*Trichodorus* and *Paratrichodorus* spp.), were obtained from infested soil collected from Docking, Norfolk site, UK, 52°54'01.7"N 0°36'32.4"E, which has a history of SRN infestation. Nematodes were extracted using Seinhorst two flask method (Bezooijen, 2006). Soil was gently mixed and washed through a 1mm sieve to remove large stones and debris that would otherwise block the flasks. The extract was then washed through 215 µm and 53 µm sieves to collect a clean suspension, which was then transferred into sample bottles. Nematodes were used to set up the assay immediately after extraction to prevent any deterioration at storage. The composition of stubby root nematodes used in this study, were identified as

Trichodorus primitivus (80%), *Trichodorus cylindricus 15%,* and *Paratrichodorus pachydermus* (5%) (Table 3), using morphological features key as described by Decraemer (1995).

Assay protocol

An *in vitro* assay was carried out by pipetting 1ml of nematode suspension containing 20 mixed stages of SRN into a 25 ml bottle. Stock solutions of each ITC were prepared using 1% dimethyl sulfoxide (DMSO). Dilutions were made to make six concentrations; 1.625, 3.125, 6.25, 12.5, 25 and 50 μg ml^{-1} for each of the ITC nad then 2 ml of test ITC added. Two controls were included i.e., distilled water and 1% dimethyl sulfoxide (DMSO) The experiment was incubated at room temperature (20±1°C) in the dark, and each treatment replicated four times and experiment repeated once. The effect of the ITC on SRN mobility was assessed after 24h, 48h and 72h exposure period in a repeated measures design. Nematodes were subjected to mechanical stimulation using a fine eyelash needle, and their locomotory response was observed and categorized as either mobile or immobile. Immobility in each treatment was expressed as number of immobile/total number of nematodes assessed. After the last assessment at 72h, the nematodes were transferred in distilled water and incubated for 48h for recovery assessment. The nematode stimulation procedure was repeated to determine whether they were dead or alive, which would indicate that the immobility effect observed was reversible or irreversible. Mortality was expressed as dead/total for each ITC.

Data Analysis

All data was analysed using R-studio software (R Core Team, 2022). A Levene test was conducted to compare the variances between the two experiments. Data from two

experiments was combined as the variances were homogeneous (P=0.24). Data on nematode mobility was analysed using generalised linear mixed effects model with concentration and ITC as fixed effects and time as a random effect (package lme4). Data on mortality was analysed by fitting a binomial generalised linear model to predict the response of mortality to the variable's concentration and ITC. The package Emmeans was then used to extract the contrast and mean estimates and for pairwise comparisons with significant differences at $P<0.05$. The package drc (dose response curve) on R-studio software was used to generate a dose response regression model for Lethal dose (LD_{50}) and effective dose (ED_{50}) values and for pairwise comparisons among the different ITCs.

Results

The effect of ITCs on SRN mobility was compared using effective dose (ED_{50}) values to determine which concentration causes 50% reduction in mobility at different exposure times (Figure 1) while nematicidal activity to determine dead nematodes after 48hours recovery in water of the different ITCs, was compared using LD_{50} values (Figure 2). Following exposure of SRN to different ITC, concentrations and exposure times, variations in SRN mobility and mortality were recorded. All the ITC tested had significantly higher immobility and mortality compared to the controls distilled water and DMSO. In general, mortality significantly increased with increase in ITC concentration and the type of ITC also had a significant effect on the mortality of SRN ($P<0.05$). There was also a significant linear positive correlation between ITC and concentration on mortality, ($R=0.73$) that was significant ($P<0.001$).

Increase in exposure time had no significant effect on nematode mobility. Immobility was significantly higher ($P<0.05$) after 24h exposure and this did not significantly

increase after 48 and 72h (Figure 1). The ED_{50} values were 7, 5 and 44 µg ml $^{-1}$ after 24h; 6 ,5 and 30 µg ml $^{-1}$ after 48h and 5.98, 4.91 and 25 µg ml $^{-1}$ for AITC, PEITC and SITC respectively. Increase in exposure time caused higher immobilisation of SRN for SITC, however the increase was not significant. A pairwise comparison of the ED_{50} values showed that AITC and PEITC were not significantly different ($P>0.05$) , while both were significantly lower compared to SITC for all the exposure times. Nematode mortality was recorded after 48h recovery assessment in distilled water. Mortality for all ITC at all tested concentrations was significantly higher ($P<0.05$) than the controls distilled water and 1% DMSO and no differences were recorded between the two controls. A steep dose response curve was obtained where a small increase in ITC concentration led to a significant increase in SRN in all the ITC tested. For AITC and SITC, the first three doses gave the same mortality effect unlike in PEITC where 6.25 µg ml^{-1} caused seven times more mortality when compared to the 1.625 and 3.125 µg ml^{-1} concentrations (Figure 2). The LD_{50} values for AITC, PEITC and SITC was 10.67, 6.91 and 24.31 respectively. PEITC had the lowest LD_{50} value which was twice and four times lower that AITC and SITC respectively. A pairwise comparison of this LD50 indicated that PEITC was significantly lower compared to both AITC and SITC. AITC was also significantly lower than SITC. At the highest concentration, 50 µg ml-1, the overall mortality was 100, 92 and 83% for PEITC, AITC and SITC respectively.

Discussion

Brassicas contain varying glucosinolates profiles which translates to distinct isothiocyanates (ITCs) with different toxicities. (Bellostas et al., 2004). This was evident in this study where significantly different EC_{50} and LD_{50} values were obtained for the different ITCs. Differences in the structure of the ITCs i.e., the chemical properties of the R side chain can confer differences in their biological activity (Lazzeri

et al., 1993). Aliphatic ITC are known to be more toxic than aromatic ITC (Lewis & Papavizas, 1971). This has been reported in a study investigating sensitivity of *Fusarium graminearum* to different ITC where aliphatic ITC i.e., allyl (AITC), methyl isothiocyanate (MITC) and ethyl isothiocyanate (EITC) were found to be more toxic compared to aromatic ITC i.e., 2-phenethyl (PEITC) and benzyl (BITC) (Ashiq *et al.*, 2021). Our study showed contrary results where the aromatic ITCs (PEITC) was more toxic than aliphatic ITC (AITC and SITC.) and is in agreement with another study that reported that aromatic ITCs were more toxic to *Meloidogyne javanica* and *Tylenchulus semipenetrans* compared to aliphatic ITC. However, the study found no relationship between ITCs structure and toxicity to the nematodes (Zasada & Ferris, 2003). This shows that variations exist depending on nematode species. Non -toxic effects have even been reported when *Caenorhabditis elegans* was exposed to SITC at doses of upto 70 ppm where the exposure instead increased the longevity of *C. elegans* (Qi et al., 2021) . The study by Wood et al. (2017) found contrary results where they reported 100% juvenile mortality of *Globodera pallida* when exposed to 50 µg ml^{-1} SITC. Our study agrees with the latter, where at highest dose of 50µg ml^{-1}, SITC caused a mortality of 83%. Toxicity of ITCs to nematodes is also known to be influenced by ITC-lipid solubility, ITCs volatility and ITCs hydrophobicity (Sarwar et al., 1998). Volatile ITC e.g., 2-propenyl are capable of dispersing evenly and effectively interacting with the target organism. Lipid soluble ITC e.g., 2-phenylethyl are able to penetrate the nematode cuticle and permeate phospholipid membranes, interacting with intercellular functions that kill the organism (Sarwar et al., 1998). The lipid solubility of PEITC might best explain its toxicity in our study. The parent glucosinolate associated with the different ITCs might also have contributed to the variations recorded, as the three different ITCs are associated with different glucosinolates i.e., sinigrin, gluconasturtiin and glucoraphanin for AITC, PEITC and

206 SITC respectively. ITC products of sinigrin, gluconapin, glucotropeolin and
207 glucodehydroerucin were more suppressive against *Heterodera schactii* at a
208 concentration of 0.5% after 48 hours exposure time, compared to those from
209 glucoraphanin and sinalbin (Lazzeri et al., 1993; Ntalli & Caboni, 2017). Brassica
210 extracts capable of producing phenylethyl and benzyl ITC recorded high mortality of
211 *Globodera pallida* juveniles compared to those containing indole-glucosinolates
212 which are unable to produce stable ITC (Buskov *et al.*, 2002). The low doses (ED_{50})
213 required to cause 50% immobility after 24h were very low for PEITC and AITC in this
214 study, which is very desirable under field conditions as ITC are very volatile in nature
215 and have a short half-life of 20-60h in the soil environment (Borek et al., 1995). During
216 a biofumigation study with *B. napus,* ITC concentration was highest within 2h and
217 90% of the production lost after 24h (Brown et al., 1991), similarly ITCs were released
218 within 30 minutes post-incorporation of *B. napus* and *B. juncea* and no ITC was
219 recovered 12 days later (Gimsing & Kirkegaard, 2006; Morra & Kirkegaard, 2002). The
220 immobility was then shown to be irreversible after recovery assessment and this is
221 shown by the similar LD_{50} and 72h ED_{50} curves. These concentrations are achievable
222 under field conditions. For instance, concentration of 100 μmol g^{-1} dry weight of 2-
223 propenyl , a glucosinolate that produces AITC, has been recorded in *Brassica nigra*
224 (Bellostas et al., 2007), 93 μmol g^{-1} dry weight in *B. carinata* (Zasada et al., 2009) and
225 90 µmol g^{-1} dry weight in *B. juncea* leaves (Ngala et al., 2015). Ideally, 2-propenyl GSL
226 concentrations above 13 µmol g^{-1} dry weight have the ability to produce at least 50 µg
227 ml^{-1} of AITC. In *Raphanus sativus*, gluconasturtiin GSL, whose derivative is PEITC,
228 has also been detected at concentrations of 53.6 μmol g^{-1} dry weight (Ngala et al.,
229 2015). Additionally, in the leaves of Indian mustard (*B. juncea*), black mustard (*B.*
230 *nigra*) and Ethiopian mustard (*B. carinata*) 14-25 µmol gluconasturtiin g^{-1} dry weight
231 has been recorded (Bellostas et al., 2007), while 15.8 µmol g^{-1} dry weight was reported

232 in the root tissue of *B. napus* (Gimsing & Kirkegaard, 2006). As for SITC GSL
233 glucoraphanin, studies indicate that concentrations achieved are dependent on plant
234 species. Concentrations of less than 3 μmol g $^{-1}$ dry weight in *Eruca sativa* have been
235 reported (Lord et al., 2011) while Ngala (2015) found higher amounts of 25.4 μmol g^{-1}
236 dry weight in the leaf tissue of *R. sativus*. The low-level production by some plant
237 species can be mitigated by careful selection of highest producing brassica species in a
238 biofumigation system. In conclusion, this study demonstrates the potential of
239 isothiocyanates in suppression of SRN at concentrations that are achievable under
240 field conditions. Brassicas such as *B. juncea* and *R. sativus* contain significant levels
241 glucosinolates that are associated with ITC tested in this study. hence these species
242 could be explored more in management of SRN. It is clear that effects of ITCs cannot
243 be generalised, as different nematodes differ in sensitivities This study focused on field
244 population of stubby root nematodes as they occur naturally in sugar beet fields.
245 Therefore, there was a mixture of SRN genera and species, although the most
246 dominant was *Trichodorus primitivus*. Other specialised tests can be conducted for
247 *Paratrichodorus* spp. which was in negligible numbers in our study.

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Tables and Figures

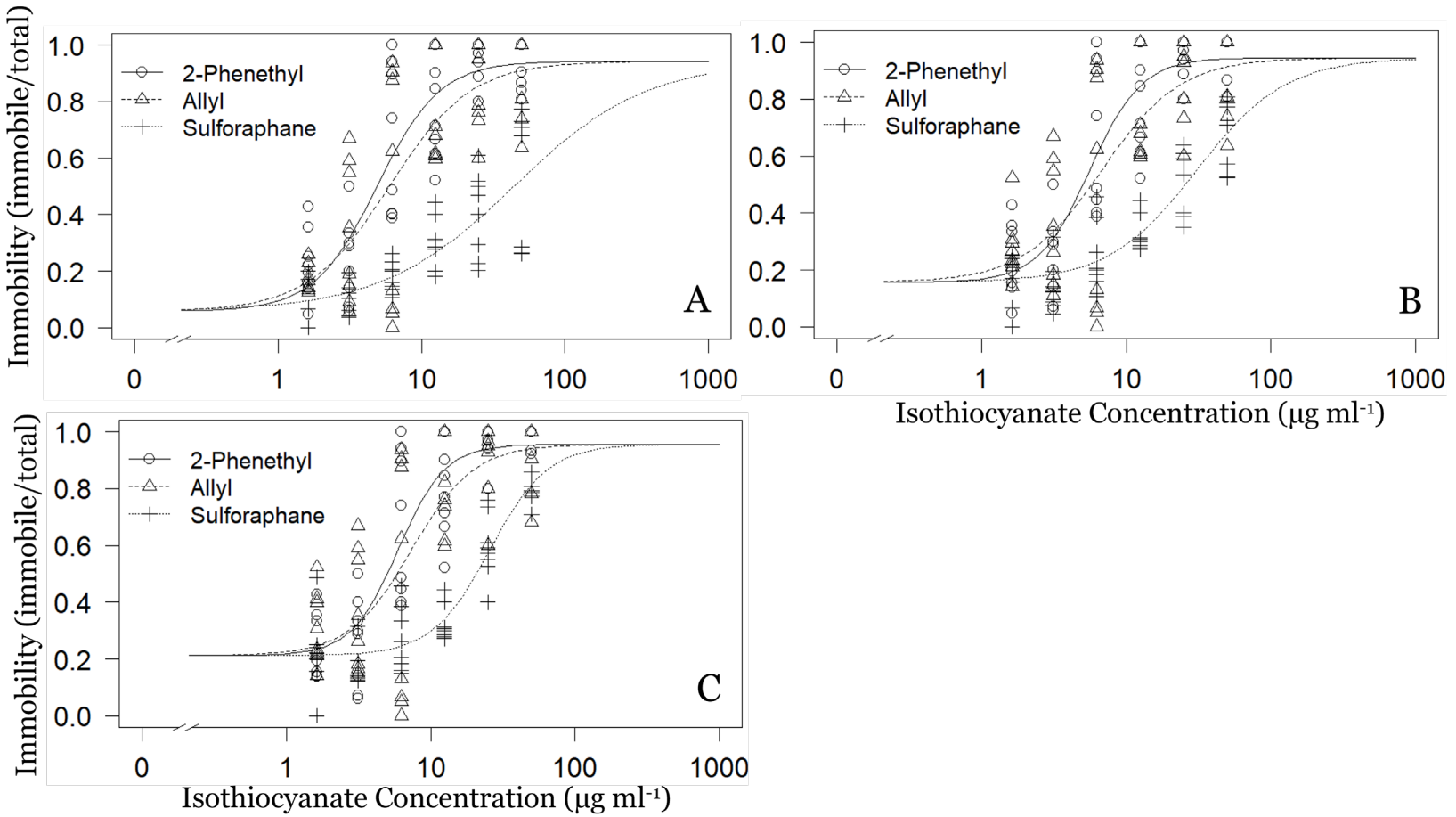

Figure 1: Dose response curves of 2-Phenethyl (PEITC), Allyl (AITC) and sulforaphane (SITC) after A: 24h; B:48h and C: 72h exposure periods on immobility of stubby root nematodes (SRN).

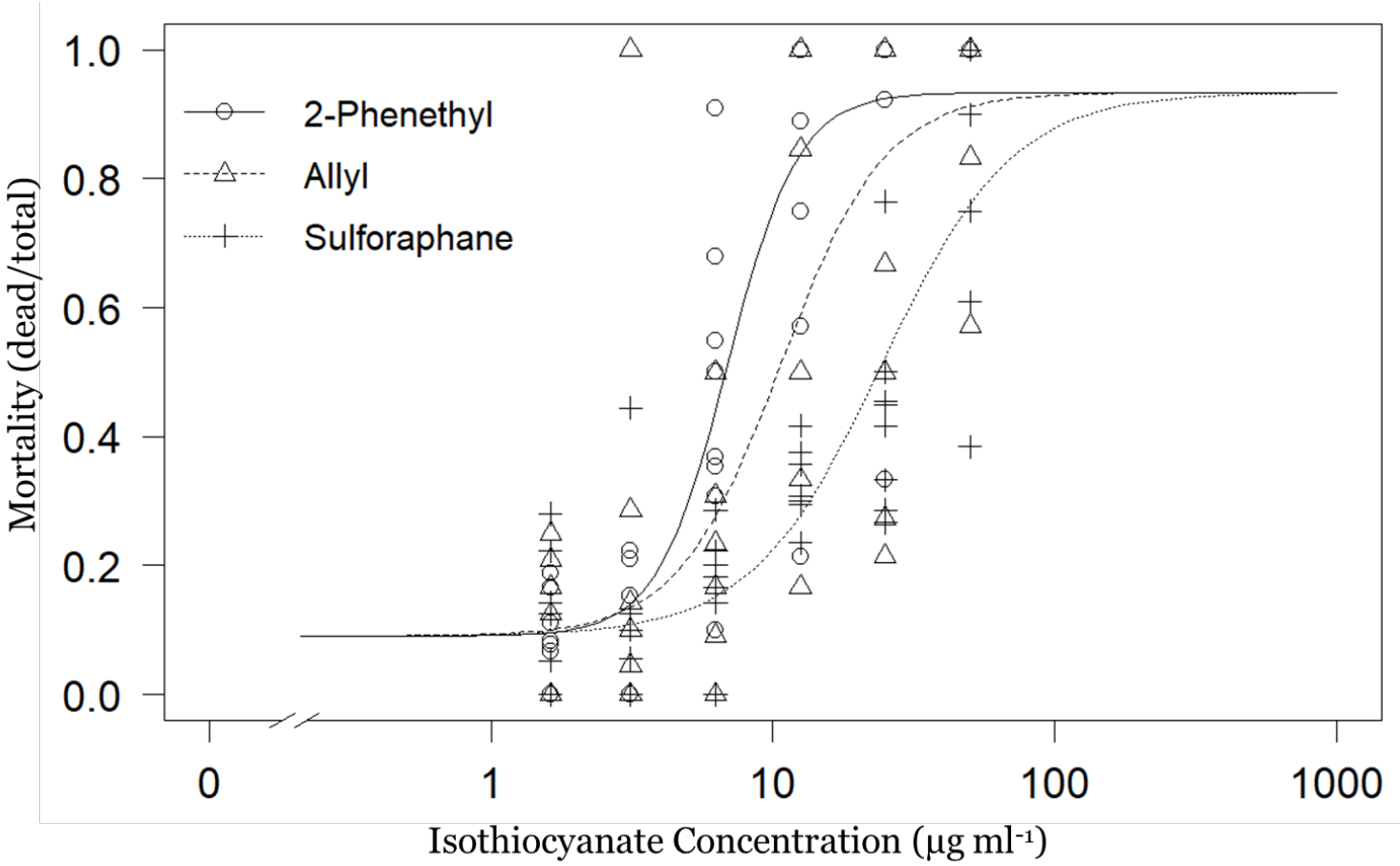

Figure 1: Dose response curve for 2-Phenethyl (PEITC), Allyl (AITC) and sulforaphane (SITC) on mortality of stubby root nematodes (SRN) after last exposure time of 72 h and incubation in distilled water for 48 h. for recovery assessment.

Table 1: Composition of average stubby root nematode species (SRN) ± SE (standard error), extracted from 200ml soil sample, n=10.

Stubby root nematode species (SRN)	Males	Females	
Trichodorus primitivus	15.5±2.20	48.4±5.11	
Trichodorus cylindricus	4±0.68	7.6±1.03	
Paratrichodorus pachydermus	1.4±0.28	2.5±0.40	
Juveniles			7.10±0.87