Concentration and Time Dependent Effects of Isothiocyanates Produced from Brassicaceae Shoot Tissues on the Pea Root Rot Pathogen *Aphanomyces euteiches*

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Abstract: Isothiocyanates (ITCs) hydrolysed from glucosinolates (GSLs) in Brassicaceae tissue are toxic to soil organisms. In this study, the effect of aliphatic and aromatic ITCs from hydrated dry Brassicaceae shoot tissues on mycelium and oospores of the pea root rot pathogen *Aphanomyces euteiches* was investigated. The profile and concentrations of GSLs in two test-Brassicaceae species, *Sinapis alba* and *Brassica juncea*, and the ITCs from the dominant hydrolysed parent GSLs were monitored. The concentrations of dominant ITCs and pathogen exposure time were evaluated in *in vitro* experiments. The greatest effect on the pathogen was observed from aliphatic ITCs hydrolysed from *B. juncea* tissue, and the effect depended on the ITC concentration and exposure time. ITCs were more effectively hydrolysed from *B. juncea* GSLs than from *S. alba* GSLs, i.e. the ITC/GSL ratio was higher in *B. juncea* than in *S. alba* tissue, giving a different release pattern. The release of phenylethyl ITC, which was common to both species, followed a similar pattern to the dominant ITC in each crop species. This suggests that traits other than GSL content, e.g. plant cell structure, may affect the release of ITCs and should therefore influence the choice of species used for bio-fumigation purposes.

Keywords: *Brassicaceae, glucosinolate, isothiocyanate, Aphanomyces euteiches, pea root rot*

INTRODUCTION

Pea root rot caused by the pathogen *Aphanomyces euteiches* Drechs is a serious problem in pea production worldwide.[1-3] The pathogen can persist in the soil for long periods without a host plant due to the thick protective cell walls of the Aphanomyces oospores. When oospores are stimulated to germinate by pea root exudates[4], a short mycelium strand and a sporangium is produced. The sporangium releases zoospores that attack the pea roots.[5] Pea root rot is difficult to control as there are no available pea cultivars with an acceptable level of resistance.[6] Further, synthetic fungicides may have a negative environmental impact, and certain agents have been prohibited, for example within the European Union[7]. The commonly recommended way to control *A. euteiches* is therefore to leave intervals (6-8 years) between pea crops in the rotation.[8]

Many plants of the family Brassicaceae (brassica) can suppress soil-borne organisms when grown as cover crops, or when added as plant residues to infested soil.[9-11] In field studies, *Sinapis alba* (white mustard) reduced the development of Aphanomyces pea root rot when used as green manure. This reduction was enhanced when white mustard was used for a second consecutive year.[12] Studies under greenhouse conditions showed that incorporating cabbage tissue after growth in contaminated soil gave close to a 10-fold reduction in pea root rot disease severity.[13] Suppressive effects of brassica tissues are associated with the glucosinolate class of chemicals (GSLs) present in the tissues.[10] The bio-fumigation concept in agricultural systems was introduced by Australian scientists in the 1990s and includes practices to relieve the pressure of soil pests and pathogens by the use of brassica crops.[14] The concept is based on the toxic volatile compounds produced from hydrolyzed GSLs.[10,13,15,16] When brassica crop tissues are damaged, GSLs are

hydrolysed by myrosinase to form volatile isothiocyanates (ITCs), thiocyanates, water-soluble nitriles and epithionitriles.[10,17] The composition and quantity of the produced substances depend on the parent GSL. The type and concentration of GSLs vary between brassica species, cultivars and vegetative parts and are also influenced by plant development stage.[16,18,19] The GSLs are classified as aliphatic, aromatic or indolyl based on their chemical structure.[10,20] Isothiocyanates suppress a wide range of organisms, including the soil-borne pathogen *A. euteiches*.[21] A study has demonstrated that volatile compounds from decomposing cabbage tissues inhibit hyphal growth of *A. euteiches*, and that the pathogen is unable to grow further when placed in fresh air after exposure, although the active substance was not specified[9]. The suppressive effects depend on the composition and concentration of ITCs.[22] The chemical structure of ITCs influences their mode of action meaning that different ITCs may show varying levels of toxicity at the same concentration.[23-25] An aliphatic structural group attached to the nitrogen in the basic ITC structure generally confers greater toxicity than an aromatic structure.[26] Generally, the toxicity of ITCs decreases as the size of the organic group increases.[27,28] Aliphatic ITCs bind directly with protein molecules inside cells and inhibit their activity whereas aromatic ITCs first interact with the cell membrane changing its electrophilic properties before entering the cell[23] and reducing cell activity.[29]

Many different brassica-derived materials have been used to study aspects of bio-fumigation, such as fresh plant tissues, rape seed meal, freeze dried plant tissues and oil extracts, but pure ITCs have also been used.[18,30-32] Shoot tissue comprises the greater part of the brassica plant material when it is used for bio-fumigation in practice. Here, we

evaluate a simple, reproducible system for studying shoot tissue effects on a plant pathogen *in vitro*, using low temperature drying of the plant material.[13,33]

Our overall aim was to discover how brassica plant tissues can be used for bio-fumigation to reduce the occurrence of root rot on peas caused by *A. euteiches.* Our specific objectives were to compare crop species with different GSL compositions to generate knowledge on the choice of brassica crop for bio-fumigation in the control of pea root rot. We tested the hypotheses that 1) ITCs from hydrolysed aliphatic-GSLs in *B. juncea* shoot tissue are more effective than ITCs from aromatic-GSLs in *S. alba* shoot tissue at reducing the ability of *A. euteiches* oospores to cause pea root rot 2) the lethal effect of aliphatic ITCs from shoot tissues on the growth of *A. euteiches* depends on the concentration of ITCs and time of pathogen exposure and 3) the release patterns of ITCs from *B. juncea* and *S. alba* are different and influence the suppressive effects on *A. euteiches*.

MATERIALS AND METHODS

Plant materials. Air dried shoot tissues of two GSL-containing plant species, *Brassica juncea* (cv. Pacific Gold) and *Sinapis alba* (cv. Architect), a non-GSL plant species, *Secale cereale* (cv. Amilo) and a water control were compared for their effect on oospores and mycelium of *A. euteiches*. The effect of volatile compounds produced from hydrated shoot tissues was studied in *in vitro* experiments. The most effective plant species was chosen for dose and exposure-time experiments, compared with the effect of chemical solutions of the dominant ITCs from the two brassica species. The composition

of GSLs in *B. juncea* and *S. alba* dry shoot tissues and of ITCs produced from hydrated tissues were analysed.

Shoot tissue production. Three cover crops, *S. alba*, *B. juncea* and *S. cereale* were grown in garden soil (Hasselfors Garden AB, Sweden) in the greenhouse. A complete nutrient solution (Cederroth International AB, Sweden) was applied at regular intervals together with additional $(NH_4)_2SO_4$ to enhance GSL production. Shoot tissues were harvested at soil level when *B. juncea* and *S. alba* reached the flowering stage. The tissues were dried at 35 °C for 72 h then ground separately four times at 6000 rpm to a fine powder. The ground shoot tissues were stored in air-tight containers and kept in a dark and dry place until use.

***Aphanomyces euteiches* inoculum.** *Aphanomyces euteiches* strain 5035:2A was used in all experiments (obtained from F. Heyman, Department of Forest Mycology and Plant Pathology, Swedish University of Agricultural Sciences, Uppsala, Sweden). The strain was maintained on corn meal agar (CMA) (Oxoid Ltd., UK) at 6 °C and was used for soil inoculation[34,35]. For the production of oospore inoculum, the *A. euteiches* strain was cultured in oat meal broth for four weeks. It was homogenised, oospore concentration was calculated then it was mixed with talcum powder (VWR International AB, Sweden) and dried. The dry inoculum material was sieved through a 1-mm mesh net and stored at 4 °C until use.

Field soil. Soil was collected from an agricultural field in Giresta, Enköping, Sweden, and tested for the absence of *A. euteiches*. The soil consisted of 46% clay, 48% silt, 2% sand and 4% organic matter. The organic matter content was estimated by loss on ignition. The soil contained 1.5 mg N/mL and the pH (H_2O) was 7.7. The soil was sieved through a 0.6-cm mesh, stored at 4 °C with soil moisture content maintained at 21% during storage. For some experiments the soil was sterilised by autoclaving twice at 121 °C for 30 min.

Oospore volatile exposure and bioassay. One gram of dry shoot tissue of *B. juncea, S. alba* and *S. cereale* was transferred separately to 220 mL plastic cups (8.9 cm diameter). Four grams of talcum powder containing about 2.8 X 10^4 oospores was spread homogenously on a filter paper placed in a 9 cm diameter sieve (mesh 1mm) and placed on top of the cup. At the start of the experiment 5 mL sterilized de-ionized water was added to the tissue and another plastic cup was immediately placed upside down on top of the sieve as a lid. The container was sealed with several layers of Parafilm (Sigma-Aldrich, Sweden AB) and placed in the dark at 24 °C for ten days. Sterilized de-ionised water was used as control and three replicates per treatment were performed.

After 10 days of incubation, the oospore-talcum powder was removed from the container and 0.8 g of the 4 g treated powder was mixed with 80 mL sterilized and non-sterilized soil. Four sub-samples, 20 mL each, of the two infested soils were transferred to 85 mL pots. One pea seed (cv. Clare) was sown on top of the infested soils in each pot and was covered with an additional 5 mL non-infested soil. The pots were placed in a greenhouse with light:dark period 14:10 h, temperature 24/19±2 °C and relative humidity

(RH) 85±5%. Soil moisture was adjusted daily to field levels in order to obtain optimal infection conditions. All pea seedling roots were evaluated for root rot disease severity three weeks after sowing. Each pea seedling was assigned a DSI (Disease Severity Index) value based on the mean disease symptoms of the individual pea seedlings in the four tubes representing each experimental unit. The scale for DSI ranges from 0 to 100 (%), but each individual plant can only be assigned one of five scores for disease severity: 0% = healthy plant; 25% = root slightly discoloured; 50% = root extensively discoloured but not shrunken; 75% = root extensively discoloured and shrunken; 100% = root partly or completely rotted or plant dead.[36]

***In vitro* exposure of mycelium to volatiles- effect of dose.** Dry shoot tissue, 60 mg, 100 mg and 140 mg of *B. juncea, S. alba* and *S. cereale* was transferred separately to 220 mL plastic cups and mixed with 5 mL sterilized, de-ionized water. A water control was included and all treatments had three replicates. The doses were determined after a pilot study with *B. juncea* shoot tissues in which a 140 mg dose completely inhibited pathogen growth. A plug (7 mm diameter) from actively growing *A. euteiches* culture was placed in the centre of a 9-cm CMA dish and placed as a lid on the top of cup immediately after water was added to the dry plant tissues. Cup and dish were sealed with Parafilm and incubated in a growth cabinet at 24 °C for four days. The colony diameter of *A. euteiches* was measured on the lid daily using a measuring scale. After four days, the culture dish lids were removed from the cups and placed in the same growth cabinet at 24 °C and observed for further growth of *A. euteiches*.

***In vitro* exposure of mycelium to volatiles- effect of exposure time.** Hydrated *B. juncea* shoot tissue (140 mg) was used to produce volatile compounds which were exposed to an actively growing *A. euteiches* plug (7 mm diameter) put on fresh CMA dishes, as in the dose experiment described above. Three exposure times were used, 40, 80 and 120 minutes, with three replicates of each. After exposure, dishes were incubated at 24 °C and the colony diameter of *A. euteiches* was measured daily using a measuring scale on the lid. After four days, the culture dish lids were removed from the cups and placed in the same growth cabinet at 24 °C and observed for further growth of *A. euteiches*.

Exposure with ITC chemical standards. An *in vitro* experiment was performed using authentic chemical standards (CS) of volatile compounds produced from *B. juncea* and *S. alba* tissues, identified as described below. Standard solution mixtures were designed to give concentrations of volatile compounds that closely resembled those recorded from hydrated dry shoot tissue by collecting volatile compounds from plant tissue and CS under the same conditions and quantifying the major components by gas chromatography (Table 1). The *B. juncea* chemical standard mixture (B.juncea-CS) contained allyl ITC 40 µg/µl (allyl ITC >98% purity, Sigma-Aldrich, Sweden AB) and phenylethyl ITC 2 µg/µl (phenylethyl ITC 99% purity, Sigma-Aldrich, Sweden AB). The *S. alba* chemical standard mixture(S.alba-CS) contained benzyl ITC 30 µg/µl (benzyl ITC 98% purity, Sigma-Aldrich, Sweden AB) phenylethyl ITC 5 µg/µl and allyl ITC 0.2 µg/µl. All chemical standard mixtures were dissolved in hexane.

One µl of each B.juncea-CS and S.alba-CS standard mixtures was placed in the centre of a filter paper in a sealed plastic cup containing an actively growing *A. euteiches*

mycelium (7 mm plug) placed on a fresh CMA dish and incubated in the dark at 22 °C. Hyphal growth was measured daily for four days using a measuring scale. The growth was compared with the growth of an *A. euteiches* volatile-exposed culture where the volatiles originated from 140 mg hydrated dried tissues of *B. juncea* and *S. alba*, using water and hexane as control treatments. For details of ITC analyses, see below.

Glucosinolate analysis. Glucosinolates were extracted from ground shoot tissue.[37] In brief, GLSs were extracted from 100 mg of dry shoot tissue by 70% MeOH which was heated to 90 °C to deactivate myrosinase and therefore avoid degradation of the GSLs. The extracts were transferred to a DEAE-Sephadex 25 column (Sigma, St. Louis, MO, USA) before de-sulfating. Desulfoglucosinolates were separated and identified using HPLC with an acetonitrile/Milli-Q water gradient. GSL detection was performed with a photodiode array detector with 229 nm wavelength. The correction factors at 229 nm to calculate the concentrations of GSLs were followed.[38,39] The peaks of desulfoglucosinolates were identified by comparison with standards on the basis of retention times and ultraviolet spectra. Different concentrations of sinigrin (2-propenyl) (Acros Organics, Fair Lawn, NJ, USA) were used as external standard and extracted following the same procedure as the shoot tissue. The levels of GSLs were calculated on the basis of dry weight of tissues.

Isothiocyanate analysis. Ground shoot tissue (140 mg) was placed in a glass dish and moistened with 5 mL Milli-Q water. The suspension was immediately placed under a bell shaped glass vessel (380 mL) with two openings and sealed with bulldog clips at 24±2 °C

205 in the dark. Volatile compounds were collected by pulling air from outlet of the jar
206 through a glass liner containing Tenax TA (50 mg 60/80 mesh, Supelco, Bellefont, PA,
207 USA).

208 A positive pressure push-pull system was used, with charcoal-filtered air pushed
209 through an inlet into the vessel at 500 mL/min and pulled out through the adsorbent at
210 350 mL/min. The greater push rate prevented entry of contaminating volatiles. All
211 glassware and Teflon tubes (connecting air flow tubes) were washed with detergent,
212 distilled water and acetone and baked in an oven at 175 °C for at least 16 hours prior to
213 the entrainment. Charcoal filters and glass liners with Tenax TA were baked at 175 °C
214 and 220 °C respectively under N_2 flow for 16 h. Volatiles were collected for periods of
215 10 min at 0-10, 30-40, 60-70 and 120-130 min after adding water to the tissue. The rest of
216 the time, the outlet of the glass vessel was open preventing the build-up of released
217 volatiles.

218 Concentrations of ITCs arising from the dosing of chemical standard (CS) solutions as
219 described above were determined by analysing one µl of either B.juncea-CS or S.alba-CS
220 mixtures put on a filter paper, placed inside a sealed plastic cup (220 mL). A glass liner
221 (with Tenax TA) was inserted through a hole in the plastic cup and air was pulled from
222 the cup at a flow rate of 350 mL/min. The aim was to sample at the peak concentration of
223 the major ITCs, which was after 60-70 min incubation for B.juncea-CS and 0-10 min for
224 S.alba-CS, as indicated by previous analysis of volatiles from *B. juncea* and *S. alba* plant
225 tissues. Volatiles were collected for 10 min and analysed by gas chromatography as
226 described below.

For quantification, collected volatiles were analysed by gas chromatography (GC) on a Agilent 6890N with a flame ionization detector, equipped with an HP-1 column (100% dimethyl polysiloxane, 50 m, 0.32 mm i.d. and 0.52 μm film thickness, J & W Scientific, USA), and fitted with an Optic 3 thermal desorption system (Atas GL Intl., Veldhoven, Netherlands). An internal standard, 50 ng of decane, was injected onto the glass liner containing the sample prior to desorption. The liner containing the Tenax with absorbed volatiles was placed directly into the injector and volatiles were thermally desorbed starting at 30 °C for 0.5 min and rising at 16 °C/sec to 250 °C. The GC temperature program was 30 °C for 3 min, 5 °C/min to 150 °C for 0.1 min then 10 °C/min to 250 °C for 15 min, using hydrogen as carrier. The amount of each compound was calculated relative to the internal standard.

For tentative compound identification, volatile samples from the treatments, *B. juncea* hydrated plant tissue, B.juncea-CS mixture, *S. alba* hydrated plant tissue and S.alba-CS mixture were collected as described above and analysed by coupled GC-mass spectrometry using an Agilent 7890N GC coupled to an Agilent 5975C mass selective detector (electron impact 70eV) fitted with an Optic 3 thermal desorption system (Atas GL Intl., Veldhoven, Netherlands). The thermal desorption and GC oven temperature programs were as described above. The carrier gas was helium with a flow rate of 1.3 mL/min. Volatile compounds were identified by comparison against a commercially available library (National Institute of Standards and Technology, NIST 08, USA) and by comparison of mass spectra and retention indices with commercially available authentic standards where available (Sigma-Aldrich AB, Sweden). Where standards were unavailable, in some cases the retention index (Kovats Index, KI) of the substance could

250 be matched with a previously published KI for the compound on the type of GC column
251 used in the current study (HP-1) (Figure 5).
252
253 **ITC/GSL ratios.** ITC/GSL ratios were calculated for selected GSLs. The GSL 2-
254 propenyl was the dominant GSL in *B. juncea* shoot tissues, and *p*-hydroxybenzyl GSL
255 was dominant in *S. alba* shoot tissues. 2-phenylethyl was detected in both plant tissues as
256 a common GSL. The detected GSLs were recorded as µmol/g dry tissue in the GC
257 analysis (Table 2) and converted to µg for the selected GSLs using their molecular
258 weights. Estimated amounts of ITCs produced from their parent GSLs in hydrated plant
259 tissues during the initial 130 minutes were calculated (Figures 5 A & B). Total amounts
260 of each ITC (µg) produced in the initial 130 min of hydrolysis from 1 g hydrated dry
261 tissue was divided by the amount of each GSL (µg) in 1 g dry tissue and multiplied by
262 100 to determine the ITC/GSL ratios.
263
264 **Statistical analysis.** Mean DSI and radial growth of hyphae were transformed to their
265 natural logarithms. The experiments were generally analysed in accordance with their
266 completely randomized design. However, the soil treatment (sterilized vs. non-sterilized)
267 in the test of disease development on peas after oospore exposure to ITC volatile
268 compounds was treated as a sub-plot factor and the cover crops as main-plot factor in a
269 split plot design. The effect of cover crop, dose of cover crops, time of exposure to
270 volatile and cover crop/chemical were treated as fixed factors in all experiments and
271 replicate was treated as a random factor. Analyses of variances were made using the

procedure linear model and least square means were compared using Tukey's test with a $P \leq 0.05$ significance limit in R version 2.15.1 (The R Foundation, 2012)

RESULTS

Effects of volatile compounds on oospores. Volatile compounds from hydrated *B. juncea* dry tissue strongly reduced the inoculum potential of *A. euteiches* oospores, shown by a significant reduction in pea root rot symptoms compared to control shoot tissues from the other species ($P<0.001$, SE ± 0.120; Figure 1). The other cover crops or soil sterilization before the experiment started had no significant effect on disease development.

Effect of volatile compounds on hyphal growth- effect of dose. *Brassica juncea* shoot tissue suppressed the hyphal growth of *A. euteiches* more efficiently than tissue from the other species ($P<0.001$) (Figure 2). The hyphal growth of *A. euteiches* was more affected by shoot tissue dose with *B. juncea* than with *S. alba* (dose X cover crop interaction, $P<0.001$). The highest dose of *B. juncea* shoot tissue completely inhibited growth, and no further growth was registered when these cultures were placed in fresh air. The volatile compounds produced from the lower doses of *B. juncea* and the highest dose of *S. alba* also reduced the hyphal growth of *A. euteiches* significantly compared with the water control. No effect was found in the *S. cereale* tissue treatment.

Effect of volatile compounds on hyphal growth- effect of exposure time. The hyphal growth of *A. euteiches* was affected by the time of exposure to volatile compounds

295 produced from hydrolysed *B. juncea* shoot tissue (P=0.002). The volatile compounds
296 from the 140 mg dose of hydrated *B. juncea* shoot tissue completely inhibited the growth
297 of *A. euteiches* after exposure for 120 min (Figure 3). No further growth was observed
298 during the following days when the cultures were placed in fresh air.
299
300 **Effects of chemical standard mixtures.** Volatile compounds arising from the chemical
301 standard mixtures suppressed the growth of *A. euteiches in vitro* (P<0.001). Volatile
302 compounds from hydrated *B. juncea* dried shoot tissue and both chemical standard
303 mixtures, B.juncea-CS and S.alba-CS, completely inhibited the growth of *A. euteiches*
304 (Figure 4). There was no further growth in the following days when the cultures were
305 placed in fresh air. Volatile compounds from hydrated *S. alba* tissue also caused some
306 reduction of hyphal growth compared to the water control. No inhibition of pathogen
307 growth was observed when exposed to the solvent hexane.
308
309 **Glucosinolates in *B. juncea* and *S. alba* shoot tissues**. Both species contained aliphatic,
310 aromatic and indolyl GSLs. Total extracted GSL concentrations in *S. alba* were higher
311 than the concentration of GSLs in *B. juncea*. *Sinapis alba* contained one more GSL
312 compound than *B. juncea*. The aliphatic GSL 2-propenyl dominated in *B. juncea* and the
313 aromatic GSL *p*-hydroxybenzyl dominated in the *S. alba* tissue (Table 2).
314
315 **Isothiocyanates from *B. juncea* and *S. alba*.** *Sinapis alba* shoot tissue produced more
316 volatile compounds and a greater total amount of ITCs, than *B. juncea*. *Sinapis alba* and
317 *B. juncea* were dominated by aromatic benzyl ITC hydrolysed from the GSLs *p*-

hydroxybenzyl and benzyl, and aliphatic allyl ITC from the GSL 2-propenyl, respectively. Both plant species produced the aromatic phenylethyl ITC (Table 3).

Pattern of ITC release from *B. juncea* and *S. alba* hydrated tissues. ITCs were released faster from the GSLs in *S. alba* than from the GSLs in *B. juncea* tissue at the beginning of hydrolysis (Figures 5 A & B). The GSLs of *B. juncea* produced ITCs over a longer time with increasing rate compared to ITCs from *S. alba* tissue. The estimated ITC/GSL ratio of the dominant aliphatic 2-propenyl producing allyl ITC in *B. juncea* was 1.82. The ITC/GSL ratio for the dominant aromatic *p*-hydroxybenzyl and benzyl producing benzyl ITC in *S. alba* was 0.56. The ratio of the aromatic 2-phenylethyl GSL producing phenylethyl ITC which was common to both species was 11.51 for *B. juncea* and 15.51 for *S. alba*.

The release patterns of phenylethyl ITC were different for the two species, even though the originating GSL 2-phenylethyl was the same in both species. Instead, it followed the same pattern as the dominant allyl ITC in *B. juncea* and benzyl ITC in *S. alba* (Figures 5 A & B).

DISCUSSION

This study shows that volatile compounds produced by brassica shoot tissue have a strong inhibitory effect on *Aphanomyces euteiches*. However the results also highlight the importance of understanding the mechanisms behind the suppression when designing systems where plants containing GSLs are used to control soil borne pathogens. We show

that brassica species, dose of ITC and time of exposure interact in determining the effect of the volatile compounds on *A. euteiches* mycelium or oospores.

We used shoot tissues from two brassica species with different GSL profiles. Although we used different cultivars than in earlier investigations, produced the plant tissue under greenhouse conditions, partly with artificial light, and dried the material to standardise dosages, the variety and concentration of GSLs in *B. juncea* and *S. alba* shoot tissues was similar to that found in earlier studies.[10,15,42-45] This indicates that the GSL composition remains consistent in the plant species despite differences in cultivar, growing conditions and low temperature drying of the plant material. As a consequence of the composition of GSLs, we found that the volatile compounds produced from *B. juncea* and *S. alba* were dominated by allyl and benzyl ITCs respectively, which is in line with earlier studies.[10] The thick walled, long lived oospores of *A. euteiches* are very different structures from the thin walled *A. euteiches* hyphae. Nevertheless, suppression by direct exposure to volatile compounds was shown for both these reproductive structures. For both mycelium and oospore exposures, volatiles hydrolysed from *B. juncea* tissue were more effective than volatiles from *S. alba* tissue. Previous investigations comparing the effects of aliphatic and aromatic ITCs tested the survival of weevil larvae.[30] The conclusion was that the aliphatic allyl ITC is likely to have greater biological activity than the aromatic ITCs, which is in line with the biological responses observed in the current study.

The *in vitro* data from the current study show that the amount of *B. juncea* tissue used was lethal to *A. euteiches*, whereas the equivalent amount of *S. alba* tissue did not completely prevent pathogen growth. This indicates that the highest dose of *S. alba* dry shoot tissue did not produce a high enough concentration of volatile compounds to

363 prevent pathogen growth. However in the experiment with chemical standards, the
364 volatile compounds released from the *S. alba* chemical mixture completely prevented
365 hyphal growth. The analysis of collected volatile compounds showed that the *S. alba*
366 chemical mixture produced 10 times more ITCs than *S. alba* hydrated dry shoot tissue. It
367 can be assumed that the concentration of ITCs contributes to their suppressive effects.
368 Our ITC analysis revealed differences in the pattern of ITC release between *B. juncea*
369 and *S. alba* hydrated dry tissues. The calculated ITC/GSL ratios indicate that the GSL 2-
370 propenyl is more efficient than *p*-hydroxybenzyl and benzyl GSLs at producing ITCs.
371 The chemical structure and size of GSLs mainly regulate their efficiency of conversion
372 into ITCs.[25,48] Our chemical analysis also shows the hydrated dry shoot tissue of *S. alba*
373 released volatile compounds more rapidly, with the highest concentration recorded within
374 a much shorter time than for *B. juncea*. The dominant ITC released by *B. juncea* tissue,
375 allyl ITC, has a lower molecular weight and lower boiling point than benzyl ITC, the
376 dominant compound released by *S. alba*. However, despite the expected higher volatility
377 of allyl ITC, its concentration in the headspace of hydrolysed powder of the respective
378 plants was comparable to that of benzyl ITC. Although it is likely that the volatility of
379 these ITCs does influence their toxicity, our results suggest that other factors inherent to
380 the dry powders themselves are also important.
381 The hydrolysis process for the dominant aliphatic GSL in *B. juncea* shoot tissue
382 seemed to follow a longer time course than the dominant aromatic GSL in *S. alba*.
383 Further, we observed that the release pattern of phenylethyl ITC differed between plant
384 species, following the same pattern as the dominant ITC for each species. The ITC/GSL
385 ratio (indicating the efficiency of production) of the species common aromatic

386 phenylethyl ITC from the parent GSL 2-phenylethyl was lower in *B. juncea* tissue than in
387 *S. alba*. This suggests that other traits inherent to the dry powders, such as plant cell
388 structure or water absorbing capacity, influence GSL hydrolysis and ITC release. These
389 factors probably interact with the characteristics of the chemicals themselves, such as
390 volatility, to determine the eventual ITRC release.[48] These factors, however, require
391 further investigation.

392 Toxic effects from incorporated *B. juncea* plant tissue in *A. euteiches* contaminated
393 soil have been shown to reduce the development of pea root rot, but an effect of ITCs has
394 not always been established.[12,46] In a closed system, volatile ITCs can easily interact
395 directly with the exposed pathogen. We show that the degree of detrimental effects from
396 ITCs was enhanced as the time of pathogen exposure increased. For *A. euteiches* growing
397 on CMA agar medium, the lethal dose of ITCs from *B. juncea* was reached after two
398 hours of exposure, showing the importance of keeping the concentration of effective ITCs
399 high for this period. For an optimal effect in a bio-fumigation process, this suggests the
400 use of a cover after incorporating plant biomass to minimise evaporation of volatiles.
401 This measure was also suggested in earlier studies.[47]

402 The current study shows that brassica plant tissue, its dominant ITCs, their
403 concentration and release pattern, and exposure time are important factors for the
404 suppression of *A. euteiches*. The results highlight the importance of choosing plant
405 species with the most effective production ratio but also high biomass production to reach
406 optimal concentration levels when GSL containing plants are used for bio-fumigation.
407 The results support the use of a cover to minimise dispersal of ITCs, which should allow

the ITCs to interact directly with the pathogen for a longer period. However, the findings from this study need further verification under field conditions.

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FIGURE CAPTIONS

Figure 1. Pea root rot symptoms registered in a bioassay depending on pre-treatment of Aphanomyces oospores with volatile compounds from hydrated dry plant tissues. Pea seedlings were rated for pea root rot three weeks after pea seed sowing. The scale for disease severity index (DSI) is 0-100 (%). 0%=healthy plant; 25% = root slightly discolored; 50% = root extensively discolored but not shrunken; 75% = root extensively discolored and shrunken; 100% = root partly or completely rotted or plant dead.[36] The value of DSI is back transformed from the natural logarithm. Control = water.

Figure 2. Effects of volatile compounds from 60, 100 and 140 mg hydrated *Brassica juncea*, *Sinapis alba* and *Secale cereale* dry shoot tissues on the hyphal growth (cm/day) of *Aphanomyces euteiches* at 24 °C, *in vitro*. Control = water.

Figure 3. Effects of volatile compounds from 140 mg hydrated *Brassica juncea* dry shoot tissues exposed for 40, 80 and 120 min on the hyphal growth (cm/day) of *Aphanomyces euteiches* at 24 °C, *in vitro*. Control = water.

Figure 4. Effects of volatile compounds from 140 mg hydrated *Brassica juncea* and *Sinapis alba* dry shoot tissues and the chemical standard mixtures, B.juncea-CS and S.alba-CS on the hyphal growth (cm/day) of *Aphanomyces euteiches* at 22 °C, *in-vitro*. Control = water.

Figure 5. Release pattern and concentration of allyl, benzyl and phenylethyl ITCs at different time (min) points after adding water to 1 g dry shoot tissue of A) *Brassica juncea* (cv. Pacific Gold) and B) *Sinapis alba* (cv. Architect).

Table 1. Major isothiocyanates detected from 140 mg hydrated dried plant tissues of *Sinapis alba* and *Brassica juncea* and their chemical standard mixtures 1 µL each (S.alba-CS and B.juncea-CS). Samples were taken 0-10 min and 60-70 min after adding water, according to the highest peaks with 140 mg dried plant tissues of *Sinapis alba* and *Brassica juncea*, respectively.

Treatment	Allyl (µg/min)	SE[e]	Benzyl (µg/min)	SE	Phenylethyl (µg/min)	SE
S. alba at 0-10 min	0.0003	0.00002	0.0245	0.00299	0.0026	0.00009
S.alba-CS[a] at 0-10 min	0.0013	0.00013	0.2051	0.05291	0.0123	0.00483
B. juncea at 60-70 min	0.3650	0.00710	nd[c]		0.0003	0.00005
B.juncea-CS[b] at 60-70 min	0.4310	0.01100	ni[d]		nd[c]	

[a]Mixture of chemicals standard for predominant ITCs from *S. alba* denoted as 'S.alba-CS'.
[b]Mixture of chemicals standard for predominant ITCs from *B. juncea* denoted as 'B.juncea-CS'.
[c]Not detected
[d]Not included in mixture
[e]SE=Standard error

Table 2. Different glucosinolates and concentrations (μmol/g) in dry shoot tissue of *Brassica juncea* (cv. Pacific Gold) and *Sinapis alba* (cv. Architect)

Compound	***Brassica juncea***	**SE**	***Sinapis alba***	**SE**
Aliphatic				
2-(S)-2-Hydroxybutenyl	nd[a]	nd	0.515	0.023
5-Methylsulphinylpentyl	nd	nd	0.008	0.005
4-Pentenyl	0.011	0.006	nd	nd
n-Butyl	0.032	0.011	nd	nd
3-Methylthiopropyl	nd	nd	0.013	0.004
3-Butenyl	0.012	0.007	0.062	0.005
2-Hydroxy-3-butenyl	nd	nd	0.018	0.001
2-Propenyl	3.909	0.497	nd	nd
Unknown	0.019	0.007	nd	nd
Aromatic				
2-Phenylethyl	0.119	0.016	0.068	0.005
Benzyl	nd	nd	0.483	0.054
p-Hydroxybenzyl	nd	nd	7.935	0.981
Indolyl				
4-Hydroxy-3-indolylmethyl	0.006	0.004	nd	nd
4-Methoxy-3-indolylmethyl	0.001	0.001	0.008	0.001
3-Indolylmethyl	0.040	0.007	0.016	0.002
1-Methoxy-3-indolylmethyl	0.008	0.002	0.009	0.002
Total	4.156		9.136	

[a]nd, not detected

Table 3. Volatile compounds (µg/min) in the first two hours after adding water in 1 g dry shoot tissue of *Brassica juncea* (cv. Pacific Gold) and *Sinapis alba* (cv. Architect)

Compound	***Brassica juncea***	**SE**	***Sinapis alba***	**SE**
Aliphatic ITC				
3-Butenyl[b]	0.0024	0.0003	0.0352	0.0104
4-Methylpentyl[b]	nd		0.005	0.0014
Allyl[a]	0.2077	0.0211	0.0016	0.0007
n-Heptyl[a]	nd		0.0005	0.0001
n-Hexyl[a]	nd		0.0024	0.0009
n-Pentyl[a]	nd		0.0008	0.0002
Sec-Butyl[c]	0.0022	0.0004	0.0007	0.0003
Aromatic ITC				
Benzyl[a]	nd		0.1851	0.0404
Phenylethyl[a]	0.0112	0.0021	0.0289	0.0046
Others				
(z)-3-Hexen-1-ol[a]	nd		0.0017	0.0002
(z)-3-Hexenal[a]	0.0019	0.0006	0.0024	0.0007
3.5-Octadien-2-one[d]	0.0014	0.0003	0.0023	0.0005
Allyl thiocyanate[b]	0.0211	0.0022	nd	
Benzaldehyde[a]	0.0024	0.0004	0.0058	0.0011
Benzyl isocyanate[a]	nd		0.0014	0.0003
2-phenylpropane[d]	0.0088	0.0047	0.0054	0.0032
Diallyl disulphide[a]	nd		0.0003	0.0001
Dimethyl disulphide[a]	nd		0.0005	0.0003
Total	0.2591		0.28	

[a]mass spectrum and retention index (Kovats Index KI) match with National Institute of Standards and Technology standard library (NIST 08) and with an authentic standard
[b]mass spectrum match in NIST08 and KI concurs with published KI[40]
[c]no satisfactory match in NIST08 but mass spectrum matches published spectrum[41]
[d]match in NIST08

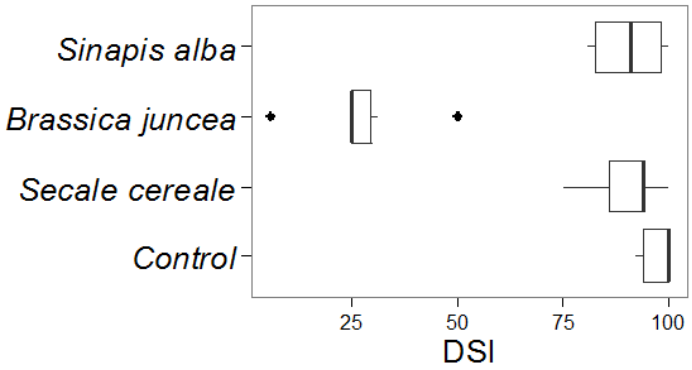
Sinapis alba
Brassica juncea
Secale cereale
Control
25
50
75
100
DSI

Figure 1

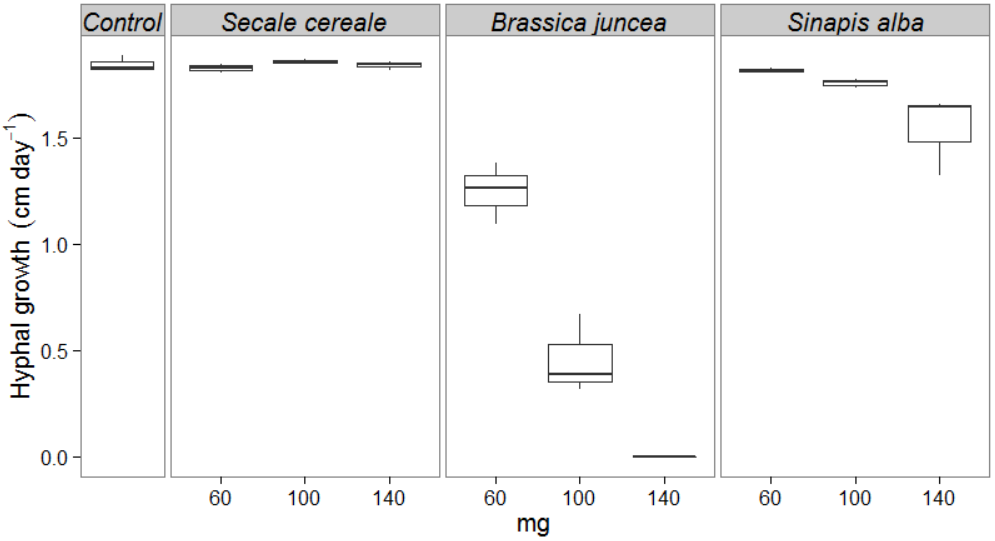
Control
Secale cereale
Brassica juncea
Sinapis alba
Hyphal growth (cm day^{-1})
0.0
0.5
1.0
1.5
60
100
140
mg

Figure 2

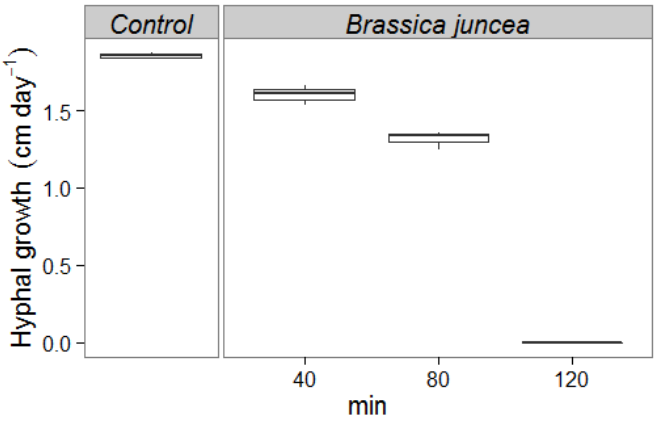
Control
Brassica juncea
Hyphal growth (cm day^{-1})
1.5
1.0
0.5
0.0
40
80
120
min

Figure 3

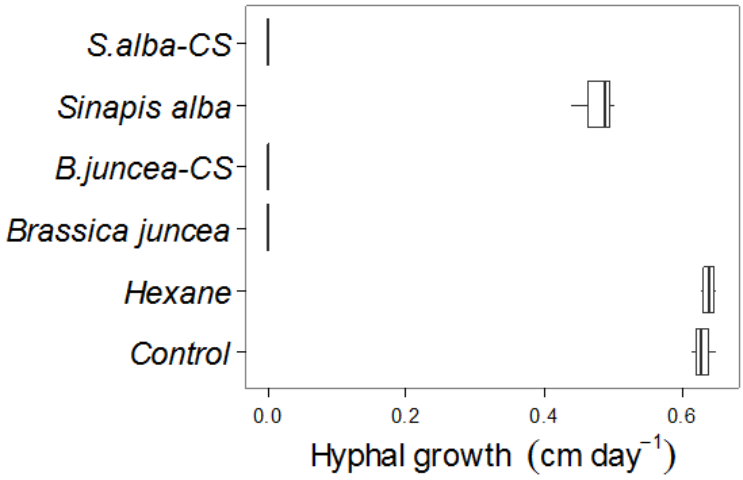
S.alba-CS
Sinapis alba
B.juncea-CS
Brassica juncea
Hexane
Control
0.0
0.2
0.4
0.6
Hyphal growth (cm day^{-1})

Figure 4

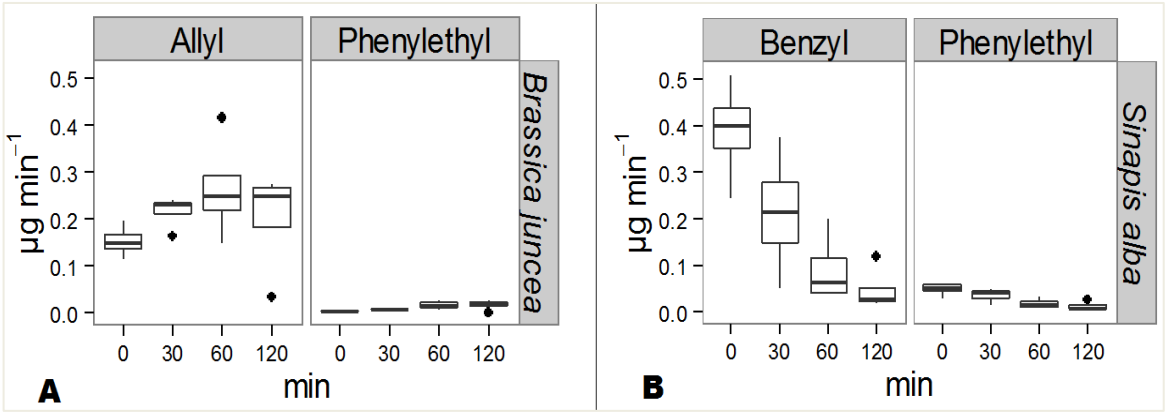
Allyl
Phenylethyl
Brassica juncea
µg min−1
0.5
0.4
0.3
0.2
0.1
0.0
0
30
60
120
min
A
Benzyl
Phenylethyl
Sinapis alba
B

Figure 5