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Soil Sand Content, But Not Solil Myrosinase Enzyme Activity, Affects MAM Efficacy in Suppressing Weed Growth

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Abstract

Mustard seed meal (MSM) has been shown to control weeds and pests in crop fields. Prior work showed that a sandy loam soil was superior to a silt loam soil in supporting velvetleaf growth suppression by MSM. To explore this difference, myrosinase enzyme activity was measured in both soils. Results showed no significant difference between the soils. Sand content in Michigan silt loam soil was manipulated in order to test the role of sand content on MSM efficacy in suppressing short-term velvetleaf growth. Results showed that sand enhanced the effect of MSM in suppressing seedling growth and germination.

Introduction

Biofumigation is a plant-based alternative to control pests in soil, developed in the 1990s in response to the phasing out of widely-used synthetic chemicals, such as methyl bromide (Martin 2003). The process involves pulverizing plant parts of the *Brassica* genus, with our choice being mustard seed meal (MSM). The molecular basis involves a reaction between components present in the plant parts: the enzyme myrosinase hydrolyzes glucosinolate in the presence of water. One of the products is isothiocyanate (ITC), the bioactive ingredient that has been shown to control pests (Angus et. al 1994; Brown & Morra 1997). ITC interacts with seedlings in a phytotoxic manner – by inhibiting their growth (Fenwick et al. 1982). The molecular basis for biofumigation is that ITC is nonspecifically and irreversibly toxic to many organisms due to its reaction with sulphur-containing proteins and amino acids (Brown & Morra 1997).

Research about biofumigation currently involves understanding how the reactants (glucosinolate) and products (ITC) of the process interact with the environment (directly affects efficacy and environmental effects). Being natural compounds, they are safe to use and they exhibit relatively fast degradation times, so there is low risk of leaching (refers to loss of nutrients from the soil – accumulation of compounds results in soil leaching). Maximizing efficacy has been shown to be possible through increasing the amount of glucosinolate hydrolyzed or preserving ITC activity in the soil (Gimsing & Kirkegaard 2006; Matthiessen & Kirkegaard 2006). But through natural means, ITC degrades and is degraded by microorganisms (Warton et. al 2003).

The field of research involving biofumigation shows promise as a natural alternative to phased-out synthetic chemicals. Although efficacy has not reached that of synthetic chemicals yet, ITC has the beneficial property of being virtually harmless to the environment. As a relatively new technique, optimization of biofumigation has been the focus of researchers at present. Maximizing efficacy can be done in numerous ways, one of which involves gaining insight into the variability

shown in the efficacy of biofumigation under different soil compositions. In an article by Gimsing et al. (2009) that discussed glucosinolate activity with variations in temperature and water used, the difference in soil composition being tested showed a noticeable difference – the glucosinolate seemed to degrade faster in soil with lower percentage of sand. Moreover, previous research from Zdor (unpublished) demonstrated increased MSM efficacy in sandy soil versus silt soil. The effect of sand in biofumigation has not been extensively tested, presenting a gap of knowledge which I hope to fill with this project. My research focuses on exploring the differences in ITC efficacy between Michigan (MI) silt loam and Missouri (MO) sandy loam by testing myrosinase enzyme activity of the soils and manipulating soils to alter levels of sand to soil content. Our hypothesis predicts that ITC efficacy will increase with increase in sand content. Furthering the knowledge in this field could lead to a natural, affordable, and effective way to control pests. If manipulation of sand composition in soil is found to be effective, it could provide an easy way to increase the efficacy of biofumigation.

Methods

Soil Myrosinase Enzyme Activity Quantification

The presence of active myrosinase enzyme hydrolyzes glucosinolate into isothiocyanate (ITC). Measuring myrosinase activity gives insight into the amount of glucosinolate being hydrolyzed, showing the ratio of reactant that was hydrolyzed into product. This shows whether the soil collected in Michigan exhibits the same enzymatic activity as the soil collected in Missouri. The glucosinolate-containing substance used in our experiment was sinigrin, derived from a *Brassica* plant. Quantification of myrosinase activity was done indirectly through a glucose assay. This method is viable due to glucose being a side product of the reaction (Fig. 1); the amount of glucose produced is directly correlated to the amount of isothiocyanate produced (Brown & Morra 1997). The procedure for running the enzyme assay was outlined by Al-Turki and Dick (2003).

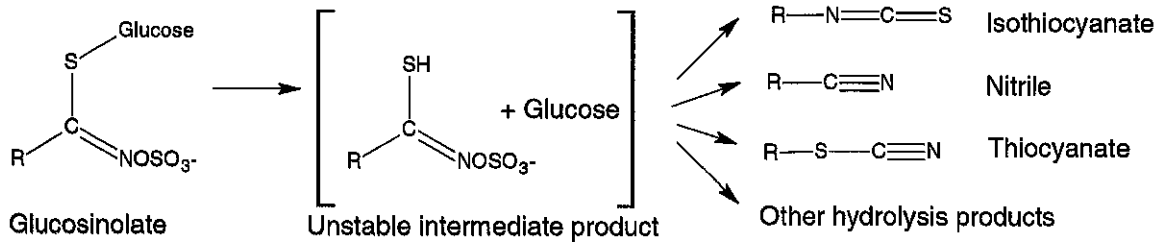


Figure 1. The hydrolysis of glucosinolate by myrosinase enzyme. Products of the reaction include glucose, isothiocyanate, nitrile, and thiocyanate. Figure from Gimsing & Kirkegaard 2009.

The types of samples tested were: Michigan (MI) silt loam with sinigrin, Missouri (MO) sandy loam with sinigrin, MI control, and MO control (control samples lacked sinigrin).

Hydrolyzing sinigrin involved 1 g of dry soil sieved through a 1 mm metal screen (MI and MO soil) and the addition of (in order) 0.2 mL toluene, 2.3 mL 0.1 M TES buffer at pH 7.0, and 0.5 mL sinigrin stock solution at 20 mM. This produced a final solution of 3 mL per sample tube. The TES buffer was prepared using TES buffer powder dissolved in deionized (DI) water, with adjustments in pH made using 10 M NaOH. The 20 mM sinigrin stock solution was prepared with sinigrin powder dissolved in DI water.

The samples were then incubated for 4 hr at 37 °C. For the experimental, sinigrin was added before the incubation, and for the control, sinigrin was added after the incubation. After incubation, the samples were centrifuged using a benchtop centrifuge at top speed to remove the soil. The supernatant was pipetted into a separate tube and then centrifuged in a floor centrifuge at 8,000 x g for 10 min. A Corning 0.2 μm SFCA filter separated out any remaining particulates in the supernatant.

The glucose assay utilized a Sigma GAGO-20 kit (contained a glucose oxidase/peroxidase capsule, *o*-dianisidine reagent vial, 0.5 mL glucose standard) and the experiment proceeded following directions of its technical bulletin. The glucose oxidase/peroxidase capsule was opened and dissolved in 39.2 mL of DI water. DI water (1 mL) was added to the *o*-dianisidine reagent vial, with

0.8 mL of it being transferred into the bottle. The various solutions for the glucose assay were prepared according to Table 1. The reagent was not be added until all the solutions are prepared. A stopwatch was be readied and started as soon as the reagent was added to a solution. The reaction was allowed to run for exactly 30 min at 37°C. When the 30 min was up, 2 mL of 12N H_2SO_4 was added into each tube to stop the reaction. The solutions were then read in a spectrophotometer at 540 nm. The glucose standard was used to create a standard curve, from which the amount of glucose can be calculated using the slope.

The experiments were performed twice. Soil samples were produced for each treatment, and each sample was replicated and assayed. Run 1 had a total of 4 soil samples: 2 replicates were produced for each of the 4 treatments – resulted in 8 glucose values. Run 2 had a total of 6 soil samples: samples for each of the experimental treatments were produced twice and each had 2 replicates, and there were 2 replicates for the control treatment – resulted in 12 glucose values.

Tube content	Water (μL)	Sample (mL)	Glucose Standard (μL)	Reagent (mL)	Final Volume (mL)
Blank	1000	--	0	2	3
Standard 1	980	--	20	2	3
Standard 2	960	--	40	2	3
Standard 3	940	--	60	2	3
Standard 4	920		80	2	3
MI soil control	--	1	--	2	3
MI soil + sinigrin	--	1	--	2	3
MO soil control	--	1	--	2	3
MO soil +sinigrin	--	1	--	2	3

Table 1. Solutions prepared to perform the glucose assay. The standards were produced using the glucose standard in the kit at different dilutions. The supernatant of the centrifuged soil samples were assayed. Reagent (2 mL) was added to start the assay reaction.

Velvetleaf Seed Germination in Varying Sand-Soil Combinations

Testing for the efficacy of MSM in varying soil compositions was done by measuring the root length of soil-grown velvetleaf seedlings. The control soil used was the MI silt loam that was analyzed to be 29% sand. Pure sand was then added to bring the MI soil to 35%, 45%, and 70% sand. The amount to be added was determined by: $pure\ sand\ (g) = \frac{\%\ sand\ desired - 29}{1-(29/100)}$. For each

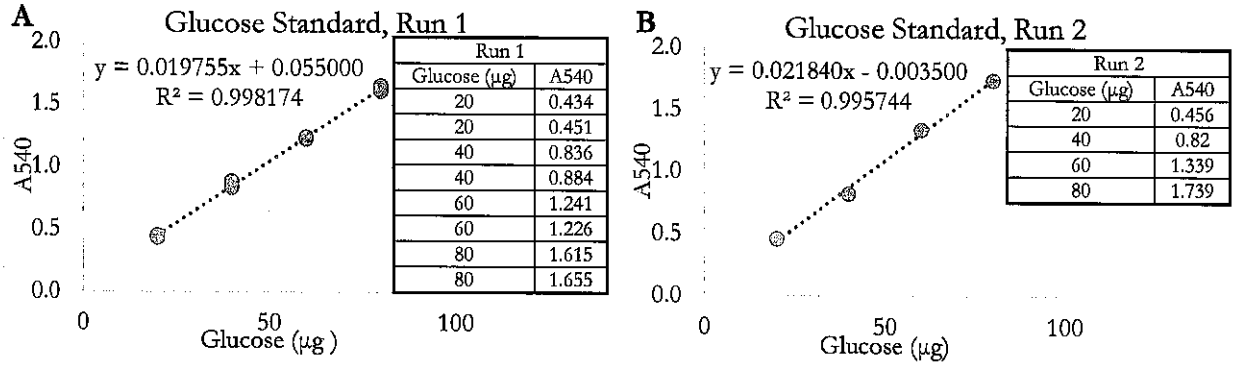
nonsterile square petri plate, 100 g of respective soil mixes were added. The experimental condition was adding in 1 g of MSM per 100 g of soil mix. The MSM (*Brassica juncea* Pacific Gold mustard, provided by Jim Davis at the University of Idaho Canola and Mustard Program) was prepared by crushing with mortar and pestle and then sieving it through a 1 mm metal screen (to be used immediately). After the soil mixes (and MSM) have been prepared in the plates, 24 mL of sterile water was pipetted evenly onto the soil and then there was a wait time of 30 min before the seeds were planted. For each plate, 24 velvetleaf seeds were planted (4 rows). The seeds were prepared (disinfested and heat shocked) by: placing seeds in a tea holder, placing holder in 50 mL 10% bleach and agitated for 3 min, rinsing by shaking holder in 50 mL sterile water for 10 s, placing holder in 50 mL 70% ethanol for 2 min, rinsing and shaking holder in 50 mL sterile water for 10 s for 10 times, placing holder in 50 mL sterile water in a 65 °C water bath for 10 min. The covers of the plates were Parafilm wrapped to their bases and then incubated at 29 °C for 48 hr. After incubation, the percent that germinated was determined and root lengths were measured using a digital caliper. The data set of root lengths was analyzed using an ANOVA test, with Bonferroni post-hoc. This test was used because there were more than two conditions that was tested for a set of means, and an ANOVA was the test that suited this purpose. To account for seeds that were not able to germinate, a germination rate for each condition was recorded. The entire experiment was performed twice (2 runs), with 768 seeds planted.

The method of counting root lengths to determine the efficacy of MSM is viable due to the nonspecific toxicity that MSM exhibits, even to seedlings and their root growth. Studies have shown that the products of biofumigation interact with seedlings by inhibiting their growth (Fenwick et al. 1982; Oleszek 1987; Petersen et al. 2001). Thus, relationship can be drawn between MSM presence and seedling root length (seedlings in MSM soil have shorter root lengths).

Results

Soil Myrosinase Enzyme Activity Quantification

The glucose standard for "Run 1" was created from duplicates for each glucose concentration, while "Run 2" only used one sample for each glucose concentration. The slope of the standard curve for "Run 1" was 0.0198 (Fig. 2A) and was 0.0218 for "Run 2" (Fig. 2B). The slope was taken from plotting absorbance values versus glucose concentration (in μg) for the glucose standard. The absorbance values ('y') for each soil sample was then substituted in. Solving for 'x' using the slope-intercept forms for the respective runs yielded the calculated sugar value (μg glucose g^{-1} soil 4 hr^{-1}). In "Run 1" each soil type and treatment was analyzed twice in separate tubes. In "Run 2" duplicates were made for each soil type and treatment and they were each analyzed twice in separate tubes as well. The difference in temperature between the runs was due to experimental error (although no noticeable difference resulted). Overall, myrosinase enzyme activity was low for both soils. Myrosinase enzyme activity was not noticeably different between the MI silt loam and MO sandy loam, with and without sinigrin. The results and samples tested for the myrosinase enzyme activity assay are summarized in Table 2 and Figure 3.



Run 1	
Glucose (μg)	A540
20	0.434
20	0.451
40	0.836
40	0.884
60	1.241
60	1.226
80	1.615
80	1.655

Run 2	
Glucose (μg)	A540
20	0.456
40	0.82
60	1.339
80	1.739

Figure 2. Glucose standard curves were produced from plotting the absorbance at 540 nm (A540) vs. μg of glucose. The amount of sugar in each prepared sample is listed on the inset table. The slope-intercept forms for the respective runs were used to calculate sugar values from absorbance values of the soil samples. A) The glucose standard curve for run 1, with a slope=0.01978. B) The glucose standard curve for run 2, with a slope=0.02184.

Soil Sample Treatments and Calculated Sugar Values

Run 1			Run 2		
Soil Type & Treatment	A540	Calculated Sugar Value (μg glucose g^{-1} soil 4 hr^{-1})	Soil Type & Treatment	A540	Calculated Sugar Value (μg glucose g^{-1} soil 4 hr^{-1})
MI+S A	0.301	12.42424	MI+S 1A	0.190	8.539377
MI+S B	0.349	14.83586	MI+S 1B	0.186	8.356227
MI con A	0.252	9.949495	MI+S 2A	0.249	11.24084
MI con B	0.261	10.41667	MI+S 2B	0.235	10.59982
MO+S A	0.266	10.65657	MI con A	0.281	12.70604
MO+S B	0.334	14.10534	MI con B	0.277	12.52289
MO con A	0.200	7.323232	MO+S 1A	0.227	10.23352
MO con B	0.211	7.867133	MO+S 1B	0.226	10.18773
			MO+S 2A	0.345	15.63645
			MO+S 2B	0.333	15.087
			MO con A	0.210	9.455128
			MO con B	0.189	8.49359

Table 2. Myrosinase enzyme assay. Soil types and treatments analyzed with a spectrophotometer by taking the absorbance at 540 nm (A540). This value is then used to extrapolate sugar values using the slope intercept form (y=mx+b) for the respective runs: (absorbance is 'y' and sugar value is 'x'). The soil types and treatments are: MI soil control (MI con), MI soil + sinigrin (MI+S), MO soil control (MO con), MO soil + sinigrin (MO+S). The numbers denote different prepared samples, while the letters indicate duplicates from same sample.

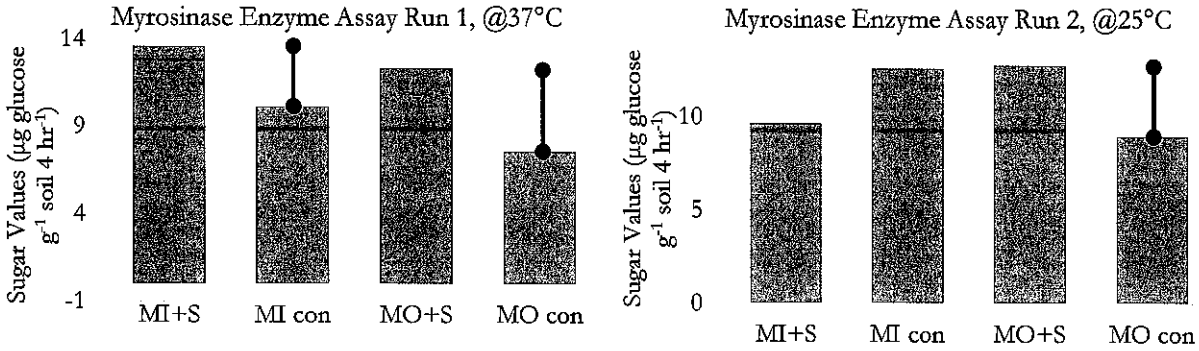


Figure 3. Soil myrosinase activity in Michigan silt loam and Missouri sandy loam soils. The '+S' indicates the presence of sinigrin, while 'con' indicates no addition of sinigrin. The lines above the bars indicate the released glucose attributed to soil myrosinase activity (with the addition of sinigrin substrate).

Velvetleaf Seed Germination in Varying Sand-Soil Combinations

The independent variables for this experiment were MSM presence and sand-soil content. The dependent variable was velvetleaf seedling length. The total amount of velvetleaf seeds planted was 768, with 685 seeds that germinated. The seeds that did not germinate were recorded but were not included in the data set for statistical analyses in order to avoid inaccurate results. In the presence of MSM, the percentage of seeds that germinated was reduced in soil containing 70% sand (Figure 4). A summary of the descriptive statistics for the different combinations is depicted on Table 3.

A factorial ANOVA showed that there are significant differences between MSM presences [$F(1, 677)=2143.627$, $p<0.001$], sand content types [$F(1, 677)=97.821$, $p<0.001$], and interaction effects between MSM presence and sand content types [$F(3, 677)=7.099$, $p<0.001$]. The results of the factorial ANOVA is shown on Table 4. Further analysis was done using 2 one-way ANOVAs, partitioning the data set into groups without MSM (MSM-) and with MSM (MSM+) to determine

the full effects of sand content on seedling root lengths. A Bonferroni post-hoc of both one-way ANOVAs showed that root lengths for MSM- showed significant difference at 70% sand content, while MSM+ showed significant difference at 45% and 70% sand content. The presence of MSM combined with higher sand content resulted in increased efficacy of MSM. The results of the data analyses can be found on Table 5.

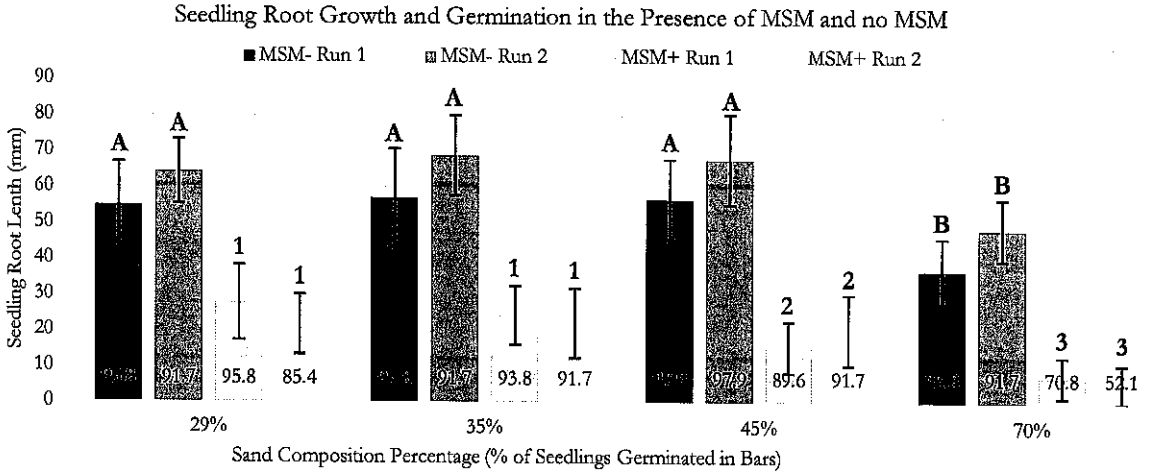


Figure 4. Velvetleaf seedling growth in soils that varied in sand content and in the presence or absence of mustard seed meal (MSM). The number in each bar is the percentage of seed germination and the height of each bar is the average length of seedlings (in mm) growing in specific soil conditions. Bars differing in numbers or letters denote significant differences in the mean root lengths between sand combinations.

Descriptive Statistics

Dependent Variable: seedling root length (in mm)

Treatment	Run	Mean	Std. Deviation	% Germ.	N
MSM-	29%	59.5388	11.61333	93.75	90
	35%	62.8946	13.79823	93.75	90
	45%	62.3218	13.10197	97.92	94
	70%	42.6024	10.50190	92.71	89
MSM+	29%	25.0121	10.02508	90.63	87
	35%	23.3175	9.03793	92.71	89
	45%	17.9731	8.94815	90.63	87
	70%	6.5303	5.60052	61.46	59

Table 3. Descriptive statistics for velvetleaf seedling length grouped by MSM presence and then sand-soil content. Included are the mean, standard deviation, percent germinated, and data set sample size.

Tests of Between-Subjects Effects

Dependent Variable: seedling root length (in mm)

Source	Type III Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	281177.781[a]	7	40168.254	343.553	.000
Intercept	945883.039	1	945883.039	8090.004	.000
MSM Presence	250632.803	1	250632.803	2143.627	.000
% Sand	34311.640	3	11437.213	97.821	.000
MSM Presence * % Sand	2489.950	3	829.983	7.099	.000
Error	79154.822	677	116.920		
Total	1414273.638	685			
Corrected Total	360332.602	684			

a. R Squared = .780 (Adjusted R Squared = .778)

Table 4. Results of the factorial ANOVA used to analyze the means of velvetleaf seedling root length, with the independent variables of MSM presence and sand content. There is significant differences in seedling root length for the independent variables of MSM presence, soil content, and interactions between MSM presence and soil content.

A

Oneway: MSM-

ANOVA

Seedling root length

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	24817.083	3	8272.361	54.37	.000
Within Groups	54618.228	359	152.140		
Total	79435.310	362			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: seedling root length

Bonferroni

(I) Sand-Soil %	(J) Sand-Soil %	Mean Difference (I-J)	Std. Error	Sig.
29%	35%	-3.35578	1.83872	.413
	45%	-2.78303	1.81905	.761
	70%	16.93642*	1.84388	.000
35%	29%	3.35578	1.83872	.413
	45%	.57275	1.81905	1.000
	70%	20.29220*	1.84388	.000
45%	29%	2.78303	1.81905	.761
	35%	-.57275	1.81905	1.000
	70%	19.71945*	1.82427	.000
70%	29%	-16.93642*	1.84388	.000
	35%	-20.29220*	1.84388	.000
	45%	-19.71945*	1.82427	.000

*. The mean difference is significant at the 0.05 level.

B

Oneway: MSM+

ANOVA

Seedling root length

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	14048.449	3	4682.816	60.69	.000
Within Groups	24536.594	318	77.159		
Total	38585.043	321			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: seedling root length

Bonferroni

(I) Sand-Soil %	(J) Sand-Soil %	Mean Difference (I-J)	Std. Error	Sig.
29%	35%	1.69454	1.32433	1.000
	45%	7.03897*	1.33183	.000
	70%	18.48173*	1.48144	.000
35%	29%	-1.69454	1.32433	1.000
	45%	5.34442*	1.32433	.000
	70%	16.78719*	1.47470	.000
45%	29%	-7.03897*	1.33183	.000
	35%	-5.34442*	1.32433	.000
	70%	11.44276*	1.48144	.000
70%	29%	-18.48173*	1.48144	.000
	35%	-16.78719*	1.47470	.000
	45%	-11.44276*	1.48144	.000

*. The mean difference is significant at the 0.05 level.

Table 5. Two one-way ANOVAs were used to analyze the means of velvetleaf seedling root length. The original data set was partitioned into different data sets by MSM presence. These were then analyzed according to sand content. A) The one-way ANOVA for soil without MSM showed that there was significant difference between the groups [$F(3, 318)=60.69$, $p<0.001$]. A Bonferroni post-hoc showed that the root length of seedlings in soil with 70% sand is significantly different from root lengths of seedlings in soil with lower percentages of sand. B) The one-way ANOVA for soil with MSM showed that there was significant difference between the groups [$F(3, 359)=54.37$, $p<0.001$]. A Bonferroni post-hoc showed that the root length of seedlings in soil with 45% and 70% sand is significantly different from root lengths of seedlings in soil with lower percentages of sand.

Discussion

The research performed helped shed some light as to why there were differences in ITC efficacy between the MI silt loam and MO sandy loam soils. The results of the myrosinase enzyme assay showed that the difference in efficacy was not correlated to differences in myrosinase enzyme activity in the soil. In MI silt loam and MO sandy loam, the myrosinase enzyme activities were low and had similar values. For context, the myrosinase enzyme assays presented in Al-Turki and Dick (2003), ranged from 71-323 μg glucose g^{-1} soil 4 hr^{-1}. In comparison, the soil samples in our experiments ranged from 7.32-15.63 μg glucose g^{-1} soil 4 hr^{-1}. The results of the velvetleaf seedling growth showed that difference in efficacy was correlated to variations in sand-soil content, with higher sand content correlating to higher ITC efficacy. A study by Bending and Lincoln (1999) found that higher amounts of both glucosinolate and ITC were found in sandy-loam soils, correlated with relatively lower microbial respiration. In another study by Price et. al (2005) that tested for ITC presence and CO_2 release in variable conditions, ITC concentration was found to be 38% higher in sandy loam soil than in clay loam soil. These studies support our result and hypothesis that soil with higher sand content is correlated with ITC efficacy. Our experiments performed proved to be simple, yet effective, in testing our hypothesis and exploring a possible basis for the differences in ITC efficacy between MI and MO soils.

A possible explanation for increased ITC efficacy with higher sand content could be due to better diffusion of the volatile ITC through the soil. Studies have shown that gas diffusion is greater in soils that contain more air pores and lower moisture levels (Currie 1984). Although sand results in higher levels of aeration, a potential problem could be poor water retention. However, the lower amount of sand content needed by ITC (shown in the experiment to be 45%) to result in significantly shorter root lengths is important, since the soil composition would be sufficient to provide well-balanced strengths in water retention and ITC propagation. Another possible

explanation could be that soils with less sand contain more organic matter, resulting in higher microorganism activity. This could lead to faster degradation of ITC, meaning that more ITC needs to be produced to reach similar levels of efficacy.

The solution of amending soil with sand to increase ITC efficacy is simple and provides a cheap method to enhance biofumigation, although not by a massive amount. Further investigation of any differences in duration of ITC between the variations of sand-soil content would be helpful. Our experiments did not measure the presence of ITC itself, we measured the amount of glucosinolate used indirectly (in the myrosinase assay) and how well ITC propagates (velvetleaf seedling growth). Investigations can also include differences in microorganisms or bacterial growth between the variations of sand-soil content, in order to test for differences in ITC efficacy in relation to microorganism populations.

Oleszek W. 1987. Allelopathic effects of volatiles from some *Cruciferae* species on lettuce, barnyard grass and wheat growth. *Plant and Soil* 102:271-273.

Petersen J, Belz R, Walker F, Hurle K. 2001. Weed suppression by release of isothiocyanates from turnip-rape mulch. *Agron J* 93:37–43.

Price AJ, Charron CS, Saxton AM, Sams CE. 2005 Allyl Isothiocyanate and Carbon Dioxide Produced during Degradation of *Brassica juncea* Tissue in Different Soil Conditions. *Hort Science* 40(6): 1734-1739.

Warton B, Matthiessen JN, Shackleton MA. 2003. Cross-enhancement: enhanced biodegradation of isothiocyanates in soils previously treated with metham sodium. *Soil Biol Biochem* 35:1123–1127.