Bioassays for Assessing the Fate of Brassicaceae-derived Biopesticides in Soil and an Analysis of the Kinetics of Myrosinase Isoenzymes from Select Brassicaceae Species

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Abstract

Seed meal of plants from the family Brassicaceae contains glucosinolates which are hydrolyzed by the endogenous enzyme myrosinase to toxic isothiocyanates which have been shown to be effective for treating a wide array of pest species. The overarching goals of this study were to understand the fate of allyl isothiocyanate (AITC) from sinigrin (2-propenyl glucosinolate) in agricultural soil. Specific objectives were 1) to explore the use of bioindicators such as wireworms, *Limonius infuscatus* for pesticide fate studies which could be assessed *in-situ* and *ex-situ*; 2) to study the kinetics of myrosinase isoenzymes from *Brassica juncea*, *Sinapis alba,* and *Limnanthes alba* using sinigrin, glucolimnanthin (m-methoxybenzyl glucosinolate), and sinalbin (4-hydroxy benzyl glucosinolate) as substrates to assess which myrosinase source will result in the highest production of biopesticide in the soil.

Two wireworm bioassays were evaluated. The first assay was used to assess the effects of soil depth and incubation period on the fate of the biopesticides. Pre weighed wireworms were used as bioindicators and isolated at specific depths in soil columns which were treated with *B. juncea* seed meal. The columns were incubated for four or eight days, then the wireworms were assessed for mass change and mortality. The soil was analyzed for AITC, glucosinolates, sulfate, pH, and electrical conductivity. The second assay assessed the effects of tarping on the efficacy of *B. juncea* seed meal against wireworms. A single pre-weighed wireworm was placed midway in a soil column which was treated with *B. juncea* seed meal. Columns were covered with a polypropylene sheet to simulate field tarping and were incubated for two days, then the wireworms were removed, and their mass, mortality, and vertical location were recorded. The wireworms were transferred to fresh soil with wheat

seeds as a food source and monitored weekly for mass change and mortality. Soil was analyzed for AITC, electrical conductivity, and pH.

In the first assay, neither depth nor incubation period significantly affected wireworm mortality and mass change; however, meal application, depth, and incubation period did significantly affect soil EC, pH, and sulfate concentration. This may be due to glucosinolate hydrolysis as well as acids and ionic compounds in the meal. In the second assay, wireworms in treated columns which were tarped showed significantly higher mortality than wireworms in treated columns which were uncovered which did not show significantly different mortality rates than those in the control columns, suggesting AITC volatilization may significantly reduce, or completely negate the effects of biopesticide. Treatment also had a significant effect on pH, and electrical conductivity.

The myrosinase isoenzyme from *S. alba* showed the highest maximum activity of the three isoenzymes with sinigrin and glucolimnanthin as substrates. The maximum activity of *S. alba* with sinigrin and glucolimnanthin was significantly higher than the maximum activity of *B. juncea*; however, *B. juncea* myrosinase had a significantly greater affinity for both sinigrin and glucolimnanthin. *S. alba* had the greatest Vmax with sinigrin as a substrate and had similar maximum activities for both glucolimnanthin and sinalbin. *B. juncea* myrosinase exhibited Michaelis Menten kinetics for sinigrin and glucolimnanthin but showed signs of substrate inhibition for sinalbin. The myrosinase extract from *L. alba* showed negligible activity and may have degraded prior to extraction.

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Dedication

This work is dedicated to my parents. Mom, Dad thank you for being my role models and for all the support you have given me. I wouldn't be who I am today without you.

Table of Contents

Chapter 1: Bioassays for Determining the Fate of *B. juncea*-Derived Biopesticides in Soil

Introduction

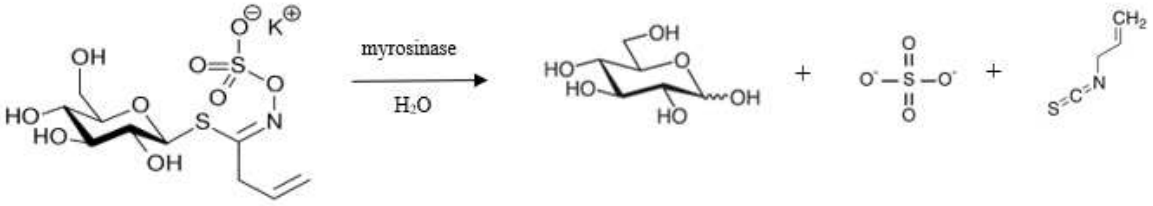
The potato cyst nematode, *Globodera pallida,* is a globally regulated pest of potatoes first detected in the United States in 2006 in Idaho (Dandurand *et al.* 2019). *G. Pallida* poses a significant threat to potato crops globally. It was estimated in 2007 that there was a 9% decrease in potato crop value per year in the U.K. because of damage caused by *G. Pallida* (Vreugdenhil and Bradshaw 2007). Potato cyst nematodes can persist in soil for up to 30 years without a suitable host (Turner 1996), and when left untreated, *G. Pallida* can reduce potato yield by up to 80% (Talavera *et al.* 1998). Currently, *G. Pallida* is limited in North America to a small area of Idaho and the island of Newfoundland in Canada ("USDA APHIS | Pale Cyst Nematode" 2020), whereas more than 60% of fields in the U.K. are infested with *G. Pallida* (Contina *et al.* 2018). It is necessary to combat *G. Pallida* to ensure the viability of farmland for potato growth.

Soil fumigants such as methyl bromide have been useful for combating plant-parasitic nematodes in the past but have been phased out due to environmental regulations (Zasada *et al.* 2010). Bio fumigation with brassica tissue has emerged as an effective alternative to chemical soil fumigants to combat plant-parasitic nematodes (Dutta, Khan, and Phani 2019). There are several methods for incorporating brassica tissue into soil. For example, upper aerial parts of brassica plants can be cut or ground and incorporated into the soil's upper layers as an amendment, or the plants could be used as cover crops or trap crops (Smith, Gray, and Koch 2004; Hafez and Sundararaj 2009). Seed meal from various Brassica plants

has also been used to combat plant parasitic nematodes (I. A. Zasada, Meyer, and Morra 2009). Allyl isothiocyanate (AITC) produced from Brassicaceae plants can be used as a biofumigant to eradicate *G. pallida* from infested fields. Research shows that juvenile *G. pallida* exposed to AITC at a concentration of 50 μL L^{-1} had 100% mortality after 24 hours (Wood, Kenyon, and Cooper 2014). AITC affects *G. pallida* mortality by inhibiting the juvenile's ability to hatch, resulting in a nemostatic effect rather than a nemotoxic effect (Forrest 1989).

AITC is produced from the hydrolysis of 2-propenyl-glucosinolate, commonly known as sinigrin, in *B. juncea* by the endogenous enzyme myrosinase, thioglucoside glucohydrolase (Cole 1976; Fenwick and Heaney 1983). Sinigrin belongs to glucosinolates, a group of specialized, anionic plant metabolites that are composed of thiohydroximates carrying an *S*-linked β-glucopyranosyl residue, an *O*-linked sulfate residue, and an amino acid derived, variable side chain (Agerbirk and Olsen 2012). During hydrolysis, myrosinase cleaves the *S*-glycosidic link, generating glucose and an unstable aglycone which can rearrange to form isothiocyanates, thiocyanates, nitriles, sulfate, and other products (Halkier and Gershenzon 2006). Some of these products, such as AITC, are effective biocides and serve as a defense mechanism for the plant (Redovnikovic *et al.* 2008; Zhu *et al.* 2020). The glucosinolates and myrosinase remain separate while the plant is intact; however, when the plant tissue cells are damaged the glucosinolates and myrosinase come in contact, and in the presence of water the glucosinolates are hydrolyzed (Rask *et al.* 2000). To generate AITC for biofumigation, defatted *B. juncea* seed meal is applied to the soil, then watered to facilitate

glucosinolate hydrolysis (Abdallah, Yehia, and Kandil 2020). The hydrolysis reaction is shown below.



An advantage of using brassica-derived isothiocyanates, isothiocyanates, as pesticides is that they are metabolized and detoxified by mammals faster than synthetic pesticides and do not accumulate in mammalian systems (Ioannou, Burka, and Matthews 1984). The rapid metabolization and detoxification of isothiocyanates in mammals minimizes the risk of any adverse effects. In fact, intact glucosinolates have a wide array of health benefits for humans and have been shown to reduce the risk of myocardial infarction and developing cancer, which makes these biopesticides a safe and beneficial alternative to pesticides like methyl bromide (Traka 2016; Hansson *et al.* 1993; Cornelis, El-Sohemy, and Campos 2007).

Widespread adoption of *B. juncea* seed meal as a biofumigant is currently hindered by numerous factors, including difficulties associated with assessing the fate of allyl isothiocyanate in soil. allyl isothiocyanate is a volatile compound, so perturbing treated soil to collect soil samples for analysis can lead to the loss of allyl isothiocyanate and would not accurately reflect in situ soil concentrations of allyl isothiocyanate (Torrijos *et al.* 2019). Two primary methods for allyl isothiocyanate analysis are based on derivatization with following high performance liquid chromatography detection or headspace analysis by gas chromatography/mass spectrometry (M. J. Morra and Kirkegaard 2002; C. W. Chen and Ho 1998). Both methods are lab based and require expensive analytical equipment, reagents, and

training to be performed. As a result, assessing the concentration of allyl isothiocyanate in soil is cost and time prohibitive and limits the ability of farmers and land managers to assess the efficacy of the biopesticide. A simple bioassay may enable farmers, land managers, and researcher to monitor the fate and efficiency of biofumigants such as allyl isothiocyanate in the field. Instead of directly quantifying the concentration of allyl isothiocyanate in soil, its fate could be indirectly studied based on the response of a bioindicator (Kamiloglu *et al.* 2021). Since allyl isothiocyanate is effective against insects , microbes, and fungi there are a plethora of suitable indicator species which could be used to assess its fate (Zhu *et al.* 2020; H. Wu *et al.* 2009; Y. Li *et al.* 2020).

Previous research has shown that allyl isothiocyanate has a significant effect on wireworm mortality and Brassica seed meal influences wireworm movement (Williams *et al.* 1993; Brown, Morra, and Borek 1991). Both these variables could serve as indicators for the presence of allyl isothiocyanate and can be qualitatively and quantitatively assessed by researchers with limited experience. If wireworm response can be quantified, it could serve as a bioindicator for pesticide fate in soil. Wireworms are the larval stage of a beetle group known as click beetles (Coleoptera: elateridae) and are ubiquitous soil pests which can be easily captured using solar bait traps which are constructed with a trash bag, a marker flag, a shovel, and wheat seeds as bait (Rashed *et al.* 2015). The larval stage for wireworms varies based on the species, but can last for several years (Finney 1946). They can be stored ex-situ in damp soil and fed with wheat seeds (Elberson *et al.* 1996). Their low maintenance and prolonged larval stage make them ideal indicators since they can be stored for extended periods and used to assess allyl isothiocyanate fate at different times throughout the year.

The objectives of this experiment were to assess whether a simple bioassay using wireworms as bioindicators could be used to assess the fate of allyl isothiocyanate in soil columns and study alternative indicators of allyl isothiocyanate production which could be measured in-situ. Two different bioassays were tested for the experiment. The first bioassay was a vertical isolation assay and was used to study whether wireworms' response to *B. juncea* seed meal differed at different depths in the soil over time. The second bioassay was used to assess whether soil tarping affects the efficiacy of allyl isothiocyanate against wireworms. Since allyl isothiocyanate is a volatile compound, it may evaporate out of the soil if the seed meal is not covered prior to, or shortly after being saturated (Torrijos *et al.* 2019). A common method to limit allyl isothiocyanate volatilization from treated soil involves covering the treated soil in a tarp, which has been shown to increase the efficacy of the pesticides considerably (Earlywine *et al.* 2010).

Materials and Methods

Wireworm Collection

Wireworms were collected using wheat seed baits during early April of 2021 in an agricultural field in Latah County, East of Moscow, Idaho. A mixture of wheat, barley, and corn was soaked in water for 48 hours for wireworm bate. A six-inch hole was dug into the ground at the collection site, and approximately 200mL of the bate was placed in the hole. The seed was buried and covered with soil until a mound protruded 3-4 inches off the ground. The mound was then covered with a 2 x 2-foot dark plastic sheet, and the edges of the sheet were covered with soil to prevent the sheet from blowing away. A blue marker flag was placed into the mound to mark the trap location. After two weeks, the plastic sheet was removed, and the mass of germinating seed along with its surrounding soil was transferred to a plastic bag (Rashed *et al.*, 2015). Wireworms were sorted by species and by length, then placed in plastic cups with sandy soil and 3, ungerminated wheat seeds. The cups were held at room temperature and maintained at approximately 17% moisture content (w/w) prior to the experiment. *Limonius Infuscatus* were used for both bioassays. The wireworms ranged in length from 10 to 22 mm and ranged in weight from 15 to 65 mg on dry weight basis.

Soil Collection

The fate of and effects of allyl isothiocyanate from *B. juncea* seed meal on wireworms was tested in Bannock loam collected from an agricultural field in Shelley, Idaho. Previous research has demonstrated the presence of potato cyst nematodes in and around Shelley Idaho (Contina, Dandurand, and Knudsen 2018). The site was selected to reflect the type of soil which the seed meal would be utilized on. Approximately 16 gallons of soil was collected from 0-20cm across 20 sampling locations within the field. The sampling locations were evenly spaced throughout the field to obtain a representative sample.

Soil Analysis

The soil was air dried in 1x1 m pans, then crushed and combined into 5-gallon buckets. Half of the crushed soil was then combined into a large sieve with a 1-cm mesh screen. The soil was sieved through the mesh three times to remove large soil aggregates as well as plant debris and mix the soil. This process was repeated with the second half of the soil as well. The 1-cm sieved soils were then mixed by combining approximately half of the soil from each batch into new buckets and re-sieving through the 1-cm mesh screen. The sieved soil was crushed again, recombined into different buckets, and filtered through a 2-mm sieve three times. The 2-mm sieved soil was recombined into different buckets after every repetition to homogenize the soil. Soil pH and electrical conductivity were determined using a 1:1 mixture of soil and deionized water. The soil pH was measured using an Orion glass electrode and electrical conductivity was measured using an Oakton conductivity electrode. Soil samples were placed measuring tins, then baked for 48 hours at 100 °C and weighed to determine bulk density. Sulfate content was determined by extracting 8 g of air-dried soil with 40 mL of a 0.6mM KH_2PO_4 solution. The mixture was shaken at room temperature for 30-min using a reciprocal shaker, then centrifuged at 3500 rpm for 10 min, and filtered through a 0.42 μm syringe driven filter (Ensminger 1954). The aqueous extract was analyzed with a Dionex Aquion ion chromatography system with a Dionex AS-AP autosampler and an ADRS 4mm suppressor. Chromatographic separation was conducted with a 4x210 mm Ion-Pac AS16 with an AG16 guard column. The flow rate was 1.00 mL min^{-1} and the mobile phase was 33 mM NaOH. Anion suppressor current was set to 82 mA and the injection volume was 25 μL. Run time was 17 min. The results were compared against a standard curve which was generated using K_2SO_4 stock solutions prepared using reagent grade K_2SO_4 from Ward's Science +, ON, Canada. All measurements were

conducted in triplicates. The soil was analyzed for phosphorous, potassium, nitrate, ammonium, and organic matter content at the University of Idaho Analytical Sciences Laboratory.

Quantification of allyl isothiocyanate in Soil

allyl isothiocyanate was extracted from the soil using the methods of Morra and Kirkegaard (2002). Once homogenized, 10 g of soil was added to 10 mL of cold methanol in a propylene tube. The sample was shaken for one hour at room temperature, then centrifuged at 4000 rpm for 10 min. A 4 mL aliquot of solution was removed from the centrifuge tube with a 5 mL glass syringe. The solution was then passed through a 0.42 µm syringe filter (25 mm Millex-GN, non-sterile, nylon) to obtain approximately 2 mL of clear filtrate. A six-hundred microliter subsample of methanol extract was added to 1 mL high performance liquid chromatography autosampler vials (8 x 40 mm) containing 600 µL of 100 mM K_2HPO_4 buffer (pH 8.6) and 200 µL of a 35 mM 1,2-benzenedithiol/1% mercaptoethanol solution. Vials were capped with Teflon-lined caps and inverted several times to mix thoroughly. The samples were incubated at 65 ^oC for one hour, then stored in the freezer before high performance liquid chromatography analysis (Morra and Kirkegaard 2002). Six allyl isothiocyanate analytical standards with concentrations of 0.02, 0.04, 0.06, 0.08, 0.10, and 0.12 mM and 3 matrix spike standards with concentrations of 0.206, 2.06, and 20.6 mM were used to quantify the concentration of allyl isothiocyanate in the soil samples. Three replicates of each matrix spike standard were prepared for analysis. Standards were prepared by adding 1.4 mL of the allyl isothiocyanate standard to 8.6 g of dry Bannock loam to bring the soil to ~16 % water content (w/w). The concentrations of allyl isothiocyanate used for the matrix spikes corresponded to 1/10, 1/1, and 10/1 times the maximum concentration of allyl isothiocyanate in the soil if all glucosinolates were hydrolyzed. The samples were analyzed

for allyl isothiocyanate with an Agilent 1200 series high performance liquid chromatography with a diode array detection system (DAD). Chromatographic separation of allyl isothiocyanate was conducted using an Agilent Zorbax 4.6x50mm, 3.5 µm column maintained at 30 °C. The injection volume was 20 µL. The mobile phase consisted of 0.1 % formic acid in deionized water (solvent A) and 0.1 % formic acid in acetonitrile (solvent B). The gradient program began with isocratic elution using 5 % of B and 95 % of A for three min, followed by a linear gradient to 70 % of B from three to ten min which was held for six seconds. Solvent B was then reduced to 5 % and held for 5.9 min. The flow rate was 0.4 mL min^{-1} and spectra were recorded from 190 to 400 nm.

Quantification of Sinigrin in Soil

Sinigrin was extracted from the soil samples as well as untreated soil using the methods from Gimsing and Kirkegaard (2006). Twenty g of soil was placed into pre-weighed, plastic centrifuge tubes containing 10 mL of 70 % aqueous methanol. The centrifuge tubes were shaken vigorously immediately after sampling. The samples were then weighed and centrifuged at 3000 rpm for 5 min. The supernatant was filtered through a 0.42 µm syringe-driven nylon filter. Another 10 mL of 70% aqueous methanol was added to the soil; the centrifuge tubes were shaken vigorously and then left to stand for 45 min shaking 2–3 times in that period. Following further centrifugation, the supernatant was filtered, and the two filtrates were combined (Gimsing and Kirkegaard 2006). Six analytical standards with concentrations of 0.02, 0.04, 0.06, 0.08, 0.10, and 0.12 mM as well as 3 matrix spike standards with concentrations of 0.206, 2.06, and 20.6 mM were used to construct a calibration curve. Matrix spike standards were prepared by adding 2.8 mL of each standard to ~17.2 g of soil to bring the soil to 16 % water content (w/w), then extracting the soil using the methods described above. Each of the matrix spike standards were prepared in triplicates.

The concentration of the matrix spike standards corresponded to 1/10, 1/1, and 10/1 times the maximum concentration of sinigrin in the soil if no glucosinolates were hydrolyzed.

The samples were analyzed for sinigrin with an Agilent 1200 series high performance liquid chromatography with a diode array detection system (DAD). Chromatographic separation of allyl isothiocyanate was conducted using an Agilent Extend C-18m 2.1x100 mm, 3.5 µm column maintained at 30 °C. The injection volume was 10 µL. The mobile phase consisted of 0.1% formic acid in deionized water (solvent A) and 0.1 % formic acid in acetonitrile (solvent B). The gradient program began with isocratic elution using 5 % of B and 95 % of A for five min, then 75 % A and 25 % B for one minute which was followed by a linear gradient to 95 % A and 5 % B over six seconds. These solvent concentrations were maintained for 5.9 min. The flow rate was 0.4 mL min^{-1} and spectra were recorded from 190 to 400 nm.

Quantification of Glucosinolate Concentration in Seed Meal

B. juncea seed meal (Pacific Gold variety) was used for the bioassay. Glucosinolate concentration in the meal was determine using the methods of Popova and Morra (2014). Mustard meal was ground in a coffee grinder and 0.1 g of ground meal was extracted with 5.5 mL of 73 % methanol on a reciprocal shaker at room temperature for one hour. Three biological replicates were extracts for analysis. The samples were then desulfated for high performance liquid chromatography analysis by adding one mL of the extract to a column containing 500 mg DEAE anion exchanger and allowed to drain freely. The column was washed twice with 1 mL of deionized water, then with 1 mL of 20 mM ammonium acetate buffer (pH 4.0). One hundred µL of a 1 mg L^{-1} sulfatase enzyme solution was added to the column. The bottom of the column was capped, and 100 µL of 0.02 mM ammonium acetate

buffer (pH 4.0) was added. The column was covered to prevent evaporation and allowed to stand with the enzyme for 12 hours, after which the samples were eluted into glass high performance liquid chromatography autosampler vials with 2 mL of deionized water ("ISO - ISO 9167-1:1992 - Rapeseed — Determination of Glucosinolates Content — Part 1: Method Using High-Performance Liquid Chromatography" n.d.; Morra and Popova 2014). Samples were analyzed with an Agilent 1200 series high performance liquid chromatography with a multi wavelength detector. Glucosinolates were separated using an Agilent extend C-18 column. Sinigrin was isocratically eluted over three min using 95 % and 5 % of water/0.1 % formic acid and acetonitrile/0.1 % formic acid respectively. The multi wavelength detector was set to 229 nm with a bandwidth of 4 nm and a reference of 360 nm with a bandwidth of 100 nm.

Quantification of Sulfate Concentration in the Seed Meal

To assess the concentration of sulfate in the seed meal, 4.0 g of meal was mixed with 40 mL of deionized water in a 50 mL falcon tube and left at room temperature for 48 hours to extract the sulfate and detoxify the meal by hydrolyzing the glucosinolates. Extractions were conducted in triplicates. The tubes were left uncapped but covered with tin foil to allow the allyl isothiocyanate to vent. After 48 hours the tubes were capped and centrifuged at 4000 rpm for 15 min to separate out the insoluble matter and the supernatant. Two mL of the supernatant was filtered through a 0.42 μm syringe driven filter to remove any particulates which were suspended in the solution. The filtrate was diluted 10-fold with deionized water then analyzed with IC using the methods previously described.

Vertical Isolation Wireworm Bioassay

The vertical isolation bioassay was conducted using cylindrical PVC pipes with inner diameter 5-cm and length 25-cm and 43 μm mesh screens to isolate the wireworms at

specific depths. A 6x6 cm piece of black acrylic was glued to the base of the pipe to prevent soil from falling out. The outside edge where the PVC and acrylic touched was sealed with silicon to prevent moisture leaking. The mesh screens were constructed by gluing a 5-cm diameter circle of 43µm mesh between two 2-mm thick acrylic rings which were 4-mm wide. Approximately 2.5-cm of dry soil was added to the column, then a single, pre-weighed wireworm was randomly chosen from a group of 48 wireworms of the same species and developmental stage using a random number generator. The wireworm was placed in the soil and an additional 2.5-cm of soil was added to the column, then sufficient deionized water was added to bring the soil to ~16 % moisture content w/w. A mesh screen was placed firmly over the top of the soil to prevent the wireworms from migrating upwards. The process was repeated three additional times; however, a screen was not placed on the top of the soil since the worm would not be able to travel any higher. Approximately 1.57 g of ground *B. juncea* seed meal was spread evenly on top of the soil (8 t ha^{-1}) and 30 mL of deionized water was added slowly to prevent pooling and to thoroughly hydrate the meal. Six treated columns were prepared for the assay and an additional six columns which did not contain meal were prepared to serve as controls. All 12 columns were then incubated in the dark at 25+/-2 °C. Sufficient water was added to maintain the columns at or above 80 % of field capacity to simulate a realistic agricultural soil moisture regime (Burt 2008). After four days, half of the columns were removed from the incubator. Wireworms were removed from each 5-cm section of the column via destructive sampling, then weighed, assessed for mortality and frozen at -20 °C for future analysis. Soil from each section was placed in an individual plastic bag, labeled, and frozen at -20 °C prior to analysis. The top 1-cm of soil was in the treated columns which contained the seed meal was removed so that the meal would not affect

measurements. The process was repeated for the remaining columns at 8 days. The soil sections were thoroughly homogenized, then analyzed for pH, EC, and allyl isothiocyanate, sulfate, and glucosinolate concentration using the methods previously described.

Tarping Wireworm Bioassay

A bioassay was conducted to determine the efficacy of field tarping using PVC columns with the same dimensions and construction as the bioassay. The tarping assay consisted of 4 separate treatments composed of 9 replicate columns per treatment for a total of 36 soil columns. Two treatments were covered with a polyethylene sheet to simulate field tarping and the remaining two were uncovered. *B. juncea* seed meal was applied to the surface of one of the covered treatments and one of the uncovered treatments. Columns were filled with 10-cm of dry Bannock loam, then a single, preweighed wireworm was placed in the column and an additional 10-cm of soil was added to the column. Wireworms were randomly selected from a group of 36 *Limonius infuscatus* using a random number generator. For *B. juncea* seed meal treated columns, ~3.14 g of ground seed meal was applied to the surface of the soil. Next, the columns were watered to ~16% moisture (w/w). Tarped columns were immediately covered with a 10x10cm polyethylene sheet, and the edges of the tarp were taped to the column with electrical tape to limit pesticidal loss from volatilization. Each treatment was then incubated in the dark at 25+/-2 °C for 48 hours.

After 48 hours, the columns were removed from the incubator, then destructively sampled in 1-cm increments to determine the vertical position of the wireworm in the column. The soil was placed in plastic bags, thoroughly homogenized, then stored at 4C for future analysis. Soil pH and EC were measured using the same methods previously described. Wireworms were weighed and classified as alive or stunned based on their

movement. Since wireworms may be stunned after exposure to pesticides, they were not classified as dead after initial measurements (Lehman 1933). The wireworms were then transferred to cups containing new Bannock loam soil watered to 16% moisture (w/w) containing 3 ungerminated wheat seeds as a food source. Cups were watered daily to replace evaporative losses. After one week, the soil was removed from the cups and destructively sampled to measure wireworm mortality and mass. The worms were then placed in fresh soil watered to 16 % moisture content (w/w) containing 3 ungerminated wheat seeds. The process was repeated for 5 weeks. Dead wireworms were treated the same as living wireworms until they began to decompose at which time they were discarded.

allyl isothiocyanate Extraction from Tarping Bioassay Soils

To determine the fate of allyl isothiocyanate within the tarped and untarped columns, an additional 36 columns using the same treatments as the bioassay were prepared without wireworms. After the columns had incubated for 48-hours they were capped with PVC caps and stored at -20 °C to limit microbial activity and allyl isothiocyanate volatilization. Within a week of the experiment the frozen soil cores were removed from the columns and transferred to plastic bags. The cores in the bags were then flash frozen in a mixture of ethanol and dry ice and cut into 5-cm subsections with a reciprocal saw. Each subsection was immediately placed in an airtight plastic bag to limit pesticide volatilization. The soil from each subsection was thawed at room temperature, then thoroughly homogenized within the plastic bag. The homogenized samples in the bags were flash frozen again prior to extraction.

Statistical Analysis

Statistical analysis of the results for both assays was conducted using R studio. The categorical variables in the vertical isolation assay were soil depth which consisted of 4 depths, treatment which consisted of *B. juncea* treated soil and control untreated soil, and

incubation period which was either 4 or 8 days. The response variables were soil pH, soil electrical conductivity, soil sulfate concentration, soil allyl isothiocyanate concentration, soil sinigin concentration, and wireworm mass change. The experiment was a completely randomized factorial design. In the tarping assay the experiment was a completely randomized design. The independent variable was treatment, and the response variables were soil pH, soil electrical conductivity, allyl isothiocyanate concentration, and 5-week wireworm mortality. One way ANOVA tests were conducted to determine the effects of the independent variables on each of the response variables, and a Tukey multiple comparison test was used to compare the effects of each of the 4 soil depths on the response variables. A significance level of $\alpha=0.05$ was used for the analysis.

Results and Discussion

Bannock Loam Bulk Density, pH, Sulfate, Phosphorous, Potassium, Nitrate + Nitrite, Ammonium, and Organic Matter

The bulk density of the untreated, ground and sieved Bannock Loam was approximately 1.27 g cm^{-3}. The pH was 7.3. The concentration of sulfate was approximately 2.86 μmol g^{-1} and the concentration of phosphorous, potassium, nitrogen from nitrate and nitrite, and nitrogen from ammonium were 19, 180, 3.0, and 8.9 μg g^{-1} respectively. Organic matter content was 1.7% w/w (Table 1.1).

Vertical Wireworm Isolation Assay: Soil Sulfate

The null hypotheses regarding the effects of the categorical variables on sulfate levels were that the concentration of sulfate in the soil will not be significantly different between treatments, soil depths, incubation periods, and the interactions between these variables. Soil sulfate was significantly affected by treatment, incubation time, the treatment:incubation interaction, and the treatment:depth interactions which had P-values of $<2.20\times10^{-16}$, 1.20×10^{-7}, 3.75×10^{-4}, and 0.0105 respectively, which are significant at $\alpha=0.05$ (Table 1.2). Therefore, the null hypotheses that the sulfate levels will not be different between treatments, incubation periods, treatment:incubation combinations, and treatment:depth combinations is rejected. The null hypotheses that sulfate levels are not significantly different at different depths, incubation:depth combinations, or treatment:incubation:depth combinations fail to be rejected. The interaction between treatment and depth was significant; however, the significance of the interaction between treatment and depth was likely due to the considerable effects of treatment, rather than any effects of depth. A Tukey comparison of means test showed that the treatment:depth combinations were only significant between the treated combinations and the control combinations, and sulfate concentrations at different

depths within the same treatment were not significantly different at α=0.05 (Tables 1.2 and 1.3). Therefore, we can conclude that sulfate was not significantly affected by soil depth.

Since the interaction between treatment and incubation period was significant, we can conclude that the mean sulfate levels were different between one or more treatment/incubation period combinations. The concentration of sulfate in the treated columns incubated for four and eight days and the control columns incubated for four and eight days were 1.50 ± 0.107, 2.48 ± 0.106, 0.229 ± 0.0986, and 0.483 ± 0.0820μmol g^{-1} respectively (Table 1.4). Sulfate levels were not significantly different between the control columns which were incubated for four days and the control columns which were incubated for eight days. Columns which were treated with *B. juncea* seed meal for eight days had significantly higher sulfate levels than treated columns which were incubated for four days, and all the treated columns had significantly higher sulfate concentrations than the control columns (Fig. 1.1 and Table 1.5). This was expected since sulfate is a major glucosinolate hydrolysis product and indicates that glucosinolates were hydrolyzed (Wu, Zhou, and Xu 2009). The fact that soil sulfate increased significantly between four and eight days of incubation in the treated columns suggests that the glucosinolates were not fully hydrolyzed after four days.

Previous research has shown that allyl isothiocyanate concentrations in soil increases rapidly within the first three hours after water is applied to *B. juncea* seed meal treated soil, but decreases considerably over the following 24 hours (L. Wang and Mazzola 2019). This suggests that the bulk of the sinigrin is hydrolyzed within the first few hours after watering; however, the results of this study show that sulfate is still generated more than four days after watering, indicating that there still may be a high concentration of intact glucosinolates in the

soil days after watering. Since the bulk density of the Bannock loam was approximately 1.27 g cm^{-3} each column was filled with 500 g of air-dried soil to reach 20-cm (Table 1.1). The columns were maintained at approximately 20 % moisture content (w/w), so the final mass of the wet soil was 625 g. Approximately 1.58 g of seed meal was used in the treated columns and the concentration of sinigrin in the *B. juncea* seed meal was 120 ± 1.68 μmol g^{-1} of meal (Table 2.3). Therefore, the maximum addition of sulfate to the soil from sinigrin hydrolysis was approximately 0.302 μmol g^{-1} if the sulfate was equally distributed throughout the column.

Other glucosinolates in the seed meal may have increased the concentration of sulfate in the treated columns as well. *B. juncea* seed meal contains a wide variety of glucosinolates which generate sulfate during hydrolysis (Sun *et al.* 2019). The total concentration of sulfate in the detoxified seed meal was 161 ± 8.01 μmol g^{-1} which would increase the sulfate concentration in the soil by approximately 0.406 μmol g^{-1} if the sulfate was evenly distributed throughout the column. However, the difference in sulfate concentration between the treated soils incubated for four and eight days and the control soils was greater than 0.406 μmol g^{-1}, so the difference in concentration cannot solely be due to sulfate from the meal. The sulfate concentration in the air dried and untreated soil was approximately 2.86 ± 0.0794 μmol g^{-1} of soil which is higher than the concentration of sulfate in any of the columns (Table 1.1). Sulfate in the soils may have been converted to other sulfur containing compounds by soil microbes during the assays which would account for the discrepancy between the sulfate in the treated and control columns and the airdried soil. If there were microbes in the soil which were converting sulfate to other compounds, the seed meal may have reduced microbial activity since allyl isothiocyanate it is a general biocide and can be

effective against microbes as well. Reduced degradation of sulfate in the seed meal treated columns may account for the additional sulfate in the treated columns.

Since sulfate is an anion and most soil minerals have a negative surface charge sulfate is easily leached through soil by water (Hoiberg 2018). The columns were watered daily to maintain the soil moisture at 80% of field capacity and water percolating through the soil may have leached the sulfate to lower depths which would account for the sulfate concentration being below the limit of detection in the top 5-cm of the control columns (Tables 1.4 and 1.26).

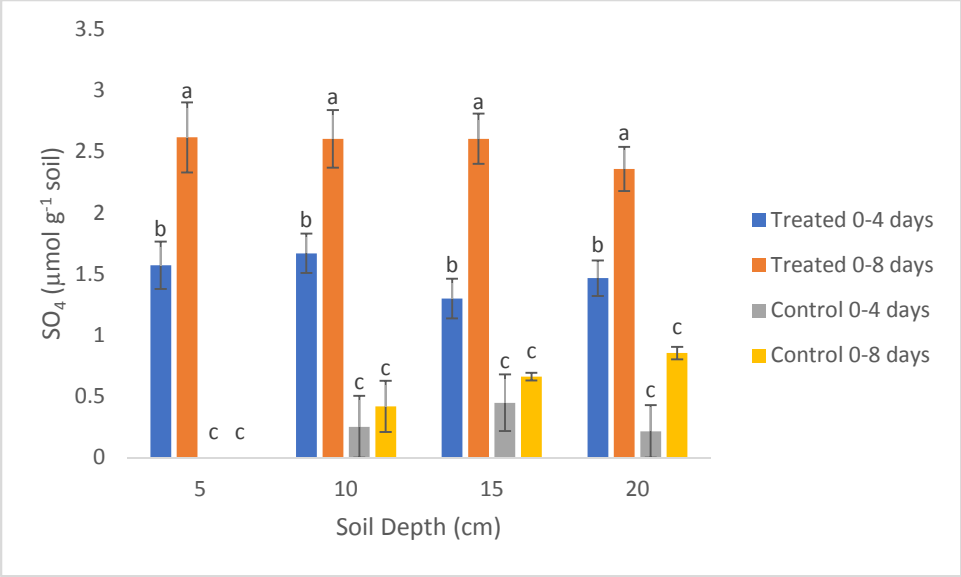


Figure 1.1 Concentration of sulfate in different column treatments for vertical wireworm isolation assay. Letters indicate significantly different concentrations of soil sulfate at α=0.05 and error bars indicate the standard error about the mean.

Table 1.1 Bannock loam chemical and physical properties

Bulk Density ($g\ cm^{-3}$)	pH	SO_4 ($\mu mol\ g^{-1}$)	P ($\mu g\ g^{-1}$)	K ($\mu g\ g^{-1}$)	NO_3 + NO_2^- ($\mu g\ g^{-1}$)	NH_4 ($\mu g\ g^{-1}$)	Organic Matter (% w/w)
1.27	7.3	2.86	19	180	3.0	8.9	1.7

Table 1.2 Statistical analysis of the effects of treatment, incubation, depth, and the interaction of these variables on soil sulfate concentration in the vertical isolation assay

Source	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Treatment	1	32.102	32.102	321.6275	2.2×10^{-16}
Incubation	1	4.571	4.571	45.7979	1.201×10^{-7}
Depth	3	0.272	0.091	0.9096	0.44733
Treatment:Incubation	1	1.577	1.577	15.8061	0.000375
Treatment:Depth	3	1.320	0.440	4.4078	0.010526
Incubation:Depth	3	0.107	0.036	0.3589	0.783082
Treatment:Incubation:Depth	3	0.253	0.084	0.8438	0.480093
Residual	32	3.194	0.100	-	-
Total	47	43.396	-	-	-

Table 1.3 Tukey comparison of means test results for sulfate concentration between different treatment and depth combinations in the vertical isolation bioassay. In the comparison columns, "C" indicates control columns and "T" indicates treated columns. The number next to the letter represents the soil depth in cm.

Comparison	Estimate	Standard Error	t-value	P
C15-C10	0.07434	0.29029	0.256	1.00000
C20-C10	0.13286	0.30020	0.443	0.99982
C5-C10	-0.40159	0.30020	-1.338	0.87852
T10-C10	1.73597	0.30020	5.783	<0.001
T15-C10	1.42081	0.30020	4.733	<0.001
T20-C10	1.51132	0.30020	5.034	<0.001
T5-C10	1.69229	0.30020	5.637	<0.001
C20-C15	0.05852	0.27582	0.212	1.00000
C5-C15	-0.47594	0.27582	-1.726	0.67088
T10-C15	1.66163	0.27582	6.024	<0.001
T15-C15	1.34647	0.27582	4.882	<0.001
T20-C15	1.43698	0.27582	5.210	<0.001
T5-C15	1.61794	0.27582	5.866	<0.001
C5-C20	-0.53446	0.28623	-1.867	0.57989
T10-C20	1.60311	0.28623	5.601	<0.001
T15-C20	1.28795	0.28623	4.500	0.00137
T20-C20	1.37846	0.28623	4.816	<0.001
T5-C20	1.55942	0.28623	5.448	<0.001
T10-C5	2.13757	0.28623	7.468	<0.001
T15-C5	1.82241	0.28623	6.367	<0.001
T20-C5	1.91291	0.28623	6.683	<0.001
T5-C5	2.09388	0.28623	7.315	<0.001
T15-T10	-0.31516	0.28623	-1.101	0.95277
T20-T10	-0.22465	0.28623	-0.785	0.99299
T5-T10	-0.04369	0.28623	-0.153	1.00000
T20-T15	0.09051	0.28623	0.316	0.99998
T5-T15	0.27147	0.28623	0.948	0.97891
T5-T20	0.18096	0.28623	0.632	0.99816

Table 1.4 Soil sulfate concentration between treatments, incubation periods and depths in the vertical isolation bioassay.

Treatment	Incubation Period (days)	Soil Depth (cm)	Mean SO_4 Concentration ($\mu mol\ g^{-1}$)	Standard Error
B. juncea	4	5	1.57	0.193
B. juncea	4	10	1.67	0.160
B. juncea	4	15	1.30	0.162
B. juncea	4	20	1.47	0.146
B. juncea	8	5	2.62	0.286
B. juncea	8	10	2.61	0.235
B. juncea	8	15	2.34	0.206
B. juncea	8	20	2.36	0.181
Control	4	5	<LOD	0
Control	4	10	0.252	0.252
Control	4	15	0.448	0.231
Control	4	20	0.215	0.215
Control	8	5	<LOD	0
Control	8	10	0.417	0.209
Control	8	15	0.662	0.0310
Control	8	20	0.854	0.0500

Table 1.5 Tukey comparison of means test results for sulfate concentration between different treatment and incubation combinations in the vertical isolation bioassay. In the comparison columns, "C" indicates control columns and "T" indicates treated columns. The number next to the letter represents the incubation time in days

Comparison	Estimate	Standard Error	t-value	P
C8-C4	0.2546	0.1396	1.824	0.276
T4-C4	1.2730	0.1396	9.119	<0.001
T8-C4	2.2527	0.1396	16.136	<0.001
T4-C8	1.0184	0.1396	7.295	<0.001
T8-C8	1.9982	0.1396	14.313	<0.001
T8-T4	0.9797	0.1396	7.018	<0.001

Vertical Isolation Assay: Soil Electrical Conductivity

The null hypotheses regarding the effects of the categorical variables on soil electrical conductivity, EC, were that the electrical conductivity of the soil in the columns would not be different between treatments, incubation periods, soil depths, or the interactions between these variables. There was a significant effect of treatment, depth, the treatment:depth interaction, the depth:incubation interaction, and the treatment:depth:incubation interaction on soil EC which had P-values of $<2.2\times10^{-16}$, 0.003493, 3.333×10^{-11}, 2.600×10^{-5}, and 2.152×10^{-5} respectively (Table 1.6). Therefore the null hypotheses that the EC will not be different between soil treatments, depths, treatment:depth combinations, depth:incubation combinations, or treatment:depth:incubation combinations is rejected. The null hypothesis that the EC will be different between soil incubation periods or treatment:incubation combinations fails to be rejected. Since the three way interaction between the categorical variables is significant we cannot conclude that there is a significant effect of any individual categorical variable and must conclude that the EC is different between one or more of the treatment:depth:incubation combinations.

Based on the results of the Tukey multiple comparison of means test, the EC of the soil from 0-5cm in the treated columns which were incubated for four days was significantly higher than the EC of any other soil treatment:depth:incubation combination (Fig. 1.2 and 1.3, Table 1.7). Other than the top 5-cm of the treated columns incubated for four days, the soil EC was not significantly different between the treated columns which were incubated for four and eight days at any depth. The EC from 10-15-cm in the treated columns which were incubated for four days was not significantly different than the EC from 5-20-cm in the control columns which were incubated for four days and the EC from 10-20-cm in the control columns which were incubated for eight days. Additionally, the EC from 15-20-cm in

the treated columns incubated for four days was not significantly different than the EC from 5-15-cm in the control soil incubated for four days and the EC from 10-20-cm in the control soil incubated for eight days. The EC from 0-10-cm in the treated soil which was incubated for four days and the EC at all depths in the treated soil incubated for eight days was significantly greater than the EC at any depth in all the control columns (Table 1.7).

The differences in EC between the treated and control columns may be due to the input of glucosinolates or sulfate and H^+ from the hydrolysis of the glucosinolates (Wu, Zhou, and Xu 2009; Tredoux, Van Der Merwe, and Peters 2009). There also may have been ionic compounds in the meal other than glucosinolates which contributed to the electrical conductivity of the soil. Previous research has shown that *B. juncea* seed meal can contain high concentrations of uric and phytic acid which deprotonate at pH's of 5.6 and 1.5 respectively, generating protons and their conjugate anions which add charge to the soil (Costello, Glonek, and Myers 1976; Sharma *et al.* 2019; Hediger 2004) Additionally, proteins are charged above or below their isoelectric point and previous research has shown that the EC of a solution increases as the concentration of some proteins increases (Alshami, Tang, and Rasco 2017; Shaw *et al.* 2001). Therefore, proteins in the seed meal may have increased the EC of the soil as well.

The high EC from 0-5cm in the treated soil which was incubated for four days suggests that ionic compounds were still being released into the soil from the meal four days after the meal was applied and watered. Although the EC's from 10-20-cm in the treated columns which were incubated for four days were not significantly different than the EC's in the treated columns which were incubated for 8 days, they were lower on average and were not significantly different than the EC's at certain depths in the control columns (Fig. 1.2 and

1.3, Tables 1.7 and 1.8). This also indicates that ions from the meal were still concentrated in the upper soil layers and had not been fully leached through the soil four days after the columns were initially watered. After eight days the mean EC was still highest in the top 5-cm of soil; however, the means at all depths were much closer, suggesting that the ions from the meal had been leached through the soil and were more equally distributed throughout the column (Fig. 1.2 amd 1.3). The results from the control columns also indicate that ions in the soil were being leached through the columns. Although the mean EC's were not significantly different at different soil depths, the mean EC's in the top 5-cm of both the control soil incubated for four and eight days were considerably lower than the EC's from 5-20-cm. As the soil was watered, ions which were naturally present were likely leached downwards, lowering the EC in the surface layer.

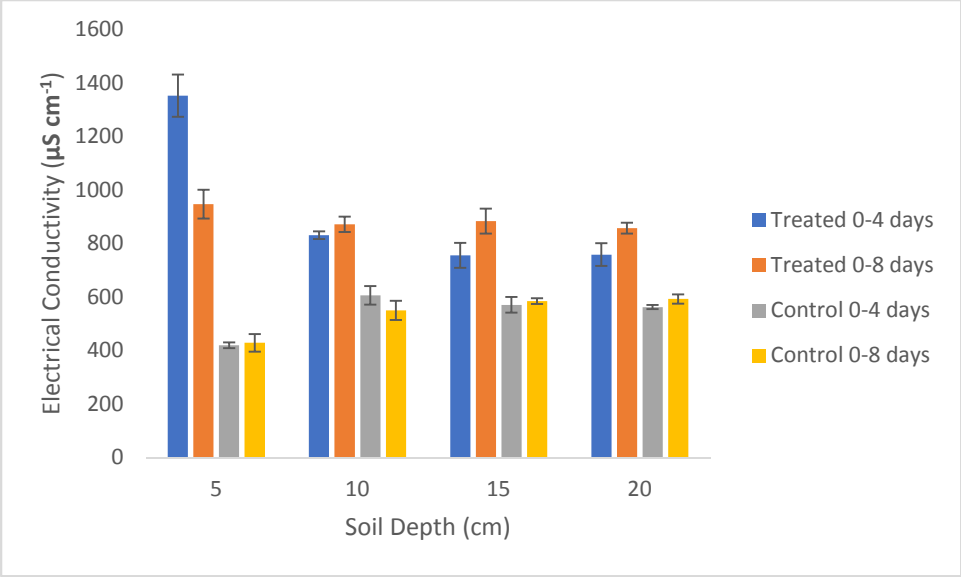


Figure 1.2 Soil Electrical conductivity in different column treatments in vertical isolation assay

Depth (cm)	Treated 4 Days	Treated 8 Days	Control 4 Days	Control 8 Days
5	1352	947	420	429
10	831	871	606	606
15	755	884	570	585
20	758	857	562	592
Average	924	890	540	553

Figure 1.3 Average soil electrical conductivity (µSiemens cm^{-1}) at different depths between different treatments and incubation periods. The color of the cells indicates the electrical conductivity with red being the highest electrical conductivity and dark green being the lowest.

Table 1.6 Statistical analysis of the effects of treatment, incubation, depth, and the interaction of these variables on wireworm soil EC in the vertical isolation assay

Source	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Treatment	1	1621778	1621778	396.8906	$<2.2*10^{-16}$
Depth	3	68046	22682	5.5508	0.003493
Incubation	1	3658	3658	0.8951	0.351192
Treatment:Depth	3	514831	171610	41.9975	$3.333*10^{-11}$
Treatment:Incubation	1	3451	3451	0.8446	0.364973
Depth:Incubation	3	142409	47470	11.6171	$2.600*10^{-5}$
Treatment:Depth:Incubation	3	145745	48582	11.8892	$2.152*10^{-5}$
Residual	32	130759	4086	-	-
Total	47	2630677	-	-	-

Table 1.7 Tukey comparison of means tests results for electrical conductivity between different treatment:depth:incubation combinations in the vertical isolation bioassay. In the comparisons column, the number in parentheses represent soil depth (cm), the number which is not in parentheses represents the incubation time (days), and the letter "T" or "C" represents treated columns and control columns respectively.

Comparison	Estimate	Standard Error	t-value	P
(10)4T - (10)4C	225.000	52.193	4.311	0.0109
(10)8C - (10)4C	-56.000	52.193	-1.073	0.9990
(10)8T - (10)4C	265.667	52.193	5.090	<0.01
(15)4C - (10)4C	-35.667	52.193	-0.683	1.0000
(15)4T - (10)4C	149.333	52.193	2.861	0.2865
(15)8C - (10)4C	-21.000	52.193	-0.402	1.0000
(15)8T - (10)4C	277.667	52.193	5.320	<0.01
(20)4C - (10)4C	-43.667	52.193	-0.837	0.9999
(20)4T - (10)4C	152.000	52.193	2.912	0.2622
(20)8C - (10)4C	-13.333	52.193	-0.255	1.0000
(20)8T - (10)4C	251.000	52.193	4.809	<0.01
(5)4C - (10)4C	-186.333	52.193	-3.570	0.0688
(5)4T - (10)4C	746.333	52.193	14.299	<0.01
(5)8C - (10)4C	-177.333	52.193	-3.398	0.0997
(5)8T - (10)4C	340.667	52.193	6.527	<0.01
(10)8C - (10)4T	-281.000	52.193	-5.384	<0.01
(10)8T - (10)4T	40.667	52.193	0.779	1.0000
(15)4C - (10)4T	-260.667	52.193	-4.994	<0.01
(15)4T - (10)4T	-75.667	52.193	-1.450	0.9809
(15)8C - (10)4T	-246.000	52.193	-4.713	<0.01
(15)8T - (10)4T	52.667	52.193	1.009	0.9995
(20)4C - (10)4T	-268.667	52.193	-5.148	<0.01
(20)4T - (10)4T	-73.000	52.193	-1.399	0.9861
(20)8C - (10)4T	-238.333	52.193	-4.566	<0.01
(20)8T - (10)4T	26.000	52.193	0.498	1.0000
(5)4C - (10)4T	-411.333	52.193	-7.881	<0.01
(5)4T - (10)4T	521.333	52.193	9.989	<0.01
(5)8C - (10)4T	-402.333	52.193	-7.709	<0.01
(5)8T - (10)4T	115.667	52.193	2.216	0.6816
(10)8T - (10)8C	321.667	52.193	6.163	<0.01
(15)4C - (10)8C	20.333	52.193	0.390	1.0000
(15)4T - (10)8C	205.333	52.193	3.934	0.0293
(15)8C - (10)8C	35.000	52.193	0.671	1.0000
(15)8T - (10)8C	333.667	52.193	6.393	<0.01
(20)4C - (10)8C	12.333	52.193	0.236	1.0000
(20)4T - (10)8C	208.000	52.193	3.985	0.0262
(20)8C - (10)8C	42.667	52.193	0.817	1.0000
(20)8T - (10)8C	307.000	52.193	5.882	<0.01
(5)4C - (10)8C	-130.333	52.193	-2.497	0.4963
(5)4T - (10)8C	802.333	52.193	15.372	<0.01
(5)8C - (10)8C	-121.333	52.193	-2.325	0.6101
(5)8T - (10)8C	396.667	52.193	7.600	<0.01
(15)4C - (10)8T	-301.333	52.193	-5.773	<0.01
(15)4T - (10)8T	-116.333	52.193	-2.229	0.6725
(15)8C - (10)8T	-286.667	52.193	-5.492	<0.01
(15)8T - (10)8T	12.000	52.193	0.230	1.0000
(20)4C - (10)8T	-309.333	52.193	-5.927	<0.01
(20)4T - (10)8T	-113.667	52.193	-2.178	0.7044
(20)8C - (10)8T	-279.000	52.193	-5.346	<0.01
(20)8T - (10)8T	-14.667	52.193	-0.281	1.0000
(5)4C - (10)8T	-452.000	52.193	-8.660	<0.01
(5)4T - (10)8T	480.667	52.193	9.209	<0.01
(5)8C - (10)8T	-443.000	52.193	-8.488	<0.01
(5)8T - (10)8T	75.000	52.193	1.437	0.9823
(15)4T - (15)4C	185.000	52.193	3.545	0.0732
(15)8C - (15)4C	14.667	52.193	0.281	1.0000
(15)8T - (15)4C	313.333	52.193	6.003	<0.01

(20)4C - (15)4C	-8.000	52.193	-0.153	1.0000
(20)4T - (15)4C	187.667	52.193	3.596	0.0647
(20)8C - (15)4C	22.333	52.193	0.428	1.0000
(20)8T - (15)4C	286.667	52.193	5.492	<0.01
(5)4C - (15)4C	-150.667	52.193	-2.887	0.2758
(5)4T - (15)4C	782.000	52.193	14.983	<0.01
(5)8C - (15)4C	-141.667	52.193	-2.714	0.3630
(5)8T - (15)4C	376.333	52.193	7.210	<0.01
(15)8C - (15)4T	-170.333	52.193	-3.264	0.1331
(15)8T - (15)4T	128.333	52.193	2.459	0.5219
(20)4C - (15)4T	-193.000	52.193	-3.698	0.0515
(20)4T - (15)4T	2.667	52.193	0.051	1.0000
(20)8C - (15)4T	-162.667	52.193	-3.117	0.1796
(20)8T - (15)4T	101.667	52.193	1.948	0.8355
(5)4C - (15)4T	-335.667	52.193	-6.431	<0.01
(5)4T - (15)4T	597.000	52.193	11.438	<0.01
(5)8C - (15)4T	-326.667	52.193	-6.259	<0.01
(5)8T - (15)4T	191.333	52.193	3.666	0.0558
(15)8T - (15)8C	298.667	52.193	5.722	<0.01
(20)4C - (15)8C	-22.667	52.193	-0.434	1.0000
(20)4T - (15)8C	173.000	52.193	3.315	0.1199
(20)8C - (15)8C	7.667	52.193	0.147	1.0000
(20)8T - (15)8C	272.000	52.193	5.211	<0.01
(5)4C - (15)8C	-165.333	52.193	-3.168	0.1622
(5)4T - (15)8C	767.333	52.193	14.702	<0.01
(5)8C - (15)8C	-156.333	52.193	-2.995	0.2263
(5)8T - (15)8C	361.667	52.193	6.929	<0.01
(20)4C - (15)8T	-321.333	52.193	-6.157	<0.01
(20)4T - (15)8T	-125.667	52.193	-2.408	0.5545
(20)8C - (15)8T	-291.000	52.193	-5.575	<0.01
(20)8T - (15)8T	-26.667	52.193	-0.511	1.0000
(5)4C - (15)8T	-464.000	52.193	-8.890	<0.01
(5)4T - (15)8T	468.667	52.193	8.979	<0.01
(5)8C - (15)8T	-455.000	52.193	-8.718	<0.01
(5)8T - (15)8T	63.000	52.193	1.207	0.9967
(20)4T - (20)4C	195.667	52.193	3.749	0.0455
(20)8C - (20)4C	30.333	52.193	0.581	1.0000
(20)8T - (20)4C	294.667	52.193	5.646	<0.01
(5)4C - (20)4C	-142.667	52.193	-2.733	0.3546
(5)4T - (20)4C	790.000	52.193	15.136	<0.01
(5)8C - (20)4C	-133.667	52.193	-2.561	0.4572
(5)8T - (20)4C	384.333	52.193	7.364	<0.01
(20)8C - (20)4T	-165.333	52.193	-3.168	0.1630
(20)8T - (20)4T	99.000	52.193	1.897	0.8586
(5)4C - (20)4T	-338.333	52.193	-6.482	<0.01
(5)4T - (20)4T	594.333	52.193	11.387	<0.01
(5)8C - (20)4T	-329.333	52.193	-6.310	<0.01
(5)8T - (20)4T	188.667	52.193	3.615	0.0625
(20)8T - (20)8C	264.333	52.193	5.065	<0.01
(5)4C - (20)8C	-173.000	52.193	-3.315	0.1205
(5)4T - (20)8C	759.667	52.193	14.555	<0.01
(5)8C - (20)8C	-164.000	52.193	-3.142	0.1706
(5)8T - (20)8C	354.000	52.193	6.782	<0.01
(5)4C - (20)8T	-437.333	52.193	-8.379	<0.01
(5)4T - (20)8T	495.333	52.193	9.490	<0.01
(5)8C - (20)8T	-428.333	52.193	-8.207	<0.01
(5)8T - (20)8T	89.667	52.193	1.718	0.9269
(5)4T - (5)4C	932.667	52.193	17.869	<0.01
(5)8C - (5)4C	9.000	52.193	0.172	1.0000
(5)8T - (5)4C	527.000	52.193	10.097	<0.01
(5)8C - (5)4T	-923.667	52.193	-17.697	<0.01
(5)8T - (5)4T	-405.667	52.193	-7.772	<0.01
(5)8T - (5)8C	518.000	52.193	9.925	<0.01

Table 1.8 Soil EC between treatments, incubation periods and depths in the vertical isolation bioassay

Treatment	Incubation Period (days)	Soil Depth (cm)	Mean Electrical Conductivity ($\mu S\ cm^{-1}$)	Standard Error
B. juncea	4	5	1352	78.7
B. juncea	4	10	831	54.2
B. juncea	4	15	755	10.5
B. juncea	4	20	758	32.6
B. juncea	8	5	947	46.3
B. juncea	8	10	872	46.9
B. juncea	8	15	884	29.4
B. juncea	8	20	857	10.7
Control	4	5	420	14.4
Control	4	10	606	28.8
Control	4	15	570	35.2
Control	4	20	562	36.1
Control	8	5	429	42.5
Control	8	10	606	20.3
Control	8	15	585	8.19
Control	8	20	593	17.4

Vertical Isolation Assay: Soil pH

The null hypotheses regarding the effects of the categorical variables on soil pH were that the pH of the soil in the columns would not be different between treatments, incubation periods, soil depths, or the interactions between these variables. Treatment had a P-value of 2.243×10^{-7} which is significant at $\alpha=0.05$ and was the only categorical variable which had a significant effect on soil pH (Table 1.9). Therefore, the null hypothesis that pH will not be different between treatments is rejected and the null hypotheses that pH will not be different between depths, incubation periods, and the two- and three-way interactions of the categorical variables fails to be rejected. The mean soil pH in the treated and control columns was 7.42 ± 0.0326 and 7.65 ± 0.0181 respectively (Fig. 1.4 and Table 1.10). The lower pH in the treated columns may be due to the release of H^+ during glucosinolate hydrolysis or deprotonation of acids in the seed meal (Wu, Zhou, and Xu 2009; Sharma *et al.* 2019). As stated before, *B. juncea* seed meal can contain high concentrations of uric and phytic acid which both have a pKa below the pH of Bannock loam (Sharma *et al.* 2019; Costello, Glonek, and Myers 1976; Hediger 2004). The soil pH is greater than the pKa of these acids, so they would deprotonate in the soil solution increasing soil acidity. Since the soil pH was not significantly lower between incubation periods in the treated columns any H^+ generated from glucosinolate hydrolysis which occurred after four days had a negligible effect on soil pH. Also, since the pH was not significantly different between depths, watering the columns to replace evaporative losses may have distributed H^+ evenly throughout the soil.

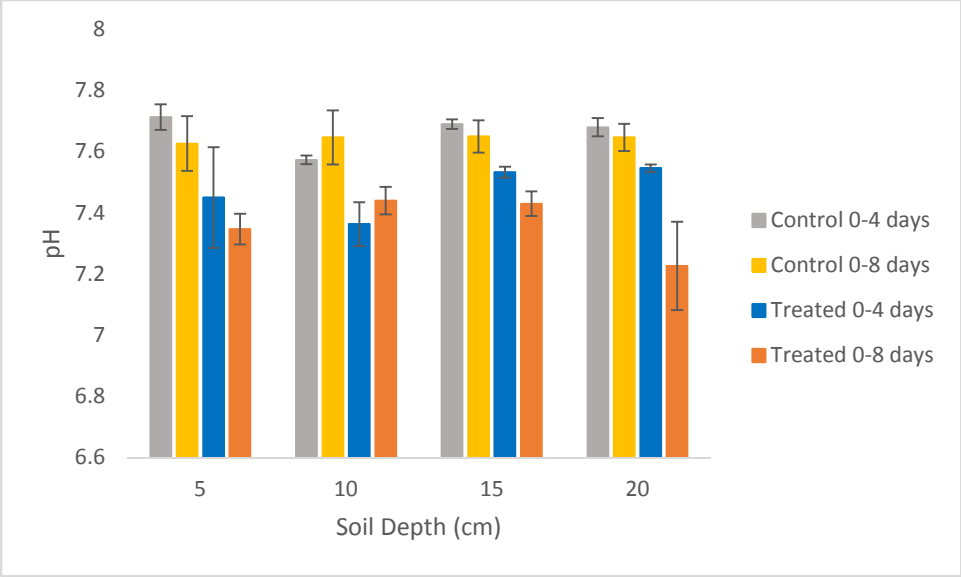


Figure 1.4 Soil pH in different column treatments. Error bars indicate the standard error about the mean.

Table 1.9 Statistical analysis of the effects of treatment, incubation, depth, and the interaction of these variables on soil pH in the vertical isolation assay

Source	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Treatment	1	0.66977	0.66977	42.8766	2.243e-07
Depth	3	0.03142	0.01047	0.6705	0.5764
Incubation	1	0.05400	0.05400	3.4571	0.0722
Treatment:Depth	3	0.01789	0.00596	0.3817	0.7668
Treatment:Incubation	1	0.02475	0.02475	1.5846	0.2172
Depth:Incubation	3	0.09899	0.03300	2.1123	0.1181
Treatment:Depth:Incubation	3	0.04011	0.01337	0.8558	0.4739
Residuals	32	0.49987	0.01562	-	-
Total	47	1.4368	-	-	-

Table 1.10 Soil pH between treatments, incubation periods and depths in the vertical isolation bioassay

Treatment	Incubation Period (days)	Soil Depth (cm)	Mean pH	Standard Error
B. juncea	4	5	7.45	0.164
B. juncea	4	10	7.36	0.0717
B. juncea	4	15	7.53	0.0176
B. juncea	4	20	7.55	0.0120
B. juncea	8	5	7.35	0.0504
B. juncea	8	10	7.44	0.0451
B. juncea	8	15	7.43	0.0404
B. juncea	8	20	7.23	0.144
Control	4	5	7.71	0.0418
Control	4	10	7.57	0.0145
Control	4	15	7.69	0.0153
Control	4	20	7.68	0.0300
Control	8	5	7.63	0.0895
Control	8	10	7.65	0.0882
Control	8	15	7.65	0.0529
Control	8	20	7.65	0.0441

Vertical Isolation Assay: Wireworm Mass Change and Mortality

None of the wireworms died during the experiment which indicates that depth, treatment, incubation period, or a combination of these variables did not have a significant effect on wireworm mortality while the wireworms were in the soil. The null hypotheses regarding the effects of the categorical variables on wireworm mass change were that the mean change in mass of the wireworms in the columns would not be different between treatments, incubation periods, soil depths, or the interactions between these variables. The treatment:incubation interaction had a P-value of 0.002738 which is significant at α=0.05 and was the only variable which had a significant effect on wireworm mass change (Table 1.11). Therefore, the null hypothesis that the mean wireworm mass change will not be different between treatment:interaction combinations is rejected and it is concluded that the wireworm mass change was different between one or more of the combinations. The null hypotheses that the mean wireworm mass change will not differ between treatments, depths, incubation periods, the treatment:depth interaction, the depth:incubation interaction, or the three-way interaction between these variables fails to be rejected.

Since the wireworms were not provided with any external source of food during their incubation, it was expected that the wireworms in all the treatments would lose weight or maintain the same weight. However, in many cases the wireworms gained weight, suggesting that the wireworms may have had a food source in the soil (Figure 1.5 and Table 1.12). In addition to living plants, wireworms may also feed on plant litter and soil organic matter (SOM) (Hemerik, Gort, and Brussaard 2003; Schaerffenberg 1942; Langenboch 1932). As previously stated, the soil which was used for this experiment was collected from the top 20-

cm of an agricultural field in Shelley, Idaho. Although the field did not contain crops and had not been seeded at the time of soil collection, there was an abundance of plant litter which remained from previous harvests. The larger pieces of plant litter were removed when the soil was dried, ground, and sieved; however, smaller pieces still remained and could have served as a food source.

The mass change of the wireworms was not significantly different between control and treated columns. This corresponds with previous research which has shown that wireworm mass is not significantly affected by some Brassica seed meals (Elberson *et al.* 1996). Since the wireworms were incubated under the same conditions and treatment did not have a significant effect on mass change, the fluctuations in mass could be due to wireworm handling. Previous research has shown that handling wireworms can cause them to lose weight (van Herk 2008). Weighing, identifying, and measuring the length of the wireworms as well as transferring them to the Bannock loam may have affected their mass. It is difficult to quantify the degree to which each wireworm was handled, and therefore ensure that all the wireworms were handled the same amount. In some cases, wireworms were lethargic and relatively easy to study, whereas other wireworms were very active and had to be restrained with forceps for analysis. The additionally handling which was required to study the active wireworms may have had an adverse effect on their mass which could have increased the variability between replicates.

The mean mass change for the treated columns incubated for four and eight days and the control columns incubated for four and eight days were -1.26 ± 1.96 %, 10.38 ± 5.14 %, 8.55 ± 4.49 %, and -7.09 ± 2.66 % respectively (Table 1.12). The results of the Tukey multiple comparison of means test shows that the wireworms in the control columns which

were incubated for four days had a significantly higher increase in mass than the wireworms in the control columns which were incubated for eight days. The Tukey test also showed that the mass change of the wireworms in the treated columns which were incubated for eight days was significantly greater than the mass change of wireworms which were incubated in the control columns for eight days (Table 1.13). These differences in mass change may be due to the depletion of SOM which was available for wireworm consumption. As previously described, all the columns were watered daily to replace evaporative losses and maintain a constant moisture content. The *B. juncea* seed meal in the treated columns may have been detoxified after several days of saturation and particulates may have leached into lower layers of the column. These particulates may have offered an additional, safe food source for the wireworms. In the control columns, no meal was present so there was no additional input of biomass to serve as a food source. Since the wireworms in the control columns which were incubated for four days gained mass on average, there may still have been biomass for consumption at that time. However, after four days the wireworms may have exhausted the SOM which was available for consumption and may have lost weight as a result.

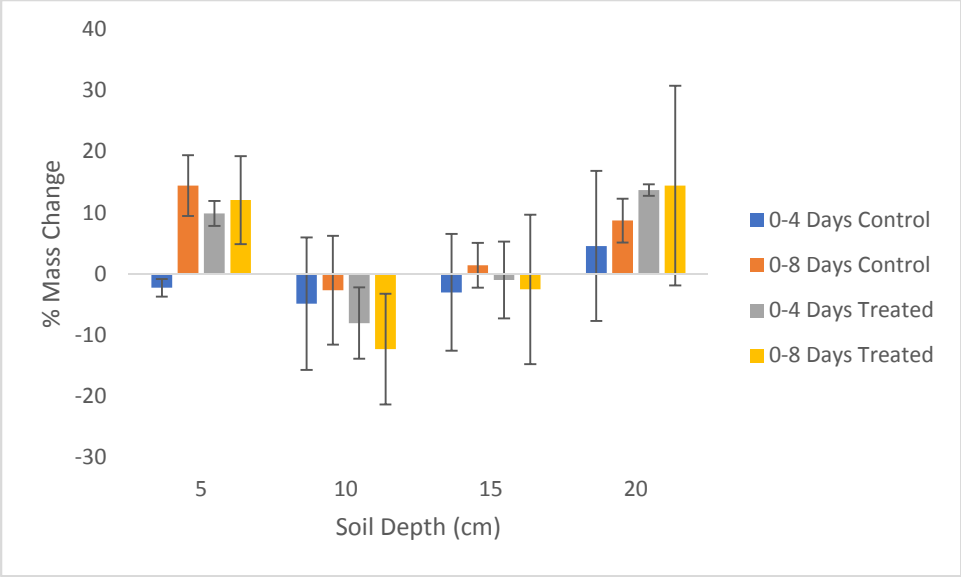


Figure 1.5 Percent wireworm mass change in different column treatments. Error bars indicate the standard error about the mean

Table 1.11 Statistical analysis of the effects of treatment, incubation, depth, and the interaction of these variables on wireworm mass change in the vertical isolation assay

Source	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Treatment	1	168.8	168.81	0.8055	0.376157
Incubation	1	44.5	44.52	0.2124	0.647989
Depth	3	304.2	101.40	0.4839	0.695844
Treatment:Incubation	1	2209.3	2209.27	10.5420	0.002738 **
Treatment:Depth	3	88.0	29.33	0.1399	0.935342
Incubation:Depth	3	94.6	31.52	0.1504	0.928660
Treatment:Incubation:Depth	3	393.5	131.15	0.6258	0.603581
Residuals	32	6706.2	209.57	-	-
Total	47	10009.1	-	-	-

Table 1.12 Percent wireworm mass change between treatments, incubation periods and depths in the vertical isolation bioassay

Treatment	Incubation Period (days)	Soil Depth (cm)	Mean % Wireworm Mass Change	Standard Error
B. juncea	4	5	-3.00	2.04
B. juncea	4	10	1.42	5.84
B. juncea	4	15	-0.971	6.27
B. juncea	4	20	-2.51	0.939
B. juncea	8	5	4.59	7.21
B. juncea	8	10	8.74	9.06
B. juncea	8	15	13.7	12.2
B. juncea	8	20	14.4	16.3
Control	4	5	-2.23	1.44
Control	4	10	14.4	10.8
Control	4	15	9.91	9.55
Control	4	20	12.1	12.3
Control	8	5	-4.84	4.97
Control	8	10	-2.65	8.87
Control	8	15	-8.01	3.66
Control	8	20	-12.3	3.58

Table 1.13 Tukey comparison of means test results for wireworm mass change between different treatment:incubation combinations. In the comparison column "C" represent control columns and "T" represent treated columns. The number next to letter indicates the incubation time in days.

Comparison	Estimate	Standard Error	t-value	P
C8 - C4	-15.495	5.361	-2.890	0.0290
T4 - C4	-9.818	5.361	-1.831	0.2727
T8 - C4	1.825	5.361	0.340	0.9863
T4 - C8	5.677	5.361	1.059	0.7159
T8 - C8	17.319	5.361	3.231	0.0122
T8 - T4	11.642	5.361	2.172	0.1471

Vertical Isolation Assay: Sinigrin and allyl isothiocyanate Concentrations

The concentrations of allyl isothiocyanate and sinigrin in the treated and control columns were below the limit of detection for all replicates and incubation periods (Table 1.26). Since the columns were surface treated with *B. juncea* seed meal and allyl isothiocyanate is a volatile it is possible that the majority of the allyl isothiocyanate generated from sinigrin hydrolysis evaporated from the columns prior to analysis. Additionally, previous research has shown that allyl isothiocyanate may have a relatively short half-life in soil, approximately 8-12 hours depending on temperature and soil characteristics (Zhu *et al.* 2020). If this were the case, then the concentration of allyl isothiocyanate in the soil would have been reduced by approximately 99.6-99.98% after four days and 99.998-100 % after eight days which would account for the low levels of allyl isothiocyanate in the soil. The low concentration of sinigrin in the soil could indicate that the majority, if not all, of the sinigrin in the meal was hydrolyzed within four days after application which could account for the high sulfate concentration in the treated columns relative to the control columns.

Tarping Bioassay: Soil Electrical Conductivity

The mean EC for the soil in the treated tarped, treated un-tarped, control tarped, and control un-tarped columns was approximately 1200 ± 24.3, 1240 ± 13.8, 877 ± 13.6, and 853 ± 27.7 μSiemens cm^{-1} respectively (Table 1.14). The null hypothesis regarding the effects of treatment on soil EC was that the mean soil EC would not be significantly different between treatments. The P-value for treatment was 4.422×10^{-16} which is significant at $\alpha=0.05$ (Table 1.15). Therefore, the null hypothesis is rejected, and it is concluded that the mean soil EC was different between one or more of the treatments. Like the vertical isolation assay, the EC was significantly higher in the columns treated with *B. juncea* seed meal than the control

columns. The EC was not significantly different between the treated tarped and treated un-tarped columns nor was it significantly different between the control tarped and control un-tarped columns (Table 1.16). As with the vertical isolation assay the difference in electrical conductivity between the columns with seed meal and the control columns was likely due to the release of ions during glucosinolate hydrolysis as well as proteins, H^+, deprotonated uric and phytic acid, and other ions from the seed meal (Wu, Zhou, and Xu 2009; Tredoux, Van Der Merwe, and Peters 2009; Sharma *et al.* 2019; Costello, Glonek, and Myers 1976; Hediger 2004; Alshami, Tang, and Rasco 2017; Shaw *et al.* 2001).

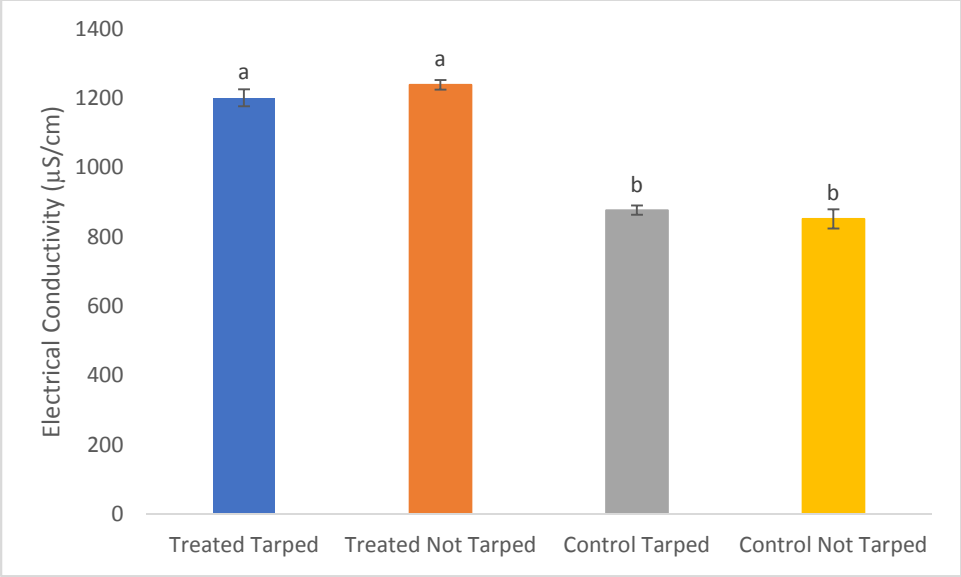


Figure 1.6 Soil electrical conductivity (µSiemens cm^{-1}) between treatments in tarping bioassay. Error bars represent the standard error about the mean and letters indicate significantly different means at α=0.05.

Table 1.14 Electrical conductivity of soil from different treatments in the tarping bioassay

Treatment	Mean Electrical Conductivity (μScm^{-1})	Standard Error
Treated Tarped	1202	24.27
Treated Un-tarped	1240	13.80
Control Tarped	877	13.58
Control Un-tarped	853	27.74

Table 1.15 Statistical analysis of the effects of treatment soil EC in tarping assay

Source	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Treatment	3	1122618	374206	95.961	4.422e-16
Residual	32	124786	3899.6	-	-
Total	35	1247404	-	-	-

Table 1.16 Tukey comparison of means test for soil EC between treatments in tarping assay. "CT", "CNT", "TT", and "TNT" indicate control tarped, control un-tarped, treated tarped, and treated un-tarped columns respectively.

Comparison	Estimate	Standard Error	t-value	P
CT - CNT	24.75	29.44	0.841	0.835
TNT - CNT	387.11	29.44	13.150	<1e-04
TT - CNT	339.97	29.44	11.549	<1e-04
TNT - CT	362.36	29.44	12.309	<1e-04
TT - CT	315.22	29.44	10.708	<1e-04
TT - TNT	-47.14	29.44	-1.601	0.392

Tarping Bioassay: Soil pH

The mean soil pH for the treated tarped, treated un-tarped, control tarped, and control un-tarped columns were 7.17 ± 0.0465, 7.34 ± 0.0221, 7.57 ± 0.0343, and 7.52 ± 0.0170 respectively (Table 1.17). The null hypothesis regarding the effects of treatment on the soil pH was that the mean soil pH would not be significantly different between treatments. The P-value for treatment was 1.215×10^{-9} which is significant at $\alpha=0.05$ (Table 1.18). Therefore, the null hypothesis is rejected and it is concluded that pH differed between one or more of the treatments. The results of the Tukey multiple comparison of means test show that the mean soil pH was not significantly different between the control tarped and control un-tarped columns, the mean soil pH in the treated un-tarped columns was significantly lower than both control treatments, and the mean soil pH in the treated tarped columns was significantly lower than all other treatments (Fig. 1.7 and Table 1.19). Like the vertical isolation assay, the soil pH in the treated columns was significantly lower than the pH in the control columns, which is likely due to the deprotonation of acids in the meal and/or the release of H^+ during glucosinolate hydrolysis (Wu, Zhou, and Xu 2009; Sharma *et al.* 2019; Costello, Glonek, and Myers 1976; Hediger 2004).

Since the same seed meal was used for both treatments, it was expected that the treated tarped and treated un-tarped columns would not have significantly different pH. However, the treated soil which was tarped had a significantly lower mean pH than the treated soil which was un-tarped. The discrepancy in soil pH may be due to carbon dioxide generated from the meal. As carbon dioxide dissolves into soil solution, it can be hydrolyzed to carbonic acid which will reduce the pH of the soil (Strawn, Bohn, and O'Connor 2019). In

the uncovered columns, CO_2 may have evaporated out of the columns rather than dissolving in soil solution; whereas in the covered columns volatilization would have been limited and the CO_2 may have moved into the soil.

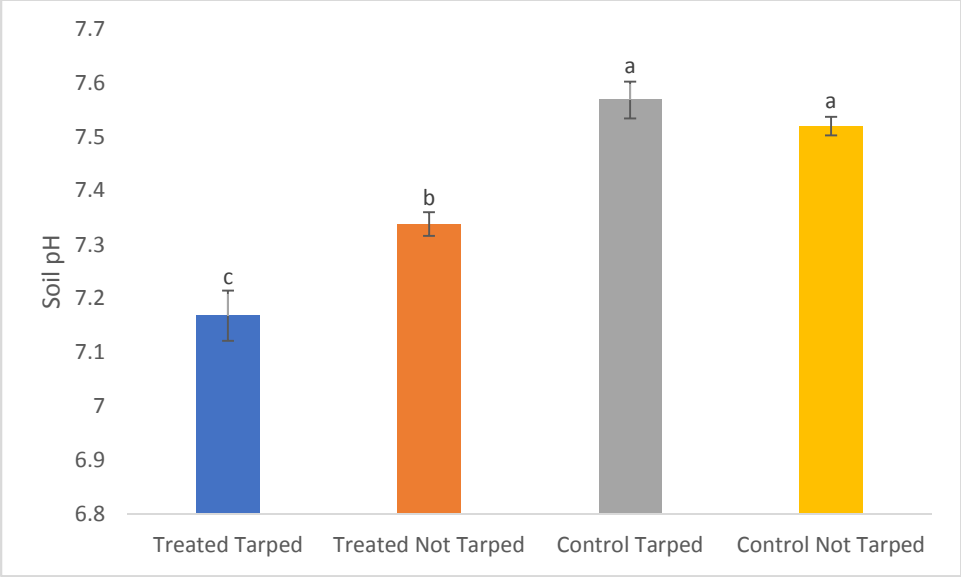


Figure 1.7 Soil pH between treatments in tarping bioassay. Error bars represent the standard error about the mean and letters indicate significantly different means at α=0.05.

Table 1.17 pH of soil from different treatments in the tarping bioassay

Treatment	Mean Soil pH	Standard Error
Treated Tarped	7.17	0.0465
Treated Un-tarped	7.34	0.0221
Control Tarped	7.57	0.0343
Control Un-tarped	7.52	0.0170

Table 1.18 Statistical analysis of the effects of treatment on soil pH in tarping assay

Source	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Treatment	3	0.87140	0.2905	31.315	1.215e-09
Residuals	32	0.29682	0.009275	-	-
Total	35	1.16822	-	-	-

Table 1.19 Tukey comparison of means test for soil pH between treatments in tarping assay. "CT", "CNT", "TT", and "TNT" indicate control tarped, control un-tarped, treated tarped, and treated un-tarped columns respectively.

Comparison	Estimate	Standard Error	t-value	P
CT - CNT	0.04833	0.04540	1.065	0.71313
TNT - CNT	-0.18194	0.04540	-4.007	0.00193
TT - CNT	-0.34417	0.04540	-7.581	< 0.001
TNT - CT	-0.23028	0.04540	-5.072	< 0.001
TT - CT	-0.39250	0.04540	-8.645	< 0.001
TT - TNT	-0.16222	0.04540	-3.573	0.00606

Tarping Assay: Wireworm Depth

The mean depths of the wireworms in the treated tarped, treated un-tarped, control tarped, and control un-tarped columns were 9.90 ± 1.58, 8.00 ± 1.24, 8.11 ± 1.25, and 11.0 ± 0.986-cm respectively (Fig. 1.8 and Table 1.20). The null hypothesis regarding the effects of treatment on the depth of the wireworms in the soil columns was that the mean depth of the wireworms would not be different between treatments. The P-value for treatment was 0.3202 which is not significant at α=0.05 (Table 1.21). Therefore, the null hypothesis that the mean depth of the wireworms in the soil columns was not significantly different between treatments fails to be rejected. The results of the analysis indicate that neither *B. juncea* seed meal, soil tarping, nor the combination of the two has a significant effect on the depth of wireworms in the soil columns (Tables 1.21 and 1.22).

Previous lab studies have shown that *L. infuscatus* wireworms migrate away from soil which contains *B. napus* seed meal, presumably in response to toxic isothiocyanates which are generated from the glucosinolates in the meal (Brown, Morra, and Borek 1991). Although sinigrin is not one of the primary glucosinolates present in *B. napus* seed meal, allyl isothiocyanate generated from sinigrin has been shown to be toxic to wireworms as well (Sang *et al.* 1984; Williams *et al.* 1993). Therefore, it was hypothesized that the wireworms would move lower in the soil column as the water percolated through in order to avoid the allyl isothiocyanate, similar to the wireworms avoiding the *B. napus* seed meal treated soil in the experiment by Brown, Borek, and Morra (1991). However, wireworms in this experiment were not significantly lower or higher in the columns between any of the treatments, which suggests that wireworms may not migrate in response to allyl isothiocyanate or other toxic

chemicals leaching from the meal. The lack of movement may also indicate that the concentration of allyl isothiocyanate and other toxic compounds leaching from the meal may have been too low to significantly affect the wireworm movement or the allyl isothiocyanate had not leached to the depth of the wireworms after two days.

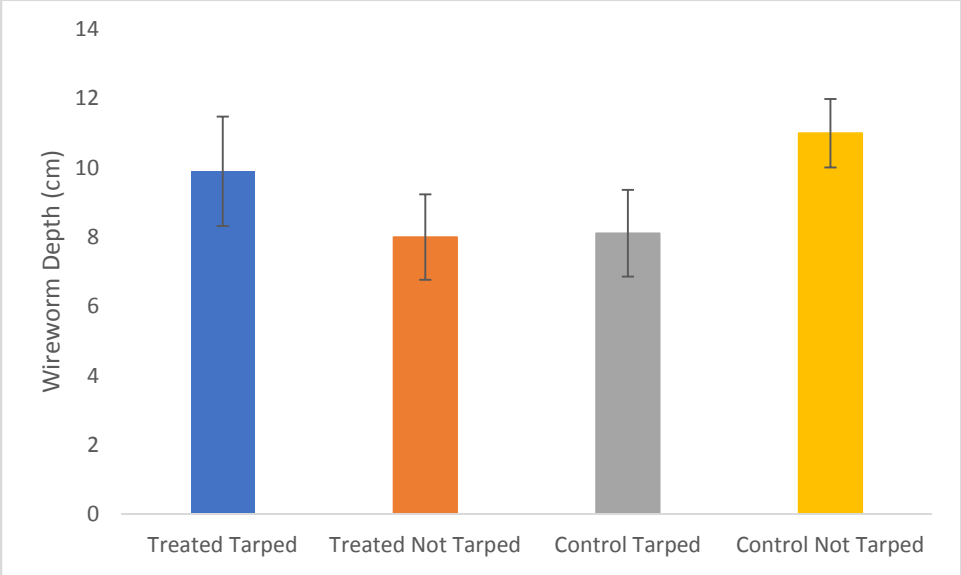


Figure 1.8 Depth of wireworms in soil columns between different treatments. Error bars indicate the standard error about the mean. Wireworm depths were not significantly different between treatments using α=0.05.

Table 1.20 Depth of wireworms in soil columns from different treatments in the tarping bioassay

Treatment	Mean Wireworm Depth (cm)	Standard Error
Treated Tarped	9.90	1.58
Treated Un-tarped	8.00	1.24
Control Tarped	8.11	1.25
Control Un-tarped	11.0	0.986

Table 1.21 Statistical analysis of the effects of treatment on wireworm depth in tarping assay

Source	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Treatment	3	53.89	17.96	1.215	0.3202
Residual	32	473.11	14.78	-	-
Total	35	527	-	-	-

Table 1.22 Tukey multiple comparison of means test for wireworm depth between treatments in the tarping bioassay. "CT", "CNT", "TT", and "TNT" indicate control tarped, control un-tarped, treated tarped, and treated un-tarped columns respectively.

Comparisons	Estimate	Standard Error	t-value	P
CT - CNT	-2.8889	1.8126	-1.594	0.396
TNT - CNT	-3.0000	1.8126	-1.655	0.363
TT - CNT	-1.4444	1.8126	-0.797	0.855
TNT - CT	-0.1111	1.8126	-0.061	1.000
TT - CT	1.4444	1.8126	0.797	0.855
TT - TNT	1.5556	1.8126	0.858	0.826

Tarping Assay: 5-Week Wireworm Mortality

Previous research by Williams *et al.* (1993) has shown that exposure of *L. Californicus* wireworms to allyl isothiocyanate concentrations as low as 240 nmol g^{-1} of soil for one day can result in 100 % mortality 5-weeks post treatment. The study also showed that small differences in allyl isothiocyanate concentration can have a significant effect on wireworm mortality. For example an allyl isothiocyanate concentration of 210 nmol g^{-1} of soil only yielded 92.5 % mortality 5-weeks post treatment and a concentration of 120 nmol g^{-1} of soil resulted in 2.6 % mortality 5-weeks post treatment (Williams *et al.* 1993). Since allyl isothiocyanate is a volatile compound it was hypothesized that un-tarped columns which were treated with *B. juncea* seed meal would have a significantly lower concentration of allyl isothiocyanate in the soil and the difference in concentration would be reflected in the 5-week mortality of the *L. infuscatus*, which are very similar to *L. Californicus* physiologically (Torrijos *et al.* 2019; Rashed *et al.* 2015).

The null hypothesis regarding the effects of treatment on 5-week post treatment wireworm mortality was that the mortality would not be significantly different between treatments. The 5-week mortality for the treated tarped, treated un-tarped, control tarped, and control un-tarped columns were 100, 11.1, 22.2, and 22.2% respectively (Fig. 1.9 and Table 1.23). The P-value for treatment was 1.801×10^{-5} which is significant at $\alpha=0.05$ (Table 1.24). Therefore, the null hypothesis is rejected, and it is concluded that the 5-week mortality was significantly different between one or more of the treatments. The results of the Tukey multiple comparison of means test showed that the wireworm mortality in the treated tarped columns was significantly greater than all the other treatments and mortality was not

significantly different between the treated un-tarped, control un-tarped or control tarped columns (Table 1.25).

The results of this experiment show that tarping soil treated with *B. juncea* seed meal has a significant effect on wireworm mortality. Tarping may have reduced allyl isothiocyanate loss due to volatilization. If so, the wireworms in the treated tarped columns may have been exposed to a higher concentration of allyl isothiocyanate than the wireworms in the treated un-tarped columns, which could account for the significantly higher mortality. Additionally, wireworm mortality in the treated tarped columns reached 100 % within one week post treatment, whereas there was no mortality in the treated un-tarped columns until 5-weeks post treatment. The mortality rate in the treated un-tarped soil was not significantly different than the mortality rate in either of the control treatments. The differences in mortality could be due to allyl isothiocyanate volatilization or degradation. Previous research by Williams *et al.* (1993) has shown, the low mortality does not necessarily indicate that all the allyl isothiocyanate from the seed meal was lost due to volatilization, and some allyl isothiocyanate may have persisted in the column which could account for the mortality (Williams *et al.*, 1993). Since neither of the control columns contained any seed meal, the observed mortality may have been due to a variety of other factors. For example, during the experiment some wireworms in the control columns had difficulty burrowing into the damp soil. Due to the lack of moisture, these wireworms typically died within several days and were desiccated prior to being transferred to fresh soil. The mortality may also be due to greater soil acidification or wireworm asfixiation due to the build up of CO_2 in the tarped columns

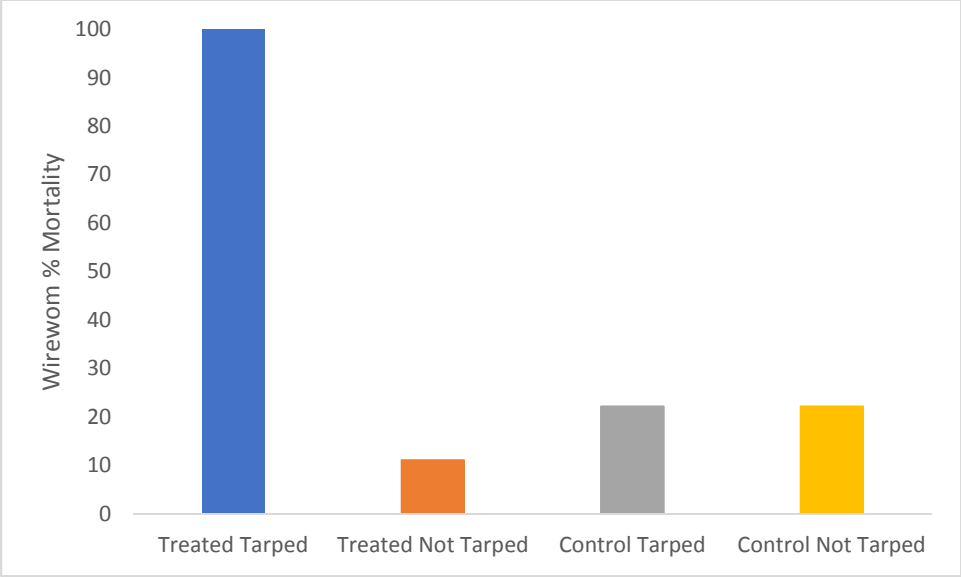


Figure 1.9 Wireworm percent mortality 5-week post treatment. All the mortality in the treated tarped columns occurred within one week post treatment, whereas mortality in the remaining treatments took place over five weeks.

Table 1.23 Mortality over time of wireworms in soil columns from different treatments in the tarping bioassay

Days Post Treatment	Mortality % by Treatment Treated Tarped	Treated Un-Tarped	Control Tarped	Control Un-Tarped
0	0	0	0	0
7	100	0	11.1	22.2
14	100	0	11.1	22.2
21	100	0	11.1	22.2
28	100	0	22.2	22.2
35	100	11.1	22.2	22.2

Table 1.24 Statistical analysis of the effects of treatment on wireworm mortality in tarping assay

Source	Degrees of Freedom	Sum of Squares	Mean Square	F	P
Treatment	3	4.5556	1.5185	12.148	$1.801*10^{-5}$
Residual	32	4.0000	0.1250	-	-
Total	35	8.5556	-	-	-

Table 1.25 Tukey multiple comparison of means test for wireworm mortality between treatments in the tarping bioassay. "CT", "CNT", "TT", and "TNT" indicate control tarped, control un-tarped, treated tarped, and treated un-tarped columns respectively.

Comparison	Estimate	Stdandard Error	t-value	P
CT - CNT	8.547e-17	1.667e-01	0.000	1.000000
TNT - CNT	-1.111e-01	1.667e-01	-0.667	0.908838
TT - CNT	7.778e-01	1.667e-01	4.667	0.000323
TNT - CT	-1.111e-01	1.667e-01	-0.667	0.908842
TT - CT	7.778e-01	1.667e-01	4.667	0.000302
TT - TNT	8.889e-01	1.667e-01	5.333	< 1e-04

Table 1.26 limit of detection of sulfate, sinigrin, and allyl isothiocyanate in bioassays

Assay	Treatment	SO_4 (µmol g^{-1})	Sinigrin (µmol g^{-1})	allyl isothiocyanate (µmol g^{-1})
Vertical Isolation	Control	0.192	0.108	3.19×10^{-5}
	Treated	0.078	0.108	3.19×10^{-5}
Tarping	Control	-	-	2.40×10^{-5}
	Treated	-	-	2.40×10^{-5}

Tarping Bioassay: allyl isothiocyanate Concentration

Due to the destructive sampling of soil columns for wireworm extraction, the measurement of allyl isothiocyanate in the same columns was not feasible as most, if not all, of the allyl isothiocyanate in the soil had volatilized. Therefore, separate columns which did not contain wireworms were prepared for the sole purpose of studying allyl isothiocyanate concentration. These columns were not destructively sampled to avoid allyl isothiocyanate volatilization. The concentration of allyl isothiocyanate in all the columns was below the limit of detection (Table 1.26). This was expected in the control columns since no seed meal was applied. As previously stated, allyl isothiocyanate can have a relatively short half-life in soil so much of the allyl isothiocyanate loss may have been due to degradation over time (Zhu *et al.* 2020). In the treated un-tarped columns loss may have also been due to volatilization as well. It is also possible that allyl isothiocyanate was lost during extraction. Although the columns were flash frozen between steps to minimize allyl isothiocyanate volatilization, the soil was crushed in order to adequately homogenize it and allyl isothiocyanate may have been released.

Summary

The objectives of this study were to determine whether a simple bioassay which used wireworms as bioindicators could be used to assess the fate of allyl isothiocyanate in soil columns and study alternative indicators of allyl isothiocyanate production which could be measured in-situ. In the vertical isolation assay, neither treatment, soil depth, incubation period, nor a combination of these variables had a significant effect on wireworm mortality when observed immediately after the wireworms were removed from the columns. This would suggest that these variables may not be effective indicators for the fate of allyl isothiocyanate. However, measuring initial mortality rather than long term mortality of the wireworms may not have been an accurate measure of the effects of allyl isothiocyanate in wireworm mortality. Previous research has shown that wireworms exposed to allyl isothiocyanate may not die for several weeks after exposure (Williams *et al.* 1993) and wireworms in the tarping assay did not show any mortality until one week post treatment.

The lack of mortality in the treated columns from the vertical isolation assay may have also been due to allyl isothiocyanate volatilization. In the tarping assay, wireworm mortality in the tarped columns treated with *B. juncea* seed meal was significantly higher than any of the other treatments and mortality was not significantly different between the un-tarped columns treated with *B. juncea* seed meal and the control columns. These results indicate that soil tarping may be an effective method for reducing allyl isothiocyanate volatilization and increasing the efficacy of *B. juncea* seed meal against wireworms, and also that allyl isothiocyanate volatilization may considerably reduce the efficacy of the meal or completely negate its effects on wireworm mortality. None of the columns in the vertical

isolation assay were tarped, so much of the allyl isothiocyanate in the treated columns may have volatilized out of the soil, reducing its effects on wireworm mortality.

In the vertical isolation assay, wireworms in the control columns which were incubated for four days gained significantly more weight than the wireworms in the control columns which were incubated for eight days. On average, the wireworms in the control columns which were incubated for eight days lost weight. The wireworms in the treated columns which were incubated for eight days gained significantly more mass or lost less mass than the wireworms in the control columns which were incubated for eight days. These discrepancies in mass change may be due to availability of SOM for consumption, rather than the present of allyl isothiocyanate. External food sources were not provided for the duration of the experiment; however, multiple wireworms in both the control and treated columns gained weight during the experiment. The wireworms may have gained weight by feeding on SOM which was present in the soil. Since, on average, wireworms in the control columns incubated for eight days lost weight and the those in the control columns which were incubated for four days gained weight, the SOM available for consumption may have been depleted between four- and eight-days post seed meal application. Wireworms in the treated columns which were incubated for eight days may have fed on detoxified seed meal which leached into the soil, causing them to gain weight on average.

In the vertical isolation assay the concentration of sulfate in the treated columns incubated for four and eight days was significantly higher than the concentration in the control columns. This was expected since sulfate is a primary glucosinolate hydrolysis product and indicates that glucosinolates in the meal were hydrolyzed. The concentration of sulfate in the treated columns incubated for eight days was significantly higher than the

concentration in the treated columns which were only incubated for four days which suggests that glucosinolate hydrolysis may occur more than four days after the seed meal was saturated. In both the vertical isolation and tarping assays, the soil electrical conductivity was higher in treated columns than in the control columns. This is likely due to the release of ionic products from glucosinolate hydrolysis as well as ionic compounds from the seed meal itself. In the vertical isolation assay, the EC form 0-5-cm in the treated soil which was incubated for four days was significantly higher than the EC at any other depth in any of the columns, and the EC form 10-20-cm in the columns which were incubated for four days was much lower and was not significantly different from the EC at several depths in the control columns. In the treated columns incubated for eight days, the EC was not significantly different between depths and was significantly higher than the EC at all depths in the control columns. This indicates that ions were still concentrated at the surface of the soil four days after the meal was saturated but leached through the soil and were equally distributed throughout the column within eight days after the seed meal was saturated.

In both the vertical isolation and tarping assays, the soil pH was significantly lower in the columns which were treated with seed meal than in the control columns. The low pH in the treated columns is likely due to the release of H^+ from glucosinolate hydrolysis as well as the deprotonation of uric and phytic acid in the seed meal, both which had a pKa which is lower than the pH of the Bannock loam used in the experiment (Wu, Zhou, and Xu 2009; Anubhuti Sharma *et al.* 2019; Costello, Glonek, and Myers 1976; Hediger 2004). In the tarping assay, the treated tarped columns had a significantly lower pH than the treated un-tarped columns. Loss of moisture in the un-tarped columns due to increased evaporation may have reduced the infiltration of H^+ from the seed meal so that there was less of a reduction in

soil pH compared to the treated tarped columns. Additionally, dissolution of CO_2 into soil solution may have increased acidification in the tarped columns (Strawn, Bohn, and O'Connor 2019). Although the decrease in pH was significant, the soil would likely buffer against large decreases in pH overtime so farmers may not require lime to restore the soil pH to its natural level.

For the tarping assay, the depth of the wireworms in the columns was not significantly different between treatments. This suggests either that the concentration of allyl isothiocyanate or other toxic compounds from the meal was low enough in the leachate that it did not significantly affect wireworm movement, or, unlike B. *napus* seed meal, wireworms do not move away from compounds released by *B. juncea* seed meal (Brown, Morra, and Borek 1991). The concentration of allyl isothiocyanate in the soil in both assays, as well as the concentration of sinigrin in the soil in the vertical isolation assay, were below the limit of detection (Table 1.26). allyl isothiocyanate has been shown to have a short half-life in soil and may have degraded prior to extraction and analysis, or it may have volatilized out during extraction (Zhu *et al.* 2020; Torrijos *et al.* 2019). The low concentration of sinigrin in the soil in the vertical isolation assay suggests that all the sinigrin was hydrolyzed prior to analysis. Since the concentration of sinigrin was below the limit of detection (Table 1.26) after both four and eight days, the increase in sulfate between four and eight days in the treated columns may be due to hydrolysis of other glucosinolates which were present in the seed meal.

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Chapter 2: Kinetics of Myrosinase Isoenzymes from *B. juncea*, *S. alba*, and *L. alba* with Sinigrin, Sinalbin, and Glucolimnanthin Substrates

Introduction

Thioglucoside glucohydrolase, commonly known as myrosinase, is a plant enzyme found in tissues of glucosinolate producing plants which catalyzes the hydrolysis of glucosinolates (Kjær 1960). Myrosinase is composed of two subunits which range in molecular weight from 60 to 70 kDa (Pessina *et al.* 1990). These subunits form a dimer which is stabilized by a Zn^{2+} ion, is heavily glycosylated, and contains a hydrophobic pocket which is ideally situated for binding of the hydrophobic sidechain of glucosinolates and two arginine residues positioned for interaction with the sulfate group of the substrate (Burmeister *et al.* 1997). During hydrolysis, myrosinase first breaks the thioglucoside linkage in the glucosinolate which releases glucose and an unstable aglycone in the presence of water. The unstable aglycone undergoes a series of rearrangements to produce products such as isothiocyanates, nitriles, thiocyanates, epithionitriles, and oxazolidines (Cole 1976; Fenwick and Heaney 1983; Sønderby, Geu-Flores and Halkie 2010; Wood 2018). Myrosinase from *B. juncea* and *S. alba* have an optimal pH of 5.9 and 6.5 respectively; however, the optimal pH is highly dependent on the source of myrosinase and has been recorded as high as 8.0 for white and red cabbage (Van Eylen *et al.* 2008; Park *et al.* 1994; Yen and Wei 1993). The optimal temperature for myrosinase activity also differs considerably between myrosinase isoenzymes; however, 37 ^{o}C is often used for activity assays (Van Eylen *et al.* 2008; Palmieri, Leoni, and Iori 1982).

Myrosinase activity is increased by L-ascorbic acid which increases activity through uncompetitive activation. However, L-ascorbic acid can inhibit myrosinase activity at higher

concentrations (Shikita *et al.* 1999; Román, Castillo, and Mahn 2018). Activity is also inhibited by sulfate and glucose which are hydrolysis products of glucosinolates (Bhat *et al.* 2015; Shikita *et al.* 1999). Sulfate inhibits myrosinase activity by competing with glucosinolates for the binding site, causing competitive inhibition which can lower the binding affinity of myrosinase for the substrate, but will not affect its maximum activity (Engelking 2015; Shikita *et al.* 1999). Finally, myrosinase has also been shown to be inhibited by fluorinated glucosinolates (Cottaz, Rollin, and Driguez 1997). However, fluorinated glucosinolates are not found in nature and would not be an obvious source for inhibition under natural conditions.

To maximize the efficacy of Brassica seed meal biopesticides and reduce application time and requirements it is critical to increase the concentration of glucosinolates in the mixtures that are applied and maximize the conversion of glucosinolates to isothiocyanates. The former can be accomplished using seed meal extract. Brassica seed meal can be extracted at a benchtop scale using 73% methanol, then concentrated by evaporation and dried by lyophilization (Popova, Dubie, and Morra 2017). The concentration of glucosinolates in the extract is significantly higher than in unextracted meal and application rates can be as low as half of the rate required to achieve the same results using meal (Popova, Dubie, and Morra 2017). However, myrosinase is quickly inactivated at temperatures above 65 ^{o}C, so the native myrosinase in the extract is inactivated during the evaporation step (Farhana, Aripin, and Surugau 2016). Therefore, unextracted seed meal must be added in conjunction with the meal extract to provide a source of myrosinase (Dandurand *et al.* 2017). Typically, mustard meal of the same species is added to the extract to provide myrosinase (Popova, Dubie, and Morra 2017). However, as stated before,

myrosinase isoenzymes differ between Brassica species and the activity as well as the optimal conditions for each enzyme can vary considerably. Therefore, it is possible that the combination of meal extract with meal of the same species may not be optimal for the conversion of glucosinolates to isothiocyanates.

The objective of this research was to determine whether myrosinase from *Sinapis alba*, *Limnanthes alba*, or *Brassica juncea* has greater specific activity for sinigrin (2-propenyl glucosinolate), sinalbin (4-hydroxybenzyl glucosinolate), or glucolimnanthin (4-methoxybenzyl glucosinolate). The hydrolysis products of these glucosinolates have been shown to have biocidal effects and increasing their hydrolysis may increase their efficacy in the field (Yu *et al.* 2007; Hansson *et al.* 2008; Intanon *et al.* 2014). As previously discussed, Sinigrin is a natural, aliphatic glucosinolate that is the primary glucosinolate found in *B. juncea* (Sun *et al.* 2019; Mazumder, Dwivedi, and Plessis 2016). Sinigrin contains a 2-propenyl side chain and is hydrolyzed by myrosinase to allyl isothiocyanate which has a wide range of biocidal effects (Ettlinger and Lundeen 1956; Zhu *et al.* 2020). Sinalbin is an aromatic glucosinolate which is the primary glucosinolate found in *S. alba* (Pihakaski and Pihakaski 1978). It contains a 4-hydroxybenzyl side chain and hydrolyzes to 4-hydroxybenzyl isothiocyanate which is unstable in aqueous solutions and degrades to thiocyanate which is a bioherbicidal compound (Borek and Morra 2005; Hansson *et al.* 2008). Glucolimnanthin is an aromatic glucosinolate which is the principle glucosinolate in *L. alba* (Intanon *et al.* 2014). It is hydrolyzed to 3-methoxybenzyl isothiocyanate which is an effective treatment for a variety of soilborne pathogens (Zasada *et al.* 2012).

Materials and Methods

Myrosinase Extraction

Myrosinase crude extract was obtained from meadowfoam (*L. alba*), white mustard (*S. alba*), and yellow mustard (*B. juncea*) seed meal. Crude myrosinase was extracted from the meal using methods similar to those described by Palmieri, Iori and Leoni (1986). Two-hundred g of seed meal was ground in a coffee grinder, then combined with two liters of deionized water and left to sit overnight at room temperature to detoxify the meal. The aqueous extract was gravimetrically filtered from the mixture, then dialyzed against deionized water for 24 hours to precipitate any insoluble proteins using Spectra/Por molecular porous membrane tubing from Fisher Scientific which selectively filtered out proteins with masses of 12-14kD. The remaining solution was filtered to remove the precipitant, then frozen at -20 ºC and lyophilized. The lyophilized extracts were stored at -20 ºC for future use. The enzyme solutions which were used for the assays were prepared by mixing approximately 0.1 g of crude extract with 8mL of deionized water. The mixtures were shaken on a reciprocal shaker for 30 min to fully dissolve the protein, then stored at -20 ºC for future use.

Soluble Protein and Sulfate Quantification in Enzyme Extracts

The concentration of soluble protein in the undialyzed aqueous meal mixture and the concentration of soluble protein in the final crude extract were quantified using a Bradford assay kit from Thermo Scientific. The solutions were diluted 10-fold, then 30µL of each solution was combined with 1.5mL of Coomassie blue stain in a polypropylene cuvette. The solutions in the cuvettes were mixed by pipetting, then left to sit at ambient temperature for 10 min to allow the color to develop. This process was repeated with ten standard solutions of bovine serum albumin which were used to generate a standard curve. The standard solutions were not diluted prior to mixing with the Coomassie blue stain. The absorbances of

the solutions were measured at 595nm using a Genesys 10S UV/vis spectrophotometer from Thermo Scientific. To quantify the sulfate concentration in the enzyme solutions, 200 µL of each solution was transferred to 1.5mL polypropylene Eppendorf tubes which were heated in a water bath at 100 ^{o}C for 10 min to denature the protein and remove any sulfate which may be bound to the active site. The extract was then filtered through a 0.42µm syringe driven filter then diluted 10-fold and analyzed for sulfate concentration with a Dionex Aquion ion chromatography system with a Dionex AS-AP autosampler and an ADRS 4mm suppressor. Chromatographic separation was conducted with a 4x210mm Ion-Pac AS16 with an AG16 guard column. The flow rate was 0.9mL/minute and the mobile phase was 33mM NaOH. Anion suppressor current was set to 82mA and the injection volume was 25µL. Run time was 7 min. The results were compared against a standard curve which was generated using K_2SO_4 stock solutions prepared using reagent grade K_2SO_4 from Ward's Science +, ON, Canada. All measurements were conducted in triplicates.

Glucosinolate Extraction and Crystallization

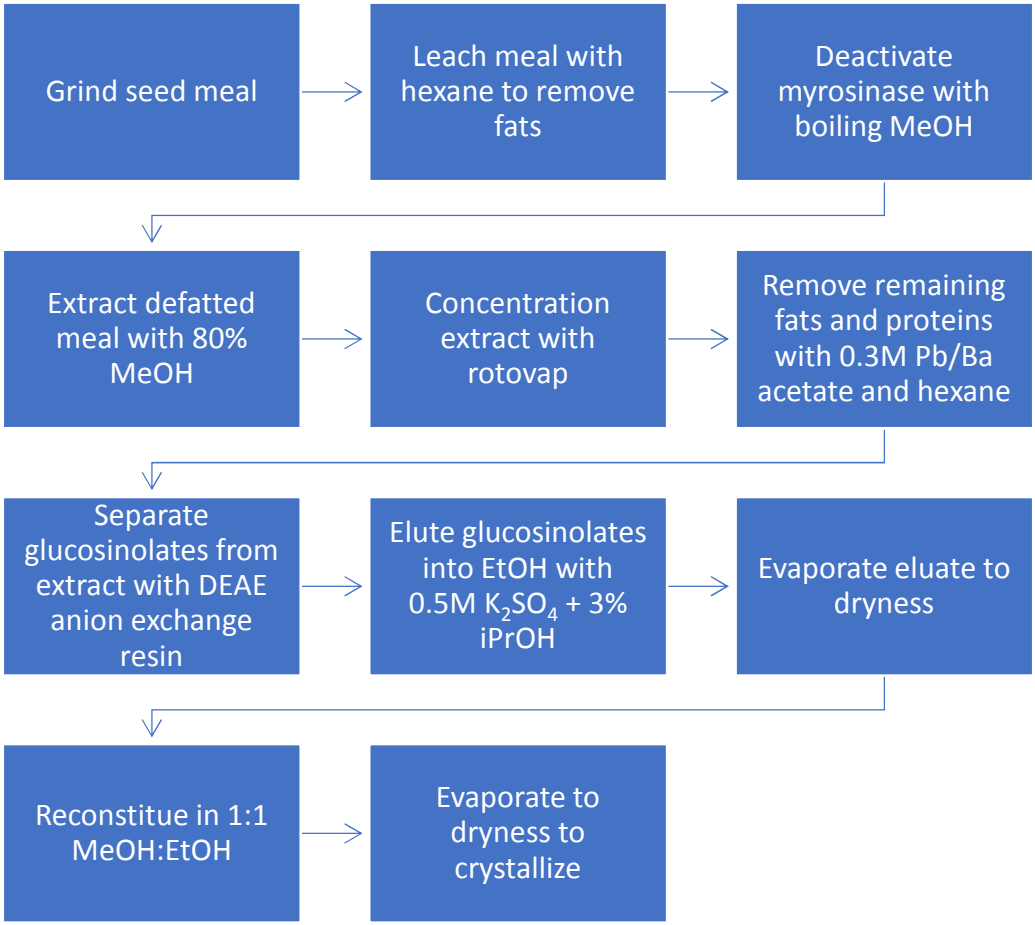

Figure 2.1 Scheme for extraction and crystallization of glucosinolates from Brassica seed meal

For the enzymatic kinetic assay, glucolimnanthin, sinigrin, and sinalbin were extracted from Brassica seed meal using a modified form of the method described by Thies (1988). Pacific Gold (*B. juncea*), Ida Gold (*S. alba*), and meadowfoam (*L. alba*) seed meal were extracted for sinigrin, sinalbin, and glucolimnanthin respectively. One hundred and 50 g of ground seed meal were combined into a Buchner funnel. Fisherbrand P8-creped filter paper was placed in the funnels to filter the extract. The Buchner funnel was leached with approximately 400mL of hexane to remove fats and oils from the meal. Once the hexane

ceased to leach through the meal gravimetrically, suction was applied for 30 min to remove the remaining hexane. Afterwards the hexane fractions were discarded. The dried meal was soaked in 160mL of boiling methanol to deactivate the myrosinase in the meal to prevent the glucosinolates from being hydrolyzed. Suction was applied to the meal to remove the methanol, then 5x100mL of 80% methanol was added sequentially to the funnel to leach the soluble glucosinolates. The methanol solution was allowed to drain gravimetrically, then suction was applied to remove the remaining methanol and water.

Once the meal had been leached with 500mL of 80% methanol the meal was discarded. The extract from the Buchner funnel was decanted into a 2L round bottom flask and evaporated to approximately 80mL using a rotary evaporator. The remaining extract was divided equally into two 50mL centrifuge tubes. Three mL of 0.3M Pb/Ba acetate was added to each of the tubes which were briefly shaken, then stored at -20 ^{o}C for 15 min to precipitate insoluble proteins. After cooling, the tubes were centrifuged at 4000rpm for 15 min to separate the insoluble proteins. Three milliliters of hexane were added to each of the tubes which were shaken, then stored at -20 ^{o}C to separate any remaining fats and oils. The organic layer was removed and discarded. The extract was then transferred to two different columns each containing 3.5 g of DEAE anion exchange resin. Half of the extract was transferred to each of the two columns. The flow through was discarded and the columns were washed with 2x30mL of deionized water. Next, the columns were leached with 3x20mL of a formic acid: isopropanol: water solution (3:2:5) to remove phenolics. Afterwards, the columns were leached with 2x25mL of deionized water and the aqueous fraction was discarded. The glucosinolates were then converted to a potassium salt by leaching with 16mL of a 0.5M K_2SO_4, 3% isopropanol solution into 50mL centrifuge tubes which contained 12.5mL of

ethanol. This was performed twice for each column so a total of 32mL was passed through each. DEAE which had only been used once was rejuvenated by sequentially adding 4mL of 30% v/v formic acid, 8mL of DI water, 4mL of 0.5M NaOH, 8mL of water, 4mL of 0.5 HCl, and finally washing with an additional 8mL of DI. The rejuvenated resin was used for additional extractions. The fractions were cooled at -20 ^{o}C for 15 min, then centrifuged at 4000rpm for 15 min to separate the insoluble salts.

The supernatant was decanted into a 2L round bottom flask and evaporated to dryness with a rotary evaporator. The soluble glucosinolates were then reconstituted in 60mL of methanol and cooled at 4C to precipitate any remaining insoluble salts. The mixture was filtered through a 0.42µm syringe driven filter and the previous two steps were repeated until no insoluble salts remained. The mixture was reconstituted in 40mL of methanol and 20mL of ethanol was added to crystallize the glucosinolates. The solution was then evaporated to dryness. The flask was cooled at -20 ^{o}C for 15 min, then the crystals were removed from the flask and transferred to a 15mL amber glass vial and stored at -20 ^{o}C for future analysis.

Glucosinolate Extract Analysis

Glucosinolate extracts were analyzed for their percent concentration by mass of glucosinolates, sulfate, and glucose and were analyzed for carbon fluorine bonds using a Nicolet is10 FTIR from Thermo Scientific. Five mM concentrations of the glucosinolate extract were prepared assuming 100% purity. These extracts were compared to pure sinigrin standards with concentrations of 5, 2.5, 1.25, 0.625, and 0.3125 mM to determine the percent w/w purity of the extracts. The glucosinolate extract solution were analyzed using the same methods described in chapter 1. Response factors of 0.5 (Buchner 1987), and 0.815 relative to sinigrin were used for sinalbin and glucolimnanthin respectively. The response factor for

glucolimnanthin was determined by comparing the response of five glucolimnanthin standards, 1, 0.5, 0.25, 0.125, and 0.0625mM to the response from five sinigrin standards of the same concentration. Sulfate concentration was quantified using the ion chromatography methods previously described. Seven mM concentrations of the glucosinolate extracts were prepared, accounting for the purity, then diluted ten-fold in deionized water. Analysis was conducted in triplicates for each extract. A standard curve for sulfate was generated using sulfate standards which were prepared with reagent grade potassium sulfate from Ward's Science, ON, Canada. Finally, the extracts were analyzed for glucose concentration using a glucose oxidase reagent set from Teco Diagnostics, 1268 N. Lakeview Ave., Anaheim, CA 92807. Three milliliters of the reagent, consisting of 15µL/mL glucose oxidase, 1.2µL/mL horseradish peroxidase, 4.0µL/mL mutarose, 0.38mM 4-aminoantipyrine, and 10mM benzene sulfonate, were combined with 100µL of 0.5mM solutions of each extract and heated in a water bath at 50 ºC for five min. Immediately after heating the absorbance of each solution was measured at 500nm using a Genesys 10S UV/vis spectrophotometer from Thermo Scientific. These values were compared to a standard curve with concentrations ranging from 25 to 1000ppm.

Seed Meal Glucosinolate Analysis

The *Limanthes alba*, *S. alba*, and *B. juncea* seed meals were analyzed for glucosinolate concentrations using the methods described by Popova and Morra (2014). Seed meal was ground in a coffee grinder, then approximately 0.1 g of the meal was combined with 5.5mL of 73% methanol and shaken on a reciprocal shaker for one hour. The extracts were then centrifuged to separate out insoluble matter. One milliliter of the supernatant from each extract was desulfated, and analyzed via high performance liquid chromatography using the

method previously described (Popova and Morra 2014). Analysis of each seed meal was conducted with three biological replicates to ensure accuracy.

Enzymatic Kinetics Assay

Between six and nine concentrations of substrate ranging from 0.14 to 7mM in 33mM phosphate buffer (pH=6.5) were used to assess the enzymatic kinetics of the different myrosinase isoenzymes with sinigrin, sinalbin, and glucolimnanthin substrates. The assays were conducted in 1mL solutions. Myrosinase extracts were thawed, then left at room temperature until the solution was no longer turbid so the proteins which precipitated out of the solution at lower temperatures would dissolve back into the solution. Fifty microliters of the crude enzyme extract solution were added to each of the samples to initiate the reaction. The solutions were inverted several times to mix, then 400µL of each solution was immediately removed and combined with 800µL of boiling methanol to deactivate the enzyme and quench the reaction. The samples were then stored at -20 ^{o}C for future analysis. The remaining solutions were incubated at 25 ^{o}C for two and a half hours, then quenched using the same method as before. The entirety of each of the quenched solutions were then desulphated using the methods described in chapter 1 and the glucosinolate concentrations were quantified with an Agilent 1200 series high performance liquid chromatography with a Zorbax extend C-18 column using the same parameters that were used for quantifying the purity of the glucosinolate extracts. Activity was determined by subtracting the final concentration of substrate from the initial concentration then dividing by the duration of the assay, in min, as well as the mass of protein used in each sample in mg. Enzymatic units were defined as the conversion of one µmol of substrate per minute. All tests were conducted in triplicates.

Determination of Enzymatic Kinetic Parameters

Enzymatic parameters for *S. alba* myrosinase and *B. juncea* myrosinase were calculated using the Michaelis Menten equation (MM), (Michaelis and Menten 1913),

$$v = V_{\max} * \frac{[S]}{K_m + [S]} \quad (1)$$

where v indicates the initial enzymatic activity, Vmax is the maximum enzymatic activity, Km is the dissociation constant which is equal to the concentration of substrate at one half of the maximum enzymatic activity. K_m is inversely related to the binding affinity constant; therefore a low K_m indicates high binding affinity(Haldane 1928). [S] is the concentration of substrate in the solution. Enzymatic parameters for *B. juncea* with sinalbin substrates were also calculated using the two binding site model (TBSM), equation two, from Lin *et al.* (2001) and the Michaelis Menten substrate inhibition model (MMSI), equation three.

$$v = V_{\max} * \frac{\left(\frac{1}{K_s} + \beta * \frac{[S]}{\alpha * K_i * K_s}\right)}{\frac{1}{[S]} + \frac{1}{K_s} + \frac{1}{K_i} + \frac{[S]}{\alpha * K_i * K_s}} \quad (2)$$

$$v = V_{\max} * \frac{[S]}{K_m + [S] + \frac{[S]^2}{K_i}} \quad (3)$$

In equation two v, Vmax, and [S] are the same parameters described in the Michaelis Menten equation and K_s is equivalent to K_m (Eun 1996). K_S and K_i are the dissociation constants of a substrate molecule from the catalytic and inhibitory sites respectively. Like K_m and K_s, K_i is inversely related to the binding affinity constant so a lower K_s indicates higher binding affinity for the inhibitory site (Eun 1996). β is the activity reduction factor used when the inhibitory site is saturated with substrate and ranges from 0 to 1, and α is an equilibrium

adjustment factor which is used when a second substrate molecule is bound (Lin *et al.* 2001). In equation three K_m, Vmax, [S], and K_i represent the same constants described in the previous two models. The mechanisms for each model are shown below:

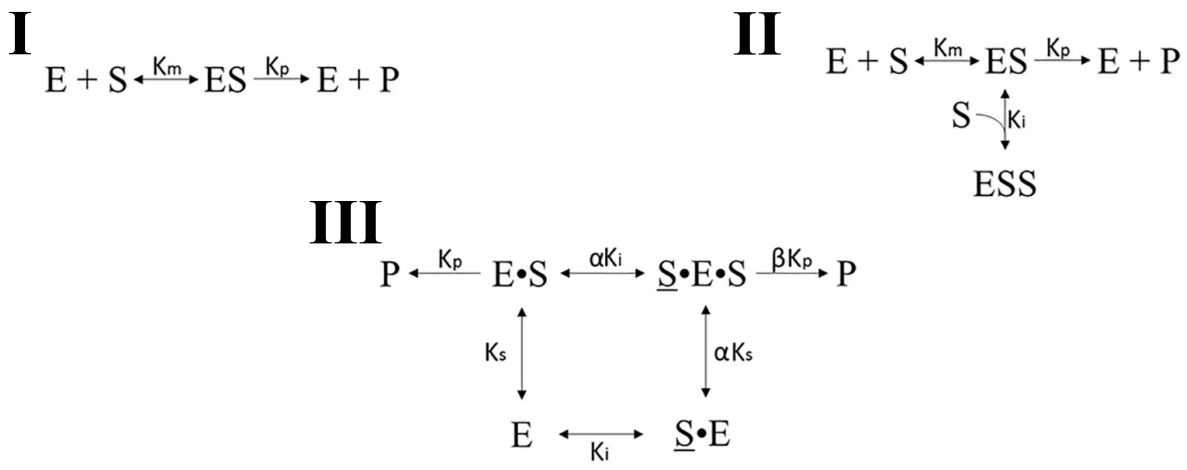

Figure (I) illustrates the mechanism described by the MM equation. In this model, the enzyme ,E, reacts with the substrate, S, to form an ES complex which converts to an EP complex that dissociates into enzyme and product, P. Figure (II) shows the mechanism for MM substrate inhibition. In this case, E and S still form an ES complex which can dissociate into enzyme and product; however, the ES complex can be inhibited from generating product by the binding of another substrate molecule which generates an ESS complex. Finally, figure (III) shows the mechanism described by the TBSM. In the model, S*E represents the enzyme substrate complex where the substrate is bound to the allosteric site and E*S represent the complex where the substrate is bound to the active site (Juan Román *et al.* 2018). In the TBSM, the substrate can bind to either the active site or allosteric site independently and may still generate product from an S*E*S complex (Lin *et al.* 2001; Miners, MacKenzie, and Knights 2010; Wu 2011). Approximate enzymatic activities were predicted as a function of [S] using the above constants as fitting parameters. Microsoft excel

solver was used to determine the values for these parameters which best fit the observed activities. Mean activities from each substrate concentration were compared using a Tukey multiple comparisons of means test in R to determine significant differences between substrate concentrations (α=0.05).

Results and Discussion

Myrosinase Extract Protein and Sulfate Concentration

Approximately 3.0, 1.2, and 3.2 g of crude myrosinase extract were obtained from the initial 200 g of *B. juncea*, *S. alba,* and *L. alba* respectively. Two enzyme solutions were used for *B. juncea* myrosinase analysis and one solution was used for the *L. alba* and *S. alba* studies each. Two solutions were used for *B. juncea* because the initial 8-mL was depleted before the experiment was finished due to the numerous trials needed to develop the assay methods. The extract used to make the second solution was the same extract used to make the first solution and had been thoroughly homogenized. The undialyzed, aqueous meal mixtures had soluble protein concentrations of 5.31 ± 0.0896, 3.81 ± 0.0913, and 1.26 ± 0.0376 mg mL^{-1} for the *B. juncea*, *L. alba*, and *S. alba* which equates to approximately 5.30%, 3.80%, and 1.30% soluble protein by mass respectively. The enzyme solutions used for the assay had concentrations of approximately 12.8, 13.0, 13.5 and 13.0 mg of crude extract per mL for the *S. alba*, *L. alba,* and the first and second *B. juncea* solutions respectively. The Bradford assay results showed soluble proteins concentrations of 0.640 ± 0.0130, 0.900 ± 0.0140, 2.60 ± 0.0240 and 1.92 ± 0.0530 mg mL^{-1} in the *S. alba*, *L. alba*, and first and second *B. juncea* solutions respectively, which equates to approximately 5.1%, 6.9%, 19% and 15% soluble protein by mass (Table 2.1). This demonstrates a considerable increase in the soluble protein concentrations for both the *B. juncea* extracts and the *L. alba* extracts which increased by factors of 3.6, 2.8 and 5.3 for the first and second *B. juncea* extracts and the *L. alba* extracts respectively, and a limited increase in the concentration of soluble protein in the *S. alba* extract which only increased by a factor of 1.4. However, the soluble protein may not be pure myrosinase and the true concentrations of myrosinase may differ from the results. Activities were normalized using soluble protein concentration to increase the accuracy of the

comparisons. Glucose concentration in the enzyme solutions was not quantified because it is a weak inhibitor and the maximum concentration of glucose in the solution, assuming all the remaining mass of the extract was glucose, would be too low to cause significant inhibition (Bhat *et al.* 2015; Shikita *et al.* 1999). The *S. alba*, *L. alba*, and first and second *B. juncea* extracts had sulfate concentrations by mass of 4.80 ± 0.100%, 1.30 ± 0.0370%, 2.70 ± 0.0380% and 2.40 ± 0.0450% respectively (Table 2.1).

Glucosinolate Extract Analysis

Extraction of glucosinolates was initially conducted using the methods described by Thies (Thies, 1988). However, these methods did not produce glucosinolate crystals, but rather generated a viscous resin which was unstable in deionized water and the glucosinolates which were extracted degraded considerably over time. The methods were modified to increase the yield of glucosinolates and form stable crystals which could be stored for prolonged periods of time. Similar to the results presented by Thies, the crystals formed with the modified method reverted to a waxy resin when stored at room temperature, but were stable when refrigerated (Thies 1988).

The modified extraction methods yielded approximately 0.630 g of crystallized glucolimnanthin extract with 73.5 ± 3.29% purity by mass and 1.3 g of 54.6 ± 0.578% pure sinigrin. Two different sinalbin extracts were used to analyze *B. juncea* activity to study the non-Michaelis Menten activity of the enzyme. The DEAE resin used in the first sinalbin extract had been previously used to extract sinigrin and was rejuvenated. The second extract was eluted through clean resin to eliminate any contaminants from previous extracts which could affect activity. The first extract yielded approximately 1.15 g of extract with 52.9 ± 1.56% purity sinalbin by mass and the second extract yielded approximately 1.40 g of 64.6 ±

2.54% pure sinalbin (Table 2.2). The second sinalbin extract was used to measure extract for both the *L. alba* and *S. alba* isoenzymes. The concentration of glucosinolates in the unextracted *B. juncea*, *S. alba*, and *L. alba* seed meals were approximately 120 ± 1.68, 100 ± 3.64, and 116 ± 10.5 μmol g^{-1} (Table 2.3). Previous research has reported concentrations of sinalbin in *S. alba* seed meal ranging from 90 to 202 μmol g^{-1} of defatted seed meal (Morra, Popova, and Boydston 2018). Other studies have documented sinigrin concentrations in *B. juncea* seed meal ranging from 128.8 to 139.5 μmol g^{-1} of defatted seed meal and glucolimnanthin concentrations in *L. alba* ranging from 45.5 to 91.0 μmol g^{-1} of defatted meal (Hebert, Mhemdi, and Vorobiev 2020; Intanon *et al.* 2014). The concentration of glucolimnanthin in the *L. alba* and sinigrin in *B. juncea* meals are slightly greater than previously reported data and the concentration of sinalbin in *S. alba* meal is within the range of previous analyses.

The measured concentration of glucosinolates in the extract equates to maximum yields of approximately 7.13, 6.97, and 8.32 g of sinigrin, sinalbin, and glucolimnanthin respectively from the 150 g of meal used for each extract. The yields for the sinigrin, glucolimnanthin, and the first and second sinalbin extracts represent 9.94%, 5.30%, 8.73% and 13.0% of the maximum yield respectively. The increase in yield between the first and second sinalbin extracts suggests that rejuvenating and reusing DEAE may lower the anion exchange capacity of the resin, reducing its efficacy. Previous research has shown that only 50% of the glucosinolates in the meal can be extracted using similar methods (Thies 1988). Therefore, the percentage of the possible yield is twice that listed above; however, the difference between possible yield and actual yield still differs by approximately 74% to 89.4%.

The low yields may have been due to thermal degradation of the glucosinolates during extraction. Prior research has shown that many glucosinolates, such as sinigrin, degrade rapidly at temperatures greater than 100 ^{o}C (Oerlemans *et al.* 2006). During the first evaporation step immediately following extraction with methanol, the solution was heated to approximately 70 ^{o}C which was sufficient to evaporate the majority of the solution and concentrate it to a final volume of approximately 80mL within 30 min. Due to the low temperature and brief exposure to heat, it is unlikely that many of the glucosinolates were degraded during this step. In the final evaporation step, there still remained a significant amount of potassium sulfate in the solution from the DEAE elution. As the sample was evaporated, the concentration of the salt in the solution increased and the colligative effects of the salt on the boiling point increased as well (Chinard 1955). Therefore, the boiling point of the solution increased as the volume decreased, so the temperature of the water bath was raised above 100 ^{o}C to evaporate the solution to dryness. This step took several hours and the prolonged heating of the extracts above 100 ^{o}C may have degraded the glucosinolates.

The concentration of glucose in the extracts was below the limit of detection and glucose was likely separated during the crystallization steps. Each extraction typically required 3-5 repetitions of the methanol/ethanol dissolution and crystallization step to fully crystalize the extract. During these steps precipitate which was formed during the prior crystallization was dissolved in methanol/ethanol. Precipitate which would not dissolve in the alcohol was filtered out prior to the next evaporation. Glucose is poorly soluble in methanol and ethanol, so glucose which formed from the degradation of the glucosinolates did not redissolve into the alcohol and was filtered out prior to the next crystallization step (Bosch, Fyles, and James 2004).

The sulfate concentrations in these extracts by mass were 0.374 ± 0.0239% and 7.31 ± 0.0195% for glucolimnanthin and sinigrin respectively, and 5.87 ± 0.168% and 6.59 ± 0.0178% for the first and second sinalbin extracts respectively (Table 2.2). The sulfate in the extracts may have been residual sulfate from the DEAE elution or may have been due to thermal degradation of the glucosinolates during the final evaporation steps. Potassium sulfate is insoluble in ethanol and other organic solvents and the presence of methanol and ethanol considerably decrease its solubility in aqueous solution (Mydlarz and Jones 1990). Therefore, sulfate remaining in the dry extract could not have formed inorganic salts with potassium. It is also unlikely that the sulfate measured in the solutions was generated by natural glucosinolate hydrolysis in the aqueous solutions since there was no detectable glucose in the solution. If glucosinolates had hydrolyzed, sulfate and glucose would both be present since both are hydrolysis products (Vaughn and Berhow 2005). Sulfate may have formed organic, alcohol soluble compounds with cationic hydrolysis products or other compounds which were present in the extract, which then degraded to sulfate and its conjugate cation in aqueous solution.

Table 2.1. Concentration of sulfate in myrosinase extracts and soluble protein concentration in extracted and unextracted meals.

Myrosinase Extract	Meal Soluble Protein Concentration (%w/w)	Extract Soluble Protein Concentration (%w/w)	Extract SO_4 Concentration (%w/w)
S. alba	1.26 ± 0.0376	5.05 ± 0.103	4.80 ± 0.104
***B. juncea* (1)**	5.31 ± 0.0896	19.3 ± 0.178	2.68 ± 0.0382
***B. juncea* (2)**	5.31 ± 0.0896	14.8 ± 0.409	2.38 ± 0.0443
L. alba	3.81 ± 0.0913	6.90 ± 0.107	1.33 ± 0.0369

Table 2.2. Concentration of glucosinolates and sulfate in glucosinolate extracts.

Glucosinolate Extract	Mass (g)	Glucosinolate Concentration (%w/w)	SO_4 Concentration (%w/w)
Sinigrin	1.30	54.6 ± 0.578	7.31 ± 0.0195
Sinalbin (1)	1.15	52.9 ± 1.56	5.87 ± 0.168
Sinalbin (2)	1.40	64.6 ± 2.54	6.59 ± 0.0178
Glucolimnanthin	0.630	73.5 ± 3.29	0.374 ± 0.0239

Table 2.3. Concentration of glucosinolates in unextracted seed meal.

Seed Meal Species	Glucosinolate	Concentration of Glucosinolate (µmol g^{-1})
B. juncea	Sinigrin	120 ± 1.68
L. alba	Glucolimnanthin	116 ± 10.5
S. alba	Sinalbin	100 ± 3.64

The data shows the mean value ± standard error about the mean.

Enzymatic Assay

Previous enzyme assays have utilized UV/vis spectrophotometry or direct UV/vis analysis with high performance liquid chromatography to quantify the change in concentration of substrate or hydrolysis products (Palmieri, Iori, and Leoni 1987; Vastenhout *et al.* 2014). In the latter experiment, commercial myrosinase and glucosinolates were used for the experiment which contain few, if any, impurities that may affect the signal and retention time of the glucosinolates and their hydrolysis products. Pure, commercial grade myrosinase and glucosinolates are cost prohibitive, so crude enzyme extracts and crystallized glucosinolate extracts were used, which may have contained phenolics or acids from the meal that may affect both the signal and retention time of the glucosinolates (Dubie *et al.* 2013; Anubhuti Sharma *et al.* 2019). Due to the additional error which may result from these compounds it is impractical to use direct UV/vis analysis with high performance liquid chromatography.

For this experiment, a novel enzymatic assay was developed and used to measure the activity of the different myrosinase isoenzymes. The enzyme solutions were quenched with boiling methanol, then the glucosinolates were desulfated and analyzed with high performance liquid chromatography to quantify the change in concentration of the substrate over time. Desulfation removes cations and neutrally charged compounds such as phenolics as well as anionic compounds such as sulfate and deprotonated acids and isolates glucosinolates from the sample which reduces the effects of compounds in the extract on the signal from the glucosinolates. Previous assays have quenched enzyme activity by heating the enzyme solutions in boiling water or by adding trichloracetic acid to denature or precipitate out the myrosinase (Stoin *et al.* 2009; Björkman and Lönnerdal 1973). However, these methods can skew the results by reducing the concentration of glucosinolates that are

present at the end of the assay or failing to completely denature the enzyme. Glucosinolates are less stable at lower pH and the addition of trichloroacetic acid could degrade the glucosinolates remaining at the end of the assay which could result in an overestimate of enzymatic activity (Jing *et al.* 2012). Increasing the heat of the solution for a prolonged time to denature the enzyme could also cause thermal degradation of the glucosinolates (Oerlemans *et al.* 2006). This may reduce the concentration of glucosinolates in the solution, potentially decreasing the accuracy and precision of the results.

Methanol and other organic solvents decrease myrosinase activity, but do not completely quench enzymatic activity so the addition of methanol alone is insufficient (Botti, Taylor, and Botting 1995). However, myrosinase is heat sensitive and begins to degrade at temperatures greater than 65 ^{o}C (Farhana, Aripin, and Surugau 2016). Since methanol boils at 65 ^{o}C it is ideal for quenching the reaction since a large volume of boiling methanol would be greater than or equal to 65 ^{o}C at atmospheric pressure ("ICSC 0057 - METHANOL" n.d.). The heat of the boiling methanol denatures most of the enzyme, quenching the reaction (Farhana, Aripin, and Surugau 2016). Any myrosinase which is not denatured by the heat will be inactivated, or have significantly reduced activity due to the high concentration of methanol (Crocoll, Halkier, and Burow 2016). Additionally, methanol will decrease in temperature faster than water would due to its lower specific heat capacity ("Heat Capacities for Some Select Substances" n.d.). This will limit thermal degradation of the remaining glucosinolates compared to quenching the reaction with boiling water since the methanol will cool faster and the glucosinolates will be exposed to high temperatures for a shorter period.

Enzymatic Kinetics of S. alba Myrosinase

S. alba myrosinase exhibited classical MM kinetics with all three substrates (Fig. 2.2-2.4). This was confirmed by testing for the difference in mean activity between the different substrate concentrations. According to the MM equation, equation (1), the activity of the enzyme should approach an asymptote at V_{max} as $[S]>>K_m$. Therefore, the enzymatic activities between lower concentrations should differ significantly, but as $[S]>>K_m$ the activities should approach V_{max} and should not be significantly different. A Tukey multiple comparisons of means test was conducted to determine whether the activities at different substrate concentrations were significantly different at a significance level of 0.05. When sinalbin was used as the substrate, enzymatic activities increased significantly from ~0.14 to 2.0mM substrate concentration, and enzymatic activities were not significantly different from approximately 2.0 to 3.1mM. The same pattern was observable with sinigrin and glucolimnanthin as substrates. With sinigrin, enzymatic activity increased significantly from ~0.14 to 1.5mM and activities were not significantly different from 1.5 to ~4.0mM. Finally, with glucolimnanthin the activities increased from ~0.14 to 2.0mM, then did not change significantly from ~2.0 to 7.5mM (Tables 2.4-2.6).

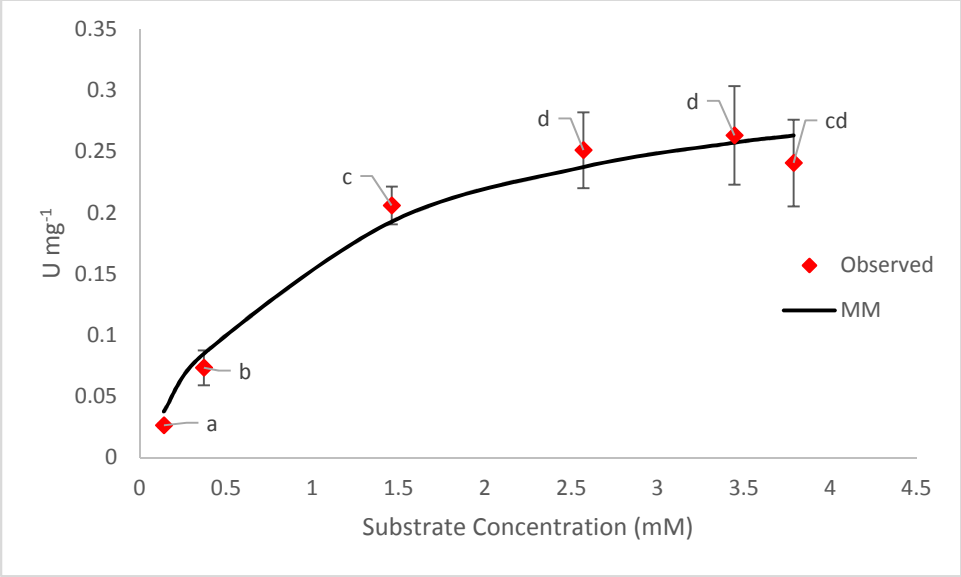

Figure 2.2. Enzymatic activity of *S. alba* myrosinase with sinigrin as a substrate. Red diamonds indicate experimental data, and the black line illustrates activities predicted using the Michaelis Menten equation. Error bars represent a 90% confidence interval calculated using a Student's t distribution. Letters indicate statistically different mean activities (α=0.05)

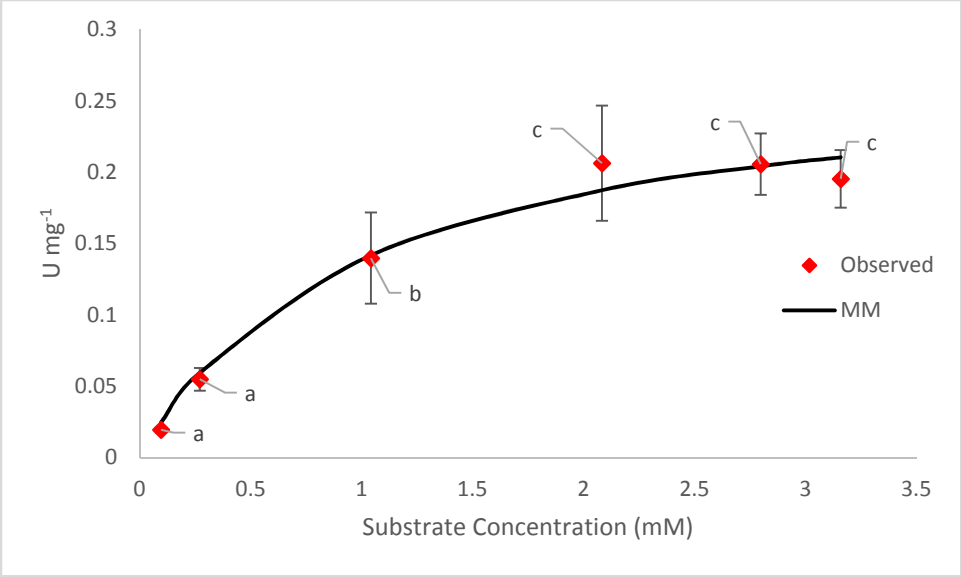

Figure 2.3. Enzymatic activity of *S. alba* myrosinase with sinalbin as substrate. Red diamonds indicate experimental data, and the black line illustrates activities predicted using the Michaelis Menten equation. Error bars represent a 90% confidence interval calculated using a Student's t distribution. Letters indicate statistically different mean activities (α=0.05)

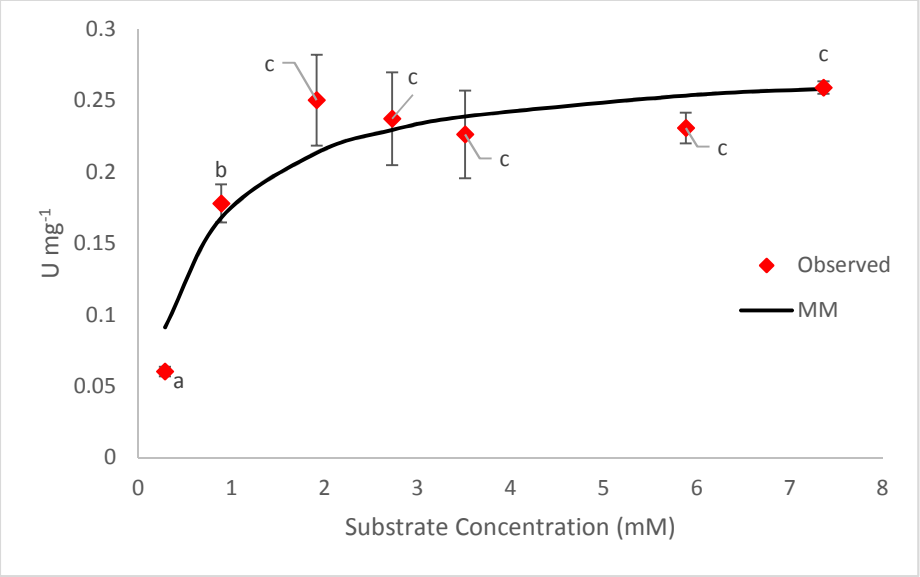

Figure 2.4. Enzymatic activity of *S. alba* myrosinase with glucolimnanthin as a substrate. Red diamonds indicate experimental data, and the black line illustrates activities predicted using the Michaelis Menten equation. Error bars represent a 90% confidence interval calculated using a Student's t distribution. Letters indicate statistically different mean activities (α=0.05)

Table 2.4. Tukey comparison of means test for *S. alba* myrosinase with sinigrin as a substrate. The numbers in the comparison columns indicate activities at different concentrations with 1 being the lowest concentration and 6 being the greatest.

Comparison	Estimate	Standard Error	t-value	P
2 - 1	0.04696	0.01292	3.634	0.03152
3 - 1	0.17966	0.01292	13.901	< 0.001
4 - 1	0.22476	0.01292	17.392	< 0.001
5 - 1	0.23687	0.01292	18.328	< 0.001
6 - 1	0.21440	0.01292	16.590	< 0.001
3 - 2	0.13270	0.01292	10.268	< 0.001
4 - 2	0.17781	0.01292	13.758	< 0.001
5 - 2	0.18991	0.01292	14.694	< 0.001
6 - 2	0.16744	0.01292	12.956	< 0.001
4 - 3	0.04511	0.01292	3.490	0.04012
5 - 3	0.05721	0.01292	4.427	0.00827
6 - 3	0.03474	0.01292	2.688	0.14908
5 - 4	0.01210	0.01292	0.936	0.92913
6 - 4	-0.01036	0.01292	-0.802	0.96174
6 - 5	-0.02246	0.01292	-1.738	0.53447

Table 2.5. Tukey comparison of means test for *S. alba* myrosinase with sinalbin as a substrate. The numbers in the comparison columns indicate activities at different concentrations with 1 being the lowest concentration and 6 being the greatest.

Comparison	Estimate	Standard Error	t-value	P
2 - 1	0.0354420	0.0118368	2.994	0.09141
3 - 1	0.1203336	0.0118368	10.166	< 0.001
4 - 1	0.1868716	0.0118368	15.787	< 0.001
5 - 1	0.1860878	0.0118368	15.721	< 0.001
6 - 1	0.1758169	0.0118368	14.853	< 0.001
3 - 2	0.0848916	0.0118368	7.172	< 0.001
4 - 2	0.1514296	0.0118368	12.793	< 0.001
5 - 2	0.1506459	0.0118368	12.727	< 0.001
6 - 2	0.1403749	0.0118368	11.859	< 0.001
4 - 3	0.0665380	0.0118368	5.621	0.00124
5 - 3	0.0657543	0.0118368	5.555	0.00138
6 - 3	0.0554833	0.0118368	4.687	0.00538
5 - 4	-0.0007838	0.0118368	-0.066	1.00000
6 - 4	-0.0110547	0.0118368	-0.934	0.92986
6 - 5	-0.0102710	0.0118368	-0.868	0.94737

Table 2.6. Tukey comparison of means test for *S. alba* myrosinase with glucolimnanthin as a substrate. The numbers in the comparison columns indicate activities at different concentrations with 1 being the lowest concentration and 7 being the greatest.

Comparison	Estimate	Standard Error	t-value	P
2 - 1	0.117695	0.010563	11.142	< 0.001
3 - 1	0.190069	0.010563	17.994	< 0.001
4 - 1	0.177079	0.010563	16.764	< 0.001
5 - 1	0.166095	0.010563	15.725	< 0.001
6 - 1	0.170612	0.010563	16.152	< 0.001
7 - 1	0.198904	0.010563	18.831	< 0.001
3 - 2	0.072374	0.010563	6.852	< 0.001
4 - 2	0.059384	0.010563	5.622	0.00106
5 - 2	0.048400	0.010563	4.582	0.00602
6 - 2	0.052917	0.010563	5.010	0.00277
7 - 2	0.081209	0.010563	7.688	< 0.001
4 - 3	-0.012990	0.010563	-1.230	0.87124
5 - 3	-0.023974	0.010563	-2.270	0.32193
6 - 3	-0.019457	0.010563	-1.842	0.54411
7 - 3	0.008835	0.010563	0.836	0.97645
5 - 4	-0.010984	0.010563	-1.040	0.93556
6 - 4	-0.006467	0.010563	-0.612	0.99527
7 - 4	0.021825	0.010563	2.066	0.41997
6 - 5	0.004517	0.010563	0.428	0.99935
7 - 5	0.032809	0.010563	3.106	0.08607
7 - 6	0.028292	0.010563	2.678	0.17500

The Vmax and K_m of the enzyme with sinigrin, sinalbin, and glucolimnanthin respectively were 0.341, 0.277, and 0.279 µmols of product produced per minute per mg of protein and 1.12, 0.988, and 0.587mM. The activity curves of *S. alba* myrosinase with glucolimnanthin, sinigrin, and sinalbin showed R^2 values of 0.8992, 0.9801, and 0.9817 respectively which indicates a significant correlation between the observed activities and those predicted by the MM equation (Table 2.14). Glucolimnanthin activity was further tested at substrate concentrations of approximately 5 and 6mM to confirm that the enzyme obeyed MM kinetics. The activities measured with ~2, 2.5, and 3.5mM glucolimnanthin showed a gradual decrease in the mean activity which is indicative of substrate inhibition (Yoshino and Murakami 2015). However, the 90% confidence intervals of the activities overlapped considerably, and the means were not significantly different, so it was likely that the mean activities could be the same and the observed difference may have been due to experimental error. The activities measured at 5 and 6mM showed that the activity did indeed plateau and did not continue to decline (Fig. 2.4).

Although sinalbin is the most abundant glucosinolate in *S. alba*, the *S. alba* myrosinase had the lowest K_m, and therefore the highest affinity, for glucolimnanthin (Pihakaski and Pihakaski 1978; Haldane 1928). This may have been due to the higher sulfate concentration in the enzyme and sinalbin solutions (Table 2.2). The concentration of sulfate was considerably higher in the sinalbin extract than in the glucolimnanthin extract. The additional sulfate may have increased competitive inhibition, increasing the K_m while not affecting Vmax (Engelking 2015; Shikita *et al.* 1999). The sinigrin extract had the lowest concentration of glucosinolates and had a higher sulfate concentration than the sinalbin and glucolimnanthin extracts (Table 2.2). Therefore, more sinigrin extract was used to make

solutions with the same concentration of glucosinolates as the sinalbin and glucolimnanthin which increased the concentration of sulfate in the sinigrin solution. The additional sulfate in the sinigrin solutions may be responsible for sinigrin having the highest K_m of the glucosinolates tested.

Previous research which utilized similar myrosinase extraction methods recorded a V_{max} of *S. alba* myrosinase with sinigrin ranging from approximately 2.57 to 4.16 U mg^{-1}, which is considerably higher than the activities observed in this experiment (Palmieri, Iori, and Leoni 1987). The discrepancy may have been due to differences in the purity of myrosinase in the extracts, difference in incubation temperature, inhibition from compounds in the myrosinase or glucosinolate extracts, or denaturation of the proteins during isolation. The extraction method described by Palmieri, Iori, and Leoni (1987) used 300 g of meal per 2L of water instead of 200 g per 2L, were dialyzed against DI water and 20mM Tris-HCl with 0.5M NaCl and purified using Con A-Sepharose affinity chromatography. The additional purification steps and greater mass of extracted meal may have increased the purity of myrosinase in the crude extract as well. The assays were also conducted at 37 ºC, whereas this experiment was conducted at 25C to emulate field application temperatures (Palmieri, Iori, and Leoni 1986). The optimal temperature for *S. alba* myrosinase activity is approximately 60 ºC, so the activity of the enzyme may have been greater for the previous assay since the incubation temperature was closer to the optimal temperature (Van Eylen *et al.* 2008).

The discrepancy in activity between this research and previous studies could also have been due to glucose inhibition; however, this is unlikely. Since the concentration of glucose in the glucosinolate extracts was insignificant, the primary input of glucose would

have come from the meal. Previous research has shown that glucose is only a weak inhibitor of myrosinase, with a K_i of 1M, and does not cause significant inhibition at concentrations below 5mM (Bhat *et al.* 2015; Shikita *et al.* 1999). The maximum concentration of glucose which could come from the enzyme extract, assuming the entire mass of the extract which was not soluble protein was glucose, would have been 3.34mM, therefore it is unlikely that glucose caused significant enzymatic inhibition. Previous research has shown that fluorinated glucosinolates may inhibit myrosinase activity as well; however, they do not occur in nature so are unlikely to have caused inhibition (Cottaz, Rollin, and Driguez 1997). Regardless, FTIR analysis of the extracts did not show a broad peak from 1000-1300 cm^{-1} which is indicative of C-F bonds (Fig. 2.5-2.8) (Bulusheva *et al.* 2017). Therefore, it does not appear that fluorinated glucosinolates were present in the extracts and could not have caused inhibition. However, the C-F peak lies in the fingerprint region of the spectrum and the presence of numerous other peaks may have covered up the C-F peak.

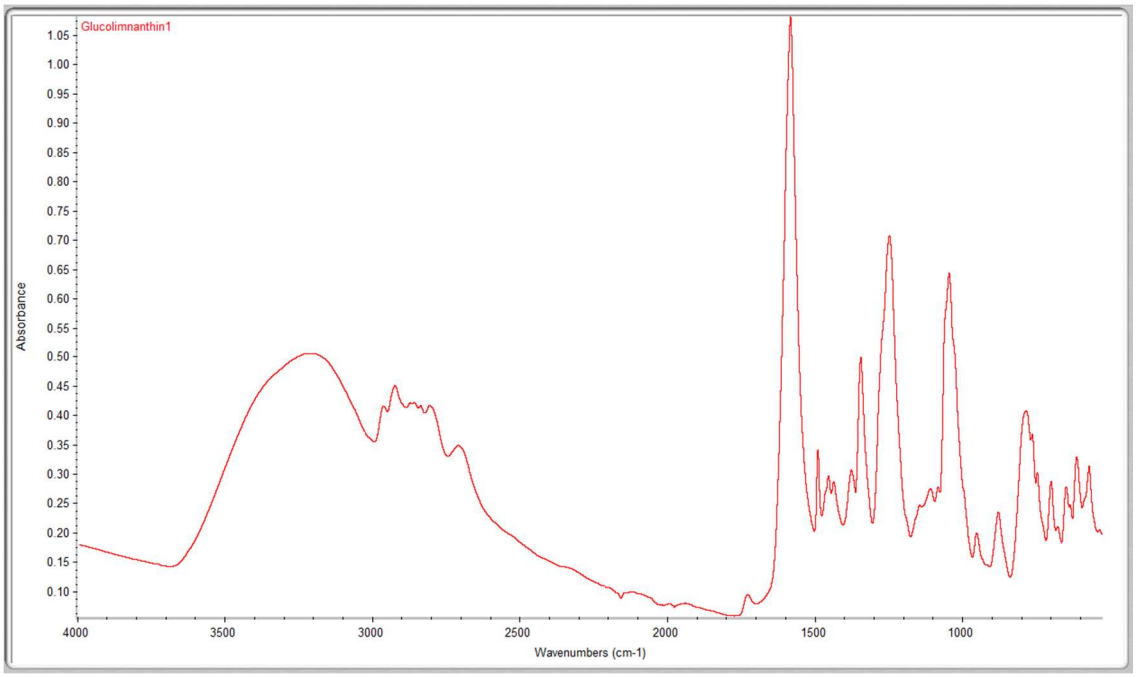

Figure 2.5 Glucolimnanthin Extract IR Spectrum

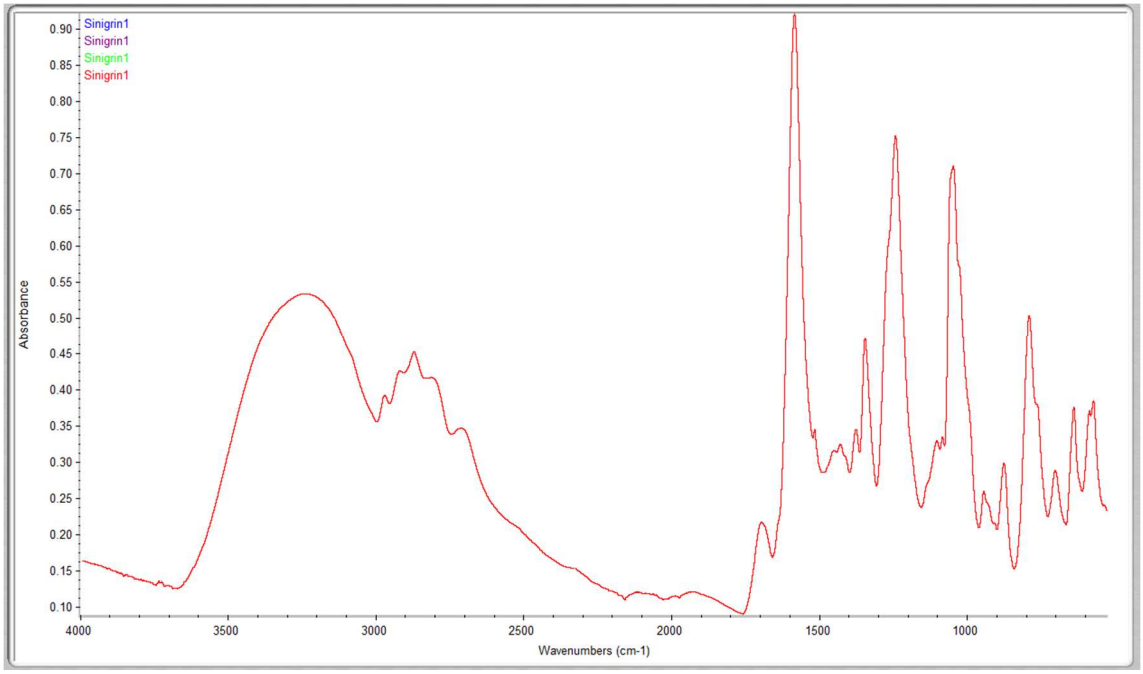

Figure 2.6 Sinigrin Extract IR Spectrum

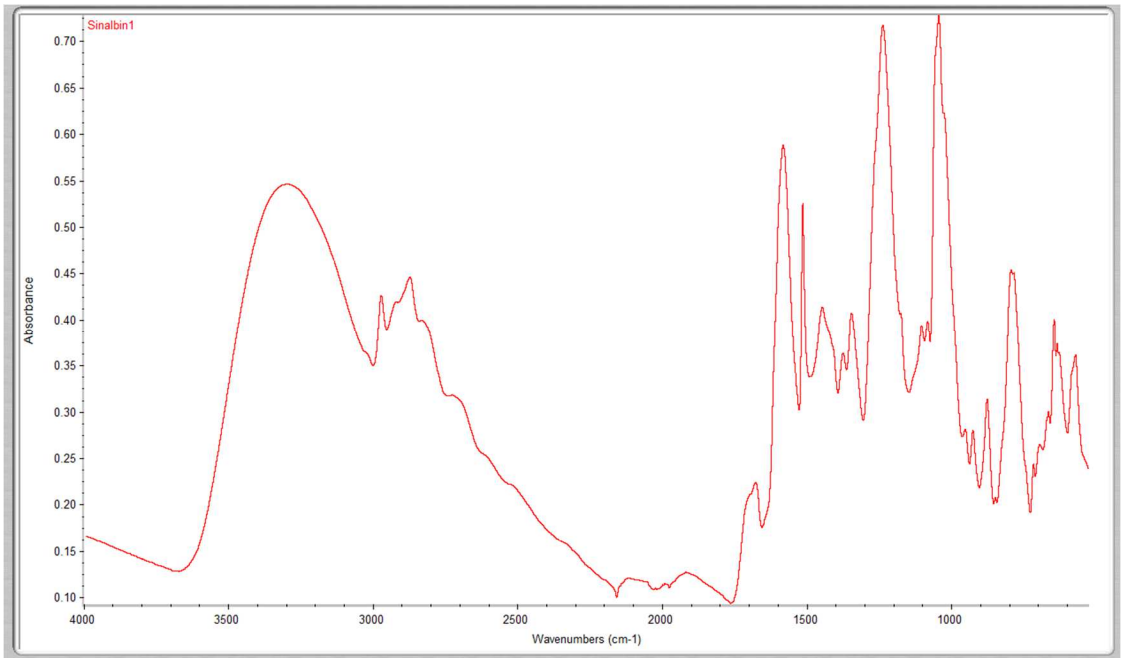

Figure 2.7 Sinalbin Extract (1) IR Spectrum

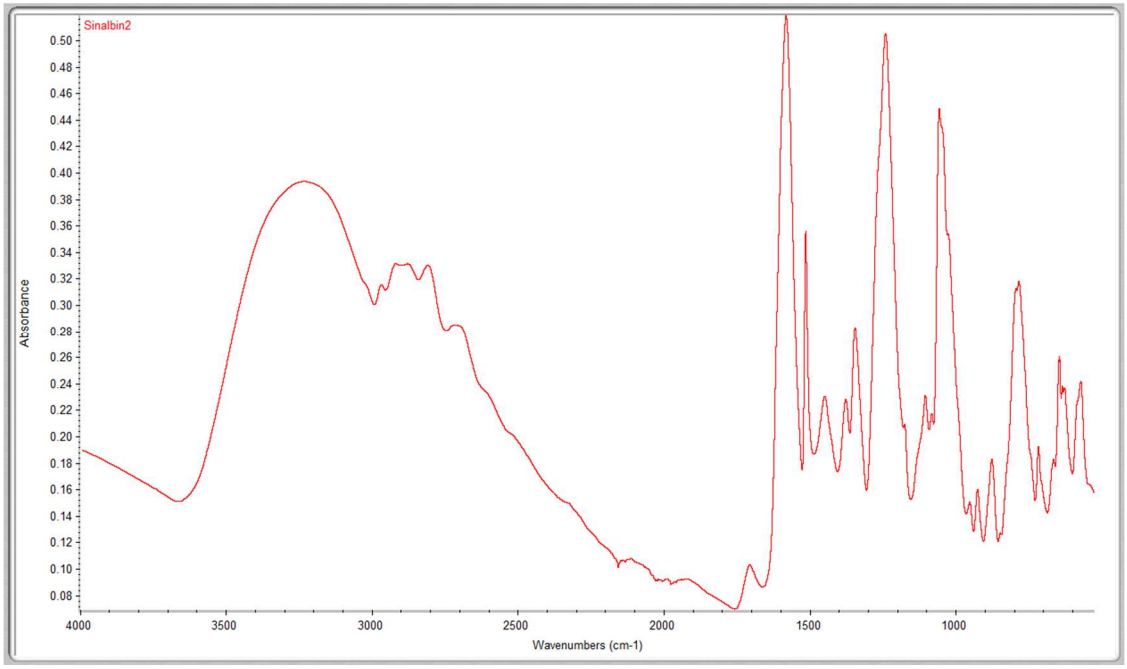

Figure 2.8 Sinalbin Extract (2) IR Spectrum

The maximum activity of the myrosinase with sinigrin was greater than the activity with either of the aromatic glucosinolates (Table 2.14). These results agree with previous research by Braschi *et al.* (2011) which showed that *S. alba* myrosinase had an activity of 63.9U with sinigrin versus 33.1U within sinalbin. The difference in activity between the aromatic and aliphatic glucosinolates suggests that *S. alba* may have greater activity with aliphatic glucosinolates. However, Braschi *et al.* (2011) also showed *S. alba* myrosinase has an activity of 113.1U with nasturtin, 2-phenylethyl glucosinolate. Since nasturtin is an aromatic glucosinolate, it does not appear that the difference in myrosinase activity between the aliphatic sinigrin and aromatic sinalbin and glucolimnanthin was due solely to the aromaticity of the substrates (Braschi *et al.* 2011). The maximum activities of the myrosinase with glucolimnanthin and *S. alba* are very similar, 0.277 and 0.279 U mg^{-1} respectively (Table 2.14). Previous research has recorded Vmax's of 1.30 and 1.38 U mg^{-1} for *S. alba* myrosinase with phenyl and benzyl glucosinolate respectively based on UV/vis absorbance at 235nm (Vastenhout *et al.* 2014). Like glucolimnanthin and sinigrin, both the phenyl and benzyl glucosinolate are aromatic and similar in structure. Therefore, it is plausible that the similarity in Vmax between phenyl and benzyl glucosinolate, and glucolimnanthin and sinalbin could be due to the similarity in structure of these pairs of glucosinolates.

Enzymatic Kinetics of B. juncea Myrosinase

B. juncea myrosinase exhibited MM kinetics with sinigrin and glucolimnanthin as substrates and showed non-MM activity with both the sinalbin extracts (Figures 2.9-2.11). Like the kinetics studies for *S. alba* myrosinase, this was confirmed by comparing the mean activities between substrate concentrations using a Tukey multiple comparisons of means test and a significance level of 0.05. *B. juncea* myrosinase activities were significantly different between lower substrate concentrations for glucolimnanthin and sinigrin, and the activities at

higher substrate concentrations were not significantly different. For sinigrin, the activities increased significantly from ~0.14 to 2.5mM. From ~2.5 to 4.0mM the activities were not significantly different (Table 2.7). When glucolimnanthin was used as a substrate the activity increased significantly from ~0.14 to 3.7mM and the activities were not significantly different from 3.7 to 7.5mM (Table 2.8). When sinalbin was used as the substrate the activity values did not plateau and decreased significantly at higher substrate concentrations (Fig. 2.9-2.12). When the first sinalbin extract was used as the substrate the activity increased significantly from ~0.15 to 0.36mM, then decreased significantly from ~0.36 to 1.2mM. Activities were not significantly different from ~1.2 to 2.8mM but decreased significantly from ~2.8 to 3.3mM. The final activity at 3.3mM was not significantly different from the activity at 0.15mM (Table 2.9). With the second sinalbin extract, activity increased significantly from ~0.12 to 0.96mM. Activities from ~1.5 to 2.6mM were not significantly different from the activity at 0.96mM, although the mean activity did peak at ~1.5mM. From ~2.6 to 3.4mM the activity decreased significantly and the activity at the final substrate concentration was not significantly different than the activity at 0.12mM substrate (Table 2.10).

The R^2 of the *B. juncea* activity curve with sinigrin as a substrate was 0.9997 relative to the curve predicted by the MM equation which indicates a nearly perfect correlation. These results agree with previous research which has also shown that *B. juncea* myrosinase exhibits MM behavior with sinigrin as the substrate (Sharma and Garg 1996). The V_{max} and K_m of *B. juncea* myrosinase with sinigrin as a substrate was 0.0226 U mg^{-1} of protein and 0.306mM respectively (Table 2.14). This demonstrates roughly a 15-fold decrease in V_{max} compared to that of *S. alba* with sinigrin (Appendix A: Figure 2.19). These results contrast

with previous research which showed that *B. juncea* exhibited higher activity than *S. alba* with sinigrin as a substrate under similar conditions (Okunade *et al.* 2015). The differences in activity between these two studies could be due to the species of myrosinase which was predominant in the *B. juncea* extract. Previous research has shown that at least two different species of myrosinase are present in *B. juncea* (Tsuruo, Yoshida, and Hata 1967). If the species of myrosinase used in these experiments were different their kinetics may differ. Additionally, the activity of myrosinase between *B. juncea* lines can vary (Li, Brown, and Eigenbrode 2002). The specific line of *B. juncea* used in this study may have lower activity than that used in the previous study. The K_m of the *B. juncea* myrosinase isoenzyme with sinigrin was considerably lower than the K_m of the *S. alba* isoenzyme with sinigrin (Table 2.14). In this case, the differences in K_m cannot be explained by differences in sulfate concentration since the concentration of sulfate in the *Brassica junce* myrosinase extract was greater than the concentration of sulfate in the *S. alba* extract. If competitive inhibition was affecting the myrosinase isoenzymes, then the K_m should have increased more in *B. juncea* than in *S. alba*. Since K_m is still lower for *B. juncea*, despite competitive inhibition, it appears that *B. juncea* myrosinase has greater affinity for sinigrin than *S. alba* myrosinase (Haldane 1928).

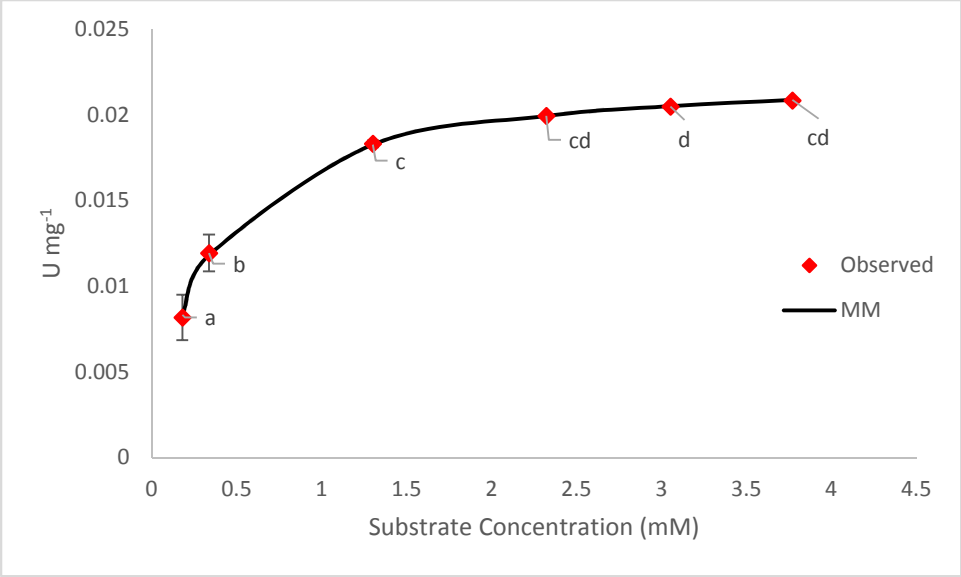

Figure 2.9. Enzymatic activity of *B. juncea* myrosinase with sinigrin substrate. Red diamonds indicate experiment results, the black line illustrates the activity curve predicted using classical Michaelis Menten Kinetics. The error bars represent a 90% confidence interval calculated using a Student's t distribution. Letters indicate statistically different mean activities (α=0.05)

Table 2.7. Tukey comparison of means test for *B. juncea* myrosinase with sinigrin as a substrate. The numbers in the comparison columns indicate activities at different concentrations with 1 being the lowest concentration and 6 being the greatest.

Comparison	Estimate	Standard Error	t-value	P
2 - 1	0.0041657	0.0011395	3.656	0.0276
3 - 1	0.0091108	0.0011395	7.995	<0.001
4 - 1	0.0110029	0.0011395	9.656	<0.001
5 - 1	0.0141926	0.0011395	12.455	<0.001
6 - 1	0.0116445	0.0011395	10.219	<0.001
3 - 2	0.0049451	0.0012182	4.059	0.0134
4 - 2	0.0068373	0.0012182	5.613	<0.001
5 - 2	0.0100269	0.0012182	8.231	<0.001
6 - 2	0.0074788	0.0012182	6.139	<0.001
4 - 3	0.0018922	0.0012182	1.553	0.6390
5 - 3	0.0050818	0.0012182	4.172	0.0111
6 - 3	0.0025337	0.0012182	2.080	0.3531
5 - 4	0.0031897	0.0012182	2.618	0.1606
6 - 4	0.0006415	0.0012182	0.527	0.9940
6 - 5	-0.0025481	0.0012182	-2.092	0.3476

The R^2 of the *B. juncea* activity curve with glucolimnanthin as a substrate was 0.8415 relative to the curve predicted with the MM equation (Table 2.14). The data did not fit the predicted curve as well as the sinigrin curve and there was considerably more variability in the activities (Fig. 2.10). Therefore, additional tests were conducted at higher substrate concentrations to confirm that the enzyme and glucolimnanthin combination did follow Michael-Menten kinetics. The V_{max} and K_m of the myrosinase with glucolimnanthin were 0.03742 U mg^{-1} and 0.2792mM respectively (Table 2.14). Like *S. alba*, the *B. juncea* myrosinase showed a greater affinity for glucolimnanthin than sinigrin which may have been due to competitive inhibition by sulfate in the extracts (Shikita *et al.* 1999). Unlike *S. alba*, the V_{max} of *B. juncea* myrosinase was greater for glucolimnanthin than sinigrin. Since the V_{max} is greater and the K_m is lower for glucolimnanthin than sinigrin as substrates, the isoenzyme is more efficient at hydrolyzing glucolimnanthin than sinigrin. This is unusual since glucolimnanthin is not a common glucosinolate in *B. juncea*, whereas sinigrin is the most abundant glucosinolate in *B. juncea* (Sun *et al.* 2019). However, evolution does not necessarily result in the enzyme having the greatest affinity for the most abundant substrate so the difference in affinitites may not be unique. Like sinigrin, the activity of *B. juncea* with glucolimnanthin was considerably lower than that of *S. alba* (Appendix A: Fig. 2.20). Again, this may be due to the species of myrosinase which was predominant in the meal and may vary based on the type of *B. juncea* meal used as the myrosinase source.

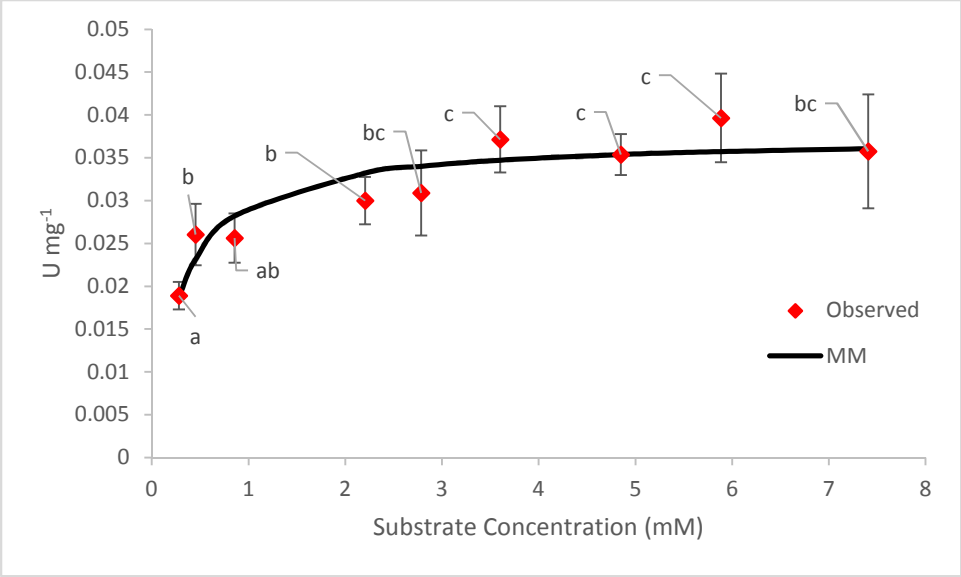

Figure 2.10. Enzymatic activity of *B. juncea* myrosinase with glucolimnanthin substrate. Red diamonds indicate experimental data and the black line illustrated activities predicted with the MM equation. Error bars represent a 90% confidence interval calculated with a Student's t distribution. Letters indicate statistically different mean activities (α=0.05)

Table 2.8. Tukey comparison of means test for *B. juncea* myrosinase with glucolimnanthin as a substrate. The numbers in the comparison columns indicate activities at different concentrations with 1 being the lowest concentration and 9 being the greatest.

Comparison	Estimate	Standard Error	t-value	P
2 - 1	0.0071248	0.0019638	3.628	0.03916
3 - 1	0.0066950	0.0019638	3.409	0.05995
4 - 1	0.0110859	0.0019638	5.645	< 0.001
5 - 1	0.0119887	0.0019638	6.105	< 0.001
6 - 1	0.0182308	0.0019638	9.284	< 0.001
7 - 1	0.0164687	0.0019638	8.386	< 0.001
8 - 1	0.0207343	0.0019638	10.558	< 0.001
9 - 1	0.0168504	0.0019638	8.581	< 0.001
3 - 2	-0.0004298	0.0019638	-0.219	1.00000
4 - 2	0.0039611	0.0019638	2.017	0.55119
5 - 2	0.0048639	0.0019638	2.477	0.30462
6 - 2	0.0111060	0.0019638	5.655	< 0.001
7 - 2	0.0093439	0.0019638	4.758	0.00376
8 - 2	0.0136095	0.0019638	6.930	< 0.001
9 - 2	0.0097256	0.0019638	4.952	0.00256
4 - 3	0.0043909	0.0019638	2.236	0.42480
5 - 3	0.0052937	0.0019638	2.696	0.21650
6 - 3	0.0115358	0.0019638	5.874	< 0.001
7 - 3	0.0097737	0.0019638	4.977	0.00239
8 - 3	0.0140393	0.0019638	7.149	< 0.001
9 - 3	0.0101554	0.0019638	5.171	0.00165
5 - 4	0.0009028	0.0019638	0.460	0.99991
6 - 4	0.0071449	0.0019638	3.638	0.03827
7 - 4	0.0053827	0.0019638	2.741	0.20100
8 - 4	0.0096484	0.0019638	4.913	0.00271
9 - 4	0.0057645	0.0019638	2.935	0.14427
6 - 5	0.0062421	0.0019638	3.179	0.09276
7 - 5	0.0044799	0.0019638	2.281	0.40023
8 - 5	0.0087456	0.0019638	4.453	0.00727
9 - 5	0.0048617	0.0019638	2.476	0.30492
7 - 6	-0.0017622	0.0019638	-0.897	0.99031
8 - 6	0.0025034	0.0019638	1.275	0.92638
9 - 6	-0.0013804	0.0019638	-0.703	0.99809
8 - 7	0.0042656	0.0019638	2.172	0.46036
9 - 7	0.0003817	0.0019638	0.194	1.00000
9 - 8	-0.0038839	0.0019638	-1.978	0.57584

As stated before, the *B. juncea* myrosinase showed an initial increased in activity with both sinalbin extracts, but the activity decreased considerably at higher substrate concentrations (Fig. 2.11 and 2.12). The maximum observed activity for *B. juncea* with the first sinalbin extract was 0.0169 U mg^{-1} at a substrate concentration of 0.383mM. The activity decreased by more than 45% from 0.383 to 3.28mM reaching a minimum of 0.00926 U mg^{-1} (Appendix B). Similar behavior was observed with the second sinalbin extract. The observed activity peaked at 0.0303 U mg^{-1} at a concentration of 1.52mM and decreased by approximately 73% to 0.00825 U mg^{-1} at 3.47mM sinalbin (Appendix B). These decreases in activity at higher substrate concentrations may be due to substrate inhibition.

Many enzymes are inhibited by their own substrates which results in activity curves which rise to a maximum, then decrease as substrate concentration increases (Reed, Lieb, and Nijhout 2010; Yoshino and Murakami 2015). Substrate inhibition is one of the most common forms of non-Michaelis Menten behavior and affects approximately 25% of all known enzymes (Reed, Lieb, and Nijhout 2010; Yoshino and Murakami 2015; Kokkonen *et al.* 2021). Substrate inhibition can play an important part in biological functions. For example, DNA methyltransferase is subject to substrate inhibition by regions of unmethylated DNA and activated by methylated cytosines on the complementary DNA strand (Flynn *et al.* 2003; Svedruzic and Reich 2005; Pradhan *et al.* 1999; Fatemi *et al.* 2001; Lorincz *et al.* 2002; Hye and Richards 2008). The inhibition of methylation by strands of unmethylated DNA ensures that unmethylated regions remain unmethylated and the activation by methylated cytosines guarantees methylation is stimulated wherever the complementary strand is methylated (Reed, Lieb, and Nijhout 2010). Glucosinolates are essential as a defense mechanism for Brassica species and require a large input of energy to produce (Martínez-Ballesta, Moreno,

and Carvajal 2013). *B. juncea* myrosinase may be inhibited by exogenous glucosinolates, such as sinalbin, so that endogenous glucosinolates are preferentially hydrolyzed to provide adequate defense for the plant. Substrate inhibition is usually attributed to the formation of an unproductive enzyme-substrate complex which forms after two substrate molecules bind simultaneously to the active site of the enzyme; however, inhibition may also result from allosteric inhibition by the substrate molecule (Kokkonen *et al.* 2021; Yoshino and Murakami 2015). Allosteric inhibition occurs when an inhibitor binds to another site on the enzyme, other than the active site, which may alter the enzyme so that it cannot efficiently convert the substrate to product (Mehrabi *et al.* 2019).

Previous research has shown that Broccoli myrosinase contains an allosteric binding site which can cause substrate inhibition (Juan Román *et al.* 2018). The unusual kinetics of *B. juncea* with sinalbin suggests that the isoenzyme may possess a similar secondary binding site. To determine which mechanism best described the observed results, the activity curves of *B. juncea* myrosinase with both sinalbin extracts were fitted using the MM equation, the MMSI model, and the TBSM from Lin *et al.* (2001), which was previously used to model substrate inhibition in Broccoli myrosinase (Michaelis and Menten 1913; Lin *et al.* 2001; Juan Román *et al.* 2018).

The activity curve of the myrosinase with the first sinalbin extract had R^2 values of 0.168, 0.696, and 0.823 for the MM, MMSI model and TBSM respectively (Table 2.15). The correlation between the data and both substrate inhibition models is considerably greater than the correlation with the curve predicted by the MM equation which suggests that it is likely that substrate inhibition is occuring. The data has the highest correlation with the TBSM which is further evidence that an allosteric binding site may exhist on *B. juncea* myrosinase.

The V_{max} and K_m predicted with the MM equation were 0.01304 U mg^{-1} and 0.04311mM respectively. Of the three models tested, this V_{max} was most similar to the maximum observed activity. The V_{max}, K_m, and K_i predicted with the MMSI model were 0.02944 U mg^{-1}, 0.3186mM, and 1.873mM respectively (Table 2.15). The V_{max} predicted by the MMSI model was approximately 80% higher than what was observed. It is possible that activity may have increased to V_{max} between 0.37mM and 1.2mM, or if the inhibition mechanism described by the model was different from the actual inhibition mechanism the parameters calculated using the MMSI model could be inaccurate. Since K_m is lower than K_i it appears that the substrate has a greater affinity for the active site than the inhibitory site.

The V_{max}, K_S, K_i, and β for the TBSM were 0.4060 U mg^{-1}, 6.819mM, 73.45mM, and 0.02042 respectively (Table 2.15). Like the MMSI model, the substrate dissociation constant is considerably lower than the inhibitor dissociation constant, indicating that the substrate has a greater affinity for the active site than the inhibitory site. The activity reduction constant, β, indicates that activity may be reduced by approximately 98% when the inhibitor sites are saturated with substrate, suggesting that *B. juncea* myrosinase is strongly inhibited by sinalbin at high concentrations. The V_{max} predicted with the TBSM was approximately 25 times greater than the maximum observed activity. Previous research which utilized this model to analyze broccoli myrosinase kinetics obtained similar overestimates (Juan Román *et al.* 2018). The TBSM model was originally used to determine the kinetic parameters for cytochrome-P450 (Lin *et al.* 2001). Myrosinase and cytochrome-P450 differ in structure considerably which may account for the differences. Additionally, the model is constrained by the α and β parameters since α must be greater than 0 and β must lie between 0 and 1. Further research is likely needed to optimize this model for myrosinase.

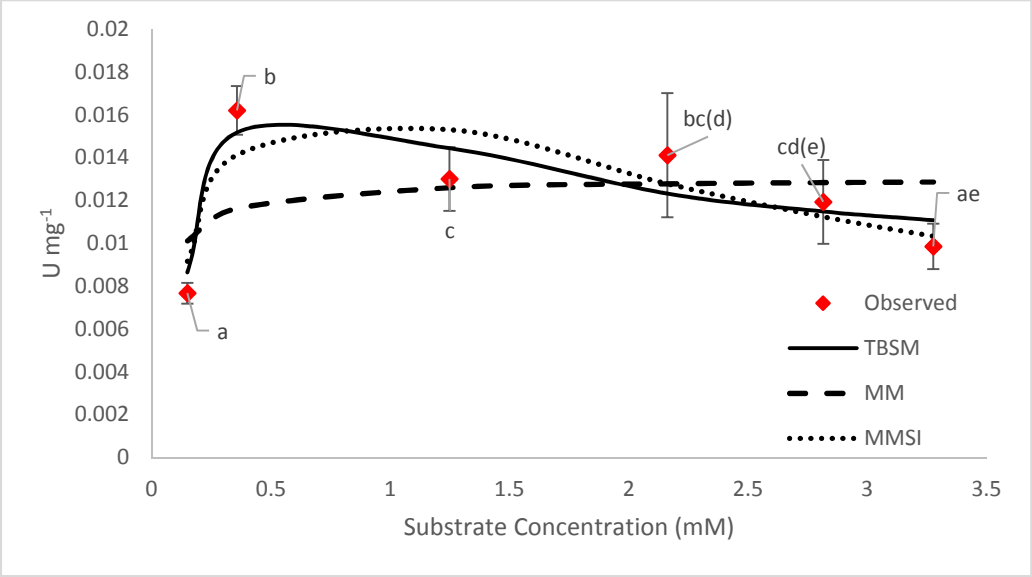

Figure 2.11. Enzymatic activity of *B. juncea* myrosinase with first sinalbin extract. Red diamonds indicate experimental data, the black line shows activities which were predicted using the TBSM, the dashed line shows activity predicted using the Michaelis Menten equation and the dotted line shows activity predicted with the MMSI model. Error bars represent a 90% confidence interval calculated using a Student's t distribution. Letters indicate significantly different mean activities (α=0.05). Letters "d" and "e" refer to the mean at the marked point which does not differ significantly from the letters outside of parentheses.

Table 2.9. Tukey comparison of means test for *B. juncea* myrosinase with sinalbin (1) as a substrate. The numbers in the comparison columns indicate activities at different concentrations with 1 being the lowest concentration and 9 being the greatest.

Comparison	Estimate	Standard Error	t-value	P
2 - 1	0.0085344	0.0008177	10.437	< 0.001
3 - 1	0.0053340	0.0008177	6.523	< 0.001
4 - 1	0.0064428	0.0008177	7.879	< 0.001
5 - 1	0.0042598	0.0008177	5.209	0.00231
6 - 1	0.0021852	0.0008177	2.672	0.15266
3 - 2	-0.0032003	0.0008177	-3.914	0.01961
4 - 2	-0.0020915	0.0008177	-2.558	0.18180
5 - 2	-0.0042746	0.0008177	-5.228	0.00222
6 - 2	-0.0063491	0.0008177	-7.765	< 0.001
4 - 3	0.0011088	0.0008177	1.356	0.75056
5 - 3	-0.0010743	0.0008177	-1.314	0.77280
6 - 3	-0.0031488	0.0008177	-3.851	0.02176
5 - 4	-0.0021831	0.0008177	-2.670	0.15336
6 - 4	-0.0042576	0.0008177	-5.207	0.00230
6 - 5	-0.0020746	0.0008177	-2.537	0.18768

The activity curve of the myrosinase with the second sinalbin extract had R^2 values of 0.5105, 0.7414, and 0.7413 for the MM equation, MMSI model, and TBSM respectively (Table 2.16). There was a greater correlation between the observed data and both the MM equation and MMSI model and a decrease in the correlation of the TBSM compared to the results from the first sinalbin extract. However, like the first sinalbin extract, there was a considerably greater correlation between the results and the substrate inhibition models than the MM equation which suggests that substrate inhibition is taking place. The V_{max} and K_m predicted from the MM equation were 0.01995 U mg^{-1} and 0.04311mM respectively (Table 2.15). Like the results from the first sinalbin extract, the V_{max} predicted with the MM equation was closest to the maximum observed activity; however, it underestimated the maximum activity by roughly 52% whereas the results from the first sinalbin extract only underestimated the maximum activity by 24%. The V_{max}, K_m, and K_i predicted with the MMSI model were 0.1326 U mg^{-1}, 1.971mM, and 0.4505mM respectively (Table 2.15). The maximum activity predicted by the model is approximately 4.4 times greater than the maximum observed activity. It is possible that there was a significant increase in activity between 0.96 and 1.5mM; however, like the previous extract the mechanism described by the model may differ from the actual inhibition mechanism which would affect the accuracy of the results. Of the the three models, the MMSI had the highest correlation with the observed results; however, the R^2 of the results of the MMSI model is only 0.0001 greater than the R^2 of the TBSM. Since the correlations are so similar it cannot be concluded that the MMSI model is a significantly better fit for the data than the TBSM.

Unlike the results from the first extract, the substrate appears to have a greater affinity for the inhibitory site than the active site based on the results from the MMSI model.

The K_i for the solutions with the second extract was 76% lower than than the solutions with the first extract and the K_m increased by 520%. The affinity of the substrate for both binding sites may have been affected by the sulfate concentration in the solutions. The concentration of sulfate was slightly greater in the second extract than the first; however, the concentration of glucosinolates was much lower in the first extract, and significantly more of the first extract was needed to prepare solutions with the same concentration as the second extract. As a result, the ratio of sulfate to sinalbin in the first extract was greater than the second extract, approximately 0.535 and 0.492 respectively, so there was a higher input of sulfate from the first extract. Additionally, the second *B. juncea* enzyme mixture was used with the second sinalbin extract and the second mixture had an 11% lower concentration of sulfate relative to the first mixture. Therefore, the solutions which were prepared with the first enzyme solution and first sinalbin extract had higher sulfate concentrations than those prepared with the second enzyme solution and second sinalbin extract. If the inhibitory site had greater affinity for sulfate than the active site, then K_i would increase more than K_m (Engelking 2015). Converseley, as sulfate concentration, and therefore competitive inhibition, decreased in the solution the affinity of the substrate for the active and inhibitory sites could both increase and converge. If the results of the MMSI model are accurate, it would appear that the inhibitory site has a greater affinity for sinalbin at low sulfate concentrations, and the active site has a greater affinity for sinalbin at higher sulfate concentrations.

The V_{max}, K_S, K_i, and β from the TBSM were 0.2441 U mg^{-1}, 3.618mM, 4.206mM, and 0 respectively (Table 2.16). The maximum activity predicted by the model is approximately 8 times greater than the maximum observed activity (Appendix A: Fig. 2.21). Though this is closer to the observed results than the maximum activity predicted from the

first sinalbin extract it still differs considerably and it likely is not indicative of the actual maximum activity. Like the MMSI model, there was a considerable decrease in the K_i compared to the assay conducted with the first extract. Unlike the results from the MMSI model, the K_s also decreased and was lower than the K_i, indicating that the substrate has greater affinity for the active site than the inhibitory site. However, the dissociation constants are very similar, indicating that the sites do not differ considerably in their affinity for the substrate. The K_s decreased by approximately 47% whereas the K_i decreased by approximately 94% which supports the results from the MMSI model which indicate that there is a disproportional effect of sulfate on the binding affinity of the inhibitory site. Beta was also lower in the second assay than in the first. Since β is 0, it would appear that substrate can cause complete inhibition of the enzyme when the inhibitory site is saturated.

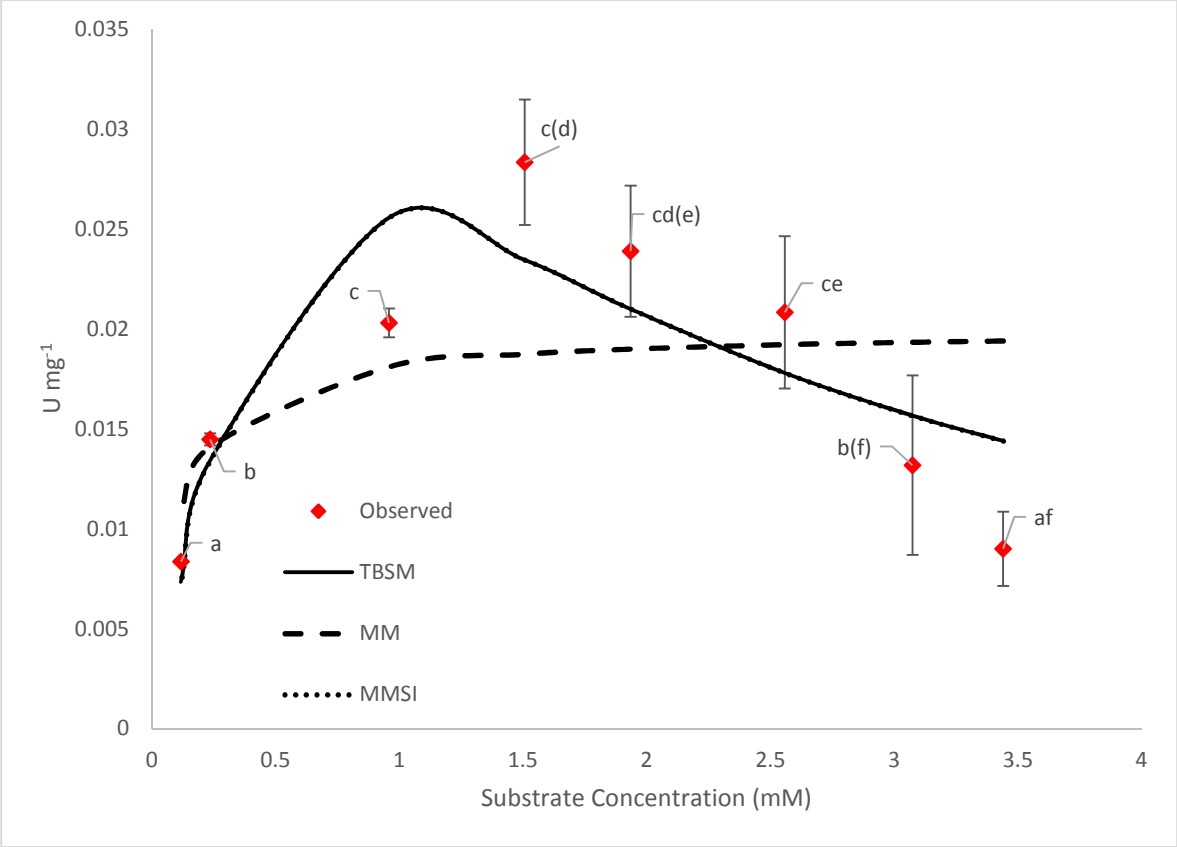

Figure 2.12. Enzymatic activity of *B. juncea* myrosinase with second sinalbin extract. Red diamonds indicate experimental data, the black line shows activities which were predicted using the TBSM and the dashed line shows activity predicted using Michaelis Menten kinetics. The activities predicted with the MM substrate inhibition model are represented by the dotted line but overlap with the values predicted by the TBSM. Error bars represent a 90% confidence interval calculated using a Student's t distribution. Letters indicate significantly different mean activities (α=0.05). Letters "d", "e", and "f" refer to the mean at the marked point which does not differ significantly from the letters outside of parentheses.

Table 2.10. Tukey comparison of means test for *B. juncea* myrosinase with sinalbin (2) as a substrate. The numbers in the comparison columns indicate activities at different concentrations with 1 being the lowest concentration and 8 being the greatest.

Comparison	Estimate	Standard Error	t-value	P
2 - 1	0.0061095	0.0013325	4.585	0.00570
3 - 1	0.0161296	0.0013325	12.105	< 0.001
4 - 1	0.0199831	0.0013325	14.997	< 0.001
5 - 1	0.0155240	0.0013325	11.650	< 0.001
6 - 1	0.0124628	0.0013325	9.353	< 0.001
7 - 1	0.0048204	0.0013325	3.618	0.03731
8 - 1	0.0006307	0.0013325	0.473	0.99964
3 - 2	0.0100201	0.0013325	7.520	< 0.001
4 - 2	0.0138736	0.0013325	10.412	< 0.001
5 - 2	0.0094145	0.0013325	7.065	< 0.001
6 - 2	0.0063534	0.0013325	4.768	0.00397
7 - 2	-0.0012891	0.0013325	-0.967	0.97295
8 - 2	-0.0054788	0.0013325	-4.112	0.01446
4 - 3	0.0038535	0.0013325	2.892	0.13968
5 - 3	-0.0006056	0.0013325	-0.455	0.99973
6 - 3	-0.0036668	0.0013325	-2.752	0.17609
7 - 3	-0.0113092	0.0013325	-8.487	< 0.001
8 - 3	-0.0154989	0.0013325	-11.632	< 0.001
5 - 4	-0.0044591	0.0013325	-3.346	0.06196
6 - 4	-0.0075202	0.0013325	-5.644	< 0.001
7 - 4	-0.0151627	0.0013325	-11.379	< 0.001
8 - 4	-0.0193524	0.0013325	-14.524	< 0.001
6 - 5	-0.0030611	0.0013325	-2.297	0.35119
7 - 5	-0.0107036	0.0013325	-8.033	< 0.001
8 - 5	-0.0148933	0.0013325	-11.177	< 0.001
7 - 6	-0.0076425	0.0013325	-5.736	< 0.001
8 - 6	-0.0118322	0.0013325	-8.880	< 0.001
8 - 7	-0.0041897	0.0013325	-3.144	0.08953

Enzymatic Kinetics of L. alba Myrosinase

The *L. alba* myrosinase did not exhibit any significant activity with sinalbin, sinigrin, or glucolimnanthin as substrates. The observed activities were near zero or negative and since myrosinase cannot generate additional substrate, the negative activities are likely due to experimental error. Positive activities were an order of magnitude, or more, lower than the lowest activity observed in the previous assays and may have also been due to experimental error. As with the previous tests, a Tukey multiple comparison of means test was used to determine whether the activities were significantly different between concentrations using $\alpha=0.05$. The results indicated that there was not a significant difference in activity between any of the substrate concentrations for all three of the substrates (Tables 2.11-2.13). There was also considerably more variability in the activity results. In many cases, replicates of the same treatment would show both positive and negative activities which were near zero. Confidence intervals were calculated for the activities at each concentration and with each substrate using a Student's t distribution and $\alpha=0.1$ to determine whether any of the activities were significantly different than zero. Zero was contained in the confidence intervals for all the treatments, so it is possible that the true means of the activities was zero (Fig. 2.13-2.15).

The lack of activity in the extract may have been due to the absence of myrosinase in the meal which was used; however, previous research has shown that active myrosinse is present in *L. alba* seed meal, so the lack of activity could be due to enzymatic degradation as well (Intanon *et al.* 2014). As previous discussed, myrosinase is thermally sensitive and is typically inactivated above 65 ^{o}C (Farhana, Aripin, and Surugau 2016). However, research has shown that the thermal stability of myrosinase varies considerably between different

Brassica species and myrosinase from green cabbage exhibits decreased activity over time at temperatures as low as 35C (Ghawi *et al.* 2012). The *L. alba* meal which was used for the crude extract had been stored in the lab at ambient temperatures for several years which could reach 35 ^{o}C or higher during the summer. The frequent exposure of the meal to temperatures ≥35 ^{o}C over the years may have inactivated the enzyme. Since the *L. alba* extract had the lowest sulfate concentration it is unlikely that its lack of activity was due to inhibition from sulfate.

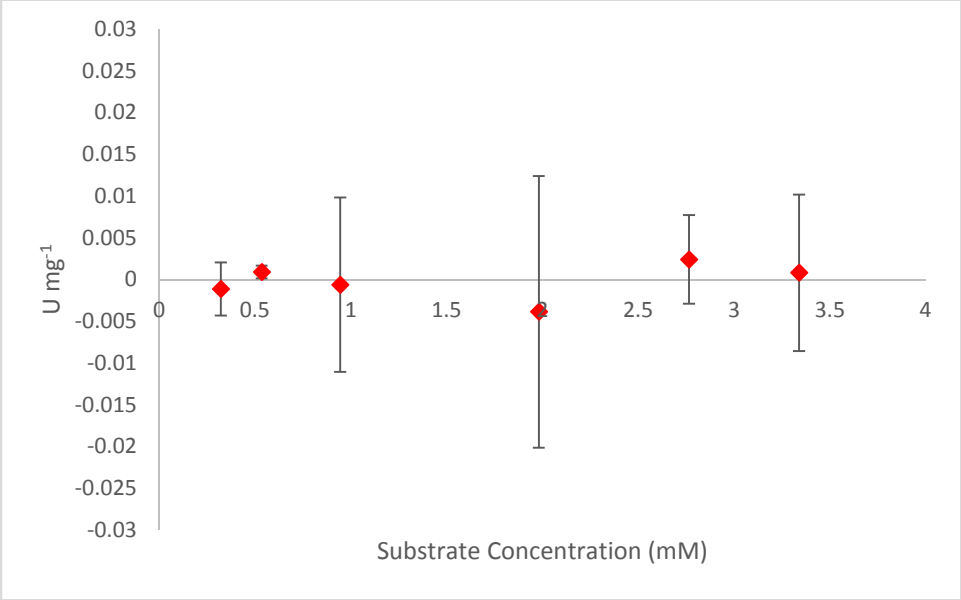

Figure 2.13. Enzymatic activity of *L. alba* myrosinase with sinigrin substrate. Red diamonds indicate observed data and error bars indicate a 90% confidence interval calculated using a Student's t distribution. None of the means were significantly different (α=0.05).

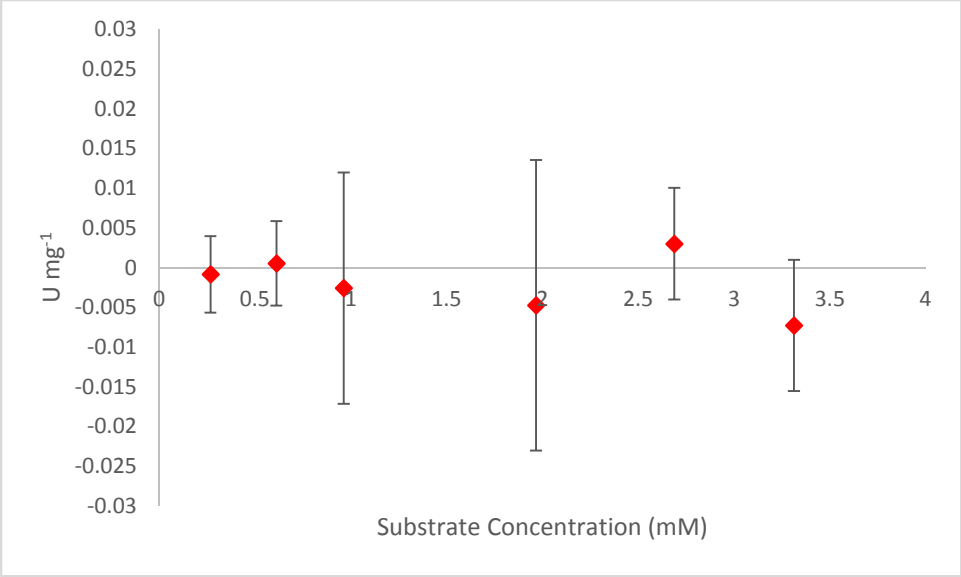

Figure 2.14. Enzymatic activity of *L. alba* myrosinase with sinalbin substrate. Red diamonds indicate observed data and error bars indicate a 90% confidence interval calculated using a Student's t distribution. None of the means were significantly different (α=0.05).

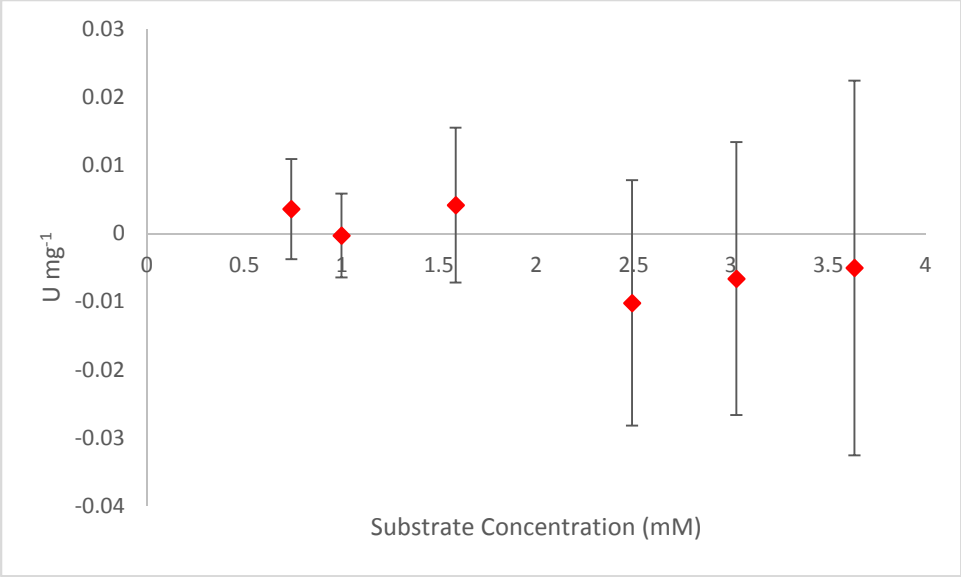

Figure 2.15. Enzymatic activity of *L. alba* myrosinase with glucolimnanthin substrate. Red diamonds indicate observed data and error bars indicate a 90% confidence interval calculated using a Student's t distribution. None of the means were significantly different (α=0.05).

Table 2.11. Tukey comparison of means test for *L. alba* myrosinase with sinigrin as a substrate. The numbers in the comparison columns indicate activities at different concentrations with 1 being the lowest concentration and 6 being the greatest.

Comparison	Estimate	Standard Error	t-value	P
2 - 1	2.004×10^{-3}	4.426×10^{-3}	0.453	0.997
3 - 1	4.938×10^{-4}	4.426×10^{-3}	0.112	1.000
4 - 1	-2.749×10^{-3}	4.426×10^{-3}	-0.621	0.987
5 - 1	3.530×10^{-3}	4.426×10^{-3}	0.798	0.963
6 - 1	1.929×10^{-3}	4.426×10^{-3}	0.436	0.997
3 - 2	-1.510×10^{-3}	4.426×10^{-3}	-0.341	0.999
4 - 2	-4.753×10^{-3}	4.426×10^{-3}	-1.074	0.883
5 - 2	1.526×10^{-3}	4.426×10^{-3}	0.345	0.999
6 - 2	-7.487×10^{-3}	4.426×10^{-3}	-0.017	1.000
4 - 3	-3.243×10^{-3}	4.426×10^{-3}	-0.733	0.974
5 - 3	3.036×10^{-3}	4.426×10^{-3}	0.686	0.980
6 - 3	1.435×10^{-3}	4.426×10^{-3}	0.324	0.999
5 - 4	6.279×10^{-3}	4.426×10^{-3}	1.419	0.716
6 - 4	4.678×10^{-3}	4.426×10^{-3}	1.057	0.889
6 - 5	-1.601×10^{-3}	4.426×10^{-3}	-0.362	0.999

Table 2.12. Tukey comparison of means test for *L. alba* myrosinase with sinalbin as a substrate. The numbers in the comparison columns indicate activities at different concentrations with 1 being the lowest concentration and 6 being the greatest.

Comparison	Estimate	Standard Error	t-value	P
2 - 1	0.001387	0.005288	0.262	1.000
3 - 1	-0.001731	0.005288	-0.327	0.999
4 - 1	-0.003895	0.005288	-0.737	0.973
5 - 1	0.003848	0.005288	0.728	0.975
6 - 1	-0.006431	0.005288	-1.216	0.821
3 - 2	-0.003117	0.005288	-0.590	0.990
4 - 2	-0.005282	0.005288	-0.999	0.910
5 - 2	0.002461	0.005288	0.465	0.997
6 - 2	-0.007818	0.005288	-1.479	0.683
4 - 3	-0.002165	0.005288	-0.409	0.998
5 - 3	0.005578	0.005288	1.055	0.890
6 - 3	-0.004701	0.005288	-0.889	0.942
5 - 4	0.007743	0.005288	1.464	0.691
6 - 4	-0.002536	0.005288	-0.480	0.996
6 - 5	-0.010279	0.005288	-1.944	0.424

Table 2.13. Tukey comparison of means test for *L. alba* myrosinase with glucolimnanthin as a substrate. The numbers in the comparison columns indicate activities at different concentrations with 1 being the lowest concentration and 6 being the greatest.

Comparison	Estimate	Standard Error	t-value	P
2 - 1	-0.0038864	0.0081605	-0.476	0.996
3 - 1	0.0005815	0.0081605	0.071	1.000
4 - 1	-0.0137756	0.0081605	-1.688	0.563
5 - 1	-0.0102049	0.0081605	-1.251	0.805
6 - 1	-0.0086447	0.0081605	-1.059	0.888
3 - 2	0.0044679	0.0081605	0.548	0.993
4 - 2	-0.0098892	0.0081605	-1.212	0.823
5 - 2	-0.0063185	0.0081605	-0.774	0.967
6 - 2	-0.0047583	0.0081605	-0.583	0.990
4 - 3	-0.0143571	0.0081605	-1.759	0.523
5 - 3	-0.0107864	0.0081605	-1.322	0.769
6 - 3	-0.0092263	0.0081605	-1.131	0.860
5 - 4	0.0035707	0.0081605	0.438	0.997
6 - 4	0.0051309	0.0081605	0.629	0.986
6 - 5	0.0015602	0.0081605	0.191	1.000

Table 2.14 Kinetic parameters of E+S pairs which exhibited MM kinetics

Myrosinase Source	Glucosinolate	V_{max} (U mg^{-1})	K_m (mM)	R^2
B. juncea	Sinigrin	0.02257	0.3064	0.9997
B. juncea	Glucolimnanthin	0.03742	0.2792	0.8415
S. alba	Sinigrin	0.3413	1.124	0.9801
S. alba	Sinalbin	0.2761	0.9880	0.9817
S. alba	Glucolimnanthin	0.2788	0.5869	0.8992

Table 2.15 Comparison of kinetic parameters for different *B. juncea* + Sinalbin (1) models

Model	V_{max}	K_m	K_s	K_i	α	β	R^2
MM	0.01304	0.04311	-	-	-	-	0.1678
MMSI	0.02944	0.3186	-	1.873	-	-	0.6957
TBSM	0.4060	-	6.819	73.45	$3.360*10^{-4}$	0.02042	0.8232

Table 2.16 Comparison of kinetic parameters for different *B. juncea* + Sinalbin (2) models

Model	V_{max}	K_m	K_s	K_i	α	β	R^2
MM	0.01995	0.09760	-	-	-	-	0.5105
MMSI	0.1326	1.971	-	0.4505	-	-	0.7414
TBSM	0.2441	-	3.618	4.206	0.05826	0	0.7413

Summary

The objective of this research was to determine whether myrosinase from *S. alba*, *L. alba*, or *B. juncea* has greater specific activity for sinigrin, sinalbin, or glucolimnanthin in order to determine which isoenzyme will generate the most isothiocyanates and thiocyanates from the substrates. The results of this study indicate that the *S. alba* myrosinase used in this experiment had considerably higher activity than *B. juncea* or *L. alba* for all the glucosinolates which were studied (Appendix A: Fig. 2.19-2.21). *S. alba* exhibited classical MM kinetics with all three substrates and showed the highest V_{max} with sinigrin as a substrate and approximately equal V_{max} for both glucolimnanthin and sinalbin. The similarity in maximum activities for glucolimnanthin and sinalbin may be due to the similarity between the structures of the glucosinolates. *S. alba* myrosinase showed the greatest affinity for glucolimnanthin, followed by sinalbin, then sinigrin; however, the differences in affinity may be due to competitive inhibition by sulfate rather than characteristics of the isoenzyme or the glucosinolates since the extracts with lower affinities also had higher sulfate concentrations.

B. juncea exhibited MM kinetics with sinigrin and glucolimnanthin as substrates and showed signs of non-MM kinetics in two assays which used sinalbin as the substrate. Both activity curves of the isoenzyme with sinalbin showed a rapid increase in activity followed by a decrease in activity at higher substrate concentrations, rather than the plateau in activity which is associated with MM kinetics. This is indicative of substrate inhibition and the results from the assays with *B. juncea* and sinalbin showed a greater correlation with substrate inhibitions models than the MM equation. Previous research has shown that broccoli myrosinase has a secondary binding site which can cause substrate inhibition and the results of this study suggest that a similar site may be present on *B. juncea* myrosinase

(Román *et al.* 2018). Since the isoenzyme did not demonstrate substrate inhibition with sinigrin and glucolimnanthin it may be selectively inhibited by certain glucosinolates.

The *B. juncea* myrosinase showed greater activity and affinity with glucolimnanthin than sinigrin which is unusual considering that sinigrin is the primary glucosinolate in *B. juncea* and glucolimnanthin is not typically found in the plant (Sun *et al.* 2019). The maximum activities of the isoenzyme with sinigrin and glucolimnanthin were approximately 12 and 7.5 times lower than that of *S. alba* myrosinase respectively; however, the *B. juncea* myrosinase showed a much higher affinity for the substrates. Like before, the difference between the affinities of the enzymes may be due to competitive inhibition from sulfate since the sinigrin extract used for the assay had a higher concentration of sulfate than the glucolimnanthin. The difference in binding affinities between the isoenzymes may also be due to competitive inhibition by sulfate since the *B. juncea* enzyme extract had approximately half the concentration of sulfate as the *S. alba*. The maximum observed activities of *B. juncea* with sinalbin were approximately 10-20 times lower than that of *S. alba* myrosinase with sinalbin (Appendix A: Fig. 2.20). For both *B. juncea* sinalbin assays, the V_{max} which was predicted using the MM equation was lower than the maximum observed activities and the V_{max} predicted with the substrate inhibition models was an order of magnitude, or more, higher. The discrepancy between the maximum activity that was observed, and the maximum activities predicted by the models suggests that the models may not be indicative of the actual mechanism of enzymatic activity and inhibition and future research is required to develop an accurate model.

In the first sinalbin assay, the results show that the inhibitory site had lower affinity for the substrate than the active site, whereas in the second assay the affinities were very

similar, or the inhibitory site had greater affinity for the substrate than the active site. The enzyme and sinalbin extracts used in the second assay had lower sulfate concentrations than those used in the first assay. The differences in affinity could be due to sulfate preferentially binding to the inhibitory site over the active site. If so, an increase in the concentration of sulfate in the solution could have a disproportionate effect on the binding affinity of the inhibitory site which would explain the results. If this is the case, then the inhibitory site may be blocked at very high sulfate concentrations. If the sulfate does not cause allosteric inhibition like the substrate, then the enzyme may exhibit MM kinetics at high sulfate concentrations since sulfate is preventing substrate inhibition. *L. alba* did not show any significant activity and the enzyme may have been inactivated over time prior to extraction. Fresh seed meal should be used in the future to thoroughly assess the kinetics of *L. alba* myrosinase.

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Conclusion

The results of the tarping bioassay from chapter one indicates that wireworms have the potential to serve as bioindicators for the fate of allyl isothiocyanate in soil. Research has shown, small differences in allyl isothiocyanate concentration in soil can have significant effects on the mortality of some wireworm species. Since allyl isothiocyanate is a volatile compound, it was hypothesized that the concentration of allyl isothiocyanate would be lower in columns treated with *B. juncea* seed meal which were un-tarped than in columns which were tarped, due to volatilization, and wireworm mortality would be greater in the tarped columns due to the higher concentration of allyl isothiocyanate. As predicted, wireworm mortality was significantly higher in treated columns which were covered with a tarp than in the un-tarped columns. allyl isothiocyanate concentration was below the limit of detection for high performance liquid chromatography analysis after 48 hours of incubation in the tarping assay (Table 1.26), therefore no conclusions about allyl isothiocyanate fate can be made using these conventional analytical methods. However, wireworm mortality indicates that allyl isothiocyanate concentration was likely higher for longer in the tarped columns. These bioassays have the benefit of allowing us to assess allyl isothiocyanate fate in soil, even after the majority has degraded or volatilized.

The difficulty associated with extracting and analyzing allyl isothiocyanate in soil can be a deterrent for biopesticide users since it is cost and time prohibitive. The results of the tarping assay show that bioassays have the potential to act as an effective and affordable alternative to conventional analytical methods which could make allyl isothiocyanate fate analysis feasible for researchers or biopesticide users with limited resources. Future research may demonstrate that alternative commercially available bioindicators could be used to assess pesticide fate as well. This would streamline assay preparation since indicators could

easily be purchased, rather than needing to be harvested manually. The principles of these experiments could easily be applied to assess the efficacy of different treatment methods in the future.

Although soil electrical conductivity and pH were both significantly affected by soil treatment in the bioassays, we cannot conclude that they are effective in-situ indicators of allyl isothiocyanate fate based on the results of the experiments. Sinigrin hydrolysis does generate ions and H^+ which affect EC and pH; however, the meal also contains ionic compounds and acids such as uric acid, phytic acid, and negatively charged proteins which may have a greater effect on EC and pH than glucosinolate hydrolysis. Future bioassays could compare EC and pH in columns treated with detoxified seed meal as well as unaltered seed meal to determine whether glucosinolate hydrolysis alone has a significant effect on EC and pH. Soil sulfate concentration in the vertical isolation assay was also significantly affected by treatment. The concentration of sulfate in the detoxified *B. juncea* seed meal was only slightly greater than the concentration of sinigrin in the meal. This indicates that sinigrin hydrolysis is the primary source of sulfate in detoxified seed meal. Therefore, changes in sulfate concentration in *B. juncea* treated soil are likely due to sinigrin hydrolysis and could be used to assess allyl isothiocyanate production in treated soil. Unlike allyl isothiocyanate analysis, sulfate analysis does not require complex and time-consuming extractions and derivatizations. Utilizing sulfate as an indicator for allyl isothiocyanate production could drastically expedite analysis and reduce costs for researchers and biopesticide users.

Based on the results of the enzyme kinetics studies in chapter two, the *S. alba* used in the experiment was a much better source of myrosinase than the *B. juncea*. The maximum activities were considerably higher, and the enzyme did not exhibit substrate inhibition with

any of the glucosinolates. The *B. juncea* was much less effective at converting the substrate to product and if it were used in the field as a myrosinase source the biopesticides may have reduced efficacy since fewer glucosinolates may be hydrolyzed. *B. juncea* myrosinase did not exhibit substrate inhibition with either sinigrin or glucolimnanthin and was selectively inhibited by sinalbin. This indicates that the inhibitory site may have selective affinity for specific glucosinolates. Previous research has shown the broccoli myrosinase also exhibits selective substrate inhibition. Like *B. juncea* myrosinase, the broccoli myrosinase shows typical MM kinetics with sinigrin as a substrate but shows substrate inhibition kinetics with glucoraphanin as a substrate. Since multiple myrosinase isoenzymes show selective inhibition by specific glucosinolates, it is possible that other myrosinase isoenzymes are also selectively inhibited. Therefore, there may not be an individual myrosinase isoenzyme which is an optimal source for all Brassica seed meal extracts. Future research is required to determine whether other myrosinase isoenzymes are selectively inhibited by specific glucosinolates. This would prevent biopesticide users from combining myrosinase isoenzymes with substrates which cause inhibition.

The goals of this research were to determine affordable alternative analytical methods for the fate of allyl isothiocyanate in soil and assess which myrosinase isoenzyme and glucosinolate combinations generate the most product so that biopesticide users can efficiently maximize the efficacy of the biopesticides and minimize the cost. The results of the bioassays demonstrate that bioindicators could be used to study the fate of allyl isothiocyanate in soil, even after the majority of the allyl isothiocyanate has degraded or volatilized. Glucosinolates are used as treatments for a wide array of pest species, therefore there are a plethora of bioindicators which may be used to study the fate of hydrolysis

products from different glucosinolates. Bioassays could reduce the cost of analysis for biopesticide users and expedite analysis so that application methods can be optimized efficiently. The kinetics studies demonstrated that myrosinase isoenzymes may be selectively inhibited by specific glucosinolates, therefore incompatible combinations of myrosinase and substrates could reduce the efficacy of the biopesticide. It is imperative that biopesticide users are aware of which myrosinase isoenzymes are incompatible with different Brassica seed meal extracts to maximize the efficacy of the biopesticide and minimize costs. If biopesticide users can utilize bioassays to determine which myrosinase sources are most effective with specific seed meal extracts, then the users can optimize application methods and the results could provide invaluable data for researchers studying the inhibition of myrosinase isoenzymes. Enabling biopesticide users and laboratory researchers to collaborate and conduct research in conjunction will expedite data collection and optimization of application methods which could make these biopesticides an appealing alternative to conventional pesticides and increase commercial adoption.

APPENDIX A: COMPARISON OF ISOENZYME ACTIVITES BETWEEN SUBSTRATES

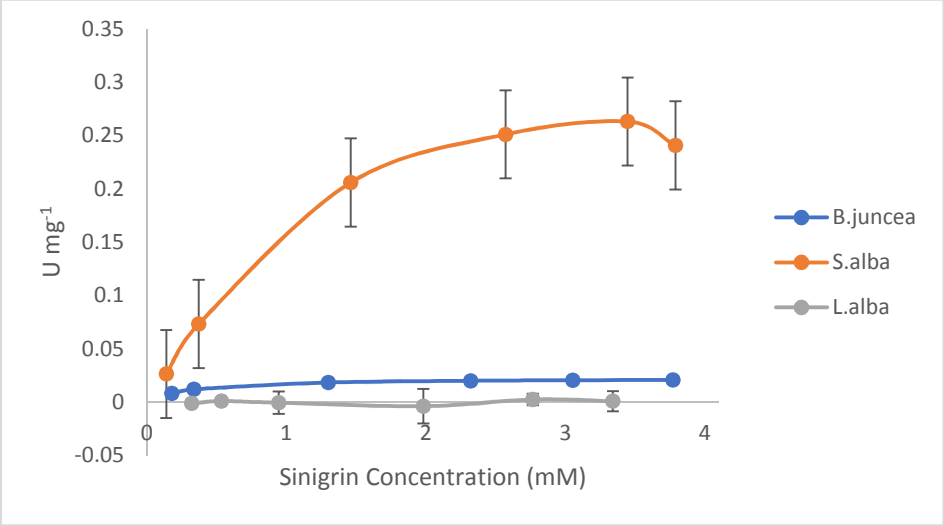

Figure 2.19. Activity curves of *B. juncea* (extract 1), *S. alba*, and *L. alba* myrosinase with sinigrin as a substrate. Error bars represent a 90% confidence interval calculated using a Student's t distribution.

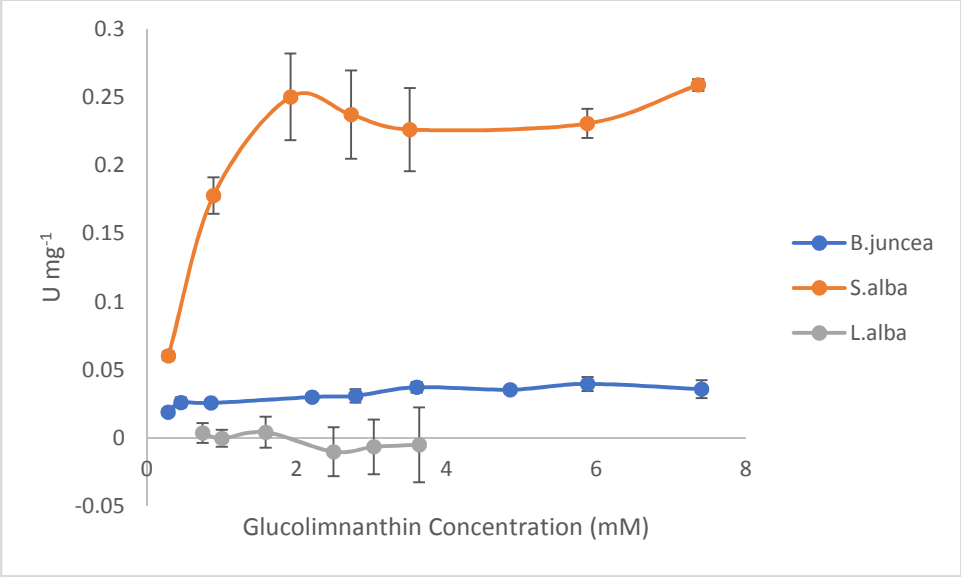

Figure 2.21. Activity curves of *B. juncea* (extract 1), *S. alba*, and *L. alba* myrosinase with glucolimnanthin as a substrate. Error bars represent a 90% confidence interval calculated using a Student's t distribution.

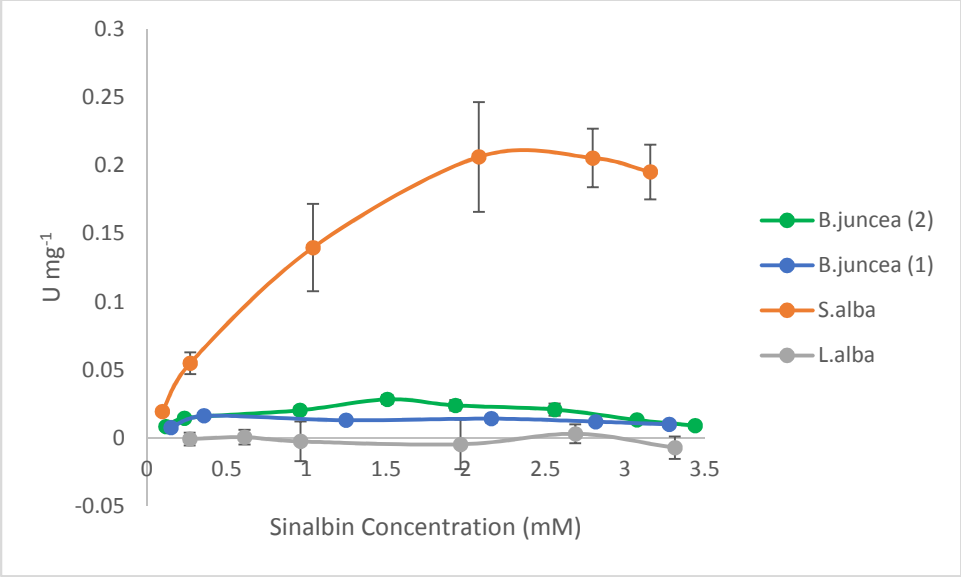

Figure 2.20. Activity curves of *B. juncea* with sinalbin extracts 1 and 2, *S. alba*, and *L. alba* myrosinase with sinalbin as a substrate. Error bars represent a 90% confidence interval calculated using a Student's t distribution.

APPENDIX B: ENZYMATIC ACTIVITY ASSAY DATA
B. juncea Myrosinase with Sinigrin Activity Data

Assay Duration (min)	Concentration of Protein per Sample (mg mL^{-1})	Initial Substrate Concentration (mM)	Final Substrate Concentration (mM)	Activity (U mg^{-1})
149	0.130	0.207	0.0286	$9.23*10^{-3}$
144.5	0.130	0.179	0.0156	$8.72*10^{-3}$
144.5	0.130	0.145	0.0197	$6.65*10^{-3}$
144.5	0.130	0.186	0.0231	$8.67*10^{-3}$
149	0.130	0.366	0.0887	0.0143
149	0.130	0.349	0.0981	0.0129
149	0.130	0.294	0.0974	0.0102
149	0.130	1.25	0.900	0.0181
149	0.130	1.32	0.951	0.0191
149	0.130	1.33	1.06	0.0150
147	0.130	2.36	1.96	0.0207
147	0.130	2.30	1.95	0.0187
147	0.130	2.30	1.95	0.0186
147	0.130	2.96	2.53	0.0223
147	0.130	3.02	2.60	0.0220
147	0.130	3.18	2.74	0.0232
145	0.130	3.86	3.47	0.0204
145	0.130	3.70	3.35	0.0185
145	0.130	3.75	3.42	0.0210

B. juncea Myrosinase with Sinalbin (1) Activity Data

Assay Duration (min)	Concentration of Protein per Sample (mg mL^{-1})	Initial Substrate Concentration (mM)	Final Substrate Concentration (mM)	Activity (U mg^{-1})
145	0.130	0.145	$6.62*10^{-3}$	$7.39*10^{-3}$
145	0.130	0.155	$5.19*10^{-3}$	$7.97*10^{-3}$
145	0.130	0.149	$4.59*10^{-3}$	$7.68*10^{-3}$
149	0.130	0.340	0.0375	0.0156
149	0.130	0.383	0.0552	0.0169
149	0.130	0.349	0.0379	0.0161
149	0.130	1.21	0.977	0.0120
149	0.130	1.15	0.888	0.0137
149	0.130	1.38	1.12	0.0133
147	0.130	2.12	1.81	0.0159
147	0.130	2.21	1.94	0.0140
147	0.130	2.16	1.92	0.0125
147	0.130	2.82	2.57	0.0131
147	0.130	2.96	2.73	0.0120
147	0.130	2.66	2.46	0.0108
145	0.130	3.26	3.06	0.0105
145	0.130	3.28	3.11	$9.26*10^{-3}$
145	0.130	3.29	3.11	$9.82*10^{-3}$

B. juncea Myrosinase with Sinalbin (2) Activity Data

Assay Duration (min)	Concentration of Protein per Sample (mg mL^{-1})	Initial Substrate Concentration (mM)	Final Substrate Concentration (mM)	Activity (U mg^{-1})
146	0.0960	0.116	$5.18x10^{-3}$	$8.27x10^{-3}$
146	0.0960	0.120	$5.22x10^{-3}$	$8.57x10^{-3}$
146	0.0960	0.116	$5.89x10^{-3}$	$8.25x10^{-3}$
146	0.0960	0.233	0.0362	0.01401
146	0.0960	0.239	0.0309	0.0149
146	0.0960	0.234	0.0300	0.0146
143	0.0960	0.964	0.599	0.0266
143	0.0960	0.948	0.633	0.0230
143	0.0960	0.965	0.636	0.0240
153	0.0960	1.52	1.11	0.0284
153	0.0960	1.52	1.07	0.0303
153	0.0960	1.48	1.10	0.0264
143	0.0960	1.94	1.60	0.0251
143	0.0960	1.92	1.63	0.0213
143	0.0960	1.94	1.60	0.0253
143	0.0960	2.53	2.25	0.0206
143	0.0960	2.56	2.31	0.0183
143	0.0960	2.58	2.26	0.0236
143	0.0960	3.02	2.85	0.0127
143	0.0960	3.07	2.90	0.0124
143	0.0960	3.12	2.93	0.0144
153	0.0960	3.41	3.27	$9.32x10^{-3}$
153	0.0960	3.47	3.35	$8.25x10^{-3}$
153	0.0960	3.44	3.31	$9.41x10^{-3}$

B. juncea Myrosinase with Glucolimnanthin Activity Data

Assay Duration (min)	Concentration of Protein per Sample (mg mL^{-1})	Initial Substrate Concentration (mM)	Final Substrate Concentration (mM)	Activity (U mg^{-1})
147	0.0960	0.270	0.0146	0.0181
147	0.0960	0.277	0.0128	0.0187
147	0.0960	0.292	0.0100	0.0200
144	0.0960	0.411	0.0850	0.0236
144	0.0960	0.474	0.0958	0.0274
144	0.0960	0.472	0.0964	0.0272
144	0.130	0.827	0.355	0.0253
144	0.130	0.905	0.391	0.0275
144	0.130	0.834	0.383	0.0241
144	0.130	1.83	1.37	0.0245
144	0.130	1.86	1.43	0.0227
144	0.130	1.84	1.38	0.0246
143	0.130	2.84	2.20	0.0343
143	0.130	2.74	2.20	0.0288
143	0.130	2.77	2.22	0.0296
143	0.130	3.59	2.91	0.0364
143	0.130	3.62	2.88	0.0397
143	0.130	3.60	2.94	0.0353
144	0.0960	4.77	4.27	0.0365
144	0.0960	4.81	4.34	0.0338
144	0.0960	4.97	4.48	0.0358
147	0.0960	5.73	5.21	0.0374
147	0.0960	5.80	5.19	0.0432
147	0.0960	6.12	5.58	0.0384
144	0.0130	7.49	6.03	0.0779
144	0.0130	7.33	6.13	0.0639
144	0.0130	7.56	6.19	0.0736

S. Alba with Sinigrin Activity Data

Assay Duration (min)	Concentration of Protein per Sample (mg mL^{-1})	Initial Substrate Concentration (mM)	Final Substrate Concentration (mM)	Activity (U mg^{-1})
142	0.0322	0.141	0.0226	0.0259
142	0.0322	0.144	0.0181	0.0275
142	0.0322	0.133	0.0162	0.0255
142	0.0322	0.321	0.0301	0.0636
142	0.0322	0.406	0.0443	0.0792
142	0.0322	0.389	0.0363	0.0772
142	0.0322	1.54	0.552	0.216
142	0.0322	1.40	0.499	0.197
142	0.0322	1.44	0.506	0.205
145	0.0322	2.58	1.36	0.261
145	0.0322	2.63	1.40	0.262
145	0.0322	2.50	1.43	0.230
145	0.0322	3.40	2.30	0.236
145	0.0322	3.52	2.21	0.282
145	0.0322	3.41	2.14	0.272
145	0.0322	3.94	2.82	0.239
145	0.0322	3.76	2.53	0.262
145	0.0322	3.67	2.64	0.221

S. Alba with Sinalbin Activity Data

Assay Duration (min)	Concentration of Protein per Sample (mg mL^{-1})	Initial Substrate Concentration (mM)	Final Substrate Concentration (mM)	Activity (U mg^{-1})
142	0.0322	0.102	$8.45x10^{-3}$	0.0205
142	0.0322	0.0970	$7.69x10^{-3}$	0.0195
142	0.0322	0.0901	$6.93x10^{-3}$	0.0192
142	0.0322	0.292	0.0234	0.0587
142	0.0322	0.243	0.0162	0.0496
142	0.0322	0.278	0.0212	0.0561
142	0.0322	0.965	0.421	0.119
142	0.0322	1.06	0.400	0.144
142	0.0322	1.10	0.387	0.156
145	0.0322	2.09	1.01	0.231
145	0.0322	2.03	1.17	0.183
145	0.0322	2.13	1.18	0.204
145	0.0322	2.75	1.84	0.194
145	0.0322	2.86	1.84	0.219
145	0.0322	2.79	1.84	0.203
145	0.0322	3.10	2.23	0.187
145	0.0322	3.21	2.23	0.209
145	0.0322	3.17	2.28	0.190

S. Alba with Glucolimnanthin Activity Data

Assay Duration (min)	Concentration of Protein per Sample (mg mL^{-1})	Initial Substrate Concentration (mM)	Final Substrate Concentration (mM)	Activity (U mg^{-1})
147	0.0322	0.276	<LOD	0.0582
147	0.0322	0.294	<LOD	0.0622
147	0.0322	0.285	<LOD	0.0603
144	0.0322	0.873	0.0535	0.177
144	0.0322	0.864	0.0726	0.171
144	0.0322	0.928	0.0634	0.186
144	0.0322	1.93	0.714	0.262
144	0.0322	1.88	0.674	0.260
144	0.0322	1.94	0.877	0.229
143	0.0322	2.63	1.63	0.216
143	0.0322	2.76	1.65	0.242
143	0.0322	2.79	1.62	0.254
143	0.0322	3.46	2.51	0.206
143	0.0322	3.49	2.41	0.233
143	0.0322	3.58	2.47	0.240
147	0.0322	5.82	4.70	0.237
147	0.0322	5.99	4.89	0.232
147	0.0322	5.84	4.78	0.224
144	0.0322	7.27	6.06	0.262
144	0.0322	7.39	6.20	0.257
144	0.0322	7.43	6.23	0.258

L. Alba with Sinigrin Activity Data

Assay Duration (min)	Concentration of Protein per Sample (mg mL^{-1})	Initial Substrate Concentration (mM)	Final Substrate Concentration (mM)	Activity (U mg^{-1})
150	0.045104	0.316	0.333	-2.40×10^{-3}
150	0.045104	0.314	0.328	-2.00×10^{-3}
150	0.045104	0.334	0.327	1.06×10^{-3}
144	0.045104	0.527	0.520	1.08×10^{-3}
144	0.045104	0.547	0.545	3.80×10^{-4}
144	0.045104	0.530	0.522	1.23×10^{-3}
144	0.045104	0.886	0.933	-7.30×10^{-3}
144	0.045104	0.972	0.968	5.80×10^{-4}
144	0.045104	0.977	0.945	4.90×10^{-3}
144	0.045104	1.99	2.03	-6.30×10^{-3}
144	0.045104	1.98	1.94	6.81×10^{-3}
144	0.045104	1.93	2.02	-0.0147
145	0.045104	2.63	2.59	6.64×10^{-3}
145	0.045104	2.69	2.66	3.93×10^{-3}
145	0.045104	2.74	2.75	-1.50×10^{-3}
145	0.045104	3.31	3.38	-0.0116
145	0.045104	3.39	3.40	-2.00×10^{-3}
145	0.045104	3.24	3.30	-8.30×10^{-3}

L. Alba with Sinalbin Activity Data

Assay Duration (min)	Concentration of Protein per Sample (mg mL^{-1})	Initial Substrate Concentration (mM)	Final Substrate Concentration (mM)	Activity (U mg^{-1})
144	0.045104	0.249	0.276	-4.10×10^{-3}
144	0.045104	0.283	0.281	4.50×10^{-4}
144	0.045104	0.270	0.263	1.14×10^{-3}
144	0.045104	0.592	0.602	-1.50×10^{-3}
144	0.045104	0.620	0.626	-1.00×10^{-3}
144	0.045104	0.624	0.597	4.18×10^{-3}
144	0.045104	0.991	0.947	6.78×10^{-3}
144	0.045104	0.962	0.990	-4.20×10^{-3}
144	0.045104	0.936	1.00	-0.0103
144	0.045104	1.99	2.03	-6.30×10^{-3}
144	0.045104	1.98	1.94	6.81×10^{-3}
144	0.045104	1.93	2.02	-0.0147
145	0.045104	2.63	2.59	6.64×10^{-3}
145	0.045104	2.69	2.66	3.93×10^{-3}
145	0.045104	2.74	2.75	-1.50×10^{-3}
145	0.045104	3.31	3.38	-0.0116
145	0.045104	3.39	3.40	-2.00×10^{-3}
145	0.045104	3.24	3.30	-8.30×10^{-3}

L. Alba with Glucolimnanthin Activity Data

Assay Duration (min)	Concentration of Protein per Sample (mg mL^{-1})	Initial Substrate Concentration (mM)	Final Substrate Concentration (mM)	Activity (U mg^{-1})
144	0.045104	0.685	0.668	$2.59x10^{-3}$
144	0.045104	0.759	0.760	$-2.00x10^{-4}$
144	0.045104	0.781	0.726	$8.40x10^{-3}$
144	0.045104	0.993	0.968	$3.85x10^{-3}$
144	0.045104	0.978	0.999	$-3.10x10^{-3}$
144	0.045104	1.03	1.04	$-1.60x10^{-3}$
149	0.045104	1.53	1.56	$-3.50x10^{-3}$
149	0.045104	1.61	1.54	$9.17x10^{-3}$
149	0.045104	1.61	1.57	$6.88x10^{-3}$
144	0.045104	2.12	2.26	-0.0225
144	0.045104	2.66	2.68	$-3.80x10^{-3}$
144	0.045104	2.70	2.73	$-4.20x10^{-3}$
149	0.045104	2.97	2.93	$4.95x10^{-3}$
149	0.045104	3.02	3.15	-0.0188
149	0.045104	3.09	3.13	$-6.00x10^{-3}$
149	0.045104	3.55	3.49	$9.09x10^{-3}$
149	0.045104	3.58	3.59	$-1.30x10^{-3}$
149	0.045104	3.77	3.93	-0.0229

APPENDIX C: SELECT CHROMATOGRAMS OF ENZYMATIC ACTIVITY

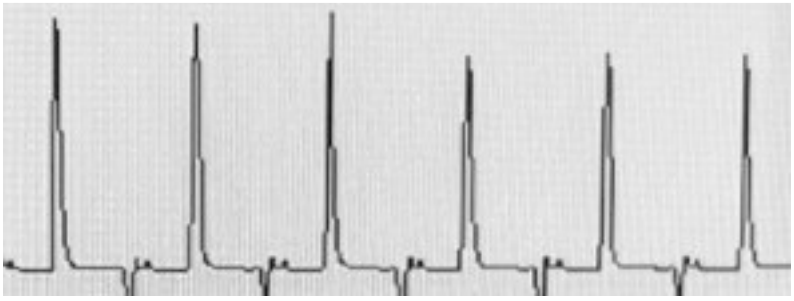

Figure 2.16. Chromatogram of sinalbin hydrolyzed by *B. juncea* myrosinase. The three peaks on the left are the initial measurements and the three lower peaks on the right are the final measurements.

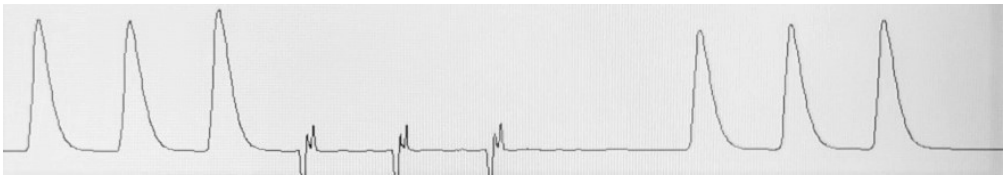

Figure 2.17. Chromatogram of glucolimnanthin hydrolyzed by *B. juncea* myrosinase. The three peaks on the left are the initial measurements and the three lower peaks on the right are the final measurements.

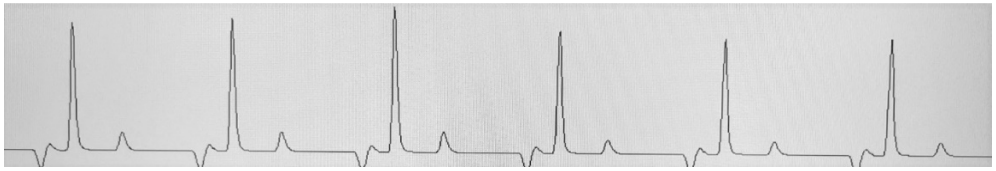

Figure 2.18. Chromatogram of sinigrin hydrolyzed by *B. juncea* myrosinase. The three peaks on the left are the initial measurements and the three lower peaks on the right are the final measurements.