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ANALYSIS OF ROOT EXUDATES FROM *BRASSICA OLERACEA VAR. CAPITATA* AND *BRASSICA RAPA SUBSP. RAPA*

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Abstract

Recent studies have established that plants compete through some form of root-driven signaling, but the underlying mechanism for this competition was not well-studied or understood. This study demonstrates that root exudation drives plant-plant competition (and cooperation) and aims to identify the specific metabolites that function to strategically aid or inhibit neighboring plants' growth. After a 30-day growth period, soil samples were collected, chemcial extraction was completed and analyzed via GCMS. Analysis showed that the levels of excretion for several metabolites fluctuated depending on their growth conditions. This study demonstrates that root exudates are potentially acting as a means of competition in plants.

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Introduction

Historically, plants were thought to grow and function independently. Now it is understood that plants communicate chemically through their root systems.[1] This is done through the excretion of root exudates into the surrounding soil. This underground method of communication is shown to vary and is dependent on the plants that are grown in proximity to one another.[1] Robertson et. al. found plants are secreting compounds from their roots to send messages to neighboring plants, microbes, and insects in the rhizosphere. This aids in competition since plants grown near those of a different variation can modulate their root growth and nutrient uptake from the soil based on the root exudates of the other plants.[2] The plants can also modulate their root exudates to aid the growth of neighboring plants of the same variation.[3]

Until recently, the concept of familial recognition—an organism's ability to recognize its biological relatives—was believed to be uniquely characteristic of animals.[4] Current research has dispelled this misconception. Plants strategically aid in the survival of their relatives by reducing individual root growth when in the proximity of family, enabling multiple family members to grow and prosper together under space and nutrient constraints.[4] Furthermore, in a striking display of competition, plants exhibit greater root growth when surrounded by nonfamilial plants.[4] The fitter of the two nonrelatives gains dominance through greater root growth, increasing its likelihood of survival and reproduction.[4] The study was designed to analyze the root scores, masses and the root exudates of *Brassica oleracea var. capitata* (cabbage) and *Brassica rapa subsp. rapa* (turnips). Due to COVID-19 restrictions only the chemical extraction of the soil samples was able to be completed. This resulted in the analysis the root exudates of when grown under kin and non-kin conditions. The objective of these experiments was to determine the differences in root exudation between plants grown in competition (turnips with cabbage) compared to plants grown near relatives. In general, plants under stress will increase root exudation to facilitate communication with surrounding organisms or defend themselves.[6] In this study it was observed that turnips released higher levels of metabolites overall compared to cabbage plants when grown under competition.

A polar extraction mixture of isopropanol/methanol/water (3:3:2 *v/v/v*) was chosen because root exudates are primarily composed of polar metabolites, and this mixture was demonstrated to extract a wide range of metabolites.[7] Samples were then derivatized to make them suitable for GC/MS analysis because most of the metabolites commonly found in root exudates, such as sugars and hydroxy acids are not sufficiently volatile. A two-step derivatization protocol was followed using methoxymation, then trimethylsilylation to make the samples more suitable for analysis. After disregarding derivative artifacts, 54 metabolites were identified and compared in the soil samples from the following compound classes: organic acids, amino acids, fatty acids, fat metabolites, sterols, nucleobases, sugars, sugar alcohols, and secondary metabolites.

Experimental Methods

Preparation and Planting

The seeds of two different plant species—*Brassica oleracea var. capitata* (cabbage) and *Brassica rapa subsp. rapa* (turnips)—were purchased from Menards in Clive, Iowa. Cabbage and turnips were identified as ideal organisms for the experiment due to their rapid growth times, similar spacing requirements, and watering needs. Cabbage seeds were planted alongside turnip seeds to analyze growth changes and increased root exudation due to interspecific competition. Seeds were grown indoors under lights on a rack, so all plants received the same amount of exposure. To control for baseline growth and root exudate levels when lacking interspecific competition, cabbage and turnip seeds were also planted alongside their own species. Lastly, a plain soil control group was implemented to control for metabolites already present in the soil prior to root exudation (Table 1). Seeds were planted in plastic pots that were approximately two cubic inches in size and had ample gaps for water drainage. In each pot, the two seeds were planted approximately one inch apart and buried in one inch of soil. For the soil, fertilizer-free topsoil, which was purchased from Menards, was used. All treatment groups, including the plain soil control, were planted in replicates of 36 to ensure that minimum conditions were met for later statistical testing.

Growth Period

Once planted, all pots—including the plain soil control pots—were placed on racks in an indoor laboratory. Grow lights were hung above each rack and set to cycle on for 12 hours a day (from 6:00 am to 6:00 pm). Each day, the pots were watered with distilled water to prevent the introduction of metabolites from municipal tap water.

Sample Collection

After 30 days of growth, the roots were harvested. Plants were removed from their pots and set aside to dry. Unfortunately, due to COVID-19 lab shutdowns, the planned analysis of growth through root scores and masses became impossible. However, soil samples were collected for chemical analysis. For the experimental pots with both cabbage and turnip plants, two soil samples were collected from each pot: one from the cabbage side of the pot, and one from the turnip side of the pot. Soil was removed from each pot and stored at -20ºC until analysis.

Soil Preparation and Extraction

Soil samples were prepared based on the method from Swenson *et al.*.[8] A subset of 10 replicates per combination was chosen at random for GC/MS analysis. Prior to extraction, the soil was sieved using a < 2 mm sieve shaker and two grams of soil was set aside for extraction. The 2 g of soil was added to 50 mL polypropylene Falcon tubes and kept on ice. Next, 8 mL of an ice-cold extraction solution composed of isopropanol/methanol/water (3:3:2 *v/v/v*) was added to each tube followed by a spike with 5 µg of the internal standard 2-amino-3-bromo-5-methylbenzoic acid (ABMBA). The samples were shaken using an orbital shaker at 200 rpm for 1 hour on ice then centrifuged at 3220 x *g* for 15 minutes. The supernatant was filtered using 0.45 µm syringe filters (Macherey-Nagel, CHROMAFIL O-45/15 MS) into 20 mL vials and dried using a Savant SpeedVac™ SPD120 (ThermoFisher). The dried extract was resuspended in 200 µL of LC/MS methanol, sonicated, then transferred to 1.5 mL centrifuge tubes and centrifuged at 5000 x *g* for 5 minutes. The supernatant was filtered using 0.22 µm centrifugal membranes (MilliporeSigma™ Ultrafree™-MC) by centrifuging at 5000 x *g* for 3 minutes. A 100 µL aliquot was taken and dried for derivatization.

Sample Derivatization for GC/MS

The preparation and analysis of soil metabolites was performed following the methods described by T.R. Sana, *et al.* and their analysis of rice soil.[5] A mixture of retention index (RI) markers was prepared using fatty acid methyl esters (FAMEs) of C8, C10, C12, C14, C16, C18, C20, C22, C24, C26, C28, and C30 linear chain length, dissolved in chloroform at a concentration of 0.8 mg/mL (C8-C16) and 0.4 mg/mL (C18-C30). One µL of the RI solution was added to each dried sample prior to derivatization. A two-step derivatization protocol was followed using methoximation followed by trimethylsilylation. A 20 µL solution concentrated at 20 mg/mL of 98+% methoxyamine hydrochloride in silylation grade pyridine was added to each sample, vortexed to mix, then heated at 30 ºC for 90 minutes to protect ketone and aldehyde groups. 90 µL of MSTFA with TMCS (1%) was added for the trimethylsilylation of acidic protons, vortexed, and heated at 37 ºC for 30 minutes. All samples underwent a ten-fold dilution with this solvent mixture (1:10 sample:solvent mixture) prior to injection.

Table 1. Plant Growth Combinations for Two Crop Species

Plants Grown in the Same Pot			
Plant 1	**Plant 2**	**Combination Name**	**# of Pots**
Cabbage	Cabbage	CC	36
Cabbage	Turnips	CT (cabbage side) TC (turnip side)	36
Turnips	Turnips	TT	36
None (Plain Soil)	None (Plain Soil)	PS	36
			Total = 144 Pots of Soil

GC/MS Data Acquisition

Derivatized samples were analyzed using an Agilent 7890 Gas Chromatograph (Santa Clara, CA, U.S.A.). A 30 m long, 0.25 µm ID Rtx5Sil-MS column (Restek Corp., Bellefonte, PA, PN 13623), 0.25 µm 5% diphenyl film with a 10 m integrated guard column and controlled by Agilent GC/MS MassHunter Acquisition software. The sample injection volume was 1 µL and the injector was operated in split mode with a split ratio of 10:1 and a split flow of 12 mL/min. The oven parameters were applied as described in Swenson *et al.* with an initial oven temperature of 50 ºC with the following gradient: ramp at 5 ºC min^{-1} to 65 ºC, held for 0.2 min; ramp at 15 ºC min^{-1} to 80 ºC, held for 0.2 min; ramp at 15 ºC min^{-1} to 310 ºC, held for 12 min. Mass spectrometry was performed using an Agilent 5977 single quadrupole mass spectrometer with a 250 ºC transfer line temperature, and the electron ionization source at 70 eV. Mass spectra were acquired in duplicates back-to-back for each sample, acquisition range: *m/z* 20-700 and blanks were run after every sample.

GC/MS Data Analysis

Samples were initially evaluated using Agilent ChemStation software then deconvoluted to obtain a pure mass spectrum using AMDIS (Automated Mass Spectral Deconvolution and Identification System) software. The FAME RI markers were evaluated between replicate runs to ensure consistency in the alignment of retention time and the sensitivity of the instrument. Total ion chromatograms were integrated in ChemStation and each integrated peak was associated with a known compound after deconvolution. The electron ionization (EI) spectra were compared against the NIST 20 NIST/EPA/NIH Electron Ionization Library, resulting in putative metabolite matches. Metabolites were not compared to authentic standards. Some isomers could not be differentiated and are both reported as is the case for l-xlyose/xylose (Figure 8). The average peak areas were recorded for 10 replicates from each growth condition. Heatmaps were constructed based on these averages which were normalized to the highest value from the plain soil in each compound class.

Statistical Analysis

ANOVA statistical tests were determined to be the most appropriate route of analysis for this study. To prepare for testing, the average peak areas of each metabolite *within treatment groups* were calculated. ANOVA tests were conducted for each metabolite of interest to determine whether there was a statistically significant difference in average peak areas among the treatment groups for

any of the metabolites. Unfortunately, no statistical significance was found for any of the metabolites (p-value > 0.05 for all metabolites).

Results and Discussion

Organic Acids

Twelve organic acids were detected in the soil samples (Figure 1). Compared to the cabbage pots, turnip competition pots have increased levels of organic acids as seven organic acids increase while only three increases for cabbage competition pots. The levels of γ-aminobutyric acid (GABA) and succinic acid are higher in Cabbage-Cabbage (CC) pots than the plain soil and competition pots. These acids are secreted by cabbage roots when grown together, but not when grown with turnips. Succinic acid is a growth promoting acid for plants.[9] The levels of succinic acid increased in CC pots only and not Turnip-Turnip (TT) pots or competition pots indicating that this is possibly released to help promote the growth of neighboring cabbage plants (figure 1A). GABA was not detected in the plain soil and is found at the highest concentration when cabbage is grown with cabbage. GABA has been demonstrated to reduce root growth in plants as from a study on *Arabidopsis* roots.[10] Plants are known to reduce root growth to cooperate with one another, however, root analysis is required to confirm if this is the case for the cabbage plants. Plants under stress increase overall root exudation to communicate between neighbors and microbes to help alleviate that stress.[3] What is interesting is that GABA levels are increased when turnips are grown in competition compared to when grown by themselves. Turnip plants may be releasing GABA to alter the root growth of themselves or the neighboring cabbage.

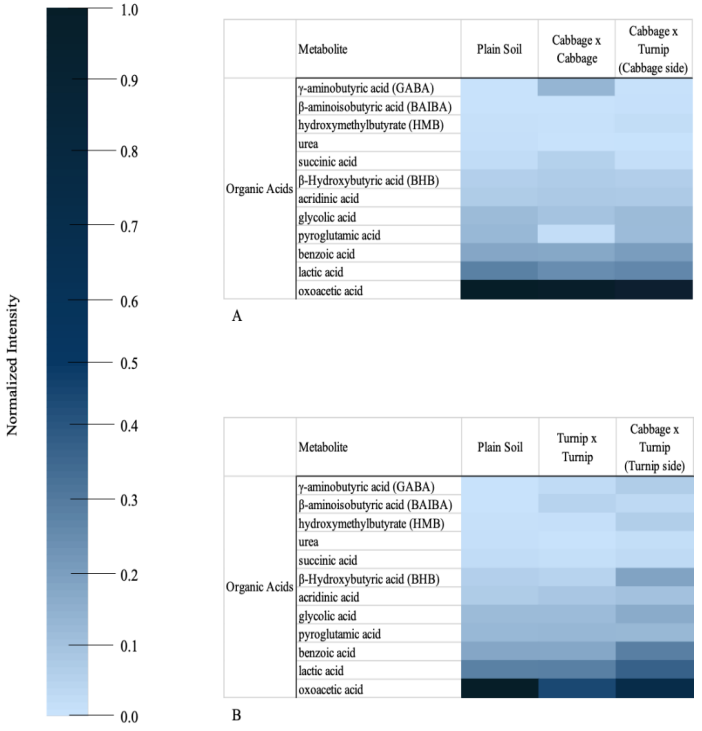

Figure 1. Heatmap of organic acids in soil samples. Twelve organic acids were detected in total and are organized based on the levels in the plain soil. Values are normalized to the largest value in plain soil for each metabolite class which is oxoacetic acid. (A) Comparison of cabbage metabolites under competition. GABA and succinic acid levels are elevated when cabbage plants are grown together and when grown in competition the levels decrease. (B) Comparison of turnip metabolites under competition. GABA and succinic acid levels are increased from the turnip side of competition pots.

Amino Acids

Ten amino acids were detected in total. Isoleucine levels increase the most compared to the other amino acids when cabbage is under competition (Figure 2). Isoleucine has been shown to inhibit root growth when plants are grown in its presence, so it is possible cabbage releases it to reduce the root growth in their neighboring turnip plants.[11/12] Isoleucine appears to play a role for cabbage plants when under competition. Isoleucine levels also increase in turnip competition pots, however not to the same level.

In response to competition, turnips release more of all amino acids except for N-N-diacetyl glycine indicating that turnips increase root exudation of amino acids when in competition (Figure 2B). Valine levels only increase when turnips are grown in competition which differs from cabbage. Valine also inhibits root growth and is increased in turnips when grown under competition indicating it may be used as defense in turnip plants against cabbage.[11/12]

Fatty Acids

Eleven fatty acids were detected in the soil samples. Fatty acids levels do not change much overall in cabbage pots for both growing conditions (Figure 3). Margaric acid, myristic acid, and nonanoic acid levels are all decreased under both growing conditions indicating these are just nutrients and do not play a role in competition (figure 3A). A similar trend emerges when turnip

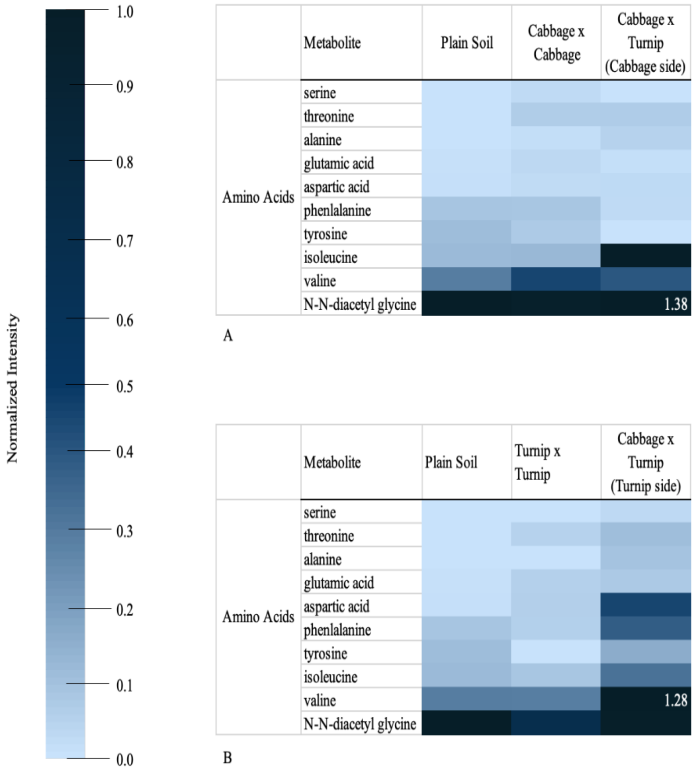

Figure 2. Heatmap of the amino acids in soil samples. Ten amino acids were detected in total and are organized based on the levels in the plain soil. Values are normalized to the largest value in plain soil for each metabolite class which is N-N-diacetyl glycine. (A) Comparison of cabbage metabolites under competition. Valine levels are increased when cabbage is grown together. Under competition isoleucine and N-N-diacetyl glycine levels are increased. (B) Comparison of turnip metabolites under competition. When turnips are grown together isoleucine and N-N-diacetyl glycine levels decrease. Under competition, all amino acid levels increase except for N-N-diacetyl glycine.

plants are grown together as there is a general decrease in fatty acid levels when turnip plants are grown together (figure 3B). In general, fatty acids do not appear to play a large role in competition between these plants.

Fat Metabolites and Sterols

Two fat metabolic breakdown products were detected in the soil samples including glycerol and glycerol monostearate. Based on figure 4 A & B, both cabbage and turnip plants follow a similar trend in that glycerol and glycerol monostearate both increase when under competition. Glycerol monostearate may be important in competition and cabbage plants release more of it than turnip plants.

Four sterols were detected including stigmasterol, campesterol, cholesterol, and β-sitosterol. Based on figure 5 A & B, the levels of root exudation of these metabolites are similar in both cabbage and turnip plants (Figure 5). The levels of cholesterol and β-sitosterol decrease in CC and TT pots indicating these are taken up by the roots. All sterol levels increase under competition with turnips releasing more than cabbage.

Nucleobases and Sugar Alcohols

Two nucleobases were detected, thymine and uracil. Based on figure 6 A & B, both cabbage and turnips release higher levels of both nucleobases when under competition. Additionally, turnips release higher levels of nucleobases then cabbage. The levels of uracil increase when cabbage plants are grown together which isn't the case for turnips.

Two sugar alcohols were detected, myo-inositol and mannitol/glucitol. Both turnip and cabbage plants uptake mannitol/glucitol from the soil when not in competition (Figure 7). Under competition cabbage plants release more myo-inositol. In turnips, mannitol/glucitol levels decrease under competition compared to when turnips were grown together indicating that the plant is taking up mannitol/glucitol when under stress from competition.

Sugars

Nine sugars were detected in total. Comparing cabbage to turnip plants xylulose, sucrose, and allose are released at higher levels in turnips. Arabinose, allose, and glucose are released at higher levels in cabbage when not in competition (figures 8 A & B). When cabbage plants are under competition, the sugar levels detected decrease for all these sugars indicating that less is released when under competition. For turnips, the opposite is true as xylulose, sucrose, and allose levels increase when under competition. Xylu-

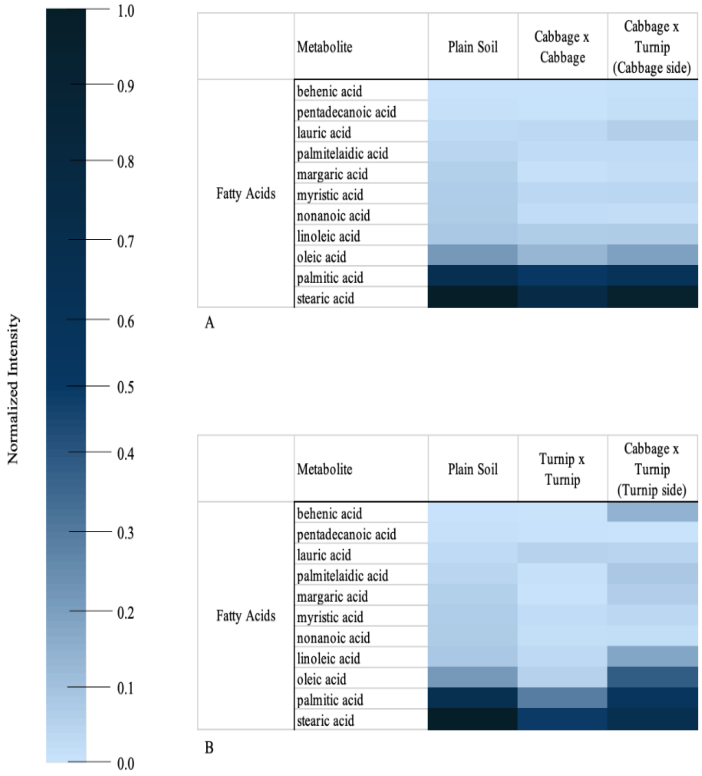

Figure 3. Heatmap of the fatty acids in soil samples. Eleven fatty acids were detected in total and are organized based on the levels in the plain soil. Values are normalized to the largest value in plain soil for each metabolite class which is stearic acid. (A) Comparison of cabbage metabolites under competition. Margaric acid, myristic acid, and nonanoic acid levels are all decreased under both growing conditions. (B) Comparison of turnip metabolites under competition. When turnips are grown together, the levels for eight out of eleven fatty acids decrease. Under competition eight fatty acids increase in levels including margaric acid.

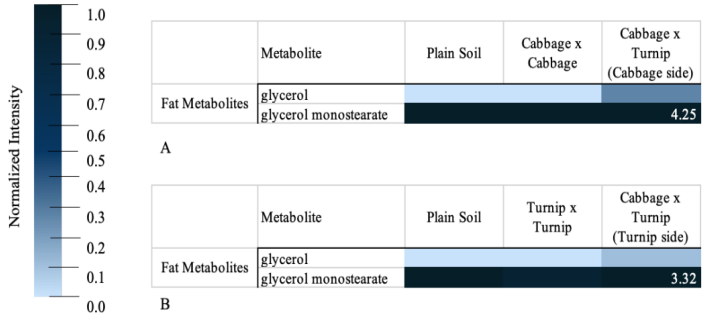

Figure 4. Heatmap of the fat metabolic breakdown products detected in soil samples. Two fatty metabolites were detected in total and are organized based on the levels in the plain soil. Values are normalized to the largest value in plain soil for each metabolite class which is glycerol monostearate. (A) Comparison of cabbage metabolites under competition. Both glycerol and glycerol monostearate levels increase when under competition though glycerol monostearate levels increase much more. (B) Comparison of turnip metabolites under competition. A similar trend can be observed for turnip plants as for cabbage.

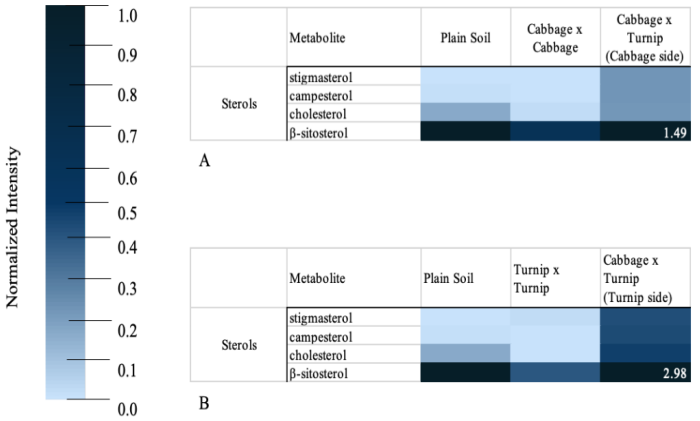

Figure 5. Heatmap of sterols detected in soil samples. Four sterols were detected in total and are organized based on the levels in the plain soil. Values are normalized to the largest value in plain soil for each metabolite class which is β-sitosterol. (A) Comparison of cabbage metabolites under competition. Both cholesterol and β-sitosterol levels decrease when cabbage is grown together while campesterol and stigmasterol levels match the plain soil. Under competition all sterol levels increase. (B) Comparison of turnip metabolites under competition. The same trend is observed for turnips though turnips release higher levels of sterols under competition compared to cabbages plants.

lose is released by turnips in much greater levels overall compared to cabbage which doesn't release this metabolite, but the role of xylulose is not known in root exudation.

Allose has been shown to reduce root growth and allose levels are increased when turnips are under competition with cabbage and may function in defense.[13] When comparing the sugar released at the highest levels in each plant, glucose is released by cabbage plants and is taken up by turnip roots while the opposite is true for sucrose. However, in both cases there isn't a great difference between competition and non-competition levels indicating these could just be commonly exuded sugars by each plant species.

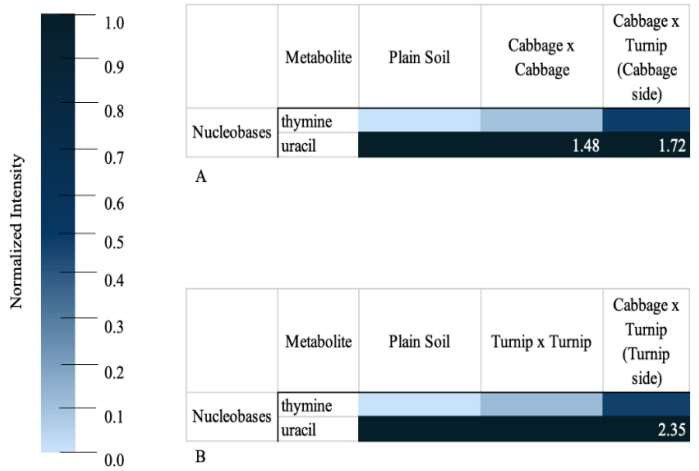

Figure 6. Heatmap of the nucleobases detected in soil samples. Two nucleobases were detected in total and are organized based on the levels in the plain soil. Values are normalized to the largest value in plain soil for each metabolite class which is uracil. (A) Comparison of cabbage metabolites under competition. Both thymine and uracil levels increase under competition. Cabbage plants release higher levels of uracil even when grown together compared to the plain soil. (B) Comparison of turnip metabolites under competition. The same trend is observed for turnip plants though the levels of uracil increase more than those for cabbage plants.

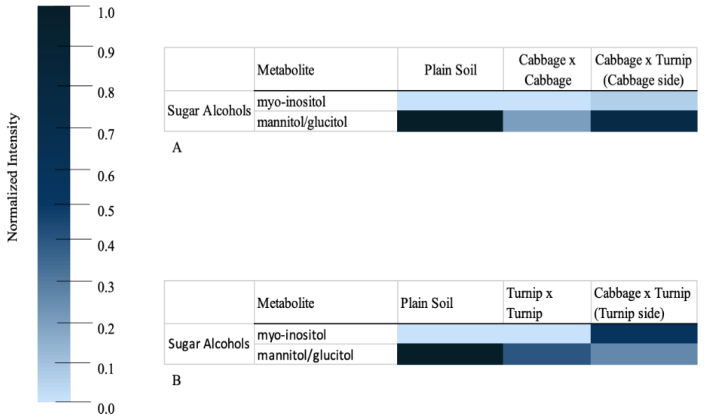

Figure 7. Heatmap of the sugar alcohols detected in soil samples. Two sugar alcohols were detected in total and are organized based on the levels in the plain soil. Values are normalized to the largest value in plain soil for each metabolite class which is mannitol/glucitol. (A) Comparison of cabbage metabolites under competition. Mannitol/glucitol levels are decreased when cabbage plants are grown together but match the plain soil under competition. Myo-inositol levels increase under competition. (B) Comparison of turnip metabolites under competition. Mannitol/glucitol levels decrease when turnips are grown together and decrease more when under competition. Myo-inositol levels increase under competition.

Secondary Metabolites

Eight secondary metabolites were detected in total. In general, when kin plants are grown together the levels of secondary metabolites decrease compared to the plain soil. There are three exceptions for cabbage plants which release slightly higher levels of isopimaric acid, α-amyrin, and asterbatanoside A when grown with kin though these all increase more when in competition (figure 9A). When turnips are grown together, α-amyrin levels are slightly increased, but again under competition they increase even more. A-amyrin has been shown to inhibit root growth in some plants so again it may function in defense.[14] Again, turnips release more metabolites under competition compared to cabbage plants.

Conclusions

Ultimately, while statistical significance was not obtained, this study presents interesting differences in soil metabolite excretion when plants of different varieties are grown together. There was a notable increase in the amount of the primary metabolites that were present in the soil of non-kin plants. In addition, there was an increase in organic acids, sugars, amino acids, and some secondary metabolites. These include GABA, valine, isoleucine, allose, and α-amyrin which are involved in root growth reduction. Overall, there was a larger increase in the root exudates of turnips compared to the cabbage when grown under non-kin conditions. This indicates that turnips are more aggressive than cabbage when grown in competition with one another. The increase in GABA and Valine production by turnips under competition demonstrates that the turnips are working to reduce the root growth in the soil. The

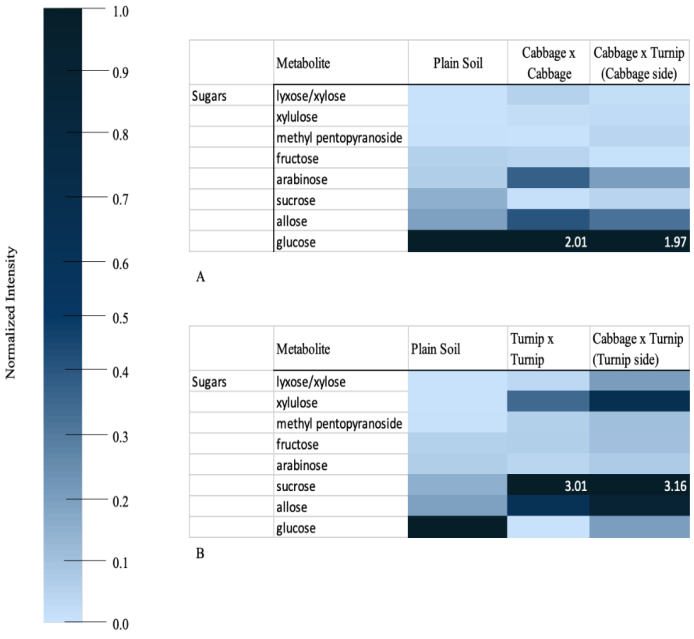

Figure 8. Heatmap of the sugars detected in soil samples. Nine sugars were detected in total and are organized based on the levels in the plain soil. Values are normalized to the largest value in plain soil for each metabolite class which is glucose. (A) Comparison of cabbage metabolites under competition. Arabinose, allose, and glucose increase in levels when cabbage is grown together while sucrose levels decrease. Arabinose, allose, and glucose levels are higher than plain soil but are lower than cabbage grown together. (B) Comparison of turnip metabolites under competition. Xylulose, sucrose, and allose levels all increase when turnips are grown together while glucose levels decrease. Under competition, sugar levels increase for all sugars except fructose and arabinose whose levels match the plain soil.

root score data could be used to draw conclusions on if the turnip root growth is affected by competition.

Small sample sizes limited the study's likelihood of achieving statistical significance. Small sample sizes require larger differences to produce low p-values. Derivatizing and analyzing more of the existing samples by GC/MS could produce statistically significant results. Furthermore, comparing to authentic standards may result in more confident metabolic ID. In addition to providing insight into "chemical warfare" in plants, this study also demonstrates novel techniques for soil metabolite extraction.

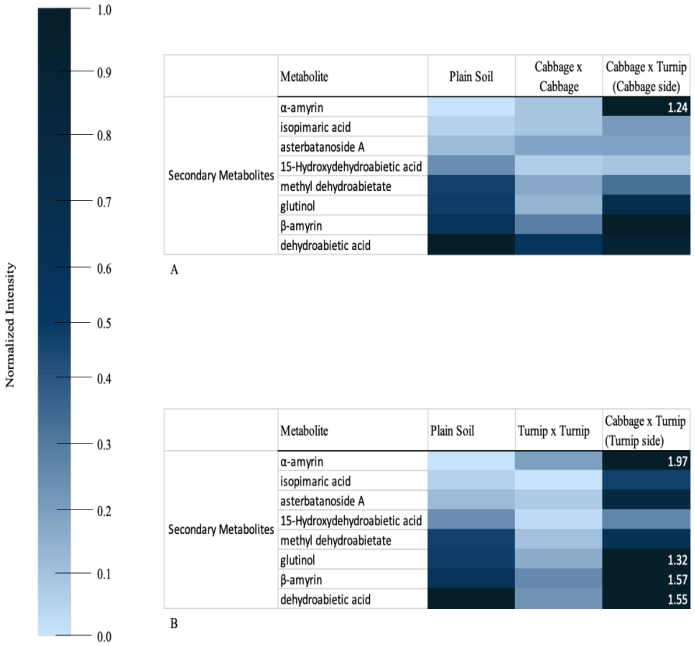

Figure 9. Heatmap of the secondary metabolites detected in soil samples. Eight secondary metabolites were detected in total and are organized based on the levels in the plain soil. Values are normalized to the largest value in plain soil for each metabolite class which is dehydroabietic acid. (A) Comparison of cabbage metabolites under competition. When cabbage are grown together, α-amyrin and asterbatanoside A increase while all other secondary metabolites decrease. Under competition, isopimaric acid, α-amyrin, and asterbatanoside A increase in levels. (B) Comparison of turnip metabolites under competition. When turnips are grown together, α-amyrin levels increase while all other secondary metabolite levels decrease. Under competition, all secondary metabolite levels increase except for 15-hydroxydehydroabietic acid which remains the same as plain soil.

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