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Natural Products In Weed Control: Allyl-Isothiocyanate Effects On In Vitro Velvetleaf Seedling Growth And The Influence Of Mustard Seed Meal On Soil Bacterial Populations

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ABSTRACT:

Past research has shown that allyl isothiocyanate (AITC) and its sister ITC compounds are effective alternatives to herbicides in reducing growth in weed seedlings. In this project the effects of mustard seed meal (MSM), a source of AITC, on plant-associated soil bacteria populations was assessed along with the effect of AITC on velvetleaf seed *in vitro* germination. Results show that MSM reduces bacterial levels in the soil. Velvetleaf seed germination was inhibited by micromolar levels of AITC. These results suggest that MSM has the potential to impact velvetleaf growth in the field as well as becoming a weed management tool.

INTRODUCTION:

The topic of weed control has long been a thorn in the side of agriculture. Weeds by definitions are plants that are unwanted and grow in competition to cultivated plants; they are both harmful to the plant (takes space and nutrients) and are difficult to remove efficiently. The weed that is the primary focus of my research is *Abutilon theophrasti* or velvetleaf.

Originating from southern Asia, velvetleaf has migrated to North America becoming an invasive species particularly in the Eastern and Midwestern United States (Dorr 2011). The weed prefers rich and cultivated soils commonly found in an agricultural environment and is harmful to agricultural crops particularly corn and soybeans (Richard et al. 1997). In the case of corn, it negatively impacts the growth rate so much so that product yield decreases by up to 34% if left untreated (Hameed et al. 2001). Not only does this weed cost millions of dollars' worth of damage control each year, it also wreaks havoc on surrounding crops. However, it has been verified through research that this weed is indeed controllable through the use of herbicides (Davis et al. 2005).

Agriculture is a vital part on our daily lives, thus the practices that surround the field must be environmentally sustainable in the long run and beneficial to our long-term health. Often times, synthetically produced chemicals that are then used as pesticides are shown to be carcinogenic. Not only this, the adverse influence spreads further by negatively impacting beneficial insect populations that assist in the pollination of plants. Thus, while natural products are not entirely devoid of these adverse effects, they pose significantly less health concerns as while being more environmentally beneficial than their synthetic counterparts (Gold 1992).

In this experiment the natural product I will be focusing on will be allyl isothiocyanate (AITC). This colorless oil is directly responsible for the pungent smell/taste of: mustard, radish, wasabi (Romanowski and Klenk 2000). In fact there are already existing studies which focus on radish and wasabi related isothiocyanate (ITC) chemicals. In one study, application of AITC from the roots of Japanese and Korean wasabi was shown to be an effective method for inhibiting bacteria (Shin et al. 2004). This experiment showed that the use of AITC against the gram negative bacterium *H. pylori* could decrease the level of bacterial activity in a dose-dependent fashion depending on the part of the wasabi plant from which the AITC was recovered. These results show that ITC based compounds possess the capability to effectively kill bacteria. This is important since soil bacteria whether that be in the roots or leaves, have significant influence on plant nutrition/health and growth.

Another study showed that plant susceptibility to ITC compounds depended mainly on seed size (Petersen et al. 2001). In this experiment, six different types of ITC compounds were made into turnip-rape mulch. In this study, the smaller seeds seemed to be more sensitive to the effects of the mulch and the general suppression of weed germination was primarily due to the high amounts of ITC present in the mulch. This study is particularly interesting because it showed that the gaseous phase (vapor) of ITC had the most effective inhibitive effects compared to other phases.

This experiment's primary focus is on mustard seed meal (MSM) and its relatedness to AITC. This is due to MSM being the source of AITC (Romanowski and Klenk 2000). More specifically, the source of meal I used for this experiment: *Brassica juncea* Pacific Gold has been shown to be an effective weed deterrent and contains AITC as the dominant ITC compound (Handiseni et al. 2001). My experiment builds upon past research because I will be exploring how the implementation of AITC as well as mustard seed meal affects various factors such as seedling germination length and bacterial populations on plant roots. Past research has already shown the herbicidal tendencies of mustard seed meal. Therefore, in addition to exploring these tendencies, I will also be seeing what other positive factors mustard seed meal has to offer, more specifically: its effect on soil bacteria levels. A variety of soil bacteria termed rhizobacteria interact with plant roots influencing plant growth. Thus it is possible that AITC-mediated alterations in the soil flora may impact the successful establishment of weeds in agricultural systems.

In order to explore the uses of MSM application as a weed control strategy, this research project will be split into two parts. The first part will be testing the presence/absence of MSM in soil and determining if it has any indirect effects on soil bacterial populations (Fig. 1). The second part will be to study the direct effects of varying levels of AITC on velvetleaf seed germination and short-term growth (Fig.1). The first aim seeks to demonstrate whether the incorporation of MSM reduces bacteria levels in the soil. The second aim is to test the inhibitory effects of the volatile AITC on weed seeds. These two aims are related to one another since both aims affect resulting velvetleaf growth.

The overall goal of this research is to compare if there is a significant difference in bacteria levels in soil containing mustard seed meal and soil lacking MSM as well as to see how the compound AITC affects the germination and growth of velvetleaf seeds.

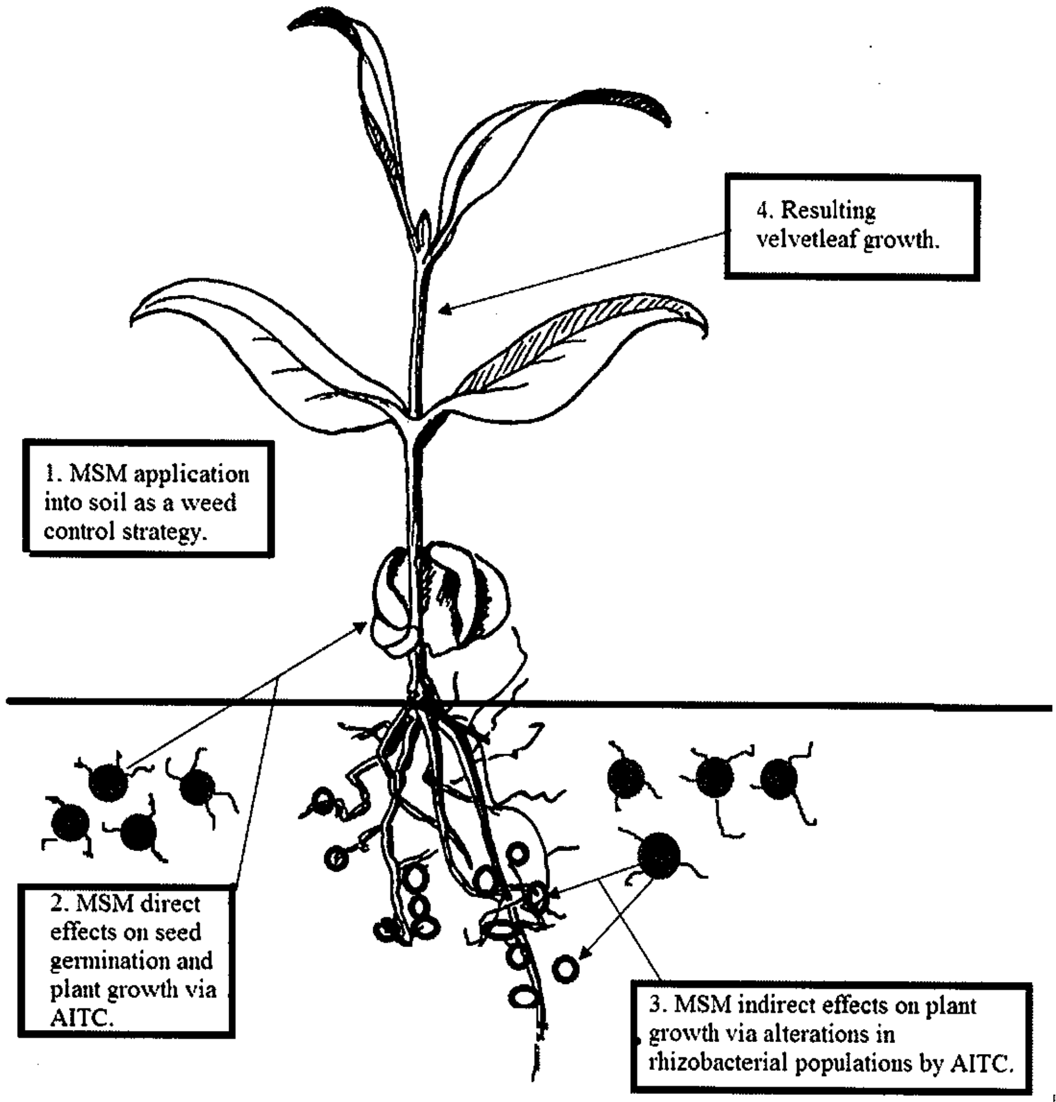

Figure 1: Possible direct and indirect effects of MSM on velvetleaf plant growth. Image taken from: <http://www.enhg.org/bulletin/b32/32_06.jpg>.

METHODOLOGY:

Assessing MSM effects on soil and seedling-associated bacterial populations:

Soil plate preparation and seed planting with/without MSM:

Air-dried silt loam sieved soil (obtained from the Andrews dairy fields) was mixed with MSM (*Brassica juncea* Pacific Gold provided by Jim Davis at the University of Idaho Canola and Mustard Program) 1 g MSM + 100g soil and transferred to square containers (6 cm x6 cm). Control plants contained soil lacking MSM. Each plate received 24 milliliters of sterile distilled water. Each plate contained a plastic divider in the center to separate soil into soil containing seedlings and soil lacking seedlings (Figure 2). Surface disinfested velvetleaf seeds were heat-shocked at 65°C for 10 minutes and then planted in two rows (~5 seeds per row) in one half of each soil plate. Once the planting process was complete, the plates were sealed with Parafilm™ and incubated for 48 hours at 28°C.

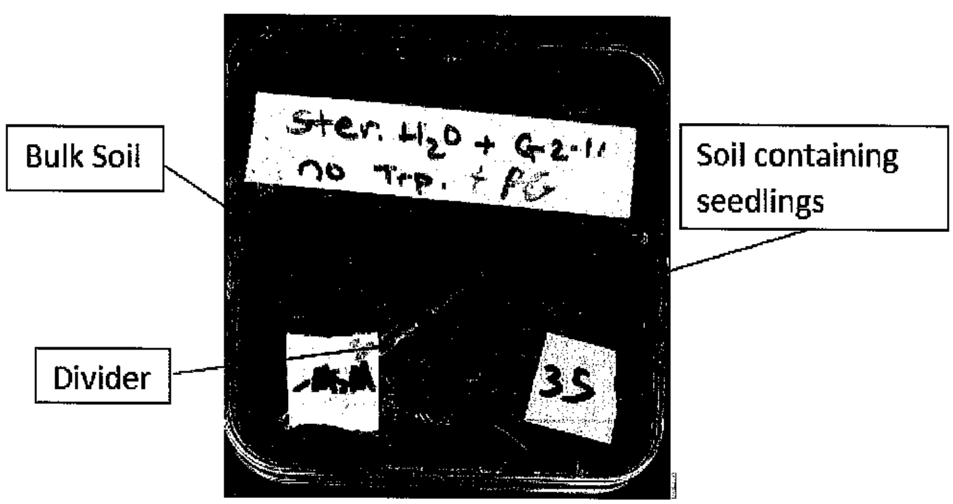

Figure 2: Sample soil plate: note the partition in the plate with bulk soil on one side and soil containing seedlings on the other side.

Enumeration of Bulk Soil & Seedling-Associated Bacterial Populations:

After incubation of the soil plates 1 gram of bulk soil was transferred from each plate into tubes of 0.1% peptone and subjected to serial dilution in this same buffer. 0.1ml aliquots from the dilution tube were spread plated in triplicate on Tryptic Soy Agar (TSA) for the non-selective cultivation of bacteria. Pseudosel Agar was used for the growth of gram negative bacteria (particularly pseudomonads), a common group of bacteria associated with plant roots. Pseudosel agar consists of 1 L of deionized waster, 20 g of protease peptone, 15 mL of glycerol, 1.5 g of anhydrous $MgSO_4$, 1.2 g of KH_2PO_4, 0.3 g of centrimide, 15 g of agar. Centrimide has an inhibitory effect on many microorganism and selects for the growth of gram negative bacteria (Lambe and Stewart 1972). To assess levels of bacteria associated with the germinated seedlings, the seedlings were removed from the soil, weighed, and transferred to a tube of 0.1% peptone and vigorously mixed. The resulting suspension was serially diluted in 0.1% peptone and spread plated in triplicate on TSA and Pseudosel agar. All plates were incubated at 28°C for 48 hours to obtain CFU/g dry bulk soil or CFU/g seedling. Some bulk soil was also dried at 80°C for 24 hours to determine the moisture content of the bulk soil. This entire experiment was performed twice.

Assessing AITC effects on velvetleaf seed germination and seedling growth:

Agar plates were prepared containing 0-200 micromolar AITC using a stock solution of AITC in 50% ethanol (2 plates/AITC concentration). Surface disinfested velvetleaf seeds were transferred to each plate in two rows (four seeds per row) and the plates incubated for 48 hours. Half of the plates were sealed in Parafilm™, the other half were unsealed. After incubation, the length of each seedling (including the hypocotyl and radicle) was measured using digital calipers. This entire experiment was performed three times.

RESULTS:

In terms of the sealed vs. unsealed results, there was generally a negative correlation in % seed germination vs. increasing AITC concentration. The percentages are based off a total of 48 seeds from 3 independent experiments. It is important to note that at all AITC concentrations the unsealed plates always had a higher rate of % germination when compared to the corresponding sealed plates (Fig. 3). There is one instance in the sealed condition at 200 μm where the % germination is higher than its previous value at 150 μm

For the treatment concerned with the effects of AITC on average root length, results were taken from 3 independent experiments. The number of plants used for each mean varied due to the different levels of AITC resulting in different germination rates. It is important to note that the general trend for both the sealed and unsealed plates is a negative correlation between mean root length and AITC concentration. There is one instance in the sealed condition at 100 μm where the % germination was higher than the germination rate of 50 μm. In all cases, the unsealed mean root lengths are always higher than the sealed root lengths values for the corresponding AITC concentration (Fig. 4).

The statistical test I ran for this condition was the one-way ANOVA. This was done in order to see if the mean root lengths differed significantly between various AITC treatments. The test showed that mean root length did differ significantly from various AITC treatments ($p<0.05$). Additionally, Tukey's test (Post hoc) was run in order to confirm where differences occurred in between the different AITC conditions. As shown in Fig. 4, means within the same condition sharing the same letter were not considered statistically significant ($p<0.05$). If no letters are shared, means were statistically significant. Sealed data was run separately from unsealed data.

For the treatment concerning the bacterial populations in bulk soil or associated soil seedlings with and without mustard seed meal (MSM), each bar represents the average of 6 replicates from 2 independent experiments (Fig. 5). Pair wise comparisons were made between different agar media with or without MSM.

In both bulk soil and seedling samples TSA supported the growth of higher populations of bacteria as compared Pseudosel. In terms of MSM+ and MSM -, all cases involving soil lacking MSM always had a higher average CFU/g soil (seedling). For the relative bacteria levels recovered from seedlings and bulk soil, bulk soil always had more CFU/g than compared to CFU/g seedling in the respective category.

The statistical test I ran for this condition, was the two-way ANOVA in order to know if populations were significantly different between (±) MSM treatments for a given soil/seedling source and media type. The test showed that populations were indeed statistically significant in the MSM soil treatments ($p<0.05$). A post hoc test could not be run for this condition because there are less than 3 groups for each comparison. However, a pairwise comparison was run between the TSA and Pseudosel plates as well as the (±) MSM. The comparison showed that while TSA and Pseudosel were statistically significant from one another ($p<0.05$), the presence or absence of mustard meal had no statistical significance ($p>0.05$).

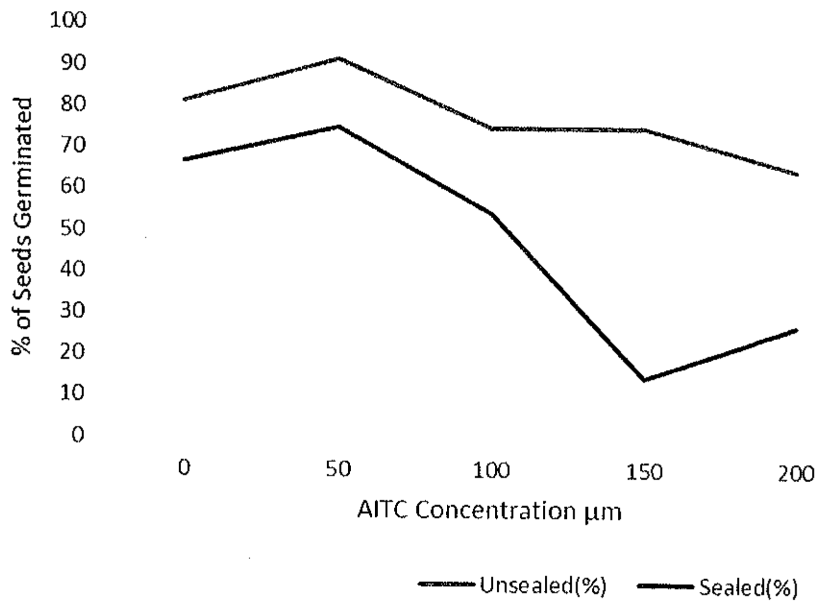

Figure 3: Velvetleaf seed germination in the presence of varying levels of allyl-isothiocyanate (AITC): Each percentage is the overall germination of a total of 48 seeds from 3 independent experiments. Seeds were germinated under sealed or unsealed plate conditions.

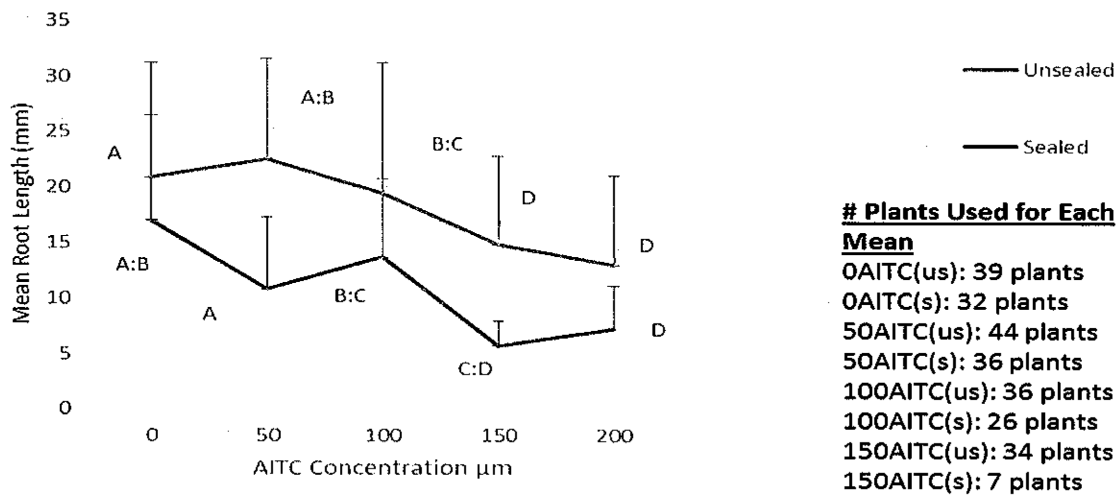

Figure 4: Velvetleaf seedling length in the presence of varying levels of allyl-isothiocyanate: Each mean is from 3 independent experiments. Means with same letter within the same category are not statistically different ($p<0.05$) by one-way ANOVA. Error bars represent the standard deviation for each mean.

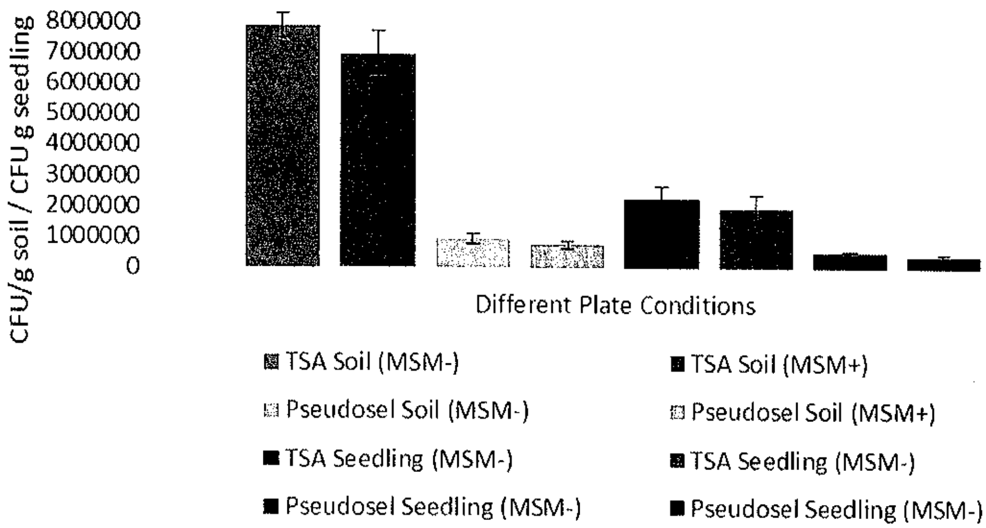

Figure 5: Bacterial populations in bulk soil or associated soil seedlings with and without mustard seed meal (MSM): Bacteria were cultured on Tryptic Soy Agar (TSA) or Pseudosel. Each bar represents the average of 6 replicates from 2 independent experiments. Error bars represent the standard deviation for each mean.

DISCUSSSION:

Based on the results, two conclusions can be made. The first conclusion is that AITC inhibits seed germination of velvetleaf in both sealed and unsealed conditions. As mentioned earlier from the results of Figure 3, the general trend line for both lines is negative. The lowest concentrations of AITC for both conditions shows the highest percentage of germinated seedlings, while steadily decreasing with each of the following AITC treatments. In terms of the sealed vs. unsealed conditions, the unsealed plates generally had higher percentages of seed germination across the board when compared to the sealed plates. This is to be expected since AITC experiences less dissipation in the sealed plates. This allows for greater and more potent effects of this volatile chemical. A study showed that the ITCs in a turnip-rape mulch was the primary reason for suppression in weed growth (Petersen et al. 2001).

As an extension to this, Figure 4 also illustrates the inhibiting effects of AITC in both the sealed and unsealed conditions. There are some exceptions on the sealed line where some of the lower concentrations of AITC have higher average root lengths than higher concentrations. Once again however, the general trend for increasing AITC concentration is the decrease of average root length. Another important trend to notice is that the number of seedlings available for use in mean root length measurement decreased as the concentration of AITC went up. This complements the data from Figure 3, as well as showing that AITC not only inhibits seed germination but also results in a decrease in the length of the seedling's root. This is supported by a study that observed the germination of peas, wheat, and rape after application of AITC. The presence of AITC was shown to change nitrogen and sugar metabolism which consequently reduced respiration and anaerobic glycolysis by ~30% (Leblová-Svobodová and Koštíř 1962).

The second conclusion that can be made is that MSM treatment of the soil decreases the amount of CFU/g in both soil and seedlings. Through the comparison of four different cases, all cases showed that plates with MSM had less CFU/ g in both soil and seedling treatments. When comparing Pseudosel plates to TSA plates, they always had a much lower CFU/ g calculation. This is not out of the ordinary however, since Pseudosel selects for only the growth of gram negative bacteria present in the soil. In all cases, soil plates had greater CFU/ g than their seedling counterparts. Analysis of bulk soil vs rhizosphere soil revealed differences in the numbers of types of bacteria present (Söderberg et al. 2004). This is consistent with our results that showed the CFU/g soil being higher in bulk soil than in the seedling samples.

In terms of significance, the two-way ANOVA showed significance for the MSM soil treatments ($p<0.05$). When using a pairwise comparison to see significance between TSA and Pseudosel agar as well as +/- MSM. The comparison showed that between agar mediums, there was significance ($p<0.05$). However, when comparing whether the absence or presence of MSM there was no significance ($p>0.05$). This doesn't mean that the use of MSM doesn't not affect the CFU/ g content. The statistical significance being absent, could result in the data needing to be dramatically different. Since the CFU/ g numbers I am working with, range in the tens of millions, extremely large differences would need to be made to bring about this statistical significance. One study showed that the addition of allyl-ITCs, there was not only a dramatic decrease in fungal populations (~85%) but also a less dramatic impact on bacterial populations. In fact there was an increase in the proportions of *Firmicutes,* which are bacteria known to be antagonistic to plant pathogens (Hu et al. 2014). This study shows that using different types of ITC can have unique impacts on soil microorganisms, enabling helpful microorganisms while targeting detrimental ones.

affected by dissipation effects. The goal would be to test how differences in dissipation effects in gas vs. liquid ITC ultimately affects mean average root length.

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