Codependence: The influence of targeted soil inoculation on vegetative growth, chemical composition, and fruit fermentation

By

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Abstract

In order to ensure food security for the steadily increasing population, intensive, carefully managed agricultural practices are a necessity. In an attempt to increase crop yields, mineral and organic fertilizers have been excessively and incorrectly applied, resulting in problems for the neighbouring water systems. To avoid the environmental damages associated with excessive fertilizer application, other means of nutrient recycling and retention have been investigated.

Biochar, a porous pyrogenic material, has been introduced to soil systems and has improved both the physical and chemical properties of the soil. Soil inoculation, or the addition of plant growth promoting bacteria, has been utilized with species specific results. The successful combination of these two techniques has produced a biochar-microbial composite that has been applied to the soil with varying results on the vegetative growth of plants. An investigation into the volatile impact of the inoculation revealed that the volatile profile of marigold shoots changed, while the nitrogen uptake by a given plant was decreased or unaffected, depending on the plant species. In order to predict how these bacterial strains might behave in a fermentation environment, wine was co-fermented with yeast and bacteria. Common and predictable differences were observed in the LC-MS profiles of red and white wine when a bacterial strain was introduced to the fermentation.

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List of Abbreviations

SOM	Soil Organic Matter
CEC	Cationic Exchange Capacity
BC	Biochar
NB	Nutrient Broth
NA	Nature Aid Fertilizer
D. tsuru	*Delftia tsuruhatenesis*
P. rho	*Pseudomonas rhodesiae*
Meth	*Methylobacterium* sp.
PR3(F)	Planter's Ridge Soil Sample 3 (Fuzzy)
PR2(P)	Planter's Ridge Soil Sample 2 (Pink)
Multi(B)	Isolated from multiple locations (Brown)
PR(Y)	Planter's Ridge (Yellow)
LALA3(C)	L'Acadie Vineyards L'Acadie Blanc Soil Sample 3 (Clear)
BBSE6(BO)	Benjamin Bridge Soil Extract 6 (Brown-Orange)

1. INTRODUCTION

1.0. Agriculture and Viticulture

Agriculture, or the practice of farming, is an essential means of food production.[1] The supply of food for the ever-growing global population is a complex, nuanced system that is constantly under development. With recent advances in the understanding of agricultural practices, sustainable methods of crop production are an absolute necessity to ensure food security. A key resource required for extensive agriculture is land.[2] In response to this demand, many countries have begun to make international land deals in an effort to increase their productivity. These international land deals have been criticised as a means of exploiting rural communities in third world countries for cheap labour and land.[3] To avoid this issue, the development of agricultural practices that increase crop productivity on smaller land masses are required. Another resource required for extensive agriculture is access to water. Water scarcity has become a global risk in recent years, and is especially damaging to agriculture.[4] To ensure food security, sustainable water use must be implemented.

One of the crops particularly impacted by water shortages are grapevines. Viticulture, the practice of grape farming, is responsible for the production of table grapes, raisins, and wine. In many countries, wine is the primary product of viticulture and rich traditions are associated with winemaking. The growth and development of both the plant and fruit are largely dependent on the environmental conditions.[5] Due to the sensitivity of the crop, it has been utilized as a model for the assessment of water stress conditions and development of future irrigation systems. Complex microbial interactions with crops have been proposed as a method to deal with space and water constraints, and

grapevines have once again been utilized as a model to study the soil community.[6] An increased understanding of the complex communication networks that exist between the plant and rhizosphere may provide an opportunity for the development of sustainable farming methods. The method of farming, conventional or organic, has also demonstrated dramatic implications for the soil microbiota that must be studied further.

1.0.1. Crop Requirements

Regardless of the implemented farming practices, agricultural crops have base requirements that must be met in order to generate produce. Space, water availability, sunlight, and sufficient nutrient supplies greatly influence food production. The three main elements associated with crop production are nitrogen (N), phosphorus (P), and potassium (K).[7] When these nutrients are not available to plants in high enough quantities, crop productivity declines and food security can become an issue as a result.

Nitrogen is important in the structure of amino acids and subsequently proteins, and as such plants are very sensitive to the concentration of N in the local environment. Plants uptake N primarily in the form of nitrate (NO_3^-) and ammonium (NH_4^+) ions as they do not have the ability to fix inorganic N, or N_2, directly from the atmosphere.[8] Phosphorous is an integral component of DNA, RNA, and proteins, making it another important nutrient for plant growth and development.[9] Plants utilize inorganic phosphates (P_i), or PO_4^{3-} ions, however the uptake of this form of P is limited due to the slow diffusion and high levels of fixation. Fixation refers to the phenomenon that occurs when P_i enters the soil and combines with main group cations, such as calcium, which results in the precipitation of the salt which renders it inaccessible to the plant. Potassium is utilized

in plants as a way to generate cell potentials, regulate osmosis, and activate enzymes.[10] Due to the important functions K is involved in, it is the third important nutrient required for plant success. K is accessed by the roots in the form of K^+, the cationic, water-soluble form of the element.

Native soils are generally not high enough in nutrients to sustain intensive agricultural practices. To stimulate plant growth and production, soluble minerals can be added to the soil to supplement the nutrient-constrained natural systems.[11]

1.0.2. Fertilizers

To improve plant growth and crop yields, fertilizers can be used to artificially increase the nutrient availability in agricultural soils. Fertilizers can be organic waste products such as degraded plant material or animal waste, or chemical in nature such as phosphate and ammonium salts. Global food production has increased significantly as a result of widespread fertilizer accessibility and use.

While the addition of these beneficial nutrients can dramatically increase crop production, there are drawbacks associated with fertilizer use that must be addressed as well. One issue associated with excessive fertilization is the oversaturation of water systems with nutrients such as nitrogen and phosphorous.[12] Enriching waterways with these elemental contaminates results in eutrophication, an environmental state where excessive plant and algal growth has occurred, resulting in highly turbid waters. Agricultural runoff that is enriched in excess fertilizer flows into waterways and rapidly enhances the growth of both multicellular and unicellular plant life. The rapid increase of primary production in the ecosystem results in an unstable system that cannot adapt to the

changes in biomass. Increased biomass of surface dwelling algal species can result in limited sunlight penetration which limits the growth of benthic plant species, which in turn lowers the available oxygen in the water system. The lowered oxygen concentration results in the death of larger marine organisms, such as fish, which can impact the food security of both humans and fishing organisms.

While the ecosystem impacts are largely apparent, eutrophication has many other implications for both terrestrial and aquatic systems. Increasing the nutrient availability in water systems can also increase the rate of bacterial growth for both pathogenic and non-pathogenic strains. Increased virus replication has also been observed in nutrient-rich water systems. The nutrient availability can also increase the abundance of pathogen hosts, which in turn increases the likelihood of human exposure to the infected hosts. These systems have demonstrated the ability to sequester more airborne contaminants due to the bacterial abundance, resulting in water systems that are heavily contaminated by the metabolites of these pollutants. These systems can also act as a sink for other chemical waste products, such as pharmaceuticals and heavy metals, which are not degraded by the existing microbiota.

Terrestrial systems also suffer as a result of excessive nutrient addition. Nitrogen addition to unmanaged systems has resulted in a decreased microbial biomass, while properly managed fertilizer addition has resulted in an increased microbial biomass.[13] This indicates that the addition of fertilizer to rhizosphere communities must be properly managed to avoid damaging the system. Fertilization can also modify the abundance of the existing plant life, which can in turn modify the microbial environment. The addition of contaminated mineral fertilizers, particularly phosphorous fertilizers, is another source

of concern.[14] Lead (Pb), arsenic (As), and cadmium (Cd) have all been detected in mineral phosphorous fertilizers at low concentrations. The repeated application of contaminated fertilizes could result in increased concentrations of these elements which can be sequestered by the plant and transferred to the consumer.

1.1. Biochar

As a potential solution to the aforementioned consequences of mineral fertilizer use, there has been a growing interest in the utilization of biochar in soil enhancement. Biochar is a porous, carbon based material that is formed from the pyrolysis of biomass in a low oxygen environment.[15] The feedstock and the conditions of the pyrolysis (temperature, oxygen levels, length of reaction) greatly impact the properties of the final product. As such, control of these factors can produce a carbon source that is tailored to the intended application. The applications of biochar vary significantly, from the fields of organocatalysis to waste water treatment.

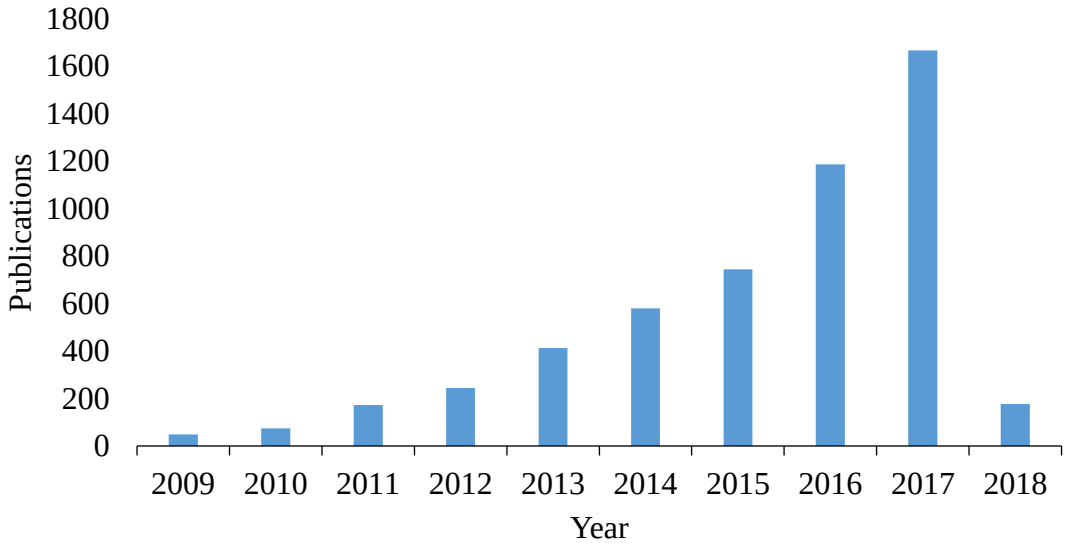


Figure 1. Publication history from ScienceDirect for the phrase “biochar”

Biochar that is produced from plant biomass generally retains some of the physical structures present in the original feedstock. These features are observable under scanning electron microscopy and provide insight into the porous structure. The pores are responsible for a number of the desired characteristics, such as the increased cationic exchange capacity (CEC) or providing adsorption sites for target compounds. The adorsption is also greatly impacted by the surface functional groups present on the material.[16] Common functional groups present include hydroxyl groups (-OH) and carboxylic acids (-COOH), however pyrolysis conditions largely govern which species are dominant.

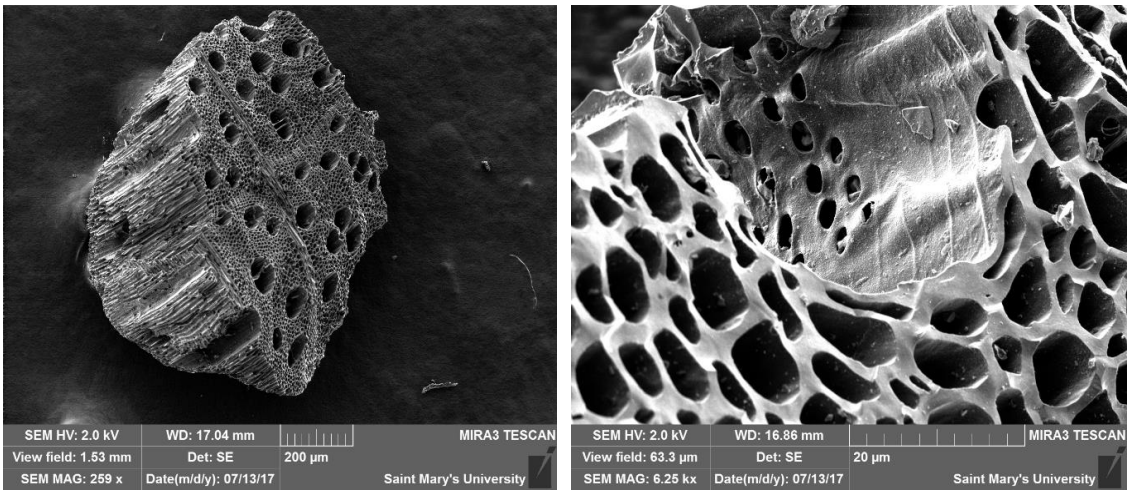


Figure 2. SEM images of forest waste biochar displaying the porous structure

1.1.1. Biochar as a Soil Amendment

One of the potential applications of biochar is as a beneficial soil additive to improve soil health.[17] Due to the high carbon content (>50%) of biochar, incorporation into soils increases the soil organic matter (SOM) and can stimulate the cycling of nutrients such as nitrogen, potassium, and phosphorous. The porous structure can also assist in water retention, increased microbial activity, and increased earthworm activity.

These biological enhancements have been reported and are associated with increased overall soil health and agricultural productivity.[18]

The application of a porous material with tailored surface functional groups also provides an opportunity to modify the chemical properties of the soil. Biochar application has demonstrated improved pH conditions which can increase the uptake of nutrients by plants. It has also been demonstrated that the CEC of soils has increased with biochar addition, which in turn increased the nutrient retention. Biochars derived from manure are nutrient enriched upon application which presents the opportunity for one material to act as a nutrient source and as a soil amendment. The reactive sites on the surface of the biochar also act as reactive pockets that can enhance the degradation of waste products, increasing the formation of new soils.

By enriching soils with biochar, favourable growing conditions for crops can be attained. However, due to the variance caused by the feedstock and formation mechanism, variable impacts on crop yield have been reported.[19] Overall, an increase of approximately 20% in crop yield is observed upon biochar addition. The increase in plant growth and yield is primarily attributed to the increased nutrient availability, adequate pH, and increased microbial biomass. The impact of biochar on the microbial biomass and soil microbial community is an area of increasing interest due to numerous potential applications.

1.2. The Rhizosphere

The rhizosphere is the area of bulk soil that is directly impacted by root exudates.[20] The activity in this volume of soil is currently thought to be primarily responsible for the decomposition of soil organic matter and as a consequence, the nutrient availability. To assist with maintaining the microbial health, plants secrete large quantities of root exudates to nourish the microbes.[21] It has also been observed that the dominant microbial strains vary with host species, and even slightly with host cultivar. This complex relationship has been extensively studied and is observed to have great impact on the health and productivity of plants.

The microbial community that inhabits the rhizosphere is incredibly diverse and unique based on the host plant and geographic location.[22] It has been estimated that in a single gram of soil there can be anywhere between 10,000 and 50,000 strains of bacteria, many of which are unidentified. The massive amount of activity that is supported contributes to plant health in complex ways which are not fully understood. To coexist, these organisms communicate utilizing low-weight molecular signals that can shape the community in a variety of ways.[23] The communication network that exists within these systems can be broken down into three main types: microbe-microbe, microbe-plant, and plant-microbe. Microbe-microbe interactions occur as a means to regulate cell density and reduce competition. Plant-microbe interactions have been documented to produce molecules that bind bacterial proteins and regulate bacterial gene expression. Microbe-plant signalling can have profound impacts on the health, productivity, and gene expression of the plant.

The benefits of the interactions with the microbial community can manifest in a number of ways. One of the most well documented interactions is the mutualistic relationship that occurs between legumes and nitrogen fixing rhizobia.[24] In this symbiosis, nitrogen fixing microbes inhabit nodules within the plant roots and increase the nitrogen content of the soils. Though nutrient cycling is an obvious benefit of the rhizosphere, it is not the only positive outcome. Disease resistance, increased productivity, and shifts in volatile profile have also been reported to be linked to interactions with the soil microbiota.

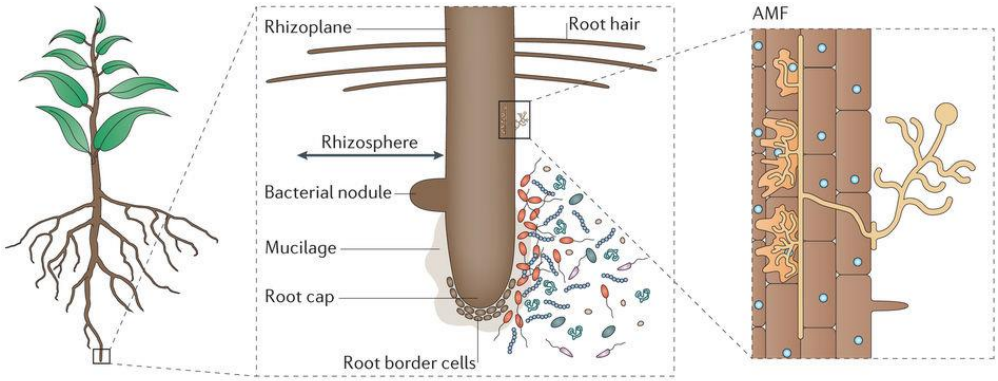


Figure 3. The rhizosphere community interacting with plant roots (AMF = arbuscular mycorrhizal fungi)[25]

1.2.1. Benefits of the Rhizosphere

The microbes that inhabit the rhizosphere have demonstrated a greater impact on the phenotypic expression of plants than was previously thought.[26] Many of the chemical profiles that were considered unique among species can actually be linked to the microbial symbionts that are present. These changes in profile and gene expression are of interest as potential bioactive fertilizers that can assist plants under both abiotic and biotic stressors.

Abiotic stresses, such as drought or salinity, pose a threat to crop productivity.[27] In response to drought conditions, the microbial community can alter the gene expression of the host plant to decrease water loss and become more drought tolerant. An example of this is the inoculation of *Arabidopsis thaliana* with *Azospirillum brasilense*, a microbe that will produce abscisic acid which is an important regulator for plants under drought stress. Similar mechanisms are observed for plants under salinity stress, where phytohormones are produced by the microbiota to help regulate plant growth under high saline conditions.

Disease resistance is another documented benefit of an enriched soil microbe community. There are several mechanisms associated with disease resistance such as competition for resources, mycoparasitism, antibiosis, and stimulation of the plant immune system.[28] Several strains of beneficial bacteria and fungi secrete siderophores, or compounds that sequester iron from the environment. The removal of iron can reduce the growth of potentially pathogenic strains by limiting their exposure to the nutrient. Mycoparasitism is a biocontrol method where a pathogenic fungus is parasitized by a beneficial fungus, thus limiting the growth of the pathogenic fungi. Antibiosis, or the production of antimicrobial compounds, is a common method of microbe signalling that occurs as an attempt to mitigate competition. Stimulating the plant immune system can result in the plant being primed against pathogens before the infection occurs. Gene regulation by the rhizosphere can also result in changes in plant physiology that produce disease resistance. This phenomenon is referred to as microbial triggered immunity and commonly occurs through the recognition of microbial elicitors, such as chitin or flagellin.[29] The recognition of these proteins initiates a signalling cascade that stimulates

the immune system of the plant and leads to reduced damage by the pathogen. In some cases, the defense response is so strong that no disease symptoms are observed. The immunity in some plants is expressed in the form of new proteins that inhibit the growth of pathogens and act in a similar manner to herbicides.

1.2.2. Stimulation of Secondary Metabolite Production

In the instances where plant-microbe interactions and microbe-plant interactions result in the production of novel secondary metabolites, an opportunity for the isolation of new active compounds is presented. Plant derived natural products have been utilized extensively for drug development for many years.[30] Natural products from microbial sources also have extensive uses, from pharmacological applications to antifungal agents. The isolation of natural products from microbes is generally easier than from plants, due to the nature of feedstock production, however products from both are still of potential interest. With the large library of bioactive secondary metabolites produced by these two taxa, it is clear to see why the stimulation of new products *via* their interaction is of interest.

In many instances, the use of applying beneficial microbes to the soil as a method of biocontrol is complex and unstable due to the intricate web of active metabolites present in the system. In an attempt to stabilize this system, fungal antagonists to common pathogenic strains have been applied *via* compost mixture to ensure the survival of the inoculant.[31] It was observed that utilizing the mixture of both compost and fungal biocontrol increased germination rates in tomatoes, indicating that increasing the survivability of the inoculant can result in the production of enough metabolites to

suppress diseases. It has also been demonstrated that the addition of environmental isolates from vermicompost (worm-formed compost) can inhibit fungal pathogens *via* the production of volatile organic compounds.[32] These compounds can further be isolated and studied in an attempt to develop new antifungal agents. The Actinomycetales, a beneficial strain of fungi, activates key genes in the jasmonate immunity pathway in *Arabidopsis thaliana.*[33] The activation of this pathway results in the increased production of jasmonic acid, which is a common method of defense against herbivores and pathogens.

1.3. Fermentation

Fermentation is the process of cellular respiration without the use of an electron transport chain.[34] To regenerate the electron carrier NAD^+, intermediate products from other biochemical pathways, such as glycolysis, are reduced. This produces the fermentation products ethanol or lactic acid. These products are widely utilized in industry for a variety of applications, whether it be the production of consumable products, degradation of hazardous waste, or renewable fuel sources.[35, 36, 37]

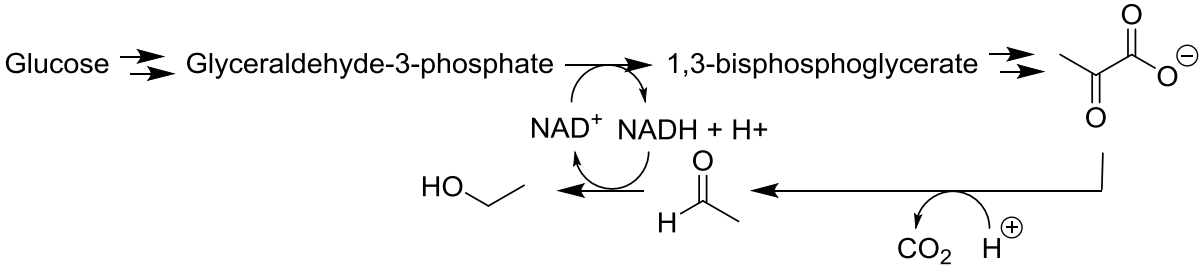


Figure 4. Mechanism of ethanol fermentation that occurs in primary fermentation[34]

The fermentation of wine and other alcoholic products occurs through ethanol fermentation carried out by *Saccharomyces cerevisiae*. This strain of yeast is commonly utilized due to the high ethanol productivity and tolerance it exhibits.[38] This fermentation pathway occurs during the primary fermentation stage, when nutrients are plentiful and cell growth is occurring at an exponential rate. The stage takes approximately three to five days, after which cell growth slows significantly due to the decreased nutrients, space, and increased ethanol in the system. After the cell density decreases, secondary fermentation, or malolactic fermentation, begins.[39] This stage can take upward of two weeks and is predominantly dominated by bacterial metabolic reactions. Many of the flavours that are associated with wine are produced during this stage of fermentation, and are highly variable depending on the bacterial strains present.

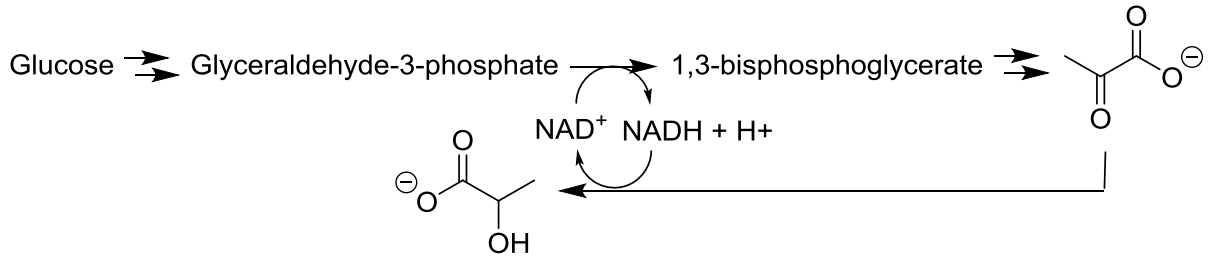


Figure 5. Mechanism of lactic acid fermentation that occurs in secondary fermentation[39]

1.3.1. Implications of the Natural Microbiota

Due to the complex microbial contribution to wine aroma and flavour, understanding the grape microbiome could result in the discovery of industry relevant fermentation strains. When grapes are crushed to form must, the must environment is high in sugars which results in intense microbial competition.[40] This competition can produce inconsistent results, so many winemakers sterilize the must with SO_2 to remove environmental microbes.[41] The desired yeast is added after SO_2 sterilization and the

fermentation is carried out. However, due to human health risks associated with the utilization of SO_2, there has been a gradual shift towards natural fermentation and other sterilization methods.

Natural fermentation is the process by which the grape must is allowed to ferment using the native microbiota present on the surface of the fruit. Several yeast strains can be found on the skin of mature grapes that ferment the must comparably to *Saccharomyces.*[42] This method of fermentation is less predictable due to the subtle changes in the microbiome caused by geographical location. The subtle differences in the microbial composite of the fruit can be traced back to the composition of the rhizosphere, which in turn produces the *terroir* of the wine.[43] *Terroir* is the flavour and aroma profile that is generated as a result of the location. By allowing the native grape microbiome to persist through the fermentation, the expression of the *terroir* may be enhanced.

While naturally present yeast strains can assist in primary fermentation, the main changes that occur in the flavour and volatile profile are triggered by the native bacterial strains. These strains contribute heavily to secondary fermentation and are responsible for most of the flavour production. Due to the large number of bacterial strains present on unsterilized fruit, the result of the fermentation is inconsistent; however, these strains have been reported to produce unique and desirable flavour profiles.

1.4. Objectives

The objective of the current research is to inoculate forest waste biochar with beneficial strains of bacteria and integrate them into the rhizosphere of various agricultural crops. The inoculation will be observed utilizing SEM imaging to determine the location and growth pattern of the bacteria. The impact of the biochar will be determined by measuring the vegetative growth and lignification of the plant during various phases of growth. The chemical impact of the biochar on the plant will be observed by collecting volatile samples and solvent extractions from leaves and analyzing them utilizing GC-MS. Differences in the generated profiles will be explored, and potentially useful compounds will be identified.

To determine the impact of soil microbes on wine fermentation, soil samples will be collected and microbes will be extracted and isolated. Pure strains will be utilized to ferment grape concentrate with and without the addition of commercially available *Saccharomyces cerevisiae.* The fermentations will be analyzed utilizing LC-MS to observe the changes that occur with different fermentative agents present.

2. MATERIALS AND METHODS

2.0. Characterization of Biochar

Forest waste biochar was obtained from the MacQuarrie Group at Cape Breton University (prepared by thermally treating biomass chips in an oxygen deficient environment followed by torrefaction and densification) and characterized *via* pH determination, elemental analysis, and infrared spectroscopy. The pH was determined by creating a suspension of biochar in deionized water and using a Sym**pH**ony B10P VWR pH probe to determine the pH. The measurements were repeated in triplicate. The C, H, and N analysis was conducted in the Centre for Environmental Analysis and Remediation by Navya Kesavan using a Perkin Elmer 2400 Series II CHN Analyzer. The infrared spectrum was obtained on a Bruker Alpha Platinum ATR using a KBr pellet.

2.1. Inoculation of Biochar

The biochar was functionalized by incubation in a bacterial suspension to colonize the recesses of the porous structure. The procedure was modified from Xiong *et al.*[44] Biochar and nutrient broth (1:1 v/v, 5 g tryptone, 3 g yeast extract, 5 g sodium chloride, 1 L water) was sterilized in a Getinge Vacuum Steam Sterilizer (Model 533Ls) at 121°C for 30 minutes. The bacterial strain of choice was transferred *via* 1 µL inoculation loop into the sterilized biochar mixture and incubated in a 30°C Amerex Instruments Gyromax 737 incubator with shaking at 100 rpm for 24 hours. The inoculated biochar was gravity filtered to remove excess broth and immediately utilized for soil inoculation. A portion of

the biochar was resuspended in nutrient broth and used to inoculate a nutrient agar plate for colony counting to determine the concentration of bacteria in the biochar.

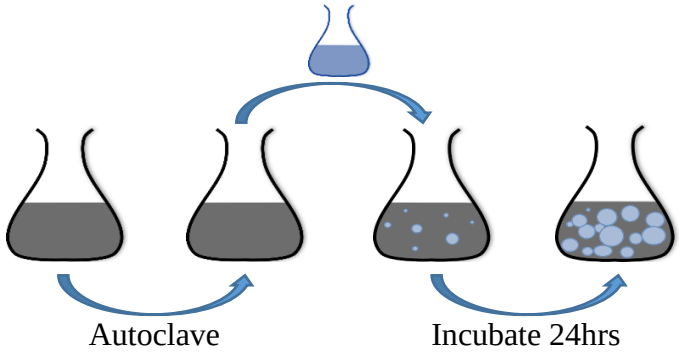


Figure 6. Graphical outline of inoculation procedure

2.2. SEM Analysis of Functionalized Biochar

Inoculated and normal biochar was imaged using a MIRA3 TESCAN Scanning Electron Microscope to observe the surface features of the biochar and the extent of bacterial colonization. The procedure for sample preparation was modified from Xiong *et al.*[44] The sample was fixed for 2 hours in a 2.5% glutaraldehyde solution (0.450 mL phosphate buffer solution with pH 7.2, 0.050 mL 25% glutaraldehyde solution). The solution was removed and the sample was dehydrated with 30%, 50%, 70%, 90%, and 100% ethanol solutions for 20 minutes at each concentration. The ethanol was removed and the sample was placed under argon before the final drying in hexamethyldisilazane (HMDS) for 10 minutes. The HMDS was removed and the sample was allowed to dry in a desiccator for at least 48 hours.

2.3. Application of Biochar

2.3.1. *Greenhouse Trials - Grapevines*

Inoculated biochar was transferred to a polystyrene biohazard bag and transported to the greenhouse for grapevine inoculation. The rhizosphere was exposed by removing approximately 5 cm of topsoil and evenly distributing the biochar on the exposed roots. The topsoil was then returned to the pot. This procedure was repeated for all treated grapevines. Thirteen different treatment groups were utilized and the conditions of each treatment are summarized in Table 1.

Plant growth was assessed by analyzing the vegetative growth of the grapevines at each sampling period. The number of leaves was counted and the main shoot length was measured from the apical meristem to the base of the shoot. During the post-inoculation sampling period, the amount of lignification to the nearest node was also measured. All values were recorded and used to assess relative vegetative growth of each trial by determining the ratio of new shoot growth ($\frac{Shoot\ Length_{Post\ Inoculation}\ (cm)}{Shoot\ Length_{Pre\ Inoculation}\ (cm)}$). The impact of the different treatments on plant development was determined using the physical plant assessment. To determine is the results were statistically significant, an ANOVA was conducted on all trials. If the calucated F value was larger than the critical F value, individual T-Tests (two sample assuming unequal variances) were conducted to determine the statistically relevant result. The reported P value was obtained from the two-tailed result on the Excel output.

Table 1. Summary of conditions for each treatment group

Treatment	Conditions
L'Acadie Blanc control	Nothing
L'Acadie Blanc biochar	5% v/v dry biochar
L'Acadie Blanc biochar with broth	5% v/v biochar soaked in nutrient broth for 48 hours
L'Acadie Blanc *Pseudomonas rhodesiae*	5% v/v biochar inoculated with *P. rhodesiae*
L'Acadie Blanc *Delftia tsuruhatenesis*	5% v/v biochar inoculated with *D. tsuruhatenesis*
L'Acadie Blanc *Methylobacterium sp.*	5% v/v biochar inoculated with *Methylobacterium sp.*
L'Acadie Blanc 50% biochar	2.5% v/v biochar inoculated with *D. tsuruhatenesis*
L'Acadie Blanc 200% biochar	10% v/v biochar inoculated with *D. tsuruhatenesis*
L'Acadie Blanc NatureAid	NatureAid Crop Booster
New York Muscat control	Nothing
New York Muscat inoculated	5% v/v biochar inoculated with *D. tsuruhatenesis*
Marechel Foch control	Nothing
Marechel Foch inoculated	5% v/v biochar inoculated with *D. tsuruhatenesis*

2.3.2. *Growth Shelf – Barley, Marigolds, Edamame*

To optimize the conditions of biochar application, barley was utilized as a model crop. Inoculated biochar was introduced into red solo cups containing 400 mL of sterile potting soil and barley seeds were planted immediately or after one week depending on the experiment. Immediately after planting, tap water was added until the pots weighed approximately 200 g. Pots were placed on a growth shelf equipped with 3000 K LED lights positioned 21" above the tops of the pots. Plants were exposed to a 12 hour light cycle beginning at 8:00 and ending at 20:00. Germination was monitored by counting the visible barley shoots on day 3, 5, 7, and 14 of the two to six week growth period. Tap water was used to water the pots when they appeared visibly dry. Upon the completion of the growth period, barley seedlings were gently removed from the soil and measured to determine the length of the shoot and longest root. After measurements, the shoots were transferred to a 55 °C fan oven and dried for 48 hours. After all water was removed from the shoots, the dry weight was recorded and the shoots were discarded. This procedure was repeated for marigold and edamame seeds with the optimized conditions of 5 % v/v biochar mixed throughout the soil. To determine if results were significant, an ANOVA followed by t-testing was utilized to determine the P value.

2.3.3. *Soil Extract Agar – Barley, Marigolds, Edamame*

To better observe the root and shoot growth, plants were cultivated in petri dishes containing soil extract agar and the biochar mixture of choice. Soil extract agar was prepared by soaking 200 mL of potting soil in 200 mL of sterile water at room temperature for 48 hours. The mixture was gravity filtered to remove the solid material and the filtrate was diluted to 600 mL with deionized water. 4.8 g of agar powder was added and the whole mixture was autoclaved for 30 minutes to sterilize the agar. The crop seed of choice was surface sterilized with 10 % bleach for 1 minute, then thoroughly rinsed with sterile water. Seeds were placed in lint-free wipes, moistened with sterile water and allowed to germinate in sterile petri dishes for 48 hours.

After radicles had begun to emerge, 20 mL of the soil extract agar was poured into petri dishes and allowed to cool but not solidify. Once the temperature decreased, inoculated biochar was introduced to the plate and thoroughly swirled to distribute the biochar. When the plate solidified, a viable seed was placed in the centre of the dish and the dish was sealed with parafilm. Dishes were covered with tinfoil and incubated facing upright at room temperature for 24 hours. After the roots penetrated the agar, the dishes were stored vertically and the tinfoil was only used to cover the roots. After 5-10 days of growth, the seedling was removed from the agar and measured with a ruler. The seedling was bisected to separate the root from the shoot and dried at 55°C for three days. After drying, the mass of the roots and shoots was recorded. Each treatment was performed in triplicate.

2.4. Volatile Plant Profile and GC-FID Analysis

The volatile composition of the grape plant foliage was analyzed using dynamic headspace sampling and gas chromatography using a modified method from Quiroz *et al.*[45] A petri dish with two holes bored in the top was used as a volatile collection chamber. Five leaves were placed in the petri dish and allowed to equilibrate for 2 hours. The volatiles were sampled by inserting a volatile trap (100mg of Porapak-Q contained within a disposable pipette and bordered by glass wool) into one of the holes and a charcoal plug (200mg of activated charcoal contained within a disposable pipette and bordered by glass wool) was inserted into the other hole. A vacuum line was connected to the Porapak-Q trap and the headspace was sampled for 1 hour with a flowrate of 1.5 L/min. The trap was eluted with 1.5 mL of diethyl ether before storage at -20 °C.

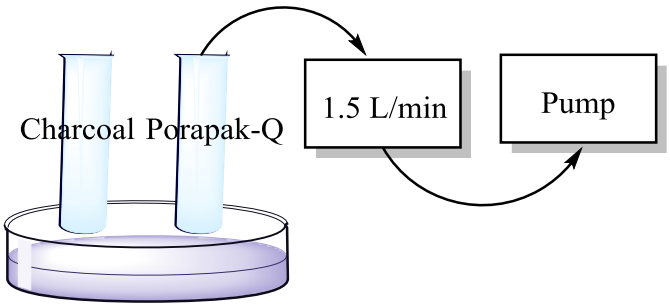


Figure 7. Schematic of dynamic headspace sampling for plant volatile analysis

GC-MS analysis of all volatile compounds was conducted on a Varian 3800 Gas Chromatography apparatus coupled to a Varian 2000 Mass Spectrometer and a Varian 3P-8400 Autosampler. The analysis method was modified from Xie *et al.*[46] A VF-5MS capillary column (Varian, CP8944, 30m x 0.25 mm, 0.25 µm) was utilized to separate the biological samples. The oven temperature was initially set to 50 °C for 2 minutes, increased to 200 °C at 10 °C/min, then increased to 250 °C at 25 °C/min and held for 3

minutes. Helium (99.9%) was used as the carrier gas and set at a constant flow rate of 1 mL/min. The injection was performed in split mode with a split ratio of 10:1 and the injection port, transfer line, and ion source were all maintained at 250 °C. Due to technical difficulties encountered with the mass spectrometer, only flame ionization was utilized to gain qualitative information about the composition of the volatile compounds.

2.5. Elemental Analysis of Plant Material

Plant samples were dried in a fan oven at 55 °C for 48 hours to remove all water. Leaves were separated and ground into a fine powder using a mortar and pestle. To assist in the grinding, liquid nitrogen was utilized to snap freeze the dried tissue. Approximately 3.5 mg of dried material was accurately weighed and analyzed using a Perkin Elmer 2400 Series II CHN Analyzer. The measurements were recorded in triplicate and averaged to observe the impact of inoculation on nitrogen uptake.

2.6. Fermentation of Grape Juice

White and red grape concentrate was obtained from Nobel Grape and used as fermentation broth for environmental isolates and commercially available yeast strains. Yeast was activated by adding the yeast pellets (0.175 g/L) to a 20 mL vial and adding deionized, filtered water (10 mL/g). The mixture was placed in a 37 °C incubator at 100 rpm and allowed to mix for 20 minutes. 16 mL of grape juice was added to the mixture, then the vial was loosely capped and allowed to ferment at 25 °C for two weeks. To determine the impact of environmental isolates, approximately 1 μL of the colony was selected and added to the yeast-juice mixture before the vial was allowed to ferment. As a comparison, approximately 1 μL of a separate colony was selected and added to grape juice without yeast. The vial was loosely capped and allowed to ferment at 25 °C for two weeks. After two weeks, the mixture was filtered through a #2 Watman filter paper and transferred to a new vial to remove the dead yeast cells. The new vial was sealed and allowed to age for 16 weeks at room temperature and ambient lighting.

2.7. Analysis of Ferment

After the fermentation was complete, 50 μL of the wine was used to inoculate a 24 well nutrient agar plate to determine if any cells were viable. To prepare the wine for LC-MS analysis, 1 mL of wine was diluted with 1 mL of HPLC methanol, acidified with one drop of 88 % aqueous formic acid, and filtered through a 0.45 μm filter. Methanol was used as a blank and quercetin dihydrate was utilized as a standard (both internal and external). The analysis was conducted on an Agilent 1100 series LC-MS equipped with a Diode Array Detector and an ion trap mass spectrometer (Agilent 110 Series LC/MSD

Trap). The sample was passed through a Bonus-RP Column (Agilent, 4.6 x 150 mm, 5 μm, 883668901) with a solvent system of 0.1 % formic acid in water as Solvent A and 0.1% formic acid in acetonitrile for Solvent B. The mobile phase began with 20 % Solvent B, increased to 45% Solvent B over 35 minutes, then increased to 90% Solvent B over 2 minutes, resulting in a 37 minute run. The needle was washed with methanol after every injection and a 5 minute post-run was completed to flush the column. The mass spectrometer was operated in both the positive and negative mode with an oven temperature of 350 °C, a nebulizer pressure of 40 psi, a dry gas flow rate of 9 mL/min, and a capillary voltage of 3.5 kV. 922.01 was used as the lock mass for the positive mode and 922.01 was used as the lock mass of the negative mode. Ions with mass to charge ratios in the range 100-1000 were recorded as masses of interest.

To find potential compounds of interest, the supplementary information from Flamini *et al.* was utilized to attempt to determine the behaviour of certain polyphenols within the wine.[48,49] The neutral exact masses were used as target masses and extracted ion chromatograms of each peak were visually assessed. Extracted ion chromatograms that presented well-defined peaks with good signal to noise ratios and reported masses similar to the neutral masses were used as indicator peaks to track the production of polyphenolic compounds in different wine samples.

3. RESULTS AND DISCUSSION

3.0. Characterization of Biochar

The pH was determined to be mildly acidic (between 6 and 7), the total C was 86.0%, total H was 2.48%, and total N was 0.32%. The IR spectrum displayed prominent peaks in the O-H stretching region (3100 – 3600 cm^{-1}), the hydrocarbon C-H stretching region (2900 – 3100 cm^{-1}) and the carbonyl stretching region (1700 – 1740 cm^{-1}).[47] These results indicate that the biochar contains hydroxyl, carboxylic acid, and carbonyl functionalities. The weakly acidic pH indicates that the O-H and C=O stretches probably belong to a mixture of carboxylic acid functionalities, hydroxyl functionalities, and carbonyl functionalities. These characteristics are common for forest waste biochar according to literature.[16]

3.1. Inoculation of Biochar

Ground biochar was demonstrated to be more effective at bacterial retention, however it was observed under SEM imaging that many of the porous structures were destroyed by the grinding process (Figure 8 and Figure 9). Several of the benefits of biochar addition have been attributed to the porous structure, so the biochar was not ground before inoculation to preserve the structural framework.[17]

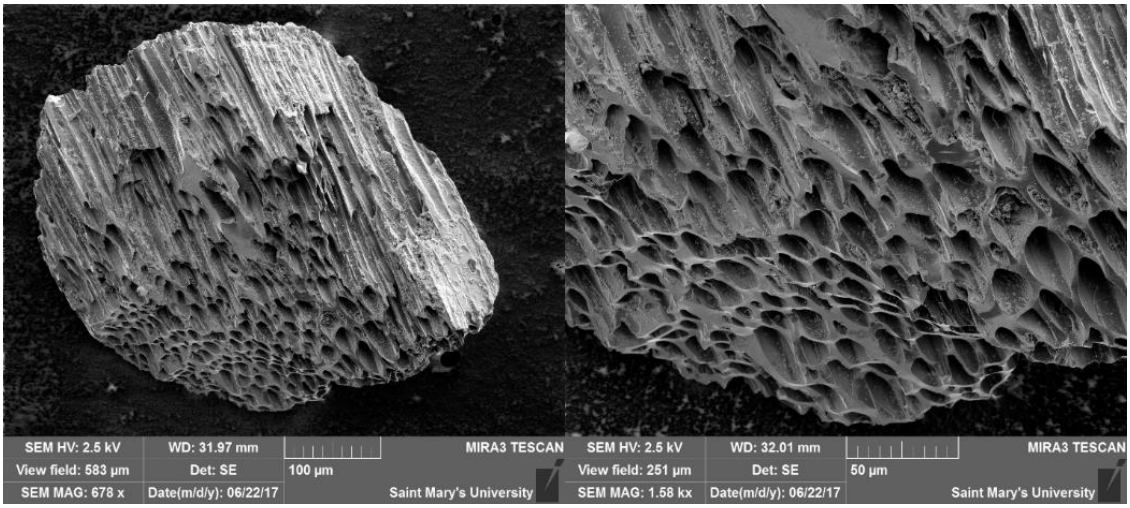


Figure 8: SEM images of biochar before grinding at low magnification (left) and higher magnification (right)

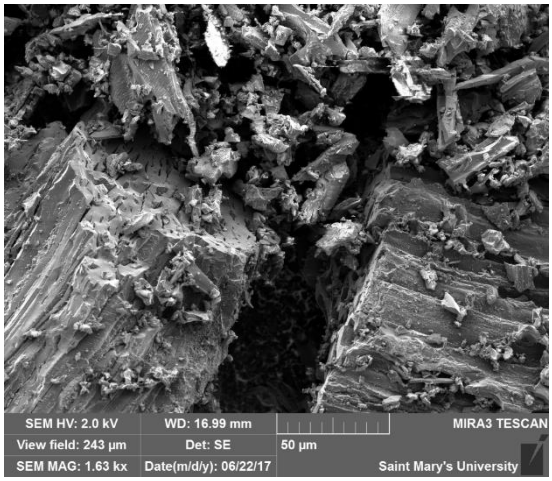


Figure 9: SEM image of biochar after grinding with a mortar and pestle

The SEM images clearly display the porous structure of the biochar. The structure of the biochar closely resembles the vascular structure of living plants (Figure 10 and Figure 11). This indicates that the plant matter converted into biochar directly impacts the resulting structure of the biochar.

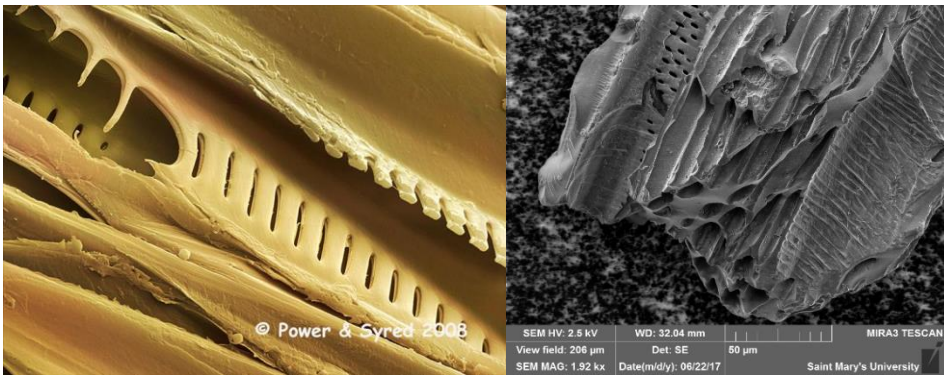


Figure 10. SEM images of xylem vessels of magnolia (*Magnolia sp.)*[50] (left) and biochar obtained from BioEnergy (Cape Breton) *via* the MacQuarrie group (right)

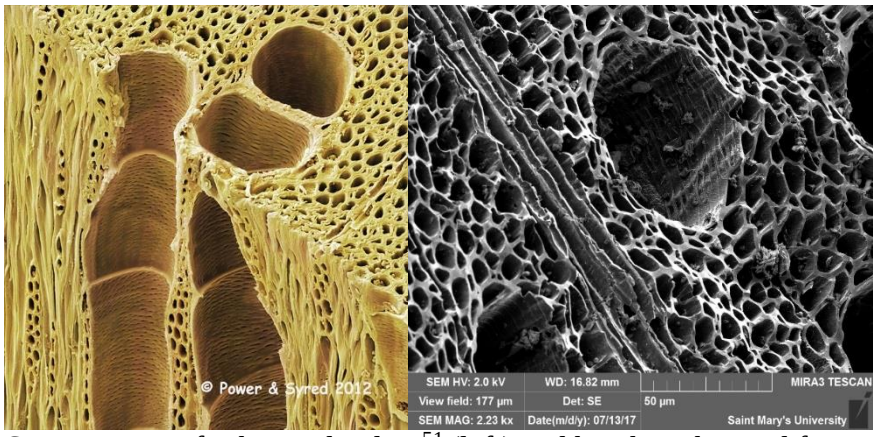


Figure 11. SEM images of ash wood xylem[51] (left) and biochar obtained from BioEnergy (Cape Breton) *via* the MacQuarrie group (right)

3.2. SEM Analysis of Functionalized Biochar

The SEM was also utilized to observe the attachment of bacterial cells to the biochar scaffold. The fixation process impacted the dispersity of the cells on the surface of the biochar (Figure 12 and Figure 13). Sample preparation that included vigorous shaking resulted in a monodispersed layer of bacterial cells that coated the surface of the biochar, while gentle sample preparation maintained the clustered nature of the cell colonies.

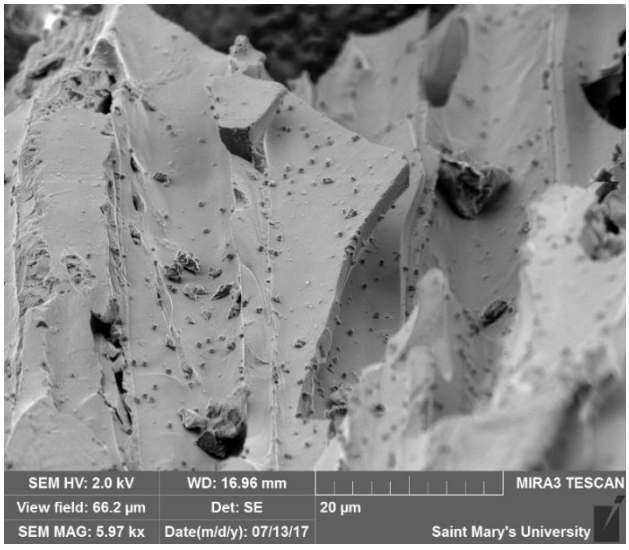


Figure 12. SEM image of monodispersed bacterial cells

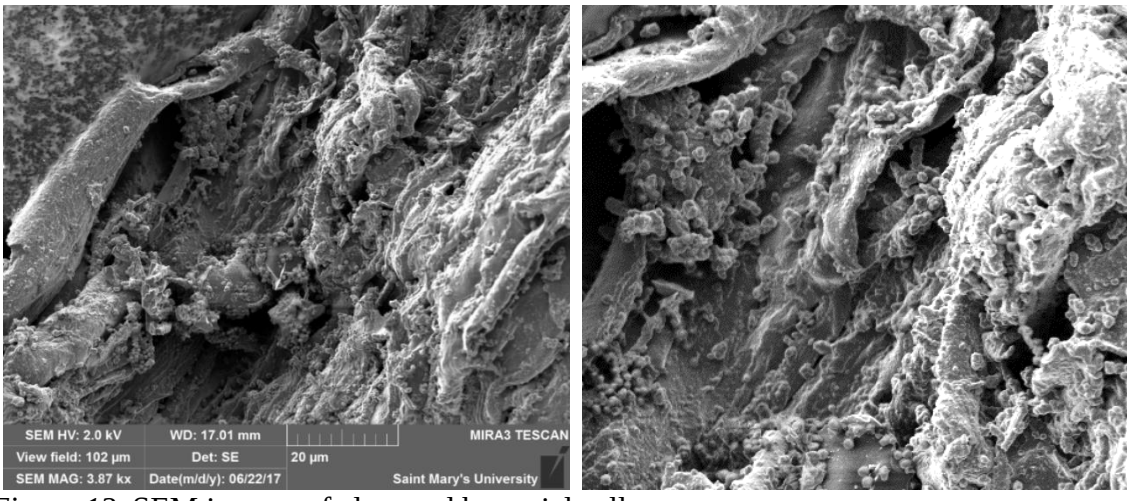


Figure 13. SEM images of clustered bacterial cells

3.3. Application of Biochar

3.3.1. Greenhouse - Grapevines

Inoculated biochar was introduced to the top of the roots of three varieties of grapevines in late August and early September. Vines were allowed to grow and mature for 8 weeks before measurements were taken to determine the impact on shoot growth. The ratio of shoot increases for all trials are presented in Figures 14 – 18.

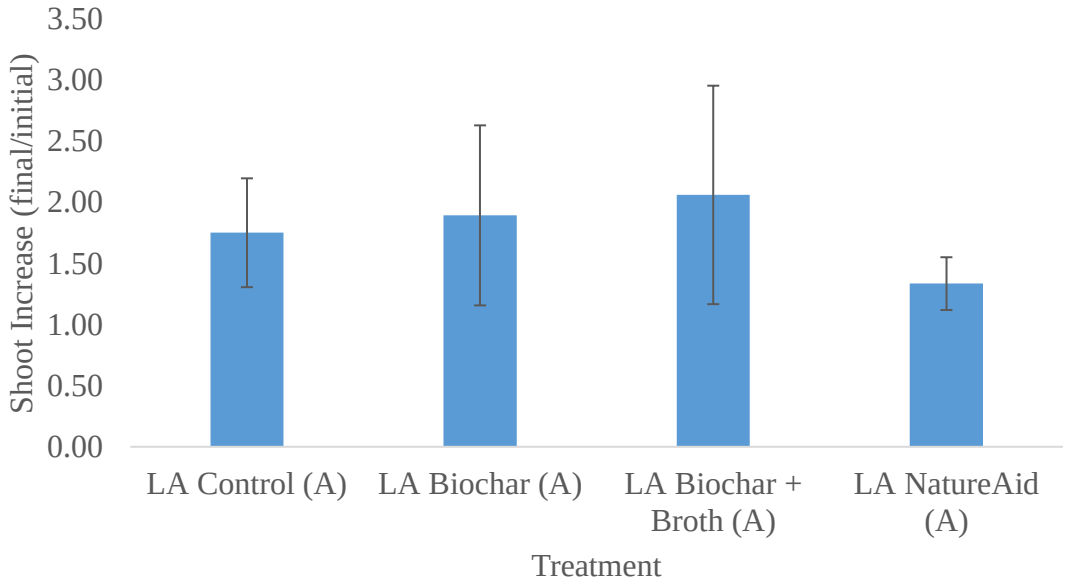


Figure 14. Shoot increase for L'Acadie Blanc control and blank trials

No significant difference was observed for the control treatments, indicating that any significant changes in vine vegetative growth was due to the bacterial strain. The blank treatments were a control, introduction of sterile biochar and introduction of sterile biochar and nutrient broth. A slight increase in vine growth was observed with the addition of these two soil amendments, however the increase was not significant and the standard deviation for these trials was very large. The large deviation indicated that these treatments generated inconsistent results when utilized. A commercially available organic fertilizer, NatureAid, was also tested to determine how the inoculation procedure

compared to industry standards. The NatureAid did not significantly increase or decrease shoot growth compared to the control, and no difference was observed between the NatureAid and other blank treatments.

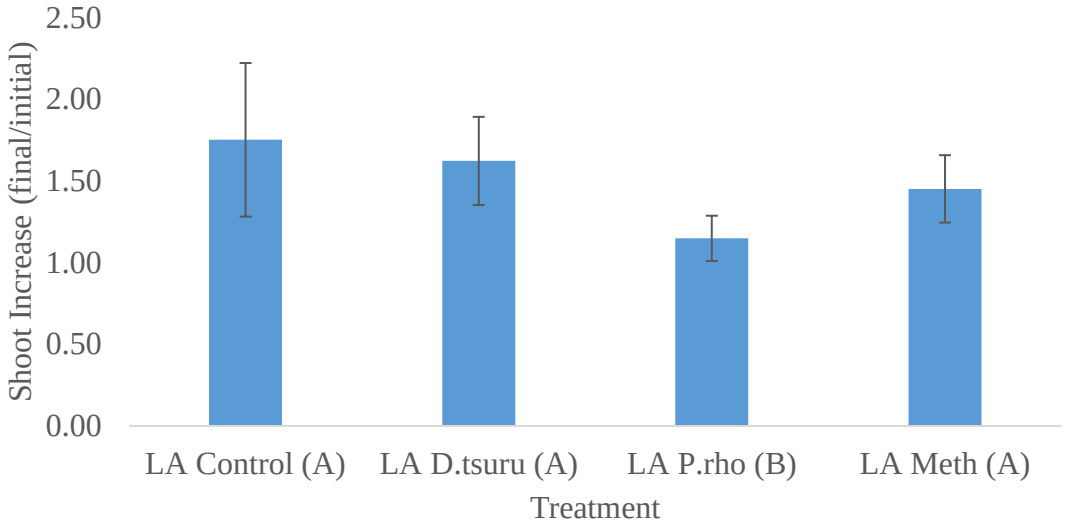


Figure 15. Shoot increase for L'Acadie Blanc inoculation trials (p = 0.0380)

It was observed that the introduction of different bacterial strains prompted a different response from the vine in terms of vegetative growth (Figure 15). When inoculated with *Delftia tsuruhatenesis*, there was minimal (p = 0.634) difference in the shoot growth observed compared to the control. When inoculated with *Pseudomonas rhodesiae*, a significant decrease (p = 0.0316) in the shoot growth was observed. When *Methylobacterium* was utilized, there was a slight (p = 0.253) decrease in the shoot growth observed. A potential explanation for these observations is physical colonization of the biochar and the secondary metabolic processes of the various strains.

When the inoculated biochar was analyzed using scanning electron microscopy, it showed how the bacteria were distributed on the surface of the biochar. When inoculated with *P. rhodesiae* and *Methylobacterium*, the surface of the biochar is extensively colonized with bacterial cells (Figure 16). The colonization pattern is fairly

straightforward with more cells present on surfaces that appeared to be rough and have many defects. This was expected as defects would aid the bacteria in their attachment to the substrate. The extensive colonization of the biochar by *P. rhodesiae* and *Methylobacterium* may account for the limited growth observed, as introducing too many cells could result in competition for the nutrients in the soil.

When *D. tsuruhatenesis* was utilized at the inoculant, the colonization was very different due to the production of a biofilm. Upon examination it appeared as though the biochar was covered in a mucus-like film that very few bacteria were attached to. The biofilm is a mixture of sugars and proteins generated by the bacterial cells as a means to aid in substrate attachment, explaining the colonization observed. The biofilm production of *D. tsuruhatenesis* may have assisted in maintaining the growth that was observed in the control as biofilms provide native bacteria with better substrate attachment and could allow them to multiply more effectively.

Another potential reason for the negative impact of the *P. rhodesiae* biochar is one of the key metabolic processes performed by the strain. While there are many plant growth promoting benefits associated with the strain, there is one major drawback with its use as a soil amendment. *P. rhodesiae* is a facultative anaerobe which allows the cell to use both oxygen and other inorganic compounds for respiration. In the presence of oxygen, O_2 will be utilized as the terminal electron acceptor in the electron transport chain due to the high electronegativity of the atoms. In the absence of oxygen, inorganic salts, specifically nitrates and nitrites, are used. When these salts are used for respiration, they are reduced to gaseous nitrogen, which exits the soil and effectively removes nitrogen. This process is known as denitrification and may account for the reduced shoot

growth that was observed. To provide a solution to this problem, inoculating the soil with lower levels of the bacteria may allow for the positive effects without resulting in denitrification of the soil.

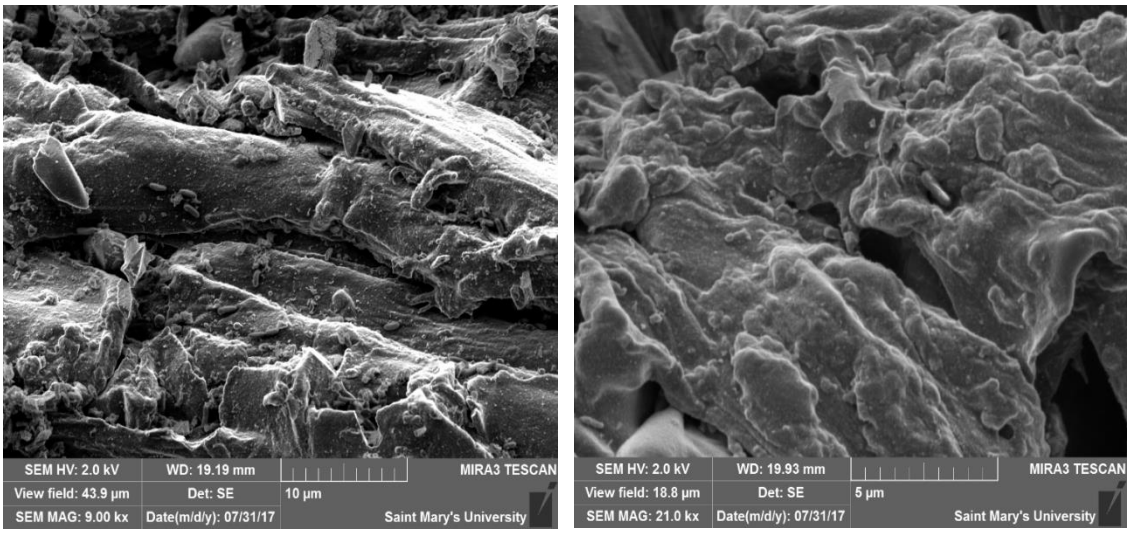


Figure 16. Pseudomonas rhodesiae inoculated biochar (left) and *Delftia tsuruhatenesis* inoculated biochar (right)

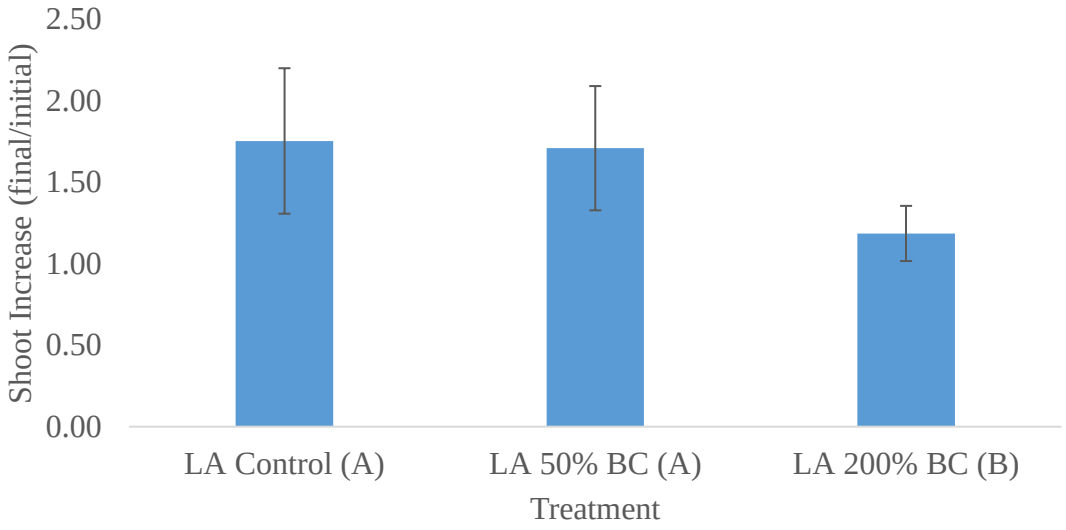


Figure 17. Shoot increase for L'Acadie Blanc with varying amounts of inoculated biochar ($p = 0.0316$)

When different amounts of inoculated biochar were introduced into the soil, the response of the plant began to change. When 2.5% v/v biochar was used and compared to 5% v/v, little difference (p = 0.883) was observed in terms of shoot increase. When the volume of biochar was increased to 10% v/v, a significant decrease (p = 0.0316) in the amount of vegetative growth was observed. This could be attributed to the amount of colony forming units introduced into the rhizosphere of the grapevine.

Bacterial communities and plants are incredibly unique in their composition and proportion of strains present. Introducing a small amount of new bacteria probably did not disrupt the community too much, however introducing a large amount of cells probably resulted in a community shift. This shift could have eliminated some of the plant growth promoting bacteria naturally found in the rhizosphere of grapes, and as a result reduced the overall growth of the plant. It was demonstrated that lowering the amount of biochar introduced to the soil effectively acts as a remedy to this issue.

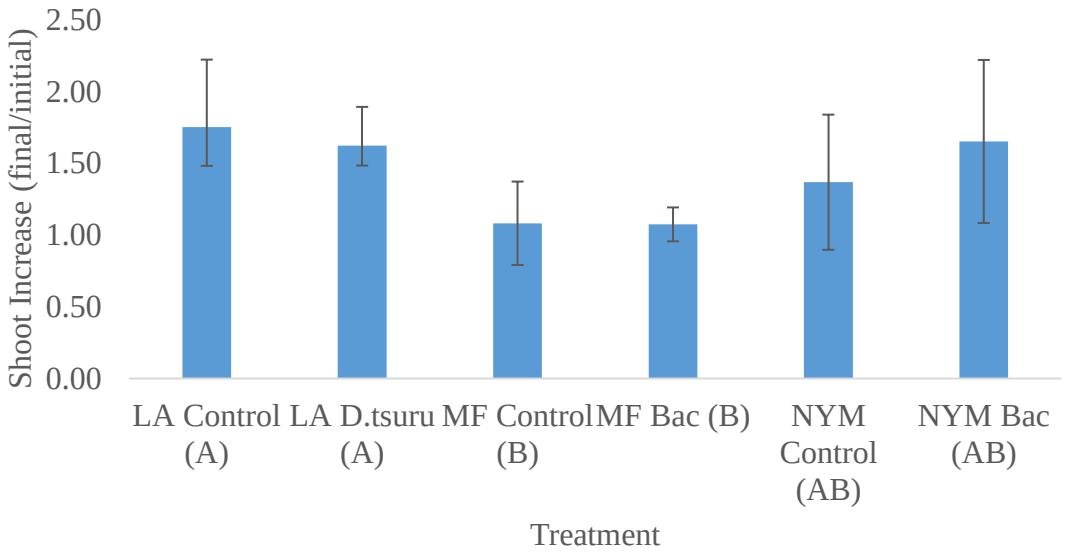


Figure 18. Shoot increase for L'Acadie Blanc, Marechel Foch, and New York Muscat when inoculated with *Delftia tsuruhatenesis* (p = 0.0271)

Different cultivars behave differently in almost all situations, which is one of the reason certain varietals are cultured in different parts of the world. It was observed that Marechal Foch grew significantly less ($p = 0.0271$) than L'Acadie Blanc in the same amount of time, effectively demonstrating that.

3.3.2. Growth Shelf – barley, marigolds, edamame

Barley was utilized as a model crop to optimize the conditions for biochar application during the winter months as the grapevines were dormant. The barley cultivar that was utilized appeared to be susceptible to wilting around day 11 of growth, and this was observed in every treatment. Despite the wilting, results were still reported due to the fact that the plants did not appear to be malnourished or stunted in their growth.

The first set of trials were the set of blanks to determine if the biochar was significantly impacting the growth without the addition of bacteria or nutrient broth (Figure 19). No statistically significant differences were observed in any of the different treatment groups, indicating that the barley did not benefit from the addition of the bacterial strain or the biochar at the addition rate of 2.5 % v/v. The amount of biochar utilized was initially decreased due to the similarities in growth demonstrated by 5% v/v and 2.5% v/v in the grape trials. The weight of the roots could not be accurately determined due to the layer of mucigel that retained soil on the root tissue, so length was utilized to determine the impact of the various treatments. The germination of the barley was also assessed by counting the seedlings on days 3, 5, 7, and 14 to determine if any of the treatments impacted the germination rate. It was observed that the germination of the barley was faster when inoculated biochar or biochar containing nutrient broth was

introduced into the soil. This could be due to the plant growth promoting effect of the bacteria, or it could be simply due to the extra nitrogen supplied by the nutrient broth. Due to the increase observed in both trials, the extra nitrogen supplied by the nutrient broth is the most likely cause of the observed germination enhancement.

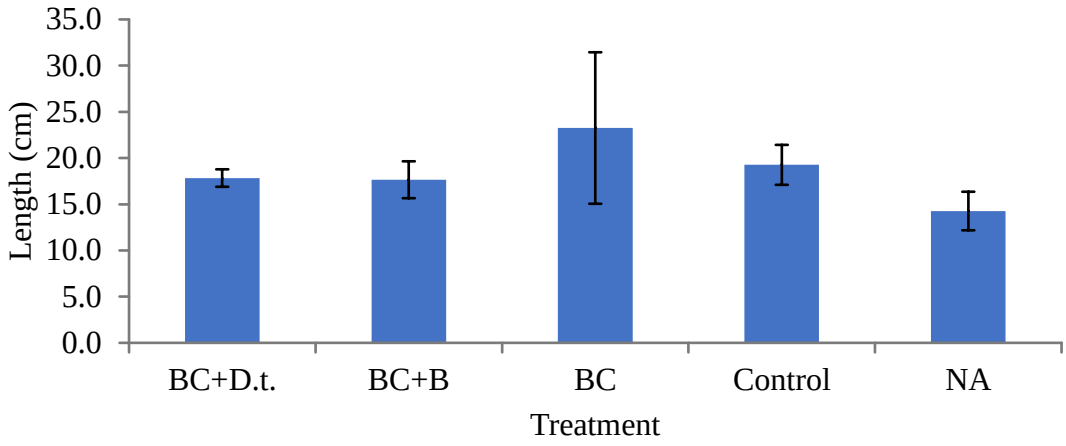


Figure 19. Root lengths of barley after 2 weeks of growth for blank treatments (BC+D.t. = inoculated biochar, BC+B = biochar with nutrient broth, BC = biochar, NA = NatureAid)

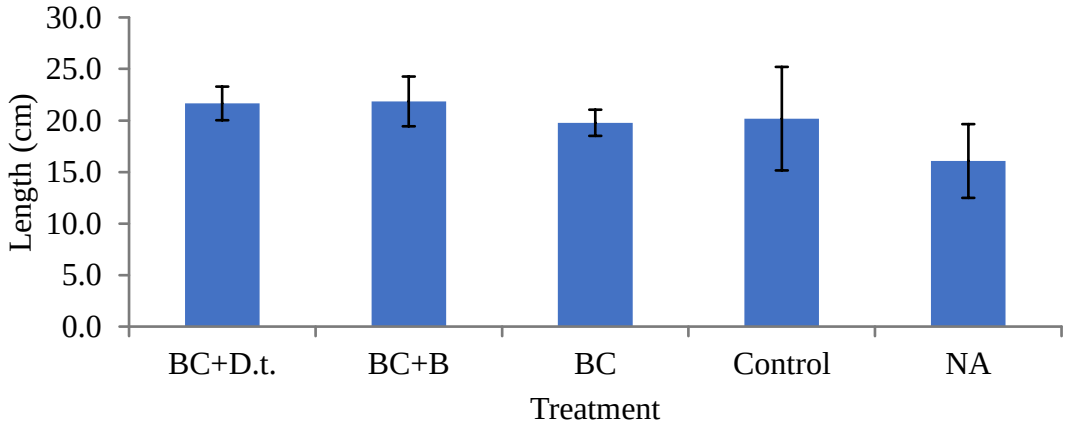


Figure 20. Shoot lengths of barley after 2 weeks of growth for blank treatments (BC+D.t. = inoculated biochar, BC+B = biochar with nutrient broth, BC = biochar, NA = NatureAid)

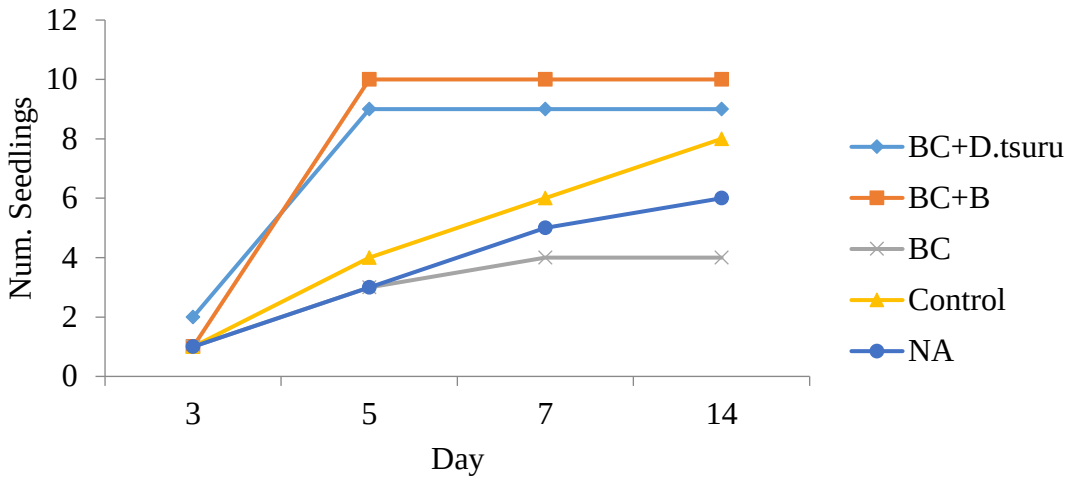


Figure 21. Germination rates of barley over a period of two weeks (BC+D.t. = inoculated biochar, BC+B = biochar with nutrient broth, BC = biochar, NA = NatureAid)

The second set of trials that was conducted was to optimize the delivery of biochar to the soil in terms of volume and placement. Three different volumes of inoculated biochar was added to the soil to observe the impact of introducing different concentrations of bacterial cells. The biochar was also introduced into different locations in the pot to see if the placement of the composite changed the growth. It was observed that introducing the biochar to the top of the soil or mixing it throughout the soil resulted in longer shoots. It was also determined that introducing 5% v/v of the inoculated biochar produced the most growth, a result consistent with the grape trials. Germination was also assessed, and it was observed that the rate of germination increased when the biochar was introduced with direct contact to the seeds.

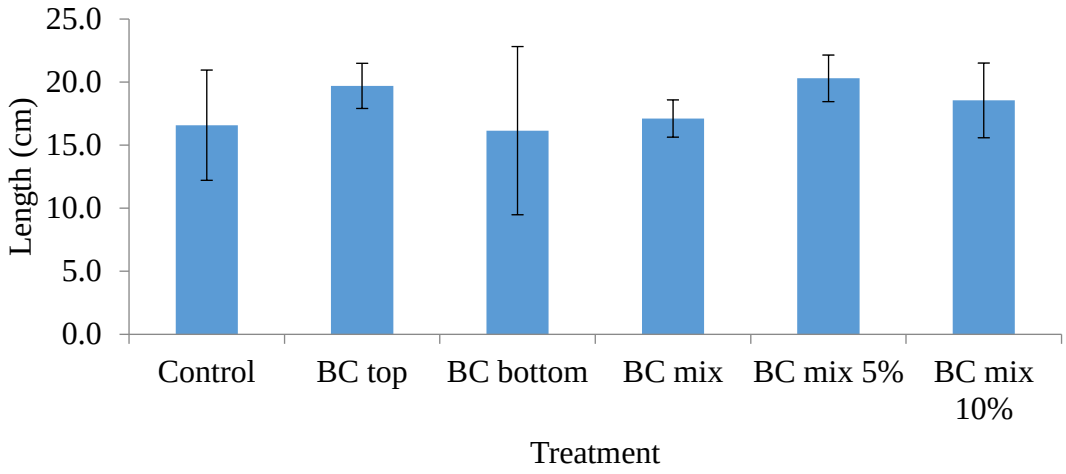


Figure 22. Root lengths of barley after 2 weeks of growth for biochar introduction optimization (BC = *D. tsuruhatenesis* inoculated biochar)

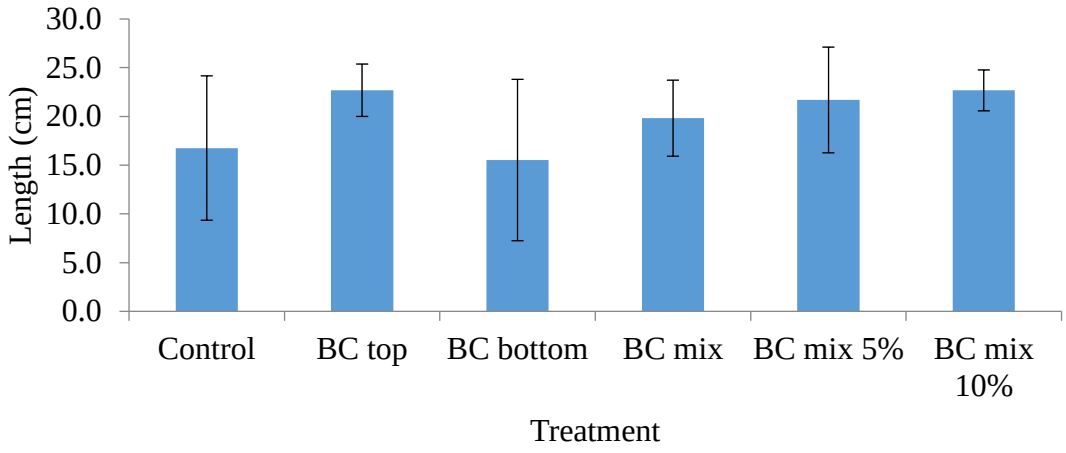


Figure 23. Shoot lengths of barley after 2 weeks of growth for biochar introduction optimization (BC = *D. tsuruhatenesis* inoculated biochar)

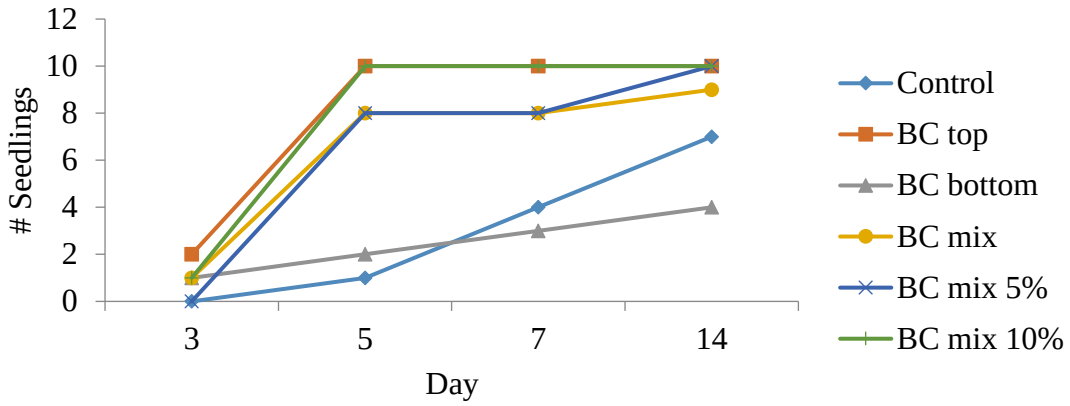


Figure 24. Germination rates of barley over a period of two weeks (BC = *D. tsuruhatenesis* inoculated biochar)

After the amount of biochar and the method of addition was optimized, an aging trial was conducted. Sterile soil was inoculated with biochar and left to age at room temperature for one week. After the week, barley seeds were planted and observations were recorded. It was noted that the germination rate of all trials suffered after aging, with zero seedlings present in the control pots after two weeks. The lack of germination in the control indicated that there was contamination from somewhere in the system that severly impacted seedling germination. Under these unfavourable conditions, inoculated trials still produced seedlings, however their numbers were reduced. It was observed that after aging, less biochar (2.5% v/v) performed the best, and the observed vegetative growth steadily decreased as the biochar loading increased. This was expected as introducing the bacteria seven days before planting gave the bacteria time to establish themselves in the soil and produce enough biomass to compete with the plant for resources.

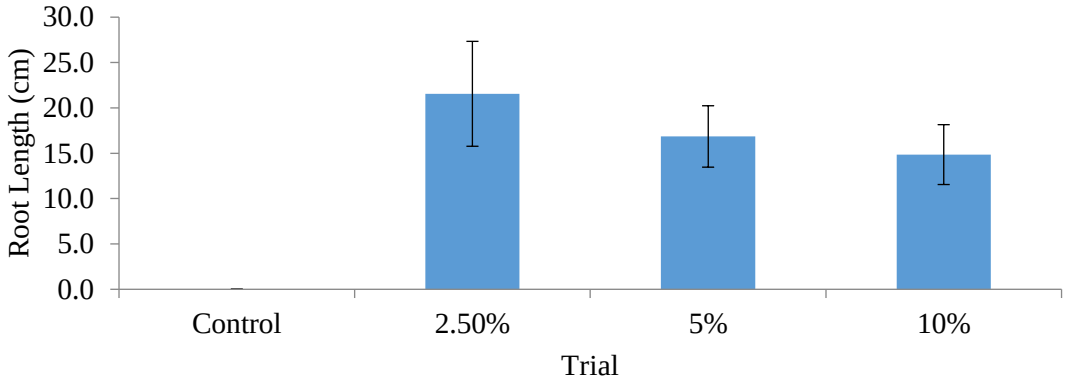


Figure 25. Root lengths of barley after 2 weeks of growth for biochar aging experiments (% = % v/v)

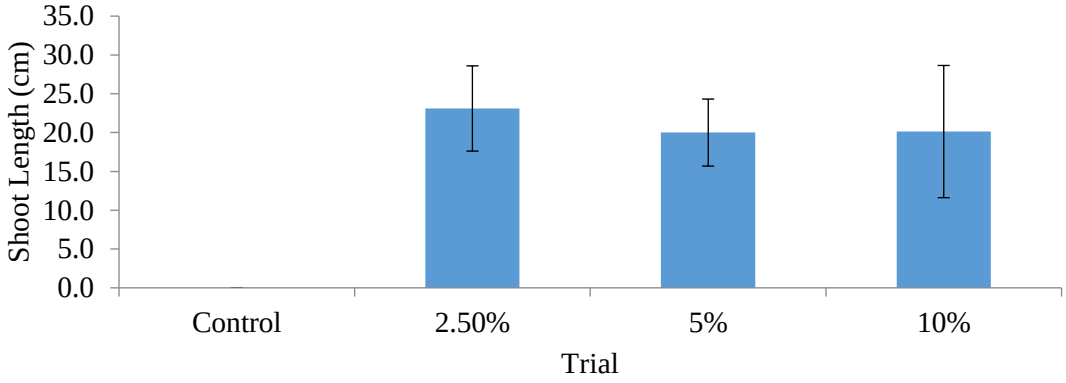


Figure 26. Shoot lengths of barley after 2 weeks of growth for biochar aging experiments (% = % v/v)

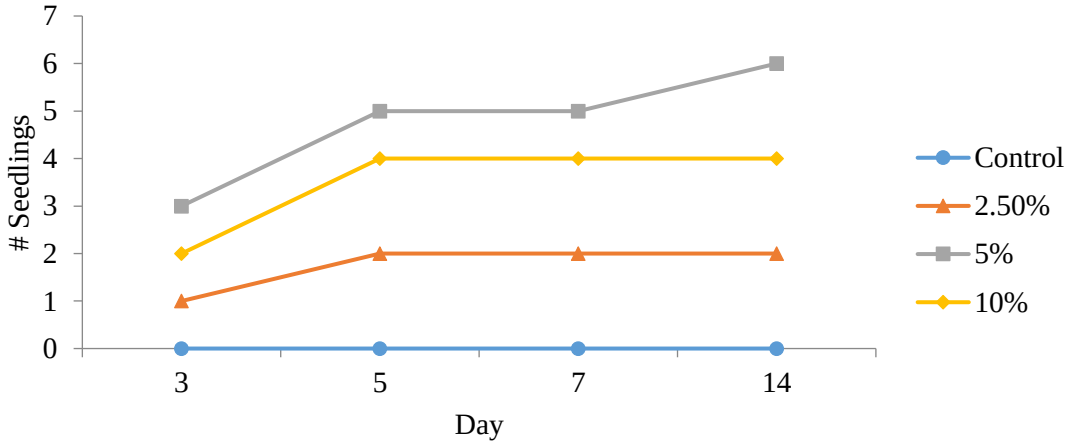


Figure 27. Germination of for biochar aging experiments (% = % v/v)

Once it was determined that aging the biochar in the soil resulted in reduced germination, one final optimization was attempted. In this experiment, the amount of nutrient broth used as the growth medium for the bacteria was steadily reduced in an effort to limit the growth promoting effect conveyed by the broth. Biochar was sterilized and soaked in varying compositions of nutrient broth before inoculation with *D. tsuruhatenesis.* After the 24 hour growth period, the biochar was introduced to the system as usual and observations were recorded. It was observed that changing the amount of nutrient broth did not greatly impact the root length of the barley and only slightly impacted the shoot length. It was also observed that the germination rate was still enhanced in the 0% nutrient broth trial (biochar was simply mixed with water and bacteria), indicating that the bacterial cells are most likely the cause of the growth enhancement, not the broth as was demonstrated previously.

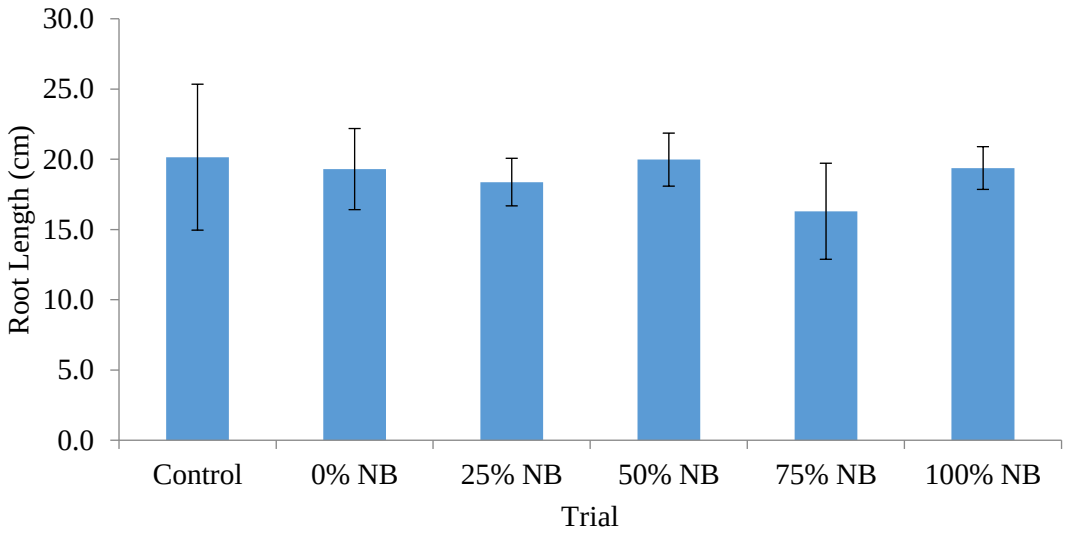


Figure 28. Root lengths of barley after 2 weeks of growth for varying nutrient broth trials

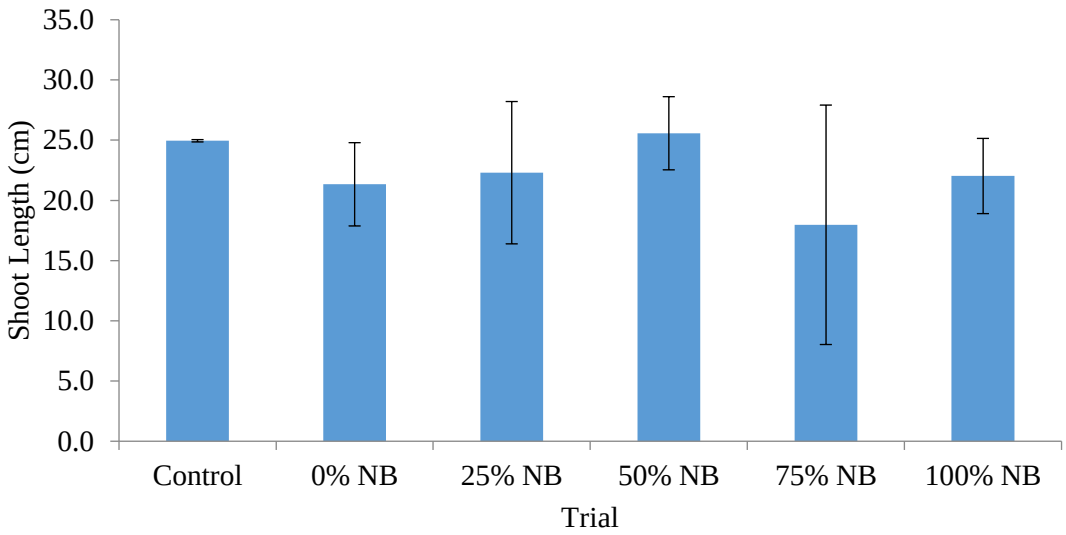


Figure 29. Shoot lengths of barley after 2 weeks of growth for varying nutrient broth trials

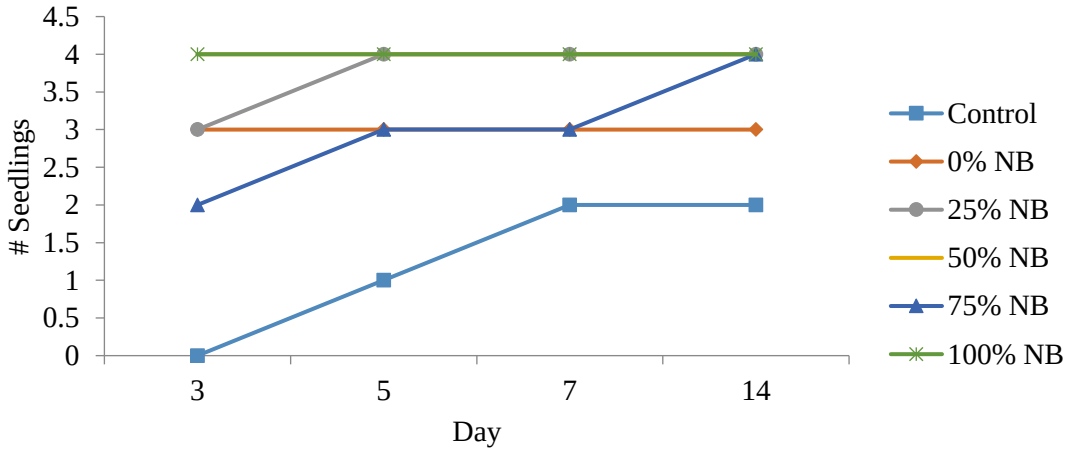


Figure 30. Germination rates of barley for varying nutrient broth trials

To assess the impact of inoculation over a longer period and on different species of host plant, three different plants were grown for six weeks. Four seeds were initially planted in each pot, but after one week of growth the seedlings were thinned to two per pot. The germination over the first week was recorded to determine how germination was impacted. After the growth period, the plants were harvested and the roots and shoots were measured. Plants were then dried for 48 hours and weighed to determine the average dry weight. Due to the delicate nature of the dry plants, separation of the samples resulted in damage so all plants were weighed and the value was averaged over the number of plants. New soil was utilized for this trial, and the germination enhancement that was observed was not as dramatic as when plants were grown in older, nutrient depleted soil. This may indicate that the inoculation procedure could be useful for crop germination as it aids in growth even when the soil is nutrient poor.

After optimization of the growth conditions, barley was grown for the six week period to see if any difference was observed after a longer growth period. It was observed that a slight increase in shoot length was observed, but no changes were statistically significant. Root length was relatively unaffected by the inoculation. The average shoot did increase with inoculation, indicating that the biomass of the plant may provide a better indication of growth than shoot length. Due to the recorded weight being an average, statistics cannot be applied to determine if the trend is significant.

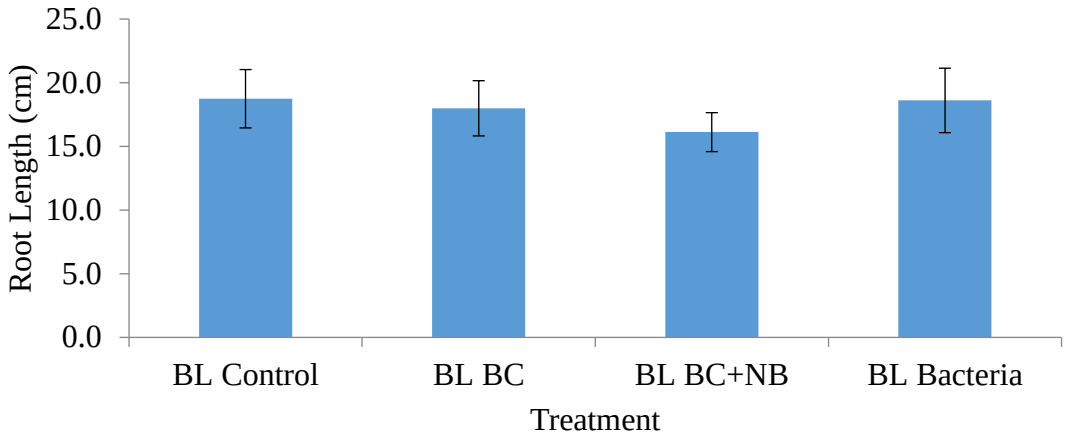


Figure 31. Root lengths of barley after 6 weeks of growth (n = 10)

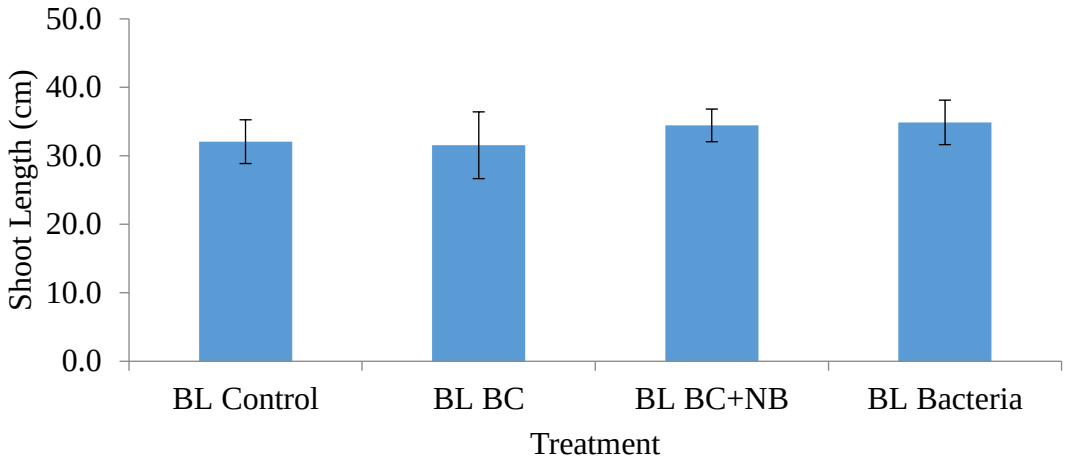


Figure 32. Shoot lengths of barley after 6 weeks for growth (n = 10)

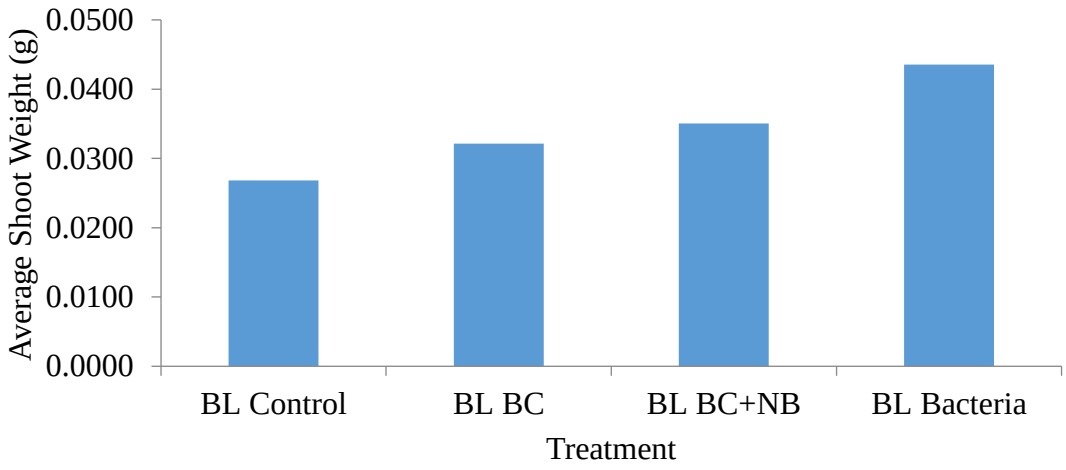


Figure 33. Shoot weight of barley after 6 weeks of growth (n = 10)

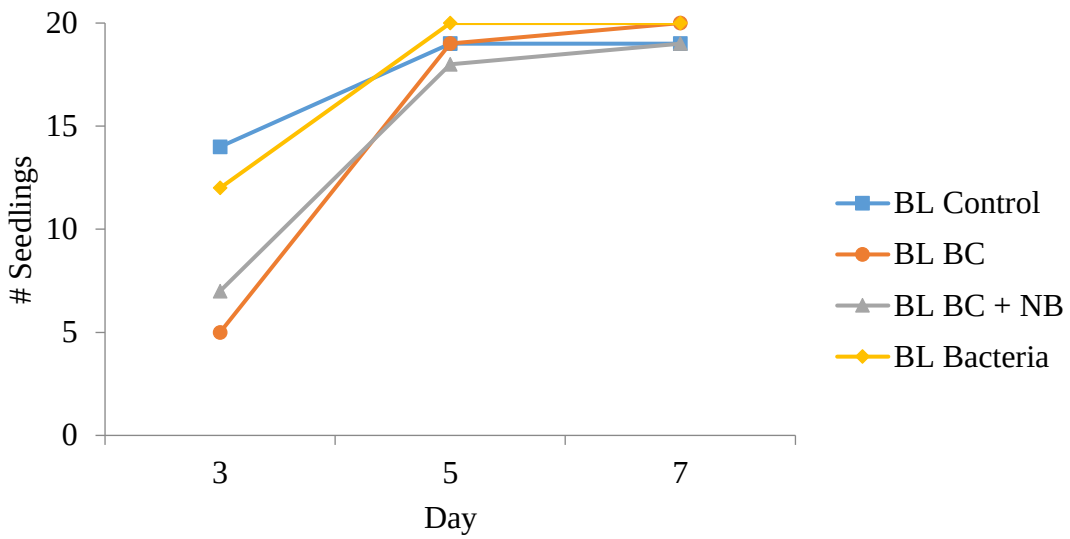


Figure 34. Germination of barley during 6 week trial (n = 20)

The next plant that was tested was the marigold. When inoculated with *Delftia tsuruhatenesis*, significantly longer roots were produced while the shoot growth showed no differences amongst all treatments. The germination rate was only slightly improved by the addition of the composite, however the addition of biochar seemed to reduce the germination that was observed.

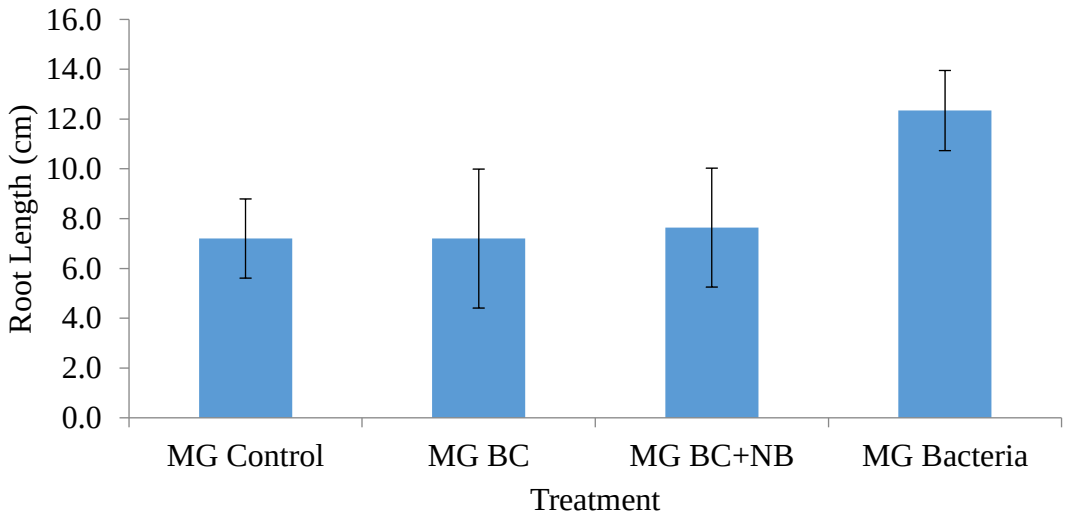


Figure 35. Root lengths of marigolds after 6 weeks of growth (n = 9-10, p = 0.02)

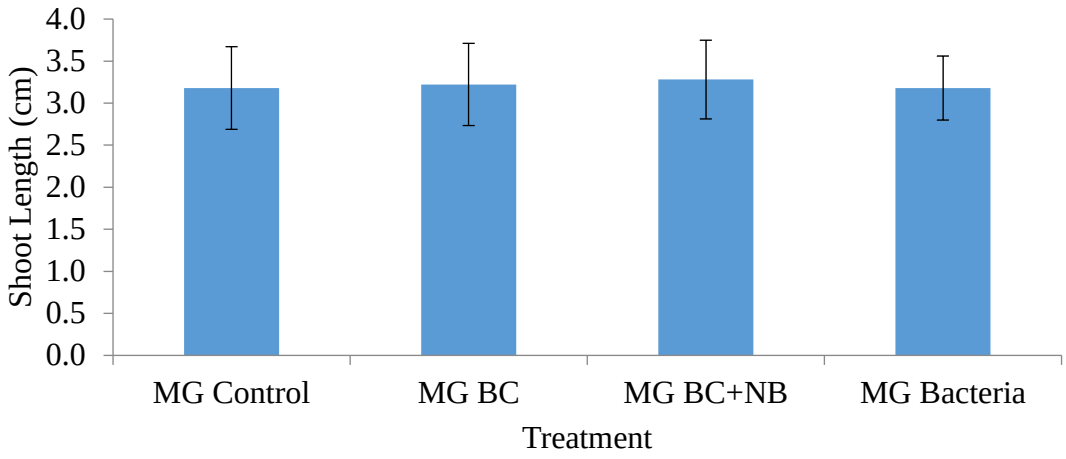


Figure 36. Shoot lengths of marigolds after 6 weeks for growth (n = 9-10)

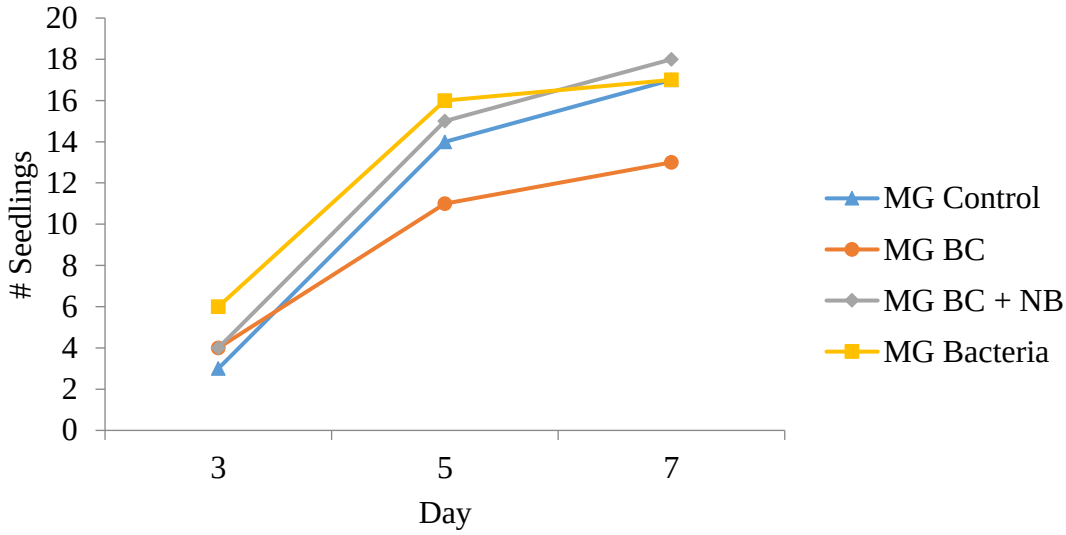


Figure 37. Germination of marigolds during 6 week trial (n = 20)

Finally, soybeans were tested as the final plant of interest. Soybeans are a species of legume, meaning they form unique interactions with specific *Rhizobium* strains to form nodules on their roots. These nodules are responsible for nitrogen fixation and their function is carefully regulated by the plant. Some evidence exists for the formation of nodules with other strains of bacteria, however no nodules were observed in these trials. The shoot and root length of the plants was not impacted by the various treatments, however germination rates increased when the soil was inoculated with *D. tsuruhatenesis.*

When the bacterium was introduced, shoot biomass actually decreased, which was unexpected. Fungal contamination was observed in almost every pot, so the trial was continued with the assumption that a fungal competitor was present. The fungal strain appeared to target seeds prior to germination, decomposing the tissue and producing an extensive network of hyphae out of the infected bean. Once the plant germinated, the fungal pathogen did not appear to inhibit growth in any way. Therefore, the increase in germination exhibited by the bacterial trial is promising as it may indicate fungal inhibition.

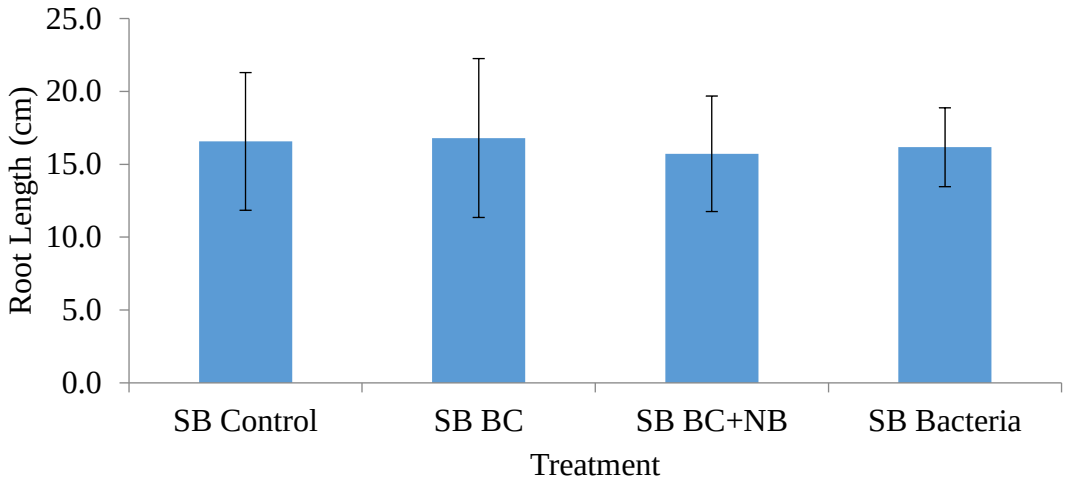


Figure 38. Root lengths of soybeans after 6 weeks of growth (n = 4-9)

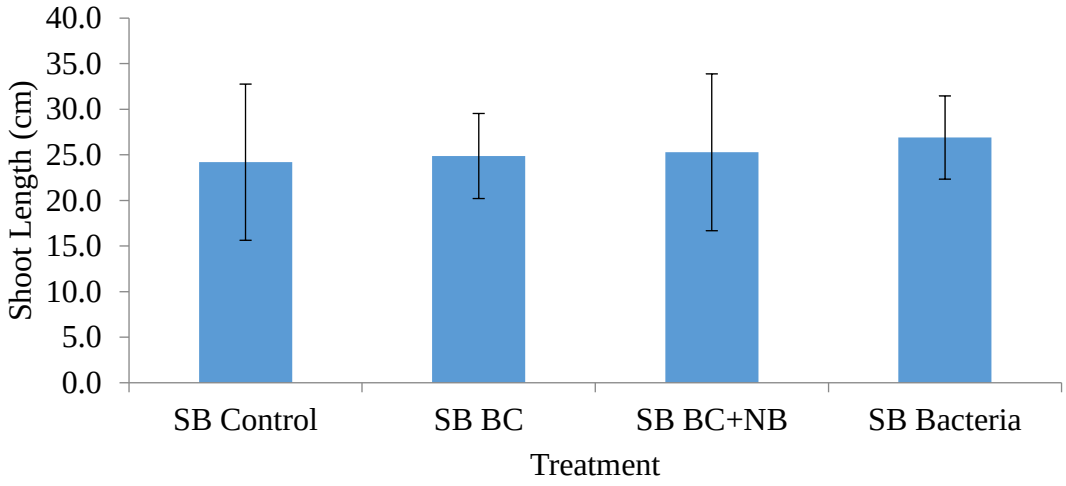


Figure 39. Shoot lengths of soybeans after 6 weeks for growth (n = 4-9)

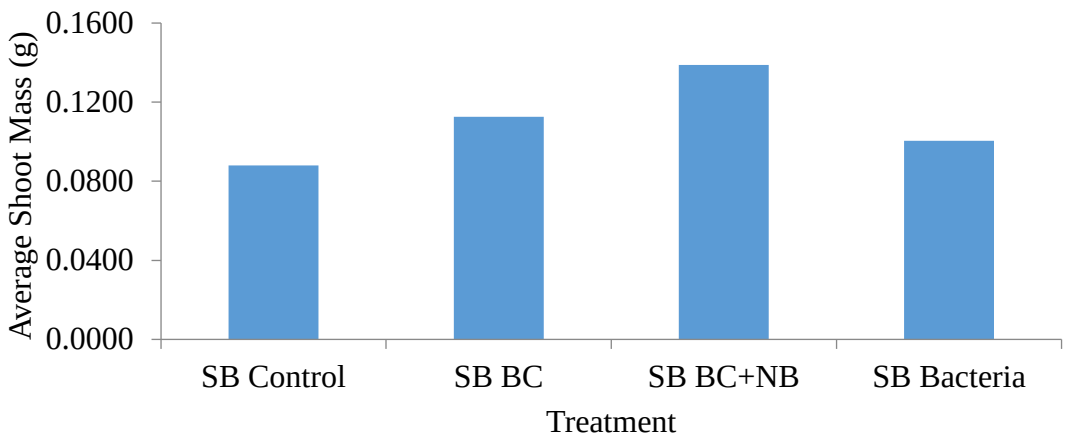


Figure 40. Shoot weight of soybeans after 6 weeks of growth (n = 4-9)

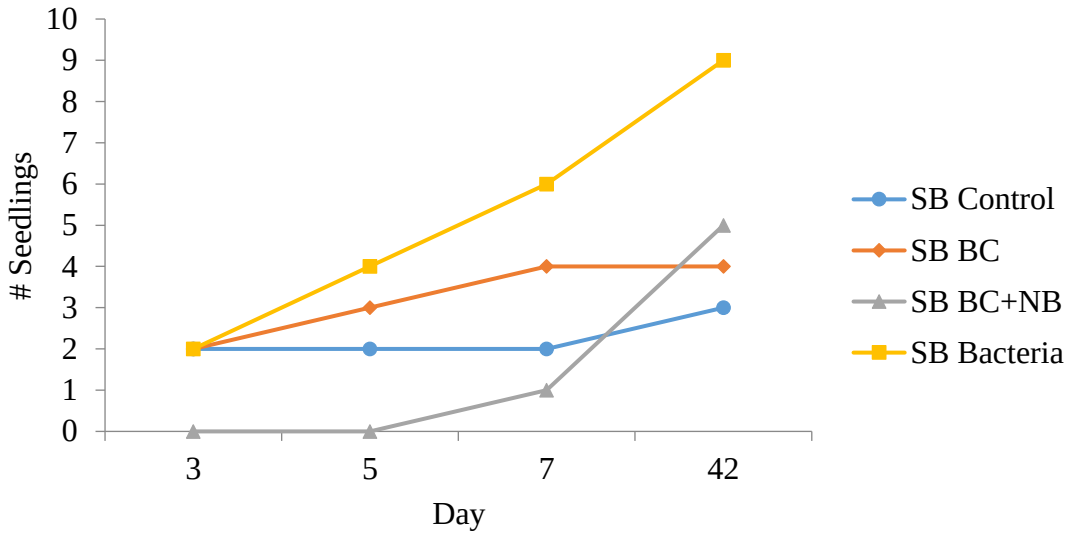


Figure 41. Germination of soybeans during 6 week trial (n= 4-9)

3.3.3. Soil Extract Agar – barley, marigolds, edamame

In an attempt to better observe the root morphology and growth of the plants, soil extract agar plates were made and used as the growth media. By introducing germinating seeds into softer agar and allowing for several days of growth, the growth could be visually assessed and the roots were clean after removal from the plate, allowing for easy weighing.

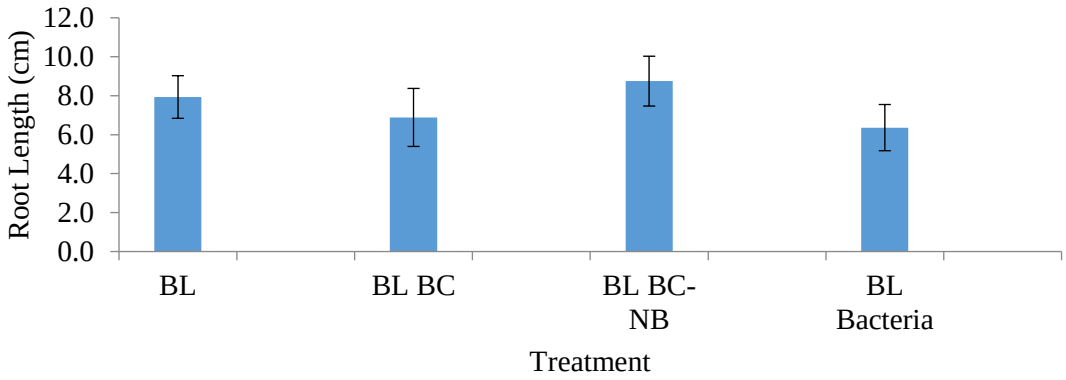


Figure 42. Root length of barley after 5 days of growth in soil extract agar (BL = barley, BL BC = barley with biochar, BL BC-NB = barley with nutrient broth and biochar, BL Bacteria = barley with inoculated biochar) (n=2).

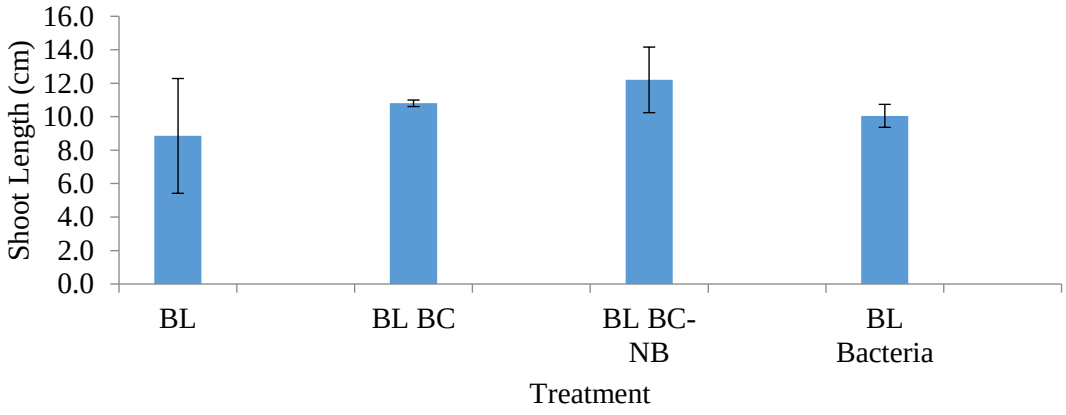


Figure 43. Shoot length of barley after 5 days of growth in soil extract agar (BL = barley, BL BC = barley with biochar, BL BC-NB = barley with nutrient broth and biochar, BL Bacteria = barley with inoculated biochar) (n = 2).

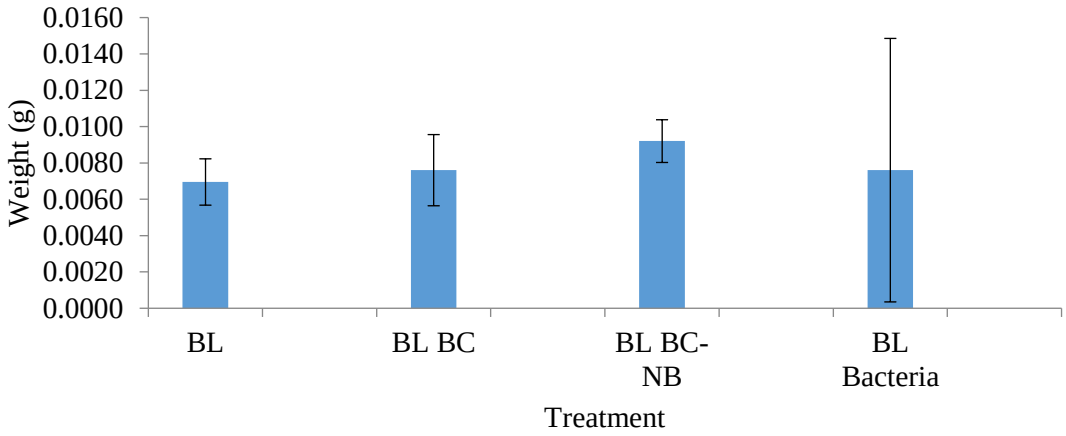


Figure 44. Root weight of barley after 5 days of growth in soil extract agar (BL = barley, BL BC = barley with biochar, BL BC-NB = barley with nutrient broth and biochar, BL Bacteria = barley with inoculated biochar) (n = 2).

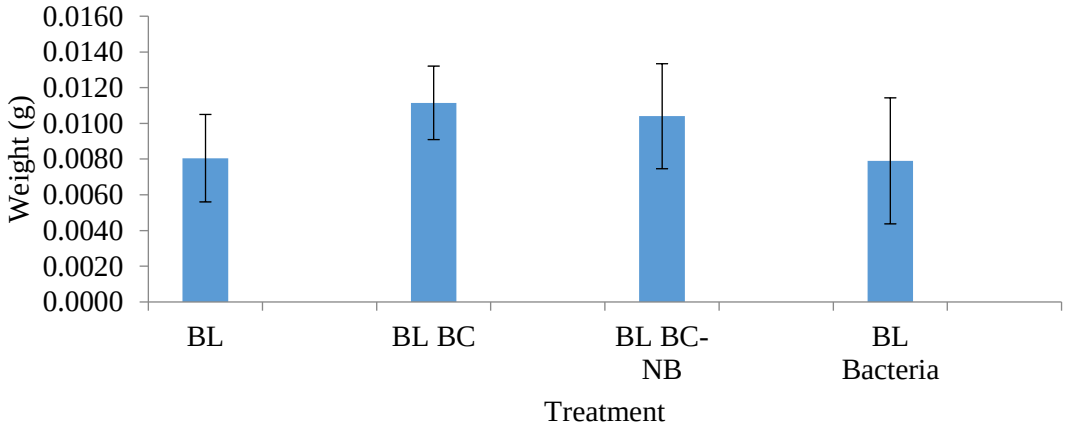


Figure 45. Shoot weight of barley after 5 days of growth in soil extract agar (BL = barley, BL BC = barley with biochar, BL BC-NB = barley with nutrient broth and biochar, BL Bacteria = barley with inoculated biochar) (n = 2).

No statistically significant differences were observed for the growth of barley under the various treatments. It was observed that the growth of the barley on the soil extract agar plates mirrored the growth demonstrated when the plant was cultivated in soil. This indicates that using soil extract agar as a means of morphological observation may be useful in situations where the plant produces mucigel or any other adaptations

that make root observation difficult. This method also allowed for the easy removal of the substrate, making root weighing significantly easier as dirt did not have to be washed away from the root tissue.

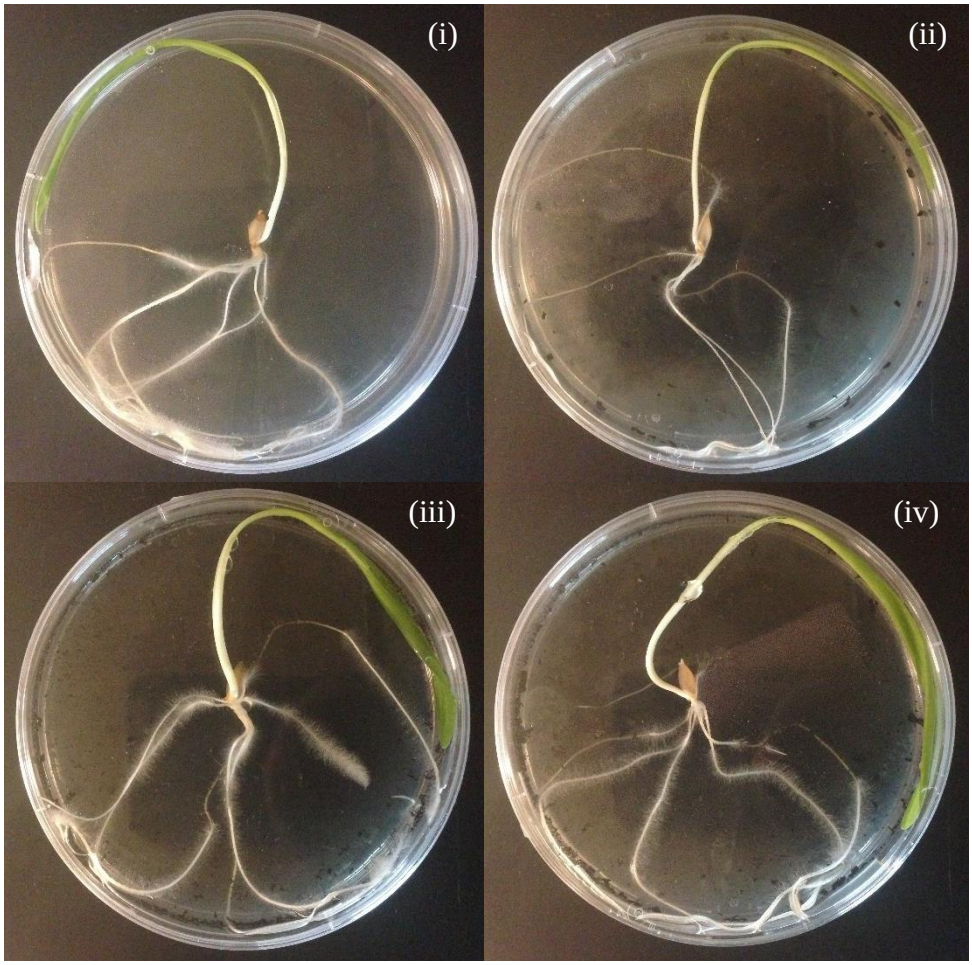


Figure 46. Barley growth after 5 days of incubation at room temperature ((i) = barley, (ii) = barley with biochar, (iii) = barley with nutrient broth and biochar, (iv) = barley with inoculated biochar)

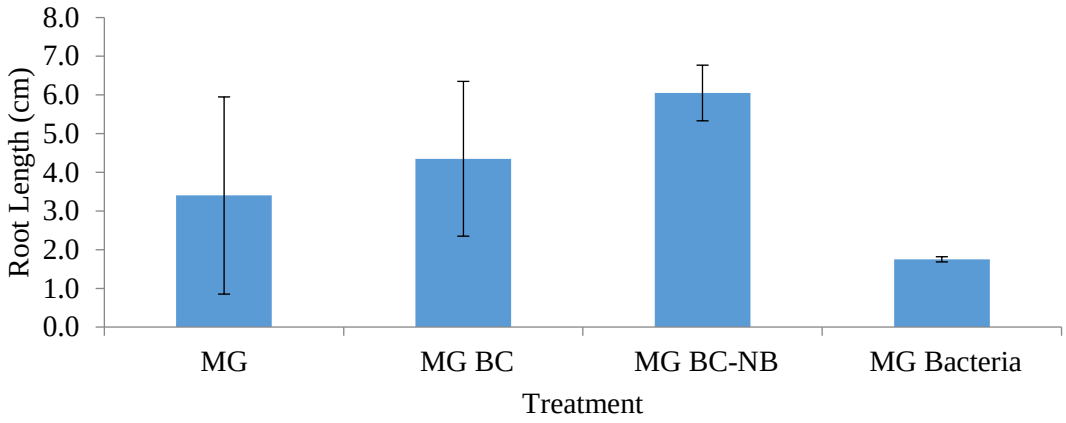


Figure 47. Root length of marigolds after 10 days of growth in soil extract agar (MG = marigolds, MG BC = marigolds with biochar, MG BC-NB = marigolds with nutrient broth and biochar, MG Bacteria = marigolds with inoculated biochar) (n = 3).

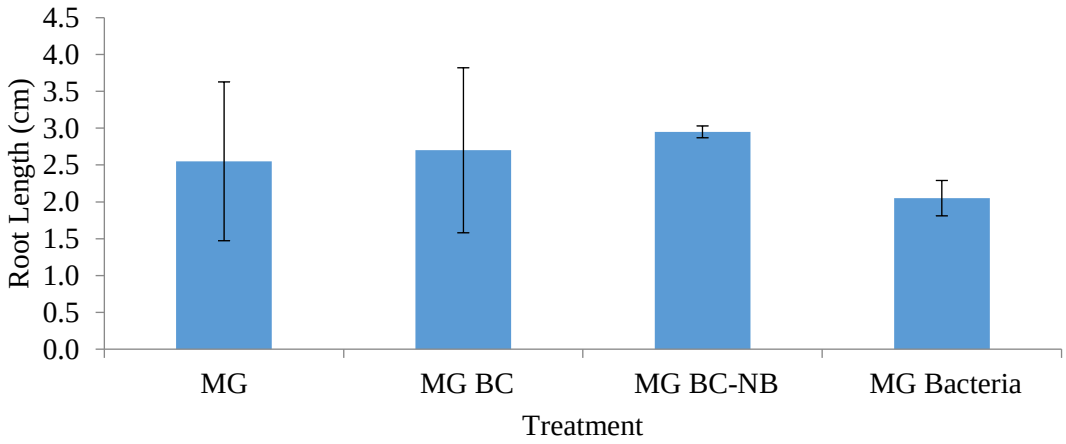


Figure 48. Root length of marigolds after 10 days of growth in soil extract agar (MG = marigolds, MG BC = marigolds with biochar, MG BC-NB = marigolds with nutrient broth and biochar, MG Bacteria = marigolds with inoculated biochar) (n = 3).

In both the root and shoot length, it was observed that the length was significantly less when bacteria was introduced compared to just nutrient broth and biochar (root length: $p = 0.0136$, shoot: $p = 0.0375$). This result demonstrates that different plants will behave differently to the same bacterial counterpart. While introducing *D. tsuruhatenesis* is not detrimental to the viability of barley and grapes, it appears to be detrimental to the growth of marigolds. The weight of the marigolds was not determined due to small size of the plants. After drying, the weight of each sample was less than 1 mg, making it difficult to get reliable mass readings. The growth in SEA was different than the growth observed in soil, so this method does not work as a model for the soil system.

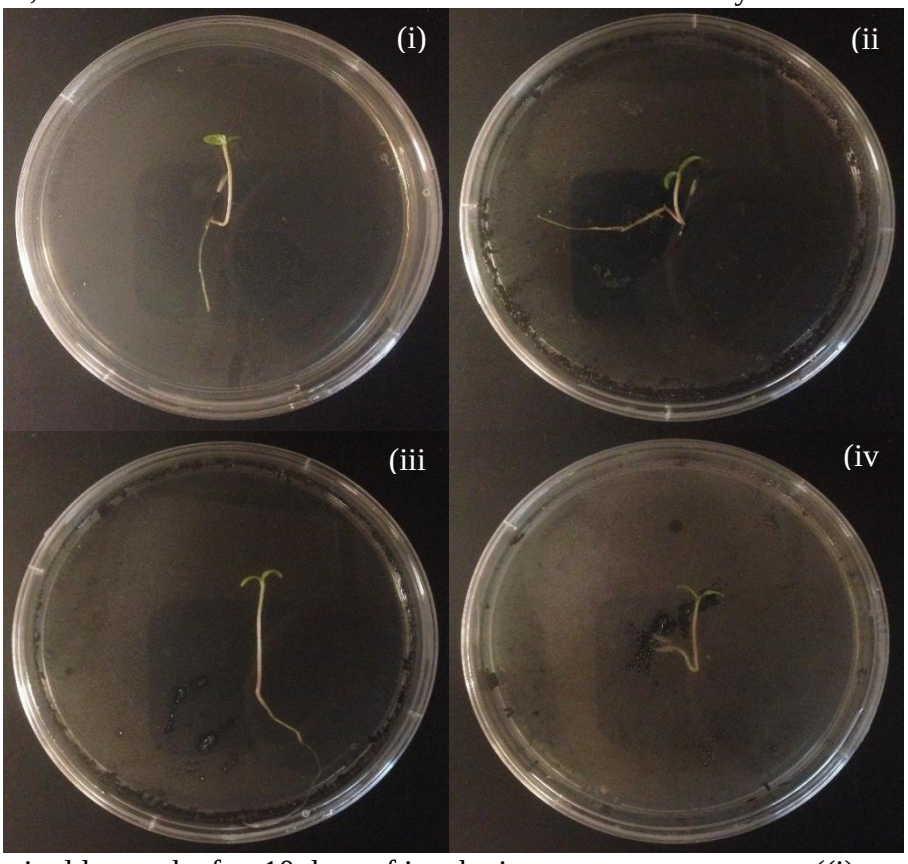


Figure 49. Marigold growth after 10 days of incubation at room temperature ((i) = marigold, (ii) = marigold with biochar, (iii) = marigold with nutrient broth and biochar, (iv) = marigold with inoculated biochar)

After marigolds were tested, the final crop left was the legume crop, the soybean. When the soybeans were sterilized and placed in lint-free wipes to germinate, it was observed that one of the beans would grow fungal hyphae and no germination would occur for any of the beans. The first time this was observed, it was regarded as simply contamination and the trial was attempted again. After a few days, the same result was obtained. In an attempt to determine the cause of the infection, an infected bean was placed on a nutrient agar plate and allowed to grow for a few days at room temperature. Within 48 hours, significant fungal growth was observed from the bean. Isolation of the fungus yielded a fast-growing white fungal strain that produced black fruiting bodies within 72 hours (SBF1).

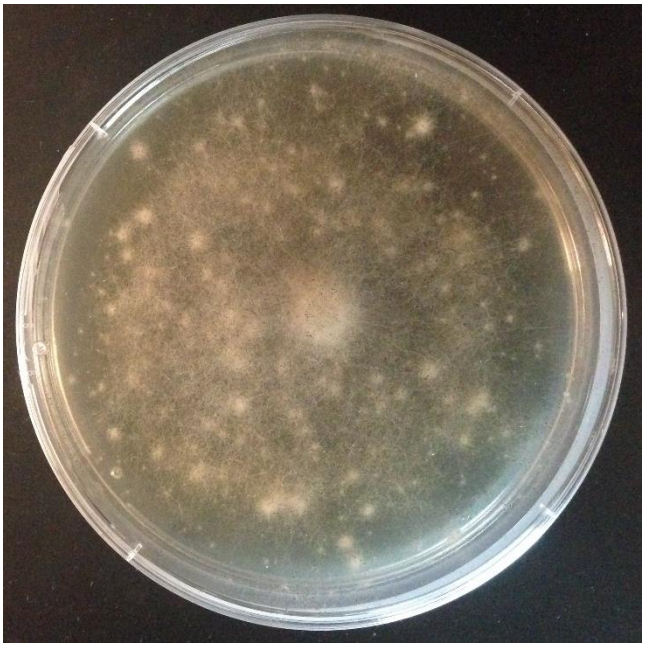


Figure 50. Soybean fungus 1 (SBF1)

To determine if the sterilization process was working, sterile and non-sterile beans were independently placed on nutrient agar and incubated at room temperature. After a few days, SBF1 was observed on the non-sterile beans, and a new white fungal strain was

observed on the sterilized beans. The new fungal strain was isolated and was observed to produce slow growing white hyphae with no visible fruiting bodies (SBF2).

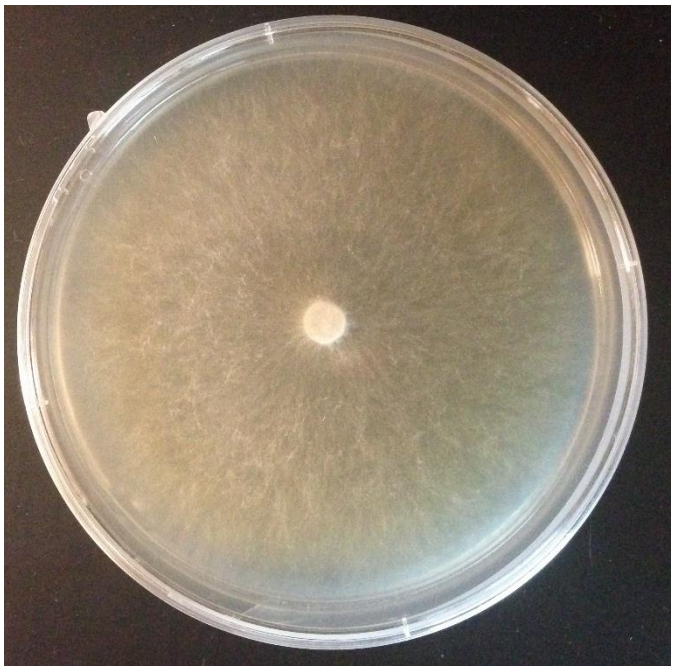


Figure 51. Soybean fungus 2 (SBF2)

In an attempt to control the growth of SBF1, co-culturing with bacterial strains derived from grape shoot tissue and soil was attempted. Eleven different strains were initially screened against SBF1 in a 12-well plate co-culture method obtained from Bertrand *et al.*[52] This co-culture method involves utilizing a 12-well plate to house four different columns of three replicates each. The first column of wells remains empty to serve as a control, the second column of wells is inoculated with the desired bacterial strain, the third column is inoculated with the fungi, and the fourth column serves as the site of co-culturing. The second and third columns are utilized as controls to observe uninhibited growth of the bacteria and fungi for comparison with the co-culture in the final column. Seven different strains exhibited some form of inhibition. The strains that exhibited inhibition were plant tissue derived, while all the strains that exhibited no inhibition were soil derived. This indicates that the plant derived strains are more potent

antifungal agent producers. This may be due to the fact that the bacterial strains present in the phyllosphere (outer surface of plant tissue) are in part responsible for immune response and assist in the defense of their host.

Of the seven strains that demonstrated inhibition, it was observed that three strains appeared to cause contact-independent inhibition of the fungal strain. To determine if a volatile compound was the cause of inhibition, the headspace was sampled using GC-MS in an attempt to detect any compounds. The type of inhibition was determined by analysing the morphology of the microbes in their columns of wells. In the case of contact-dependent inhibition, the fungal growth was only inhibited when the bacterial strain was inoculated in the same well. The growth of both the bacteria and fungi in their control wells remained unchanged, indicating that the inhibitory agent was diffusing through the agar. In the case of contact-independent inhibition, the fungal growth was inhibited in all wells. Due to the inhibition of the fungi in the control column of wells, it can be determined that the inhibitory agent must be present in the gas phase as there is no agar to facilitate the diffusion of a soluble compound. Inhibition of the co-culture column may be due to a soluble inhibitory agent as well as the volatile agent, however further work must be done in order to isolate and characterize metabolites present in the system.

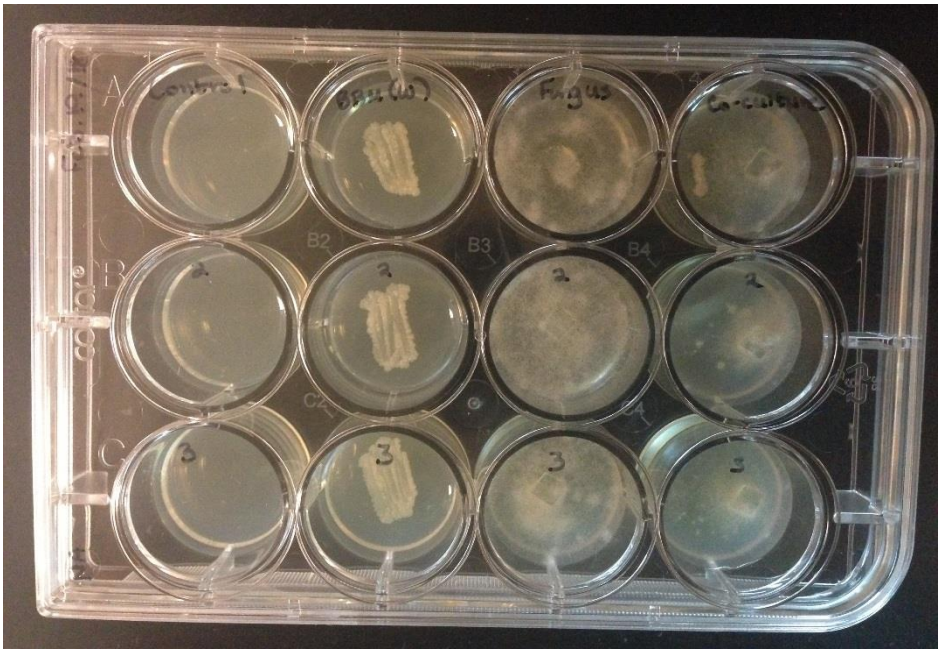


Figure 52. Unsuccessful co-culture experiment (no inhibition observed)

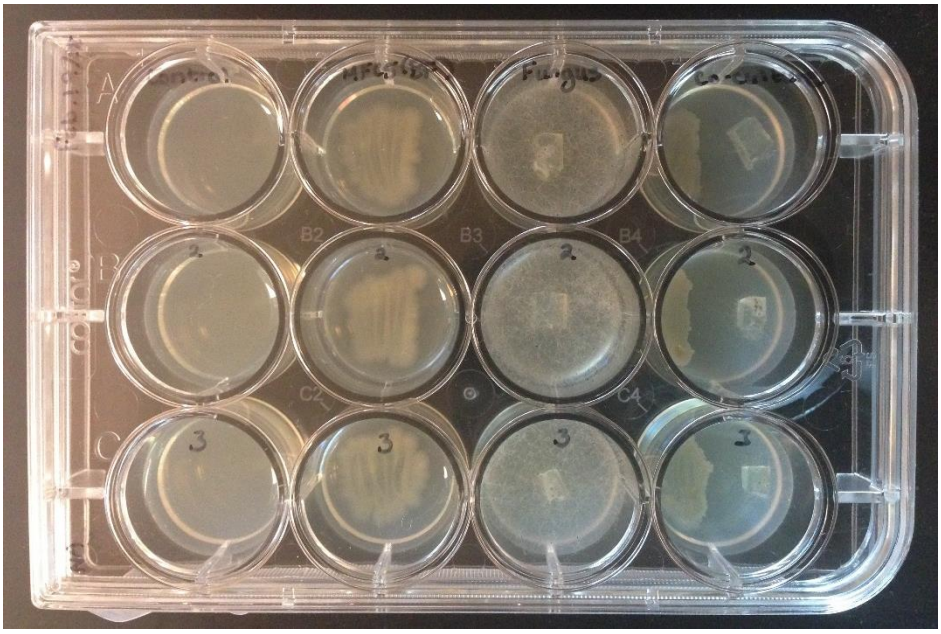


Figure 53. Successful co-culture experiment (contact dependent inhibition observed)

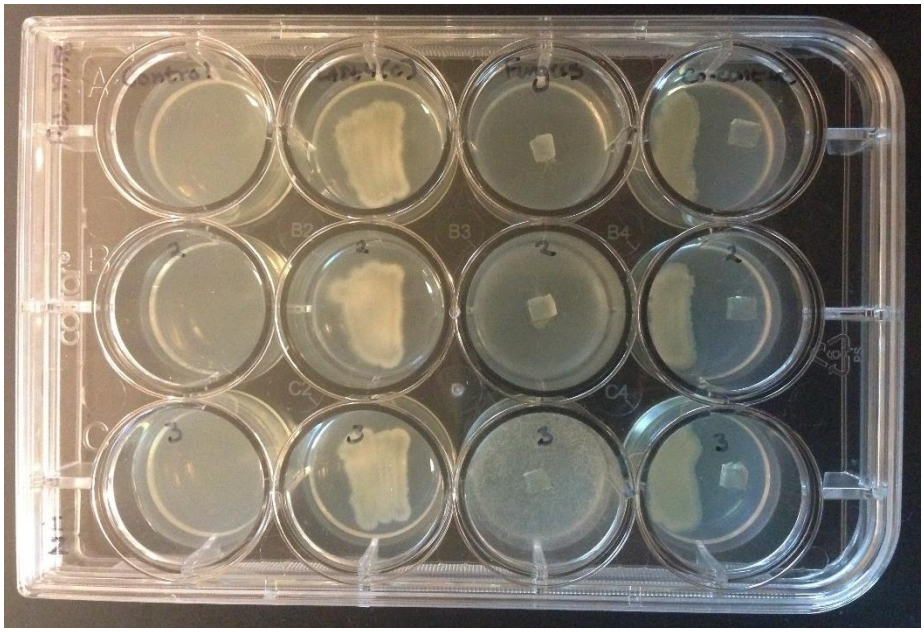


Figure 54. Successful co-culture experiment (contact independent inhibition observed)

3.4. Volatile Plant and Microbe Profiles and GC-MS Analysis

When plants are placed in different growth conditions, whether the conditions be harmful or beneficial, the volatile profile that they produce can change. In an attempt to analyze the headspace, samples of leaves were collected and analyzed *via* GC-MS. 5-10 fresh leaves were placed in a volatile collection chamber (modified petri dish with two holes bored in the lid) and allowed to equilibrate for 2 hours at room temperature. After two hours passed the headspace was sampled and the resulting volatiles were eluted from a Porapak-Q column and injected into the GC-MS.

Volatile headspace sampling revealed that inoculation increased the volatile production of marigold shoots. Significant peaks at 11.36 and 11.67 minutes appeared in the chromatogram when a bacterial partner was introduced. An increase in volatile production could indicate that the plant was stressed due to the inoculation, but peak assignments based of MS fragmentation are required. Due to incorrect cleaning of the volatile traps, only the headspace of marigolds was successfully assessed.

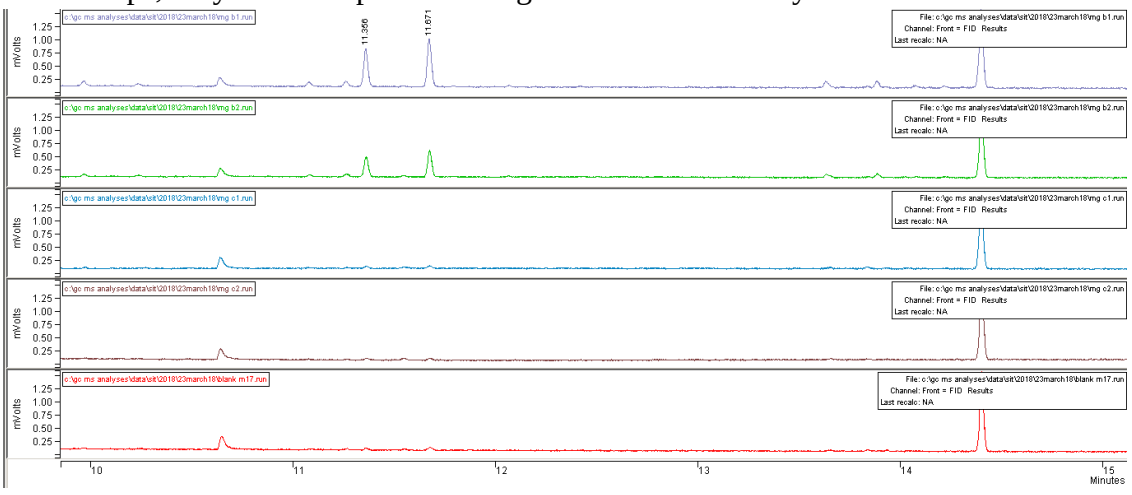


Figure 55. GC chromatogram of marigold volatiles (top two: inoculated marigolds, middle two: control marigolds, bottom: system blank)

During the fungal co-culture experiments, it was observed that three different strains of bacteria demonstrated contact independent inhibition of the fungus. To capture the volatiles responsible, a modified lid (12 well plate lid with two holes bored in it) was used to replace the top of the 12 well plate and the headspace was sampled for two hours. After sampling, the analysis was conducted the same way as the plant samples. One of the three strains, LAC2(PP), produced a new peak at 18.278 minutes that was not present in the blank of the solvent or the nutrient agar. Due to technical difficulties with the instrument, mass spec data could not be obtained, however it could be determined based on the method that the boiling point of the unknown compound was about 250 °C. Due to the high boiling point, the unknown compound may be polar or have a very high molecular weight. This could be indicative of a multi-ring system or a peptide.

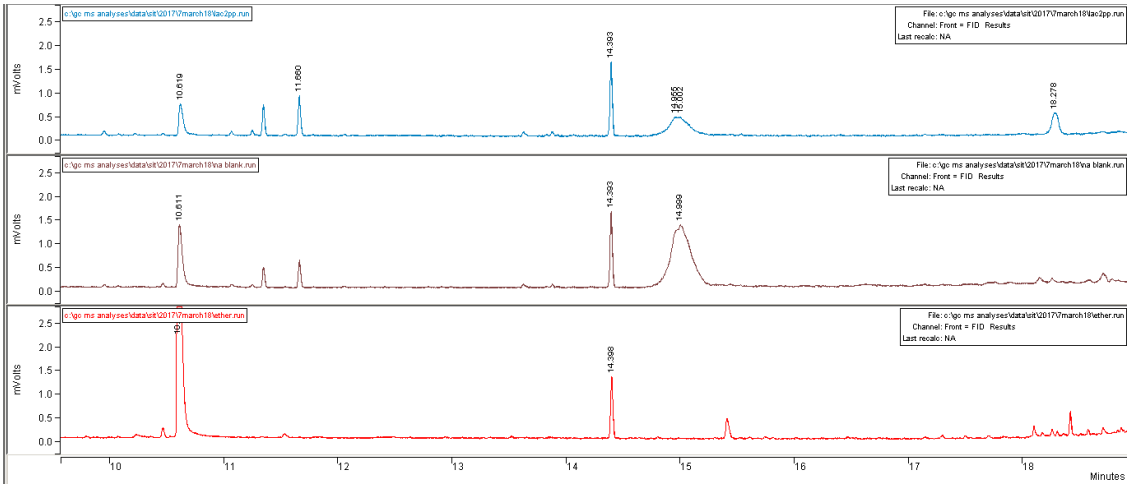


Figure 56. GC chromatogram of volatile inhibition (top: LAC2(PP) co-culture, middle: nutrient agar, bottom: diethyl ether)

The other two bacterial strains that demonstrated inhibition did not produce any new peaks when analyzed. This could be a result of the low concentration of volatile compounds or the nature of the compounds themselves. If the boiling point of the compounds are above 250 °C, they would not appear during the currently utilized

method. If the compounds are too polar, a similar issue would be encountered. Concentration of the sample or longer sampling of the headspace may correct the concentration issue, while a new method would have to be developed if the issue is the molecular structure.

3.5. Elemental Analysis of Plant Material

Nitrogen is one of the crucial elements required for proper plant growth and function. With low nitrogen uptake, the plant can suffer in a variety of ways. Nitrogen fertilizer is often applied to the soil in the form of soluble nitrates and nitrites, but another important source of nitrogen is soil bacteria. To assess the impact of inoculation of nitrogen uptake, leaves of barley and soybeans were dried for 48 hours at 55 °C, ground into a fine powder and submitted for CHN elemental analysis.

It was observed that when *Delftia tsuruhatenesis* was introduced into the soil, nitrogen uptake of barley significantly ($p < 0.001$) decreased while no significant difference was observed for soybeans. This could indicate that the bacterial strain was actually competing with the plant for nutrients, resulting in the lower total nitrogen content.

Table 2. Elemental analysis data for barley and soybean leaves

Sample	C	Stdev	H	Stdev	N	Stdev	C:N	Stdev
SB Control	43.30	0.17	6.10	0.09	8.32	0.06	5.20	0.05
SB Bacteria	42.89	0.81	5.99	0.10	8.22	0.14	5.22	0.06
BL Control	34.32	0.29	5.01	0.11	7.52	0.07	4.56	0.05
BL Bacteria	33.92	0.68	4.92	0.17	5.90	0.04	5.75	0.08

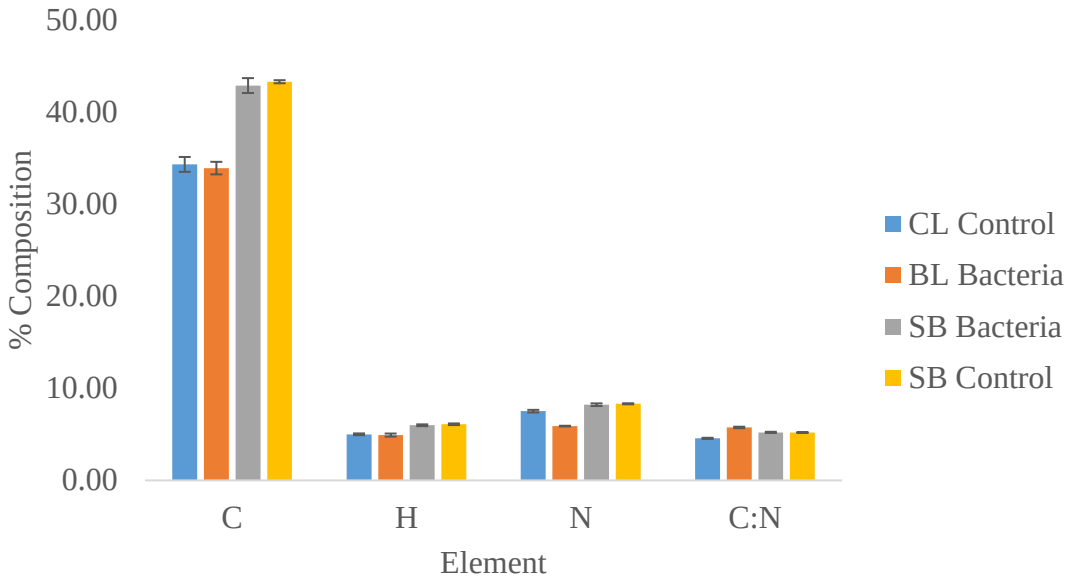


Figure 57. Elemental composition of barley and soybeans leaves (error bars represent standard deviation)

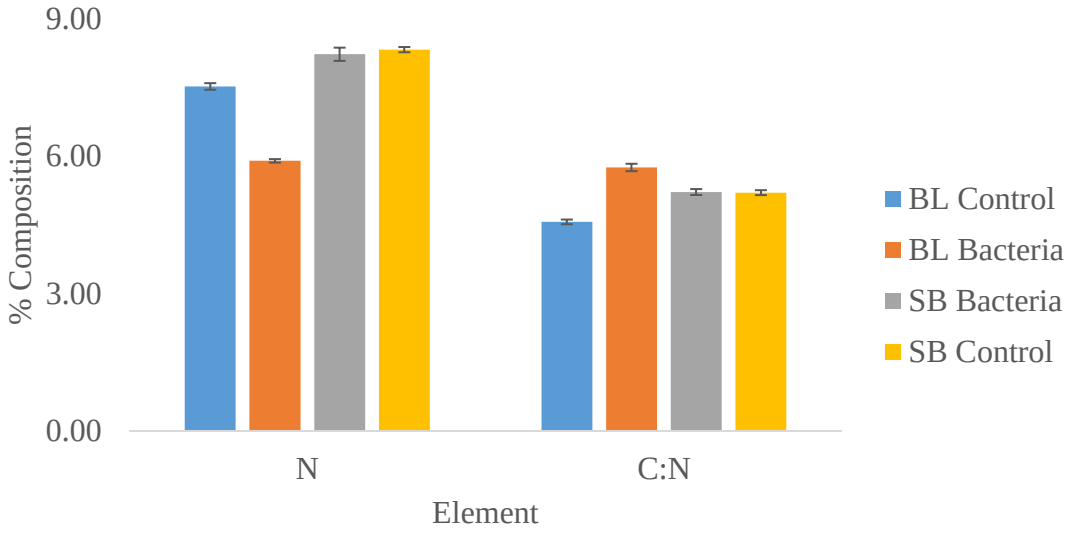


Figure 58. Nitrogen composition and C:N ratio of soybean and barley leaves (error bars represent standard deviation)

3.6. Fermentation

Zarraonaindia *et al.* examined the microbial community from various grape tissues and determined that the majority of the community found on the vegetative plant tissue was originally soil derived.[53] As such, it is not a stretch to assume that any bacterial strain in the soil may wind up on the grapes themselves and be incorporated into the fermentation. In an attempt to qualitatively assess the impact of these bacterial strains, soil and plant based bacterial isolates were screened through pseudo-fermentation conditions and incorporated into grape juice to observe their role in the fermentation process. Six different bacterial strains were utilized for the fermentation: BBSE6(BO), LALA3(C), PR(Y), Multi(B), PR2(P), and PR3(F).

The first two weeks of fermentation proceeded with lots of gas production in the ferments containing yeast over the first few days. This was expected as yeast reproduce rapidly and have a high rate of metabolism in a high sugar environment. After day five, the gas production slowed significantly and a layer of dead yeast cells (the lees) had accumulated on the bottom of the vial. Filtering through a Whatman #2 filter paper and racking into a new vial successfully removed the majority of the lees. The new vials were sealed and the wine was allowed to age for 16 weeks before samples were taken for subsequent LC-MS analysis.

To try and better understand the impact of the bacterial strains, a set of fermentations were prepared with only the bacteria as the inoculum. These trials did not produce any visible gas during the primary fermentation phase and did not result in an accumulation of dead cells. To be consistent throughout the experiment, these trials were also filtered and racked before aging. After a few weeks, it was discovered that the

bacterial cells were not completely removed during the filtering process, and many of them were still viable. This was concluded after a vial in the aging process containing only bacteria detonated after the bacteria produced enough gas to increase the pressure of the vial. After this revelation, all bacterial ferments without yeast were discarded to avoid potential damage.

After the wine samples were aged, they were analyzed *via* LC-MS for differences in the polyphenolic content. During method development, it was determined that operating the mass spectrometer with a mobile phase of >80% aqueous solvent resulted in large background spikes due to difficulties with electrospray ionization. After this discovery, the mobile phase was modified accordingly and the background noise was significantly reduced.

Utilizing the database from Flamini *et al.*, the grape derived compounds that showed reproducible deviation from the control were tentatively assigned. Figure 61 displays the compounds themselves, along with their molecular weight, while Figures 62-68 display the chromatograms obtained after LC-MS analysis of white wine. The red wine results are displayed in Figures 69 – 75.

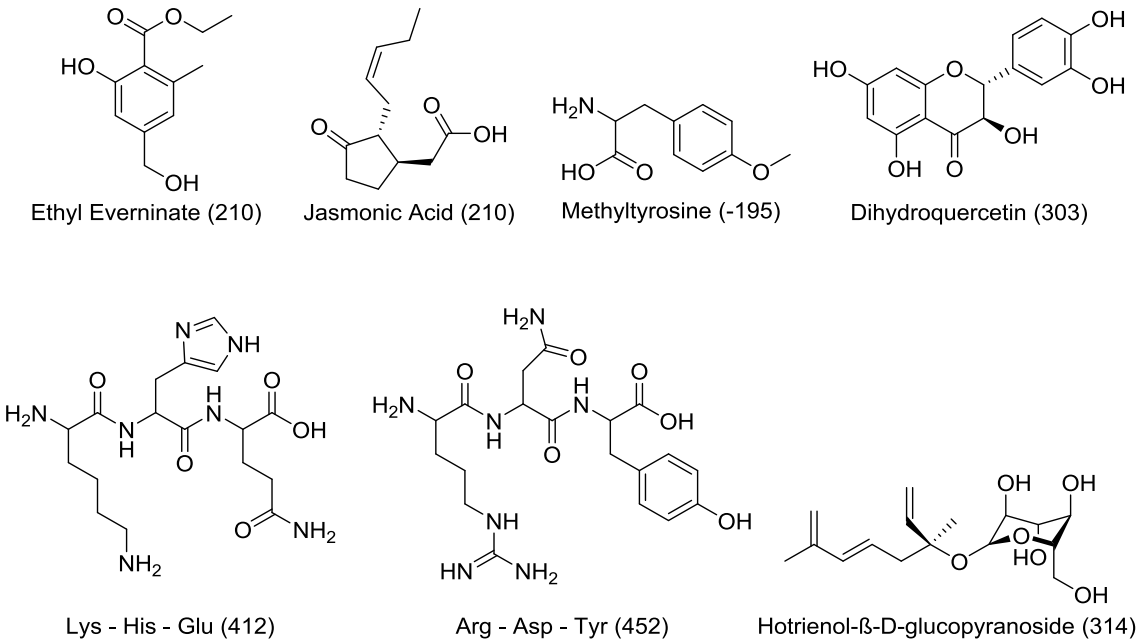


Figure 59. Wine compounds that exhibited changes with bacterial inoculation detected in white wine with their respective molecular weights

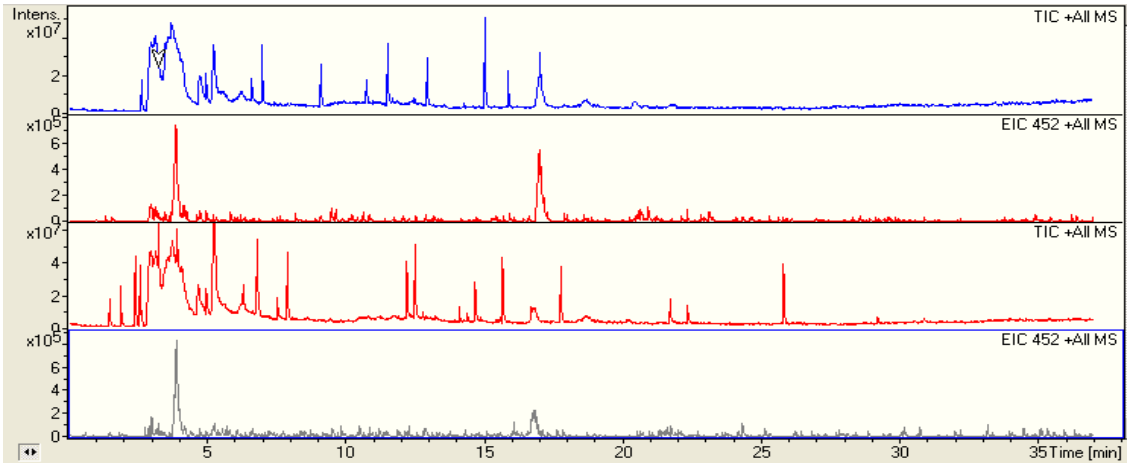


Figure 60. Extracted ion chromatogram of molecular peak 452 in the positive mode and total ion chromatograms of white wine samples (top: PR(Y), bottom: yeast)

One large peak at 3.7 mins and a small peak at 16.9 mins are present in the white wine control. In the sample containing bacteria, the peak at 16.9 mins has increased in intensity.

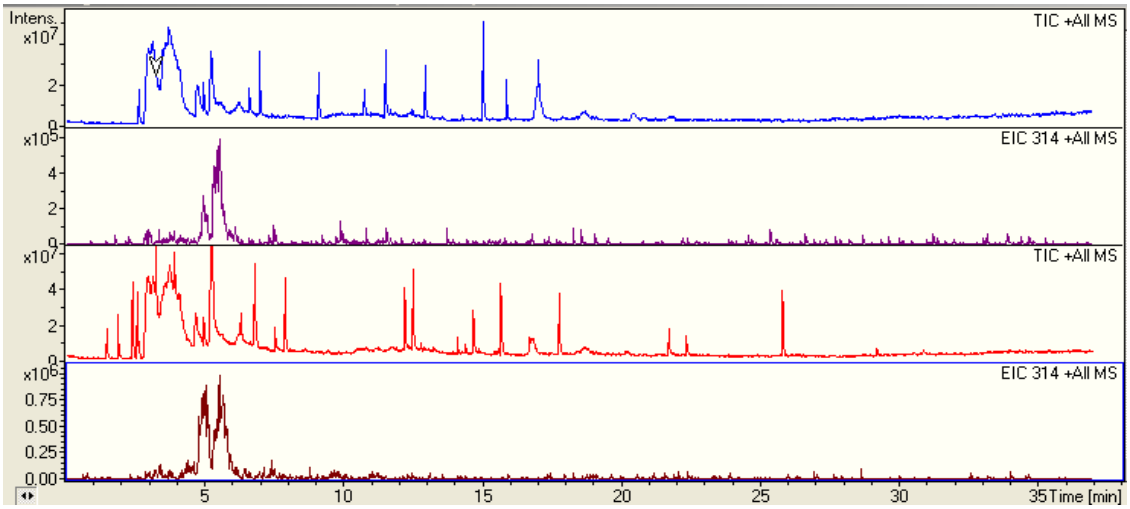


Figure 61. Extracted ion chromatogram of molecular peak 314 in the positive mode and total ion chromatograms of white wine samples (top: PR(Y), bottom: yeast)

Two prominent peaks at 5.0 mins and 5.5 mins are present in the extracted ion chromatogram for the control. In the inoculated trial, the first peak at 5.0 mins has decreased in intensity.

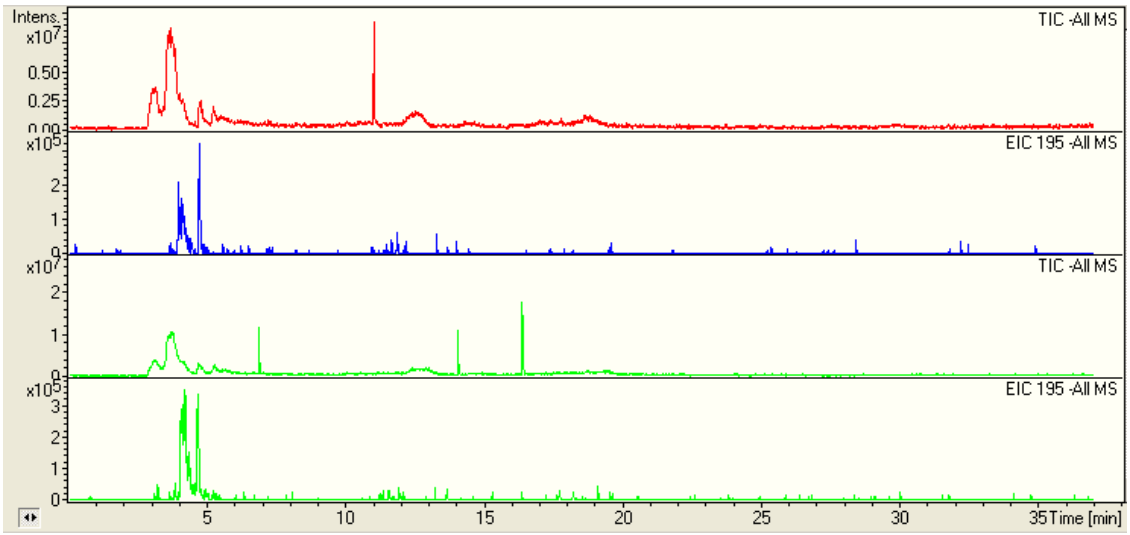


Figure 62. Extracted ion chromatogram of molecular peak 195 in the negative mode and total ion chromatograms of white wine samples (top: PR(Y), bottom: yeast)In the both samples, a prominent peak at 4.1 mins is observed. In the bacterial sample, the intensity of the peak has decreased compared to the control.

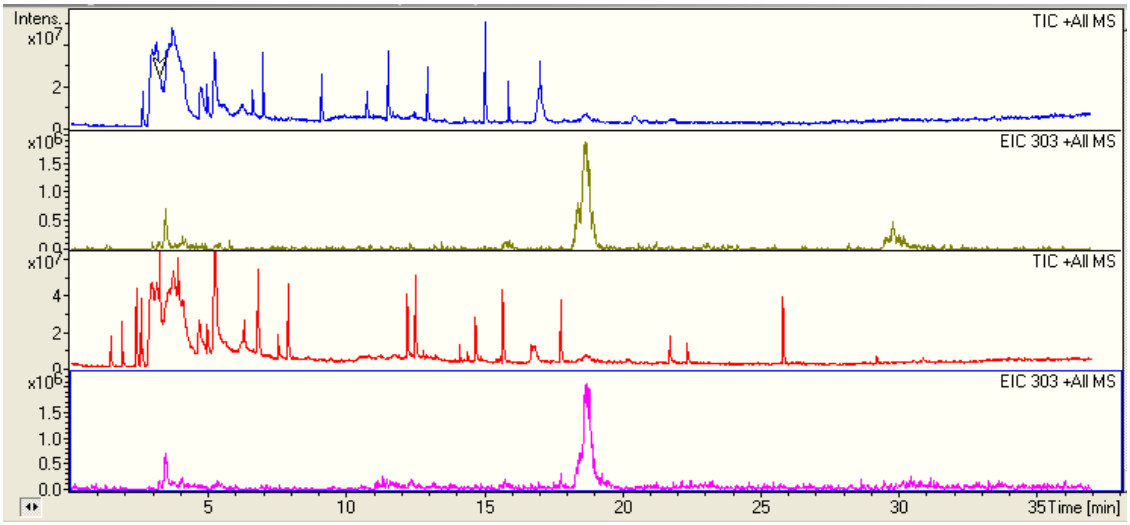


Figure 63. Extracted ion chromatogram of molecular peak 303 in the positive mode and total ion chromatograms of white wine samples (top: PR(Y), bottom: yeast)

In the control, one prominent peak at 18.7 mins is observed and one small peak at 3.4 mins is observed. In the bacterial sample, an additional peak at 29.7 mins is emerging.

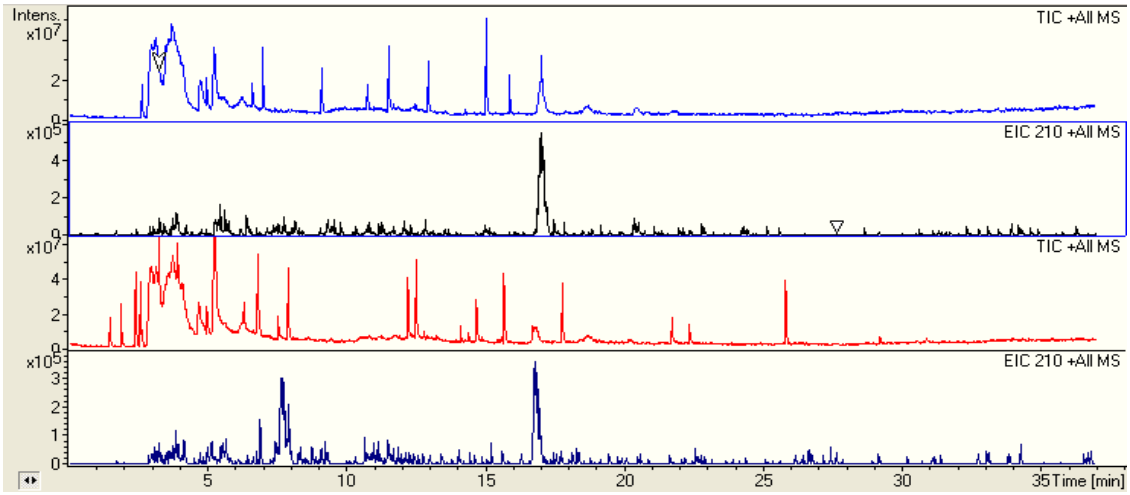


Figure 64. Extracted ion chromatogram of molecular peak 210 in the positive mode and total ion chromatograms of white wine samples (top: PR(Y), bottom: yeast)

In the control wine, two peaks at 7.6 mins and 16.8 mins are observed. In the bacterial sample, the peak at 7.6 mins has decreased to the point of being indistinguishable from the background.

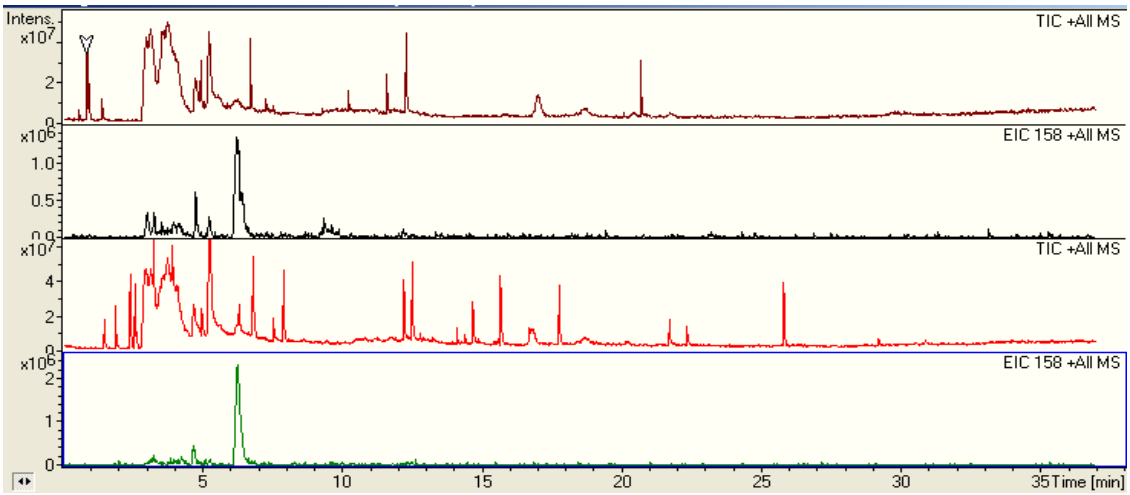


Figure 65. Extracted ion chromatogram of molecular peak 158 in the positive mode and total ion chromatograms of white wine samples (top: LALA3(C), bottom: yeast)

In the control sample, a prominent peak at 6.2 mins is observed. In the bacterial sample, the peak at 6.2 mins is still present, along with some small new peaks around 4 – 5 mins and 10 mins.

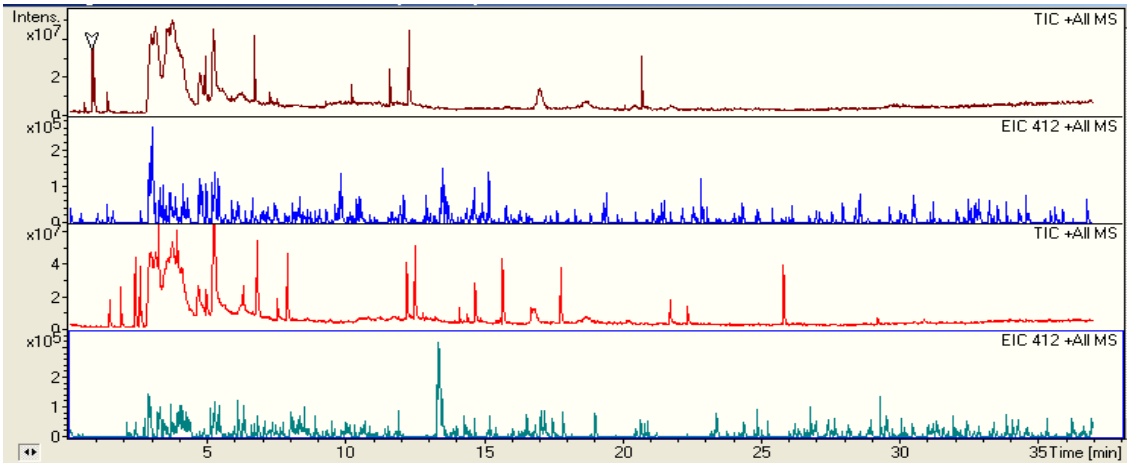
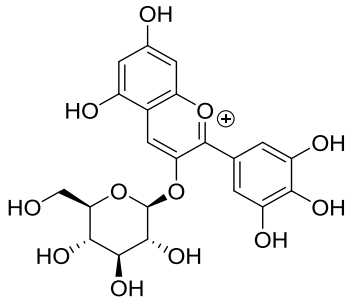


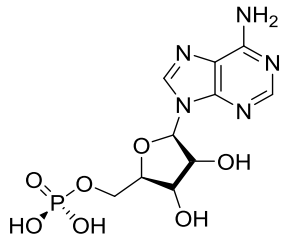
Figure 66. Extracted ion chromatogram of molecular peak 412 in the positive mode and total ion chromatograms of white wine samples (top: LALA3(C), bottom: yeast)

In the control sample, a signal is distinguishable at 13.3 mins. In the bacterial sample, the signal at 13.3 mins in buried in the baseline and a new peak at 2.8 mins has emerged.

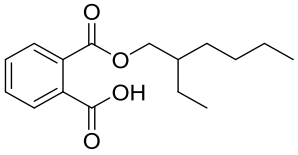
The peaks that were presented are representative of deviations that were present in two or more wine samples. Differences in EIC peaks were observed between the different inoculation strains, however due to time constraints they were not assigned. For the samples where peaks began to emerge in previously quiet regions, there is the possibility that the bacterial strain is producing a similar compound (possible in the case of the small peptides detected) or producing derivatives of existing polyphenols (possible in the case of quercetin). For the samples with peak reduction being observed, it is possible that the bacterial strain is utilizing the compound as a nutrient source or it is being degraded for some other purpose. The reduction of one of the peaks for 314 (hotrienol-β-D-glucopyranoside) is especially interesting as this molecule contains glucose. The loss of a peak corresponding to this molecule could indicate the bacterial strain is utilizing the compound as a nutrient source due to the sugar.



Delphinidin-3-O-monoglucoside (465)



Adenosine monophosphate (347)



Phthalic acid mono-2-ethylhexyl ester (278)

Figure 67. Red wine compounds that exhibited changes in their extracted ion chromatograms when a bacterial inoculant was introduced

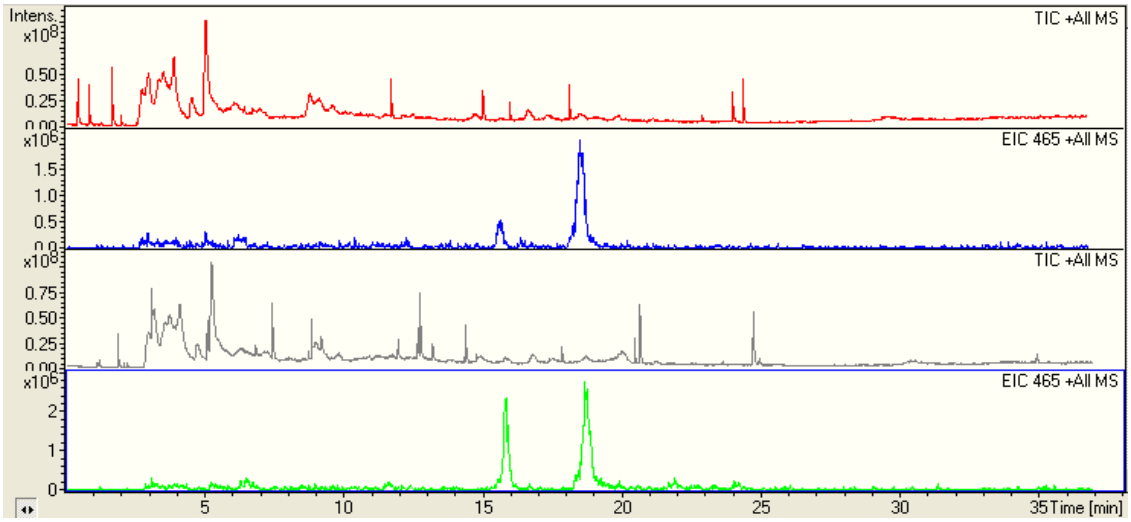


Figure 68. Extracted ion chromatogram of molecular peak 465 in the positive mode and total ion chromatograms of red wine samples (top: PR(Y), bottom: yeast)

In the control, two prominent peaks at 15.8 mins and 18.6 mins of approximately equal intensity are observed. In the bacterial trial, the peak at 15.8 mins has decreased in intensity.

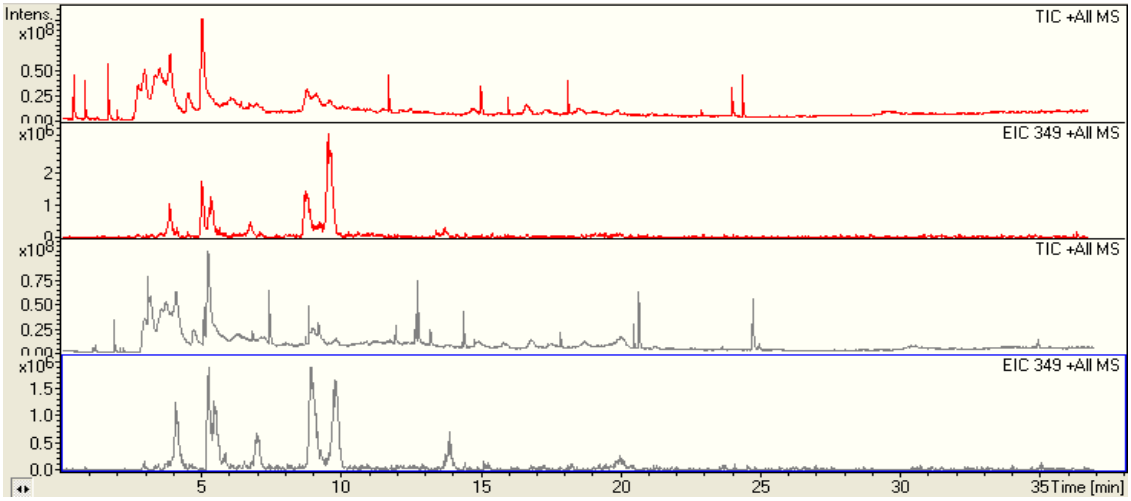


Figure 69. Extracted ion chromatogram of molecular peak 349 in the positive mode and total ion chromatograms of red wine samples (top: PR(Y), bottom: yeast)

In the control sample, several peaks at 4.0 mins, 5.2 mins, 5.4 mins, 6.9 mins, 8.9 mins, and 9.8 mins are produced. When bacterial cells are present, the peak at 8.9 mins decreases.

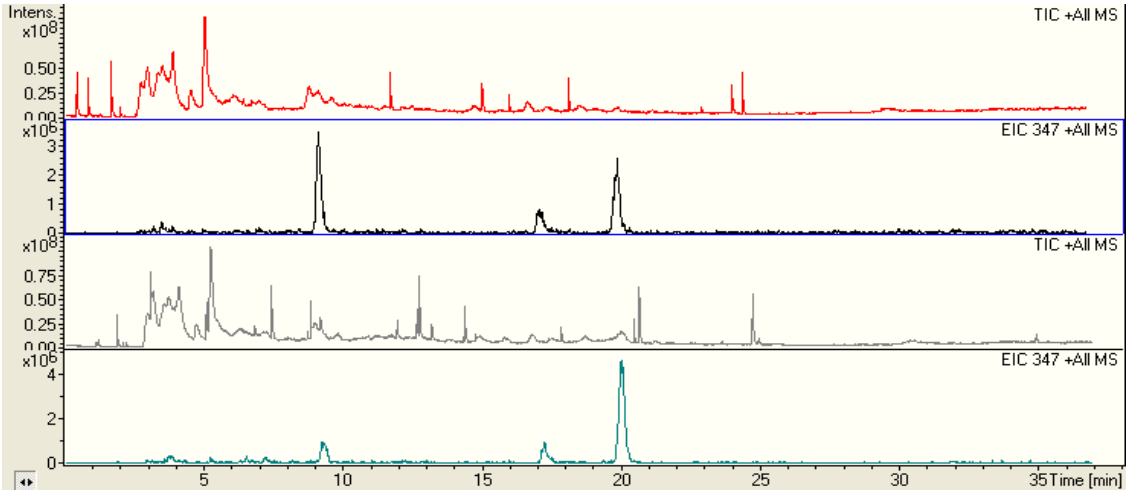


Figure 70. Extracted ion chromatogram of molecular peak 347 in the positive mode and total ion chromatograms of red wine samples (top: PR(Y), bottom: yeast)

In the yeast only trial, one prominent peak at 20.0 mins, along with two small peaks at 9.2 mins and 17.2 mins, are present. In the bacterial trial, the peak at 9.2 mins has increased while the peak at 20.0 mins has decreased in intensity.

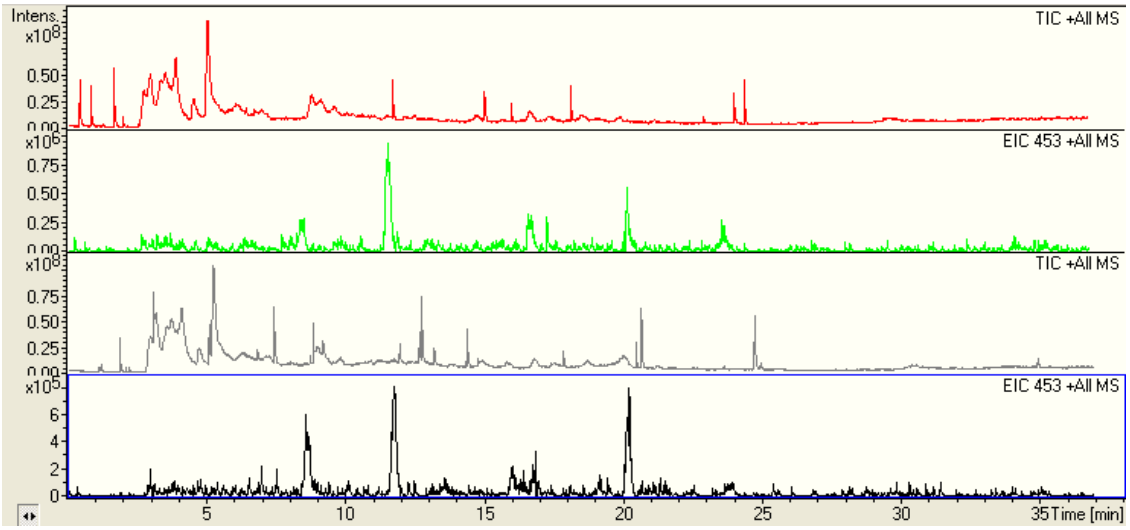


Figure 71. Extracted ion chromatogram of molecular peak 453 in the positive mode and total ion chromatograms of red wine samples (top: PR(Y), bottom: yeast)

In the control, three large peaks at 8.5 mins, 11.7 mins, and 20.2 mins are observed. When bacteria are introduced to the fermentation, the two peaks at 8.5 mins are 20.2 mins shrink.

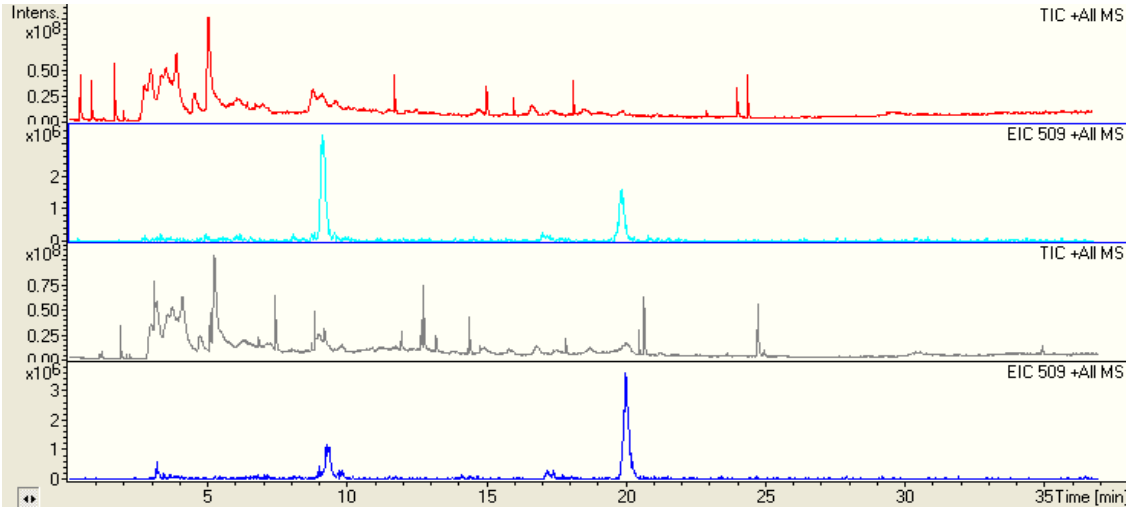


Figure 72. Extracted ion chromatogram of molecular peak 509 in the positive mode and total ion chromatograms of red wine samples (top: PR(Y), bottom: yeast)

In the control, a small peak at 9.2 mins and a large peak at 20.2 mins are produced. In the bacterial sample, the peak at 9.2 mins has increased while the peak at 20.2 mins has decreased.

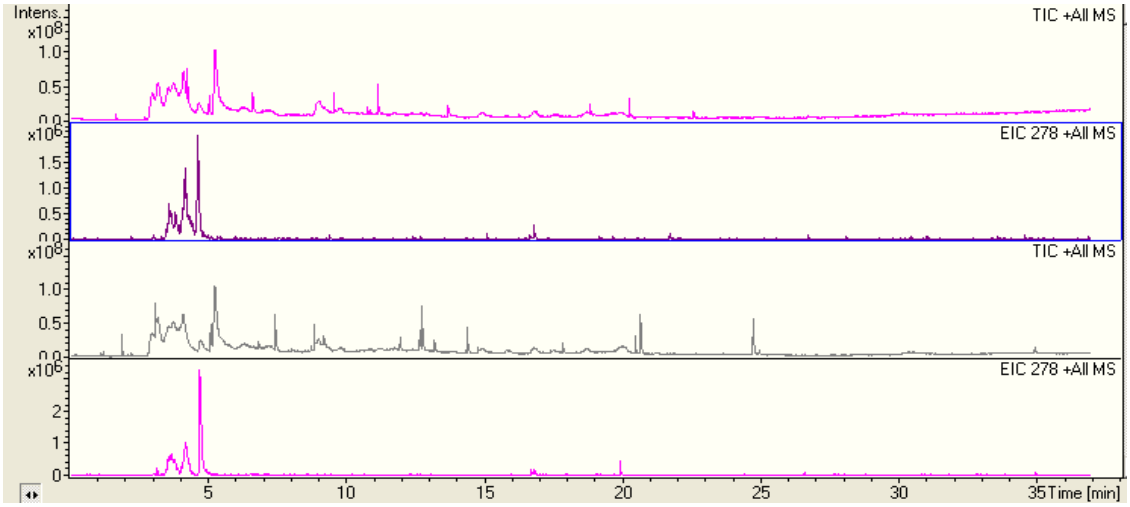


Figure 73. Extracted ion chromatogram of molecular peak 278 in the positive mode and total ion chromatograms of red wine samples (top: PR3(F), bottom: yeast)

In the standard wine, two small peaks at 3.6 mins and 4.1 mins, along with a large peak at 4.6 mins, are observed. In the bacterial trial, the peaks at 3.6 mins and 4.1 mins have increased in intensity.

In the analysis of red wine, it was observed that half of the extracted ion chromatograms belonged to compounds that were not grape derived. Further work should be done to identify these metabolites (most likely of yeast or bacterial origin) by cross referencing them with databases.

4. CONCLUSIONS

In summary, it was observed that biochar could be functionalized with bacteria and successfully delivered to the rhizosphere of plants. The amount of bacteria within the biochar remained fairly consistent between different preparations. Introducing the biochar-microbial composite into the soil of grapes resulted in significantly reduced growth when *Pseudomonas rhodesiae* was utilized at 5 % v/v and when *Delftia tsuruhatenesis* was utilized at 10 % v/v. The growth of different varieties of grapes was observed to be significantly different and slight trends in the growth indicated that the varieties may behave differently to inoculation.

Introducing the biochar into indoor trials resulted in the optimum conditions of 5 % v/v biochar mixed throughout the soil, 50% nutrient broth used for the growth medium, and immediate sowing of the seeds. The average shoot weight of barley was increased with the addition of the microbial composite and the root weight of marigolds significantly increased. The shoot weight of soybeans decreased when bacteria were introduced compared to the biochar and nutrient broth treatment. Total nitrogen of barley significantly decreased with inoculation while the nitrogen of soybeans remained unaffected. The volatile profile of marigold shoots changed when inoculation occurred.

Soil extract agar plates served as a useful proxy for potting soil in the case of barley, allowing for the easy observation of root morphology. This technique also allowed for the weighing of root systems that are usually covered by a layer of mucigel which prevents cleaning. Fungal contamination was observed on the purchased soybeans, and co-culturing with grape derived bacteria resulted in the identification of seven strains that demonstrated inhibition against one of the fungal strains. Headspace sampling of the

co-cultures that demonstrated volatile inhibition produced one new peak after GC-FID analysis. The boiling point of the molecule was approximately 250 °C, but MS data was not obtained.

Fermentation of wine with different bacterial strains produced predictable peak shifts for both grape derived and unidentified compounds.

5. FUTURE WORK

To continue this project, a thorough investigation into the use of waste material, such as vegetative agricultural waste, as a growth substrate for the bacteria could provide an even greener alternative to nutrient broth. This technique would allow for the reuse of a waste product with the potential to increase food production.

Different bacterial strains should also be tested with a variety of different host plant species in an attempt to identify strains that are compatible. Longer trials should also be conducted to observe the impact of the inoculation on produce production. Determining the impact of the microbial composite on the successful growth of fresh grape cutting should also be investigated.

Further investigation into the volatile profile of inoculated plants would be useful as volatile profiles are associated with pest infestations. Work should also be done to try and identify the changes in the non-volatile profile, as any changes in the chemical composition could be transferred to the crop.

The unidentified peaks that shifted in the LC-MS data of the wine should be investigated *via* comparisons with databases. Different bacterial strains should be screened in an effort to observe severe changes in the wine, and the LC-MS method should be improved to provide better separation for easier analysis.

6. REFERENCES

1. Stephens EC, Jones AD, Parsons D. Agricultural systems research and global food security in the 21st century: An overview and roadmap for future opportunities. *Agric. Sys.* **2017.** DOI: 10.1016/j.agsy.2017.01.011

2. Santangelo G. The impact of FDI in land in agriculture in developing countries on host country food security. *J. World Bus.* **2017.** DOI: 10.1016/j.jwb.2017.07.006

3. Schoneveld G. Host country governance and the African land rush: 7 reasons why large-scale farmland investments fail to contribute to sustainable development. *Geoforum.* **2017,** *83,* 119-132. DOI: 10.1016/j.geoforum.2016.12.007

4. Xinchun *et al.* Assessing water scarcity in agricultural production system based on the generalized water resources and water footprint framework. *Sci. Total Envrion.* **2017,** *609,* 587-597. DOI: 10.1016/j.scitotenv.2017.07.191

5. Fraga H, de Cortazar Atauri G, Santos JA. Viticulture irrigation demands under climate change scenarios in Portugal. *Agric. Water Manag.* **2018,** *196,* 66-74. DOI: 10.1016/j.agwat.2017.10.023

6. Likar *et al.* Ecological and conventional viticulture gives rise to distinct fungal and bacterial microbial communities in vineyard soils. *Appl. Soil Scol.* **2017,** *113,* 86-95. DOI: 10.1016/j.apsoil.2017.02.007

7. Xu *et al.* Nutrient requirements for maize in China based on QUEFTS analysis. *Field Crops Res.* **2013,** *150,* 115-125. DOI: 10.1016/j.fcr.2013.06.006

8. Xuan W, Beeckman T, Xu G. Plant nitrogen nutrition: sensing and signaling. *Curr. Opin. Plant Biol.* **2017,** *39,* 57-65. DOI: 10.1016/j.pbi.2017.05.010

9. Wang *et al.* Molecular mechanisms of phosphate transport and signaling in higher plants. *Semin. Cell Dev. Biol.* **2017.** DOI: 10.1016/j.semcdb.2017.06.013

10. Wang Y, Wu W-H. Regulation of potassium transport and signaling in plants. *Curr. Opin. Plant Biol.* **2017,** *39,* 123-128. DOI: 10.1016/j.pbi.2017.06.006

11. Nkebiwe *et al.* Fertilizer placement to improve crop nutrient acquisition and yield: A review and meta-analysis. *Field Crops Res.* **2016,** *196,* 389-410. DOI: 10.1016/j.fcr.2016.07.018

12. Smith VH, Schindler DW. Eutrophication science: where do we go from here? *Trends Ecol. Evolut.* **2009,** *24***(4),** 201-207. DOI: 10.1016/j.tree.2008.11.009

13. Geisseler D, Scow KM. Long-term effects of mineral fertilizers on soil microorganisms – A review. *Soil Biol. Biochem.* **2014,** *75,* 54-63. DOI: 10.1016/j.soilbio.2014.03.023

14. Jiao *et al.* Environmental risks of trace elements associated with long-term phosphate fertilizers applications: A review. *Environ. Pollut.* **2012,** *168,* 44-53. DOI: 10.1016/j.envpol.2012.03.052

15. Tan *et al.* Returning biochar to fields: A review. *Appl. Soil Ecol.* **2017,** *116,* 1-11. DOI: 10.1016/j.apsoil.2017.03.017

16. Qambrani *et al.* Biochar properties and eco-friendly applications for climate change mitigation, waste management, and wastewater treatment: A review. *Renew. Sust. Energ. Reviews.* **2017,** *79,* 255-273. DOI: 10.1016/j.rser.2017.05.057

17. Agegnehu G, Srivastava AK, Bird MI. The role of biochar and biochar-compost in improving soil quality and crop performance: A review. *Appl. Soil Ecol.* **2017,** *119,* 156-170. DOI: 10.1016/j.apsoil.2017.06.008

18. Munoz *et al.* Environmental hotspots in the life cycle of a biochar-soil system. *J. Clean Prod.* **2017,** *158,* 1-7. DOI: 10.1016/j.jclepro.2017.04.163

19. Yin *et al.* Effect of biochar and Fe-biochar on Cd and As mobility and transfer in soil-rice system. *Chemosphere.* **2017,** *186,* 928-937. DOI: 10.1016/j.chemosphere.2017.07.126

20. Ge *et al.* Stability and dynamics of enzyme activity patterns in the rice rhizosphere: Effects of plant growth and temperature. *Soil Biol. Biochem.* **2017,** *113,* 108-115. DOI: 10.1016/j.soilbio.2017.06.005

21. Jiang *et al.* Plant cultivars imprint the rhizosphere bacterial community composition and association networks. *Soil Biol. Biochem.* **2017,** *109,* 145-155. DOI: 10.1016/j.soilbio.2017.02.010

22. Dance A. Soil ecology: what lies beneath. *Nature.* **2008,** *455,* 724-725. DOI: 10.1038/455724a

23. Venturi V, Keel C. Signaling in the Rhizosphere. *Trends Plant Sci.* **2016,** *21***(3),** 187-198. DOI: 10.1016/j.tplants.2016.01.005

24. Bruning B, Rozema J. Symbiotic nitrogen fixation in legumes: Perspectives for saline agriculture. *Environ. Exper. Bot.* **2013,** *92,* 134-143. DOI: 10.1016/j.envexpbot.2012.09.001

25. Philippot *et al.* Going back to the roots: the microbial ecology of the rhizosphere. *Nature Rev.* **2013,** *11,* 789-799. DOI: 10.1038/nrmicro3109

26. Pineda A, Kaplan I, Bezemer TM. Steering Soil Microbes to Suppress Aboveground Insect Pests. *Trends Plant Sci.* **2017,** *22***(9),** 770-778. DOI: 10.1016/j.tplants.2017.07.002

27. Kumar A, Verma JP. Does plant-Microbe interaction confer stress tolerance in plants: A review? *Microbiol. Res.* **2018,** *207,* 41-52. DOI: 10.1016/j.micres.2017.11.004

28. Ghorbanpour *et al.* Mechanisms underlying the protective effects of beneficial fungi against plant diseases. *Biol. Control.* **2017.** DOI: 10.1016/j.biocontrol.2017.11.006

29. French *et al.* Mechanisms of quantitative disease resistance in plants. *Sem. Dev. Biol.* **2016,** *56,* 201-208. DOI: 10.1016/j.semcdb.2016.05.015

30. Atanasov *et al.* Discovery and resupply of pharmacologically active plant-derived natural products: A review. *Biotechnol. Adv.* **2015,** *33***(8),** 1582-1614. DOI: 10.1016/j.biotechadv.2015.08.001

31. Dukare *et al.* Evaluating novel microbe amended composts as biocontrol agents in tomato. *Crop Prot.* **2011,** *30***(4),** 436-442. DOI: 10.1016/j.cropro.2010.12.017

32. Mu *et al.* Biocontrol potential of vermicompost through antifungal volatiles produced by indigenous bacteria. *Biol. Control.* **2017,** *112,* 49-54. DOI: 10.1016/j.biocontrol.2017.05.013

33. Hirsch AM, Valdes M. *Micromonospora:* An important microbe for biomedicine and potentially for biocontrol and biofuels. *Soil Biol. Biochem.* **2010,** *42***(4),** 536-542. DOI: 10.1016/j.soilbio.2009.11.023

34. Willey, Sherwood & Woolverton. *Prescott's Microbiology* (10ed.). **2017.** New York: McGraw Hill Education.

35. Wang *et al.* Pretreatment of corn stover by solid acid for D-lactic acid fermentation. *Bioresour. Technol.* **2017,** *239,* 490-495. DOI: 10.1016/j.biortech.2017.04.089

36. Lemos *et al.* Selection and application of nontoxic solvents in extractive ethanol fermentation. *Biochem. Eng. J.* **2017,** *127,* 128-135. DOI: 10.1016/j.bej.2017.08.003

37. Chen *et al.* Understanding the mechanisms of how poly aluminium chloride inhibits short-chain fatty acids production from anaerobic fermentation of waste activated sludge. *Chem. Eng. J.* **2018,** *334,* 1351-1360. DOI: 10.1016/j.cej.2017.11.064

38. Auesukaree C. Molecular mechanisms of the yeast adaptive response and tolerance to stresses encountered during ethanol fermentation. *J. Biosci. Bioeng.* **2017,** *124***(2),** 133-142. DOI: 10.1016/j.jbiosc.2017.03.009

39. Cappello *et al.* Linking wine lactic acid bacteria diversity with wine aroma and flavour. *Int. J. Food Microbiol.* **2017,** *243,* 16-27. DOI: 10.1016/j.ijfoodmicro.2016.11.025

40. Perrone *et al.* Investigation of the dominance behavior of *Saccharomyces cerevisiae* strains during wine fermentation. *Int. J. Food Microbiol.* **2013,** *165***(2),** 156-162. DOI: 10.1016/j.ijfoodmicro.2013.04.023

41. Raposo *et al.* Sulfur free red wines through the use of grapevine shoots: Impact on the wine quality. *Food Chem.* **2018,** *243,* 453-460. DOI: 10.1016/j.foodchem.2017.09.111

42. Ma *et al.* Performance of selected *P. fermentans* and its excellular enzyme in co-inoculation with *S. cerevisiae* for wine aroma enhancement. *LWT – Food Sci. Tech.* **2017,** *86,* 361-370. DOI: 10.1016/j.lwt.2017.08.018

43. Foroni *et al.* The smell of *terroir!* Olfactory discrimination between wines of different grape variety and different *terroir*. *Food Qual. Prefer.* **2017,** *58,* 18-23. DOI: 10.1016/j.foodqual.2016.12.012

44. Xiong *et al.* Enhanced biodegradation of PAHs in historically contaminated soil by *M. gilvum* inoculated biochar. *Chemosphere.* **2017,** *182,* 316-324. DOI: 10.1016/j.chemosphere.2017.05.020

45. Quiroz *et al.* Host-plant chemicals and distribution of *Neuquenaphis* on *Nothofagus. J. Chem. Ecol.* **1999.** *25***(5),** 1043-1054. DOI: 0098-0331/99/0500-1043/$16.00/0

46. Xie *et al.* The GC/MS analysis of volatile components extracted by different methods from *Exocarpim Citri Grandis. J. Anal. Methods Chem.* **2013,** *2013:* 918406. DOI: 10.1155/2013/918406

47. Field *et al.* Organic Structures from Spectra (5th Ed.). **2015,** *John Wiley & Sons.* Markono Print Media Pte Ltd, Singapore, 16-20.

48. Flamini *et al.* An innovative approach to grape metabolomics: stilbene profiling by suspect screening analysis. *Metabolomics.* **2013,** *9***(6),** 1243-1253. DOI: 10.1007/s11306-013-0530-0

49. Flamini *et al.* Study of grape polyphenols by liquid chromatography-high-resolution mass spectrometry (UHPLC/QTOF) and suspect screening analysis. *J. Anal. Methods Chem.* **2015**. DOI: 10.1155/2015/350259

50. PSmicrographs. Magnolia xylem vessels. https://psmicrographs.com/sems/flowers-plants/ (accessed November 2017)

51. PSmicrographs. Ash wood xylem (*Fraxinus excelsior*). https://psmicrographs.com/sems/flowers-plants/ (accessed November 2017)

52. Bertrand *et al.* Multi-well fungal co-culture for *de novo* metabolite-induction in time-series studies based on untargeted metabolomics. *Mol. BioSyst.* **2014.** DOI: 10/1039/c4mb00223g

53. Zarraonaindia *et al.* The soil microbiome influences grapevine-associated microbiota. *Meriot Microbiome.* **2015,** *6***(2).** DOI: 10.1128/mBio.02527-14