Testing the efficacy of a microbial inoculant to increase cold tolerance in corn

Jonathan Zajonc

Department of Plant Science

McGill University, Montréal

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Abstract

Climate change is increasing the severity of abiotic stresses that can reduce plant growth and crop yield. Furthermore, modern agricultural practices have degraded soil microbiota, thus limiting their ability to alleviate abiotic stress in plants. Due to climate change, irregular temperature events, such as periods of cold during spring planting or fall harvest, may reduce crop yield and even render it unfit for market. Annually, in the province of Quebec and throughout Canada, low temperature stress causes significant losses of staple crops such as corn. It has been shown that inoculation with a microbial consortium can improve soil microbial diversity and reduce the effect of abiotic stresses such as high temperature and salinization on crops. However, there has been limited research on their efficacy in alleviating low temperature stress. While there are many agricultural biostimulants available, none are specifically targeted for cold stress. This project aimed to test the efficacy of an existing commercial bacterial inoculant to promote the growth of corn under low temperature stress. The expected mode of action for this to be achieved is phosphorus and calcium solubilization, and iron chelation. The temperatures tested were 25 °C as the optimal temperature, and 20 °C and 15 °C as the low temperatures. The ability of the consortium to alleviate cold stress was assessed through a series of germination and biomass accumulation experiments done in a highly controlled environment along with plant tissue nutrient analysis. The response of the rhizosphere to low temperatures and the addition of the microbial inoculant was characterized by community profiling using 16S rRNA sequencing data. The results showed that there was no significant effect of the consortia on germination rate or early plant growth. There was also no effect on plant height throughout the V0 to V4 growth stages for the range of temperatures tested, although there was a detectable yet insignificant increase in biomass at the V4 stage for the treatment group at the optimal temperature. This suggests that the inoculant may have slightly promoted plant growth under ideal conditions. The nutrient analysis showed a higher concentration of phosphorous, calcium, and metals such as iron and zinc in the plant tissue of the treatment group at the optimal temperature, further supporting this finding. The results of the rhizosphere microbiome community profiling showed that the addition of the inoculant resulted in no detectable effect on community structure at any of the temperatures tested, although the temperature differences resulted in significantly different microbial communities. Overall, the inoculant seemed to have a positive yet statistically insignificant effect on plant growth at the optimal temperature tested only.

Résumé

Le changement climatique augmente la sévérité des stress abiotiques qui peuvent réduire la croissance des plantes et le rendement des cultures agricoles. De plus, les pratiques agricoles modernes ont dégradé le microbiote du sol, limitant ainsi la capacité des plantes à atténuer le stress abiotique. En raison du changement climatique, des événements de température irréguliers, tels que des périodes de froid pendant les semences du printemps ou la récolte d'automne, peuvent réduire le rendement des cultures agricoles et même les rendre impropres à la commercialisation. Annuellement, au Québec et partout au Canada, le stress lié aux basses températures entraîne des pertes importantes de cultures de base comme le maïs. Il a été démontré que l'inoculation avec un consortium microbien peut améliorer la diversité microbienne du sol et réduire l'effet des stress abiotiques tels que les températures élevées et la salinisation imposée sur les cultures. Cependant, peu de recherches ont été menées sur leur efficacité à atténuer le stress lié aux basses températures. Bien qu'il existe de nombreux biostimulants agricoles disponibles, aucun n'est spécifiquement ciblé contre le stress dû au froid. Ce projet visait à tester l'efficacité d'un inoculant bactérien commercial favorisant la croissance du maïs soumis á de basses températures. Le mode d'action choisi a été la solubilisation du phosphore et du calcium et la chélation du fer. Les températures testées étaient de 25 °C comme température optimale, et de 20 °C et de 15 °C comme basses températures. La capacité du consortium à atténuer le stress dû au froid a été évaluée grâce à une série d'expériences de germination et d'accumulation de biomasse réalisées dans un environnement hautement contrôlé, ainsi qu'à l'analyse des éléments nutritifs des tissus végétaux. La réponse de la rhizosphère aux basses températures et à l'ajout de l'inoculant microbien a été caractérisée par le profilage de la communauté à l'aide des données de séquençage de l'ARNr 16S. Les résultats ont montré qu'il n'y avait pas d'effet significatif des consortiums sur le taux de germination ou la croissance précoce des plantes. Il n'y a pas eu non plus d'effet sur la hauteur des plantes tout au long des stades de croissance V0 à V4 pour les températures testées, bien qu'il y ait eu une augmentation détectable mais insignifiante de la biomasse au stade V4 pour le groupe de traitement à la température optimale. Cela suggère que l'inoculant peut avoir légèrement favorisé la croissance des plantes dans des conditions idéales. L'analyse des éléments nutritifs a démontré une concentration plus élevée de phosphore, de calcium et de métaux tels que le fer et le zinc dans les tissus végétaux pour le groupe de traitement à la température optimale, ce qui confirme davantage cette découverte. Les résultats du profilage de la communauté du microbiome de la rhizosphère ont montré que l'ajout de l'inoculant n'avait entraîné aucun effet détectable sur la structure de la communauté à aucune des températures testées, bien que les différences de température aient entraîné des communautés microbiennes significativement différentes. Dans l'ensemble, l'inoculant semble avoir un effet positif mais statistiquement non significatif sur la croissance des plantes testées à la température optimale de 25 ˚C.

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List of Abbreviations

ABA – Abscisic acid

ACCd – Aminocyclopropane-1-carboxylic acid deaminase

COR – Cold responsive genes

Ctl – Control

GHG – Greenhouse gas

IAA – Indole-3-acetic acid

PCA – Plate count agar

PCR – Polymerase chain reaction

PGPB – Plant growth promoting bacteria

PPM – Parts per million

T0 – Initial timepoint

TF – Final timepoint

Uly – Treatment with Era Boost Pro consortium

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Contribution of the Author

All experiments were designed by Jonathan Zajonc with input, recommendations, and advice from Dr. Smith, Dr. Pagé, Dr. Backer, and Dr. Subramanian. Members of the Smith lab and the Dastmalchi lab occasionally helped to keep the plants watered. The 16S library prep was done by Jonathan Zajonc with help from Catherine Blanchard. Data collection and analysis was done by Jonathan Zajonc. Dr. Pagé provided help with statistical analysis of the microbial community composition. Jonathan Zajonc wrote the entirety of this thesis and incorporated the suggestions and edits of Dr. Smith and Dr. Pagé.

Chapter 1: Introduction

8.3 General Overview

The global population has risen rapidly over the past century. While a range of improvements in agricultural practices and technologies has contributed to this growth, the green revolution is predominantly responsible for this rapid rise (Harwood, 2019). The ability to industrially produce synthetic nitrogen fertilizer has removed the growth restrictions imposed by this once limiting nutrient and has led to major crop yield increases. However, the over-production, over-application, and over-reliance of synthetic fertilizers has come at extreme costs to both local and global environments (Smith & Gregory, 2013).

Our population is projected to continue growing for the next half century. This presents an enormous challenge for how to sustainably feed everyone since, simultaneously, crop yield increases have begun to slow, indicating that the current system of crop production is reaching its limits. Rather than expanding the remaining amount of arable land, new advances in sustainable agricultural technologies and practices are needed. Regenerative agriculture and regionally based closed loop food systems that can produce enough food while providing environmental benefits exist but are slow to catch on. More immediate solutions are needed.

Plant growth promoting microbial inoculants are a promising and readily available way to promote crop growth while reducing the amount of synthetic fertilizers needed to achieve the same yields. Research in this field has occurred for only about 30 years and commercialization of these products is even younger, indicating that there is great potential for optimization (Lyu et al., 2020).

On top of slowing yields, many of today's crops are under extreme environmental pressure due to anthropogenic climate change. With warmer than average temperatures, Canadian farmers are opting to grow once traditionally southern crops further north. This practice comes with a cost, however, since farmers are sowing their crops extremely early to make up for the short growing season. This exposes the crops to low temperature stress early in the season, potentially limiting crop growth and even making the harvest unmarketable. Due to climate change, cold snaps, both early in the season and near harvest in the late growing season, are harder to predict and are becoming more intense (Francis & Skific, 2015).

Along with reducing the need for synthetic fertilizers, some microbial inoculants can alleviate crop abiotic stress. While many products have been shown to work for heat or drought stress, none so far have been commercialized to alleviate low temperature stress. However, there is ample evidence that certain microbial taxa have this function.

In this study, a commercial microbial inoculant was tested for its ability to alleviate low temperature stress in corn. The product had not yet been tested for this, although the same microbial species have been shown to solubilize phosphorus and calcium and chelate iron molecules, mechanisms shown to alleviate cold stress. Furthermore, some of the same species present in the consortium have been shown to alleviate low temperature stress in a range of crops in studies from around the world. It was hypothesized that due to its functional capabilities, the consortium could potentially help alleviate cold stress.

1.2 Research Objectives and Hypotheses

There were two main objectives this research sought to address. The first was to determine whether the inoculant Era Boost Pro from the company Ulysse Biotech could promote corn growth under low temperature stress. The second objective was to investigate how inoculation of the consortium affected the rhizosphere microbial structure and composition of the corn plants.

The first hypothesis of this experiment addressed whether, if corn is inoculated with the consortium, then it will experience significant growth promotion compared to the uninoculated control when exposed to low temperature stress.

The second hypothesis of this experiment contains two parts. The first is that the structure of the rhizosphere microbial community of the inoculated corn will be significantly different than that of the uninoculated (control) corn. The second part is that with increasingly severe low temperature stress, the inoculated corn will have a greater proportion of the inoculant's microbial population present in the rhizosphere microbial community compared to the control, uninoculated plants.

Chapter 2: Literature Review

2.1 Climate change and modern agriculture

In the face of global climate change, the current agricultural system cannot sustainably produce enough food for a global population that is projected to reach 9.7 billion by 2050 and will only peak in the mid 2080s (McKersie, 2015; DESA, 2019). While modern agriculture is capable of producing large quantities of staple crops, it does so at a considerable cost to the environment. Current agricultural practices rely heavily on the overapplication of fertilizers, pesticides, and irrigation, negatively affecting both the local and global environments (Smith & Gregory, 2013).

The over-application of nitrogen and phosphorus fertilizers has led to severe leaching with unused nutrients traveling in ground or surface water to large bodies of water, eventually causing algal blooms and dead zones (United States Environmental Protections Agency [US EPA], 2017; Hart et al., 2004). About 1-2% of added nitrogen fertilizer is transformed into nitrous oxide, a greenhouse gas (GHG) that is approximately 300 times more effective at trapping heat in the atmosphere compared to carbon dioxide (Oertel et al., 2016). The production of nitrogen fertilizer is also extremely energy intensive, releasing enormous amounts of GHGs into the atmosphere (US EPA, 2017). Although new technologies can potentially reduce the energy consumption and GHG emissions of fertilizer production, nitrogen continues to be over-applied in agricultural systems (Kyriakou et al., 2020).

Additionally, practices such as tillage worsen soil quality by degrading its structure and reducing biodiversity (Tsiafouli et al., 2014). By identifying and describing the "soil health gap", new management strategies can attempt to restore the soil microbiome to a functionally productive state (Maharjan et al., 2020).

Crop genetic engineering, precision agriculture, and biostimulants are examples of practices that can lessen the environmental impacts of agriculture while still increasing yields (Lotz et al., 2020; Adamchuk, 2017; Banerjee et al., 2019; Koutsos and Menexes 2019). Plant growth-promoting microbial inoculants have recently received growing attention (Lyu et al., 2020). They can be used to revitalize a degraded soil microbiome through rhizosphere engineering by modifying the rhizosphere's microbial community composition, improving

community structure and functioning to increase crop yield under both optimal and stressful conditions (Del Pozo, 2020). Backer et al. (2018) have outlined a framework for developing and commercializing inoculants as an increasing number of companies implement these technologies and bring their microbial inoculant products to market (Parnell et al., 2016).

As microbial inoculants become more widely used, maximizing their efficiency while restoring and maintaining soil health will be crucial. Along with plant growth promotion, increasing soil biodiversity by introducing or reintroducing beneficial microbes and bolstering native microbial taxa are equally important factors in restoring nutrient cycling.

While employing many new technologies will be crucial to improve the sustainability of modern agriculture, more fundamental changes are needed to address the numerous environmental catastrophes currently looming. Recently, there has been renewed interest in adopting sustainable practices such as regenerative agriculture. Regenerative agriculture relies on several fundamental principles and practices that focus on rebuilding soil health (Newton et al., 2020). These include no till or minimal tillage (also known as conservation tillage), cover cropping, and incorporating diverse crop rotations and/or intercropping strategies (Lal, 2020). Finally, livestock integration, when appropriate for the ecosystem, can also be incorporated (Lal, 2020).

Relying less on monocrop agricultural systems and cultivating crop diversity can improve the food system's resilience to environmental stress. Incorporating native crops that are regionally adapted can further improve local climate resiliency (Shelef et al., 2017). This practice prevents biodiversity loss and preserves traditional knowledge systems. Growing crops in regions where they have evolved and have been domesticated can return specific ecosystem functions that have been lost (Kramer et al., 2019).

A large fraction of yields from some monocrop systems goes to the production of biofuels. For example, approximately 40 % of corn grown in the United States is used for ethanol production. There are many positive aspects of using biofuel as it can be less harmful than extracting coal or liquified natural gas and can reduce greenhouse gas emissions from the transportation sector (Lee et al., 2021). However, there are important limitations and environmental impacts that need to be considered, especially from a soil health perspective.

Fields of corn require large amounts of inputs for adequate yields; these inputs take energy to produce and have negative downstream effects, while simultaneously reducing soil biodiversity and health when under continuous cultivation (Figuerola et al., 2015). Even though they have many of their own environmental challenges, a greater reliance on wind, solar, hydro, and even nuclear energy would immensely alleviate the pressure to grow crops for biofuel, allowing more land to be used for food production (Murphy et al., 2011).

Another 40 % of the total U.S. corn crop is used for animal feed, which is both wasteful and harmful to human health if consumed in large quantities (Rust et al., 2020). Meat can alternatively be produced as part of a regenerative agriculture system that can reduce greenhouse gas emissions (Lal, 2021). While it is currently a privilege of those wealthy enough to afford such high-quality meat, the affordability of conventionally produced meat is in no small part to due to massive government subsidies given to the current agricultural industry complex (Smith, 2019).

An even more basic solution to improve the sustainability of current food systems and feed our growing population is to eliminate food waste. The U.S., for example, wastes 30-40 % of its food supply each year. Wealthy nations waste about 6 times more food than poor nations, with large fractions being lost due to poor food storage facilities (Chen et al., 2020). This is unacceptable considering the amount of energy, resources, and environmental harm that comes along with our current food system. Addressing this issue would not only benefit the health and wellbeing of the neediest, but also the entire environment.

These issues are largely structural and political. Because it may take generations for these fundamental changes to occur, immediate solutions must be capitalized on. Using microbial inoculants, for example, is one way to address many of the problems the agricultural industry faces (Trivedi et al., 2021). They can increase soil biodiversity, promote plant growth while reducing the need for synthetic inputs, and improve crop resilience in the face of intensifying climate change.

2.2 Low temperature stress

Anthropogenic climate change is not only making average global temperatures warmer, storms stronger, soils saltier, and droughts longer, but it is also having more subtle effects at regional scales. Low temperature stress, or cold stress, is increasingly becoming a more prevalent issue for northern regions of the globe, especially in the context of agriculture (Francis & Skific, 2015). The lower mainland of British Columbia, a regional agricultural hub, had its coldest spring on record in early 2022. The Canadian prairies had their record coldest spring in 2013, however it was still an exceptionally cold spring in 2022, greatly delaying planting dates. This late spring cold spell was attributed to a polar vortex event, which are becoming less predictable due to climate change (Mitchell et al., 2012).

In other regions, a less consistent snow cover throughout the winter can drop soil to below average temperatures in northern latitudes which can last into spring (Comerford et al., 2013). This, coupled with an early snow melt and unexpected frost in the spring, may negatively affect perennial crops and trees that rely on snow insulation (Kreyling et al., 2012).

In Canada, as agricultural land expands northward into previously uncultivated regions to satisfy the rising global demand for food, crops will need to be produced in shorter growing seasons (Tang et al., 2013; Francis & Vavrus, 2012). In an effort to increase yield, corn farmers have increasingly been planting their crops earlier (Kucharik, 2006). Furthermore, due to average warmer temperatures, farmers further north have been able to plant crops traditionally grown in more southern regions such as corn (Baum et al., 2020). These trends create a risky situation for Canadian corn growers. Due to a shorter growing season further north, seeds are sown as early as possible to make the most of the warm months. This exposes the seedlings to cold stress if an unpredicted cold snap occurs. Modern corn varieties were originally domesticated in Central America and are for the most part not cold tolerant (Ali et al., 2018). While this is not the case for land races such as Gaspé Flint, which is native to northern New England and Eastern Canada, most hybrid cultivars will be irreversibly damaged by cold stress (McCaw et al., 2016).

For the past few decades, research has focused on elucidating genetic mechanisms in above ground plant tissues that detect and respond to cold temperatures as part of the process

of senescence. However, only recently have researchers studied the mechanisms occurring in the root tissue (Ambroise et al., 2020). An understanding of the belowground genetic and metabolic pathways is needed because the predicted lack of snow covering on perennial roots throughout winter in the future requires methods to improve freezing stress tolerance in vulnerable crops (Ambroise et al., 2020).

When cold stress is detected, a plant's cold responsive (COR) genes are activated. These trigger a complex cascading network of proteins that change the plant's metabolism and produce antifreeze compounds (Ding et al., 2019). During the process of senescence, and the associated loss of leaves, plants change their sugar content to reduce their freezing point.

The genetic modification of crops or the use of speed breeding, as seen in the development of cold hardy crops, may be potential routes for overcoming cold stress (Yi et al., 2020). However, as the complex network of COR genes are still not fully understood, this is not currently a viable option (Rihan et al., 2017). In addition, genetically modified crops that are resistant to cold stress may be unappealing to some organic farmers. The use of plastic mulch to warm soils in northern Canada has been shown to improve the yield of corn, however this practice cannot scale to full industry levels and contributes to microplastic pollution in soils (Kwabiah, 2004; Huang et al., 2020). Microbial inoculants that alleviate cold stress can be an environmentally beneficial solution for organic growers.

The mechanisms by which cold stress affects corn physiology are well documented (Ambroise et al., 2020). However, there has only been one investigation to date regarding its effect on the corn root microbiome (Beirinckx et al., 2020). As such, there have been several calls for further research in this field, in the hopes of discovering a microbial inoculant capable of alleviating cold stress in corn.

2.3 Assessing commercial inoculants for novel purposes

Bioprospecting for cold tolerant plant growth promoting bacteria is a reasonable approach, especially if they can be used in local systems. However, a plethora of commercial products are already available. It makes most sense to first assess preexisting products for the potential to alleviate cold stress prior to bioprospecting to formulate an entirely new consortium. One example of a plant growth promoting microbial consortium is the product Era

Boost Pro from the Quebecois company Ulysse Biotech (see Materials & Methods below). This bacterial consortium consists of three *Bacillus* species: *B. megaterium*, *B. licheniformis*, and *B. velezensis*. Its mode of action for promoting plant growth is through the solubilization of phosphorus and calcium, and the chelation of iron molecules.

While this consortium was designed for general growth promotion rather than cold stress alleviation, there is ample evidence to suggest that it may indeed serve this purpose as well. Experiments have shown that some specific *Bacillus* species are able to alleviate low temperature stress in a range of crops. For example, *B. velzensis* has promoted the early growth of wheat under cold stress by affecting plant metabolic function and stress response as well as through phosphorus solubilization (Abd El-Daim et al., 2019; Zubair et al., 2019). Furthermore, several experiments have shown the efficacy of *B. velezensis*, *B. licheniformis*, and *B. megaterium* to promote corn growth by phosphorus solubilization and iron chelation along with the production of exogenous phytohormones (Katsenios et al., 2022; Lipková et al., 2021, Efthimiadou et al., 2020). Finally, the company Ulysse Biotech has evidence from a field trial of corn growth promotion during wet and cool growing conditions, although corn grows better in warm and dry conditions. Since abiotic stresses in the field cannot be easily teased apart into their individual components, a controlled environment experiment is needed to show which environmental stress(es) the consortium is effective for. This is necessary if the company wants to market their product for alleviating a particular stress. There is an increasingly large market in Canadian corn producers for a product that could alleviate low temperature stress.

When testing the efficacy of an inoculant to alleviate a specific type of stress, it is useful to measure its effect on the growth at early developmental stages of the desired crop. Using this shortened growth period is a rapid way to do many iterations of an experiment and come to a more accurate conclusion. There is also a strong correlation, and therefore predictive power, between growth promotion at early life stages and harvest. These results should then be validated in a larger controlled environment trial where the crop is grown to harvest and with a field trial, if possible, after the initial tests.

Among the various early growth measurements such as germination rate, radical length, and plant height for the first few growth stages, dry weight is the most important measure of

later yield increases due to a microbial inoculation. Dry weight has been correlated with grain yield in corn (Shafer & Wiggans, 1941). It has also been shown that wet weight is an unreliable measurement for assessing growth promotion compared to dry weight (Huang et al., 2017).

When testing a consortium with a known mechanism of growth promotion such as nutrient solubilization, another invaluable measurement to confirm its efficacy is to assess the plant's nutrient composition. If it is different when inoculated, it can be inferred that the consortium had the desired effect.

Plants will change their root exudate profile to recruit a different associated microbial community when under abiotic stress. For example, if a plant is phosphorus limited, it will preferentially select for phosphorus solubilizing bacteria (Brisson et al., 2022). Likewise, if under low temperature stress, a microbial community able to alleviate this pressure will be selected for. It is in this way that plant microbiomes can extend their phenotypic plasticity under a range of conditions (Kolodny & Schulenburg, 2020). Using microbial community profiling by 16S rRNA gene sequencing is an affordable way to determine which microbial members are there and how the microbial community composition changes with stress, plant stress response, and plant growth promoting microbial inoculation. The 16S ribosomal subunit is a highly conserved genetic region across all bacterial and archaeal taxa. However, within the general structure there are highly conserved genetic regions and those that are more variable. For example, the V4 and V5 hypervariable sections can be used to phylogenetically distinguish one microbial taxa from another during classification (Větrovský & Baldrian, 2013). For a comprehensive understanding of how a microbial inoculant affects a host plant and the associated microbiome, it is essential to view this living system as a holobiont.

2.4 Holobionts

In 1943, the German theoretical biologist Adolf Mayer-Abich introduced the concept of the holobiont (Baedke et al., 2020). A holobiont consists of a host eukaryotic organism and the entirety of the microorganisms associating with it. The complex interactions between plants and their microbiomes, known as the phytomicrobiome, are the result of coevolution between several kingdoms of life (Guerrero et al., 2013). Evolutionary pressures have acted upon the plant and its microbiome as the unit of selection. Natural selection acts upon the phenotype of

the holobiont, which ultimately alters the hologenome - the complete genomic information of the host and its associated microbiome (Morris, 2018; Roughgarden et al., 2018). The hologenome is also adaptive to the local environment, with the microbial populations shifting in response to stimuli. In this sense, the microbiome plays an active role in extending host phenotypic plasticity (Kolodny & Schulenburg, 2020).

Phytomicrobiomes play a major role in facilitating a plant's ability to colonize new environments by mediating both abiotic and biotic stress responses (Rosenberg & Zilber-Rosenberg, 2018; Vandenkoornhuyse et al., 2015). The associated phytomicrobial community is present on or in all parts of the plant host including the above ground leaves, stem, reproductive organs, belowground roots, and area surrounding the roots known as the rhizosphere (Hawkes et al., 2021). Microbes are also present within the plant, known as the endosphere, including the vasculature, cellular apoplast and symplast, and in specific microbe housing organs such as nodules (Berg et al., 2016; Dastogeer et al., 2020).

The phytomicrobiome is acquired horizontally from the environment and vertically through the seed (Nelson, 2018; Vandenkoornhuyse et al., 2015). The soil in which a plant develops supplies the greatest source of microbial diversity available to associate with the plant. Which microbes are present is heavily dependent on soil characteristics including pH, salinity, and texture (Lauber et al., 2009; Ilangumaran & Smith, 2017; Bell et al., 2019). Both competition and environmental influence naturally shape microbial population diversity, however due to anthropogenic climate change and land management practices, there is a trend towards microbial species homogeneity and reduced functional capacity (Guerra et al., 2021).

The plant host imposes selective pressures on the microbial community by releasing root exudates, rhizodeposits, and phytohormones, which act as filters of microbial diversity by promoting the growth of some species while inhibiting that of others (Cordovez et al., 2019). The fraction of the phytomicrobiome that is inherited vertically tends to have coevolved more closely with the host than those acquired horizontally, suggesting that those organisms play a vital role in the plant's survival and fitness (Nelson, 2018).

The phylogenetic relatedness of two plant species corresponds to the amount of variation found between their microbiomes; closely related species within a given environment

will share similar microbiomes (Kaplan et al., 2020). Therefore, examining the microbiomes of indigenous plants that are related to modern crops will help identify potentially beneficial taxa that may have been lost during the domestication and agricultural intensification processes (Alcaraz et al., 2018; Porter & Sachs, 2020).

The symbiotic coevolution between a host and its microbiome may have been facilitated by the ability of the microbes to induce the cooperation of its host (Lewin-Epstein & Hadany, 2020). For example, rather than selecting for certain taxa, plants may instead select for genomic islands, or core genes in the microbial population, that code for specific functions such as the induction of host cooperation by the rhizospheric community (Baltrus, 2017; Jia & Whalen, 2020; Finan, 2002). However, the host must retain the ability to regulate its associated microbiome. If it does not, the randomness of ecological microbial drift could introduce deleterious taxa, which may drive the host to extinction (Foster et al., 2017).

Microbial members of the phytomicrobiome that consistently associate with and are present among all organisms of a host species constitute the core microbiome (Berg et al., 2020). Microbes that only associate with a host in a particular biogeography or regional environment are known as the accessory microbiome (Vandenkoornhuyse et al., 2015). The plant functional core microbiome is the portion of the core microbiome that serves a particular function required for host survival (Lemanceau et al., 2017; Compant et al., 2019). For agricultural crops, these were likely inadvertently selected for alongside other phenotypic traits during domestication (Tkacz et al., 2020; de la Fuente Cantó et al., 2020). The conserved nature of the core microbiome suggests that it fulfills a specific role, or set of roles, for its host. Studying core microbial taxa under biotic and abiotic stress can elucidate the function of the core microbiome (Vandenkoornhuyse et al., 2015). To better understand its role, it has been suggested that future research in plant ecology, evolution, and sustainable agriculture focus on the holobiont rather than plants and microbes independently (Saikkonen et al., 2020).

A novel, even more inclusive, way to think about holobionts is that of the eco-holobiont (Singh et al., 2020). An eco-holobiont encompasses one holobiont interacting with another holobiont. For example, a plant and its phytomicrobiome can interact closely with a pollinator and its microbiome, enabling the sharing of microbial species (Lui et al., 2019). The eco-

holobiont concept introduces a greater level of fluidity between the macro individual and the community, all linked by related microbial species. Applying microbiome-based thinking and analysis to community ecology increases our understanding of regional nutrient cycling and functional interdependence.

2.5 Soil microbial community composition

As agricultural lands expand into previously uncultivable regions due to the shifting of climactic zones or to increase production, crops will be exposed to new soil microbial communities (Harvey & Pilgrim, 2011; King et al., 2018). Certain generalist microbial taxa present in bulk soil, where they are not influenced by crop roots, will likely associate with and benefit the newly sown plants (Lyu et al., 2020; Ramirez et al., 2019). Microbial communities in bulk soil have a large amount of species diversity, albeit with a relatively limited community structure (Shi et al., 2016). This diversity is essential for determining which taxa will be available to associate with the crops (Bakker et al., 2015).

The rhizosphere, on the other hand, has less microbial diversity since a plant's root exudate profile selects for a certain community composition based on its growth stage, among other exogenous factors (Singh et al., 2007). Plant root exudates give structure to rhizosphere microbial communities, leading to greater functional connectivity (Shi et al., 2016). These phytocompounds are central to both plant-microbe and microbe-microbe signaling (Bhatt et al., 2020).

The phytomicrobiome is composed of hub taxa that are important for local interspecies interactions, and keystone taxa that are integral for the functioning of the broader community (Agler et al., 2016). Some keystone taxa are rare yet still have a disproportionately important role (Jousset et al., 2017). Various keystone taxa fulfill numerous roles within the microbiome; the more specific its role, the more valuable it is for proper community functioning (Banerjee et al., 2018). Examples of these roles include fixing atmospheric nitrogen, oxidizing carbon monoxide, and suppressing plant diseases (Lin et al., 2019; Trivedi et al., 2017).

Although the function of a microbial community is notoriously difficult to interpret, let alone predict based on composition, research combining metagenomics, metaproteomics, and metatranscriptomics can identify community members and their functions to better

understand these highly complex networks (Nannipieri et al., 2020). Additionally, theoretical work is needed to explain phenomena such as species functional redundancy and niche overlap (Jia & Whalen, 2019).

Technologies that observe microbial community dynamics may help elucidate the intricacies of these networks (Wei et al., 2019). “Microbiome-on-a-chip” technologies use microfluidic techniques to analyze microbial interactions in the presence of root exudates (Stanley & van der Heijden, 2017; Aleklett et al., 2017). A semi-realistic analysis of rhizospheric interactions in a soil environment can be attained using a “SoilBox” that integrates spatially relevant associations with genotypic and environmental variation (Bhattacharjee et al., 2020).

2.6 Plant – microbe interactions

Plants manipulate their associated microbial communities throughout their growth and development to suit their needs (Foster et al., 2017). The plant microbiome is a closely regulated system in which community composition and structure is largely dictated by plant genetics (Chen et al., 2020; Bodenhausen et al., 2014). Plant root exudates and rhizodeposits play a key role in selecting for beneficial mutualisms with the soil microbial community (Jain et al., 2020; de la Fuente Cantó et al., 2020). Plants change their root exudate profiles to recruit specific microbes in response to abiotic and biotic stress (Williams & de Vries, 2020; Estendorfer et al., 2017; Wei et al., 2019). As an example, in response to drought stress, plants have been found to recruit a higher percentage of *Actinobacteria* that promote greater drought stress tolerance (Bell et al., 2019).

Beneficial plant-associated bacteria produce a range of phytohormones and signaling compound precursors that promote host growth and survival (Foo et al., 2019; Khan et al., 2020). They promote plant growth by improving soil nutrient availability through biological nitrogen fixation, phosphate solubilization, and the production of siderophores that bind to and make iron available to plants (Compant et al., 2019). There are relatively large amounts of phosphorus present in most soil ecosystems, however the majority is not readily available to plants. Either the phosphorus is in its inorganic form and bound to calcium, aluminum, or iron, or it is in its organic form phytic acid, neither of which are accessible to plants. Phosphorus solubilizing bacteria, however, can produce organic acids, such as oxalic acid, that makes the

inorganic phosphorus bioavailable (Backer et al., 2018). Furthermore, certain microbial taxa use tryptophan, originating from root exudates, as a precursor in the production of indole-3-acetic acid (IAA), which induces growth promotion when detected by plants (Vacheron et al., 2013). In exchange, as plants grow, they increase the production of root exudates and rhizodeposits – nutrients that nourish microbial communities (Souza et al., 2015).

In response to abiotic and biotic stress, plants increase their endogenous levels of the phytohormone ethylene to induce senescence as part of their defense response (Dubois et al., 2018). Certain microbes can reduce ethylene concentrations in the plant by producing 1-aminocyclopropane-1-carboxylate deaminase (ACCd), degrading the precursor to ethylene (ACC) and enabling growth under suboptimal conditions (Kumar et al., 2019). Finally, beneficial microbes can reduce plant susceptibility to pathogens by producing antibiotics and preventing pathogen establishment by competing for the same ecological niche (Compant et al., 2019). This is the foundation of a positive feedback loop in which the root exudates and rhizodeposits influence the rhizosphere community, whose phytoactive products, in turn, positively affect the host plant (Bell et al., 2019). A greater diversity of microbial taxa in the soil infers more resilience in the face of biotic and abiotic pressures, highlighting the importance of soil health.

2.7 Influence of agriculture on phytomicrobiomes

Domestication has changed how soil microbial communities interact with their host plants (Porter & Sachs, 2020). Modern crop varieties have been bred to grow under optimal conditions, often involving excessive nutrient input and irrigation (Schneider & Lynch 2020). Excessive fertilizer use modifies the soil environment by artificially enhancing growth conditions through nutrient availability, replacing the need for diverse microbial communities that could fulfill this function (Hartman et al., 2018; Wattenburger et al., 2019).

A reduction in microbial diversity can occur in response to specific cropping practices such as tillage, sowing methods, and the amount and type of fertilizer applied (Hartman et al., 2018; Huang et al., 2020). Plants will modify their root exudate profiles in response to the addition of fertilizers, consequently affecting the composition of their microbiome (Zhu et al., 2016). For example, the rate of biological nitrogen fixation found in microbes associated with switchgrass decreased as more inorganic nitrogen fertilizer was applied (Bahulikar et al., 2019).

Furthermore, the addition of inorganic nitrogen fertilizer weakens the ability of the microbial community to mineralize other nutrients from the environment (Wattenburger et al., 2019). The type of nitrogen fertilizer applied, whether organic or inorganic, will also alter the predicted composition and function of the microbial community differently (Caradonia et al., 2019).

Different types of fertilizers and cropping system practices have been shown to affect the presence of keystone microbial species as well (Lin et al., 2019; Hu et al., 2020). Due to their importance within a microbial community, the loss of keystone species will result in a shift in community functioning and can ultimately reduce ecosystem function, such as carbon sequestration or nitrogen fixation (Herren & McMahon, 2018; Jousset et al., 2017). The loss of rare microbial taxa in the rhizosphere, especially those that may be keystone species, could also have significant effects on the host plant (Gera Hol et al., 2015). For example, some rare taxa can influence the flowering time of their host plant as well as its growth patterns, impacting its reproductive life cycle traits (Astorga-Eló et al., 2020; Gera Hol et al., 2015).

Shifts in rhizospheric microbial communities have been shown to last for generations in plant populations (Bell et al., 2019). A small percentage of beneficial microbes are inherited through the seed; however, most that constitute the phytomicrobiome originate from the surrounding soil (Rodríguez et al., 2019; Allard et al., 2016). Agricultural practices should, therefore, strive to optimize soil health by increasing soil microbial diversity and community connectedness (Hartman et al., 2018).

2.8 Plant growth promoting bacteria

Beneficial plant growth-promoting taxa can be reintroduced in the form of an inoculant into soil communities that have lost microbial species (Wallenstein, 2017). Microbial consortia of several different taxa or strains appear to outperform single strain inoculants in plant growth promotion under field conditions because of their functional connectivity and resilience during establishment (Menéndez & Paço, 2020). Microbial inoculants promote plant growth by improving nutrient uptake, phytohormone production, and resistance to abiotic and biotic stresses (Calvo et al., 2014). The advantage of using microbial consortia for pest management remains inconclusive, although their ability to promote plant stress response hormone levels is well documented (Gadhave et al., 2016).

The application of plant growth-promoting consortia should seek to bolster the preexisting diversity and ecosystem services of beneficial soil microbes while promoting plant growth and resilience (Hartman et al., 2018; Compant et al., 2019). As inoculants become more integrated into agricultural practices, a greater emphasis on soil microbial ecology at multiple biogeographical scales must be taken into account to formulate the most efficient consortium (Baltrus, 2020). Consortia should be designed to work within a specific environment, as it is a combination of environmental and edaphic conditions, crop genotype, and cropping systems that drive microbial community composition and function (Busby et al., 2016). Microbial inoculants should ideally be crop-specific and modeled after the microbiome of their wild relatives (Oyserman et al., 2018). These considerations should also expedite the formulation of the inoculants (Pérez-Jaramillo et al., 2016).

New crop varieties can be bred to support microbial relationships by producing specific root exudates to attract beneficial microbes. These new varieties may benefit more from inoculation with microbial consortia than current cultivars if they actively recruit and support their persistence (Bender et al., 2016). Microbial inoculants should be designed to achieve a specific function based on the crop genotype, core microbiome, and expected environmental conditions (Toju et al., 2018). Incorporating the spatial linkage of the microbial taxa during the formulation of a consortium will optimize the functioning of the community (Ben Said et al., 2020). Hence, consortia should include microbes known to stimulate and give structure to rhizosphere communities, such as keystone taxa (Mattarozzi et al., 2020; Oyserman et al., 2018). Taxa that play a key role in ecosystem functioning from the local environment should be included to limit the loss of beneficial or rare species (Guerrieri et al., 2020).

A microbial inoculant must successfully establish to affect the native soil community and promote plant growth (Ambrosini et al., 2016). The microbial community, including natural immigrants and pre-existing taxa, may outcompete inoculants and prevent them from establishing (Dini-Andreote & Raaijmakers, 2018). Increasing the number of taxa in a consortium can improve its chances of establishment (Rivett et al., 2018). Establishment and association with the host can be further improved using advances in seed coating technologies (Ma, 2019). During the inoculation process, soil amendments such as compost or manure can

help the new microbial communities establish (Shahzad et al., 2017). If consortia are inoculated into soils along with an additional source of nutrients, a new inoculant-specific niche will be created, helping their population establish (Mallon et al., 2015; Wallenstein, 2017). Microbial consortia can also be used alongside other biostimulants such as botanically or microbially derived compounds or polymers to improve plant growth promotion (Woo & Pepe, 2018; Vassilev et al., 2020).

It is nevertheless challenging to achieve the intended purpose of an inoculant; in fact, unintended consequences can occur, such as a reduction in microbial diversity and community functioning or the spread of antibiotic resistance (Trabelsi & Mhamdi, 2013; Ramakrishna et al., 2019). Testing the efficacy of consortia in laboratory and greenhouse settings and in non-sterile soils is necessary, although long-term field trials are also needed to confirm that the desired effect will occur without unintended consequences (Stanley & van der Heijden 2017; Vorholt et al., 2017). Precision agriculture techniques can facilitate field trials by using sensory technologies to screen for effective inoculants (Roupheal et al., 2018).

The growing use of microbial inoculants is improving the sustainability of agricultural practices by reducing the amount of synthetic nitrogen fertilizer applied to fields. This is especially true for those containing diazotrophic species that fix atmospheric nitrogen into plant available forms. Globally, nitrogen is biologically fixed at 60 Tg y^{-1} (Vitousek et al., 2013). The reduction of inorganic nitrogen fertilizer will result in a more diverse rhizosphere community that can provide more nutrients to the plant (Bloch et al., 2020; Wattenburger et al., 2019). Furthermore, for leguminous crops that contain biological nitrogen fixing microbes in their root nodules, the addition of phosphorus solubilizing bacteria has been shown to promote growth, thereby reducing the need to add phosphorus fertilizers (Shome et al., 2022).

Field and crop-specific growth-promoting microbial inoculants are already being advanced (Awasthi, 2019; Bell et al., 2019). Microbial community profiles from various soil types and geographic regions will soon be used to formulate plant growth-promoting inoculants for specific crops in a local environment (Mitter et al., 2019). The combination of precision agriculture with microbial inoculants will further bolster the impact of these practices (Sergaki et al., 2018; Del Pozo, 2020).

2.9 PGPB Inoculants for Cold Stress Alleviation in Crops

For non-motile organisms such as plants (including agricultural crops), abiotic stress can be an inescapable environmental pressure. Plants native to historically cold regions, such as those of high elevation or altitude, have evolved to rely on associated microbial organisms to alleviate cold and freezing stress (Pandey & Yarzábal, 2019). Non-native crops, especially those that have not been bred to be cold hardy, can easily succumb to cold stress. For much of the world, crop loss due to a period of cold weather early in the growing season can lead to serious food shortages and economic loss. In fact, an unexpected freeze during any stage of a crop's development, even post-harvest, may render it undesirable, and reduce its potential yield (Gu et al., 2008).

Just as microbial inoculants and biostimulants can aid crop growth when faced with biotic and abiotic stresses such as drought and salt stress, the application of psychrotolerant microbes, biostimulants, or a combination of both, can be used to improve a crop's response to cold stress (Wang et al., 2016; Askari-Khorasgani et al., 2019). Biostimulants containing free amino acids, such as proline, have been shown to reduce the impact of cold stress on crops (Gavelienė et al., 2018). Successful application of microbial inoculants to alleviate cold stress has been shown in a wide variety of crops such as wheat, bean, canola, grape, and corn, with many of these crops showing higher levels of proline after inoculation (Bandara et al., 2021).

Microbes capable of conferring tolerance to cold and freezing stress do so by inducing a plant's antioxidant production, regulating its hormones, and mediating its cellular osmotic balance (Acuña-Rodriguez et al., 2020). As part of a plant's natural response to cold stress, reactive oxygen species (ROS) such as O_2 and H_2O_2 accumulate and can be detrimental to its cellular structure and genetic contents (Rihan et al., 2017). Several plant-associated fungal and bacterial species have been shown to produce antioxidants which reduce ROS and improve cold tolerance. Microbial production of phytohormones, such as salycilic acid, jasmonic acid, and IAA, also promote plant growth during suboptimal temperatures (Subramanian et al., 2016). The accumulation of sugars such as trehalose and amino acids like proline produced by microbes also regulate plant cellular osmotic stress induced by cold temperatures (Acuña-Rodríguez et al., 2020).

More general mechanisms for cold stress alleviation in plants by their microbial symbionts include phosphorus solubilization, which increases trehalose content, calcium solubilization that is involved in ROS crosstalk, and iron chelation important for ROS scavenging enzymes (Askari-Khorasgani et al., 2019). Other known mechanisms to alleviate cold stress include enhancing root growth and contributing to cell membrane modifications (Bandara et al., 2021).

The bulk of research studying how microbes induce cold stress tolerance in crops has been done on fungi, leaving much to be done on bacteria. This research is necessary since studies have shown that fungal-induced cold resistance only occurs when there are sufficient nutrients present in the soil, indicating that their use may be limited under nutrient stressed conditions in cold regions (Acuña-Rodríguez et al., 2020).

Microbes that can survive and produce growth-promoting compounds at low temperatures should be included in microbial inoculants to ensure functioning throughout the growing season (Yadav et al., 2017). Current commercial plant growth promoting microbial consortia may not function properly when temperatures are suboptimal as they are designed to work under ideal conditions (Trivedi et al., 2012).

Psychrotolerant bacteria isolated from several plant species native to high elevation areas have been used as PGPB to the common bean (Tiryaki et al., 2019). These microbes were able to confer cold stress resistance by producing exogenous ACCd, which reduces the plant's ethylene levels, and reducing the ROS in plant leaf tissue (Tiryaki et al., 2019). This demonstrates that microbes can associate with and have a positive effect on specific plant species.

Chapter 3: Methods

3.1 Overview

A series of experiments were carried out in order to test whether a commercial microbial inoculant could promote the growth of corn under low temperature stress. Specifically, the corn seeds tested were Dekalb hybrid (DKC46-17RIB). Growth promotion was assessed from the earliest to mid-development growth stages at an optimal temperature and at two low temperatures; trends from these stages correlate strongly with later plant development and economically important traits such as grain yield (Shafer & Wiggans, 1941). Germination rate and radical growth was measured in the first experiment followed by emergence, shoot height, and dry weight in the second experiment. A detailed nutrient analysis was conducted on the corn biomass from the second experiment to compare any differences that might be due to inoculation with the consortium. The second experimental setup was repeated with lettuce (organic butterhead), a dicot as opposed to a monocot. Emergence, leaf area, and leaf dry weight were measured, however a nutrient analysis was not conducted on the lettuce tissue. This was done in order to test for any similarities with the corn data. In order to evaluate what effects the consortium might have had on the soil microbiome, bulk and rhizosphere samples were taken from the second corn experiment. 16S rRNA gene sequencing was conducted to analyze the bacterial community profile and to detect any changes in the resulting community composition after inoculation. The first and second corn experiments, including microbial DNA extraction, occurred from April to September, 2021. The lettuce experiment occurred from January to April, 2022, and 16S rRNA sequencing occurred during the month of June, 2022.

3.2 Bacterial strains

The microbial consortium used for all experiments was the commercially available inoculant Era Boost Pro from Ulysse Biotech, a company based out of Trois-Rivières, Quebec. This product is a probiotic, meaning it contains live bacterial cells, and a biostimulant designed to promote the growth and yield of the crops to which it is applied to as well as the proliferation of other soil microbes surrounding the rhizosphere (https://ulysse-

biotech.com/en/products/era-boost-pro). The modes of action by which the consortium promotes growth are by solubilizing calcium and phosphorus, thereby increasing their bioavailability, and by chelating iron molecules through the production of siderophores. Finally, bacterially produced enzymes from the consortium contribute to the soil microbial community structure and function by forming a biofilm in the rhizosphere in combination with plant root exudates, constructing a niche for highly specialized plant-microbe symbioses.

Era Boost Pro is composed of five different strains of plant growth promoting rhizobacteria (PGPR). There are two strains of *Bacillus velezensis*, two strains of *Bacillus megaterium* (this taxon has now been reclassified as *Priestia megaterium* (Gupta et al., 2020; Biedendieck et al., 2021), however for simplicity, and to follow how the company has marketed Era Boost Pro, it will be referred to by its previous name), and one strain of *Bacillus licheniformis*. The consortium contains 80,000,000 viable spores per gram, or colony forming units (CFU), of each strain with a combined total of 400,000,000 CFU per gram. The dosage prescribed by Ulysse Biotech for field and greenhouse use is 0.5mL of consortium per liter of water. This was the concentration used for all experiments unless otherwise indicated.

Bacillus velezensis is a gram-positive spore forming PGPR with important functional roles in shaping rhizosphere microbial community composition. *B. velezensis* contributes to the production of a biofilm to surround the rhizosphere. It can shape the rhizo-microbiome by selecting for beneficial taxa while limiting the growth of plant pathogens (Rabbee et al., 2019). For example, when *B. velzensis* was inoculated to promote the growth of cucumber, researchers discovered a syntrophic cooperation with *Pseudomonas stutzeri* in the occurring biofilm. They were then able to show that salt stress was alleviated in cucumber only when both bacterial species were present (Sun et al., 2022). Other known plant growth promoting mechanisms of *B. velezensis* include iron chelation and the production of the phytohormone analog IAA (Meng et al., 2016).

Bacillus megaterium, now *Priestia megaterium*, has been known to have beneficial properties that promote plant growth for the past 20 years. It serves as a PGPR for a range of crops such as tomato, corn, and soybean. Three main modes of action have been described for *B. megaterium*. First, the bacteria are capable of solubilizing phosphorus in the soil by secreting

a range of organic acids. Secondly, *B. megaterium* produces a range of exogenous phytohormones such as IAA and ABA, which stimulate growth and improve plant resilience to abiotic stress. Studies have also shown that *B. megaterium* influences endogenous phytohormone signaling such as cytokinin and ethylene (Ortíz-Castro et al., 2008; López-Bucio et al., 2007). Finally, *B. megaterium* has several antipathogenic properties such as chitase production, protection against fungal pathogens, and various quorum quenching mechanisms, suppressing attacks from bacterial pathogens (Biedendieck et al., 2021).

Bacillus licheniformis is a well-studied and characterized PGPR. It is gram positive and spore forming with several strains shown to solubilize calcium phosphate and produce siderophores. Many strains are also known to produce phytohormones such as IAA and gibberellins (Mpofu et al., 2019). Its plant growth promotion capabilities have been shown in a range of crops such as tomato, pepper, mungbean, and broccoli (Lim & Kim, 2009; Katsenios et al., 2021).

3.3 Germination rate and total radical length experiments

The consortium Era Boost Pro from Ulysse Biotech was tested for its efficacy to promote the germination and early growth of corn at low temperatures. An initial experiment was conducted to validate the viability of the bacterial cells present in the consortium. Twenty-four petri dishes of plate count agar (PCA) with a pH of 7.04 were created. Twelve plates were used for the treatment group and another 12 served as the control. Ninety-six corn seeds were soaked in 100% bleach for 1 minute and then thoroughly rinsed 5 times with sterilized water. Forty-eight of these seeds, acting as the control, were then soaked in sterilized water for 10 minutes, while the 48 seeds of the treatment group were soaked in a 0.5 mL L^{-1} dilution of the consortium for 10 minutes. Four seeds were removed from either the treatment or control solutions and placed on each of the 12 plates and sealed with parafilm. The plates were then placed in a dark environment at 25 °C for 48 h and checked for bacterial growth every 24 h. All plates in the treatment group showed bacterial growth consistent with *Bacillus* species morphs while there was no bacterial growth present in the control after 48 h (Supplemental figure 1). This confirmed that the dilution of the consortium recommended by the company was adequate to have viable cells present and capable of colonizing corn seeds.

To assess the efficacy of the consortium to promote germination at low temperatures, 10 seeds were placed on petri plates (Cat.no. 431760, sterile 100 × 15 mm polystyrene Petri dish, Fisher Scientific Co., Whitby, ON, Canada) that were lined with filter paper (09-795D, QualitativeP8, porosity coarse, Fisher Scientific Co., Pittsburg, PA, USA). A dilution of the consortium was prepared in sterile water at a concentration of 0.5 mL L^{-1}. Each seed in the treatment group was inoculated with 10 mL of the diluted consortium and each seed in the control was inoculated with the same amount of sterile water. In total there was 100 mL inoculated on each plate. There were four replicate plates per treatment and therefore a total of 40 seeds per treatment. The petri plates were then sealed with petri tape that allowed for gas exchange and were placed in a dark environment at either 25, 20, or 15 °C, with 25 °C considered to be the optimal temperature.

The seeds were checked every 24 h and the number that had germinated per plate was recorded. The average was calculated for the number of seeds that had germinated per plate across the four replicates for each day. This was done for each temperature until all seeds from either the treatment or control group had germinated.

At each temperature, when the first experimental group reached 100 % germination, both groups were removed from their growth chamber and the radical lengths were measured (in millimeters). For each replicate petri plate, the radical lengths were summed. The average was taken across the four replicates following modified methods from Naamala et al (2022).

The same experimental set up was repeated, but this time seeds were inoculated with only 5 mL of either the diluted consortium for the treatment group or sterile water for the control group, totaling 50 mL per plate.

The resulting radical length and germination data were represented in bar plots using Microsoft Excel, and results were tested for significance using students t-test with least square means in Excel. Results were considered significant using a p-value ≤ 0.05.

3.4 Growth chamber experiments

The consortium Era Boost Pro from Ulysse Biotech was tested for its ability to promote corn growth at low temperatures. The plants were grown until the V4 growth stage in a PGR15 Conviron growth chamber located in McGill's MacDonald Campus phytorium at the optimal

temperature, 25 °C, or at the two stressfully low temperatures, 20 or 15 °C. For all growth trials, the daily light intensity was kept at 300 micromoles m^{-2} s^{-1} for 15 h, followed by 9 h of darkness. The temperatures tested for each growth trial were kept constant throughout each experiment.

For each temperature growth trial, 46 pots of 15.25 cm diameter were filled with approximately 600 g of moistened G6 Agro Mix potting media (www.fafard.ca/) and were each placed on individual saucers. Each pot was fertilized with 200 mL of 20-20-20 N-P-K fertilizer (www.plantprod.com/) at a concentration of 100 ppm. The pH of the soil was 6.0 and the pH of the fertilizer added was approximately 6.2. Five non-genetically engineered corn (i.e. non-BT corn) seeds were planted at a depth of approximately 3 cm in 34 of the 46 pots. The remaining 12 pots were kept seedless and served as bulk soil replicates.

A dilution of the Era Boost Pro consortium was made at a concentration of 0.5mL L^{-1} of sterile water. The treatment group of 23 pots was inoculated by soil drenching with 250 mL of the diluted consortium while the other 23 pots were inoculated with 250 mL of sterile water.

Following a randomized complete block design, 20 of the treatment pots and 20 of the control pots (each group had 3 pots without seeds and 17 pots with seeds) were placed with their saucers in the growth chamber. The seedlings were checked every 24 h for emergence. After 72 h, if no new emergence was observed, the average number of days for each pot and then between the control and treatment group were calculated. Seedlings were thinned from 5 to 3 plants, selecting for uniformity within each pot for each temperature when the plants were between emergence and the V1 growth stage. At 25 °C, thinning occurred on day 6 after planting. At 20 °C, thinning occurred on day 9 after planting, and at 15 °C, thinning occurred on day 16 after planting.

The 6 remaining pots of soil with no plants (3 inoculated treatment and 3 control) that were not placed into the growth chamber were sampled for microbial community analysis. This represented the initial timepoint, or T0 soil sample. The soil was sampled using sterilized utensils and weighing boats. The top 3 cms of soil was harvested from each pot following a standard quartering method (Campos & Campos, 2017), the sample being fractioned and thoroughly mixed. Quartering was repeated until approximately 2.5 g of soil remined for each pot (n = 3). The soil samples were then stored in sterile falcon tubes and frozen at -80 °C.

All pots in the growth chamber were top watered until the plants emerged, after which they were bottom watered directly in the saucer. When the plants reached each subsequent growth stage (V1, V2, V3, and V4), they were fertilized with 200 mL of 20-2-20 N-P-K at a concentration of 100 ppm with a pH of 6.2. For the 25°C growth trial, the next growth stage and fertilizer application occurred every 7 days. For the 20 °C growth trial, fertilization occurred every 10 days. For the 15 °C growth trial, fertilization occurred every 16 days. For the 25 and 20 °C trials, the pots were watered approximately every other day with 200 mL of water. For the 15 °C trial, the pots were watered every third day. For all temperatures, when the plants reached the V3 growth stage, water dosage increased to 300 mL. By the V4 growth stage there was no drought stress although mild phosphorus stress, which was expected due to the fertilizer composition of 20-2-20, was observed (Supplemental figure 2). This was imposed to coax the plant to shift its root exudate profile in order to recruite more phosphorus solubilizing bacteria, such as those in the consortium.

The experiment was repeated a second time for the 15 °C temperature trial as it was hypothesized that any effect the consortium might have on plant growth under low temperature stress would be most obvious under the most stressed conditions. The data from both 15 °C trials were combined for analysis.

3.5 Harvesting plant and rhizosphere material

Four pots were removed in destructive sampling from both the treatment group and control group at each growth stage (V1-V4), resulting in data for the 4 time-points. Plant height was measured in centimeters for each of the 3 plants in each pot from the soil surface to the tip of the newest leaf with a fully developed collar. The average was taken for each pot, and then across the 4 pots for both the treatment and control group. For the first three timepoints, the soil was washed completely from the roots. The plants were then placed in paper bags and put in drying ovens at 65 °C for 48 h. The dry plant tissue was then separated into roots and shoots and weighed. The average dry weight was then calculated for the 3 plants in each pot and then again across the 4 replicates for the treatment and control group. Time till emergence, plant height, and dry weights were represented in Excel using bar plots and analyzed using students t-test and least square means in Excel. Results were considered significant at $p \leq 0.05$.

For the 4th, and final, time-point harvest, the plant heights and dry weights were measured following the same procedure. Bulk soil was removed by vigorously shaking the plant roots by hand for 10 minutes, following a modified protocol from Benitez et al. (2021). Any soil that remained adhered to the shaken roots was determined to be the rhizosphere. The rhizosphere was removed from the roots of each plant using a sterilized utensil and thoroughly homogenized, resulting in 1 sample from each of the replicate pots. If more than approximately 2.5 g of soil was collected from the rhizosphere, the same quartering method was followed until this weight was reached, at which point all the samples were collected in sterile falcon tubes and stored at -80 °C. The rhizosphere was collected from the 25, 20, and 15°C growth trials, however it was only collected from the first of the two 15 °C growth trials.

The 6 pots of soil alone (3 treatment and 3 control) remaining at the end of the growth experiments were harvested (TF) at the same time as the T4 plant harvest. Soil from the top 3 cm was harvested from each pot and the same quartering method was followed to obtain a volume of 25 g. The soil samples were then placed in sterile falcon tubes and stored at -80 °C.

3.6 Plant tissue nutrient analysis

The final time-point harvested from all three temperatures was washed to remove all excess soil after the rhizosphere was collected, and dried at 65 °C for 48 h before the roots and shoots of each plant were weighed. The three plants from each pot from both the treatment and control groups were separated by roots and shoots and placed in labeled paper bags. The dry plant tissue was sent to A&L Canada Laboratories (London, Ontario) for nutrient analysis following in-house standardized protocols including inductively coupled plasma – optical emission spectrometry (ICP-OES), colorimetric analysis, and thermal conductivity (https://www.albiologicals.com/services).

Nutrient concentration data for both roots and shoots from each pot were generated and returned for the following nutrients: nitrogen, sulfur, phosphorus, potassium, magnesium, calcium, sodium, boron, zinc, manganese, iron, copper, and aluminum. The averages were calculated for both the treatment and control roots, shoots, and combined plant total (n = 4) and represented in bar plots using Excel. The averages were compared between the treatment

and control group for each temperature using students t-test using least square means. Results were considered significant at $p \leq 0.05$.

3.7 Growth chamber experiment with lettuce

The biomass experiment conducted with corn, a monocot, was repeated with lettuce to see if the microbial consortium Era Boost Pro from Ulysse Biotech would have similar results and potentially promote growth at low temperatures in a dicot. The temperatures tested were, again, 25, 20 and 15 °C. The lettuce variety grown was organic butterhead and the seeds came from Norseco (Laval, Quebec).

For the 25 and 20 °C trials, forty 15.25 cm diameter pots were filled with approximately 600 g of moistened G6 growing media. Five seeds were sown in each pot approximately 5 mm deep. Following the same experimental methods as the corn trial, 200 mL of 20-20-20 N-P-K fertilizers was applied at 100 ppm to each pot. The control group (n = 20 pots) was then given 250 mL of sterile water. The treatment group (n = 20 pots) was inoculated with 250 mL of the microbial consortium at a concentration of 0.5 mL L^{-1}.

The rate of emergence was measured by adding up the total number of seedlings per treatment group every 24 h until no more new plants emerged for 48 h. Seedlings were thinned from 5 plants to 3, selecting for uniformity within each pot when the plants had 3 leaves (two cotyledons and one true leaf). Thinning occurred on day 8 for the 25 °C trial, on day 11 for the 20 °C trial, and on day 18 for the 15 °C trial. The pots were then fertilized with 200 mL 20-2-20 N-P-K fertilizer at a concentration of 100 ppm.

Following Ulysse Biotech's protocol for applying Era Boost Pro to lettuce, a second inoculation was performed by soil drenching. At 25 and 20 °C, 10 pots from the treatment group were inoculated with 250 mL of the consortium at a concentration of 0.5 mL L^{-1} and 10 pots from the control were inoculated with 250 mL of sterile water. Ten pots for both treatment and control did not receive a second inoculation to test whether one inoculation would have an effect. These remained in the growth chamber for the duration of the experiment, but the biomass data was not collected since no effect was visible. For the 15 °C trial, all 20 treatment pots were inoculated with the same dose of consortium, and all 20 control pots were inoculated with the same amount of sterile water. The 25 °C trial was

inoculated a second time on day 15, the 20 °C trial was inoculated a second time on day 18, and the 15 °C trial was inoculated a second time on day 32.

The aboveground plant tissue from the treatment and control groups that had been inoculated twice was then harvested. Harvest occurred on day 21 for the 25 °C trial, day 29 for the 20 °C trial, and day 42 for the 15 °C trial. The cumulative leaf area for each lettuce from each pot was measured in cm^2 using a LI-COR LI-3100C leaf area meter (LI-COR, Lincoln, Nebraska) within two hours after harvesting. The average was then calculated for each treatment group (n = 10 for 25 and 20 °C, n = 20 for 15 °C). The leaf tissue was then dried in an oven at 65 °C for 48 h before being weighed. Both the leaf area and dry weight results were represented with bar plots and compared using students t-test using least square means. Results were considered significant at $p \leq 0.05$.

3.8 Microbiome analysis

Microbial DNA from the corn growth experiment was extracted from both the soil samples (T0 and TF) without plants grown (i.e. bulk soil) and the rhizosphere soil that was collected from the final timepoints of the corn growth trials at 25, 20, and 15 °C. There were 42 samples in total. The microbial DNA was extracted using Qaigen's Dneasy PowerSoil Pro kit (qaigen.com) following the manufacturer's instructions. A 10 µL aliquot was taken of the resulting 75 µl of DNA suspended in buffer and was quantified using a Qubit fluorometer (Thermo Fisher Scientific). The remining 65 µL were stored at -80 °C and used for sequencing.

16S rRNA gene library preparation and sequencing was carried out at the National Research Council (NRC) at Royalmount in Aquatic and Crop Resource Development Facilities. A V4/V5 region of the 16S rRNA gene was PCR amplified using forward primer 515Y (5'-GTGYCAGCMGCCGCGGTAA-3') and reverse primer 926R (5'-CCGYCAATTYMTTTRAGTTT-3'). PCR mixes were as followed: 1X KAPA HiFi Hot Start ReadyMix and 1X KAPA 2G mix (Kapa Biosystems) with 0.5 mg/ml of bovine serum albumin and 0.6 µM each of forward and reverse primers. PCR conditions were as followed: 3 min at 95°C, 25 cycles of 30 sec at 95°C, 30 sec annealing at 55°C, 30 sec at 72°C, and a 5 min final elongation at 72°C. The final PCR products were quantified by PicoGreen, had their concentration normalized to 4 ng μl^{-1}, before sequencing libraries were prepared using a 500 cycles MiSeq Reagent Kit v2 and following

Illumina's 16S Metagenomic Sequencing Library Preparation (Part # 15044223 Rev. B, available at support.illumina.com/documentation). The samples were then sequenced using an Illumina MiSeq instrument (San Diego, California).

Data from the 16S rRNA gene amplicon sequencing was processed using the National Research Council's (NRC) in-house AmpliconTagger pipeline which removed contaminant and unpaired reads, trimmed and removed primer sequences, and clustered filtered reads to 97% similarity creating Operational Taxonomic Units (OTUs) (Tremblay and Yergeau, 2019). OTUs were assigned taxonomic groups using the latest SILVA rRNA database (SILVA 138, www.arb-silva.de/) and were rarified using the software QIIME (qiime.org/). Beta diversity for the different variables tested was calculated using Bray-Curtis dissimilarity matrices and visualized using Principal Coordinate Analysis (PcoA). All PERMANOVA analyses used the function adonis from the R package Vegan (Oksanen et al., 2020). The taxonomic profiles representing the most abundant taxa present were created using the NRC website Taxa Profiler (jtremblay2.shinyapps.io/taxonomy/). Variation in the relative abundance of taxa was tested for significance using the t.test() function in R and results were considered significant at $p \leq 0.05$. P – values were not corrected for multiple testing due to the small sample size. Although this may result in false positives, this test is helpful in verifying the qualitative observations seen in the taxonomic profiles.

Chapter 4: Results

4.1 Overview

Most of the results from this study revealed very little, if any, effect due to the consortium. At 25 °C, the optimal temperature, plant measurements such as germination, radical length (in the 10 mL treatment), dry weight, and nutrient concentrations showed a positive effect due to the inoculation, however this was not always significant. At the two low temperatures, 20 and 15 °C, no effect, or even a negative effect, was observed, suggesting slight, but insignificant, growth inhibition. The lack of statistical significance may have been due to the small sample size of the experiments. The lettuce experiment followed the same trends as that of the corn with a detectable effect on plant growth at the optimal temperature, and no or an insignificant negative effect at low temperatures.

Results from the rhizosphere microbiome data indicate that the consortium did not impact community structure as much as time (i.e. T0 vs. TF) or temperature. The class or genera to which the bacterial strains in the inoculant belong to were not observed among the most abundant taxa from the rhizosphere microbial community.

4.2 Early Growth Experiments

4.2.1 Germination rate

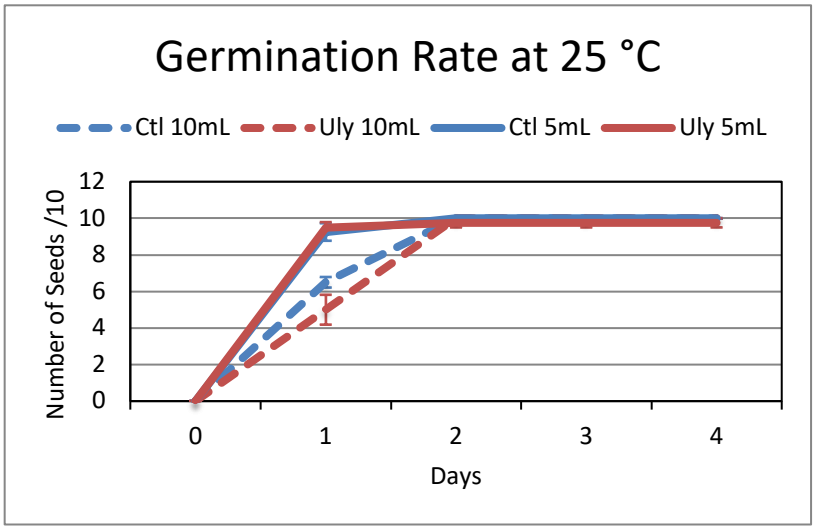

Table 1. P-Values for corn germination at 25 °C**.**

Germination Rate 25°C P-Value		
	Volume	
Day	5 mL	10 mL
1	0.670	0.024
2	0.356	NA

Figure 1. Corn seed germination rate for two volumes tested at 25 °C. Uly represents the treatment and Ctl represent the control group**.** Error bars show standard deviation.

There was very little difference in germination rate at 25 °C for the 5 mL treatment (Uly 5 mL) and control (Ctl 5 mL) groups. However, there was a significant ($p = 0.024$) difference for seeds given 10 mL of either the control (Ctl 10 mL) or the treatment (Uly 10 mL) with an average of 6.5 control seeds and 5 treatment seeds, respectfully, germinating by the first day. Both treatment and control groups, at both volumes tested, reached an average of 9.75 seeds germinating by the second day.

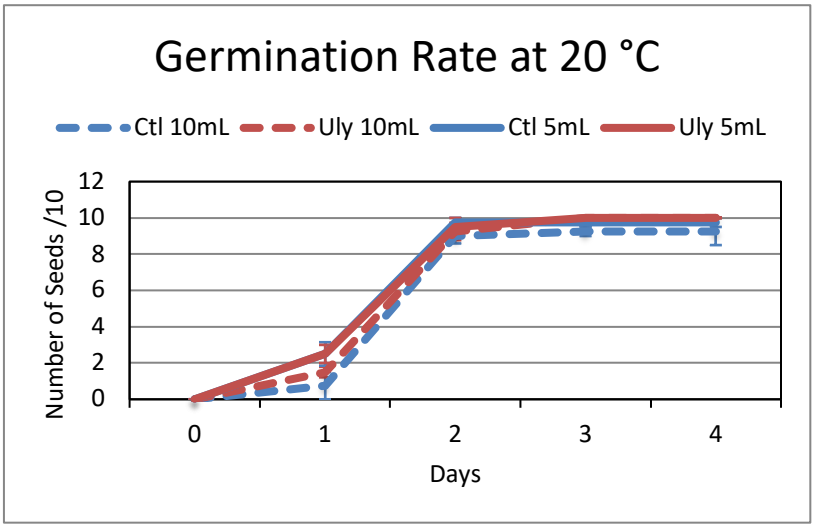

Table 2. P-Values for corn germination at 20 °C**.**

Germination Rate 20°C P-Value		
	Volume	
Day	5mL	10mL
1	1	0.387
2	0.670	0.705

Figure 2. Corn seed germination rate for two volumes tested at 20 °C. Uly represents the treatment and Ctl represent the control group**.** Error bars show standard deviation.

The different volumes (5 mL vs 10 mL) had less of an effect for seed germination at 20 °C but again both the treatment and control groups given 5mL rather than 10mL had slightly more seeds germinate on day one. By day two, all groups for both volumes tested had finished germinating. The addition of the consortium did not have a significant effect.

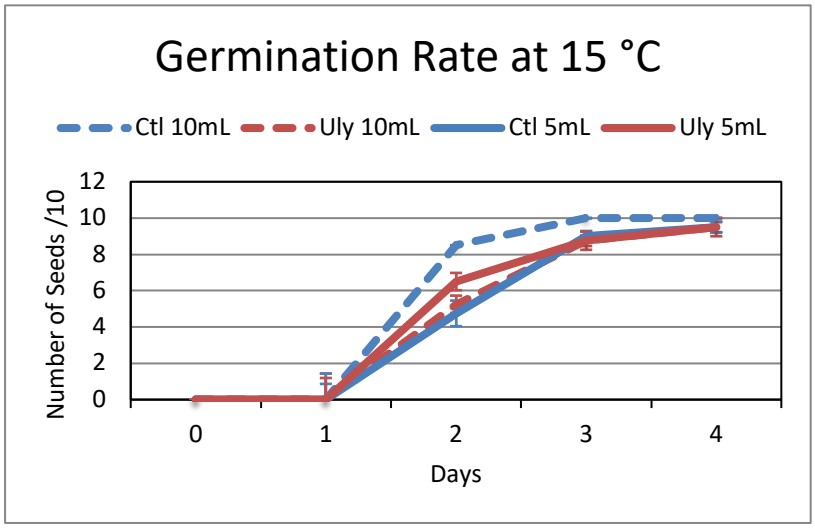

Table 3. P-Values for corn germination at 15 °C**.**

Germination Rate 15°C P-Value		
	Volume	
Day	5mL	10mL
2	0.384	0.101
3	0.780	0.040
4	1	0.134

Figure 3. Corn seed germination rate for two volumes tested at 15 °C. Uly represents the treatment and Ctl represent the control group**.** Error bars show standard deviation.

At 15 °C, all seeds in both treatments at both volumes only began to germinate on day two and completed germination on day four. Interestingly, the 10 mL control group germinated at a higher rate than the 5 mL control. At 10 mL, the treatment group had an average of 5.25 seeds germinate while the control had an average of 8.5 seeds germinate within the first 24-h period. At 5 mL, the control had on average fewer seeds germinate within the first 24-h period compared to the treatment with 4.75 seeds and 6.5 seeds, respectively. A significant ($p = 0.04$) difference was observed during the second 24 h period of germination in which the 10 mL control group had more seeds germinate than the treatment.

4.2.2 Radical length

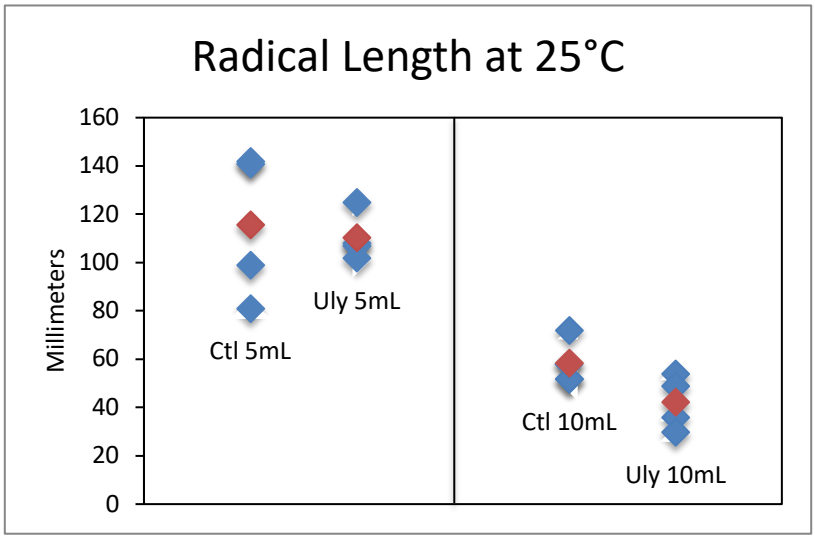

Figure 4. Total radical lengths for two volumes tested at 25 °C. Blue represents the total length for each plate while red represents the average between plates.

The radical lengths were longer for both 5 mL groups (control (Ctl) and treatment (Uly)), with averages at 115.75 mm and 110.5 mL, respectively, compared to the control and treatment group at 10 mL with values of 58.5 mm and 42.25 mm, respectively. This difference likely reflects the variation in germination rate due to the different volumes tested rather than the addition of the consortium. The control group for both volumes had on average longer radical lengths, however this was insignificant.

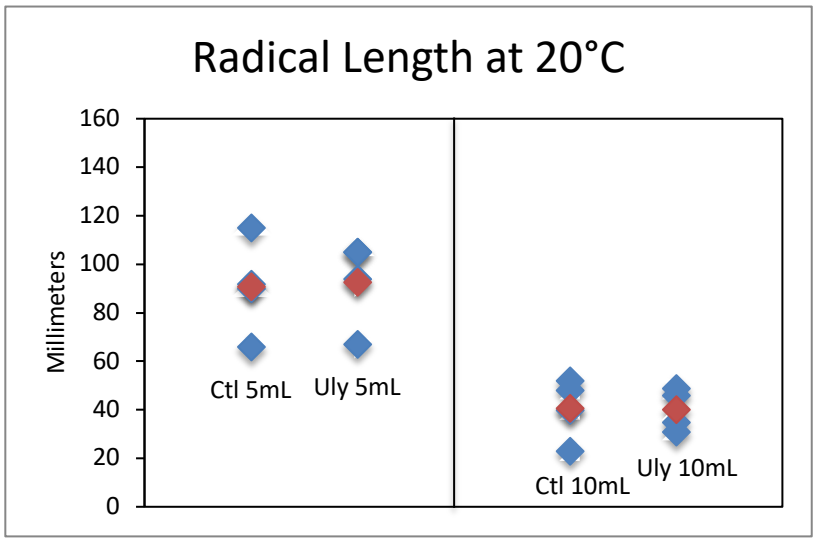

Table 4. P-Values for corn radical lengths**.**

Radical Length P-Value		
	5mL	10mL
25°C	0.756	0.068
20°C	0.887	0.951
15°C	0.548	0.127

Figure 5. Total radical lengths for two volumes tested at 20 °C. Blue represents the total length for each plate while red represents the average between plates.

Radical lengths at 20 °C followed a similar pattern to those at 25 °C, however there was even less of a difference between the averages of the treatment and control groups. Given 5 mL, the control group had an average of 90.75 mm and the treatment was slightly longer at 92.75 mm. Given 10 mL, the average control length was 40.75 mm while the treatment average was 40.25 mm. These slight differences in average length were insignificant.

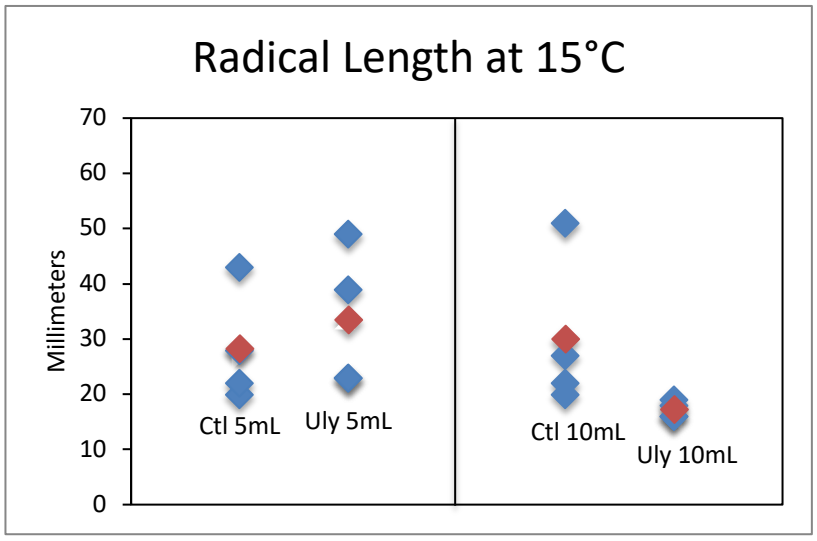

Figure 6. Total radical lengths for two volumes tested at 15 °C. Blue represents the total length for each plate while red represents the average between plates.

For the 15 °C trial, the average radical length was slightly longer for the treatment group given 5 mL with a length of 33.5 mm compared to 28.25 mm in the control, however this was insignificant. With 10 mL, the average control radical length was greater than the treatment at 30 mm compared to 17.25 mm. This difference was not significant.

4.3 Growth chamber experiments

4.3.1 Emergence

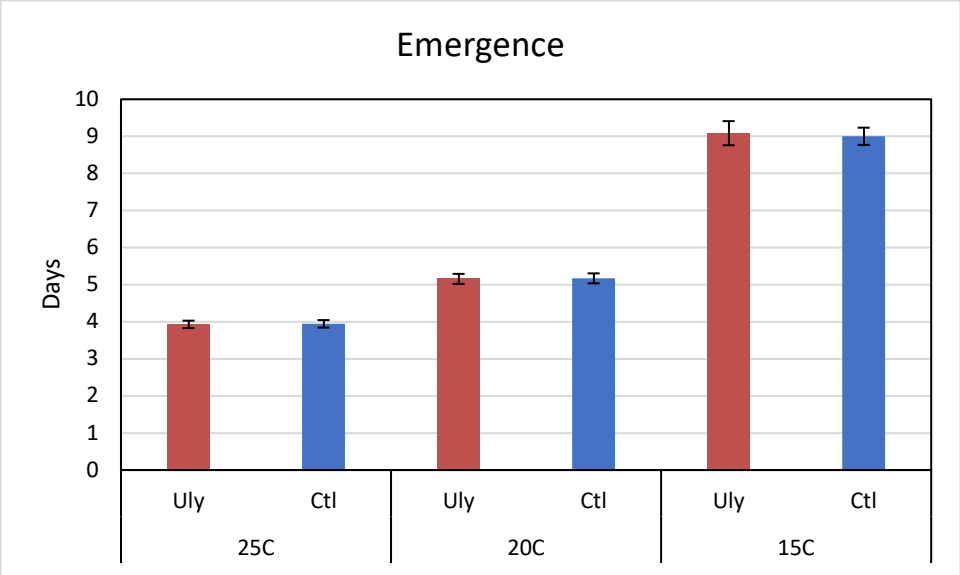

Figure 7. Number of days till corn seed emergence for treatment and control groups at the three temperatures tested. Error bars indicating standard deviation are shown, n=17.

Addition of the inoculant did not appear to influence the rate of emergence across the range of temperatures tested. At 25 °C, the average emergence took 3.9 days for the inoculated group (Uly) while the uninoculated control (Ctl) took 3.9 days. At 20 °C, the average number of days before emergence was 5.1 and 5.1 for the treatment and control, respectively. Finally, at 15 °C, the treatment took an average of 9.0 days while the control took an average of 9.0 days to emerge. Differences were insignificant for all temperatures.

4.3.2 Plant height

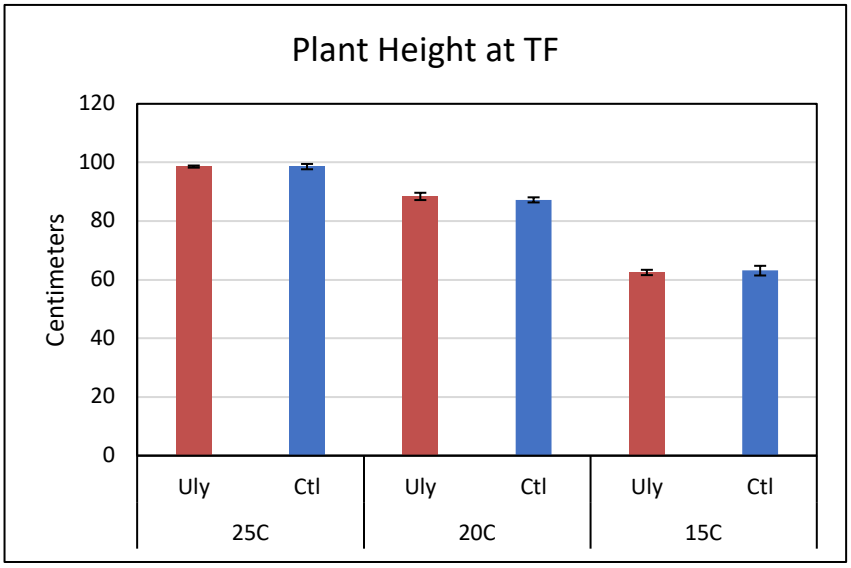

Figure 8. Average plant height (cm) for treatment and control groups at the three temperatures tested (25, 20, and 15 °C). Error bars indicating standard deviation are shown, n=4.

At the final timepoint, plant height differences between the consortium and control treatment were not significant within the three temperatures tested. The treatment (Uly) group was 0.01 % taller at 25 °C, 1.36 % taller at 20 °C, and 0.99 % shorter at 15 °C compared to the control (Ctl), although insignificantly so.

4.3.3 Plant dry weight

The next three figures show the differences in shoot and root dry weight between the treatment (Uly) and control (Ctl) groups throughout the four timepoints taken during the experiment.

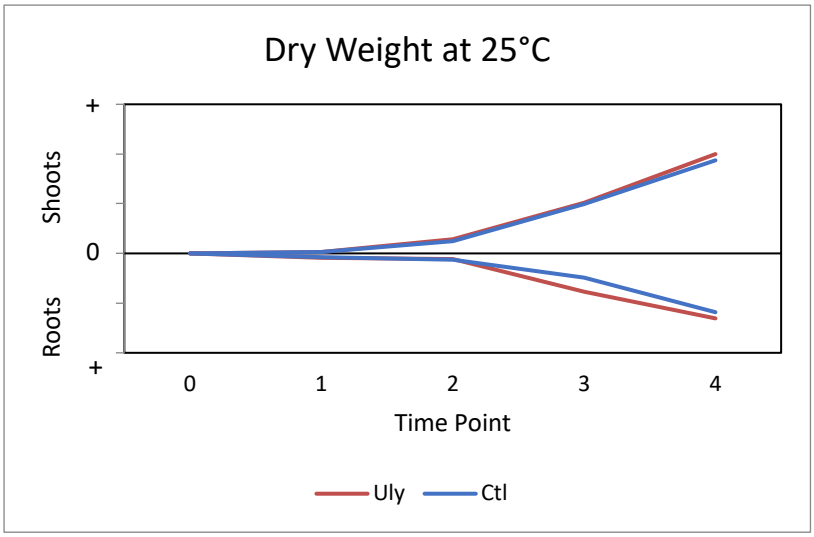

Figure 9. Average dry weight (g) at 25 °C for treatment and control groups across timepoints.

At the 25 °C temperature, differences can be seen by the third timepoint, which corresponds with the third harvest at the V3 growth stage, when inoculated plants in the treatment group had greater root dry weights, although insignificantly so (p-value of 0.0628), and by the fourth timepoint the treatment plants had both greater root and shoot dry weight.

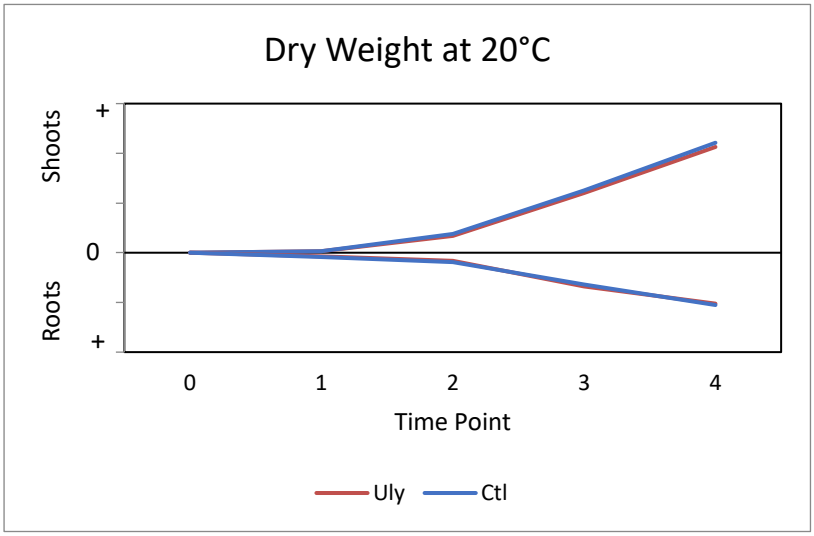

Figure 10. Average dry weight (g) at 20 °C for treatment and control groups across timepoints.

At 20 °C, the differences between treatment shoot and root dry weights across the first few timepoints is less obvious, with the control having a slightly greater shoot dry weight at the final timepoint.

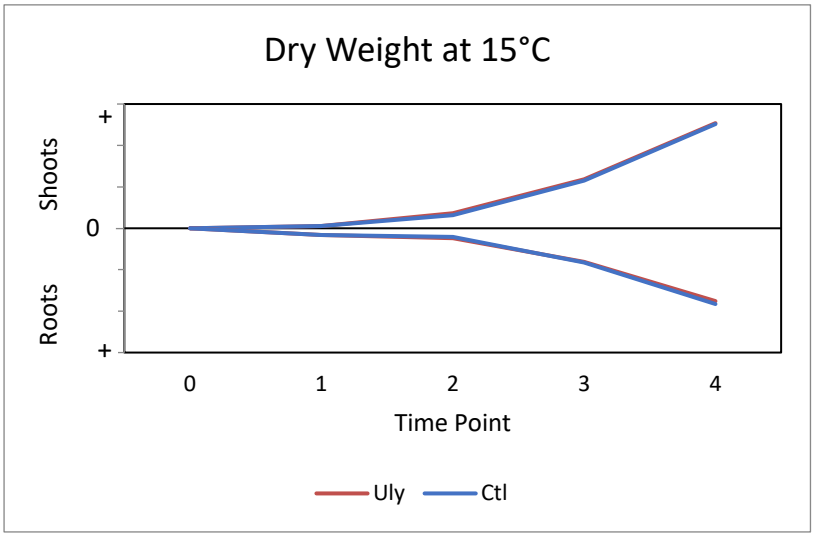

Figure 11. Average dry weight (g) at 15 °C for treatment and control groups across timepoints.

At 15 °C, the differences between treatment dry weights for both shoots and roots is almost indistinguishable, although the control group was found to have a slightly greater root dry weight on day 4.

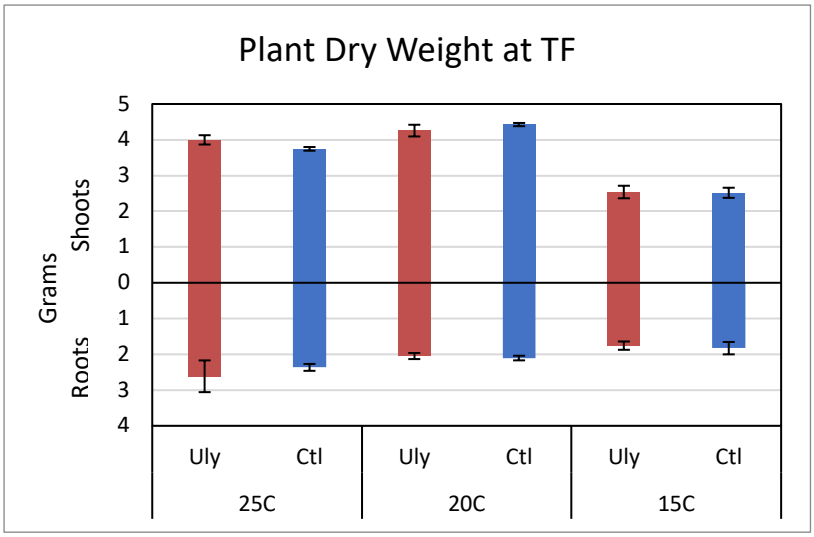

Figure 12. Average shoot and root dry weight for treatment and control groups at the three temperatures tested. Error bars indicating standard deviation are shown, n=4.

A closer look at plant shoot and root dry weight at the final timepoint suggests that plant growth was promoted at 25 °C. The average shoot dry weight was 6.71 % and root dry

weight 10.52 % greater for the treatment group (Uly) compared to the control (Ctl). Inoculation of the consortium led to a total dry weight increase of 8.18 % at 25 °C. The percent differences in dry weight between treatments were insignificant, however. At 20°C, shoot, root, and combined dry weight were 3.97, 2.73, and 3.57 % smaller in the treatment group than the control, respectively, although these differences were also insignificant. At 15 °C, shoot dry weight was 0.86 % greater in the treatment group while root dry weight was 4.02 % less. The overall dry weight was 1.14 % less, but this was insignificant

Table 5. Percent difference of shoot height, shoot, root, and total dry weight for all temperatures. DW stands for dry weight

Percent Difference				
	Shoot Height	Shoot DW	Root DW	Total DW
25°C	0.01%	6.71%	10.52%	8.18%
20°C	1.36%	-3.97%	-2.73%	-3.57%
15°C	-0.99%	0.86%	-4.02%	-1.14%

Table 6. P-Value of shoot height, shoot, root, and total dry weight, and emergence for all temperatures. DW stands for dry weight.

P-value					
	Shoot Height	Shoot DW	Root DW	Total DW	Emergence
25°C	0.995	0.322	0.603	0.429	0.832
20°C	0.354	0.513	0.620	0.331	0.874
15°C	0.552	0.886	0.640	0.871	0.545

4.3.4 Plant tissue nutrient analysis

Nutrient concentration data for both roots and shoots were obtained for nitrogen, sulfur, phosphorus, potassium, magnesium, calcium, sodium, boron, zinc, manganese, iron, copper, and aluminum. However, since the inoculant's mode of action for plant growth promotion is thought to be phosphorus and calcium solubilization and iron chelation, particular focus was placed on those elemental analyses. Other differences that are not statistically

significantly different are not discussed although the data are presented. Error bars indicating standard deviations are shown, n=4.

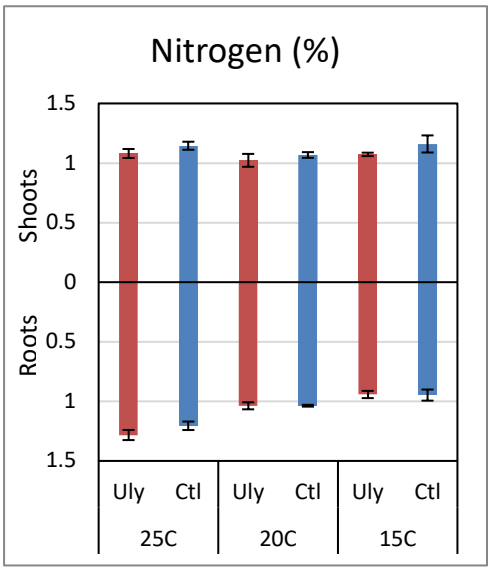

Figure 13. Shoot and root percentage of nitrogen for treatment and control at all temperatures. Standard deviation is shown.

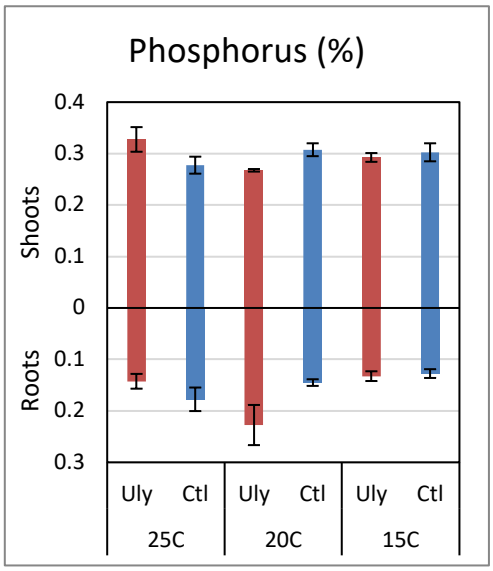

Figure 14. Shoot and root percentage of phosphorus for treatment and control at all temperatures. Standard deviation is shown.

At 25 °C, the average phosphorus content for plant shoots was higher in the treatment group (Uly) at 0.33 % versus 0.28 % in the control (Ctl). For roots however, the treatment group had less phosphorus at 0.14 % than 0.18 % for the control. This trend led to the treatment group's total plant average phosphorus content to be 0.01 % greater than the control. Differences in shoot, root, and total phosphorus content at 25 °C were insignificant. At 20 °C, phosphorus content was significantly less ($p = 0.02$) in the shoots of the treatment than those of the control. The phosphorus content in the treatment roots was greater at 0.23 % than that of the control at 0.15 %, although this difference was insignificant. Total plant phosphorus content was 0.5 % for the treatment group and 0.45 % for the control group, although again this was insignificant. Phosphorus content at 15 °C followed the same trend as that of 20 °C although the differences were even smaller for shoots and roots, and there was no difference for total phosphorus. No values for phosphorus content at 15 °C were significantly different.

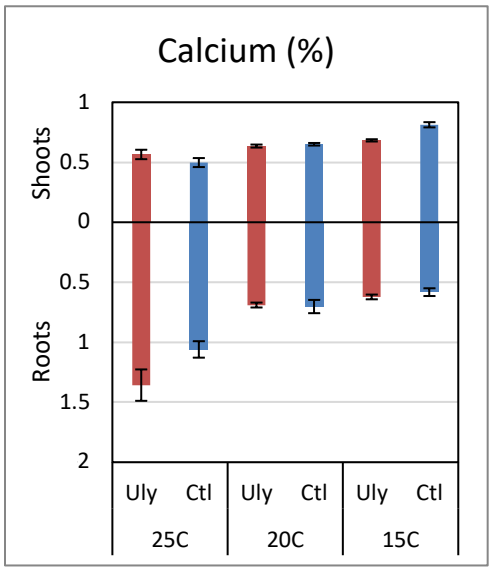

Figure 15. Shoot and root percentage of calcium for treatment and control at all temperatures. Standard deviation is shown.

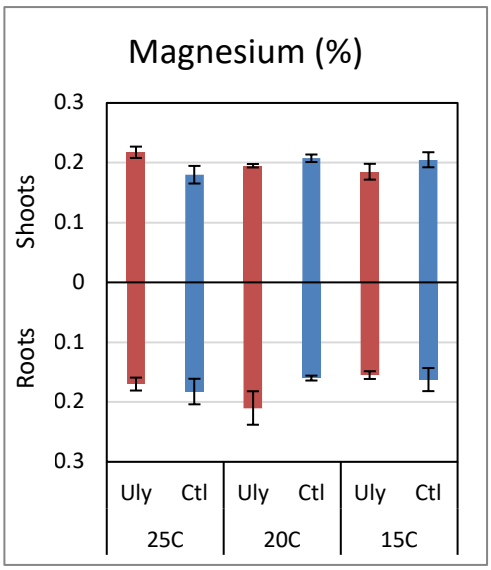

Figure 16. Shoot and root percentage of Magnesium for treatment and control at all temperatures. Standard deviation is shown.

At 25 °C, the average calcium content for the whole plant was significantly greater ($p \leq 0.05$) in the treatment than the control, but specific differences between shoots and roots of the treatment and control were insignificant. At 20 °C, differences in calcium content between the treatment and control were not significant for shoots, roots, or both. At 15 °C, total average calcium content was not significantly different, but it was significantly lower ($p = 0.002$) in the shoots of the treatment plants than those of the control. There was no significant difference in root calcium content at 15 °C.

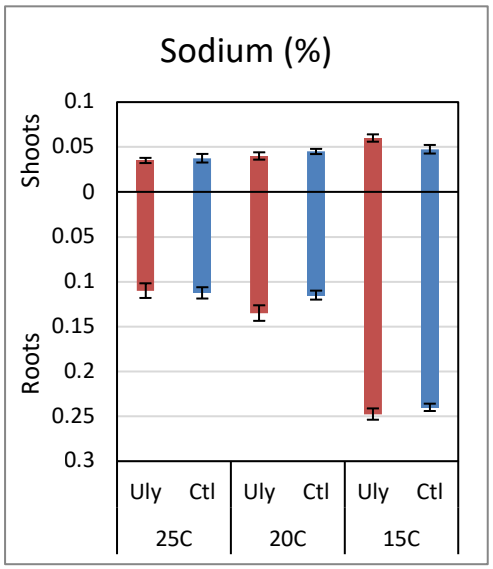

Figure 17. Shoot and root percentage of sodium for treatment and control at all temperatures. Standard deviation is shown.

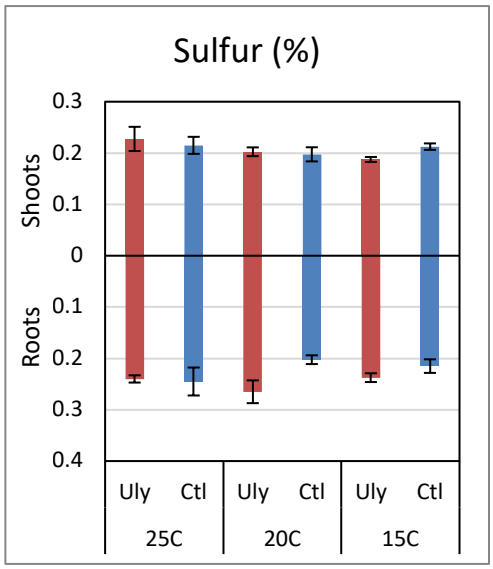

Figure 18. Shoot and root percentage of Sulfur for treatment and control at all temperatures. Standard deviation is shown.

While there was no significant difference in sulfur content at 25 °C, it was significantly ($p = 0.039$) higher in treatment roots at 20 °C, and significantly ($p = 0.02$) lower in treatment shoots at 15 °C.

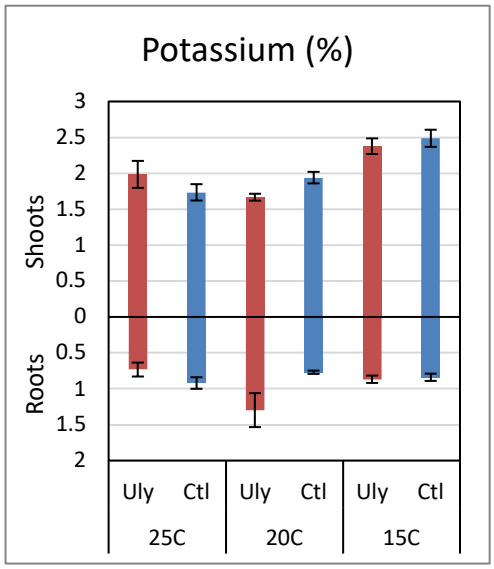

Figure 19. Shoot and root percentage of potassium for treatment and control at all temperatures. Standard deviation is shown.

Total plant potassium content was higher in the treatment groups at 25 and 20 °C but lower at 15 °C, although this was insignificant. The control group had a significantly ($p = 0.027$) higher potassium content in the shoots at 20 °C.

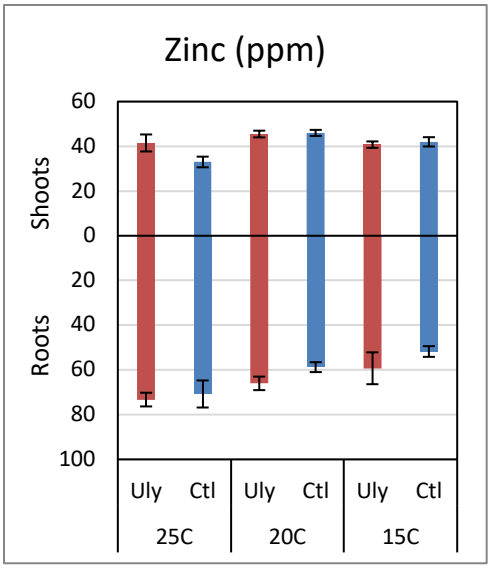

Figure 20. Shoot and root concentration of zinc in ppm for treatment and control at all temperatures. Standard deviation is shown.

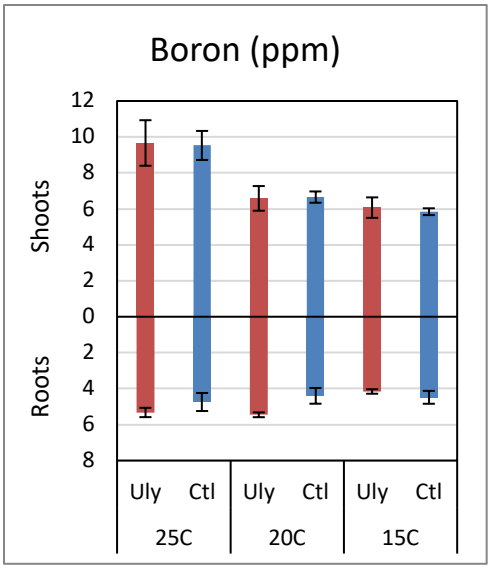

Figure 21. Shoot and root concentration of boron in ppm for treatment and control at all temperatures. Standard deviation is shown.

Zinc, manganese, and aluminum were all at higher levels (total plant contents) in the treatment group at 25 °C than the control, but the total plant content was lower in the treatment group than the control at 20 °C and was variable at 15 °C.

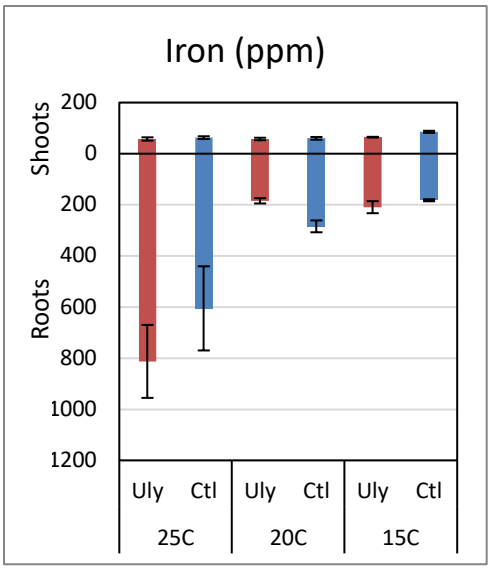

Figure 22. Shoot and root concentration of iron in ppm for treatment and control at all temperatures. Standard deviation is shown.

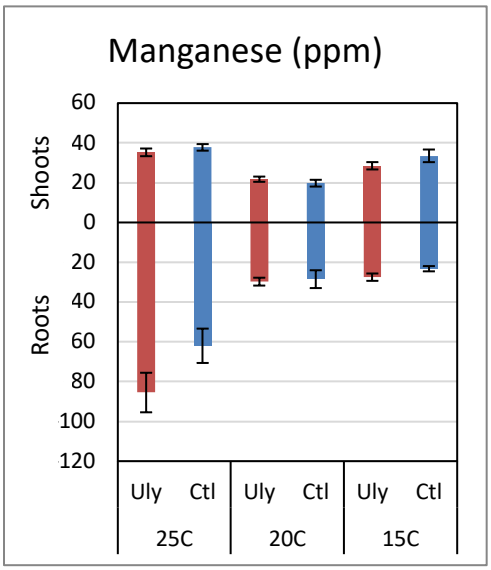

Figure 23. Shoot and root concentration of manganese in ppm for treatment and control at all temperatures. Standard deviation is shown.

Iron levels in roots at 25 °C were higher in the treatment group at 812.75 ppm compared to the control at 605.25 ppm, although this difference was not statistically significant. Significant differences were observed for average iron content at 20 °C for the total plant ($p = 0.014$) and for the roots ($p = 0.008$), with the control having a higher concentration. At 15 °C the average iron content was significantly ($p = 0.003$) higher in the shoots of the control while there was no significant difference for total plant content or content of roots alone.

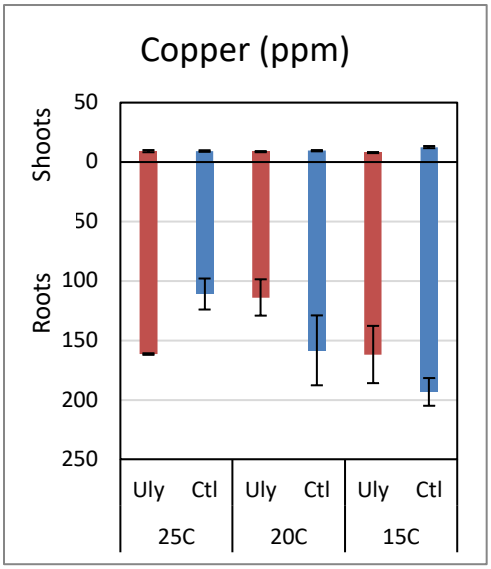

Figure 24. Shoot and root concentration of copper in ppm for treatment and control at all temperatures. Standard deviation is shown.

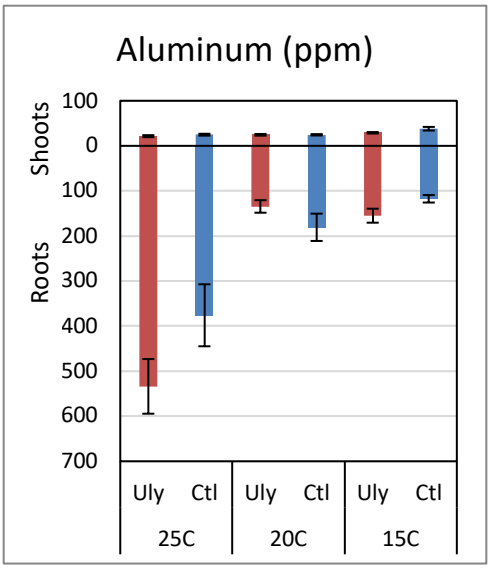

Figure 25. Shoot and root concentration of aluminum in ppm for treatment and control at all temperatures. Standard deviation is shown.

The total amount of copper was significantly ($p = 0.011$) higher for the treatment group at 25°C than the control. This was largely due to the content in the roots, which was significantly ($p = 0.008$) different. This trend did not hold for the other temperatures however, with the control roots having higher amounts at 20 and 15 °C. Furthermore, the shoots in the treatment group had a significantly ($p = 0.004$) lower copper content than the control at 15 °C.

Table 7. Total plant average amount of nutrients for all temperatures.

Total Plant Average								
		Nitrogen %	Sulfur %	Phosphorus %	Potassium %	Magnesium %	Calcium %	Sodium %
25 °C	Uly	2.36	0.47	0.47	2.72	0.39	1.92	0.15
	Ctl	2.35	0.46	0.46	2.66	0.36	1.56	0.15
20 °C	Uly	2.06	0.47	0.50	2.97	0.41	1.33	0.18
	Ctl	2.10	0.40	0.45	2.71	0.37	1.35	0.16
15 °C	Uly	2.02	0.43	0.43	3.25	0.34	1.31	0.31
	Ctl	2.11	0.43	0.43	3.33	0.37	1.40	0.29

		Boron (ppm)	Zinc (ppm)	Manganese (ppm)	Iron (ppm)	Copper (ppm)	Aluminum (ppm)
25 °C	Uly	14.99	114.75	120.75	870.25	170.67	555.25
	Ctl	14.27	103.75	99.75	668.00	120.27	400.75
20 °C	Uly	12.04	111.50	51.50	242.00	122.66	159.00
	Ctl	11.06	104.75	48.25	344.25	167.94	205.25
15 °C	Uly	10.23	100.00	56.00	273.75	169.85	184.00
	Ctl	10.33	93.75	56.75	267.75	205.94	155.50

Table 8. P-Values for all nutrients total, shoots, and roots at all temperatures.

P-value								
		Nitrogen	Sulfur	Phosphorus	Potassium	Magnesium	Calcium	Sodium
25 °C	Total	0.880	0.888	0.728	0.848	0.410	**0.050**	0.730
	Shoots	0.246	0.680	0.136	0.298	0.076	0.260	0.670
	Roots	0.212	0.865	0.243	0.187	0.620	0.091	0.816
20 °C	Total	0.495	0.083	0.351	0.401	0.228	0.691	0.059
	Shoots	0.475	0.768	**0.020**	**0.027**	0.121	0.401	0.356
	Roots	0.875	**0.039**	0.082	0.068	0.128	0.839	0.092
15 °C	Total	0.385	0.914	0.852	0.721	0.400	0.112	0.108
	Shoots	0.277	**0.020**	0.626	0.521	0.315	**0.002**	0.094
	Roots	0.932	0.203	0.708	0.716	0.725	0.331	0.356

		Boron	Zinc	Manganese	Iron	Copper	Aluminum
25 °C	Total	0.698	0.237	0.182	0.387	**0.011**	0.140
	Shoots	0.930	0.107	0.363	0.557	0.938	0.324
	Roots	0.342	0.725	0.124	0.378	**0.008**	0.136
20 °C	Total	0.310	0.213	0.645	**0.014**	0.216	0.222
	Shoots	0.927	0.813	0.388	0.744	0.307	1.000
	Roots	0.060	0.101	0.807	**0.008**	0.228	0.215
15 °C	Total	0.908	0.463	0.895	0.811	0.229	0.217
	Shoots	0.717	0.639	0.222	**0.003**	**0.004**	0.082
	Roots	0.424	0.357	0.110	0.301	0.284	0.077

4.4 Growth chamber experiments with lettuce

4.4.1 Lettuce emergence

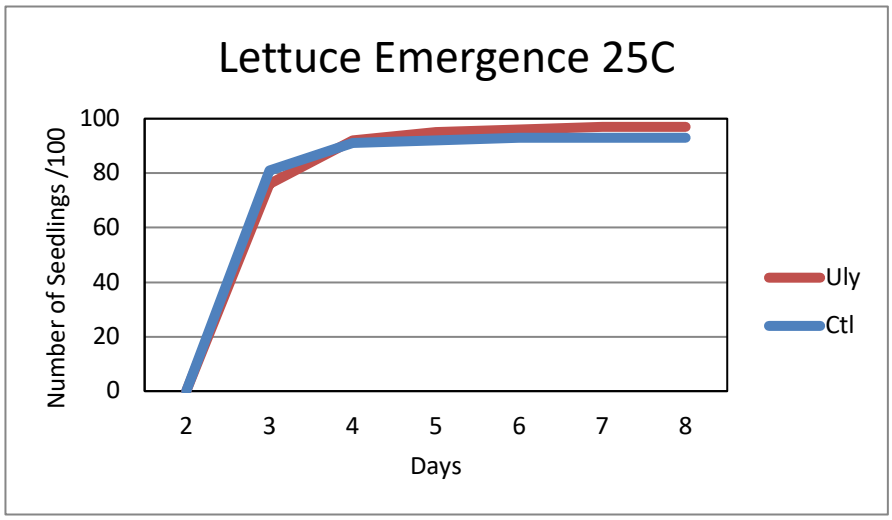

Table 9. P-Values for lettuce emergence at 25 °C.

Emergence at 25 °C	
Day	P-Value
3	0.447
4	0.807

Figure 26. Number of days till lettuce emergence for treatment and control groups at 25 °C.

At 25 °C there was little difference in seedling emergence rate between the treatment (Uly) and control (Ctl) groups, although 93/100 seeds emerged in the treatment group compared to 97/100 in the control. This is likely due to the expected failure of germination of a small percentage of the seeds, as described by the seed pack's 95 % success rate.

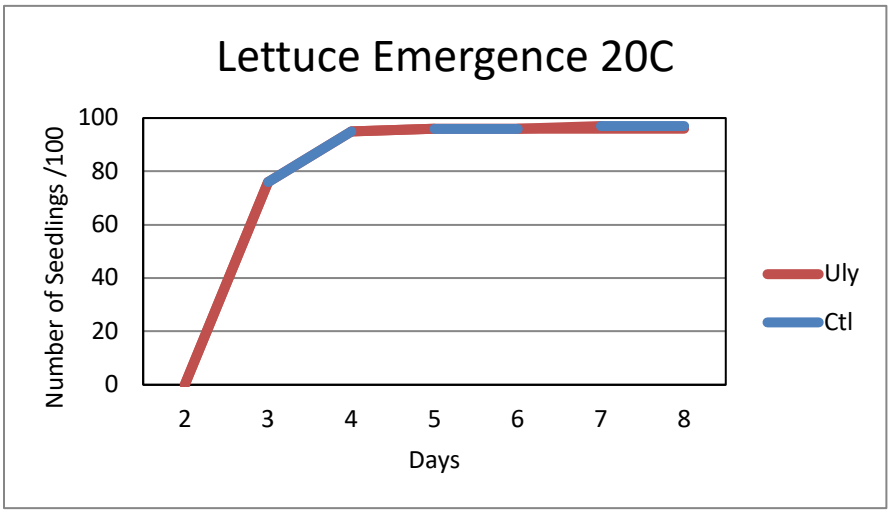

Table 10. P-Values for lettuce emergence at 20 °C.

Emergence at 20 °C	
Day	P-Value
3	1
4	1

Figure 27. Number of days till lettuce emergence for treatment and control groups at 20 °C.

At 20 °C, there was no difference in the emergence rates between the control and treatment.

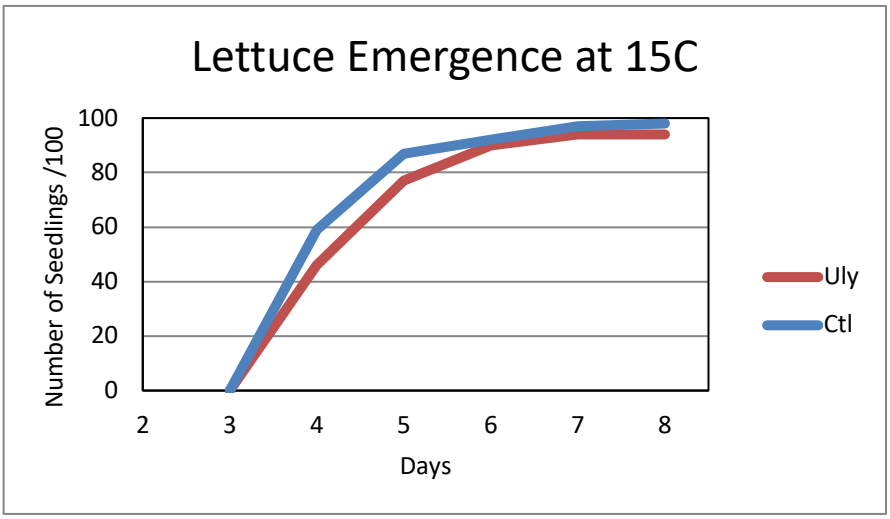

Table 11. P-Values for lettuce emergence at 15 °C.

Emergence at 15 °C	
Day	P-Value
4	0.061
5	0.040
6	0.627
7	0.329

Figure 28. Number of days till lettuce emergence for treatment and control groups at 15 °C.

At 15 °C, inoculation of the treatment group appeared to slightly slow the emergence rate compared to the control. On the first day of emergence, the control group had 13 % more seedlings and 10 % more on the second day, although these differences were only significant on the second day of emergence.

4.4.2 Lettuce dry weight and leaf area

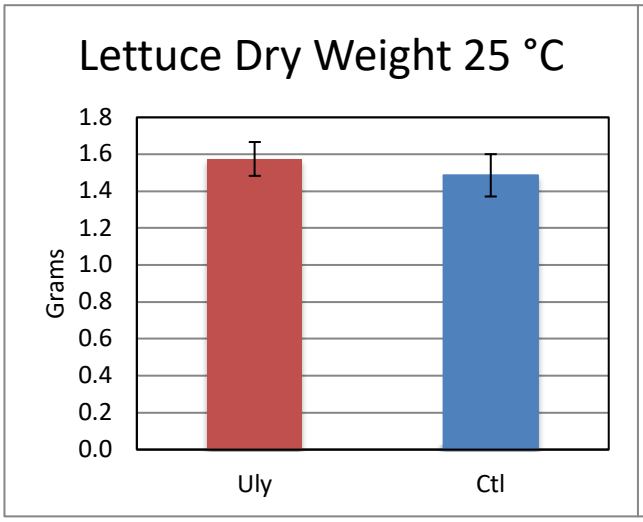

Figure 29. Lettuce dry weight at 25 °C. Standard deviation is shown.

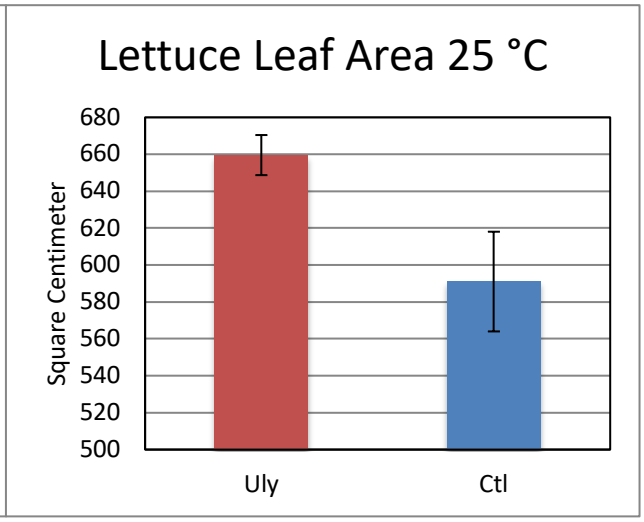

Figure 30. Lettuce leaf area at 25 °C. Standard deviation is shown.

Table 12. Percent difference and P-Values for lettuce dry weight and leaf area at 25 °C.

	Percent difference		P-values	
	Dry Weight	Leaf Area	Dry Weight	Leaf Area
25 °C	5.98	11.60	0.352	0.002

At 25 °C, lettuce dry weight was nearly 6 % greater in the treatment (Uly) group compared to the control (Ctl), although this was insignificant. The leaf area, however, was 11.6 % greater in the treatment than the control, which was a significant ($p = 0.002$) difference.

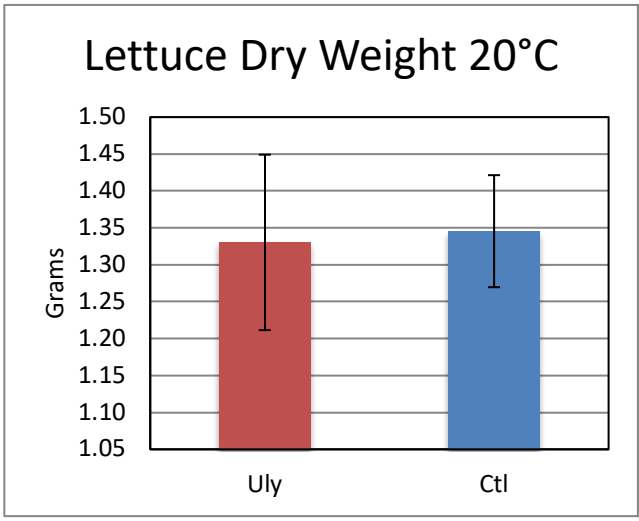

Figure 31. Lettuce dry weight at 20 °C. Standard deviation is shown.

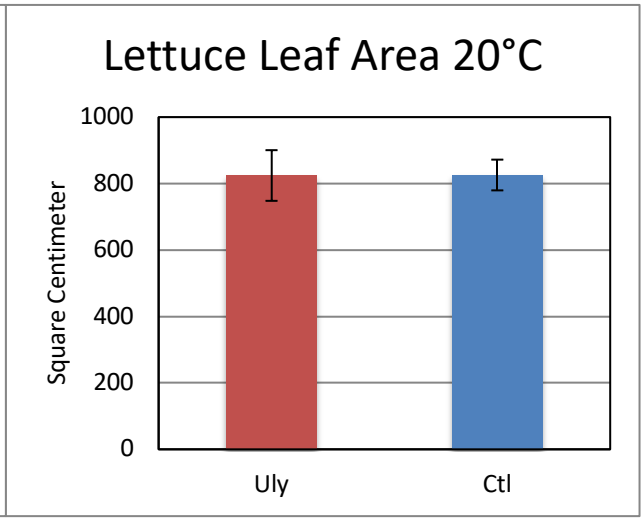

Figure 32. Lettuce leaf area at 20 °C. Standard deviation is shown.

Table 13. Percent difference and P-Values for lettuce dry weight and leaf area at 20 °C.

	Percent difference		P-values	
	Dry Weight	Leaf Area	Dry Weight	Leaf Area
20 °C	-1.14	-0.18	0.867	0.979

At 20 °C, both plant dry weight and leaf area were slightly lower for the treatment group compared to the control, although insignificantly so.

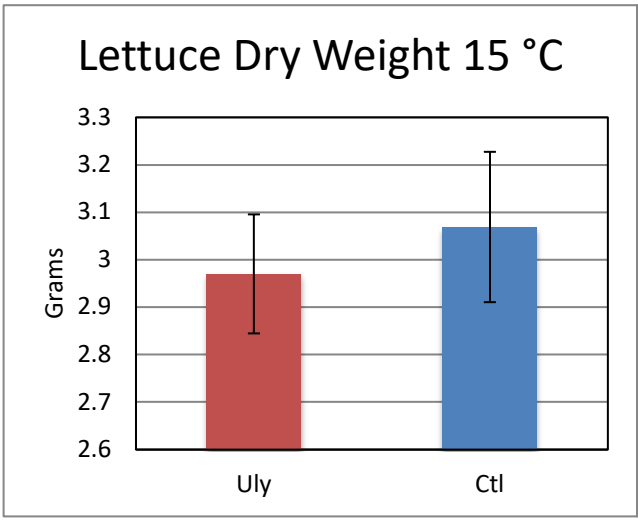

Figure 33. Lettuce dry weight at 15 °C. Standard deviation is shown.

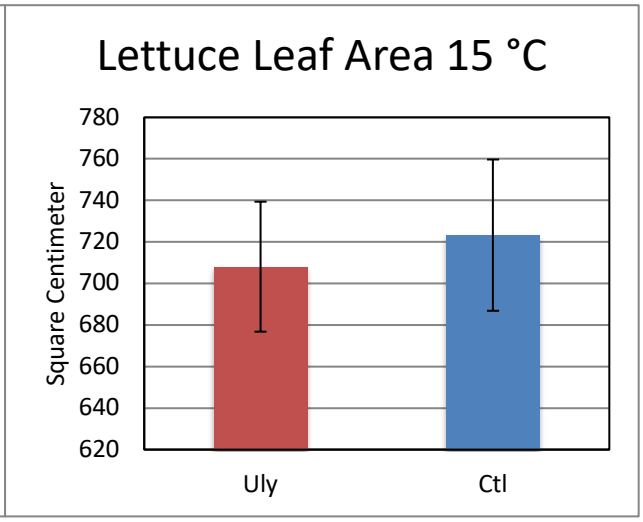

Figure 34. Lettuce leaf area at 15 °C. Standard deviation is shown.

Table 14. Percent difference and P-Values for lettuce dry weight and leaf area at 15 °C.

	Percent difference		P-values	
	Dry Weight	Leaf Area	Dry Weight	Leaf Area
15 °C	-3.33	-2.14	0.281	0.484

At 15 °C, both dry weight and leaf area were again insignificantly smaller in the treatment than the control group.

4.5 Microbial analysis

4.5.1 Beta diversity

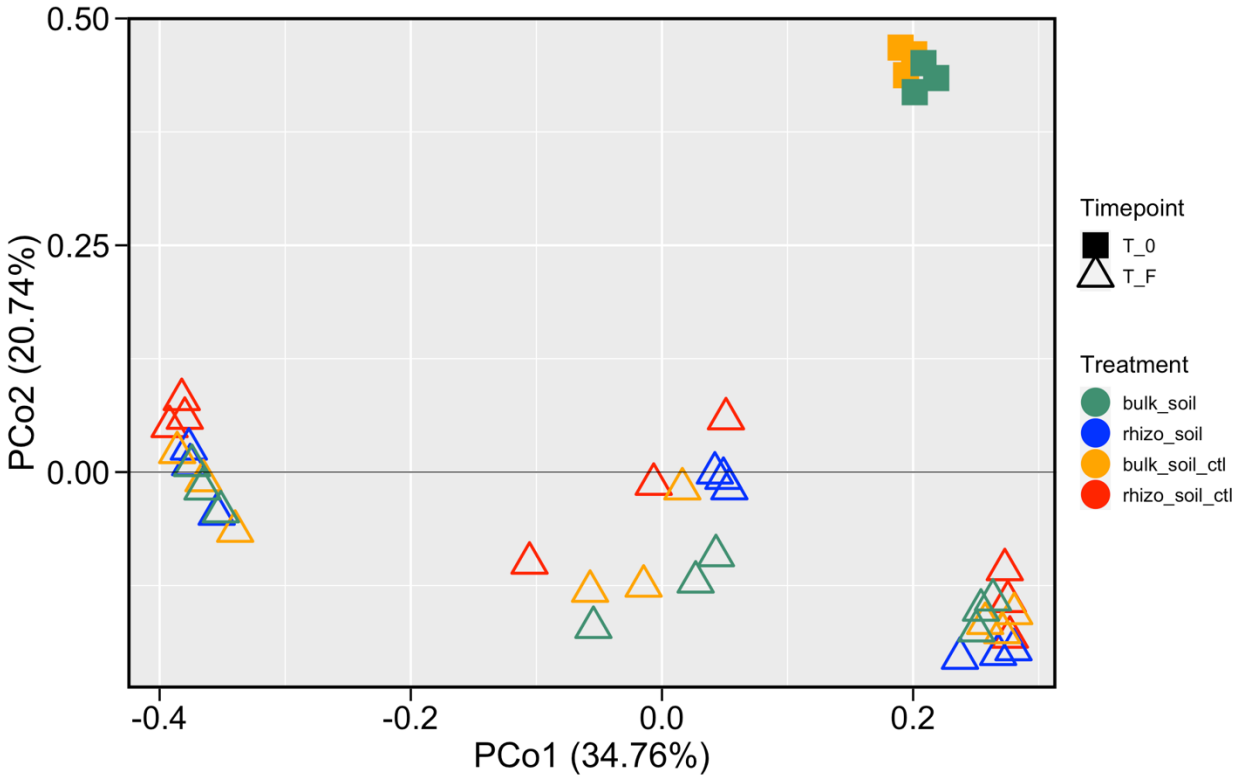

Figure 35. Principal coordinate analysis differentiating between timepoints of all data.

Table 15. Results of PERMANOVA for timepoints and between T0 samples.

Variable	R^2	P-value
Timepoint	0.212311	$1* 10^{-4}$
Treatment T_0	0.219268	0.3

An initial look at the entire microbiome data set shows significant clustering due to sampling time (day 0 (T0) vs day Final (TF)). Bulk soil, inoculated with the consortium, and bulk soil control at T0 were insignificantly different from each other, however each of the samples had significantly changed in community structure by the end of the experiment.

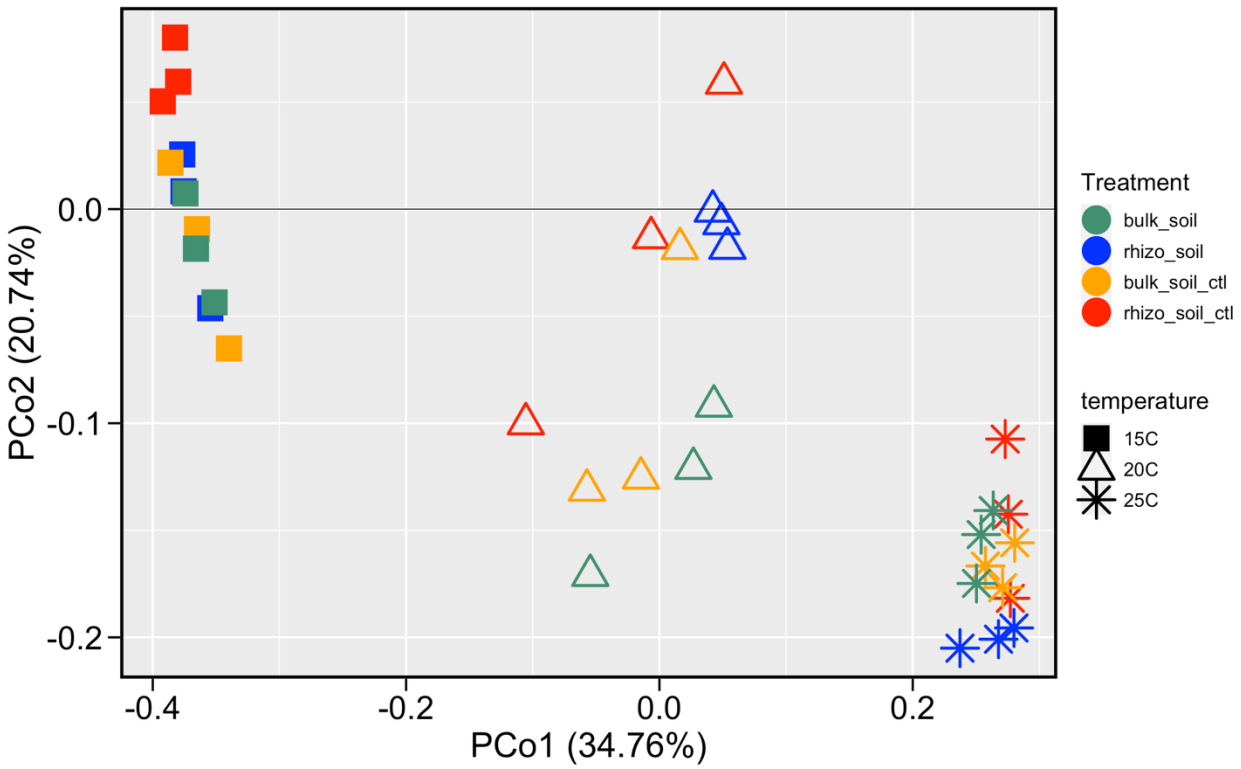

Figure 36. Principal coordinate analysis differentiating between temperatures of final timepoint samples.

Table 16. Results of PERMANOVA for temperature.

Variable	R^2	P-value
Temperature	0.62154	$1* 10^{-4}$

When considering samples from the final timepoint only, the effect of temperature significantly shaped a combination of treatment bulk and rhizosphere soils as well as control bulk and control rhizosphere soils.

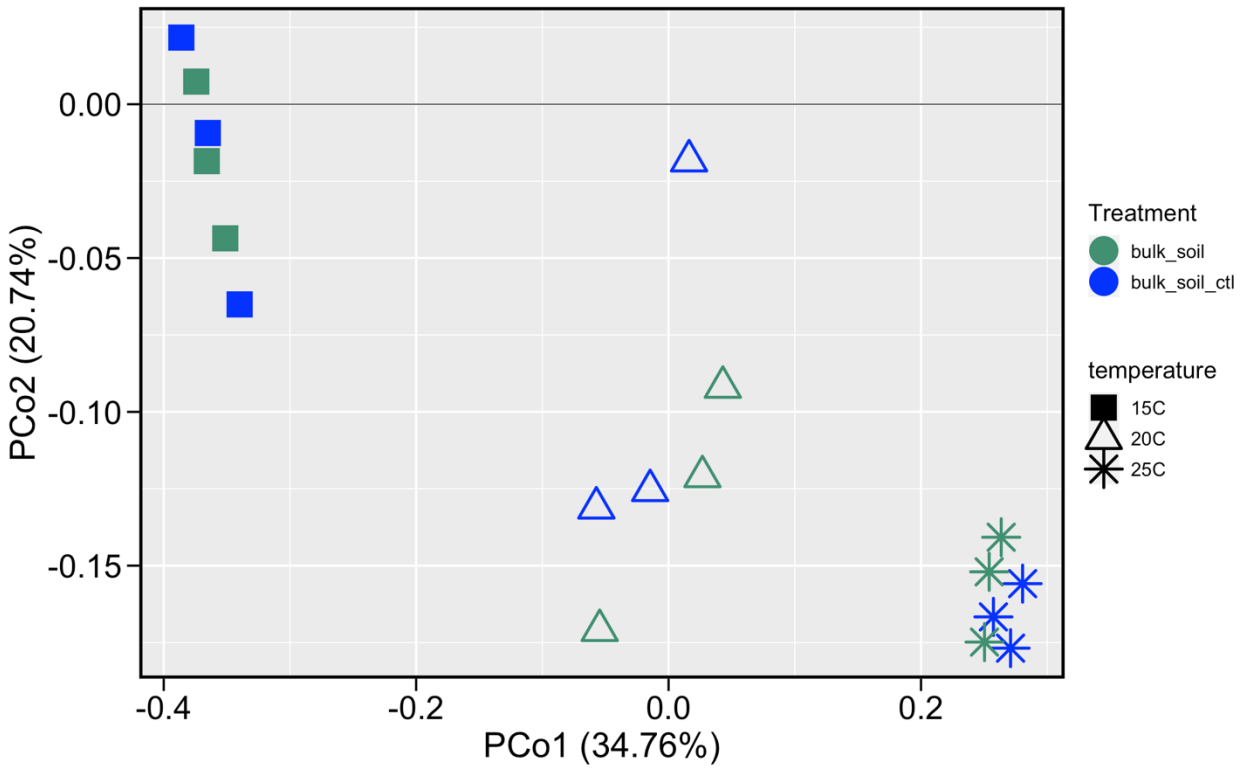

Figure 37. Principal coordinate analysis of treatment and control bulk soil samples at all temperatures.

Table 17. Results of PERMANOVA for bulk soil samples.

Variable	R^2	P-value
Treatment Bulk	0.03443	0.7428

The effect of the inoculant did not have a significant impact on shaping the bulk soil microbial community structure. While clustering due to the effect of temperature is apparent, the treatment did not cause any discernible clustering within the three temperatures.

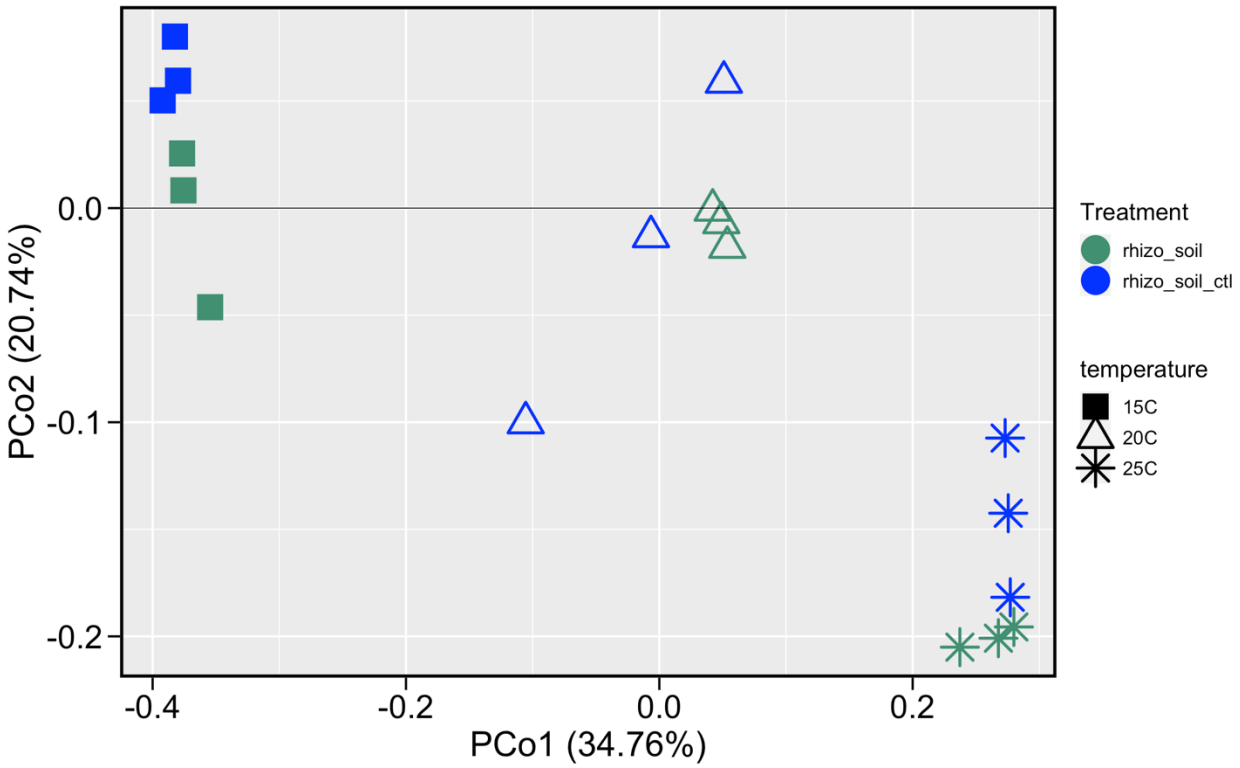

Figure 38. Principal coordinate analysis of treatment and control rhizosphere soil samples at all temperatures.

Table 18. Results of PERMANOVA for rhizosphere soil samples across and within temperatures.

Variable	R^2	P-value
Treatment Rhizo	0.03176	0.754
15°C Rhizo	0.31486	0.1
20°C Rhizo	0.28337	0.1
25°C Rhizo	0.41528	0.1

The effect of the consortium also did not have a significant impact on community structure in rhizosphere soil, although more distinct clustering is notable between the treatment and control at each temperature compared to the bulk soils. When focusing on each specific temperature, the effect of the consortium was still found to be statistically insignificant.

4.5.2 Microbial community composition

Changes in the relative abundance of microbial taxa due to the experiment variables (time, temperature, and treatment) were tested and can be seen in figures 39-42. The complete results including the p-values and means (n=3) for each group are presented in tables in the supplementary material. Only taxa that were significantly different ($p \leq 0.05$) were included in the tables.

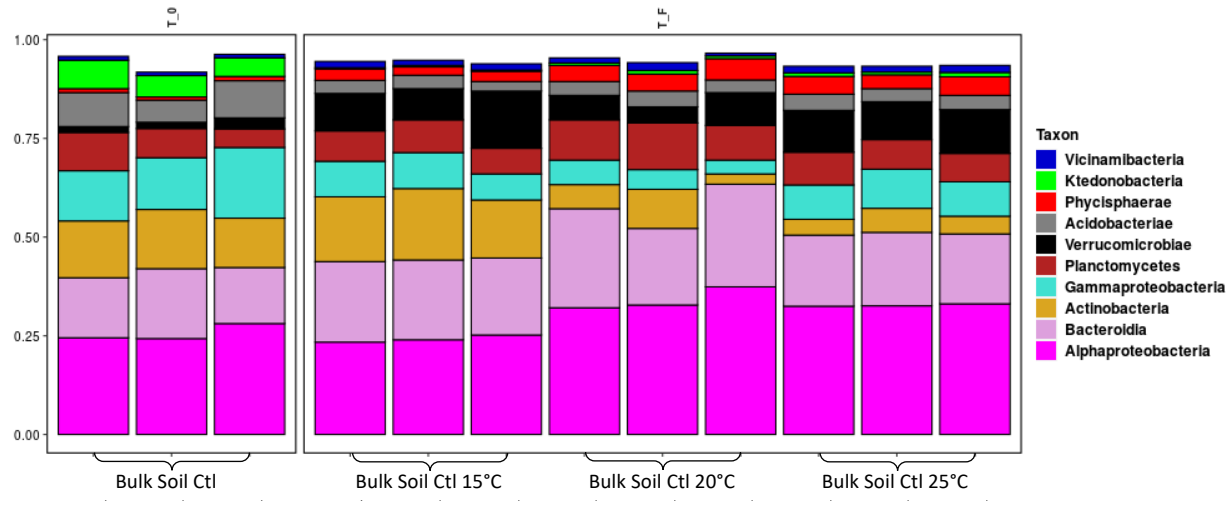

Figure 39. Relative abundance of control soil bacterial communities at the class level of initial and final timepoints for all temperatures. The 10 most abundant microbial classes are shown. The initial community composition of uninoculated bulk soil at T0 is compared to the final timepoint uninoculated bulk soil at the three experimental temperatures.

The effect of temperature and time on community composition at the class level can be seen in Figure 39. The class *Ktedonobacteria* was significantly reduced in abundance compared to the control (on day 0) at all three temperatures by the end of the experiment. *Acidobacteriae* was significantly reduced at 25 °C, while *Verrucomicrobiae* significantly increased in abundance at both 15 and 25 °C and insignificantly at 20°C. *Phycisphaerae* significantly increased in abundance at all temperatures. *Actinobacteria* significantly decreased in abundance at 25 °C. *Bacteroidia* significantly increased at both 20 and 25 °C while *Gammaproteobacteria* was significantly reduced compared to the control.

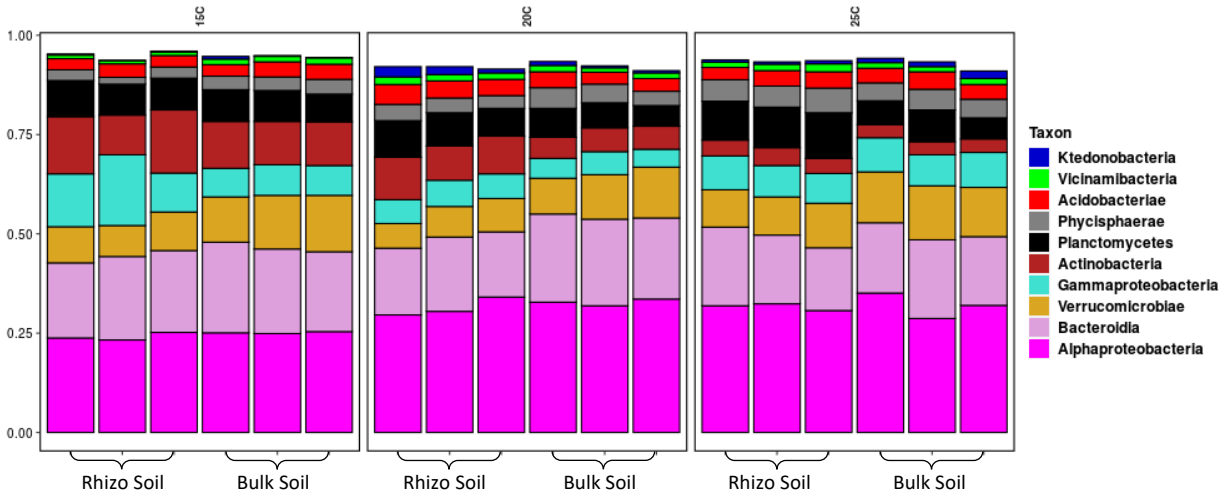

Figure 40. Relative abundance of soil bacterial communities at the class level for treatment bulk and rhizosphere soil samples at all temperatures. The 10 most abundant microbial classes are shown.

For the bulk and rhizosphere inoculated treatments, slight rhizosphere effects were detectable (Figure 40). For all temperatures there was less abundance of *Verrucomicrobiae* in the rhizosphere than in the bulk soil but this was only significant at 15 and 25 °C. Conversely, *Actinobacteria* was found at higher abundances in the rhizosphere at 20 °C. *Planctomycetes* was also found in higher abundances in the rhizosphere than the bulk soil across all temperatures, but only significantly at 25 °C.

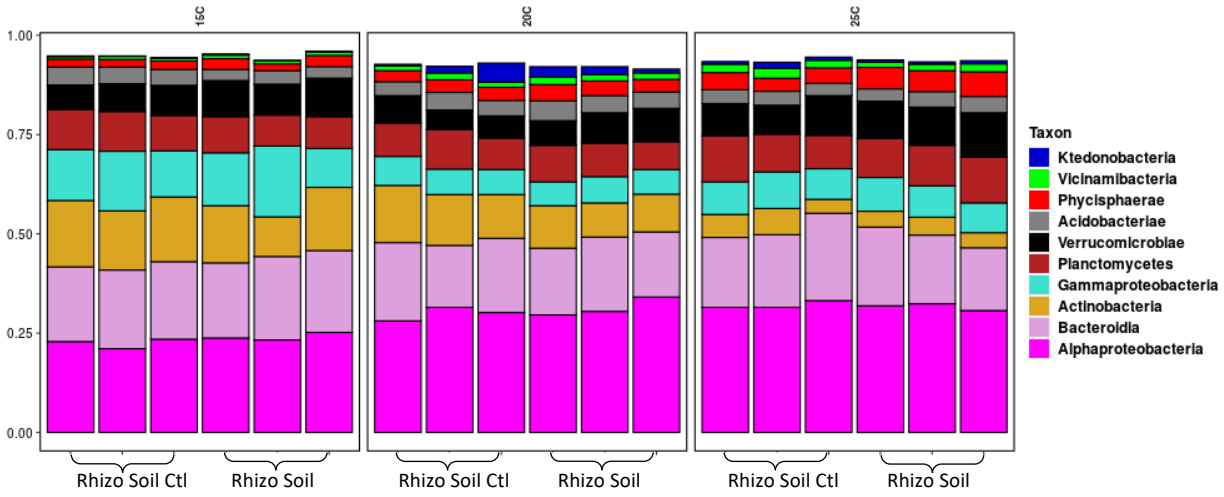

Figure 41. Relative abundance of rhizosphere bacterial communities at the class level for treatment and control groups at all temperatures. The 10 most abundant microbial classes are shown.

At the class level in the rhizosphere soil, very slight differences in microbial community composition between the treatment and control were detected at all temperatures tested (Figure 41). Variation in relative abundance of the top ten most abundant taxa was largely insignificant. *Acidobacteriae* was significantly less abundant in the treatment rhizosphere at 15 °C while *Phycisphaerae* was significantly more abundant at 25 °C in the treatment rhizosphere.

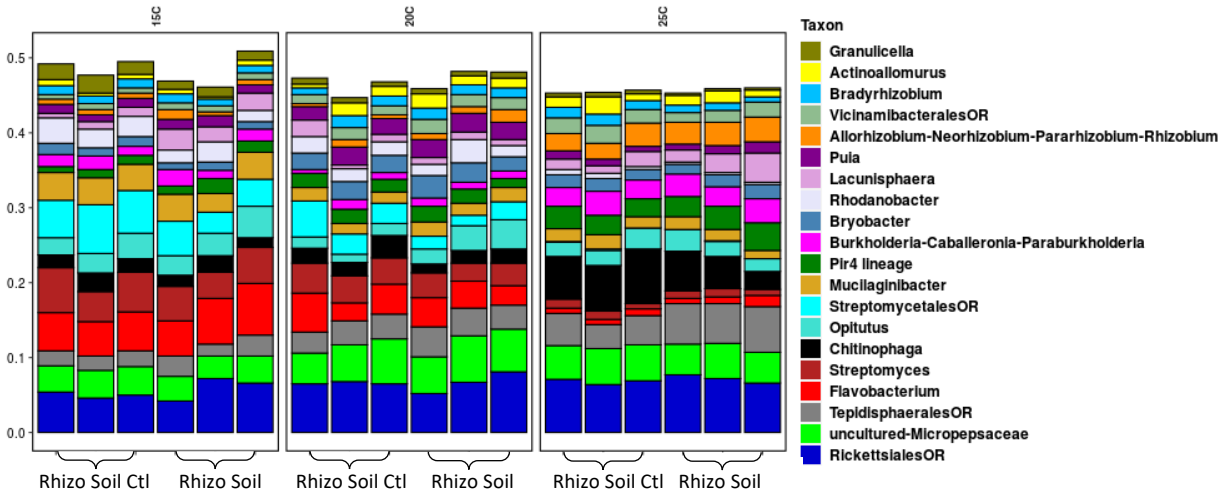

Figure 42. Relative abundance of rhizosphere bacterial communities at the genus level for treatment and control groups at all temperatures. The 20 most abundant microbial genera are shown.

Effects of temperature were much more noticeable on community composition at the genus level in both the treatment and control groups (Figure 42). *Tepidisphaerales* were present in greater relative abundance at 20 and 25 °C , however the difference in relative abundance between the treatment and control groups was only statistically significant at 25 °C. Both *Streptomyces* and *Flavobacterium* decreased in abundance at 25 °C while *Chitinophaga* increased, but there was no significant difference due to inoculation. *Mucilaginibacter* decreased in relative abundance at both 20 and 25 °C. The *Burkholderia* and *Rhizobia* clades were present in greater abundances at 25 °C while *Granulicella* was less abundant. *Granulicella* was significantly less abundant in the treatment group compared to the control at both 15 and 25 °C. Both *Vicinamibacterales* and *Rhodanobacter* was less abundant at 20 and 25 °C. *Pula* was most abundant at 20 °C. At 20 °C, *Streptomyces* was less abundant and *Opitutus* more so in the treatment group than the control, although insignificantly so. At 15 °C, *Streptomycetes* was significantly less abundant in the treatment group while *Lacunisphaera* was more abundant in the treatment group, although this was insignificant.

Enrichment of certain microbial taxa in the rhizosphere at temperatures where plant growth promotion was measured suggest the involvement of those taxa, however the data is not sufficient to demonstrate a direct causal effect. For this, further research using metagenomics or metabolomics, or a combination of both, would be needed.

Chapter 5: Discussion

5.1 Germination and radical length

Microbial inoculants are often tested for their ability to alleviate stress or promote crop growth at early plant growth stages since it is a rapid and reliable way to screen candidate strains. Germination rate and radical length are common techniques used to quantify growth promotion at these early growth stages since they are strong predictors for later developmental traits such as yield. It can be challenging, however, to distinguish if the germination of a plant is responsible for promoting microbial growth through root exudates or if the microbes are spurring the seed to germinate, although evidence suggests that the microbes may influence germination (Eldridge et al., 2021). The cotyledons supply much of the energy and nutrients needed for initial growth, but microbes may supplement the seedling with external nutrients and phytohormones, and may also regulate seed and seedling physiology, thus stimulating growth under stressful conditions.

Microbial inoculants have been tested this way on corn, and specifically phosphorus solubilizing bacteria have been tested for their ability to improve plant germination this way. Some of the bacterial species present in the Ulysse Biotech consortium Era Boost Pro, such as *Bacillus megatarium*, have been tested this way. More than a 20% increase in germination was observed when *B. megatarium* was inoculated on corn seeds in petri plate conditions (Bákonyi et al., 2013). Furthermore, bacterial taxa intended to alleviate low temperature stress have also been tested this way on other monocots such as wheat (Abd El-Daim et al., 2019). Another study found that co-inoculation of a *Penicillium* fungus and the micronutrients zinc and manganese was able to promote the accumulation of corn biomass under low temperature stress (Gómez-Muñoz et al., 2018). Together, all of this suggests that the experimental approach was appropriate to initially test the efficacy of a commercial inoculant for its ability to promote corn growth under low temperature stress.

While there was a noticeable effect on germination of the volume (5 and 10 mL) at 25°C, it was less noticeable at 20 °C and not apparent at 15 °C (Figure 1-3). These two volumes were tested because the petri plates were sealed with tape that allowed for gas exchange. Upon initial tests, volumes less than 5 mL dried out too quicky at 25 °C, whereas with 5 mL there was

still some moisture remaining when the seeds had finished germinating. At volumes greater than 10 mL, there was an excess amount of water. With 10 mL, there was sufficient evaporation that by days 3 and 4, the filter paper at 15°C remained appropriately moist.

There was a clear effect of the lower temperatures on delaying germination, which is consistent with other findings (Meng et al., 2022). The consortium, on the other hand, largely did not influence germination rate at the temperatures tested. Where there was any impact was in actually slowing the germination rate compared to the control (i.e. at 25 and 15 °C).

These major trends bear out in the radical length measurements, with the volume (5 vs 10 mL) being the strongest influence (Figure 4-6). At both 25 and 20 °C, the groups given 5 mL had longer radical lengths than those given 10 mL. This is consistent with the higher volume slightly delaying germination at the same temperatures. The effect of volume was more variable for germination at 15 °C, and its effect on radical length was not apparent.

As expected, temperature greatly influenced radical length with average lengths being longest at the optimal temperature of 25 °C, and increasingly shorter at 20 and 15 °C. Radical length was measured after all seeds in one treatment had germinated. At 25 °C, the radical length was measured after 2 days while at 20 °C it was measured after 3 days and at 15 °C after 4 days. This indicated that even after a shorter duration, the radicals at 25 °C were able to grow the longest. The inoculant had no significant effect on radical length at all three temperatures. At 25 °C it slightly reduced radical length at both volumes tested, while at 20 and 15 °C the effects were variable, with the average length of the control less at 5 mL and more at 10 mL than the inoculated treatment.

Interestingly, across all temperatures and volumes tested, except for 5 mL at 15 °C, the treatment group had a narrower distribution of total radical lengths than the control. Even though the effect of the consortium seemed to reduce radical length at most temperatures and with both volumes the narrow range of radical lengths could potentially have been caused by the inoculant. If the consortium led to similar growth results, albeit limiting initial growth, it could be useful for field uniformity at crop establishment. This would require much larger trials across a range of abiotic and biotic stressors, and ultimately field trials, to test this hypothesis.

It is not uncommon to see little difference in seed germination when testing microbial effects, as has been reported by several studies on corn (Schmidt et al., 2018; De Zutter et al., 2022). In this experiment, it is possible that the lack of observable effects might be due to the concentrations of the inoculated consortium being too low. To address this challenge, several studies soak sterilized seeds in undiluted consortium after the bacteria have been grown to a desired optical density. However, for this experiment, since several colony forming units developed when seeds were germinated on plate count agar at the optimal temperature (25 °C) following the same inoculation methods and consortium concentration, it was assumed that the bacteria were present in high enough concentrations to proliferate during the germination experiment. Furthermore, while those studies testing high concentrations of inoculant may show an effect, they are less useful for functional applications as inoculant companies mainly require that their product be greatly diluted to have economic viability. Dilution allows for the inoculant to be applied to soil, or crop foliage, as a spray compatible with existing farm machinery.

Another potential reason that this consortium might not have promoted significant seed germination and radical growth might be that they could not sufficiently establish either before or after germination. For this germination experiment, the seeds were not surface sterilized since they were kept on filter paper without added nutrients. This was done as fungal contamination was not expected to be an issue, and not observed in preliminary trials, since no extra nutrients were present on the filter paper. This also kept the seed microbiome intact prior to the addition of the consortium to somewhat replicate field conditions. Even though the two volumes tested, 5 and 10 mL, should have been enough for the consortium to establish, it is still possible that they were outcompeted by the initial microbial community. In a field setting, if this consortium was applied as a spray after germination, the introduced microbial community would have to compete with the native soil microbial population – a community much more complex than the starting seed microbiome. Furthermore, since a seed only releases minimal root exudates in the spermosphere until it germinates, a possible nutrient source that the consortium may have needed for proliferation may not have been sufficiently present (Schiltz et al., 2015). Providing an additional source of nutrients during inoculation has been shown to

improve the success inoculant establishment in field settings (Albright et al., 2022). Without the addition of an external nutrient source it is possible that the consortium was not able to establish at high relative abundances during this experiment and could have been outcompeted by the starting microbial community.

Another reason that there may have been little effect could be that the applied bacteria were not able to perform their desired function. Since their mode of action is phosphorus and calcium solubilization and iron chelation, the lack of these elements in the petri plate environment renders their predicted mode of plant growth promotion challenging, since filter paper is made strictly of cellulose. If growth promotion had been observed in this experiment, it may have rather been an effect of the *Bacillus* strains producing exogenous phytohormones, or microbe-to-plant signals that has been documented for other strains of the same species (Backer et al., 2018).

It is worth noting that an experiment using the same strains of *Bacillus* from the company Ulysse Biotech as individual inoculants, rather than a consortium, was able to show an improvement in corn seed germination when testing the application of the product's bacterial cell free supernatant (Yaghoubian et al., 2022). The cited experiment tested the individual strain's efficacy to promote germination and early growth of corn under salinity stress. While an effect was shown for promoting germination under optimal conditions, a greater effect was seen for the high salinity environments. This shows that the individual *Bacillus* strains do produce phytoactive compounds such as phytohormones which can promote germination and early growth of corn. The experiment failed, however, to test if the same effects could be seen when supernatant from the strains was combined as a consortium. This could affect the bacterial metabolite composition due to species-species interactions which might change their growth promotion efficacy.

5.2 Growth chamber experiments

The initial data from the series of growth chamber experiments aligns closely with that of the germination experiments. While there was no significant difference in days till emergence between the consortium and control, at 15 °C the inoculated treatment group had taken, on average, a slightly longer time to emerge than the control (Figure 7). This is consistent

with the germination experiment where the treatment group had a slower germination rate and radical length at 15 °C when given 10 mL of the diluted inoculant. Temperature, again, had the strongest effect, with 25 °C, the optimal temperature, taking the shortest amount of time to emerge and 20 and 15 °C taking progressively longer.

Shoot height is another reliable and often used measure to assess the efficacy of a biostimulant to promote plant growth under various stress conditions (Calvo et al., 2017). It correlates strongly with desired crop traits such as yield, and when traits such as yield cannot be measured, as in the case of this experiment due to limited space and time, it is a suitable alternative. Regardless, all results should ideally be confirmed in field settings with crops grown to harvest.

Emergence appears to have been an accurate predictor of shoot height with many of the initial measurements being consistent at the later growth stage, but still with no significant differences between the treatment and control groups (Figure 8). The plant heights were slightly greater for the treatment groups at 25 and 20°C and slightly smaller at 15 °C, corroborating the observed slight differences in germination times at the three temperatures. Overall, inoculating the seeds by means of soil drenching after they have been sown with the recommended field concentration of the product did not significantly impact emergence or shoot height.

Inoculation did, however, seem to increasingly promote biomass accumulation across the timepoints at the optimal temperature of 25 °C (Figure 9). This trend was reversed for both 20 and 15 °C, with the control group accumulating more over time, although the differences were even smaller (Figure 10-11).

Dry weight, as opposed to wet weight, was measured for this experiment since it has been shown to be a more reliable indicator of plant growth promoting rhizobacteria efficacy (Huang et al., 2017). Dry weight measurements taken at the final timepoint at 25 °C showed the treatment group having greater than an 8% more total biomass than the control (Figure 12). These weights can be compared to the results of the company Ulysse Biotech (ulysse-biotech.com/en/case-studies). In a field trial, with the same variety of corn seed tested as in this experiment, the company noticed a range in efficacies for growth promotion when

comparing the dry weight of seeds from 5 inoculated plants to 5 control plants. Depending on the type of soil the corn was grown in and the date of planting, the company showed that their product had an efficacy range of -2 to 20.5 %, with a median of 9.25 %. Statistical significance was reported on the company's website for most cases of plant growth promotion. The lack of statistical significance in the present study might be at least in part due to the limited number of replicates. If the experiment was able to continue until the plants reached maturity, it is likely that the differences between the treatment and control dry weights would have continued with the same trend, potentially yielding greater differences and more significant results at later growth stages, although this is speculation and should be the subject of further research.

At 20 and 15 °C however, the differences in average dry weight at the final timepoint between the treatment and control group were smaller, with the treatment group weighing less than the control except for shoot dry weight at 15 °C, although these differences were insignificant (Figure 12). The trend of the treatment group having slower or less growth at the lower temperature was seen here as well with lower total biomass than the control.

The results from the growth chamber experiment remain inconclusive, given that the differences were not statistically significant. Still, the data shows that there was potentially some growth promotion at the optimal temperature compared to the lower temperature conditions. This is contrary to what the company Ulysse Biotech has shown in their field trials, where there was greater growth promotion under more stressful abiotic conditions. It is important to note that the abiotic stress in a field setting is much more complex, with the effect resulting from a culmination of several environmental factors. A larger controlled environment experiment in a greenhouse with more replicates would be needed to conclusively show whether the consortium does or does not help alleviate low temperature stress in corn.

5.3 Plant tissue nutrient analysis

While basic measurements such as emergence, plant height, and dry weight are important data to include in the evaluation of a microbial inoculant, a more thorough analysis including nutrient profiles *in planta* is often essential for corroborating minor effects. This was the case for this experiment, where subtle hints in plant traits like dry weight suggest growth

promotion at optimal temperatures, although insignificantly so. This approach also makes sense for detecting the efficacy of this specific inoculant as it has a known mode of plant growth promotion. If the consortium is effective, phosphorus, calcium, and iron should all be readily available for, and therefore present at greater concentrations in, inoculated plants than in the uninoculated plants. Overall, this is what was observed, although not always significantly so.

The fertilizer regime caused the plants to experience phosphorus limitation stress at the later growth stages, as indicated by the purple coloration visible on the tips of the mature leaves (Supplemental Figure 2). This was done to encourage the plants to shift the composition of their root exudate profile to specifically recruit phosphorous solubilizing microbes such as those present in the consortium. It has been shown that even throughout the corn domestication process, like teosinte, modern hybrid varieties still have this capability (Brisson et al., 2022).

Phosphorus is quicky immobilized when added to soil, rendering a large fraction inaccessible to the plant. Depending on the pH of the soil, it will absorb to or otherwise bind with various other elements such as calcium, aluminum, or iron. At a low pH (i.e. below 6), it is more likely for phosphorus to bind with aluminum, and since aluminum phosphates are less soluble in water than for example calcium phosphate, it renders this essential nutrient inaccessible (Bhattacharya, 2019). Phosphorus solubilizing microbes can help mineralize this fraction of phosphorus (Gulnaz et al., 2017). Since there was enough phosphorus present in the system from the initial fertilization, with much of it binding to the substrate, it was expected that the treatment group would have a significantly higher amount of phosphorus in the plant tissues than the control. Furthermore, phosphorus solubilizing bacteria have been shown to alleviate low temperature stress in corn, so it was also expected that along with higher phosphorus tissue content, there would be significant growth promotion at the low temperatures tested (Jha and Mohamed, 2022).

One important caveat is that the corn seeds were coated in a fungicide. While this is a practice that can greatly reduce the rate of fungal pathogenic infection during early growth stages, it remains unclear how it might affect a plant's interactions with non-pathogenic fungi such as mycorrhizae (Hage-Ahmed et al., 2019). It has been shown that corn plants associated

with mycorrhizal fungi accumulate higher levels of phosphorus, and those mycorrhizae capable of interacting with phosphorus solubilizing bacteria enable the host plant to obtain even higher levels of the nutrient (Battini et al., 2017). Several studies have shown that corn seeds coated with fungicides are still able to interact with mycorrhizae and are minimally affected in their nutrient content by this practice (Burrows & Ahmed, 2007; Jin et al., 2013; Cameron et al., 2017). An interesting future research direction would be to test if this commercial bacterial inoculant has an improved efficacy if applied simultaneously with a mycorrhizal inoculant.

Total phosphorus content in the plant tissue was largely the same between the treatment and the control groups at 25 °C (Table 7). The amount of phosphorus that had accumulated in the treatment group was only 0.01 % greater than the control, however where it was distributed within the plant varied. In the treatment group, 70 % of phosphorus was found in the shoots and 30 % in the roots. The control group, on the other hand, had 61 % of its total phosphorus content located in the shoots and 39 % in the roots. The greater amount of phosphorus found in the shoots of the treatment group was not statistically significant, and neither was the lower percentage in the plant roots of the treatment group (Table 8). This contrasts starkly with the 20 °C trial which had the opposite effect. At 20 °C, the treatment group allocated 54 % of its total phosphorus to its shoots and 46 % to its roots compared to the control plants with 68 % of phosphorus in its shoots and 32 % in its roots. Neither of these patterns were truly noticeable at 15°C where both treatment and control groups had approximately the same level of phosphorus and similar distributions within the plant. When comparing the total phosphorus content between the optimal and lowest temperatures, the phosphorus content decreased slightly in both the treatment and control groups, suggesting that the addition of the consortium did not promote growth under stressful conditions through phosphorus solubilization (Figure 14).

The range of optimal pH for corn growth is generally accepted to be 5.8-6.2. The initial pH of the soil (G6 media) was approximately 6.0 and with subsequent additions of both 20-20-20 and 20-2-20 fertilizers consistently measured approximately 6.2. When soil pH is above 6, phosphorus readily absorbs with calcium instead of aluminum. Since calcium solubilization is

another mode of action by the consortium, it was predicted that the treatment groups would have higher rates of this element in their tissue.

This, in fact, is what was seen at the optimal temperature. The treatment group had a significantly higher total amount of calcium than the control group (Table 8). However, this was not the case for the two low temperatures tested. At 20 °C, no significant difference due to the consortium was seen on calcium content. At 15 °C, there was no significant effect on total calcium content but there was a significantly higher amount of calcium present in the shoots of the control plant, suggesting that the consortium may have hindered its accumulation. Unlike the distribution of phosphorus at the three temperatures tested, calcium had roughly the same distribution between the shoots and the roots for both treatment and control groups. This is consistent with earlier findings for plant dry weight. The addition of the consortium could have promoted plant growth at the optimal temperature by increasing calcium accumulation. It might be expected that through calcium solubilization, phosphates should also be released, making phosphorus accessible to the plant and increasing its concentration. This may indeed have been the case as seen in the higher concentration of shoot phosphorus in the treatment group at 25 °C, although this was insignificant. The calcium contents at 20 and 15 °C are further in line with earlier results of this study where at sub optimal temperatures the addition of the consortium did not promote corn growth and in some instances may have hindered it.

Iron content at optimal temperatures followed a similar trend with a higher concentration in the treatment group compared to the control, although this was insignificant. There was a significantly smaller total concentration of iron of the inoculated group at 20 °C and significantly less in the shoots at 15 °C (Table 8). This again suggests that the addition of the consortium could have possibly had an effect in chelating iron molecules and making them more plant accessible. Furthermore, several other metals such as manganese and aluminum followed very similar patterns where, while accumulating at a much higher rate at optimal temperatures for both the control and treatment groups, the treatment group seemed to amass even higher amounts even though the difference was insignificant.

Copper and zinc were two other elements which were found to be in higher concentrations in the treatment group at 25 °C (Figures 20 & 24). Copper was at significantly

higher concentrations in the treatment group, and specifically in the roots, than the control (Table 8). Zinc was also present in greater amounts in the shoots of the treatment group, although insignificantly so. Total zinc content was greater, again insignificantly, in the treatment group than the control at all temperatures tested. However, copper did not follow the same trend and at lower temperatures the treatment group had lower total concentrations than the control. Iron, manganese, copper, and zinc are most available for plant uptake at slightly acidic pH levels. Microbially produced siderophores and other chelating agents excreted by bacteria such as *Bacillus megaterium* then capture these nutrients, delivering them to plant roots (Orr et al., 2020; Arceneaux et al., 1984; Bar-Ness et al., 1992).

Finally, the sodium content of the inoculated plants is important to note (Figure 17). Although no values were significantly altered by treatment for this nutrient, at the two low temperatures, 20 and 15 °C, the treatment group had higher total sodium contents than the control. At 15°C, the roots in both the treatment and control group had much greater levels of sodium than plants at 25 and 20°C. Tolerance to sodium accumulation has been observed in non-corn plant species in conjunction with low temperature stress (Shamustakimova et al., 2017). In Arabidopsis, the salt tolerance-related protein (STRP) has been shown to mediate low temperature stress (Fiorillo et al., 2020). The results of this nutrient analysis indicate that corn may experience the same phenomenon of higher salt tolerance at low temperature however more research is needed to show this. The ability of the inoculant to enable corn to accumulate higher levels of sodium is in line with previous research, however only the cell free supernatant has been tested (Yaghoubian et al., 2022).

Ultimately, slight effects of growth promotion at the optimal temperature of 25 °C was observed. Although these findings were largely insignificant, evidence from some nutrients, such as calcium, suggest an underlying mechanism. The lack of significance may be due to the limited sample size of the growth chamber experiment and the short duration of the experiment. Growth promotion was not detected at the low temperatures tested and in some cases trends of slight growth limitation were observed. Further research is needed at larger scales, both in sample number and duration, to supplement these preliminary findings.

5.4 Lettuce experiment

A secondary growth chamber experiment was performed to confirm the findings from the corn experiment, this time using lettuce. Due to their long evolutionary divergence, corn, a monocot, and lettuce, a dicot, are extremely different in their morphology, life history traits, and associated microbial compositions, although both are domesticated agricultural crops – perhaps leading to a slight convergence in their microbiomes due to anthropogenic influences. In order to solely test the effect of the consortium and its potential ability to alleviate low temperature stress, the growth conditions were kept identical for the lettuce experiment except for a second inoculation of the consortium as per the company's instructions. Since the company Ulysse Biotech has reported positive results on lettuce yield in field conditions, a similar response was expected in a growth chamber environment, and it was expected that any effects at low temperature would also be observed.

There was no effect on lettuce emergence at 25 °C or at 20 °C, however a significant effect was observed on day 5 at 15 °C in which the treatment group had significantly fewer seedlings emerge (Figure 26-28). Lettuce dry weight and leaf area were used as measurements to assess growth promotion. At 25 °C, the treatment group had higher amounts of dry weight compared to the control, although this was insignificant (Figure 29). Before the plants were dried, the total leaf area was measured and the treatment group was significantly greater than the control (Figure 30). The company was able to show growth promotion of approximately 23 %, however this experiment was only able to show an 11.6 % increase. At 20 °C, both dry weight and leaf area were marginally smaller showing no effect of the consortium (Figure 31-32). At 15 °C, there was the largest difference in dry weight in which the treatment group had smaller averages than the control, although this was still insignificant (Figure 33). This was also reflected in the smaller leaf area in the treatment group at 15 °C (Figure 34). This is consistent with the delayed emergence rate in the treatment group, suggesting a potential hinderance in growth. These results align with those observed in the corn growth chamber experiment where marginal growth promotion was observed at the highest temperature and none or even slightly less was observed at low temperatures.

5.5 Microbiome analysis

5.5.1 Principal coordinate analysis

Differences in microbial community composition between the inoculated treatment and uninoculated control plants were analyzed to determine whether any effects of the plant growth promoting inoculant could be detected. It is important to note that function cannot readily be assumed from 16S rRNA data alone, although this is improving with recent computational approaches, observing how the microbial community composition shifts in response to a variable can help identify potential causes of variation in phenotypic expression (Franzosa et al., 2015; Breitkreuz et al., 2021). To truly have an understanding of community function, a more complete microbial analysis involving metagenomics or a combination of metagenomics and metabolomics would be required. The analysis of microbial communities using 16S rRNA was used to investigate how the inoculant potentially impacted the taxonomic community structure in a highly controlled environment.

There was a clear effect of time causing significant shifts in community structure from the start to the end of the experiment (Figure 35). There was a significant difference between the starting control bulk soil and the final timepoint control bulk soil, showing that succession in the microbial communities occurred. This could have been affected by the watering every few days to keep the soil moist, or to the addition of a fertilizer four times throughout the experiment that provided nutrients and slightly altered the soil's pH. The root exudates from pots containing plants could have influenced the microbial community over time, and in both the bulk and rhizosphere treatment groups, the microbial communities were expected to have been influenced by the inoculant.

No significant shift was detected in the starting soil when the inoculant was added. While a tight clustering of samples from the inoculated and control soil samples were seen in the PCoA (Figure 35), the communities were not found to be significantly different. When the starting soil community was sequenced neither the control nor treatment groups were found to have *Bacilli* within their 20 most abundant genera (Supplemental Figure 3). This indicates that for the treatment group, either the T0 sample was not taken long enough after the consortium was added to sufficiently establish, or the concentration that the consortium was inoculated at

(0.5 mL L^{-1}) was too low to detect. Other native *Bacilli* in the control T0 sample were also not within the 20 most abundant taxa. *Bacillus* species and strains are among the most abundant bacteria in soil ecosystems, however this appears not to be the case for the G6 growing media (Walters et al., 2018).

The data indicated that temperature had a significant effect on community succession. There was tight clustering of each temperature within the treatment and control samples (Figure 36). This was expected since along with pH, which slightly increased from 6 to nearly 6.2, temperature has been found to be the biggest driver of microbial community structure.

A deeper analysis of these results is needed to identify any effect of the inoculant on community structure. Starting with the inoculated and uninoculated bulk soil, the two treatments were not different from each other (Figure 37). While the effects of temperature are still clear, the inoculant did not change the community structure. This could possibly be because the consortium was only added once at the beginning of the experiment. Due to the low concentration of inoculant bacterial cells, it is likely that they were not able to establish and were outcompeted by the starting population. Even though nutrients were added several times throughout the experiment, they could have been used by the dominant soil microbial community instead of the consortium.

Next, the rhizosphere community structure was also not significantly affected by the inoculant, but again the effect of temperature was the greatest factor shaping the structure. There was more clustering, however, between the treatment and control groups in the rhizosphere samples compared to the bulk soil samples at all three temperatures (Figure 38). This suggests that, while insignificant, the addition of the consortium may have slightly altered the structure of the microbial community in the rhizosphere. One potential mechanism for this could be that the added *Bacilli* were in their spore stage and the immediate soil environment was not optimal for growth. When the corn seed germinated and started releasing root exudates, the environment would have been more hospitable for the *Bacillus* strains. However, because *Bacilli* were not found in the microbial data in detectable abundances, any potential effect directly from them cannot be confirmed. Furthermore, the small sample size likely

contributed to the lack of significance between the rhizosphere samples at the three temperatures.

5.5.2 Microbial community composition

A comparison of the most abundant taxa found within the bacterial communities revealed significant differences due to temperature and time in the control bulk soils (Figure 39). The ten most abundant taxa at the Class level were compared. The overarching differences between the control bulk soil community at T0 with those at TF are most likely due to these variables. Notably, *Ktedonobacteria* was virtually absent at the end of the experiment while the abundances of *Acidobacteriae* at 15 °C and *Gammaproteobacteria* at 15 and 20 °C were greatly reduced. Focusing on the TF control bulk soils in order to determine the effect of temperature revealed that this variable did strongly influence community composition. *Actinobacteria*, for example, was significantly reduced at 25 °C, greatly reduced at 20 °C, and maintained its starting abundance at 15 °C. This established a baseline profile for all the temperatures tested. Any discrepancies from these observed shifts could be most likely attributed to the addition of the inoculant (bulk soil without plants), presence of roots (uninoculated plants), or the interaction between the inoculant and the roots (inoculated samples with plants).

The rhizosphere effect was not as evident in microbial community composition as anticipated (Figure 40). The beginning of the rhizosphere is clear since it emanates from the rhizoplane, but by its nature, where it ends remains ill-defined. The key characteristic of the rhizosphere is that there is a significant reduction in microbial diversity with increasing proximity to the rhizoplane. While there were marginal differences noticed at the class level for the three temperatures, such as more *Actinobacteria* at 20 °C and less *Verrucomicrobiae* at both 15 and 25 °C in the rhizosphere, these differences were not as stark as those reported in other experiments (Bakker et al., 2015). A potential reason for this could be the method of rhizosphere extraction. This experiment relied on physical shaking of the roots to dislodge any loosely bound soil particles. Any that remained after 10 minutes of vigorous shaking were assumed to be part of the rhizosphere since root exudates and bacterial extracellular polysaccharides bind the soil aggregates to root surfaces. This contrasts to other methods of rhizosphere extraction such as shaking roots while submerged in a phosphate buffer or a low

concentration NaCl solution after the removal of the bulk soil (Simmons et al., 2018; Barillot et al., 2013). Any soil collected in these solutions afterwards is then considered to be the rhizosphere. Other more recent experiments, however, have used the same method as the current experiment to isolate the rhizosphere (Benitez et al.,2021). No standardized and widely accepted method for corn rhizosphere extraction has been published yet. A further limitation to the method used in the current study was that the roots of the three plants grown in each pot were so bound that, while most of the bulk soil had been removed, there is a chance that some may have remained as the roots acted like a net, which would have likely been an issue regardless of the method of rhizosphere extraction used. Since only subtle differences are seen in the microbial community composition between the rhizosphere and bulk soils, the rhizosphere may have been sampled further away from the rhizoplane than intended, with the microbial community experiencing less selective pressure from the root exudates. The rhizosphere soil was thoroughly homogenized before DNA extraction which should have resolved any discrepancy caused by the location of the soil sampling. However, if the bulk soil (i.e. soil that had adhered to the roots and was not influenced by root exudates) was present in the homogenization process, the resulting DNA extraction would have been less specific.

When analyzing the differences between the rhizosphere samples of the control and inoculated groups, several key trends were observed (Figure 41). First, there was the strong effect of temperature as seen with the abundances of *Actinobacteria* and *Phycisphaerae*. Secondly, the rhizosphere consisted of taxa that appear to be highly conserved in the corn rhizosphere. *Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Planctomycetes,* and *Verrucomicrobiae*, for example, have been identified in corn rhizospheres across geographic regions, climates, soil types, and management strategies (Deng et al., 2021). While a core corn microbiome has yet to be formally defined, these taxa have all been shown to be highly heritable and are likely to be the dominant taxa (Walters et al., 2018). Of note is the increase in *Verrucomicrobiae* when comparing the inoculated and uninoculated rhizospheres. While the correlation between specific microbial taxa and nutrient content was not performed, other research has shown a strong correlation between nutrient content and the enrichment of this taxa, as well as *Gammaproteobacteria* and phosphorus content in the plant tissue (Aguirre-

von-Wobeser et al., 2018). An investigation into the corn rhizosphere composition revealed that *Bacilli* did not reliably contribute to the top ten most abundant taxa at the class level, indicating that their functional role may be more closely aligned to those of rare taxa in shaping microbial community structure and resiliency to environmental perturbation, since numerous other studies have reliably shown growth promotion across a range of crops when several strains were added (Rubin et al., 2017). In this experiment, the *Bacilli* class was not represented in the top 10 most abundant taxa in either the treatment rhizosphere or bulk soils. This suggests that the inoculant had not established well enough to become a dominant taxon in these habitats (Figure 40).

Although the composition of the rhizosphere at higher taxonomic levels generally mirrors what is seen in field soils from around the world, it is important to note that the bulk soil had approximately the same composition as the rhizosphere soil at the class level (Figure 40). The ability of the plant to select for beneficial symbionts by altering its associated microbial community, as well as the role the inoculant plays in promoting these associations, are the forces that drive the response of the host plant to abiotic stresses, and in the case of this experiment, phosphorus-limitation stress more so than low temperature stress.

An even closer analysis of the rhizosphere community composition and differences between control and treatment groups at the genus level revealed that the biggest differences in composition occurred at 25°C and that there were practically no changes at 20 or 15 °C (Figure 42). Most notably, the treatment group had on average less *Chitinophaga*. This is a genus which some research has associated with pathogenicity, although other studies have found that this taxon may actually alleviate fungal pathogen pressure through the production of chitinase (Yin et al., 2021; Carrión et al., 2019). *Lacunisphaera* and *Tepidisphaerales* were both enriched in the treatment group as well. *Lacunisphaera* is a newly described genus and a functional role in plant health has yet to be identified (Rast et al., 2017). *Tepidisphaerales*, on the other hand, has been associated with improved phosphorus uptake in soybean (Ahmed et al., 2021). This study's data indicated that a similar effect might have occurred in this experiment, although it was not found to be significant, and further research using corn as a model is be needed for confirmation.

Even though the inoculant was not found in the top ten most abundant classes, nor in the top 20 most abundant genera, it does not necessarily mean the added *Bacilli* did not persist at a basal level. Both the rhizosphere of the control and treatment groups contained *Bacilli* at very low abundances (Supplemental Figure 3). A possible mechanism for how the inoculant could still be influential is through shaping the early rhizosphere community structure. Alternatively, the inoculant may have remained in low abundances yet still had an effect as a set of rare taxa. The lower abundance of *Bacilli* in the treatment group was unexpected but is a finding consistent with earlier research (Overbeek, 2020). Using the same consortium, corn inoculated in a field experiment experienced slight growth promotion. However, when a microbial analysis of the soil was conducted, inoculation did not noticeably alter the community structure or composition. In that experiment, *Bacilli* were present at a lower abundance in the treatment group compared to the control (Overbeek, 2020).

A possible solution to the low levels of *Bacilli* relative abundance could have been to assess their population by using quantitative polymerase chain reaction (qPCR). This method would have more accurately detected extremely small populations of the inoculant that may have persisted in the rhizosphere and it could have been used to quantify the consortium in response to low temperature stress. This method was not pursued, however, since it would not have yielded information regarding the structure of the entire rhizosphere microbial community in response to inoculation and low temperature stress. Furthermore, it was expected that there would be high amounts of endogenous *Bacilli* present in the starting soil that, at the genus level, would not be distinguishable from the inoculant.

5.6 Final Discussion

The ultimate goal of a company with a commercial plant growth promoting microbial inoculant is to increase plant growth and yield. Along with prior research, the current study suggests that this was achieved at optimal temperatures under highly controlled conditions, although the evidence is not strong. Claims of improving plant uptake of phosphorus, calcium, and iron are supported at optimal temperatures, although not always significantly so. This study did not find that the commercial inoculant could alleviate low temperature stress in a

highly controlled environment since there was either no change or even reduced growth (although with no statistically significant changes) at the lowest temperatures tested.

Recommendations for further experiments using this consortium should start with tracking how the inoculant alters the rhizosphere community composition of each crop that it is applied to. If a trend emerges across crop species, such as an enrichment in *Verrucomicrobiae*, for example, a potential mechanism for indirect growth promotion could be further investigated. This experiment is not the first to find a lack of evidence of inoculant strains promoting growth in corn (De Zutter et al., 2022). If the inoculant promoted growth by improving phosphorus, calcium, and iron contents through modification of the microbiome, this might mean that its efficacy would at least partially depend on the diversity of the initial soil microbial population. Elucidating, on a field-by-field basis, the starting microbial composition could be an important step in determining whether growth promotion due to PGPR inoculation would be expected. This scenario is supported by inconsistent results of commercial microbial inoculants in field trails which face the added difficulty of different weather occurring year-by-year during long term trials (Li et al., 2022). In line with this suggestion, it would be valuable to repeat the current experiment using field soil. Northern soils of Quebec tend to more acidic rendering phosphorus to be more limited. Under controlled conditions, one could observe how the inoculant affects the starting soil microbiome and how together, as a holobiont, the the system responds to different low temperature stress.

Since Ulysse Biotech has reported that their product promotes plant growth across several crop species, as is confirmed in this experiment for corn and lettuce at optimal temperatures, the inoculant may shape microbial community function rather than composition. The phosphorus limitation imposed in this experiment could have further promoted this. While it was expected that nutrient stress would result in the recruitment of inoculant members, another potential mechanism could be that the inoculant instead recruited other taxa capable of alleviating this stress, thereby shaping the community's function. This could explain why only minimal shifts were seen in the rhizosphere of inoculated plants. This would have to be confirmed using techniques such as metagenomics and metabolomics to identify functional gene expression, which may be similar across diverse microbial taxa.

Finally, future research should assess how inoculating crops with a higher concentration of the consortium than is currently suggested by the company would affect soil microbial communities. Samples taken at different timepoints and from rhizospheres in both field and highly controlled conditions could be used to establish a baseline, in order to assess the succession of the microbial population, and how this is affected by the amount inoculant added.

5.6.1 Revisiting hypotheses

The experimental results were not able to support the proposed hypotheses of the study. In summary, it was hypothesized that the consortia would promote corn growth under low temperature stress. However, this was not supported by the corn germination, radical length, emergence, plant height, shoot and root dry weight, and tissue nutrient analysis data. While there was slight growth promotion and nutrient accumulation at the optimal temperature, it was not always significant and it was also not observed for the two low temperatures tested. The lack of statistical significance may have been due to the small sample size and short duration of this experiment. A larger and longer controlled experiment is needed to confirm these results, however the secondary experiments using lettuce seem to offer some support for these findings.

The hypotheses regarding the corn rhizosphere microbial community composition were not supported either. First, it was hypothesized that there would be a significant change in the microbial community structure, although this was not detected. There was a significant change in the structure between the timepoints and between the three temperatures tested. No significant change was observed with the addition of the consortium. The second hypothesis was that the inoculants' populations would increase in abundance with lower temperatures. This was not observed since *Bacilli* (the class and genera) were not dominant in the 10 most abundant microbial classes or in the 20 most abundant genera. *Bacilli* were even detected at lower abundances in the treatment than control groups (Supplemental Figure 3). A functional analysis using metabolomics could help identify the inoculants' effect instead of relying on observable changes in microbial community structure and composition.

Chapter 6: Conclusions

Although there was evidence in the literature to suggest that the microbial strains present in the consortium could have alleviated low temperature stress in corn due to their functional capabilities, this was not supported by the data. However, further research using larger sample sizes and testing different concentrations of the consortium is needed to confirm this study's findings. If the inoculant can establish in the rhizosphere at a higher concentration, then the results may have been different from what was observed.

The first objective of this project testing for corn growth promotion at low temperatures with the consortium Era Boost Pro was assessed using early growth and subsequent growth chamber experiments. Germination rate and radical length were measured at optimal and cold temperatures during the earliest stages of corn growth. Seedling emergence, plant height, shoot and root dry weight, and plant tissue nutrient concentrations at optimal and cold temperatures were measured during early growth (up to the V4 stage) to detect any effect of the consortium. Furthermore, results were confirmed by running similar experiments with lettuce plants. The results showed that the inoculant does not promote corn growth under low temperature stress.

The second objective of this research was achieved by conducting microbial community profiling of the bulk and rhizosphere soil using 16S rRNA gene sequencing. The microbial community structure in both bulk and rhizosphere soil was not significantly affected from the inoculation of the consortium at the concentrations tested. Rather, it was the variation in the three temperatures tested that had the most significant effect in shaping microbial community structure. Slight variation in microbial community composition due to inoculation was detected, while again, temperature had a more noticeable effect.

Even though the tested microbial consortium did not have a clear effect in alleviating low temperature stress, it is still a very important and useful product. There are many other abiotic stresses that in a field setting can negatively affect crop growth and yield. Since, for example, there is evidence that the consortium alleviates salt stress and there is other evidence of closely related taxa helping with heat and drought stress, its (and other products like it) implementation will be vital in securing crop yields in a rapidly changing climate.

Low temperature stress receives much less attention than other more prevalent abiotic pressures, however it is becoming an increasingly serious threat. In Canada, this stress will greatly impact farmers, especially as crops are grown further north. Since there is evidence that microorganisms can alleviate low temperature stress in a range of crops, it is likely that there will soon be an inoculant that can achieve this goal.

While microbial inoculants are a promising solution for alleviating many, if not yet all, abiotic stresses for agricultural crops, they only represent an immediate fix to the larger problem at hand. Biostimulants will undoubtably play an essential role in making future agriculture more sustainable and climate resilient, however they can only be part of the solution. Fundamental change is needed in which less synthetic fertilizers are used, and a greater emphasis is placed on soil health and agricultural biodiversity. Regenerative practices and regionally based food systems are necessary, but a diverse array of innovations is also needed to accomplish fundamental change.

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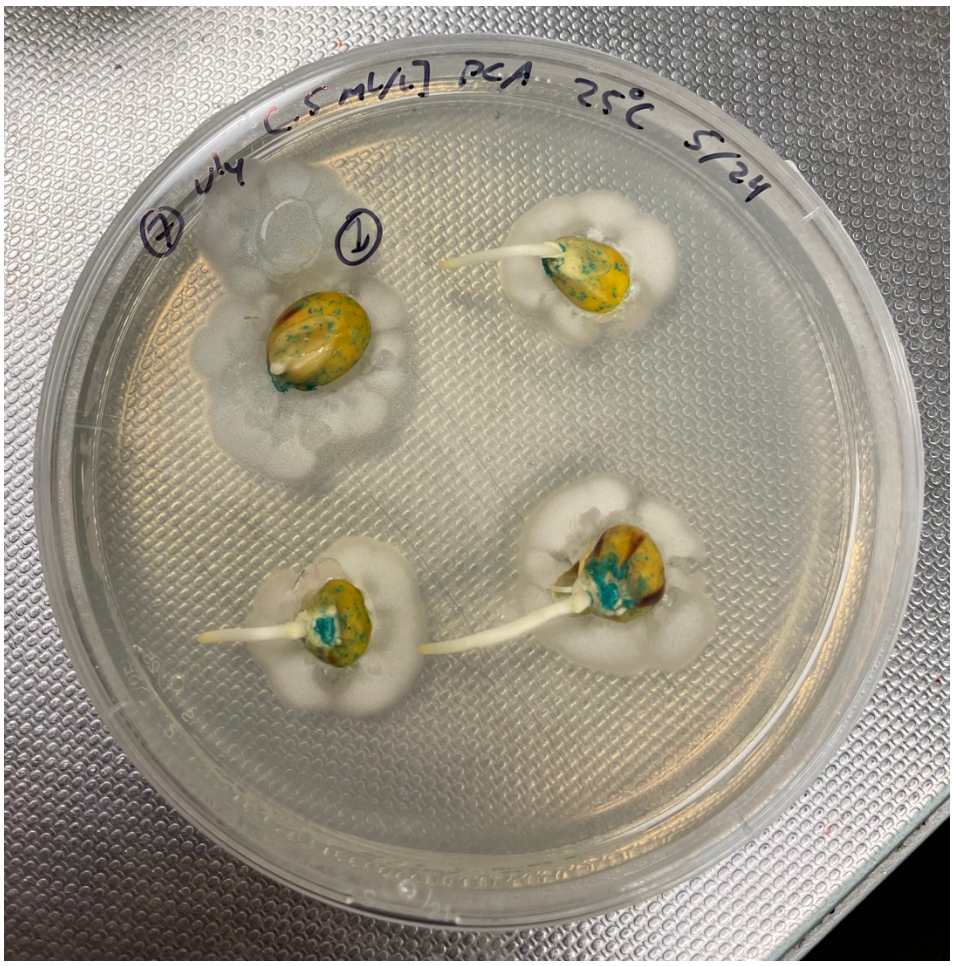
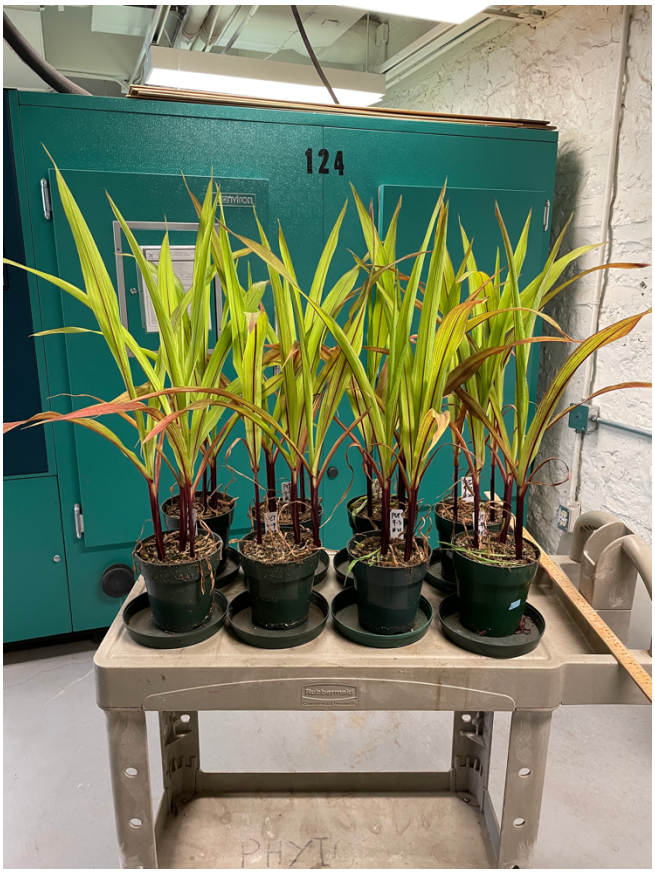
Supplementary Material

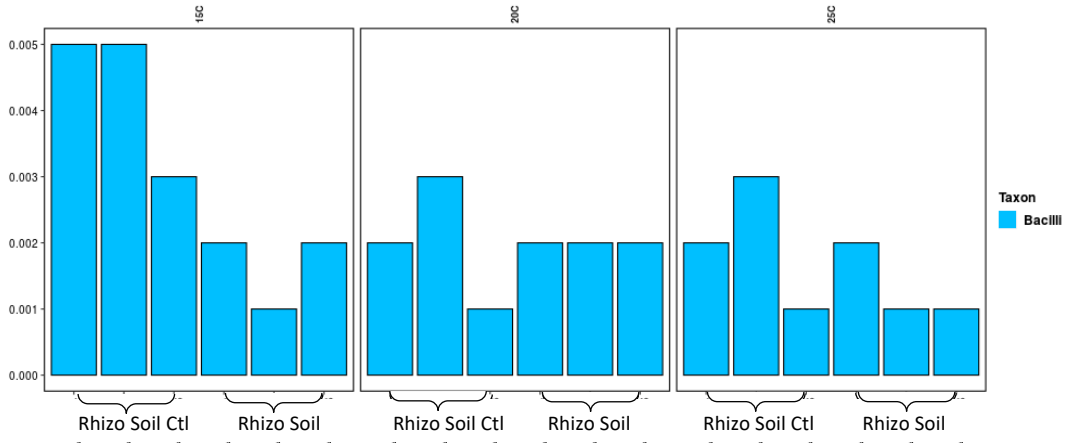
Supplementary figures



Supplementary figure 1. *Baccili* growth 24 h after inoculation on sterile corn seeds at 25 °C.



Supplementary figure 2. Mild phosphorus stress visible during the final timepoint sample.



Supplementary figure 3. Low abundance of *Bacilli* in treatment and control rhizosphere samples at all three temperatures.

Supplementary tables

Supplementary table 1. P-value for top 10 classes in bulk soil control groups at the initial (T0) and final timepoint (TF).

P-value for top 10 classes in bulk soil ctl at T0 vs TF			
Taxa	P-Value	Mean T_0	Mean T_F
15 °C			
Acidobacteriae	0.0444	0.0787	0.0303
Bacteroidia	0.0454	0.1570	0.2003
Gammaproteobacteria	0.0444	0.1457	0.0823
Ktedonobacteria	0.0181	0.0577	0.0033
Phycisphaerae	0.0128	0.0097	0.0253
Verrucomicrobiae	0.0437	0.0197	0.1063
Vicinamibacteria	0.0210	0.0093	0.0150
20 °C			
Alphaproteobacteria	0.0176	0.2563	0.3410
Bacteroidia	0.0444	0.1570	0.2350
Gammaproteobacteria	0.0156	0.1457	0.0490
Ktedonobacteria	0.0200	0.0577	0.0073
Phycisphaerae	0.0095	0.0097	0.0460
25 °C			
Actinobacteria	0.0009	0.1397	0.0487
Alphaproteobacteria	0.0267	0.2563	0.3273
Ktedonobacteria	0.0206	0.0577	0.0090
Phycisphaerae	0.0096	0.0097	0.0423
Verrucomicrobiae	0.0002	0.0197	0.1043
Vicinamibacteria	0.0076	0.0093	0.0167

Supplementary table 2. P-value for top 10 classes in the treatment bulk soil and rhizosphere soil groups at the final timepoint (TF).

P-value for top 10 classes in bulk soil TF vs rhizo soil TF			
Taxa	P-Value	Mean bulk	Mean rhizo
15 °C			
Verrucomicrobiae	0.0193	0.1303	0.0887
Vicinamibacteria	0.0043	0.0147	0.0087
20 °C			
Actinobacteria	0.0155	0.0570	0.0960
Bacteroidia	0.0112	0.2147	0.1730
25 °C			
Planctomycetes	0.0187	0.0643	0.1050
Verrucomicrobiae	0.0189	0.1293	0.1007

Supplementary table 3. P-value for top 10 classes in treatment rhizosphere soil (rhizo soil) and control rhizosphere soil (rhizo soil ctl) at the final timepoint (TF).

P-value for top 10 classes in rhizo soil TF vs rhizo soil ctl TF			
Taxa	P-Value	Mean rhizo	Mean rhizo ctl
15 °C			
Acidobacteriae	0.0081	0.0303	0.0423
20 °C			
NA	NA	NA	NA
25 °C			
Phycisphaerae	0.0115	0.0563	0.0383

Supplementary table 4. P-value for top 20 genera in treatment rhizosphere soil (rhizo soil) and control rhizosphere soil (rhizo soil ctl) at the final timepoint (TF).

P-value for top 20 genera in rhizo soil TF vs rhizo soil ctl TF			
Taxa	P-Value	Mean rhizo	Mean rhizo ctl
15 °C			
Granulicella	0.0419	0.0120	0.0207
Lacunisphaera	0.0096	0.0237	0.0093
Streptomycetales_genus	0.0397	0.0367	0.0573
20 °C			
NA	NA	NA	NA
25 °C			
Bradyrhizobium	0.0255	0.0090	0.0137
Granulicella	0.0198	0.0030	0.0053
Tepidisphaerales_genus	0.0132	0.0560	0.0380