Organic fertilizers influence the rhizosphere bacteria and soybean growth under heat and water stress

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List of Abbreviations

Abbreviation	*Full Description*
ACC	1-aminocyclopropane-1-carboxylate
ACC deaminase	1-aminocyclopropane-1-carboxylate deaminase
ANOVA	Analysis of variance
ERF	Ethylene response factor
MIQE	Minimum Information for Publication of qPCR Experiments
PGPR	Plant growth promoting rhizobacteria
QY	Quantum yield
ROS	Reactive oxygen species
SAM	*S*-Adenosyl methionine
SWHC	Soil water holding capacity
ΔCq	Change in quantitation cycle between reference gene and gene of interest

Abstract

Soybean responds to heat and water stress by producing ethylene, a plant growth hormone that reduces soybean biomass accumulation and shortens its lifespan. Soybean rhizosphere bacteria can degrade the precursors of growth-suppressing ethylene with the enzyme 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase). Greater ACC deaminase activity of soybean rhizosphere bacteria is expected when soybean grows in soil that receives a complete, nutrient-rich organic fertilizer than N fertilizer only. The objectives of this thesis were 1) to determine the morphological responses of organic-fertilized soybean plants exposed to heat and water stress, 2) to evaluate the ethylene production by soybean and the ACC deaminase activity of soybean rhizosphere bacteria in soil receiving organic fertilizer versus N fertilizer only and 3) to determine if fertilizer sources alter the composition of the soybean rhizosphere bacterial community. I hypothesized that soybean will accumulate more biomass and their rhizosphere bacteria will produce more ACC deaminase when soybean is fertilized with organic fertilizer than with N-only fertilizer, when soybean is exposed to heat or water stress. If the fertilizer source changed the ACC deaminase activity, then I expected to see differences in the composition of the rhizosphere bacterial community, as different taxa have different ACC deaminase production capacities. Soybean were grown in soil amended with organic fertilizer (biosolids, anaerobic digestate or compost) or N fertilizer for 9 wk in a greenhouse, and exposed to stress (no stress, heat stress, water stress or heat plus water stress) for 7 d. Then, I measured plant metabolic processes (photosynthetic efficiency, ethylene concentration), morphological variables and bacterial communities in the rhizosphere and bulk soil under soybean. As expected, soybean plants exposed to heat or water stress were taller and produced more aboveground biomass ($p < 0.05$) when grown in soil with organic fertilizer, such as anaerobic digestate or biosolids, than in soil that received N fertilizer. There was greater ACC

deaminase activity ($p < 0.05$) in the rhizosphere soil amended with biosolids than with N fertilizer, and ACC deaminase activity was negatively correlated with the ethylene concentration (r = -0.40, $p < 0.05$). This suggests biosolids may stimulate the growth of ACC deaminase producing bacteria in the soybean rhizosphere, which could reduce the growth-suppressing effects of ethylene on soybean. This possibility is supported by biosolids cultivating a unique bulk soil bacterial community (ANOSIM = 0.4045, $p = 0.002$), which includes increased proportions of taxa such as *Chryseobacterium, Scopulibacillus, Ralstonia, Ideonella* and *Asticcacaulis spp*, which may be due to biosolids inoculating the soil with live heterotrophic bacteria or by supplying additional essential nutrients which were not measured here. Additionally, biosolids and N fertilizer treatments led to unique soybean rhizosphere bacterial communities, which may be due to biosolids increasing the surface area for root adhesion, or by altering soybean gene expression and root exudation pattern. Further work is needed to determine the ACC deaminase production capacity of these indicator species in the soybean rhizosphere. In conclusion, organic fertilizer like biosolids can increase soybean tolerance to heat and water stress by supporting more plant growth and greater ACC deaminase activity, perhaps by changing the composition of the rhizosphere bacterial community associated with soybean.

Résumé

Le soya répond au stress thermique et hydrique en produisant l'éthylène, une hormone de croissance végétale capable de réduire sa biomasse et raccourcir sa durée de vie. Les bactéries qui occupent la rhizosphère possèdent la capacité de dégrader le précurseur d'éthylène avec l'enzyme 1-aminocyclopropane-1-carboxylate déaminase (ACC déaminase). Une plus grande activité enzymatique est attendue quand le soya pousse dans un sol qui reçoit un engrais organique complet qui est riche en nutriments, comparativement à ce qui reçoit l'engrais azoté uniquement. Les objectives de cette thèse étaient de 1) déterminer les réponses morphologiques du soya fertilisé avec un engrais organique quand il est exposé au stress thermique et hydrique, 2) d'évaluer la production d'éthylène par le soya et la production d'ACC déaminase par les bactéries présentes dans la rhizosphère d'un sol qui reçoit un engrais organique comparé à l'engrais azoté et 3) de déterminer si le type d'engrais modifie la composition de la communauté bactérienne de la rhizosphère. J'ai émis l'hypothèse que le soya accumulera plus de biomasse et les bactéries de sa rhizosphère produiront plus d'ACC déaminase quand le soya est fertilisé avec un engrais organique qu'avec l'engrais azoté, et lorsque le soya est exposé au stress thermique ou hydrique. Si le type d'engrais change l'activité d'ACC déaminase, je m'attends à observer une différence dans la composition de la communauté bactérienne de la rhizosphère, car des taxons ont de différentes capacités à produire l'ACC déaminase. Alors, le soya a été cultivé dans un sol qui a reçu un engrais organique (biosolides, digestat anaérobie ou compost) ou l'engrais azoté (l'urée) pendant 9 semaines dans une serre, et a ensuite été exposé à un stress (aucun stress, stress thermiques, stress hydrique ou thermiques+ hydriques) pendant 7 jours. J'ai ensuite mesuré les processus métaboliques (efficacité photosynthétique, concentration d'éthylène) et les paramètres morphologiques du soya et la composition des communautés bactériennes dans la rhizosphère et

le sol en vrac. Comme prévu, le soya exposé au stress thermique ou hydrique était plus haut et a produit plus de biomasse hors terre ($p<0,05$) lorsqu'il est cultivé dans un sol qui a reçu un engrais organique comme le digestat anaérobie ou les biosolides, que celui qui a reçu l'engrais azoté. Il y avait plus d'ACC déaminase dans la rhizosphère du sol qui a reçu des biosolides que dans celle qui a reçu l'engrais azoté, et l'activité d'ACC déaminase a été négativement corrélé avec la concentration d'éthylène ($r=-0.40$, $p<0,05$). Ceci suggère que les biosolides peuvent peut-être stimuler la croissance des bactéries dans la rhizosphère qui sont capables de produire l'ACC déaminase, qui pourrait réduire les effets de suppression de la croissance que l'éthylène porte sur le soya. Cette possibilité est appuyée par le fait que les biosolides ont cultivés une communauté unique dans le sol en vrac (comparé à l'engrais azote) (ANOSIM = 0.4045, p = 0.002), qui incluaient des plus hautes proportions de *Chryseobacterium, Scopulibacillus, Ralstonia, Ideonella* et *Asticcacaulis spp*. Ceci montre peut-être que les biosolides inoculent le sol avec des bactéries hétérotrophes vivants ou qu'ils fournissent des nutriments essentiels supplémentaires qui n'ont pas été mesurés ici. De plus, les traitements avec des biosolides ou engrais azoté ont développés des communautés bactériennes uniques dans la rhizosphère du soya, ce qui peut être dû au fait que les biosolides augmentent la surface d'adhérence des racines ou qu'ils modifient l'expression des gènes du soja et l'exsudation racinaire. Des travaux supplémentaires sont nécessaires pour déterminer la capacité de ces taxons dans la rhizosphère à produire l'ACC déaminase. En conclusion, les engrais organiques, comme les biosolides peuvent augmenter la tolérance du soya au stress thermique et hydrique en supportant plus de croissance végétale, plus d'activité ACC déaminase, peut-être en modifiant la composition de la communauté bactérienne de la rhizosphère associée au soya.

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Contributions of Authors

This thesis consists of a General Introduction, which outlines the context of my research project, a literature review chapter and two chapters written in manuscript format in accordance with the guidelines of the McGill Graduate and Postdoctoral Studies Office, a General Discussion, which provides direction for future research and General Conclusions, which summarizes the findings of this thesis.

The first chapter is a literature review of the previous work and knowledge gaps about organic fertilizers and their impacts on soybean and the rhizosphere bacterial communities. The three objectives of my thesis are presented at the end of this chapter.

Chapter 2 presents the results from a greenhouse experiment that addresses the first two research objectives. Chapter 3, also based on the same greenhouse experiment, addresses the third objective. Chapter 2 is followed by a transition paragraph that explains the connection between Chapters 2 and 3.

The Chapter 1 literature review was written by the candidate and edited by her two supervisors Dr. Cynthia Kallenbach and Dr. Joann K. Whalen. Both Chapters 2 and 3 were co-authored by the candidate, Dr. Kallenbach and Dr. Whalen.

The greenhouse experiment was designed by the candidate with the guidance of Dr. Whalen. The soybean care, sampling and sample processing were carried out by the candidate, with the help of several occasional assistants. The gas sampling was analyzed by the candidate with the help of Mr. Yvan Gariepy. With the guidance of Dr. Kallenbach and Dr. Whalen, the candidate carried out all laboratory experiments and statistical analyses for the experiments. The data interpretation and preparation of both manuscripts was done by the candidate under the supervision of Dr. Whalen and Dr. Kallenbach.

General Introduction

Fertilizer increases soybean yields. Organic fertilizers, such as those derived from municipal wastes, are a complete fertilizer that contains all essential elements for plant growth. Soybean yield increased by 11–31% with the application of 120 kg N/ha of biosolids, an organic fertilizer, compared to unfertilized controls (Currie et al., 2003). In addition to providing plant-available nutrients, organic fertilizers contains carbon-rich substrates and other nutrients, which stimulate the growth of heterotrophic bacteria in soil (Esperschütz et al., 2007; Seaker & Sopper, 1988). Furthermore, organic fertilizers contain exogenous bacteria that may survive after application to agricultural soil, adding to the diversity of the indigenous soil bacterial community (Gandolfi et al., 2010; Wolters et al., 2018). Thus, applying organic fertilizer to soybean is expected to improve crop nutrition and growth, while supporting a larger and more diverse population of soil bacteria.

The rhizosphere bacterial community is composed almost exclusively of bacteria recruited from the bulk soil (Sugiyama, 2019). However, I expect differences in the composition and functions of bacteria found in these two soil compartments. Soybean specifically curates the rhizosphere bacteria to support its needs, thus, its rhizosphere bacterial community is generally less diverse and represents a subset of indigenous bacteria present in the bulk soil (Marilley et al., 1998). Soybean is known to attract plant growth promoting bacteria from the bulk soil to the rhizosphere through the release of specific organic carbon and signal biomolecules (Pérez-Jaramillo et al., 2016; Sugiyama, 2019), presumably to aid the plant with one or more functions which it cannot do for itself, such as nutrient acquisition and abiotic stress tolerance (Pérez-Jaramillo et al., 2016). Thus, identifying whether organic fertilizers provide exogeneous bacteria that are recruited into the soybean rhizosphere bacterial community is a first step to understanding

if there is a biological basis for organic fertilizers to enhance soybean tolerance to sub-optimal growing conditions.

It is desirable for soybeans to cultivate a rhizosphere bacterial community that can assist it in combating abiotic stress. Some rhizosphere bacteria produce 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase), an enzyme that degrades 1-aminocyclopropane-1-carboxylate (ACC); the precursor to ethylene. Soybean produces ethylene to suppress its growth under stressful conditions, such as heat or water stress (Arraes et al., 2015). Ethylene causes a positive feedback by inhibiting photosynthesis and other metabolic functions, resulting in a shorter lifespan, which reduces the soybean meal and oil yield (Djanaguiraman & Prasad, 2010b). Soybeans that have ACC deaminase-producing bacteria in their rhizosphere should experience less growth inhibition when exposed to abiotic stress. Organic fertilizers may increase the ACC deaminase activity of soybean rhizosphere bacteria because they supply organic carbon and nutrients that can be metabolized by ACC deaminase-producing bacteria in soil, which is expected to increase bacterial counts (Lazcano et al., 2013), and they might be an exogenous source of ACC deaminase-producing bacteria. Consequently, the ACC deaminase-producing bacteria in the soybean rhizosphere should have more tolerance to heat and water stress in soil receiving organic fertilizer than in soil with no fertilizer or N fertilizer only.

Less soybean growth inhibition due to abiotic stress is expected when soybean is planted in soil receiving a complete, nutrient-rich organic fertilizer than with N fertilizer only. Additionally, greater ACC deaminase activity in the soybean rhizosphere and a unique composition of bacterial communities in bulk and rhizosphere soils under soybean is expected in soils amended with organic fertilizer than with N fertilizer only. The general objective of my thesis is to determine if organic fertilizers protect soybean from the negative consequences of heat and

water stress by supporting the activity of ACC deaminase-producing bacteria in their rhizosphere bacterial community. The specific objectives of this thesis were 1) to determine the morphological responses of organic-fertilized soybeans exposed to heat and water stress, 2) to evaluate the ethylene production by soybean and the ACC deaminase activity of soybean rhizosphere bacteria in soil receiving organic fertilizer versus N fertilizer only and 3) to determine if fertilizer sources alter the composition of the soybean rhizosphere bacterial community.

Chapter 1

Soybeans undergoing heat and drought stress produce ethylene which can negatively alter crop development and yield. New approaches, specific to soybean need to be considered to determine how stress ethylene can be decreased and prevent yield losses.

1.1 Soybean: A Plant That Experiences Abiotic Stress

Soybean is an economically important crop in Canada that generated $5.8 billion of revenue in 2014 (MNP LLP, 2016). This profitable oil seed contains approximately 20% oil, which is extracted for human consumption, and the protein-rich residue is then crushed into meal to feed livestock. Soybean is the fourth most planted crop by acreage in Canada (Statistics Canada, 2019). From 2015 to 2019, the national average soybean production was 6.85 million metric tonnes grown by more than 31 000 Canadian farmers (Soy Canada, 2019; Statistics Canada, 2019). Soybean is grown primarily in Ontario and Quebec with growers in both provinces producing, on average, 1.40 and 1.28 t ha^{-1} in 2018, which is greater than the national average of 1.16 t ha^{-1} (Statistics Canada, 2019). However, climate change is expected to affect soybean productivity in Eastern Canada because this crop is susceptible to heat and drought stress, both of which can reduce yield (Arraes et al., 2015; Djanaguiraman & Prasad, 2010b). For example, a warmer than average July in 2019 was responsible for a 14% reduction in soybean yields on farms in Ontario and Quebec (Statistics Canada, 2019). It is predicted that each increase of +1°C in global temperature will reduce global soybean production by 3.1% (C. Zhao et al., 2017).

The impacts of global warming will likely be negative for Canadian soybean producers in Ontario and Quebec. The expected increase in average and maximum temperatures predicted in this region, coupled with drier summers (The Prairie Climate Centre, 2019), will reduce soybean

growth. In addition, more frequent and severe fluctuations in temperature and longer intervals between precipitation events will reduce soybean yields. At the present time, 26-43% of the year-to-year variability in soybean yield produced in the top five soybean-producing countries (U.S.A, Argentina, Brazil, India and China) can be explained by variability in weather (Ray et al., 2015).

Nutrient deficiencies may also exacerbate soybean yield losses during hot, dry growing seasons (da Silva et al., 2011). Typically, soybean is under-fertilized (relative to non-leguminous grain crops) because it can fix N_2 through biological nitrogen fixation and is expected to absorb the residual nutrients left from the corn or cereal crops grown the previous year. Future weather projections for Canada suggest that there will be more precipitation during the winter and spring months (The Prairie Climate Centre, 2019), which could increase water-borne nutrient losses and therefore diminish the residual nutrient supply for soybean. Future summers are predicted to be hotter and drier, and a lack of soil moisture is expected to decreases the rate of nutrient diffusion from the soil matrix to the root surface where nutrient absorption occurs (Chapin, 1991), thus limiting nutrient uptake by soybean roots. Additionally, the rate at which nutrients can be translocated within the plant is also reduced under low-water conditions. Therefore, soybean is vulnerable to stress not only due to the high heat and low water conditions, but also because it may become nutrient deficient. Consequently, climate change will likely increase heat and water stress on soybeans and should continue to be closely monitored.

1.1.1 Soybean Response to Heat Stress

Heat stress is a sudden rise in the temperature in the environment (air, soil) that affects physiological and biochemical processes of a plant. The severity of heat stress is a function of the intensity (temperature) and the duration of exposure (Colombo & Timmer, 1992). Low to moderate heat stress (caused by either low intensity temperatures for a long period of time or high

temperature for a short period of time) leads to abnormal plant growth and earlier maturation, which tends to lower the yield (Kitano et al., 2006). Extreme heat stress (caused by high temperatures for too long) is associated with permanent wilting and death of the plant (Deryng et al., 2014).

1.1.1.1 Heat Stress Affects Soybean Photosynthesis

The photosynthetic capacity of soybean is affected by heat stress. High temperature initiates a molecular signal cascade that produces reactive oxygen species (ROS) such as superoxide radicals and H_2O_2. The ROS damage the photosystem II in thylakoid membranes, which reduces photosynthetic capacity. In addition, the ROS affect lipid peroxidation and thus increase the permeability of mitochondrial and plasma membranes, alters the electrochemical gradient and resulting in cell death (Nahar et al., 2011). Dead cells are incapable of providing photosynthate. At the flowering stage, soybean under heat stress (38°C daytime /28°C nighttime) for 2 d had a 7.4% lower photosynthesis rate and those exposed to the stress for 10 d had 19.6% lower photosynthesis rates compared to soybean grown at 28°C daytime /18°C nighttime conditions (Djanaguiraman, Prasad, & Al-Khatib, 2011). When there is a reduction in photosynthate production during flowering, an early reproductive growth stage, soybean may abort its young flowers, seeds and pods. Thus, heat stress alters the phenology of soybean and shortens its lifespan.

1.1.1.2 Heat Stress Affects Soybean Yield

Depending on the time of onset and duration, heat stress can have drastic consequences on the whole plant and thus the yield (Hasanuzzaman et al., 2013). After germination, the optimal temperature for soybean growth is 20-30°C (Schlenker & Roberts, 2009). Soybean vegetative

growth is optimal at 30°C whereas reproductive phases (R1-R8) have an optimal temperature of 26°C (Hatfield et al., 2011). Although soybean is a relatively heat resistant crop, it is susceptible to stress during the reproductive stages between flowering and seed filling (R1-R6). For example, soybean exposed to day/night temperatures of 38°C/28°C during pod set stage (R4) for a period of 14 d produced ≥ 70% less oilseed (Djanaguiraman & Prasad, 2010b). Furthermore, heat stress during the R1-R6 reproductive stages is associated with a 7.2% increase in seed protein content, a 3.4% decrease in oil content and alters the proportions of the fatty acid components of the oil (Dornbos & Mullen, 1992; GunHo et al., 2012). In contrast, soybean exposed to heat stress at full maturity (R8 reproductive stage) have lower reductions in protein and oil content and overall yield since the soybeans are senesced (Teramura et al., 1990). Consequently, heat stress is going to have more drastic effects on the final oilseed yield when the heat stress occurs during the R1-R6 reproductive stages than at other times during soybean development.

1.1.2. Soybean Response to Drought Stress

Drought stress is associated with low soil moisture content, resulting in an inadequate water supply for the normal physiological and biochemical processes in a plant. Drought stress can be intermittent, occurring for short periods when evapotranspiration exceeds the water supplied by precipitation and irrigation. However, prolonged drought stress is not uncommon, particularly in dryland agriculture systems without irrigation. Low to moderate drought stress, is associated with changes in plant physiology and can result in marginally higher or lower yields. In contrast, when the drought duration is extended and intensity is more severe, this can result in altered phenology and a shorter lifespan or the plant may reach its permanent wilting point and die (Szabó & Basal, 2018).

1.1.2.1 Drought Stress Affects Soybean Photosynthesis

Drought stress, like heat stress, impacts the soybean photosynthetic capacity and thus limits the growth of cells, tissues and organs. When soybean plants do not acquire enough water, they lose turgor pressure between the cell wall and membrane. To preserve water, the stomates close, which limits gas exchange. The limited amount of CO_2 entering the leaves slows photosynthesis and reduces the quantity of carbohydrates available for cellular growth and reproduction. The lack of CO_2 also exposes chloroplasts to excess energy excitation, producing ROS (Anjum et al., 2011; Muller et al., 2011; Rucker et al., 1995). Hence, photosynthesis is impaired because of the ROS damage to their chloroplasts and photosystem II (Fahad et al., 2017). The plant's dilemma is how to conserve water while permitting gas exchange to acquire enough CO_2 to support its growth. The duration and intensity of drought stress will determine the growth rate and lifespan of soybean (Colom & Vazzana, 2001).

1.1.2.2 Drought Stress Affects Soybean Yield

Depressed photosynthesis rates and accumulation of ROS during drought events will ultimately lead to a yield reduction in all crops, including soybean. However, drought-associated yields losses also depend on when plants are exposed to drought during their life cycle. In soybeans, the effects of drought are most detrimental during the reproductive stages (R1-R6) from flowering to seed filling. Drought stress during these reproductive stages limits the size of seeds and the number of seeds produced. Brown et al. (1985) found that four soybean cultivars exposed to drought stress at the R2 stage had 16.0–22.8% fewer seeds and up to 6.4% smaller seeds on average, while the same cultivars that experienced drought stress at the R4 stage had 14.2–51.4% fewer seeds and an 5.6–19.2% reduction in seed size. Since soybean that produce fewer, smaller

seeds have lower yields, drought stress during the reproductive stages could reduce soybean yield by 30-80% (Brown et al., 1985; Desclaux et al., 2000).

1.1.3 Soybean Response to Combined Heat and Drought Stress

1.1.3.1. Soybean Physiological Response to Combined Heat and Drought Stress

Abiotic stress caused by two or more individual stressors can exacerbate the negative outcomes for soybean (Cohen et al., 2019). Each abiotic stress evokes a unique change in expression of regulatory genes in the plant (Zandalinas et al., 2018), and it is commonly reported that combined heat and drought stress alter the gene expression, and thus the metabolism of the plant, in a way that is distinct from heat stress alone or drought stress alone (Rizhsky et al., 2002; Xu & Zhou, 2006). Although heat and drought stress both produce ROS within cells, the ROS tends to originate from different organelles. For instance, ROS comes from the chloroplasts of plants under heat stress whereas drought stress is associated with ROS production in chloroplasts, mitochondria and peroxisomes (Fahad et al., 2017; Laxa et al., 2019).

In addition, combined heat and drought stress may cause contradictory signals in the plant as it responds to alleviate stress. For example, soybean experiencing heat stress will open their stomates and transpire to cool their leaves. However, soybean under drought stress must close their stomates to prevent water loss (Signorelli et al., 2015). Thus, combined heat and drought stress is expected to be more damaging for soybean than each stress alone, since the plant may not be able to use its most efficient or preferred mechanism to alleviate the stress.

1.1.3.2. Combined Heat and Drought Stress Affect Soybean Yield

There are no studies about the soybean response to combined heat and drought stress. In other field crops, it appears that combined heat and drought stress is more damaging than exposure

to these stressors individually. Greenhouse grown barley exposed to both elevated temperature (40°C for 6h/d) and drought for a period of 10 d had a 30% reduction in grain weight, which is more severe than the 5% reduction in grain weight of barley grown under the elevated temperature only and the 20% reduction in grain weight of barley exposed to drought only (Savin & Nicolas, 1996). The negative synergy of combined heat and drought on corn yield under field conditions was demonstrated by Obata et al. (2015), who tested 10 maize cultivars with variable tolerance to heat and drought during two growing seasons. In the first year, heat stress reduced corn grain yield by 12.9%, drought lowered corn grain yield by 54.3% and the combined heat and drought treatment had 94.3% less corn grain yield than the non-stressed corn. In the second year, heat stress was mild and did not affect the corn grain yields, so the combined heat and drought stressed plants had similar yield to corn grown with drought stress (Obata et al., 2015). Thus, a low level of a particular abiotic stress may not induce any difference in yield when evaluated alone or in combination with another stress. Since combined heat and drought stress can be highly detrimental to the yield in other crops, we may expect soybean to respond similarly or more severely. As soybean is a C3 plant we might expect more consequences than C4 plants, such as corn, under combined stress as the C4 physiology allows for higher photosynthetic capacity at high temperatures and has a higher photosynthetic use efficiency of water under drought stress than C3 plants (Lattanzi, 2010).

1.1.4 Soybean Produces Ethylene in Response to Stress

Plants respond to stress through various physiological and biochemical pathways, but universally respond to stress by producing the phytohormone ethylene. Stress that affects leaf functions, such as lower photosynthetic capacity due to elevated temperature (heat stress) result in ethylene production (Djanaguiraman, Prasad, Boyle, et al., 2011). Drought stress has a two-fold effect – first, it is detected in the roots due to lack of water and second, leaves are notified by root-

leaf signaling via transpiration streams, which leads to stomatal closure to reduce water loss (Flexas et al., 2002). Although the plant senses heat and drought stress differently, both elicit ethylene production. The production of ethylene is a signal to the plant to down-regulate energy-demanding reactions and therefore conserve energy. When the stress is prolonged or severe, ethylene production can be responsible for changes in the plant's phenology, including a shorter lifespan and smaller yield.

Interpretation of the heat and drought stress effects on ethylene production in soybean can be confounded by other abiotic stresses. For instance, deficiencies in P, Fe, K, S and B lead to ethylene production in other plants including other Fabaceae (Martín-Rejano et al., 2011; Maruyama-Nakashita et al., 2006; Romera, 1999; Shin & Schachtman, 2004; Zhang, 2003). In other crops, both N deficiency and excess N may trigger ethylene synthesis (Tian et al., 2009; Zheng et al., 2013), and plants are more sensitive to ethylene production when they have a N deficiency (Schmelz et al., 2003). Similar responses may also occur in soybean. I predict that nutrient deficient conditions will trigger soybean to produce more ethylene and/or exacerbate the damage due to heat or drought stress, but this needs to be confirmed experimentally.

1.1.4.1 Production of Ethylene

Ethylene synthesis begins with the transformation of the amino acid methionine to *S*-adenosyl-L-methionine (SAM) by the enzyme SAM synthase. This product is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by the enzyme 1-aminocyclopropane-1-carboxylate synthase which is the rate limiting step (Dubois et al., 2018).

Ethylene is a signal for a plant to start or stop many of its developmental functions. For instance, ethylene is responsible to starting functions like germination, flowering and fruit development, while similarly being responsible for stopping functions related to flowering and

nodulation (Abeles, 1973; Ferguson et al., 2013; Locke et al., 2000). Additionally, when plants experience heat or drought stress, ethylene production is a mechanism that can cause a vegetative plant to cease vegetative growth and begin reproductive growth. The intensity and duration of the stress impacts the quantity and duration that ethylene is produced for and thus impacts how the plant responds. Initially, the concentration of stress ethylene increases slightly in the first 1-3 d of stress exposure. This period does not last long, as the small increase in ethylene is due to pre-existing ACC, its precursor, and is rapidly depleted. Once ACC is produced by the organ experiencing stress, it is transported in the xylem to the plant tissues that need to conserve energy, where it is converted into ethylene by 1-aminocyclopropane-1-carboxylate synthase (S. F. Yang & Hoffman, 1984). This small increase triggers the transcription of genes that allow the plant to prepare and defend itself (Robison et al., 2001). If stress continues past this threshold, a larger, and better-detectable increase in ethylene concentration occurs (Gamalero & Glick, 2012). This second peak reduces a plant's growth rate and initiates senescence or abscission of plant organs to conserve resources (Druege, 2006; Ozga et al., 2016). In this way, ethylene signaling affects the growth of leaves and shoot by reducing their photosynthesis rate (Pettigrew et al., 1993). Plants in reproductive growth stages that are exposed to ethylene will stop flowering and initiate seed set, since ethylene targets immature flowers, pods and seeds (Prasad et al. 2002), and causes leaf senescence. The plant thus begins prioritizing its reproduction, in case resources deplete (Glick et al., 2007). Thus, the plant shifts from growth to reproduction, thereby shortening its lifespan.

Ethylene signaling due to heat or drought stress can have similar, as well as, opposing outcomes on plant growth and development. For example, ethylene produced by heat and UV tends to initiate premature leaf senescence (Djanaguiraman & Prasad, 2010b). Conversely in drought, the production of ethylene causes leaf abscission to minimize water loss from leaves (Oh et al.,

1997) and halts shoot growth (Sharp & LeNoble, 2002). Heat and drought stress ethylene signaling do target some organs the same way. Most importantly, in both heat and drought stress, immature flowers and seeds will be aborted (Liu et al., 2004; Prasad et al., 2002).

1.1.4.2 Activation of Ethylene

Low doses of ethylene elicit unique plant responses, depending on the type of stress, which help the plant to achieve energy conservation in relation to the particular stress. It begins with ethylene binding to one of the five types of ethylene receptors on the membrane of target cells (Arraes et al., 2015). Ethylene binding to a specific receptor induces the expression of two primary transcription factors responsible for expression of a many ethylene response factors (ERFs). Complete functions of the ~68 genes involved in ethylene signal transduction in soybean is still being discovered (Arraes et al., 2015) but in general, they initiate numerous signaling cascades that lead to stress-specific outcomes for the plant (Müller & Munné-Bosch, 2006). For example, ERF8, an ERF that strongly inhibits cell division and growth in leaves is stimulated specifically under drought stress conditions (Dubois et al., 2018). While this is a fascinating example of plant stress adaptation, the study of ethylene response factors is beyond the scope of this thesis.

1.1.5 Heat and Drought Stress Produce Ethylene in Soybean

Ethylene stress response and signaling varies among crop species, and there are relatively few studies that relate directly to soybean (Arraes et al., 2015; Djanaguiraman & Prasad, 2010a). However, soybean will probably exhibit similar patterns to other plants. Arraes et al (2015) studied the similarity of soybean ethylene signaling with other plant species. They found that soybean has 178 genes related to ethylene synthesis and signal transduction. Yet, only 38.3% of the genes responsible for signal transduction in soybean were important in other model organisms like

Arabidopsis (Arraes et al., 2015). This suggests that soybean may have unique ethylene signaling pathways, although genetic divergence does not necessarily imply a different functional response of soybean to ethylene, compared to other plants.

In their study, Djanaguiraman and Prasad (2010a) confirmed the importance of ethylene in signalling heat and drought stress in soybean during R3. Soybeans under heat stress (38°C daytime/28°C nighttime) for 6 d produced 3.4-fold more ethylene after 1 d of stress and 1.7-fold more ethylene after 6 d of stress compared with those under normal temperature conditions (28°C daytime/18°C nighttime) (Djanaguiraman & Prasad, 2010a). This suggests that ethylene production in soybean causes an immediate cession of the most critical functions, and the lower level of ethylene production in soybean with ongoing heat stress assures that critical functions remain shut down. Furthermore, the ethylene production was greater in soybean leaves (6.11 nmol g^{-1} fresh weight h^{-1}) than in pods (1.54 nmol g^{-1} fresh weight h^{-1}) and flowers (205 pmol g^{-1} fresh weight h^{-1}) (Djanaguiraman & Prasad, 2010a). This suggests that leaf functions were the most energy-demanding process in soybean at the R3 growth stage and need to be down-regulated. This study also demonstrated that stress during R3 that reduces photosynthate in the leaves consequently also diminishes yield by reducing the number of pods per plant and the number of seeds per pod.

Since Djanaguiraman and Prasad (2010a) did not quantify ethylene production from soybean roots, this remains an intriguing possibility for future study for two reasons. The quantity of ethylene produced by the root system is of interest as it is likely the only ethylene that may come into direct contact with the root microbiome. Some bacteria located in the rhizosphere are able to degrade the ethylene precursor and thereby increase the threshold of stress (and ACC) required to create negative physiological and phenological changes for soybean (Glick, 1995).

1.2 Soybean-Microbial Interactions in the Rhizosphere

1.2.1 The Soybean Rhizosphere

The soybean root system grown in loamy soil in a humid continental climate has a total root length of ~680 m, giving a large surface area in contact with the soil matrix (Arya et al., 1975). As a dicotyledonous species, the soybean root system is composed of a main taproot that may extend in the soil to a depth of 1.8 m, surrounded by shallow fibrous roots that extend laterally 0.25-0.50 m from the taproot at angles of 40-60° (Andjelkovic & Putnik-Delic, 2018; University of Wisconsin, 2015). The root-associated soil (up to 80 mm away from the root surface) is called the rhizosphere (Koo et al., 2005). Nodule protrusions that contain N_2-fixing bacteria are an additional morphological feature that distinguishes the root system of soybeans and other legumes from the root system of non-legume plants.

The rhizosphere is a dynamic environment. Throughout the plant lifespan, root tips elongate continuously. Evapotranspiration pulls water and nutrients towards the fine roots, where they are absorbed into the root cortex through symplastic and apoplastic processes. The hydrated, nutrient-rich environment of the rhizosphere has distinct chemical and biological properties. Soybeans release 5-20% of their organic carbon into the rhizosphere through rhizodeposition (Shamoot et al., 1968). Organic carbon compounds attract microbes and support elevated biological activity and approximately two times larger bacterial populations and greater diversity in the rhizosphere compared to the bulk soil (Breidenbach et al., 2016). These rhizosphere microbes may be free-living or associated with the roots as endophytes. The rhizosphere contains three zones inhabited by microbes: the endorhizosphere (inside root tissue, within the endodermis and cortical layers), the rhizoplane (the root surface and epidermal tissues) and the ectorhizosphere (in the soil near the root) (Lynch, 1987).

1.2.2 Root Microbiome of The Soybean Plant

The bacteria, fungi, and archaea that inhabit the rhizosphere, collectively called the root microbiome, contribute to the proper nutrition of the plant and reduce its stress responses while ensuring their own survival. Rhizosphere microbes are a specialized community and those that provide direct benefits to the plant are called plant growth-promoting rhizobacteria (PGPR). These PGPR may occur naturally (in the soil, in the seed) or be added artificially through the use of inoculants.

The soybean rhizosphere soil bacteria community composition is not stagnant and is susceptible to changes in the plant's development and the environment. First, the plant recruits most of its rhizosphere bacteria from the bulk soil, so the rhizosphere bacteria community depends on the inherent soil bacteria community (De Ridder-Duine et al., 2005; Koranda et al., 2011). Second, the nature of the relationship between the plant and specific microbes changes during the plant's life, depending on the growth stage. Sugiyama (2019) noted that the soybean rhizosphere bacteria community and diversity change from the vegetative stages to maturity) (Sugiyama, 2019). During soybean development, Proteobacteria increase in abundance while the number of Acidobacteria and Firmicutes decrease (Blaha et al., 2006; Sugiyama et al., 2014). Although soybean cultivars may initially have distinct bacterial community structure, their rhizosphere bacteria community tends to become similar as they mature (Chaparro et al., 2014). Third, climate and abiotic stress can influence the type of bacteria recruited into the root microbiome, suggesting that soybean may secrete specific molecules that foster root colonization by specific bacterial species, to improve its stress tolerance (Marasco et al., 2012; Sugiyama, 2019).

Like all plants, soybean controls its rhizosphere bacteria community by modulating the kinds of root exudates (stimulatory or inhibitory) it releases, which alters the bacterial populations

and their quorum sensing behaviour (Gao et al., 2003). However, the symbiotic association with N_2-fixing bacteria in soybean further distinguishes the soybean rhizosphere bacterial community from other plants (Reinhold-Hurek et al., 2015). Unlike non-legume plants, the soybean root microbiome often contains large proportions of rhizobia (free-living and within nodules). The two most common bacterial phyla in the soybean root microbiome are Proteobacteria (which includes rhizobia) and either Actinobacteria or Planctomycetes, according to studies done on field-grown soybean in Brazil, Japan and China (Liang et al., 2018; Mendes et al., 2014; Sugiyama et al., 2014). Furthermore, Sugiyama et al. (2014) and Liang et al. (2018) identified the core genera of their soybean rhizosphere samples to be *Bradyrhizobium*, *Steroidobacter*, *GP6*, *GP4*, *Comamonmadaceae*, *Sphingomonadaceae* and *Sphingomonas*. Many of the bacteria in those soybean rhizosphere samples are PGPR, however, the rhizosphere may still contain plant pathogens and neutral bacteria who provide no benefit nor disadvantage to the plant (Sugiyama, 2019).

Soybean rhizosphere bacteria are known to modulate at least 28 functions in the soybean plant, including disease defence, increasing the availability of various nutrients such as N, P and K and stress mitigation, such as through ACC deaminase activity (Mendes et al., 2014; Rousk et al., 2009). The population size and relative abundance of particular bacterial taxa can be indicative of their functions. For example, some *Bradyrhizobium*, which are abundant in the soybean rhizosphere possess the ability to degrade soybean's stress ethylene through ACC deaminase production. The approximate functional potential of bacterial genera may be inferred from marker genes using bioinformatic tools such as PICRUSt2 or FAPROTAX (Douglas et al., 2019; Louca et al., 2016). Although the presence of bacteria does not confirm that they are actively contributing to the plant's health, the fact that they co-exist in the rhizosphere may lead us to assume that they

are contributing benefits at some point during the plant's lifespan. Thus, shifts in the rhizosphere bacteria community will likely impact plant functions and are relevant for my thesis research.

1.2.3. ACC Deaminase and its Function in the Soybean Rhizosphere

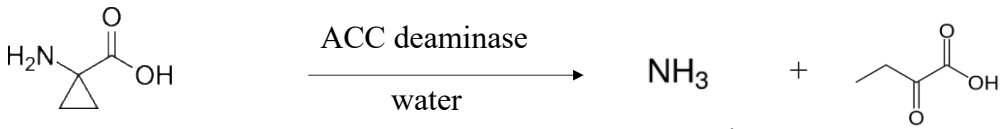
Plants experiencing abiotic stress release a variety of chemicals into the rhizosphere, including ethylene (Glick, 2012). In response, some PGPR secrete enzymes into the rhizosphere that help mitigate the plant's stress-induced physiological response. In their metagenomics survey of 12 soybean rhizosphere soil bacteria samples, Mendes et al. (2014) found that 2.7% of rhizosphere bacteria had functional genes for stress tolerance. The primary way that rhizosphere bacteria reduce stress is through the production of extracellular enzymes such as ACC deaminase, which reduces ethylene produced due to abiotic stress.

1.2.4.1 ACC Deaminase Reaction with ACC (Ethylene)

Many PGPR produce ACC deaminase, particularly when they are exposed to elevated ACC concentration in the root tissue or the rhizosphere (including the soil solution around the roots). ACC deaminase is capable of cleaving ACC, the ethylene precursor, into ammonium and α-ketobutyrate Figure 1.1). The affinity of ACC for ACC deaminase is quite low with a Km of 1.5-17.4 mM at a pH of 8.5 (Honma & Smmomura, 1978). Hydrolysis of ACC by ACC deaminase prevents the production of ethylene, reducing the plant's stress response in the rhizosphere and allows root growth to continue and prevent the shortening of soybean's lifespan.

The ACC deaminase produced by rhizosphere soil bacteria degrades ethylene until it is no longer available. The bacterial gene acdS encodes for ACC deaminase production and has sophisticated regulation, which ensures that it is produced in the presence of ACC and that it is not

synthesized in excess when it is not required. Thus, in the absence of stress, ACC deaminase secretion decreases to before-stress levels.



1-Aminocyclopropane-1-carboxylic acid
disubstituted cyclic α-amino acid

α-ketobutyrate

Figure 1.1 Reaction of ACC hydrolysis by ACC deaminase.

1.2.4.2 Soybean Rhizosphere Bacteria Possessing the acds Gene and ACC Deaminase Activity

The bacterial gene acdS that encodes for ACC deaminase production is relatively common across bacterial phyla. However, many bacteria that possess acdS do not express the gene and thus do not synthesize ACC deaminase in the presence of ACC (Li et al., 2015). Selected proteobacteria cultured *in vitro* do secrete ACC deaminase, including *Pseudomonas*, *Enterobacter*, *Kluyvera, Rhizobium, Burkholderia,* and *Azospirillum* (Blaha et al., 2006; Burd et al., 1998; Glick, 1995; Penrose & Glick, 2003; Wang et al., 2001). Additionally, *Pseudomonas* spp, *Rhizobium* spp display ACC deaminase activity in the rhizosphere of pea plants (Arshad et al., 2008; Ma et al., 2003). Although ACC deaminase activity in the soybean rhizosphere has yet to be quantified, its rhizosphere is colonized by PGPR in the Proteobacteria phyla, some of which exhibit ACC deaminase activity in the rhizosphere of other plants.

Rhizobia are dominant bacteria in the soybean root-microbiome and are also known to be ACC deaminase producers. Their production of ACC deaminase is thought to stem from the fact that elevated ethylene levels inhibit the establishment of nodules and may have occurred

evolutionarily independently from the ACC deaminase producing activity of other producers who are singularly free-living (Ma et al., 2003). However, the level of ACC deaminase they produce under desirable conditions varies substantially but is generally lower than that of other free-living PGPR (between 10-100 fold less) (Nascimento et al., 2016). Nevertheless, since rhizobia are prevalent in the rhizosphere of legumes, their ACC deaminase activity when in free-living form could be quite important for the stress tolerance and survival of the plant.

Although ACC deaminase activity is often associated with PGPR, some exceptions exist. Some opportunistic plant root pathogens also possess the acdS gene and ACC deaminase activity. By producing ACC deaminase, root pathogens will eliminate ACC and prevent ethylene production in the rhizosphere, which allows for continued root growth for the benefit of the pathogen (Singh et al., 2015). Furthermore, some fungi, such as *Penicillium citrinum and Trichoderma asperellum* also have the acdS gene and exhibit ACC deaminase activity (Singh et al., 2015). It should be noted that my thesis research could identify bacterial root pathogens or fungi contributing to ACC deaminase production.

1.3 Organic Wastes as Organic Fertilizers for Soybean Production

As in other parts of the world, municipalities in Quebec are looking for sustainable solutions for disposing of organic wastes. By 2020, organic wastes will no longer be incinerated or accepted at landfills in Quebec (Taillefer, 2014; Villeneuve & Dessureault, 2011). A viable option for disposal of these various organic wastes is to transform them into nutrient-rich organic fertilizer and apply them to agricultural land. Organic fertilizers are a valuable input to the soil because they provide nutrients for plants and soil microbial communities and, unlike inorganic fertilizers, a source of C for heterotrophic microbial communities, which are often C-limited in agricultural soils. Plants require mineralizable forms of N such as NH_4^+-N and NO_3^-N, to support

their growth. However, all heterotrophic microbes require both organic C and organic N to carry out their metabolism. Soil contains plant-available nutrients, which are absorbed by plants, as well as organic matter that microbes degrade as a slow-release source of plant-available nutrients. Microbially-mediated nutrient mineralization is partly regulated by the quantity and chemistry of organic C compounds. Organic fertilizers from different sources and treatment processes will vary in the composition of mineralizable nutrients and organic matter (Bustamante et al., 2010), which has consequences for microbial activity and the production of plant-available nutrients.

Biosolids are a type of organic fertilizer that originate generally from sewage sludge and are mainly composed of metabolized human wastes. On average, biosolids contain 4.74% total N, 2.27% total P and 0.31% total K (Stehouwer et al., 2000). However, biosolids have high levels of organic matter, meaning that much of their nitrogen and carbon are in organic forms. Biosolids from wastewater recovery plants must be treated before they are safe to handle and apply to agricultural land. Mechanical shearing with lime stabilization are often used to break down the raw sludge into nutrient forms that plants and microbes can metabolize, while providing the necessary sanitation of the waste (Lystek Int., 2018). Digestion is another common treatment for biosolids and may occur under either anaerobic or aerobic conditions. Aerobic digestion usually results in organic fertilizers that have more mineralized N (36.1-60.8% of total N added) than anaerobically digested fertilizers (13.7-25.2% of total N added) (Magdoff & Chromec, 1977). Composts, such as those generated from food scraps and yard waste, are another type of organic fertilizer. They have high organic matter content (>40% total dry weight) and N, P and K account for approximately 1%, 0.4% and 0.3% of the total dry weight (Furrer & Gupta, 1983). A large proportion of the N in compost is organic N, with typically 3.0– 8.1 % mineral N, mainly NH_4-N (Selim et al., 2012; Sullivan et al., 2018).

Biosolids and compost are beneficial for soybean production, since soybean cannot obtain enough N from N_2 fixation to reach a maximum yield (approximately 40–75% of N in soybean tissue is derived from soil (Harper, 1974)). In contrast to inorganic N fertilizers, which are generally applied in small doses to meet the crop requirements for the current growing season, organic fertilizer contain mineral N for this growing season, plus organic N that will be mineralized in a future growing season. Currie et al. (2003) found that soil fertilized with a one-time application of biosolids produced greater soybean yield in that year and for the next three years (11.2–17.1% greater yield), compared to the unfertilized control (Currie et al., 2003). Thus, organic fertilizers like biosolids and compost are alternatives to inorganic N fertilizers for soybean production.

1.3.1. Organic Fertilizers Affect Soybean Rhizosphere Bacteria

Organic fertilizers may affect the soybean bulk soil bacterial community. These fertilizers may provide additional benefits over chemical fertilizers as they increase soil microbial biomass (Esperschütz et al., 2007), microbial activity (Bailey & Lazarovits, 2003) and allow for changes in the bacterial community composition of the bulk soil (Marschner, 2003). There are 2 main ways organic fertilizers may directly affect the bacterial community of the bulk soil.

Firstly, the chemical components and physical structure of organic fertilizers can feed and support the naturally occurring bulk soil microbes. Many of these microbes are heterotrophs and require breaking down organic carbon compounds to obtain energy. Unlike chemical fertilizers, organic fertilizers contain organic matter, which can feed the microbes' metabolic demands and support their population growth (Towers & Horne, 1997). Seaker and Sopper (1988) found that reclaimed mine sites amended with biosolids had much larger populations of aerobic heterotrophic bacteria (4.09-63.67x 10^6 g^{-1} soil) than mine sites amended with chemical fertilizer and lime (3.06 x 10^6 g^{-1} soil). These authors justify that the increased microbial counts and activity is due to the

high organic matter content of the biosolids. Additionally organic fertilizers, such as biosolids, contain micronutrients like zinc, copper and molybdenum that are required for soil bacteria (Canadian Council of Ministers of the Environment, 2012b; Perron & Hébert., 2007). Some micronutrients, such as zinc are required by many bacterial cofactors and thus for enzyme function (Gupta et al., 2016). Barron et al.(2009) for example, found that molybdenum is an essential cofactor for free-living and symbiotic N_2 fixing bacteria (Barron et al., 2009). Thus, the potential for organic fertilizers to increase the supply of organic C, micronutrients and organic N and thus influence the bulk soil and enrich community compositions may facilitate the enrichment of the rhizosphere bacterial community.

Secondly, organic fertilizers may impact the bulk soil community through the addition of new microorganisms to the environment. Organic fertilizers are breeding grounds for lots of heterotrophic bacteria that thrive in soil environments (Qin et al., 1995). Little (2020) found that biosolids contained their own microbiome, that include PGPR taxa previously documented to possess ACC deaminase activity. Additionally, Gandolfi et al. (2010) report that the microbes present in compost are able to colonize the soil and influenced the soil microbial community composition and its functions (Gandolfi et al., 2010).

Organic fertilizers directly impact soybean growth, which will also affect its rhizosphere bacterial community. Organic fertilizers alter soil physical and chemical properties leading to increases in the soil water holding capacity and allow soybeans to grow larger root systems (Reyes-Cabrera et al., 2017). Larger soybean roots have a larger surface area and thus have a greater volume of rhizosphere soil which microbial populations can inhabit. A larger rhizosphere soil bacteria population is able to provide more functions for the plant such as greater nutrient acquisition and thus reciprocally, help the plant grow and mature (Pathma & Sakthivel, 2012).

Larger and deeper root systems are also capable of acquiring more water. A moister environment also allows microbes to carry out more enzymatic activity and contribute more to the plant's success (Borowik & Wyszkowsk, 2016).

Viti et al. (2010) suggest that organic fertilizers like compost will increase the abundance of some PGPR in rhizosphere soils, and their functions include indole-3-acetic acid (IAA) and siderophore production. However, there is no information about the abundance of PGPR that produce ACC deaminase in the rhizosphere of soybean grown in soil amended with organic fertilizers. This will be an important topic in my thesis.

1.4 Conclusions and Thesis Objectives

Root-associated bacteria that produce the enzyme ACC deaminase are capable of decreasing ethylene, a stress hormone that is released from the root system of soybean experiencing heat and drought stress, by degrading its precursor, ACC. Heat stress is most often sensed by the leaves, whereas drought stress signaling begins with a lack of plant available water sensed by the roots (Figure 1.2). Once these organs perceive the stress, they respond with the production of ACC which travels to the organs that will manifest responses to the stress. Upon arrival at the destined organs (the leaves, shoots, flowers and pods), ethylene will initiate signal cascades that restricts the soybean's growth. The continuation of the stress or its intensification leads to the soybean altering its phenology, which shortens its lifespan. A decrease in the stress ethylene concentration will prevent the shortening of the plant's lifespan, which may lead to fewer reductions in grain yield.

The ability of associated ACC deaminase producing bacteria to reduced soybean's stress will likely depend on the type of stress (Figure 1.3). Little is known about the possibility of long-distance signaling and transportation of ACC produced distally in shoots or leaves to the root

environment which would need to occur in heat stress (de Poel & Van Der Straeten, 2014; McManus, 2012). If even possible, ACC relocation from shoot or leaves to root areas via transportation, would be considered long distance this would need to occur through travel in the phloem or xylem (if there is a lack of oxygen), and thus ACC deaminase producing bacteria perhaps would only be able to marginally lower ACC levels and thus the soybean's stress (de Poel & Van Der Straeten, 2014; Finlayson et al., 1991). Therefore, while ACC deaminase production from bacteria may help reduce ethylene levels, its effect on alleviating the soybean stress response will likely depend on the type of stress.

The prolonging of a stressed plant's lifespan by ACC deaminase producing bacteria has been studied in many crops under elevated ethylene concentrations. However, there is a gap in the literature about how heat and drought stress alter soybean's rhizosphere microbial community's ability to produce ACC deaminase and thereby decrease ethylene concentrations and prevent premature morphological and phenological changes to soybean (Figure 1.4 a).

Typically, soybean receives minimal fertilizer inputs but well-fertilized soybean is expected to develop an extensive root system that sustains a large population of rhizosphere bacteria, some of which are ACC deaminase producers. Furthermore, fertilizers that contain organic matter and micronutrients may be beneficial to the growth and activity of ACC deaminase producers. Still, there is a gap in research knowledge about how organic fertilizers alter the rhizosphere bacterial community's composition and, whether they can promote the activity of the ACC deaminase-producing bacteria in the soybean rhizosphere, and therefore degrade a greater proportion of the ACC that soybean produces when it experiences heat and drought stress (Figure 1.4 b).

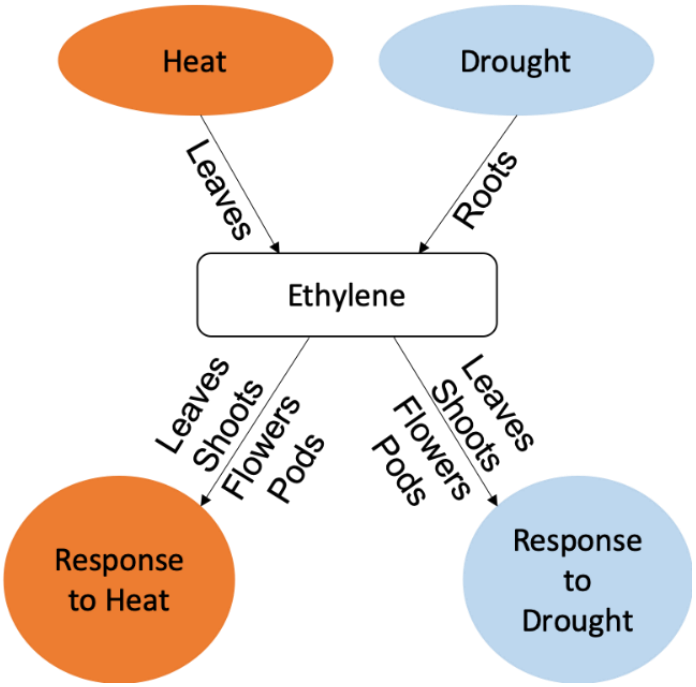


Figure 1.2 Organs involved in sensing stress and producing ethylene in response to the stress. Heat stress is most often sensed by the leaves, whereas drought stress signaling begins with a lack of plant available water sensed by the roots. Once these organs perceive the stress, they respond with the production of 1-aminocyclopropane-1-carboxylic acid (ACC) the precursor of ethylene. ACC travels to the organs that will elicit responses to the stress. Upon arrival at the destined organs (the leaves, shoots, flowers and pods), ethylene will initiate signal cascades that alter the phenology of the plant, shortening its lifespan. This has consequences for the quantity of soybean meal and oil produced. Arrows indicate outcomes.

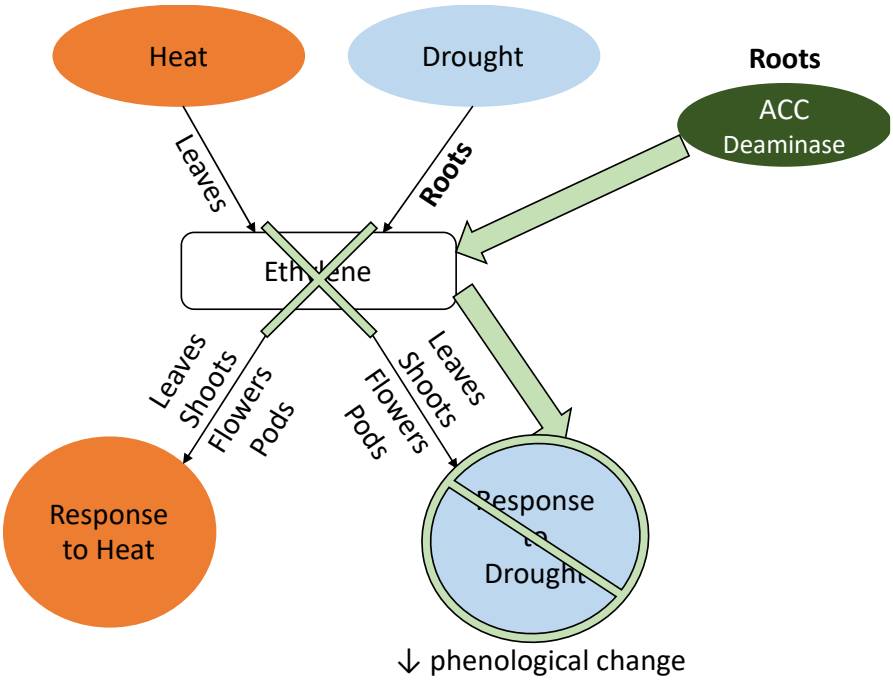


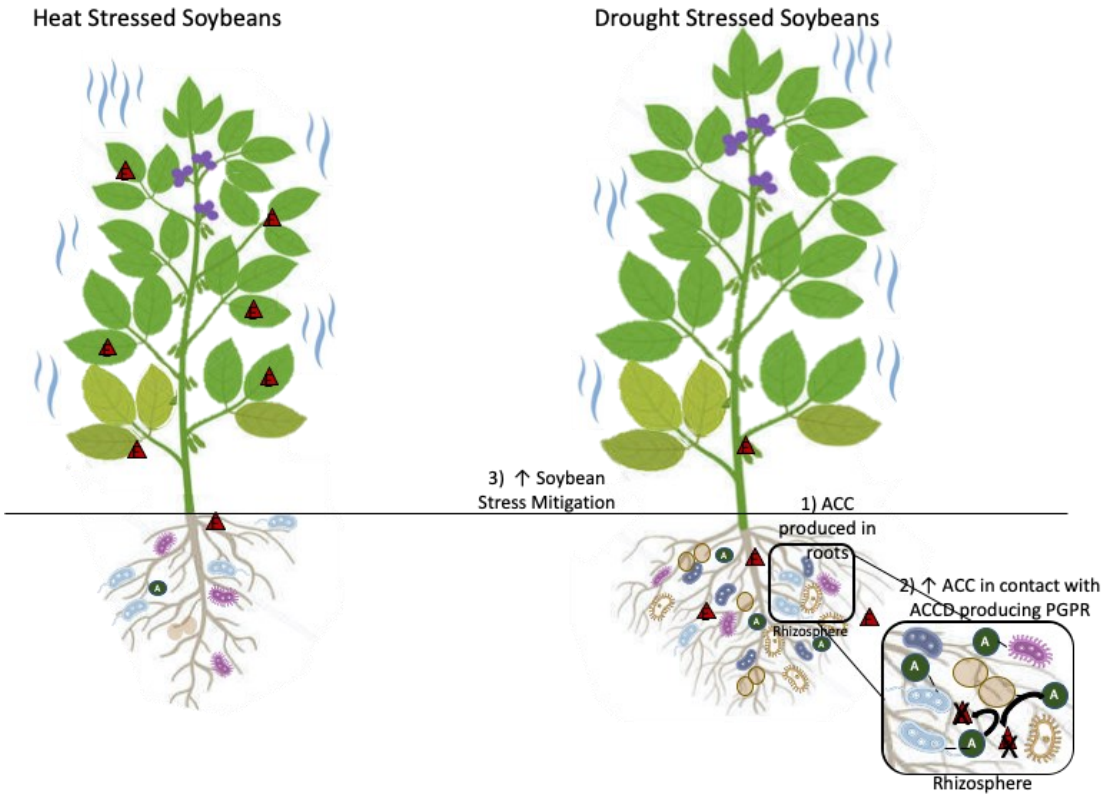
Figure 1.3. Drought stressed plants may be more protected from the impacts of stress ethylene than heat stressed plant. Heat stress is most often sensed by the leaves, whereas drought stress signaling begins with a lack of plant available water sensed by the roots. Bacteria that produce ACC deaminase (ACC deaminase) live in proximity to the roots and may come in contact with more 1-aminocyclopropane-1-carboxylic acid (ACC) produced from roots experiencing drought stress than ACC produced by leaves during heat stress. Consequently, ACC deaminase may provide more protection to drought stressed soybeans and may require higher intensities of stress (and ACC) to experience negative physiological and phenological changes. Thin arrows indicate outcomes.

My thesis will address these knowledge gaps by answering the following research questions:

1) Do soybeans with elevated ACC deaminase production in their rhizosphere have lower ethylene concentrations and do they display differences in their morphology in response to heat or drought?
2) Does organic fertilizer increase the ACC deaminase activity in the rhizosphere of soybean experiencing heat and drought stress?
3) How do organic fertilizer alter the composition of the soybean bulk and rhizosphere bacteria communities relative to N fertilizer?

Conceptual Diagram

A



B

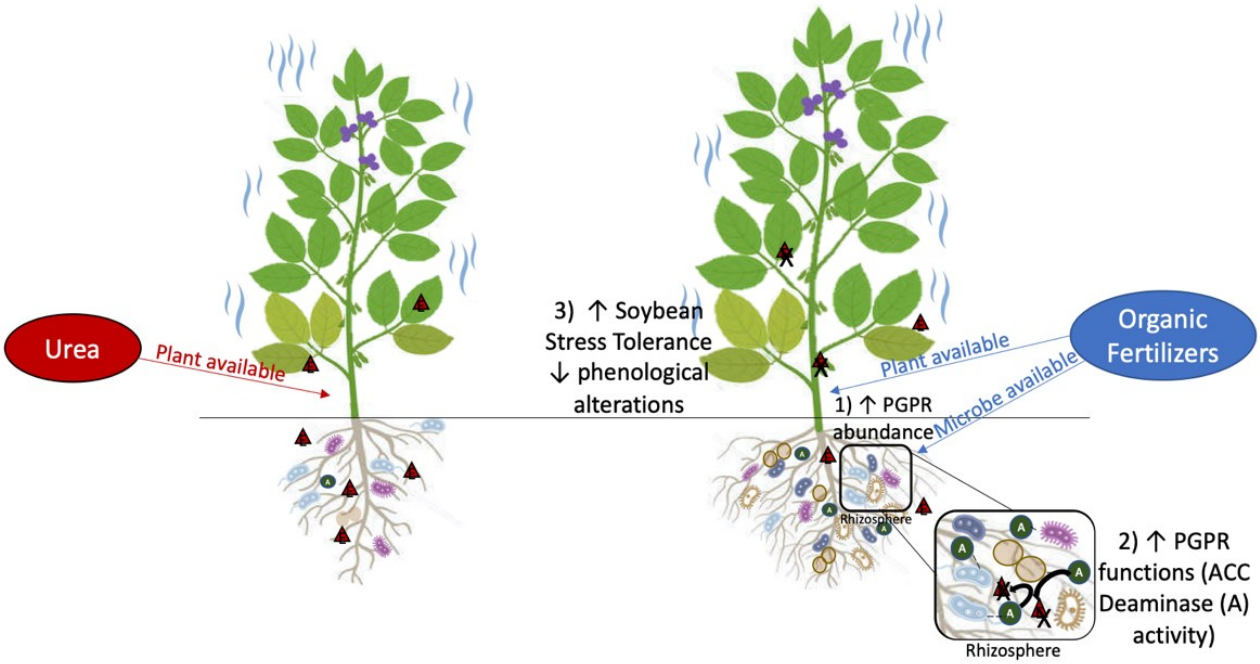


Figure 1.4 a) Left: Heat stressed soybeans produce 1-aminocyclopropane-1-carboxylic acid (ACC) (ethylene precursor) from leaves that detect heat stress. ACC must diffuse to roots to come in contact with ACC deaminase producing PGPR in the rhizosphere. Thus, I hypothesize low ACC deaminase production under heat stress. Right: Drought stressed soybeans produce ACC mainly from roots. Thus, ACC is secreted from the roots, close to the ACC deaminase producing PGPR, which will hydrolyze ACC and prevent ethylene formation.

Figure 1.4 b) Drought stressed soybean with two nutrient sources (urea versus organic fertilizer). Lack of plant available water through the root system signals the production of ACC (ethylene precursor) in the roots. Microbes in the rhizosphere will upregulate ACC deaminase production to consume ACC and prevent phenological alterations. It is hypothesized that organic fertilizers, which contain organic matter and nutrients, will increase the abundance of rhizosphere bacteria and PGPR in the rhizosphere, compared to inorganic fertilizers. Larger populations of PGPR will produce more ACC deaminase and consume more ACC. As a result, the soybean receiving organic fertilizer will have fewer phenological alterations that reduce its lifespan and yield.

Connecting Paragraph

Chapter 1 illustrates that it is desirable for soybean to have rhizobacteria that can degrade 1-aminocyclopropane-1-carboxylate (ACC) because ACC is a precursor of ethylene, a plant growth hormone associated with phenological changes that reduce soybean yield. Applying organic fertilizer in soybean production systems is expected to directly improve crop nutrition and growth, while supporting a large population of rhizosphere bacteria, and thus may increase 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) produced by these bacteria. Thus, Chapter 2 will 1) investigate if organic fertilizers improve the soybean tolerance to heat, water or combined heat plus water stress by supporting more ACC deaminase-producing rhizobacteria, resulting in greater ACC deaminase activity, and 2) whether this translates in lower ethylene concentration and fewer changes in soybean morphology, relative to the N fertilizer treatment.

Chapter 2

Soybean Abiotic Stress Tolerance Is Improved by Beneficial Rhizobacteria in Biosolids Amended Soil

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2.1 Abstract

Soybean produces ethylene when exposed to heat and water stress, which reduces biomass accumulation, but soybean that receives an organic fertilizer (containing all essential plant elements) is expected to be more tolerant of these abiotic stresses. This could be due to bacteria in the rhizosphere of soybean that receive organic fertilizer, since some rhizobacteria can prevent ethylene production by hydrolyzing its molecular precursor with the enzyme 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase). The objective of my study was to determine if organic-fertilized soybean exposed to heat, water or heat plus water stress will have less morphological change, elevated ACC deaminase activity in their rhizosphere and produce less ethylene than soybean that received N fertilizer only. Specifically, I expected less morphological change and higher ACC deaminase production in soybean exposed to water stress than heat stress, because a soil water deficit is first detected by roots and the associated rhizobacteria that produce ACC deaminase, whereas heat stress is detected by leaves. Soybean plants were grown in soil amended with organic fertilizer (biosolids, anaerobic digestate or compost) or with N fertilizer (urea) for 9 wk in a greenhouse, then exposed to the following stress treatments: no stress, heat stress, water stress or heat plus water stress. Biosolids-amended soybean was more resilient to heat or water stress as their aboveground biomass was larger ($p < 0.05$). There was greater ACC deaminase activity ($p < 0.05$) in the rhizosphere soil of biosolids-amended than N fertilized soybean, and ACC deaminase activity was negatively correlated with the ethylene concentration

($r = -0.40$, $p < 0.05$). I conclude that applying an organic fertilizer like biosolids to improve soybean growth may also increase the ACC deaminase activity of its rhizobacteria and potentially improve its tolerance to abiotic stress.

2.2 Introduction

Soybean can generally acquire ample N for growth from N_2 fixation, however fertilization often increases soybean yield. Organic fertilizers, such as biosolids derived from municipal organic wastes, supply plant-available nutrients to soybean, and can result in 11–17% higher soybean yield when 120 kg N/ha is supplied by biosolids, compared to unfertilized controls (Currie et al., 2003). Many organic fertilizers contain all the essential elements for plant growth and organic matter, which improves soil physicochemical properties and ultimately soybean growth. The labile organic substrates and nutrients in organic fertilizer are readily metabolized by soil microbial communities, so applying organic fertilizer will also support larger bacterial and fungal populations with more microbial biomass (Esperschütz et al., 2007; Seaker & Sopper, 1988). Furthermore, organic fertilizers may add microorganisms that colonize the rhizosphere for the benefit of the soybean crop (Gandolfi et al., 2010). Therefore, applying organic fertilizer in soybean production systems is expected to directly improve the crop nutrition and growth, while supporting a large population of rhizosphere microorganisms.

It is desirable for soybean to have rhizobacteria that can degrade 1-aminocyclopropane-1-carboxylate (ACC) because ACC is a precursor of ethylene, a plant growth hormone that is associated with phenological changes (e.g., early onset of flowering, premature senescence) that reduce soybean yield (Nahar et al., 2011). In soybean, synthesis of ACC followed by ethylene production occurs when the plant experiences a sudden environmental change. The most common environmental triggers for ethylene production in soybean are heat stress, related to the number of

days that air temperature exceeds 30°C, and inadequate water supply (< 30% Soil Water Holding Capacity (SWHC)) during the growing season (Dubois et al., 2018). However, soybean is most susceptible to heat and water stress during the R2–R6 reproductive growth stages (Ferris et al., 1998). After 1–3 days of exposure to elevated ethylene, the plant will prematurely flower or initiate seed set, which shortens its lifespan and reduces the soybean meal and oil yield (Djanaguiraman & Prasad, 2010b). An active community of rhizobacteria that produce ACC deaminase, the enzyme that degrades ACC, could prevent soybean growth inhibition. Therefore, soybean plants that have ACC deaminase-producing bacteria in their rhizosphere are expected to be more tolerant of heat and water stress.

Organic fertilizers are expected to increase the ACC deaminase activity of rhizobacteria associated with soybean, for several reasons. Organic fertilizers are a complete source of essential plant elements that directly promote soybean growth by fulfilling its nutritional needs and thus promote the development of a large root system (Reyes-Cabrera et al., 2017). Large root systems provide a large surface area for rhizobacteria to colonize and produce compounds that may assist the plant during periods of stress. Furthermore, organic fertilizers contain organic substrates that support large, active soil microbial populations (Canadian Council of Ministers of the Environment, 2012b; Seaker & Sopper, 1988). Finally, organic fertilizer may contain ACC deaminase-producing microbes that can survive in bulk soil and potentially colonize the rhizosphere (Gandolfi et al., 2010). Thus, soybean fields fertilized with organic fertilizers may have a large community of ACC deaminase-producing rhizobacteria with potential to increase the stress tolerance of soybean.

Soybean stress response depends upon the type of stress experienced by the plant. Water limitation is detected by roots, which leads to the production of ACC, ethylene's precursor, and its

secretion in exudates from root tip cells (Finlayson et al., 1991; McManus, 2012). The exuded ACC molecules may be degraded by ACC deaminase-producing rhizobacteria living near the root tip, or ACC may diffuse through the root tissue and elicit a response from the rhizobacteria inhabiting the epidermal tissues and root surface of the fine root system. Excess ACC will be transported through the phloem or xylem (if there is a lack of oxygen) to the shoot and leaves, which can biosynthesize ethylene (de Poel & Van Der Straeten, 2014). In contrast, heat stress is mostly sensed by the leaves and elicits ACC production followed by ethylene biosynthesis in leaves (Djanaguiraman & Prasad, 2010b). It is not known whether ACC produced distally in shoots or leaves can be transported to the roots and hydrolyzed by rhizobacteria (de Poel & Van Der Straeten, 2014; McManus, 2012), but it is unlikely to be a rapid or effective way to inhibit ethylene biosynthesis in leaves. Therefore, the type of stress will determine what plant organ produces ACC and the likelihood that ACC deaminase-producing rhizobacteria will reduce ethylene biosynthesis in soybean (Figure 2.1).

The objective of this study was to determine if organic fertilizer would improve the soybean tolerance to heat, water or combined heat plus water stress by supporting more ACC deaminase-producing rhizobacteria, resulting in greater ACC deaminase activity, lower ethylene concentration and fewer changes in soybean morphology, relative to the N fertilizer treatment.

2.3 Materials and Methods

2.3.1 Soil and Soybean

Soil for my greenhouse experiment was collected from the Emile A. Lods Agronomy Research Centre in Sainte- Anne-de-Bellevue, Québec (latitude: 45°25'N; longitude: 73°55'W; 39 m elevation) in November 2018 after corn was harvested from the field. The soil was is a sandy loam in the Macdonald soil series and had a pH of 5.0. Prior to 2012, the field was planted with

forages and since 2012, the field had been cultivated with annual crops (soybean, wheat and canola) and managed with conventional farming practices (spring disc harrow, fall moldboard plow and mineral fertilization) or left fallow (2017 growing season). For my greenhouse experiment, I selected a commercial soybean variety [*Glycine max* (L.) Merr. cv. Altitude R2] that contains the proprietary Roundup Ready 2 Yield® genetic modification. I tested the germination of 50 seeds (subsampled at random from the bag), which had a 96% germination rate, before using non-germinated seeds from the same batch in my experiment.

2.3.2 Experimental Design and Fertilizer Treatments

The greenhouse experiment was designed as a full factorial with 4 fertilizer sources and 4 stress conditions with 4 replicates of each (n=4), for a total of 16 experimental units. The experimental unit was a pot (diameter=18cm, height=15cm, volume= 3817cm^3) that contained 50 g of Ottawa sand at the bottom to facilitate drainage. Next, ~0.2 kg of soil (sieved <2 mm) was placed over the sand, then 0.6 kg of soil mixed with fertilizer was added and covered with 0.4 kg of unfertilized soil (5 cm deep).

I tested four fertilizer sources: N-based fertilizer (urea) and three more nutrient complete organic fertilizers (Table 2.1). Anaerobic digestate (hereafter referred to as digestate) was produced from the anaerobic digestion of municipal source-separated organics (SSO), retail food waste and other unidentified organic waste by BIO-EN (Elmira, ON). Lystegro was a treated biosolids produced by Lystek Inc. (Guelph, ON) from municipal water treatment facilities in the Greater Toronto Area and smaller communities near the Lystek plant in Dundalk, Ontario. Compost was aerobically decomposed from source separated organics by the AIM Environmental Group (Stoney Creek, ON). Fertilizers were applied according to the N fertilizer recommendation

Table 2.1. Nutrient content of the four amendments applied to experimental pots. All analyses were measured on a dry weight basis.

Parameters	Amendment N Fertilizer	Digestate	Biosolids	Compost
Dry Weight (g kg^{-1})	1000	7	140	910
Ammonia N (g kg^{-1})	460	422	27	2
Total N (g kg^{-1})	460	433	38	27
P (g kg^{-1})	0	8.6	24.5	15.3
Ca (g kg^{-1})	0	14	10.6	50.4
K (g kg^{-1})	0	124	18.7	7.3
Mg (g kg^{-1})	0	2.9	5.2	0.8
Organic Matter (g kg^{-1})	0	360	450	586
C:N	0.4	0.4	5.9	9.7

for soybean, which is 30 kg N/ha (CRAAQ, 2010), so each fertilizer was applied to provide an equivalent mass of total N according to the surface area of the pot (76 mg N/ 255 cm^2 pot). To ensure no nutrient deficiency, 0.275 g of P_2O_5 (equivalent to 80 kg P_2O_5 /ha) in the form of diammonium phosphate (DAP) and 50 mg of K_2O (providing about 20 kg K_2O/ha) in the form of KCl was added to each pot.

Next, 5 seeds were planted in each pot at a depth of 4 cm, and these were thinned to keep 3 representative soybean plants per pot after 2 wk. During the 9 wk that soybean grew in a greenhouse, the average daily temperature was 22.0°C ± 0.2°C (range 14.7-42.2°C) and soybean was exposed to a 16h: 8h daytime: night-time through the use of supplemental fluorescent lighting (800 $\mu mol^{-2}\ s^{-1}$ of photon flux density) after sunset. The hottest temperatures in the greenhouse occurred on 3 sunny spring days that temporarily increased the greenhouse temperature > 35°C for a few hours at mid-day and 1 very cold night when the greenhouse temperature dropped <15°C. The soil water holding capacity (SWHC) was measured for each amendment-soil combination. Each pot was watered every second morning to 50% of its total SWHC (based on predetermined weights). Every 4 wk, the volume of water added to maintain the SWHC was adjusted to account for the extra soybean biomass. Pots were placed randomly on the greenhouse bench and were rotated every second day after watering.

2.3.3 Heat and Water Stress Treatments

After 9 wk, soybean pots from each fertilizer treatment were randomly assigned to one of the four stress treatments: no stress, heat stress, water stress or heat plus water stress. No stress meant that the average daily temperature was kept at 22.0°C ± 0.2°C and pots were watered to 50% SWHC every day. These pots were placed in growth chambers for the duration of the stress period to maintain constant temperature and light (800 $\mu mol^{-2}s^{-1}$ photon flux density for 16 h a

day). Based on The Prairie Climate Centre's (2019) prediction that the duration of heat waves (days in a row ≥ 30°C) in Ontario and Quebec will increase 2.5-3.0 times in the next three decades, I set the heat stress condition at 30°C for 7 d. However, the temperature in the greenhouse varied depending on the time of day (>30°C at mid-day but falling <30°C at night). Based on temperature readings logged every 4 h, the soil temperature averaged 29.7±1.2°C and ranged from 22.9-47.1°C during the heat stress event. Pots were watered every day to maintain 50% SWHC. The water stress condition reduced the SWHC from 50% to 8% for 7 d, based on The Prairie Climate Centre's (2019) prediction of a longer interval between precipitation events during summer months in the future, and considering that 8% of SWHC produced water stress in the rhizosphere of wheat (Azarbad et al., 2018). Pots under water stress were placed in growth chambers to keep humidity at 8% SWHC while maintaining constant temperature (22.0°C) and light (800 $\mu mol^{-2} s^{-1}$ of photon flux density for 16 h a day). Combined heat plus water stress meant that the heat (29.7±1.2°C) and water stress (8% SWHC) occurred simultaneously for 7 d under greenhouse conditions.

2.3.4 Plant Stress Measurements

After 7 d exposure to the stress treatment, I identified the fully-emerged trifoliate leaf on the 3rd node of each plant. Then, I measured the quantum yield of photosynthesis, a measure of photosystems II efficiency, on three of the leaflets of each plant in the experimental pot using a Fluorpen FP 100 (Photon Systems Instrument, Drasov, Czech Republic).

Next, to determine ethylene production, each pot was placed in a plastic bucket, which was capped with a lid and then sealed around the edge with parafilm. Headspace (~3700 mL) was estimated by subtracting the volume of the pot and volume of aboveground biomass (calculated by dividing the aboveground weight by the density of soybean tissue: 0.35g/cm^3) (Iqbal et al., 2013). After 2 h, I withdrew three 0.1 mL gas samples (analytical replicates) from the headspace.

I then measured the ethylene concentration in each analytical replicate on a Hewlett Packard gas chromatograph model 5890A equipped with a flame ion detector (FID). The column used was a 1.22 m long x 3.175 mm diameter and had Poropak N 80/100 mesh packing, with helium (carrier gas) flow set at 165kPa, an injector temperature of 200°C, oven isothermal temperature of 40°C, and the FID detector set at 200°C. The instrument was calibrated with an analytical standard (NorLAB, ID, USA) containing 27 μL L^{-1} of C_2H_4 in synthetic air. Ethylene peaks were detected within approximately 1 min and quantified with 32bit Peak420 software (SRI Instruments, CA, USA). The ethylene concentration (nM ethylene g^{-1} h^{-1}) was calculated by converting the volumetric gas concentration from ppm to nM with the ideal gas law, then dividing by total dry biomass (g) and the incubation time (h) (Rochette & Hutchinson, 2005).

2.3.5 Plant Sampling

After measuring the ethylene concentration, the pots were destructively sampled. I measured the length of the shoots, counted the number of nodes on shoots (within 1 h of sampling) and the number of rhizosphere nodules (within 1 wk) of all three plants in each pot. I determined the % of N_2 fixing-nodules visually by determining the proportion of nodules that were red on the inside. Shoots were cut at the soil surface, rinsed with distilled water and oven-dried (50ºC for 48 h). Roots were rinsed with distilled water and stored at 4ºC until analysis. I measured root diameter and length with WinRHIZO Reg2008b 32-bit software (Regent Instruments Inc., Québec, QC, Canada). I then dried all components of the biomass (nodules, roots, shoots) at 55°C for 48 h and weighed them to determine the dry root biomass and total dry biomass for each experimental unit.

2.3.6 Soil Sampling

After removing the plant biomass, I collected bulk and rhizosphere soil from each pot. Soil and roots were separated manually. I used a fine brush to collect the soil that remained attached to the roots and classified it as rhizosphere soil (it could include soil from the endorhizosphere, rhizoplane and ectorhizosphere, according to the definition of Pinton, Varanini, & Nannipieri (2001)). Bulk soil was soil in the pot that was not in direct contact with the roots. Bulk and rhizosphere soil samples were immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

2.3.7 Soil Chemistry Analysis

Soil analysis was conducted on the bulk soil from each pot. Soil pH was measured with an Accumet research ar10 pH meter (Fisher Scientific, Pittsburg, PA, USA). The NH_4, NO_3 and dissolved organic carbon concentrations were determined in 0.5 M K_2SO_4 extracts (1:4 soil:extractant). I quantified NH_4 and NO_3 on a microplate reader at 650 nm (Sims et al., 1995). The 0.5 M K_2SO_4 sample extracts were diluted (1:3 extractant:water) to measure salt-extractable dissolved organic carbon on a Sievers InnnovOx laboratory TOC analyzer (GE Analytical Instruments, Boulder, CO, USA). Total organic C and total N was determined on ground (<1 mm mesh), oven-dried soil (55°C for 48 h) by combusting at 900°C with a Thermo Finnigan Flash EA 1112 Series C/N Analyzer (Carbo Terba, Milan, Italy). I quantified extractable P using the Olsen P method by adding 2 g of soil to 40 mL 0.5M $NaHCO_3$ (pH 8.5). Solutions were placed on a shaker for 30 min and subsequently filtered (<5 μm, Q5 filter paper). Dissolved inorganic phosphorus was measured according to Jeannotte et al. (2004) on a μQuant microplate reader (BioTek Instruments Inc., Winooski,VT, USA).

2.3.8 acdS Gene Abundance

Total DNA was extracted from rhizosphere soil using the DNAeasy PowerSoil kit (Qiagen, Germantown, MD, USA) and following the manufacturer's instructions. I quantified the Genomic DNA using a Qubit 4 Fluorometer (Invitrogen, Carlsbad, Ca, USA) and Qubit ™ ds DNA HS Assay Kit (Invitrogen, Carlsbad, CA, USA). I normalized the *acdS* gene by comparing the quantification cycle value (C_q) of each sample to the C_q of the 16S gene in the same sample. The reference gene, 16S was amplified using the primer set 515F-Y (5′-GTGYCAGCMGCCGCGGTAA and 926R (5′-CCGYCAATTYMTTTRAGTTT) (Parada et al., 2016). I amplified a 133bp section of the *acdS* gene with the primer sets acdSF5 (5'-GGCAACAAGMYSCGCAAGCT) and acdSR8 (5'-CTGCACSAGSACGCACTTCA) described by (Bouffaud et al., 2018) for the 145bp and 278bp regions of the *acdS* gene of *Burkholderia cenocepacia*. I used a Biorad CFX96 Real-Time System (Biorad, Hercules, CA, USA) and CFX Maestro Software for qPCR reactions.

I validated the qPCR reaction conditions according to the MIQE guideline (Bustin, 2009). I first validated the primer sets using a thermogradient of 54°C to 70°C, primer concentrations of 250 ng μL^{-1} and a genomic DNA dilution of 1/10 in a total reaction volume of 10 µL. The best melting temperature (Tm) for the acdS primers and 16S primers was 67ºC. Next, I identified the optimal primer concentration by preparing reactions for both the gene of interest and the reference gene at 100 ng μL^{-1}, 200 ng μL^{-1} and 250 ng μL^{-1}. A concentration of 250 ng μL^{-1} was optimal for both primer sets, based on the low C_q for gene copies and high reproducibility of technical replicates (CV=0.0130). Finally, I constructed 8 point standard curves for both genes to determine the optimal genomic DNA concentrations for the reactions, which resulted in a standard curve for the 16S reaction with an R^2 of 0.998 and an efficiency of 105.5%, and a standard curve for the

acdS reaction with an R^2 of 0.994 and efficiency of 100.8%. Efficiency values between 90 and 110% are considered acceptable for qPCR reactions.

I then quantified the gene of reference (16s rRNA) and the gene of interest (*acdS*) from 64 bacterial DNA extracts from soil. Optimized PCR conditions for the *acdS* gene and 16S gene reactions were as follows: initial denaturation 95°C for 30 s, followed by 39 cycles of denaturation at 95°C for 15 s, annealing at 67°C for 30 s, followed by elongation at 65°C ± 0.5°C /cycle to 95°C for 5 s and then held at 4°C. For each sample I combined the following: 5 µL Ssoadvanced™ Universal Sybr Green Kit (Biorad, Hercules, CA, USA), 2 uL of water and either 1 µL 250 ng μL^{-1} of the *acdS* primers or 1 µL of 250 ng μL^{-1} of the 16s primers. Based on the standard curve results, I chose DNA dilutions of 1:16 for both the 16s and *acdS* reactions and added 1 µL of this diluted genomic DNA to each reaction. I set the C_q threshold to the lowest fluorescence value that produced parallel amplification curves (on a log scale). Two no-template DNA controls for each primer set were run to ensure no contamination, and all samples were analyzed in duplicate.

I used the mean of the technical replicates to quantify the proportion of the *acdS* gene in the genomic DNA. I calculated the ΔC_q by subtracting the C_q of the 16S gene from the C_q of the *acdS* gene for each experimental unit (n=64).

2.3.9 ACC Deaminase Activity

To assess ACC deaminase activity, I measured the concentration of the hydrolysed ACC product, α-ketobutyrate (Penrose & Glick, 2003). I mixed 0.2 g of thawed rhizosphere soil from each experimental unit with 10 mL of LB broth and incubated it at 30°C and 200 rpm for 24 h. After centrifuging the suspension at 4000 *g* for 10 min, I removed the supernatant and resuspended the pellet in 4.5 mL of DF salts (Dworkin & Foster, 1958) containing 50 mM of ACC as the only

N source. I prepared the DF media by combining the following: 2 g glucose, 2 g citric acid, 4 g KH_2PO_4, 6 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$. 0.1 mL of a solution containing 100 mg of $FeSO_4•7H_2O$ in 100 mL of water. I also added 0.1 mL of a solution of following micronutrients to the media: 10 mg H_3BO_3, 11.19 mg $MgSO_4•7H_2O$, 124.6 mg $ZnSO_4•.7H_2O$, 78.22 mg $CuCl_2$, 10 mg Na_2MoO_4. Then, I autoclaved the final media. After incubating at 30 °C and 200 rpm for 48 h, I centrifuged the DF salts bacteria solution at 4000 *g* for 10 min. Then, I removed the supernatant and resuspended the pellet in 1 mL of 0.1 M tris-HCl pH 7.6 in 1.5 mL microcentrifuge tubes. Following centrifugation of the tubes at 8000 *g* for 10 min, I repeated the washing step once more before removing the supernatant and freezing the pellet at -20 °C until further analysis. Later, the samples were thawed, and I suspended them once again in 1 mL of 0.1M tris-HCl pH 7.6. Next, I centrifuged the sample at 16 000 *g*, removed the supernatant and added 565 µL of 0.1 M tris-HCl pH 8.5 to buffer the solution and 30 µL toluene to kill microbial cells to prevent them from metabolizing ACC. I vortexed the tubes to resuspend the pellet. I added 105 µL of 0.3 M ACC to each sample (for a final ACC concentration of 50 mM). I then vortexed the sample and incubated it for 15 min to allow for the microbial ACC deaminase present in solution to hydrolyze the ACC. The reaction was stopped by adding 1 mL of 0.56 N HCl and centrifuging the tube at 16 000 *g* for 5 min. I removed and kept the supernatant and resuspended the pellet in another 800 µL of 0.56 N HCl. The acidified supernatant was combined and then mixed with 300 µL of 0.2 % 2,4 dinitrophenylhydrazine in 2M HCl, vortexed and incubated at 30 °C for 30 min before adding 2 mL of 2 N NaOH to develop the final colour. After a final vortex to thoroughly mix the solution, the absorbance was measured at 540 nm on a BioTek Synergy H1 96 well plate reader (BioTek Instruments Inc., Winooski, VT, USA). I subtracted the absorbance readings of reagents without sample bacterial suspension from sample absorbance values. I then calculated the α-ketobutyrate

concentration based on absorbance values of α-ketobutyrate standards, and normalized these values based on soil dry mass (μM α-ketobutyrate g^{-1} soil). ACC deaminase concentrations were inferred from α-ketobutyrate concentrations, as their ratio in the reaction was 1:1.

2.3.10 Statistical Analyses

I verified homoscedasticity for all plant and microbial parameters with Levene's test. If the assumptions for homoscedasticity were not met, the data were log transformed. Consequently, I log transformed the values obtained for the number of nodes on shoots and the number of root nodules. Normality of the residuals for all measured plant parameters (shoot length, number of nodes, root diameter, root lengths, root weights, number of nodules, % N_2-fixing nodules, total dry biomass, QY and ethylene) was tested using the Shapiro-Wilk Goodness of Fit Test. Means and standard errors were calculated. Plant and microbial responses to the stress and fertilizer treatments, as well as the fertilizer × stress interaction, were determined with a two-way analysis of variance (ANOVA). If the assumptions for normality were not met ($p < 0.05$), I evaluated the treatment effects with a Kruskal-Wallis non-parametric test. If the individual stress or fertilizer treatments were significantly different for a particular dependent variable, I computed pairwise comparisons with Bonferroni's correction. However, if the fertilizer × stress interaction was significant for a particular dependent variable, I computed the pairwise comparisons with a post-hoc Tukey's Honest Significant Difference test.

Relationships between ethylene, acdS gene copy number and ACC deaminase activity, together with plant parameters were assessed with Pearson correlation coefficients (n=64). Coefficients indicating an association between variables that were ≥ |0.2| were considered to be meaningful.

Changes in soil chemistry dependent variables (pH, NO_3, NH_4, TOC, total organic C, total N and inorganic P) in response to fertilizer treatments and stress were determined by two-way ANOVA. Furthermore, I computed Pearson correlation coefficients to describe associations between soil chemistry dependent variables and the plant and microbial dependent variables (total biomass, shoot length, shoot nodes, root lengths, root diameter, root biomass, nodules, $\%N_2$-fixing nodules, quantum yield (QY), ethylene, acdS gene copy, ACC deaminase activity. All statistical analyses were done with RStudio version 3.6.1.for Apple.

2.4 Results

Soybean were damaged by the combined heat plus water stress treatment, which caused cession of the photosynthetic activity and resulted in leaf desiccation and death after 7 d. None of the fertilizer treatments were able to protect the soybean plants from exposure to the combined heat plus water stress, as all plants died in this treatment. Since the goal of the experiment was to investigate soybean plants that were impacted, but still functional, after exposure to heat and water stress, this treatment was excluded from further analysis. The mean values for soybean and soil properties in the heat plus water stress treatment are presented, for information, in Appendix Tables 1-3.

2.4.1 Biosolids and digestate increase soybean shoot growth, while compost and digestate increase root growth

Biosolids and digestate increased soybean shoot biomass by 20–22%, resulting in soybean plants that were 17–20% taller than those receiving N fertilizer ($p < 0.05$, Bonferroni test; Table 2.2). The number of shoot nodes was also higher in biosolids and digestate treatments (8.8–9.0 nodes) relative to compost and N fertilizer (7.6–7.7 nodes) indicating their greater growth rates (*p*

< 0.05, Bonferroni test; Table 2.2). Ethylene production was not affected by fertilizer treatments ($p > 0.05$, Table 2.3)

Compost and digestate increased soybean root biomass by 30–50%, and the root diameter was 15–69% greater with digestate than with N fertilizer under no stress ($p < 0.05$, Tukey HSD; Table 2.2). Soybean grown in the compost-amended soil had >2.5 fold more nodules and approximately 50% more N_2-fixing nodules, than soybean that received N fertilizer ($p < 0.05$, Bonferroni test; Table 2.2). However, root length and diameter did not differ between the fertilizer treatments ($p > 0.05$, Table 2.2) and this was likely due to limited space for root growth in the 3817 cm^3 pot rather than a fertilizer effect.

2.4.2 Soybean plants exposed to water stress have more aboveground biomass and larger root diameter in soil amended with biosolids and digestate

Heat stress and water stress inhibited soybean growth, resulting in less aboveground biomass and lower photosynthetic activity, smaller roots and root diameters, and fewer nodules when exposed to these stresses ($p < 0.05$, Table 2.2). For example, the N fertilized soybean was 7–10% shorter when exposed to heat or water stress than non-stressed soybean receiving N fertilizer ($p < 0.05$, significant fertilizer × stress interaction, Table 2.2). However, these stress effects were less pronounced for soybean grown in soil that received organic fertilizer than N fertilizer (Fig. 2.1). Under heat stress, the aboveground biomass of N fertilized soybean plants were 10% lighter and 19–24% shorter than biosolids- or digestate-fertilized soybean ($p < 0.05$, significant fertilizer × stress interaction, Table 2.2). Water stress was equally or more damaging, since aboveground biomass of N fertilized soybean plants was 18% lighter and 19-26% shorter than in the biosolids- and digestate-fertilized soybean ($p < 0.05$, significant fertilizer × stress interaction; Table 2.2).

Table 2.2 Soybean parameters measured after 7 days of stress. Values are the mean (±standard error) *n*=4

Stress	Fertilizer	Shoot Biomass (g)	Shoot Length (cm)	Shoot Nodes (#)	Root Length (cm)	Root Diameter (cm)	Root Biomass (g)	Nodules (#)	% N_2 Fixing Nodules	QY[1]
Control	N Fertilizer	19 (0.23) ^{c}	42 (2.0) ^{b}	8.3 (0.30)	32 (3.1)	1.3 (0.10) ^{c}	1.0 (0.061)	33 (8.30)	17 (3.9) ^{c}	0.77 (0.020)
	Digestate	23 (0.60) ^{a}	50 (2.5) ^{a}	8.9 (0.28)	26 (2.4)	2.2 (0.13) ^{a}	1.3 (0.14)	19 (5.6)	32 (7.5) ^{a}	0.74 (0.021)
	Biosolids	23 (0.45) ^{a}	49 (1.6) ^{a}	8.8 (0.21)	28 (1.8)	1.7 (0.081) ^{ab}	1.3 (0.091)	30 (4.4)	19 (2.8) ^{c}	0.77 (0.010)
	Compost	20 (0.28) ^{c}	39 (1.7) ^{b}	7.8 (0.16)	31 (1.6)	1.5 (0.050) ^{bc}	1.5 (0.070)	120 (6.5)	25 (2.3) ^{b}	0.79 (0.010)
Heat	N Fertilizer	19 (0.18) ^{c}	38 (0.77) ^{b}	7.5 (0.29)	26 (2.4)	1.5 (0.051) ^{bc}	1.2 (0.021)	21 (6.0)	22 (4.0) ^{b}	0.74 (0.031)
	Digestate	21 (0.27) ^{ab}	50 (1.8) ^{a}	9.3 (0.25)	25 (3.1)	1.6 (0.054) ^{ab}	1.1 (0.11)	37 (5.7)	35 (3.7) ^{a}	0.76 (0.030)
	Biosolids	21 (0.24) ^{ab}	47 (0.67) ^{ab}	8.8 (0.21)	30 (2.4)	1.5 (0.13) ^{bc}	1.3 (0.070)	25 (5.7)	55 (15) ^{a}	0.61 (0.104)
	Compost	19 (0.81) ^{c}	41 (2.4) ^{b}	7.8 (0.40)	27 (3.8)	1.2 (0.051) ^{bc}	1.2 (0.081)	95 (45)	15 (5.6) ^{c}	0.58 (0.084)
Water	N Fertilizer	18 (0.25) ^{c}	39 (0.55) ^{b}	7.4 (0.16)	29 (1.8)	1.3 (0.091) ^{bc}	0.81 0.030)	27 (4.3)	24 (2.5) ^{b}	0.70 (0.043)
	Digestate	22 (0.45) ^{a}	53 (1.9) ^{a}	8.9 (0.16)	23 (3.0)	1.3 (0.13) ^{bc}	1.2 (0.011)	14 (1.9)	42 (3.8) ^{a}	0.70 (0.034)
	Biosolids	22 (0.20) ^{ab}	48 (2.7) ^{ab}	8.8 (0.10)	25 (4.2)	1.2 (0.12) ^{bc}	0.92 (0.040)	8.8 (4.3)	7.6 (5.1) ^{c}	0.78 (0.011)
	Compost	19 (0.23) ^{c}	38 (0.89) ^{b}	7.1 (0.16)	27 (1.3)	1.3 (0.092) ^{bc}	1.2 (0.091)	85 (10)	23 (3.5) ^{b}	0.62 (0.050)
Source of variation	d.f.[y]									
Fertilizer	3	***	***	***	NS	**	**	***	***	NS
Stress	2	***	NS	NS	NS	***	***	**	*	*
Fertilizer*Stress	6	*	*	NS	NS	**	NS	NS	*	NS

[y]d.f., degrees of freedom; [1] QY= quantum yield; *, **, *** Significant at P<0.05, P<0.01, P<0.001, respectively: NS, not significant; superscript letters denote significantly different fertilizer x stress values

Table 2.3. Rhizosphere soil bacterial acdS gene possession, ACC deaminase activity and plant ethylene concentration measured after 7 days of stress. Values are the mean (±standard error) n=4

Stress	Fertilizer	acdS gene (ΔCq)[a]	ACC deaminase (μM g^{-1} dry soil)	Ethylene (nM g^{-1} biomass hr^{-1})
Control	Urea	2.9 (0.53)	0.16 (0.051)	14 (0.73)
	Digestate	4.4 (0.32)	0.33 (0.13)	9.1 (0.30)
	Biosolids	4.0 (0.30)	0.40 (0.10)	10 (0.99)
	Compost	5.6 (0.42)	0.36 (0.062)	14 (3.1)
Heat	Urea	3.0 (0.67)	0.40 (0.071)	11 (0.56)
	Digestate	3.0 (0.58)	0.21 (0.030)	12 (0.98)
	Biosolids	3.6 (0.40)	0.31 (0.11)	12 (2.4)
	Compost	5.6 (0.43)	0.29 (0.091)	12 (1.1)
Water	Urea	3.6 (0.53)	0.18 (0.084)	13 (1.2)
	Digestate	4.5 (0.83)	0.13 (0.052)	12 (0.43)
	Biosolids	3.8 (0.27)	0.46 (0.081)	11 (0.61)
	Compost	6.5 (0.20)	0.24 (0.02)	12 (0.62)
Source of variation	d.f.[y]			
Fertilizer	3	***	*	NS
Stress	2	NS	NS	NS
Fertilizer*Stress	6	NS	NS	NS

[y]d.f., degrees of freedom; [a] (ΔCq) Change in quantitation cycle between reference gene and acdS; *, **, *** Significant at $p < 0.05$, $p < 0.01$, $p < 0.001$, respectively: NS, not significant

Water stress also damaged soybean root systems, as root biomass was reduced by 19% ($p < 0.05$, Bonferroni test). Root diameter was smaller in water stressed soybean plants, and this negative effect was not alleviated by any fertilizer source ($p > 0.05$, fertilizer × stress interaction; Table 2.2). The proportion of N_2-fixing nodules was also altered by fertilizer and stress, since digestate application produced more active nodules under no stress, heat or water stress than the other fertilizers at each stress level ($p < 0.05$, significant fertilizer × stress interaction; Table 2.2).

2.3.3 Biosolids-Amended Soybean Rhizosphere Generated the Most ACC deaminase

Rhizobacteria possessing the *acdS* gene and having demonstrated ACC deaminase activity were affected by the fertilizer treatments ($p < 0.05$), but not by exposure to stress ($p > 0.05$, Table 2.3). Compost-amended soil had the largest proportion of rhizobacteria possessing the *acdS* gene, which was 46% greater than in soil with N fertilizer ($p < 0.05$, Bonferroni's test; Table 2.3). However, a higher proportion of rhizobacteria possessing the *acdS* gene did not translate into higher ACC deaminase activity ($p > 0.05$; Figure 2.3). Rhizosphere soil from the biosolids-amended pots had 43% more ACC deaminase activity than the digestate-amended soil and 37% higher ACC deaminase activity than soil receiving N fertilizer ($p < 0.05$, Bonferroni test; Table 2.3). Although ethylene production was not affected by fertilizer or stress treatments ($p > 0.05$, Table 2.2), plants that produced the lowest ethylene concentration had the highest ACC deaminase activity ($r = -0.4$, $p = 0.016$; Figure 2.2). Additionally, pots with the lowest ethylene concentration had the largest aboveground and total biomass ($r = -0.4$, $p < 0.001$ and $r = -0.3$, $p = 0.001$; Figure 2.2).

Figure 2.1. Soybean plants after 7 d of water stress. The left pot received N fertilizer whereas the right one received biosolids.

2.3.4 Soil properties were affected by fertilizer treatments, and associated with soybean growth

Organic fertilizers had a persistent effect on soil properties that was observable 10 wk after the fertilizer application. For instance, compost-amended soil had higher pH, total organic C and total N than N fertilized soil ($p < 0.05$, Bonferroni test; Table 2.4). In addition, the NO_3, DOC and dissolved P concentrations varied among pots, due to fertilizer × stress interactions ($p < 0.05$, significant fertilizer × stress interaction), possibly related to different patterns of nutrient acquisition and root exudation / decomposition of soybean exposed to variable fertility and stress conditions. Higher soil pH was correlated with higher nodulation ($r = 0.6$, $p < 0.001$; Figure 2.3), and associated with a greater proportion of rhizobacteria possessing the *acdS* gene ($r = 0.6$, $p < 0.001$). The NH_4 concentration was greater in biosolids-amended than N fertilized soils ($p < 0.05$, Bonferroni test; Table 2.4), and was correlated with greater aboveground biomass ($r = 0.3$, $p = 0.048$).

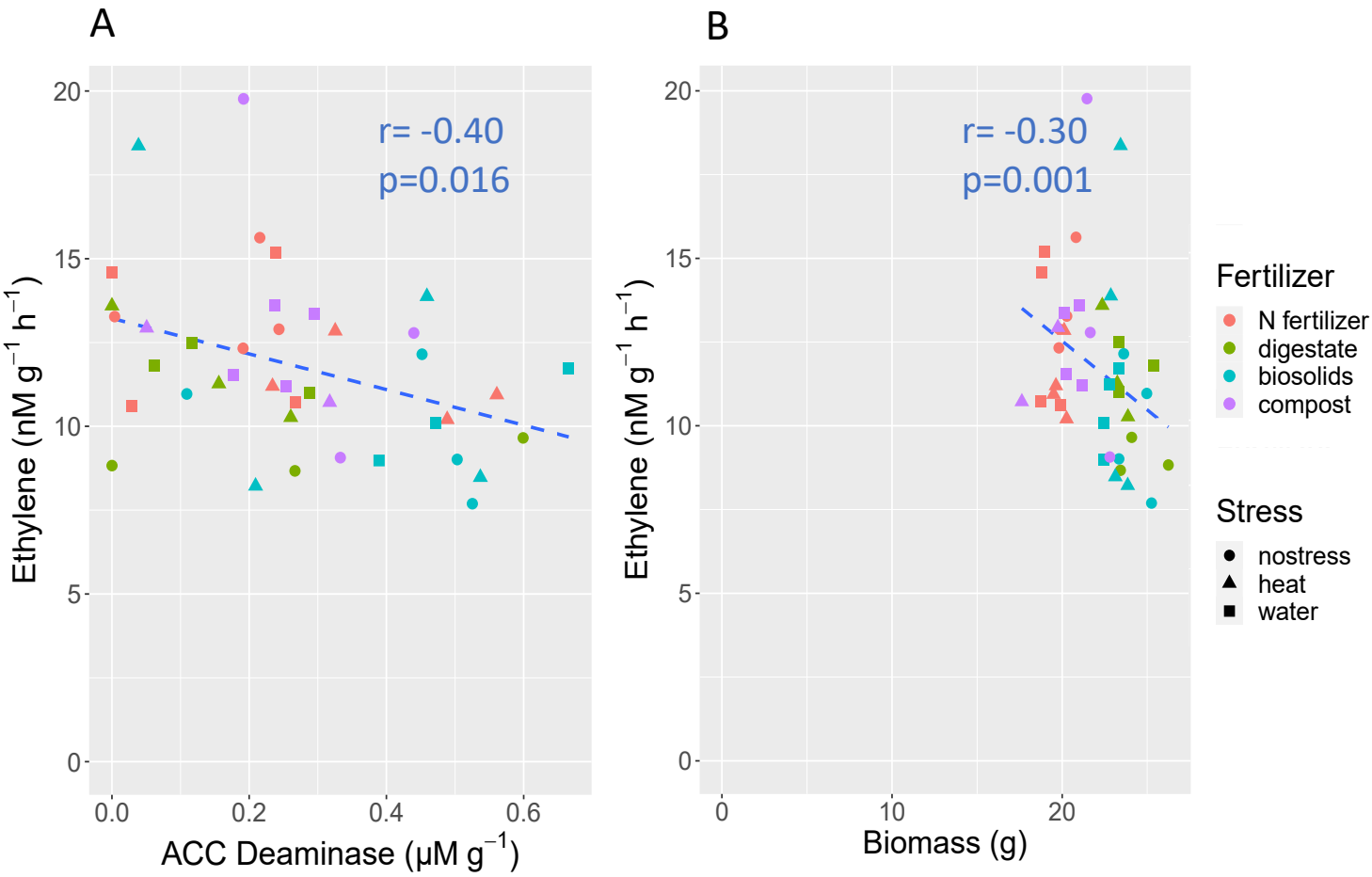

Figure 2.2. Relationship between (A) ethylene production and ACC deaminase activity, and (B) soybean biomass and ethylene production. The significant ($p < 0.05$), negative linear trends were described by Pearson correlation coefficients (r value, n=48).

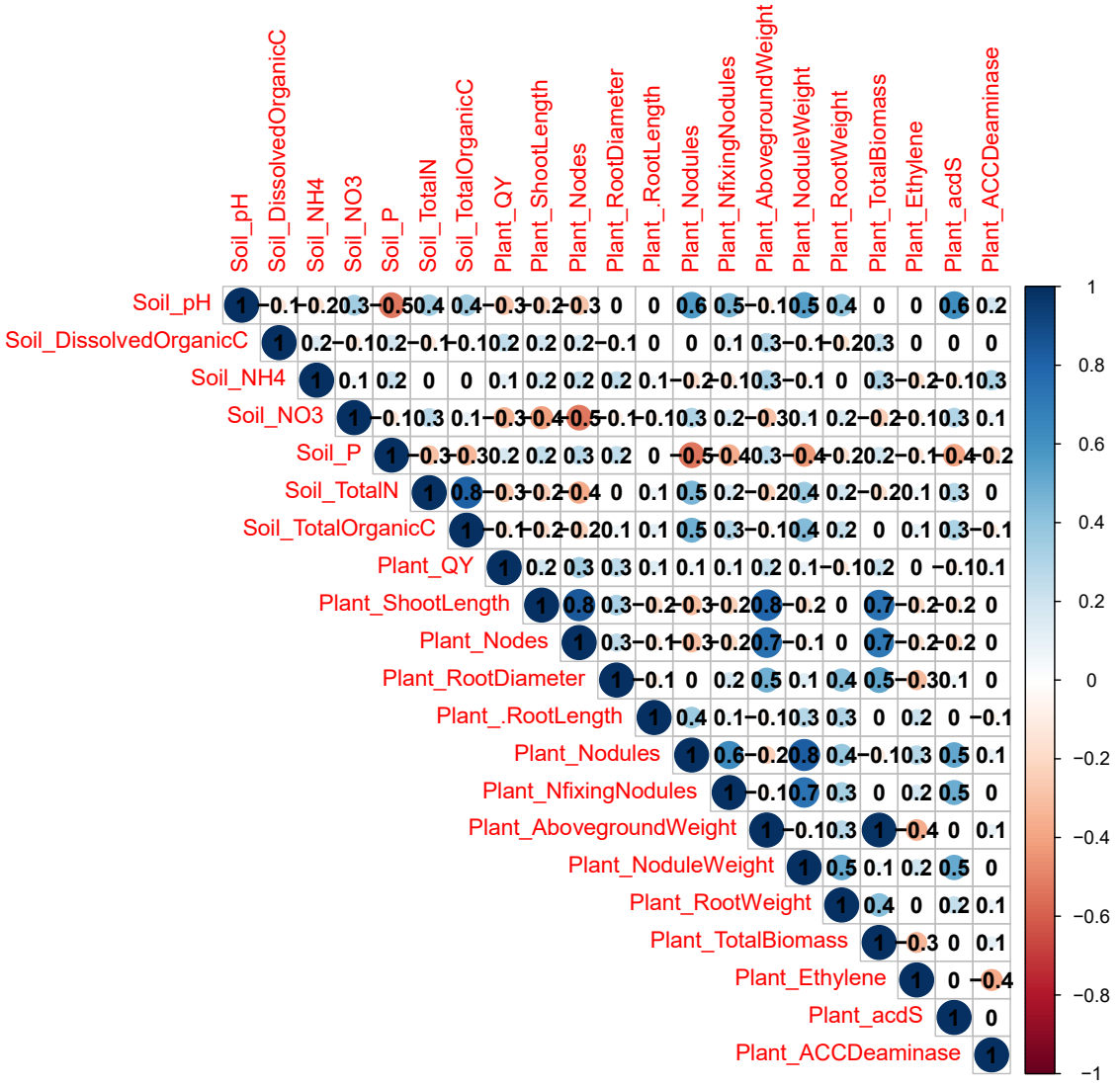

Figure 2.3. Relationships between soybean and rhizosphere bacteria responses in soil amended with N fertilizer and organic fertilizer, measured after 7 d of exposure to stress (heat, water, no stress). Values are the Pearson correlation coefficients (r values, n=48).

Table 2.4 Nutrient concentration and pH in bulk soil amended with N fertilizer (urea) or organic fertilizer (digestate, biosolids, compost) for soybean production, measured after 7 d of exposure to stress (heat, water). Values are the mean (±standard error) n=4

Stress	Fertilizer	pH	NO_3 (mg g^{-1} dry soil)	NH_4 (mg g^{-1} dry soil)	Dissolved Organic Carbon (mg kg^{-1})	Dissolved P (mg g^{-1} dry soil)	Total N (%N)	Total Organic C (%C)
Control	N Fertilizer	4.9 (0.010)	1.3 (0.33)	0.33 (0.08)	8.07 (0.67)	0.34 (0.050)	0.091 (0.015)	1.4 (0.12)
	Digestate	4.8 (0.10)	0.69 (0.29)	0.19 (0.02)	8.1 (0.67)	0.28 (0.010)	0.10 (0.012)	1.4 (0.10)
	Biosolids	4.9 (0.081)	11 (4.49)	2.6 (0.50)	8.1 (1.52)	0.22 (0.030)	0.10 (0.011)	1.3 (0.071)
	Compost	5.3 (0.15)	6.2 (0.24)	0.22 (0.01)	10.0 (0.49)	0.14 (0.021)	0.12 (0.011)	1.6 (0.11)
Heat	N Fertilizer	4.8 (0.020)	9.0 (0.55)	0.60 (0.22)	7.56 (0.84)	0.32 (0.032)	0.10 (0.013)	1.2 (0.062)
	Digestate	4.9 (0.061)	3.4 (0.90)	0.24 (0.01)	9.62 (1.03)	0.27 (0.041)	0.080 (0.0014)	1.2 (0.011)
	Biosolids	4.9 (0.071)	3.7 (0.61)	3.2 (1.5)	10.6 (0.68)	0.18 (0.061)	0.10 (0.014)	1.3 (0.074)
	Compost	5.3 (0.15)	8.8 (1.1)	0.24 (0.0012)	2.0 (2.0)	0.14 (0.020)	0.12 (0.015)	1.5 (0.062)
Water	N Fertilizer	4.7 (0.091)	6.8 (2.3)	0.41 (0.17)	10.1 (0.84)	0.30 (0.031)	0.091 (0.011)	1.3 (0.082)
	Digestate	5.0 (0.04)	6.2 (1.7)	0.85 (0.10)	11.3 (1.44)	0.25 (0.043)	0.093 (0.0022)	1.3 (0.031)
	Biosolids	4.9 (0.11)	0.80 (0.37)	1.0 (0.041)	12.4 (1.19)	0.14 (0.074)	0.091 (0.012)	1.3 (0.054)
	Compost	5.3 (0.15)	15 (2.5)	0.32 (0.013)	10.7 (2.14)	0.20 (0.083)	0.14 (0.024)	1.6 (0.12)
Source of variation	d.f.[y]							
Fertilizer	3	***	***	***	NS	***	**	***
Sterss	2	NS	NS	NS	***	NS	NS	NS
Fertilizer*Stress	6	NS	***	NS	**	***	NS	NS

[y]d.f., degrees of freedom; *, **, *** Significant at p <0.05, p <0.01, p <0.001, respectively: NS, not significant

2.5 Discussion

2.5.1 Organic fertilizers enhance the growth of soybean growth exposed to heat or water stress

As I hypothesized, applying organic fertilizer to soil before planting soybean improved its growth relative to N fertilizer. Furthermore, when soybean was exposed to heat or water stress, some of the organic fertilizers – notably the biosolids and digestate resulted in 19–26% taller and 10–18% more aboveground biomass than N fertilizer. The most obvious reason is that these organic fertilizers supplied additional nutrients for soybean growth that were not provided by urea. The biosolids and digestate are liquids (>85% water content) with high plant-available N content, and the application rates used supplied 2.28 mg NH_4 g^{-1} dry soil with biosolids and 0.42 mg NH_4 g^{-1} dry soil with digestate (calculated from Table 2.1). In contrast, the compost amendment supplied 0.26 mg NH_4 g^{-1} dry soil and the N fertilizer supplied 0.45 mg NH_4 g^{-1} dry soil, but these solid fertilizers must be dissolved before the NH_4 can be absorbed by the crop. Furthermore, biosolids and digestate contain appreciable quantities of water-soluble P, K, Ca, Mg (Table 2.1), as well as S and micronutrients (data not available).

Another reason that biosolids and digestate may improve soybean growth is by increasing the water retention capacity. I observed that soybean plants exposed to heat or water stress were less wilted and had wetter soil the day after watering when grown in pots receiving biosolids and digestate than in the compost-amended and N fertilized pots (Figure 2.3). This suggests that biosolids and digestate might contain water absorbent compounds, such as clays and aluminosilicate minerals (e.g., zeolites) that are commonly used to stabilize and deodorize such wastes (Ajenifuja et al., 2012; Nissen et al., 2000) but we do not have access to this proprietary information. It is also possible that the water-soluble organic matter and nutrients in the biosolids

and digestate caused changes in soil micro-porosity (Pagliai & Vittori Antisari, 1993), leading to greater water retention, but this needs to be determined in a future study.

Soybean exposed to heat or water stress produced roots with similar biomass, length, diameter and number of nodules, regardless of the fertilizer source applied. However, there were more N_2-fixing nodules in heat-stressed soybean that were grown in soil receiving biosolids or digestate than with N fertilizer. As well, there were more N_2-fixing nodules in water-stressed soybean grown in the digestate-amended soil than with N fertilizer. This was unexpected, since soybean grown in the compost-amended soil had >2.5-fold more nodules than the other fertilizer treatments. Soil pH was favorable for nodulation in the compost soil (on average, pH 5.3) compared to the other treatments (on average, pH 4.9), which is consistent with greater nodulation in soils with pH >5 (Ferguson et al., 2013) due to increased abundance of α-proteobacteria, some of which are rhizobia (Rousk et al., 2009; Wan et al., 2020). However, the compost-amended soil was not conducive to N_2 fixation in soybean nodules (no difference in proportion of N_2-fixing nodules between the compost-amended and N fertilized soil). The activation of N_2 fixation in soybean nodules requires a low plant-available N concentration (i.e., NH_4 plus NO_3), but the compost-amended soil had the highest or second highest plant-available N concentration after 10 wk (depending on the stress condition), which probably inhibited nitrogenase and prevented N_2 fixation in nodules (MarÃti & Kondorosi, 2014). This suggests that conditions in digestate- and biosolids-amended soils favored N_2 fixation in heat- and water-stressed soybean but identifying the reasons for such is beyond the scope of this thesis and remains to be confirmed.

2.5.2 Organic fertilizers can increase ACC deaminase-producing rhizobacteria, but do not affect ethylene production

I expected organic fertilizers would increase the ACC deaminase-producing rhizobacteria, and their higher abundance would be associated with reduced ethylene production by soybean. The first part of the statement is supported, because there were more *acdS* genes in rhizobacteria that were exposed to compost > biosolids = digestate > N fertilizer ($p < 0.05$, Bonferroni's), with more ACC deaminase activity in rhizosphere soil of soybean treated with biosolids > compost = N fertilizer = digestate ($p < 0.05$, Bonferroni's). Since rhizobacteria differ in their ability to produce ACC deaminase (Nascimento et al., 2016), this suggests the biosolids amendment created the right growth conditions for the active ACC deaminase-producing rhizobacteria. Although biosolids treated bulk soil had similar macronutrient levels to N fertilizer soil (dissolved/organic C, N P), biosolids contains other nutritive substances that can promote the growth of ACC deaminase-producing rhizobacteria, such as zinc and molybdenum. For instance, biosolids can contain 294 –2132 mg Zn kg^{-1} and 14.3 – 84.9 mg Mo kg^{-1} (Wijesekara et al., 2016); and ACC deaminase-producing rhizobacteria require 708 mg Zn kg^{-1} and 99.1 mg Mo kg^{-1} soil (as required for culturing by (Penrose & Glick, 2003).

Another possibility is that the most active ACC deaminase-producing rhizobacteria were present in the biosolids and introduced unintentionally when biosolids was mixed with the soil. Biosolids contain their own microbiome and are able to inoculate the bulk soil with novel bacteria (Little et al., 2020), which could colonize the rhizosphere as the soybean root system developed during this 10 wk study. In the future, I will need to culture or sequence bacterial isolates from biosolids and the soil to determine what proportion of the bacterial community was introduced by the biosolids application.

While pots with higher ACC deaminase activity did have lower ethylene production ($r = -0.38$, $p < 0.05$; Figure 2.2), this was not due to a particular fertilizer treatment nor was it induced

by the heat stress or water stress. Ethylene production declined with increasing plant biomass and was negatively correlated with root diameter (r = -0.3, $p < 0.05$, Figure 2.2), suggesting that larger soybean plants did not produce more ethylene. Since ACC deaminase activity was not associated with any plant variable measured in this study, I cannot conclude that larger plants had more capacity to degrade ACC via their ACC-producing rhizobacteria. Thus, I deduce that larger soybean plants probably did not produce much ethylene at all. This reasoning is consistent with the fact that larger plants can tolerate stress better than smaller plants by altering source-sink relationships to counteract heat stress, or acquire more water with large-diameter roots (White et al., 2016; Zamski, 1996).

There is no standard method to quantify the ethylene production from soybean, so my approach was to measure the ethylene emitted by an intact plant-soil system (i.e., the ethylene concentration in the headspace of a closed chamber containing the undisturbed pot). Unfortunately, this does not allow me to distinguish the ethylene produced in the root (~5% of the total biomass) from the shoot (~95% of the total biomass). According to my measurements, ethylene production and ACC deaminase activity were similar in soybean that experienced water and heat stress, and in the unstressed controls ($p > 0.05$, Bonferroni's test). Based on my procedure, it will be difficult to detect a small decline in ethylene production from the rhizosphere because the total ethylene concentration in the plant-soil system is highly skewed by ethylene emitted from the leaves. To overcome this methodological limitation, I recommend using a pot design that can be closed over the root-soil compartment and sampled through an air-tight septum, since the roots and associated soil are the habitat of ACC deaminase-producing rhizobacteria. My measurement of ACC deaminase activity required me to isolate and grow enrichment cultures of ACC deaminase-producing rhizobacteria before quantifying the ACC deaminase activity (Penrose & Glick, 2003).

Thus, my ACC deaminase activity data is not necessarily indicative of a stress response, since it reflects the magnitude of potential ACC deaminase activity in soil from the soybean rhizosphere. Since all pots contained the same soil and experienced minimal stress for the first 9 wk of the study due to the fertilizer treatments, it appears that the short-term (1 wk) of water or heat stress did not disrupt the potential ACC deaminase activity established by the fertilizer sources (biosolids > compost = N fertilizer = digestate; $p < 0.05$, Bonferroni's). I recommend that a method be developed to directly quantify ACC deaminase from rhizosphere soil, without an enrichment step, to reflect the actual ACC deaminase activity in response to experimental treatments, including water and heat stress.

2.6 Conclusions

Although soybean plants are capable of N_2-fixation, they obtain 40-75% of their N from soil and fertilizers applied to the soil. Soybean often relies on residual soil fertility rather than fertilizer inputs, which may increase its susceptibility to heat and water stress. In this study, fertilizing soybean plants with organic fertilizers such as biosolids and digestate produced more aboveground biomass than with N fertilizer, particularly when the soybean was exposed to heat or water stress. Compost increased the proportion of rhizobacteria possessing the acdS gene, while biosolids increased the activity of the ACC deaminase enzyme. Although water and heat stress did not affect the ACC deaminase-producing rhizobacteria or ethylene production, greater ACC deaminase activity was associated with less ethylene production from the soybean-soil system. These findings suggest that well-fertilized, large soybean plants, which were produced in the biosolids-amended soil, was more tolerant of heat and water stress than soybean grown in soil that received N fertilizer. Methodological refinements and field testing will be helpful to confirm this result.

Chapter 2 References

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Connecting Paragraph

In Chapter 2, I found that organic fertilizers like biosolids and anaerobic digestate increased the accumulation of soybean aboveground biomass. Water stress suppressed soybean growth, but this inhibitory effect was less pronounced for soybean grown in biosolids-amended soil than in soil receiving N fertilizer. Although soil chemical parameters were virtually identical in the biosolids-amended and N fertilized soils after 10 wk, biosolids increased the bacterial ACC deaminase activity significantly ($p < 0.05$). This suggests that biosolids may have altered the rhizosphere bacterial community by physically changing the soil environment for root growth or have increased the biodiversity by adding exogenous bacteria that were recruited into the rhizosphere community. Thus, Chapter 3 will 1) investigate if differences exist in bacterial communities of the soybean bulk and rhizosphere soil compartments and 2) determine if the fertilizer source (biosolids or N fertilizer) alters the composition of these bacterial communities.

Chapter 3

Biosolids change the composition of the rhizosphere bacterial community of soybean

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3.1 Abstract

Soil in the rhizosphere generally has a more specific, less diverse bacterial community than bulk soil. Organic fertilizer that contains carbon-rich substrates and nutrients may stimulate the growth of soil heterotrophic bacteria and add exogenous bacteria, thus, I hypothesize the composition of bulk and rhizosphere bacterial communities will depend on the fertilizer source added to soil. The objective of this study is 1) to determine if distinctive bacterial communities exist in the bulk soil and soybean rhizosphere soil and 2) to determine how bacterial communities in these soil compartments are affected by biosolids or N fertilizer (urea) sources. Soybean was grown in fertilized soil for 10 wk in a greenhouse, then bulk and rhizosphere soil samples were collected, genomic DNA was extracted and the 16S rRNA bacterial genes were sequenced via Illumina high-throughput sequencing. Biosolids and N fertilizer led to unique bulk and rhizosphere soil bacterial communities (ANOSIM = 0.4045, $p = 0.002$; Figure 3.1). A greater proportion of core taxa such as *Luteibacter* and *Burkholderia* were found in the rhizosphere soil of pots with N fertilizer than biosolids, while seven other taxa were indicators of biosolids-treated soil (enriched in bulk soil: *Chryseobacterium*, *Scopulibacillus, Ideonella, Ralstonia*; enriched in rhizosphere soil: *Cytophaga, Asticcacaulis, Holophaga*). Pots receiving N fertilizer had distinct bacterial communities between the bulk and rhizosphere soil in terms of evenness (Simpson evenness, $p < 0.05$) and composition (ANOSIM = 0.4045, $p = 0.002$), whereas pots receiving biosolids had similar bacterial diversity and evenness between the bulk and rhizosphere soil (Simpson evenness, $p > 0.05$). I conclude that organic fertilizer like biosolids lead to the development of unique bacterial communities, relative

to N fertilizer, but biosolids equalize the bacterial communities in bulk and rhizosphere soils, possibly by improving the habitat for bacteria in the bulk soil or by supporting greater bacterial colonization of roots.

3.2 Introduction

The soybean rhizosphere is colonized by a diverse bacterial community. Some of these bacteria in the soybean rhizosphere include a core group of taxa referred to as PGPR such as *Bradyrhizobium*, *Sphingomonadaceae* and *Sphingomonas* (Bhattacharyya et al., 2018; Seo et al., 2009). The composition of the soybean rhizosphere bacterial community, that often includes PGPR, is expected to be distinct from the bacterial community of the surrounding bulk soil, in part because of the selective force of compounds exuded by soybean roots. Soybean releases organic carbon and signal biomolecules in its root exudates that selectively attract bacteria from the bulk soil into the rhizosphere (Sugiyama, 2019; Whipps, 1990). Further, bacteria that colonize the rhizosphere must be motile to respond to these signals (i.e., exhibit chemotaxis) and have compatible growth traits for this plant-soil interface, such as an ability to adhere to the root epidermal cells and to grow as a biofilm on the root surface (Saleh et al., 2019). Consequently, the bulk soil community is often more diverse, less dominated by a select few taxa (higher evenness) and contains fewer PGPR (Marilley et al., 1998; Smalla et al., 2001; Y. Yang et al., 2017). In some instance however, studies have found fewer differences between these two communities (Cui et al., 2019). While several factors could explain why observations vary in whether differences or similarities exist between rhizosphere and bulk communities, one factor which may create differences is the fertilizer source.

Fertilizer sources alter soil bacterial communities, with greater changes attributed to organic fertilizers than mineral N fertilizers. Since organic fertilizer contains carbon-rich

substrates and nutrients, they stimulate the growth of soil heterotrophic bacteria, which is why Marschner (2003) reported that organic fertilizers increase soil microbial biomass and Yu (2015) found greater bacterial diversity in the bulk soil relative to both unfertilized bulk soil and fertilized rhizosphere soil. Further, organic fertilizers contain exogenous bacteria that can be incorporated into the indigenous soil bacterial communities. Gandolfi (2010) and Wolters (2018) determined that bacteria present in organic fertilizers can successfully colonize the bulk soil. Little (2020) found that treated biosolids have a microbial community with large amounts of Firmicutes, Actinobacteria and Proteobacteria such as PGPR genera *Bradyrhizobium* and *Devosia*. As the soybean recruits its rhizosphere bacterial community from the bulk soil, applying an organic fertilizer that increases the size of the bulk soil bacterial community is expected to increase the probability that more bacteria in the bulk soil will be recruited into the rhizosphere bacterial community. This may have consequences for soybean's ability to tolerate abiotic stress, since greater diversity is associated with higher resistance to disturbance (Allison & Martiny, 2008).

Additionally, organic fertilizers may directly impact the rhizosphere bacterial community. As larger root diameters and overall root biomass are expected from soybean fertilized with biosolids relative to N fertilizer (Chapter 2), This higher volume of rhizosphere soil may increase the likelihood of recruiting uncommon taxa and thus increase rhizosphere bacterial diversity. Alternatively, differences in the soybean rhizosphere bacterial community, may be due to biosolids altering plant gene expression (Chowdhury et al., 2019) and thereby the root exudation pattern (Koo et al., 2006). Thus, the potential for biosolids to alter the bulk soil microbial recruitment pool, increase the volume of the rhizosphere environment or modulate the soybean's selection of its rhizosphere community may increase rhizosphere evenness and diversity and result in more similar communities between the bulk and rhizosphere soil.

The objective of this study is 1) to determine if distinctive bacterial communities exist in the bulk soil and soybean rhizosphere soil and 2) to determine how bacterial communities in these soil compartments are affected by biosolids or N fertilizer (urea) sources. I expect the fertilizer source (biosolids or N fertilizer) will modify the composition of bulk and rhizosphere bacterial communities, and specifically biosolids will encourage a higher diversity in both soil compartments through increasing the pool of different microbes in the bulk soil and by modifying the soybean's selection of the rhizosphere community. Additionally, I hypothesize the soybean rhizosphere soils across both fertilizer sources to have less diverse bacterial communities than bulk soil because they are colonized by a subset of the bacteria present in the bulk soil. However, I expect that the relative difference between bulk and rhizosphere soil community diversity will be minimized with biosolid application because applying an organic fertilizer increases the size of the bulk soil community and thus, the probability that more bacteria in the bulk soil will be recruited into the rhizosphere community.

3.3 Materials and Methods

3.3.1 Experimental Materials, Experimental Design and Treatments

Soils collected for this study are a subset of the experiment described in Chapter 2, which is fully described in sections 2.2.1- 2.2.2 of this thesis (*Soil and Soybean*, *Experimental Design and Fertilizer Treatments*). Briefly, the experiment involved mixing fertilizer treatments (biosolids or N fertilizer) with soil and growing soybean in fertilized soil for 10 wk. The pots selected for this study did not experience heat or water stress during the experimental period (described in Chapter 2 as the no stress treatment). Bulk soil was not in direct contact with the roots. It was considered to be the soil remaining in the pot after soybean was harvested and roots were removed

from the pot. After the root mass was taken out of the pot, the rhizosphere soil was collected manually by using a fine brush to collect the soil that remained attached to the roots (it could include soil from the endorhizosphere, rhizoplane and ectorhizosphere, according to the definition of Pinton, Varanini, & Nannipieri (2001)). Any soil removed with the roots, but not tightly adhering to the roots, was discarded. All soil was immediately frozen with liquid nitrogen and stored at -80ºC until analysis.

3.3.2 Bacterial Community Sequencing

I extracted total DNA from bulk and rhizosphere soil using the Qiagen DNAeasy PowerSoil extraction kit (Cat. No 12888-100) from all four fertilizer treatments. I verified the amplification of the V3-V4 region of 16S rRNA gene of bacteria by PCR and gel electrophoresis imaging using the primer sequence 515F-Y (5′-GTGYCAGCMGCCGCGGTAA) and 926R (5′-CCGYCAATTYMTTTRAGTTT) (Parada et al., 2016). The DNA of 16 rhizosphere and bulk soil samples and 1 negative control were then sent to Genome Quebec for Illumina MiSeq high throughput sequencing (250 bp pair end reads) using the primers listed above. All raw sequencing data (with primers removed) are accessible on the NCBI Sequence Read Archive (SRA) under the submission PRJNA681400 (http://www.ncbi.nlm.nih.gov/bioproject/681400).

3.3.3 Sequence Analyses

I obtained 1 799 564 raw reads from 16 samples: 8 rhizosphere soil samples (4 fertilized with biosolids, 4 fertilized with N fertilizer) and 8 bulk soil samples (4 fertilized with biosolids, 4 fertilized with N fertilizer). The sequences were processed using the DADA2 package (version 1.14) in RStudio (Callahan et al., 2016). First, primers were removed using the cutadapt tool

(Martin, 2011). Next, the reads were dereplicated by combining all identical sequencing reads. I then merged pair-end reads together, where the contig length was 413 base pairs. The sequence processing generated a total of 813 211 reads with an average of 50 825 reads per sample. After removing chimeric sequences, I assigned taxonomy using the SILVA version132 database (Quast et al., 2013). The amplicon sequence variants (ASV) threshold of 100% similarity was used to differentiate sequences into 35 326 ASV that represent unique organisms. Next, I used the RAM package (1.2.1) to remove all ASVs with < 5 reads and rarefy my data (SFigure 3.1). This left me with 18 528 ASVs that were classified at the phylum level.

3.3.4 Data Analyses

I calculated alpha diversity indexes with the RAM package function OTU.diversity to determine at a local scale how many taxa inhabited the bulk and rhizosphere regions of soil that received either biosolids or N fertilizer. The true diversity indices selected for this study (Shannon's True Diversity and Simpson's True Diversity) rely upon linear data that represents the actual number of taxa, making them more intuitive than the logarithmic diversity indices (Blackwood et al., 2007). Simpson indices of diversity are generally more influenced by dominant taxa, while Shannon indices are more influenced by rare taxa (Keylock, 2005). I computed evenness indices (Shannon's Evenness and Simpson's Evenness) to measure the numerical balance of the abundance of each taxa in a given sample. Chao1 is a non-parametric method that determines the total taxa in a sample by extrapolating from the sequence data to infer the presence of undetected rare taxa. The alpha diversity indices of bacterial communities were compared in the rhizosphere and bulk soils (soil type), and between the biosolids and N fertilizer treatments (fertilizer source), with a non-parametric Kruskal-Wallis test or a non-parametric Wilcoxon test due to non-normal distribution of the data. When the main effects of soil type or fertilizer source

were significant, I compared the mean values of the alpha diversity index with a pairwise.t.test using Bonferroni's adjustment.

Soil type and fertilizer source could influence the variability of the bacterial community in pots with soybean, which was evaluated with a Bray-Curtis dissimilarity matrix using the Analysis of Similarity (ANOSIM) after Hellinger transformation of the ASV tables with the decostand function in the vegan package (version 2.5-6; Oksanen et al., 2008). Results were visualized with a nonmetric multi-dimensional scaling (NMDS) ordination since nulls were sparse in my ASV tables (ASVs present in some samples but absent in many others). Briefly, I used the Bray-Curtis dissimilarity matrix to calculate the ranks of the distances among samples, determine the best configuration of the samples based on these ranks and plot them in a reduced number of dimensions (k=2).

To compare the composition of bacteria in the rhizosphere and bulk soils (soil type), and between the biosolids and N fertilizer treatments (fertilizer source), I calculated relative abundances from Hellinger transformed data using the tax.abund function (from the RAM package), then compared the relative abundances with a pairwise.t.test function with a Bonferroni's adjustment.

Indicator taxa are those which have high specificity ('the probability that the surveyed site belongs to the target site group given the fact that the species has been found') and fidelity ('the probability of finding the species in sites belonging to the site group') to a particular experimental factor (De Cáceres & Legendre, 2009). Here, I employed the group.indicators function from the RAM package (using a specificity of >80%, $p < 0.05$, and fidelity of >80%, $p < 0.05$) to identify genera that were statistical indicators of a particular soil type or fertilizer source ($p < 0.05$) (Chen & Simpson, 2014). Additionally, the relationship between taxa that were indicators of biosolids-

fertilized rhizosphere soil, ACC deaminase gene and functional activity and root biomass (which were measured in Chapter 2 sections 2.2.8 and 2.2.9) was studied via a correlation analysis.

Statistical analyses were done with R version 3.6.1 (R Core Team, 2019) and data visualization of the ASV tables was done with ggplot2, corrplot, RAM, phyloseq and vegan packages following standard procedures (e.g., Chen & Simpson, 2014; McMurdie & Holmes, 2013; Oksanen et al., 2008; Wickham, 2016).

3.4 Results

3.4.1 Bacterial genera present in soil planted with soybean

Approximately 51% of sequences or 9449 ASVs were classified to the bacterial genus level, resulting in 432 different genera in 26 distinct phyla. About 73% (315 of 432 bacterial genera) were found in more than one sample and 24% (105 of 432 bacterial genera) were present in all soil samples (n=16) from pots planted with soybean. Proteobacteria, Actinobacteria and Acidobacteria were the most common phyla in all soil, while the five most common genera were *Burkholderia, Sphingomonas, Gemmatimonas, Byrobacter* and *Massilia* (SFigure 3.2).

3.4.2 Biosolids modified the bulk and rhizosphere soil bacterial communities

The bulk soil and rhizosphere soil communities were different between biosolids and N-fertilizer treatments (ANOSIM = 0.4045, p = 0.002; Figure 3.1). More *Luteibacter* (5.3%) and *Burkholderia* (13.2%) were present in the rhizosphere soil from pots receiving N fertilizer than bulk soil or the biosolids-fertilized soil (*Luteibacter*; 0.03–0.3%, *Burkholderia* 1.5–4.4%).

3.4.3 Indicator taxa detected in soil amended with biosolids

Seventeen genera were identified as the main taxa that drive the differences between the communities of bulk and rhizosphere soil fertilized with biosolids or N fertilizer (based on the

group indicators test), namely: *Chryseobacterium, Scopulibacillus, Deinococcus, Ideonella, Asticcacaulis, Cytophaga, Holophaga, Steroidobacter, Rudaea, Dyella, Rhodopseudomos,*

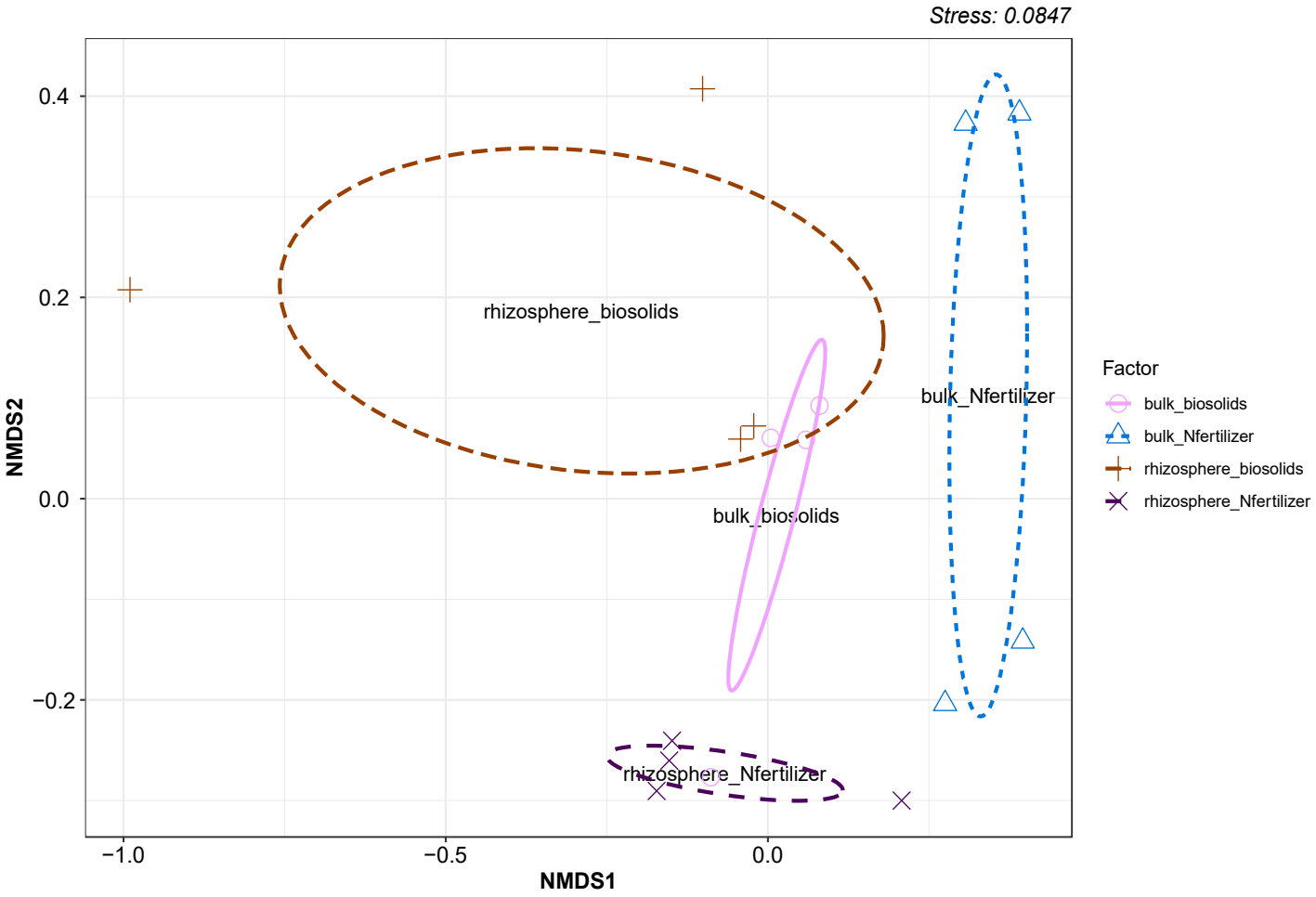

Figure 3.1. Bacterial communities (beta diversity) in rhizosphere and bulk soil from pots fertilized with biosolids or N fertilizer (urea), segregated with nonmetric multidimensional scaling (NMDS) ordination based on Bray-Curtis dissimilarity matrix. k= 2 dimensions

Ralstonia, *Pedobacter*, *Xenophilus, Aquicella, Chthoniobacter, Kribbella* and *Microvirga*. The relative abundance of 5 of these taxa were higher in the bulk soil from pots that received biosolids rather than N fertilizer (Figure 3.2); *Chryseobacterium* (16-fold higher), Scopulibacillus (23-fold higher), *Ralstonia* (10-fold higher), *Ideonella* (4-fold higher) and *Asticcacaulis* (not detectable in N fertilized bulk soil). Whereas, the relative abundance of 3 indicator taxa were higher in rhizosphere soil from pots that received biosolids rather than N fertilizer; *Asticcacaulis* (92-fold higher), *Cytophaga* (75-fold higher) and *Holophaga* (16-fold higher). Further, *Cytophaga* and *Asticcacaulis*, which were enriched in soil that received biosolids and highest in the rhizosphere soil that received biosolids, were correlated with acdS gene possession (*Cytophaga*: r=0.47, $p < 0.01$; *Asticcacaulis*: r=0.50, $p < 0.01$), ACC deaminase activity (*Cytophaga*: r=0.57, $p < 0.01$; *Asticcacaulis*: r=0.67, $p < 0.01$) and root weight (*Cytophaga*: r=0.47, $p < 0.01$; *Asticcacaulis*: r=0.59, $p < 0.01$) (SFigure 3.3).

3.4.4. Bacterial community was more even with biosolids than N fertilizer

Difference in bacterial diversity between the bulk and rhizosphere soil depended on the fertilizer treatment. Biosolids-fertilized soil had similar bacterial communities in both bulk and rhizosphere compartments (Figure 3.1). Soil that received biosolids also had similar diversity, evenness and richness in the bacterial community between bulk and rhizosphere compartments (Shannon true diversity, Shannon evenness, Simpson true diversity, Simpson evenness, Chao1 richness all $p > 0.05$; Table 3.1) but the biosolids-fertilized rhizosphere was more even than that of N fertilizer (Simpson evenness; $p > 0.05$; Table 3.1). However, N-fertilizer had distinct bacterial communities in bulk and rhizosphere compartments (ANOSIM = 0.4045, $p = 0.002$; Figure 3.1). Pots with N fertilizer had higher taxa evenness in the bulk soil than their rhizosphere of ($p < 0.05$), but similar diversity and taxa richness ($p > 0.05$; Table 3.1).

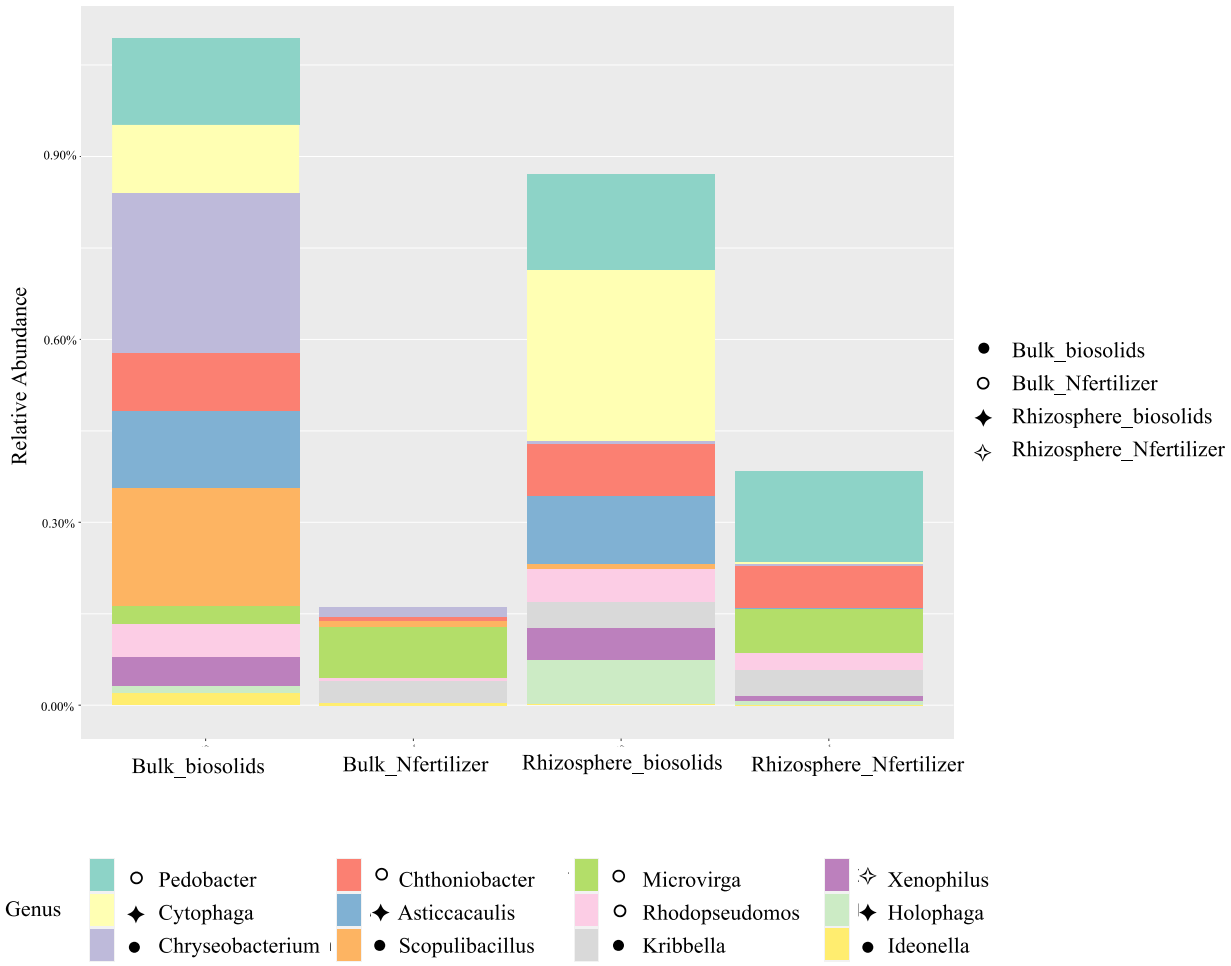

Figure 3.2. Indicator taxa that were present in significantly different proportions in bulk and rhizosphere soil fertilized with either biosolids or N fertilizer (n = 4 soil samples). Symbols indicate the soil-treatment group in which the particular taxon was highest in abundance.

Table. 3.1 Alpha diversity indexes (diversity, evenness and richness) (± standard deviations) of bacterial communities associated with soybean bulk or rhizosphere soil that were fertilized with either biosolids or N fertilizer (n=4).

	Biosolids		N Fertilizer	
	Bulk	Rhizosphere	Bulk	Rhizosphere
Shannon True Diversity	$3.56x10^3$ ($6.66\ x10^2$)	$3.16x10^3$ ($8.61x10^2$)	$3.48x10^3$ ($1.05x10^3$)	$2.98x10^3$ ($1.13\ x10^3$)
Shannon Evenness	$9.44\ x10^{-1}$ ($1.51\ x10^{-2}$)	$9.53\ x10^{-1}$ ($2.46x10^{-3}$)	$9.52x10^{-1}$ ($1.43\ x10^{-2}$)	$9.14\ x10^{-1}$ ($3.31x10^{-3}$)
Gini-Simpson True Diversity	$2.13\ x10^3$ ($7.31x10^2$)	$2.14x10^3$ ($5.0\ x10^2$)	$2.41x10^3$ ($1.04\ x10^3$)	$1.17x10^3$ ($1.09\ x10^3$)
Gini-Simpson Evenness	$3.70x10^{-1}$ ($1.09x10^{-1}$) **ab**	$4.61x10^{-1}$ ($5.02x10^{-2}$) **a**	$4.58x10^{-1}$ ($1.27x10^{-1}$) **a**	$1.84x10^{-1}$ ($1.55x10^{-1}$) **b**
Chao 1 Richness	$6.33x10^3$ ($5.71x10^2$)	$5.06x10^3$ ($1.58\ x10^3$)	$5.61x10^3$ ($1.44x10^3$)	$6.87x10^3$ ($4.72\ x10^2$)

Letters denote significantly different values at a significance of $p < 0.05$.

Regardless of the fertilizer treatment, there was more *Burkholderia* in the rhizosphere (4.4–13.2%) than bulk soil (1.5–2.6%) but fewer *Sphingomonas* and *Masillia* in the rhizosphere (*Sphingomonas*; 3.5–6.4%, *Masillia*; 1.0–2.4%) than in the bulk soil (*Sphingomonas*;6.9–8.9%, *Masillia*; 2.4–3.0%).

3.5 Discussion

3.5.1 Biosolids amendment supported unique bacterial communities in the bulk soil and rhizosphere of soybean, relative to N fertilizer

I accept my hypothesis, that biosolids encourage the development of a distinct bulk soil bacterial community relative to N-fertilizer, as this community was not the same as N-fertilized bulk soil (ANOSIM = 0.4045, $p = 0.002$; Figure 3.1) and many taxa, such as *Chryseobacterium, Scopulibacillus, Ralstonia, Ideonella* and *Asticcacaulis* were enriched (Figure 3.2). There are two reasons that biosolids amendment may favor the development of a unique community. First, there may be exogenous bacteria that were inoculated when biosolid was applied to the soil. Little (2020) reported that biosolids contain their own microbiome and Gandolfi (2010) determined that taxa originating from organic amendments can successfully colonize the soil. Exogenous bacteria from biosolids could become an indicator of biosolids- treated bulk soil since some *Chryseobacterium, Ralstonia, Ideonella* and *Asticcacaulis spp.* are known to exist in wastewater sludge (Bornscheuer, 2016; Carey & McNamara, 2014; Nguyen et al., 2019; Q. Zhao & Liu, 2019) and in this study were also detected in higher relative abundance. However, soil bacterial communities are highly resilient to disruption and tend to exclude exogenous bacteria from niches already occupied by the indigenous population (Allison & Martiny, 2008; Lourenço et al., 2018), and this will reduce the likelihood of biosolid community establishment. Additionally, I would expect a higher total richness in the biosolids treatment, but this was not significant. Thus, I need to conduct additional

experiments to confirm that exogenous bacteria from biosolids can persist in soils, and this would be done by streak-plating biosolids and soil, then identifying surviving colonies through Sanger sequencing the 16S rRNA gene.

Another potential explanation for my observations of these indicator taxa in the biosolids-amended soil is that the provision of carbon-rich substrates and nutrients in biosolids influences the size and functions of microbial communities (Esperschütz et al., 2007). This reasoning seems less likely to explain the presence of unique taxa in this treatment, since I determined (in Chapter 2) that biosolids did not change soil fertility parameters like total organic C, dissolved C, total N, dissolved P and pH, compared to the N fertilizer treatment. However, biosolids supply other nutritive substances such as boron, copper and zinc (Chambers et al., 2003), which would likely impact plant or microbial growth if there were limited amounts of these elements in the soil. Future studies should include a comprehensive soil chemical analysis of all 17 essential plant elements (before and after the experiment and additional sampling timepoints for the microbial available C) and a plant tissue digestion should be conducted to confirm the effects of biosolids on soil and plant chemistry.

Additionally, the NMDS demonstrates that the rhizosphere soil bacterial community was not the same in biosolids and N fertilizer treatments. *Cytophaga* and *Holophaga* were between 16- and 75-fold higher in the rhizosphere of pots that received biosolids than N fertilizer, despite these taxa being present in all bulk soil, regardless of fertilizer. This suggests these taxa were indeed indigenous to the soil in these pots and that biosolids promoted the soybean's selection of this taxa to the rhizosphere. Chapter 2 determined that soybean had larger root diameters and overall root biomass when soybean is fertilized with biosolids relative to N fertilizer, thus this treatment increases the volume of rhizosphere soil. Therefore, biosolids may have increased the likelihood

of roots interacting with (or NGS tools identifying) rarer taxa or more total different taxa. To support this, two of these taxa which were enriched in biosolids-treated rhizosphere, *Cytophaga* and *Asticcacaulis,* were correlated with larger roots (SFigure 3.3). However, larger root diameters can be more anatomically advanced and possess a suberized layer (Wilson & Peterson, 1983). As suberized roots generally release smaller quantities of root exudates than finer non-suberized root tips (Hoffland et al., 1989; McDougall & Rovira, 1970), some of the bacteria in the biosolids' rhizosphere were perhaps not selected through exudation but rather may have spontaneously encountered the root. Alternatively, biosolids could have altered the soybean's gene expression (Chowdhury et al., 2019), thereby modifying the root exudates. Koo (2006) determined that biosolids are capable of altering the types and quantities of organic acid that plants release into the rhizosphere. As soil bacteria consume these compounds, this suggests that here biosolids could have altered the plant's selection of its rhizosphere community to support the current needs of the plant, such as improving abiotic stress tolerance. Two of these taxa, which were enriched in biosolids-treated rhizosphere, *Cytophaga* and *Asticcacaulis,* were also correlated with acdS gene possession and ACC deaminase activity which were assessed in Chapter 2. Additionally, *Cytophaga* spp. are known to possess the acdS gene (Nikolic et al., 2011). Thus, this finding, that biosolids led to differences in the rhizosphere community may allude to how this treatment was capable of producing more bacterial ACC deaminase in Chapter 2. Perhaps this correlation analysis suggests an association that needs to be tested in future experiments. Further, more research into whether biosolids alter root exudation through chemical or physical means is needed.

3.5.2 Bacterial communities have similar diversity in the bulk soil and rhizosphere of soybean

Although I hypothesized, the soybean rhizosphere bacterial community is less diverse than the bulk soil bacterial community because they are colonized by a subset of the bacteria present in

the bulk soil, the alpha diversity was the same in the soil compartments, regardless of the fertilizer. However, the evenness of the bacterial community was lower, and the composition of the bacterial community differed in the rhizosphere and bulk soil of pots receiving N fertilizer (Simpson evenness, $p < 0.05$; Table 3.1; ANOSIM = 0.4045, p = 0.002; Figure 3.1). Smalla (2001) found that the rhizosphere has lower taxa evenness suggesting that fewer taxa thrive in this selective environment, which requires the bacteria to move (i.e. exhibit chemotaxis), to grow on root epidermal cells and to protect themselves (i.e. with a biofilm) (Saleh et al., 2019). Bacteria found in the rhizosphere are expected to be better competitors for carbon-rich substrates secreted by soybean (Sugiyama, 2019). The most abundant PGPR such as *Burkholderia* and *Luteibacter* were adapted to thrive for this selective environment (Bach et al., 2016; Guglielmetti et al., 2013) and thus were relatively more abundant in the rhizosphere than bulk soil of the N fertilized pots (*Burkholderia*; 9-fold increase, *Luteibacter*; 171-fold increase (SFigure 3.2). Thus, despite many studies reporting that the rhizosphere will select for a subset of bacteria from the bulk soil, and is thereby a less diverse community, this study cannot support this (Cui et al., 2019; Marilley et al., 1998). However, the similar diversities here may be due to the low sample number and increasing the sample size in a future study may allow for discernment.

3.5.3 Biosolids equalized the bulk and rhizosphere bacterial communities

I accept my hypothesis that the biosolids treatment leads to more similar bulk and rhizosphere communities since there was no difference in any diversity or evenness indicator and the community composition of these two compartments were not statistically different (Table 3.1; Figure 3.1). As mentioned above, this may also be due to biosolids increasing the probability that more bacteria in the bulk soil will be recruited into the rhizosphere community. The root biomass was greater in the biosolids-amended pots than the N fertilized plots (Table 2.1 from Chapter 2),

meaning that the volume of rhizosphere soil was higher and thus may have increased the likelihood of additional taxa establishing in this area and being identified by sequencing. As this biosolids-fertilized rhizosphere community is more even (and likely has higher functional redundancy), this could mean the soybean is at a lower risk for the essential functions provided by its rhizosphere community being affected by environmental fluctuations (Balvanera et al., 2005). This is an intriguing possibility, since I did observe higher ACC deaminase activity with biosolid rhizosphere communities. This possibility needs to be investigated by evaluating the bacterial metabolic capacities via metagenomics and by studying the community's response to environmental changes, such as heat and water stress.

3.6 Conclusion

Biosolids, an organic fertilizer, cultivated a unique bulk soil bacterial community and that included increased proportions of taxa such as *Chryseobacterium, Scopulibacillus, Ralstonia, Ideonella* and *Asticcacaulis spp.*, which may be due to biosolids inoculating the soil with live heterotrophic bacteria or by supplying additional essential nutrients which were not measured here. Additionally, biosolids and N fertilizer treatments led to unique soybean rhizosphere bacterial communities. While not part of this study, this may be due to biosolids increasing the surface area for root adhesion, or by altering soybean gene expression and root exudation pattern. Additionally, the composition of bulk and rhizosphere soil bacterial communities are generally distinctively different, as the rhizosphere community undergoes selective pressures from the plant, while the bulk compartment has a chemical composition that allows for a larger variety of taxa to prosper. Accordingly, the soybean rhizosphere bacterial community was less even here in soil fertilized with N fertilizer. However, biosolids, led to a similar bacterial diversity and evenness in bulk and

rhizosphere soils, which may further indicate biosolids provided carbon-rich substrates and nutrients that improved the habitat for bacteria in the bulk soil and may have increased the surface area of root structures and thereby the size of the rhizosphere bacterial community. Although I cannot determine how biosolids altered these bacterial soil communities, my results demonstrate that fertilizer sources can influence microbial communities and interactions with the soybean rhizosphere.

Chapter 3 References

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General Discussion

My thesis provides evidence that soybean have better tolerance of abiotic stresses when grown in soil that received organic fertilizer than with N fertilizer only. Biosolids appeared to be the best choice of organic fertilizer for soybean growth in stressful conditions. The major shortcoming of my study is that I did not investigate the multiple reasons why biosolids improved soybean growth, increased bacterial ACC deaminase activity and altered the bulk and rhizosphere bacterial communities. I recognize that biosolids could influence the soil physical, chemical and/or biological processes, and I will explain how these effects could be tested in the future.

First, biosolids may have improved the physical structure of the soil by improving the soil's water retention capacity. I observed that soybean plants exposed to heat or water stress were less wilted and had wetter soil the day after watering when they received biosolids instead of the compost or N fertilizer. Biosolids might contain water absorbent compounds, such as clays and aluminosilicate minerals (e.g., zeolites) that are commonly used to stabilize and deodorize such wastes (Ajenifuja et al., 2012; Nissen et al., 2000) It is also possible that the water-soluble organic matter and nutrients in the biosolids caused changes in soil micro-porosity (Pagliai & Vittori Antisari, 1993), leading to greater water retention. Thus, as a future experiment, the presence of water absorbent compounds and the micro-porosity should be measured in soil amended with biosolids and other fertilizer sources.

Second, biosolids may have altered the soil nutrient concentrations. My results showed that the total N, mineral N, dissolved P, total organic C and dissolved organic C content in biosolids-amended and N fertilized soils was similar after the 7 d stress event. However, biosolids also contain Ca, Mg and S and trace elements such as B, Cu and Zn (Chambers et al., 2003), which could impact plant or microbial growth if any of these nutrients were inadequate to meet biological

needs during the experiment. Thus, in the future a more comprehensive soil chemical analysis of soil (before, during and after the experiment) and a plant tissue digestion should be conducted to confirm the effects of biosolids on soil and plant chemistry. Additionally, a more comprehensive chemical analysis of all 17 essential plant elements in the organic fertilizers should be done prior to the experiment to ensure every pot had the same quantity of biologically relevant nutrients. Hoagland solution could then be added to equalize the nutrient contents in all pots (Hoagland & Arnon, 1950).

Third, biosolids could have inoculated the bulk soil with bacteria, some of which the soybean may have selectively recruited to the rhizosphere through root exudates. As mentioned earlier, biosolids and other organic fertilizers contain exogenous bacteria, some of which are PGPR (Little et al., 2020), that colonize the bulk soil compartment (Gandolfi et al., 2010). To confirm my theory that biosolids altered the rhizosphere community by increasing the diversity of exogenous bacteria and that some of these bacteria were capable of ACC deaminase activity, I could conduct an additional experiment to determine if the increased ACC deaminase production in biosolids-amended rhizosphere soil was attributed to specific members of the rhizosphere bacterial community and whether these members were exogenous bacteria that originated from the biosolids. This could be done by streak plating biosolids and soil. If any colonies emerge, I would 1) test for ACC deaminase activity and 2) identify the bacteria through Sanger sequencing of the 16S rRNA gene.

In this study, the ethylene production was measured from the planted pot, which includes the entire soybean plant (roots and shoots) as well as the soil. While this is a holistic measurement, it is difficult to reconcile with the ACC deaminase activity for the following reasons. Firstly, ethylene can be produced in all plant tissues, not just the root, but the ACC deaminase activity in

rhizosphere soil can only degrade the precursors of ethylene that are present in the rhizosphere. Secondly, soybean plants were not uniform in size and morphology; they varied in their aboveground and belowground biomass, photosynthetic efficiency and other parameters due to the fertilizer treatments and stress effects. Soybean plants with a larger root system could presumably have more ethylene production and/or more degradation of ethylene precursors due to ACC deaminase activity in the rhizosphere. However, the lack of differences in ethylene production across treatments suggests that the measured ethylene concentration was not able to distinguish the root-derived ethylene production from other ethylene sources. Thirdly, in addition to plants, a small proportion of soil bacteria can produce ethylene (Nagahama et al., 1992). Measuring the ethylene production from the pot, which contains ~ 1 kg of soil, did not permit me to distinguish the ethylene that is coming from the plant with that contributed by the soil. Thus, future work should focus on developing a method to measure ethylene only from the roots. Perhaps this could be done by creating a closed system around the mouth of the pot thereby enclosing the root-soil environment. An additional pot containing only soil could be measured and the baseline ethylene concentration subtracted from the experimental pots. In addition, I focused on ethylene production as the most likely mechanism for accelerated ageing in soybean plants that encounter heat and water stress. However, I acknowledge that there are other negative outcomes induced by heat and water stress (such as increased oxidative stress, or gene expression of heat shock proteins) that warrant consideration in future studies.

Another possible limitation of this study was that I separated the nodules containing rhizobia from the rhizosphere soil (in order to quantify the active and total nodules) and thus I only quantified ACC deaminase production from free-living bacteria in the rhizosphere soil. Rhizobia are a large constituent of the soybeans' rhizosphere community and are capable of producing ACC

deaminase at low levels. Approximately 10% of the soybean bacterial rhizosphere community may fix N_2 and produce ACC deaminase (Rascovan et al., 2016). Uchiumi (2004) reported that some rhizobia in the bacteroid, or nodular form, can produce ACC deaminase at low levels. Additionally, Nascimento (2016) mentions that the products of the ACC hydrolysis; ammonia and α-ketobutyrate, are readily metabolized by the rhizobia and the plant. However, I question whether nodule-bound rhizobia would hydrolyze ACC, since release of excess ammonia into the nodule will inhibit nitrogenase activity and thus inactivate the N_2 fixation function of the nodule. Additionally, not all rhizobia can produce ACC deaminase and those who do, produce 10-100X less than free-living bacteria in rhizosphere soil (Glick & Stearns, 2011; Nascimento et al., 2016). More work is needed to resolve the question about whether ACC deaminase activity occurs in nodules that are actively fixing N_2.

The procedure I followed to measure ACC deaminase required me to first enrich the microbial populations through culturing, so it is a potential activity measurement rather than the actual ACC deaminase activity during the stress period. Initially, I tried the ACC deaminase assay by directly adding the substrate to 0.2 g of soil, without first enriching the community via culturing, but I did not detect any ACC deaminase activity. In the future, it would be beneficial to develop another method to measure the realistic activity of the bacterial community directly from soil instead of from a culture. Thus, I suggest a starting point would be to try with 5-10x more soil and then attempting to quantify ACC deaminase *in situ*.

My study proved that combined heat plus water stress on soybean killed all soybeans in less than 7 d, regardless of the fertilizer treatment they received. Although soybean biomass was similar for plants under no stress, heat stress and water stress, combined heat plus water stress significantly reduced aboveground and belowground biomass ($p < 0.05$ relative to non-stressed or

heat stressed). At the onset of water stress, plants increase root growth to compensate for the lack of soil moisture (Chaves et al., 2002). However, since the effects of the combined heat plus water stress caused sudden and severe changes to the plant's physiology such as wilting and loss of active N-fixing nodules, I could not include the results in Chapter 2. Although I can confidently state that combined heat plus water stress with temperatures of 29.7±1.2°C (22.9-47.1°C) and a reduction of SWHC to 8% for a period of 7 d will kill soybean plants, this is not very useful to evaluate stress tolerance. Thus, future studies on the combined effects of heat plus water stress on soybean should reduce the intensity of water stress or opt for a shorter exposure period (2-4 d).

In general, my findings from a controlled greenhouse experiment suggest several research questions that would be worthwhile to test in a field setting. The heat and water stress that crops undergo in a field over a growing season are likely different than the conditions I tested here. Further, the composition of the rhizosphere bacteria community associated with soybean changes during its growth under field conditions (Sugiyama et al., 2014), however, here I chose to study soybeans at one particular developmental stage. Thus, the results could be different if the stress period occurred at a different developmental stage, which would be worth investigating. Additionally, despite soybean having higher susceptibility to nutrient deficiencies under stressful conditions, it is much less susceptible to climate change than other crops. For example, climate change poses a bigger risk to canola yields (Qian et al., 2018) and thus may be beneficial to conduct a field study with other crops as well.

In addition to recreating this experiment under field conditions, conducting this experiment over multiple growing seasons would improve the confidence that biosolids provide a net positive. In this study I did not investigate the negative side effects biosolids may have had on long-term soil or plant health, however, biosolids are often described as containing anthropogenic chemicals

such as triclosan and emerging substances of concern (ESOCs) such as hormones and personal care products (PPCPs), which may persist in soil or bioaccumulate in the crop (Canadian Council of Ministers of the Environment, 2012b; Pannu et al., 2012). Thus, determining the long-term impacts of biosolids application would be wise before providing it as a recommendation to growers of how they can improve the sustainability of their operations.

General Conclusions

A significant finding of Chapter 2 was that soybean grown in soils amended with biosolids and anaerobic digestate maintained its aboveground biomass when exposed to 7 d of heat or water stress. In this study, soybean under heat or water stress had aboveground biomass that was 19–26% taller and 10–18% heavier when fertilized with biosolids or anaerobic digestate than with N fertilizer. This finding may have agronomic implications for Ontario and Quebec growers, as summers in Eastern Canada are expected to become hotter and drier (The Prairie Climate Centre, 2019) and under-fertilized soybeans are at risk of developing nutrient deficiencies in low soil moisture conditions (Chapin, 1991). In a controlled environment, heat or water stressed soybeans grow better in soil that received organic fertilizer. However, a field experiment, including yield measurements, must be conducted before providing growers with the recommendation to use biosolids or anaerobic digestate to maintain soybean growth when they are exposed to heat and water stress.

Additionally, I found that the soybean rhizosphere bacterial community produced 37% more ACC deaminase when fertilized with biosolids than N fertilizer. Growing soybeans that possess a large, specialized rhizosphere bacterial community capable of ACC deaminase activity is desirable to reduce ethylene production, and thereby prevent premature senescence and yield losses. Since this apparently was not due to a change in the soil chemistry and fertility, it might be attributed to a biological change in the rhizosphere, as biosolids contain exogenous bacteria include PGPR capable of ACC deaminase activity (Little et al., 2020). I postulate that these exogenous bacteria colonized the soybean rhizosphere and contributed to the ACC deaminase activity of the rhizosphere bacterial community when abiotic stresses occurred after 10 wk of soybean growth. However, I will need to culture and sequence bacterial isolates from biosolids

and the soil to determine what proportion of the rhizosphere bacterial community was introduced by biosolids application and to verify their ACC deaminase activity before providing this explanation to the scientific community.

Despite this study indicating that biosolids increased microbial stress mitigation and curated a soybean rhizosphere bacterial community that was different from that which received N fertilizer, I cannot affirm the mechanisms through which this happened. My method of ethylene detection in Chapter 2 did not allow me to determine if this treatment reduced the stress ethylene produced by the soybean roots. Thus, I recommend to the scientific community, that in order to investigate the influence of the rhizosphere bacterial community on a plant's production of stress ethylene, ethylene should be measured exclusively from the root environment and that a new method for doing so should be developed. In Chapter 3, biosolids may have elicited compositional differences in the bulk, and subsequently rhizosphere soil, by inoculating the soil with live bacteria (Wolters et al., 2018). Alternatively, differences in the soybean rhizosphere bacterial community, may be due to biosolids altering soybean gene expression (Chowdhury et al., 2019) and thereby the root exudation pattern (Koo et al., 2006). Additionally, biosolids may have created an equalizing condition leading to similar bacterial evenness in bulk and rhizosphere compartments which may further indicate biosolids provided carbon-rich substrates and nutrients that improved the habitat for bacteria in the bulk soil and may have increased the surface area of root structures and thereby the size of the rhizosphere bacterial community. Thus, much more work by the scientific community is required to fully understand the mechanisms through which biosolids are altering these microbial communities and whether these alterations cause meaningful differences in soybean's nutrition and stress mitigation.

Thus, organic fertilizers appear to be an integral part of a more sustainable soybean production. This thesis adds to the scientific community's knowledge of how organic fertilizers improve the growth of soybean in heat or water stressed conditions and that the unique rhizosphere microbial community associated with their application may improve soybean's tolerance to these conditions.

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Appendix Chapter 2

Supplemental Table 2-1. Soybean parameters measured after 7 days of heat plus water stress. Values are the mean (±standard errors) *n*=4

Stress	Fertilizer	Total Biomass (g)	Shoot Length (cm)	Shoot Nodes (#)	Root Length (cm)	Root Diameter (cm)	Root Biomass (g)	Nodules (#)	% N Fixing Nodules	Ethylene (nmol/g biomass/hr)
Heat plus water	N Fertilizer	19 (0.22)	38 (2.2)	5.5 (0.10)	26 (3.4)	1.1 (0.14)	0.85 (0.05)	22 (2.3)	18 (6.5)	13 (0.72)
	Digestate	20 (0.39)	38 (2.5)	5.9 (0.44)	20 (3.3)	1.0 (0.09)	0.78 (0.05)	20 (11)	14 (3.8)	14 (1.5)
	Biosolids	23 (0.24)	46 (1.8)	7.0 (0.36)	27 (3.2)	1.2 (0.12)	1.2 (0.07)	12 (5.3)	13 (7.7)	13 (3.1)
	Compost	19 (0.73)	34 (2.0)	5.9 (0.34)	25 (2.6)	1.2 (0.19)	1.1 (0.20)	40 (14)	16 (3.5)	11 (0.77)

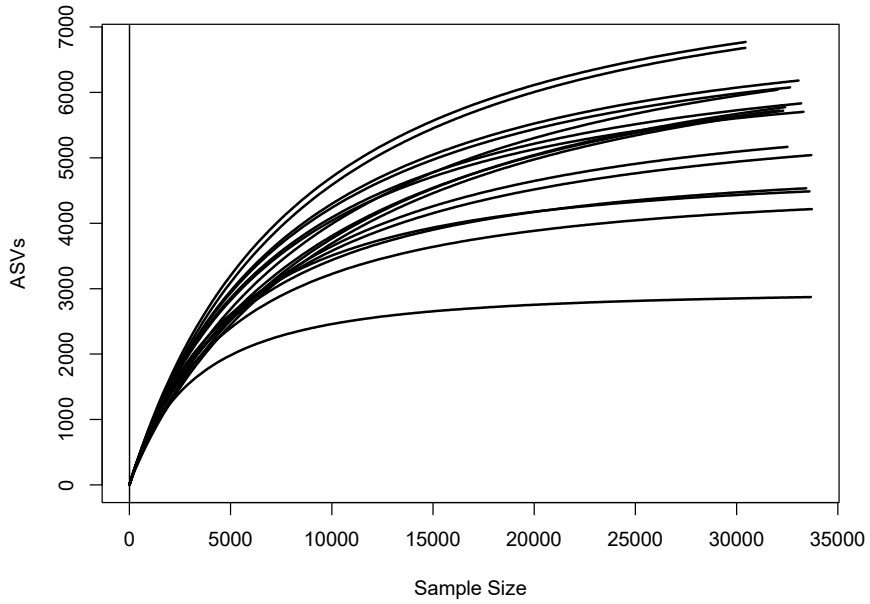
Supplemental Table 2-2. Rhizosphere soil bacterial acdS gene possession and ACC deaminase activity measured after 7 days of stress. Values are the mean (±standard errors) n=4

Stress	Fertilizer	acdS gene (ΔCq)[a]	ACC deaminase
Heat plus water	N Fertilizer	3.67 (0.89)	0.22 (0.15)
	Digestate	5.28 (0.46)	0.26 (0.08)
	Biosolids	3.79 (0.11)	0.46 (0.10)
	Compost	5.02 (0.51)	0.44 (0.08)

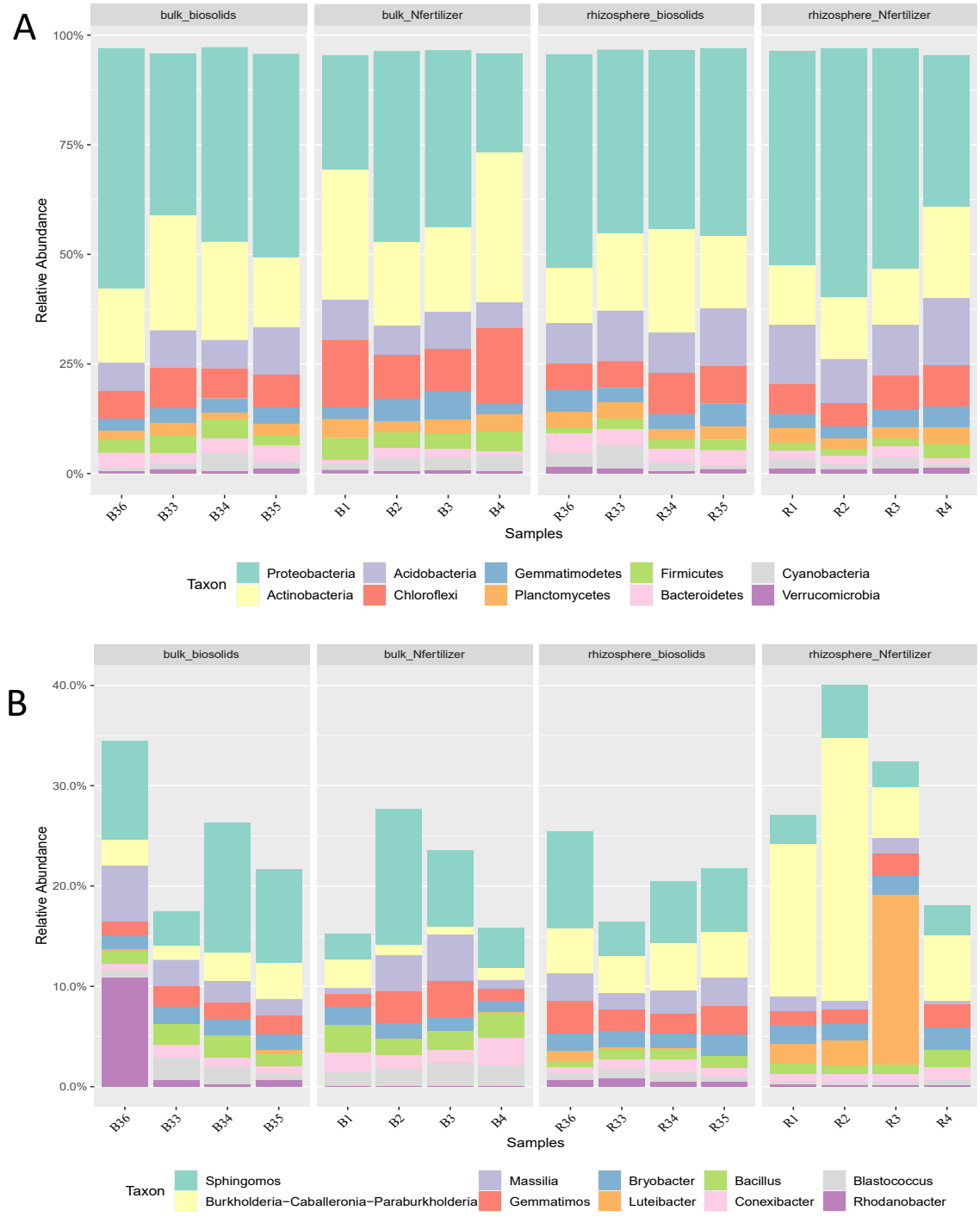
Supplemental Table 2-3. Nutrient concentration and pH in bulk soil amended with N fertilizer (urea) or organic fertilizer (digestate, biosolids, compost) for soybean production, measured after 7 d of exposure to combined heat plus water stress

Stress	Fertilizer	pH	NO_3 (mg g^{-1} dry soil)	NH_4 (mg g^{-1} dry soil)	Dissolved Organic Carbon (mg kg^{-1})	Dissolved P (mg g^{-1} dry soil)	Total N (%N)	Total Organic C (%C)
Heat plus water	N Fertilizer	4.86 (0.10)	12.1 (5.86)	0.25 (0.03)	9.03 (1.08)	0.29 (0.02)	0.10 (0.01)	1.33 (0.07)
	Digestate	4.91 (0.13)	11.2 (3.30)	2.01 (0.51)	10.7 (1.70)	0.24 (0.01)	0.10 (0.01)	1.29 (0.04)
	Biosolids	5.06 (0.08)	3.93 (1.22)	1.49 (0.10)	8.29 (1.79)	0.13 (0.01)	0.11 (0.01)	1.54 (0.28)
	Compost	5.14 (0.11)	13.4 (2.82)	0.30 (0.01)	10.7 (0.94)	0.20 (0.08)	0.14 (0.02)	1.58 (0.15)

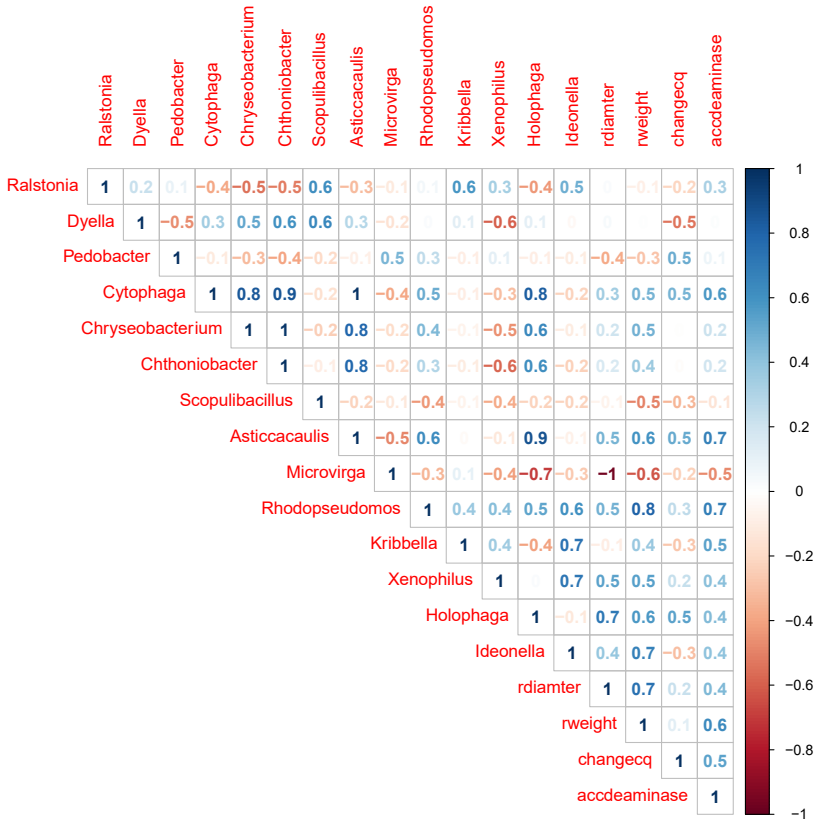
Appendix Chapter 3



SFigure 3.1. Rarefaction curve of all the samples.



SFigure 3.2. The top 10 most abundant Phyla (A) and genera (B) in bulk and rhizosphere soils fertilized with biosolids or N fertilizer for each sample unit



SFigure 3.3 Correlation coefficient matrix displaying the relationship between indicator taxa, gene and functional levels of ACC deaminase and soybean root size