Investigation of enhancement of growth in *Sorghum bicolor* by inoculation with plant growth-promoting microbes

By

Sanjeewa Niroshan Perera Thewage

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Approved - Dr J. Kevin Vessey

(Supervisor)

Approved - Dr Zhongmin Dong

(Committee Member)

Approved - Dr Clarissa Sit

(Committee Member)

Approved - Dr Yousef Papadopoulos

(Committee Member)

Approved - Dr Philippe Seguin

(External Examiner)

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Abstract

Hybrids of *Sorghum bicolor* (L.) Moench have potential as second-generation biofuel feedstock crops in Canada. This study's objective was to screen five plant growth-promoting microbial strains (*Gluconacetobacter diazotrophicus* PAL5T, *G. azotocaptans* DS1, *Azospirillum lipoferum* N7, *A. brasilences* N8 and *Penicillium bilaii*) for growth-enhancing effects on four sorghum genotypes. Two greenhouse studies and one field study were conducted. *Glocanoacetobacter diaztrophicus* (PAL5T)-treated CSSH 45 cultivar showed a significant increase in shoot dry weight by 19%, in N content by 27% and in P content by 37% compared to uninoculated control plants in one of the greenhouse studies. None of the microbial inoculants significantly increased growth of the sorghum in the field, although *Azospirillum brasilense* (N8) treated genotypes consistently had the highest mean shoot dry weight. According to the overall results of the study, PAL5T and N8 may offer the greatest potential for use as growth-promoting bacteria in sorghum production.

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List of Abbreviations

AAFC	Agriculture and Agri-Food Canada
ADP	Adenosine diphosphate
AERC Inc.	Agricultural Environmental Renewal Canada
ATP	Adenosine triphosphate
Ca	Calcium
CFSH 30	Canadian Forage Sorghum Hybrid 30
cfu	Colony Forming Unit(s)
cm	Centimeter(s)
CO_2	Carbon dioxide
CSSH 45	Canadian Sweet Sorghum Hybrid 45
Cu	Copper
DNA	Deoxyribonucleic Acid
DS1	*Gluconacetobacter azotocaptans*
Fe	Iron
g	Gram(s)
GLM	Generalized Linear Model
H^+	Hydrogen Ion
H_2	Hydrogen gas
ha	Hectare
$HgCl_2$	Mercury Chloride
IAA	Indole-3-Acetic Acid
K	Potassium

Kg	Kilogram(s)
m	Meter(s)
Mg	Magnesium
mL	millilitre
mm	Millimetres
N	Nitrogen
N_2	Nitrogen Gas
N7	*Azospirillum lipoferum*
N8	*Azospirillum brasilense*
NH_3	Ammonia
$NHCO_3$	Sodium bicarbonate
^oC	Celsius
OD	Optical Density
P	Phosphorus
P.bilaii	*Penicillium bilaii*
PAL5T	*Gluconacetobacter diazotrophicus*
PGPR	Plant Growth Promoting Rhizobacteria
Pi	Phosphate ion
UV	Ultraviolet
Zn	Zinc
µL	Microliter(s)

1. Introduction

The world energy demand still depends on fossil fuel, though it causes harmful environmental damages such as climate change. Fossil fuel combustion is the main source of greenhouse gas emission (Fu et al. 2019). The whole world is looking for alternative energy sources. Biofuel is a promising alternative energy source (Ameen et al. 2017). The selection of the best biofuel feedstock for biofuel production is the most important task in the field. Among suitable feedstock types, sorghum is becoming a more suitable crop for biofuel feedstock due to its specific growth, physical and chemical characteristics (Almeida et al. 2019). Also, sorghum can establish various microbial symbiotic interactions to speed their growth. With all the features, sorghum could be an excellent biofuel feedstock that can be grown in the field with low input (Ameen et al. 2017; Briand et al. 2018)

Some jurisdictions, including the USA, Canada, European countries, and Brazil, have started producing biofuel to fulfil their daily energy requirements. Some countries have already used regulations and mandates to implement biofuel uses. For example, five Provinces in Canada already have renewable fuel mandates. Both Ontario and British Columbia require 5% bioethanol blends with gasoline for vehicle fuel. However, Canada still buys a large amount of ethanol from the USA for this purpose (GOC 2017). Therefore, Canada needs to develop a greater bioethanol supply without using agricultural lands reserved for food. Since sorghum can produce high biomass even in extreme environmental conditions, this plant can be grown in marginals lands to be used as feedstock for bioethanol production. Also, Nova Scotia can play a major role in this case because the province has over 100,000 ha of underutilized agricultural lands that could support a sustainable biomass energy industry while providing additional income to agricultural producers (GOC 2017).

The first part of the following literature review focuses on *Sorghum bicolor* and its important uses. Details related to *Sorghum bicolor,* including general facts, phylogenetic information, growing characteristic and different uses, have been broadly described. The importance of sorghum as a biofuel feedstock is described in detail in section two. Since one of the study's objectives is to develop sorghum as a biofuel feedstock in Nova Scotia, a separate section has been used to describe the value of sorghum as a biofuel feedstock. The third section is used to describe different microbial relationships which are common in the sorghum plant. Three main sorghum microbial relationships (rhizospheric, arbuscular mycorrhizal and endophytic) have been discussed in this section. The fourth and fifth sections are allocated for descriptions of different microbial strains and different sorghum plant varieties. Those plant varieties and strain types have been tested during the experiment to identify the best combinations as the research outcome. Hence, in the fourth section, five different microbial strains are discussed, including their general information and potential to be used as symbiotic microorganisms with sorghum. The fifth section focuses on developing four sorghum genotypes developed as potential biomass feedstock crops.

2. Literature review

2.1 *Sorghum bicolor*

Sorghum is a C4 herbaceous annual grass species with wide adaptability (Rao and Kumar 2013). It is the fifth-largest grown cereal crop in the world (Song et al. 2019). Sorghum can be grown in different climatic regions, including tropical, subtropical, temperate and semi-arid. However, this plant is more popular in marginal rainfall areas in the tropics and subtropics (Song et al. 2019). Thirty-one sorghum species have been identified based on their morphological characteristics and ecological diversity (Song et al. 2019). Phylogenetic information about sorghum is still not clear, but some information is available. According to that information, sorghum belongs to the family Poaceae, tribe Andropogoneae, subtribe Sorghinae and genus *Sorghum* Moench (Arendt and Zannini 2013). A sub-generic classification is also available for sorghum, but the classification is based on only morphological features of the plant such as node, panicle, spikelet appearance and not phylogenetic information (Rao and Kumar 2013). According to the sub-generic classification, genus sorghum has been divided into the five subgenera of Chaetosorghum, Heterosorghum, Parasorghum, Stiposorghum and Eusorghum (Song et al. 2019; Rao and Kumar 2013).

Among different sorghum species, this literature review primarily focuses on *Sorghum bicolor* species, which belongs to the Eusorghum sub-generic group (Arendt and Zannini 2013). Various common names are used to identify this plant in different countries and regions, such as great millet or guinea corn in West Africa, kafir corn in South Africa, dura in Sudan, mtama in Eastern Africa, jowar in India, kaoliang in China, milo or milo maize in the USA (Rao and Kumar 2013). *Sorghum bicolor* is a grass species with broad flat leaves which is mainly cultivating from seeds. Round shaped or elliptically shaped heads contained seeds can be observed during the maturity

stage. Sorghum generally reproduces via self-pollination, but under some specific conditions, this plant can do cross-pollination. However, the occurrence of cross-pollination is low (4-10%) (Rao and Kumar 2013). *Sorghum bicolor* grain is rich in different nutrients, including protein, vitamins and minerals such as Ca, Fe, K, Mg, P, Zn (Motlhaodi et al. 2018). Also, in sweet sorghum varieties, plant stalk sap or juice is highly concentrated with soluble sugar forms such as sucrose, fructose and glucose (Fu et al. 2019; Rao and Kumar 2013).

Sorghum bicolor has some specific growth characters that make this plant better than other crop species for using as biofuel feedstock such as corn. Sorghum has low nutrient requirements compared to other crop plants (Tang et al. 2018). For instance, sorghum needs only 36% of the N fertilizer required for corn (Briand et al. 2018). Nitrogen is a crucial mineral for C4 plant productivity (Makino and Uino 2018), but higher N fertilizer applications maycause harmful environmental impacts such as global warming and eutrophication (Tang et al. 2018). The low N requirement of sorghum compared to other crops make it an ideal biomass fuel feedstock (Tang et al. 2018; Rao and Kumar 2013).

Sorghum can survive waterlogged condition and can produce good yields even under water stress. Sorghum needs less than half of the water required for corn and is known as "Camel among crops" (Briand et al. 2018). The optimum rainfall range required for sorghum is between 550 mm to 800 mm (Rao and Kumar 2013). During long dry periods, sorghum can stay dormant until environmental conditions are favourable for growing again (Rao and Kumar 2013). Sorghum can also tolerate a vast temperature range (12-37^{o}C), but the optimum range lies between 32^{o}C to 34^{o}C (Rao and Kumar 2013).

Sorghum can be successfully grown in marginal land due to its saline and alkaline tolerance ability (Fu et al. 2019). Therefore, *Sorghum bicolor* has higher productivity compared to other

plants under poor soil conditions. For example, Miscanthus gives 1.8-3.6 ton ha^{-1} biomass in low-quality soil and low fertilizer applications, whereas sorghum gives 2.5-4.0 ton ha^{-1} biomass (Rao and Kumar 2013).

Sorghum has many other features which increase the value of this plant, such as fitting with normal rotation cycle with corn and soya bean, low production cost, multiple harvests per season (depends on the management and region) (Rao and Kumar 2013), good adaptability to climate change effects (Arendt and Zannini 2013) and yield compensation (Berenguer and Faci 2001). Sorghum yield compensation mechanisms are different, depending on the nature of damages and growing stages. For instance, water deficits during flowering stages can reduce the number of grains per panicle, but sorghum can recover this damage by increasing weight per grain (Berenguer and Faci 2001).

Cereals are the primary food source for the world because they provide the necessary nutrients required for life. Sorghum is one such cereal type with wide adaptability to different climatic regions (Arendt and Zannini 2013). It is the second most important food source for southern African people. Sorghum helps to maintain nutritional security in sub-Saharan Africa (Motlhaodi et al. 2018). Sorghum grain has great nutritional value, being composed of 68% carbohydrates, 10% protein, 2% fat and 10% dietary fibre. Sorghum grain is used to make different food products such as bread, cookies, expanded snacks, pasta, and breakfast cereal (Arendt and Zannini 2013). In addition to a food source, sorghum can be used as animal feed for ruminants, pigs, and poultry. As fodder, sorghum has great potential due to its rapid growth, good quality, and high yield (Arendt and Zannini 2013). Sorghum grain's pericarp layer is rich with various chemical compounds (antioxidants and phenolic compounds), and those compounds may have beneficial health properties such as antimicrobial, reduced oxidative stress, inflammatory and

anticancer activity (Rao et al. 2018). Due to high biomass production, stress tolerance ability and metal accumulation ability, sorghum is a potential plant for phytoremediation (Phieler et al. 2015). Also, sorghum grain can be used for making alcohol, malt beer, co binders for metal casting and ore refining grits as packing materials. Sorghum stem can be used for making broom, weaving, and building fences. Sorghum plants are also used as cover crops and windbreaks (Arendt and Zannini 2013). Different products from sorghum such as vegetable oil, adhesives, waxes, dyes are also commercially available in the market. Most importantly, sorghum can produce a high biomass yield (Fu et al. 2019). Sweet sorghum has been identified as an ideal potential bioenergy crop due to its ability to feed into various energy production systems and its adaptability to different growth conditions (Han et al. 2012). Therefore, sorghum has a high potential for use as a biofuel feedstock compared to other annual crops (Silva et al. 2018).

2.2 Sorghum bicolor as a biofuel Source

World energy demand has been increasing with the rapid development of the global economy. Today, fossil fuels are the primary contributor to the energy supply (Fu et al. 2019). Different gas types, mainly CO_2, are released to the atmosphere during the combustion of fossil fuels. Among various greenhouse gas forms, CO_2 is the leading source of the greenhouse effect (Almeida et al. 2019). Increased global temperature is the ultimate outcome of greenhouse gas emissions (Almeida et al. 2019). Therefore, a special concern is building regarding this issue, and it forces the use of energy sources with low environmental impact (Almeida et al. 2019). For example, the report composed by governors of 11 mid-Atlantic and northeast states in the USA to develop a low carbon fuel standard states that "The transition to lower carbon fuel provides important energy security, climate change and economic benefits in the region" (Briand et al.

2018). Also, fossil fuels prices are increasing due to high demand (Malobane et al. 2018). Hence, alternative energy sources with better features such as renewability, sustainability, profitability and safety, must replace fossil fuel (Silva et al. 2019).

Today, biofuel is a widely used alternative energy source in the world. Biofuel contributes to 10% of the global energy supply (Fu et al. 2019). The USA is the largest biofuel producer in the world (Almeida et al. 2019). Among the different biofuel types, ethanol is the most popular and commercialized energy product (Silva et al. 2019). Also, ethanol-based fuel improves air quality in the urban area, and it involves reducing carbon emission to the atmosphere (Briand et al. 2018).

Most importantly, biofuels can be generated from different sources (feedstocks). Hence, this allows each country to produce their energy requirement locally (Malobane et al. 2018). Biofuel feedstocks have been categorized into two categories as first and second-generation fuels based on the source of biomass. Food-based crops such as vegetable oil-producing plants (e.g., canola) and grain crops (e.g., corn) represent the first-generation biofuel crops, whilst lignocellulosic plants such as sorghum and perennial grasses (e.g., switchgrass) represent the second-generation biofuel crops (Malobane et al. 2018). However, the conflict between food security and fuel (i.e., the "Food vs fuel debate) has limited the use of first-generation biofuel crops for bioenergy production (Fu et al. 2019). Therefore, second-generation biofuel crops have become the most suitable candidate in the bioenergy field (Fu et al. 2019).

Among different second-generation bioenergy crops, sorghum is becoming a promising crop due to various reasons, including its versatility, high yield potential and growth characters (Almeida et al. 2019; Malobane et al. 2018). Sorghum has similar or better performances compared to first-generation biofuel crops. For example, energy ratios (output/ input) of sorghum and sugarcane

are 2.23 and 2.42, respectively. Both amounts are quite similar, and it is comparatively higher than the energy ratio of corn (1.30) (Briand et al. 2018).

Most bio-based energy crops require resources such as land, water, nutrients for their growth, similar to food crops (Fu et al. 2019). This becomes a significant drawback in the bioenergy industry due to the limitations of the above resources. For example, water scarcity is the third largest risk in the world (Fu et al. 2019).

Sorghum can successfully grow under harsh environmental conditions such as drought and high heat conditions. This plant can grow in different soil types, from heavy clay soil to light sand, and it can tolerate a wide pH range (5.8 – 8.5) (Ameen et al. 2017). Also, sorghum can survive in saline conditions (Fu et al. 2019). Hence, sorghum can grow in marginal lands and abandoned agricultural lands well (Ameen et al. 2017; Tang et al. 2018). Factors such as surviving in less productive lands, requiring low water and nutrients and absorbing N with high efficiency ensures less competitiveness of sorghum with food crops (Ameen et al. 2017). Also, the use of sorghum for bioenergy production is economically feasible, and the short growing cycle (150-180 days) of sorghum fits with many other crop's offseason in tropical and temperate zones. For example, sorghum is a viable crop to grow during the sugarcane offseason (Almeida et al. 2019). The efficiency of absorption of solar radiation and conversion of CO_2 into biomass in sorghum is greater than the sugarcane (Silva et al. 2019).

Some sorghum species stalk is rich in different types of sugars such as sucrose, glucose and fructose. Those sugars can be easily converted to ethanol. Also, bagasse (i.e., the biomass after soluble sugar extraction) is rich in cellulose and hemicellulose, which can be converted to ethanol through enzymatic reactions (Fu et al. 2019). Under optimum conditions, *Scacromyces cerevisiea* can produce one litre of ethanol from 87g of sorghum stalk juice. 72g of sorghum

bagasse is enough for one litre of ethanol production. Hence, a large volume of ethanol can be ultimately produced from one hectare of harvested sorghum (Efendi et al. 2018).

Knowledge about the chemical composition of bioenergy crops is important in the bioenergy field because the plant's chemical composition directly influences the conversion process of the biomass to biofuels (Almeida et al. 2019; Tang et al. 2018). For instance, the optimum energy conversion of cellulosic biomass can be gained at a low level of lignin. A high level of ash content can negatively affect the thermochemical conversion of biomass into fuel (Tang et al. 2018). The chemical composition of sorghum is favourable for lignocellulosic bioethanol production (Tang et al. 2018). The presence of high fibre content addition to high sugar content makes sorghum chemically feasible for bioethanol production (Almeida et al. 2019). The growing environment and genotype can affect the sorghum's biomass yield and chemical composition (Tang et al. 2018). Some sorghum genotypes have a more favorable chemical composition than others. For example, some sorghum genotypes contain the BMR gene, which influences the expression of low lignin levels in cell walls (Tang et al. 2018).

2.3 Common sorghum-microbial interaction types

2.3.1 Arbuscular mycorrhizal interactions

Mycorrhizal interaction is a structural relationship between fungal species and host plants based on nutritional symbiosis (Cobb et al. 2016). The mycorrhizal relationship can be divided into two major subgroups based on the fungal colonization location: ectomycorrhiza (intercellular in the host) and endomycorrhiza (intracellular in the host). Arbuscular mycorrhiza (AM) belongs to the endomycorrhiza subgroup (Badri et al. 2009). Among mycorrhizal types, arbuscular mycorrhizal are common in land plants (Dodd 2000). Seventy to ninety per cent of land plants maintain this

relationship (Symanczik et al. 2018). Arbuscular mycorrhizal fungal species belong to the order Golmales and has six different genera. Among them, the most frequently identified arbuscular mycorrhizal fungi from previous scientific studies are from the genus *Glomus* (Dodd 2000). Arbuscular mycorrhizal fungi form a specialized structure called arbuscules inside the root when establishing their colonization with specific host plants. Researchers believe that arbuscules act as the main site for transferring nutrients between plant and fungus (Dodd 2000).

Arbuscular fungi express different responses to different plants in term of colonization. During the colonization process, fungi detect exudates compounds released by the plant as a signal molecule. Due to genetic diversity, the plant can produce different signal molecules with different compatibility to arbuscular mycorrhizal fungi. Therefore, fungal responses can be depend on the plant genetic make-up (Cobb et al. 2016).

Due to this symbiotic relationship, the plant receives various benefits from arbuscular mycorrhizal fungi. Arbuscular mycorrhizae allow the plant to access more nutrient (Cobb et al. 2016; Cobb et al. 2018). Fungal hyphae associated with plant roots help extend the area where plants absorb nutrients (Cobb et al. 2016). Fungal hyphae enable the transport of water and nutrients through mycelium to the plant. This also helps maintain the water balance of the plants in addition to supplying nutrients (Silva et al. 2015). The majority of land plants obtain their phosphorus and some trace minerals requirements through arbuscular mycorrhizal fungi (Cobb et al. 2016). Phosphorus is the second most crucial mineral element for plants growth (Ehteshami et al. 2017). However, plants cannot absorb a sufficient amount of phosphorus by themselves from the soil due to its insolubility in water (Ehteshami et al. 2017). Therefore, this relationship with arbuscular fungi is essential for plants to receive sufficient amount of P (Ehteshami et al. 2017).

Arbuscular mycorrhizal fungi also help increase the plant defence mechanism in various conditions (Cobb et al. 2016). For example, when the plant gets injured due to water stress, arbuscular mycorrhizae help increase the plant's physiochemical and biochemical defence mechanisms (Silva et al. 2015). Also, arbuscular mycorrhizae can increase the plant's ability to tolerate different environmental stress conditions (Pedroso et al. 2018). When a plant grows in soil contaminated with heavy metal, arbuscular mycorrhizae can absorb water and nutrient from the soil through hyphae and transfer them to the plant. Also, fungi can absorb and immobilize heavy metal by making a heavy metal complex with glycoprotein (glomalin), released by the fungus (Pedroso et al. 2018). Some mycorrhizal fungi can increase plant tolerance against drought conditions. According to the study conducted by Symanczik et al. (2018), plants with arbuscular mycorrhizae have a higher tolerance to drought compared to plant without arbuscular mycorrhizae association.

In the agricultural sector, producers can benefit economically and environmentally by having their crops associated with arbuscular mycorrhizal fungi (Cobb et al. 2016; Cobb et al. 2018)]. Agricultural fertilizers can negatively impact the environment (reduce water quality and natural ecosystem function) (Cobb et al. 2016). Improving the fungal association in the agricultural system can reduce nitrogen and phosphorus fertilizer application (Cobb et al. 2018). Previous studies have shown that sorghum plants are highly responsive to arbuscular mycorrhizae. Also, this combination improves plant growth and grain production especially in low fertility soil (Cobb et al. 2018).

2.3.2 Endophytic Interactions

Endophytic microorganisms can be defined as organisms, either bacteria or fungi, which can live inside the plant tissue without causing damages to the host plant (Govindasamy et al. 2017; Wilson 1995). Endophytes can colonize different plants parts, including roots, stems, leaves, flowers, fruits and seeds (Puri et al. 2018). Many endophytes have positive effects on their host plants (Mareque et al. 2108). Bacterial endophytes have different beneficial mechanisms to promote plant growth, either directly or indirectly (Govindasamy et al. 2017). Bacterial strains from different phyla, including Proteobacteria, Firmicutes, Actinobacteria and Bacteroidetes, have been identified as endophytes from various plants (Mareque et al. 2108). Most endophytic bacteria are from the phylum Proteobacteria (Mareque et al. 2108). As mentioned, plants receive various benefits from this association, such as producing phytohormone, fungicidal and bactericidal substance, enhancing mineral availability, stimulating plants secondary metabolites (Mareque et al. 2108).

Endophytic bacteria can produce different phytohormone such as auxin, cytokinin and gibberellins (Mareque et al. 2015). Also, endophytes can enhance the plant synthesis of hormones, especially auxin, to initiate lateral and adventitious root formation and root elongation (Govindasamy et al. 2017). Some endophytic bacteria can produce an enzyme called 1-amino cyclopropane -1- carboxylate deaminase, which can reduce the ethylene production in plants in response to different abiotic stress (Govindasamy et al. 2017). Increasing plant nutrient availability is another direct plant growth-promoting activity related to endophytes (Govindasamy et al. 2017). Some endophytes called diazotrophs can fix atmospheric nitrogen as plant usable nitrogen compounds (Govindasamy et al. 2017). Endophytes can convert atmospheric nitrogen to ammonia using an oxygen-sensitive catalytic enzyme called nitrogenase.

The host plant provides nutrients and low oxygen conditions to the endophytes to achieve. optimal nitrogen fixation activity. The conversion process can be shown in the following equation (Bhattacharjee et al. 2008).

$$N_2 + 8H^+ + +8e + 16ATP \xrightarrow{\text{Nitrogenase}} 2NH_3 + H_2 + 16ADP + 16Pi$$

Also, some endophytes help to solubilize minerals (phosphorous) to increase their availability (Mareque et al. 2015). Some endophytes release metal-chelating substances such as iron-chelating siderophores, which helps plants increase mineral uptake, including Fe, Zn and Cu (Govindasamy et al. 2017). Siderophores can also induce systemic resistance against some plant pathogens and induce systemic tolerance against nutrient stress (Govindasamy et al. 2017). Endophytic fungi can also provide benefits to the host plants as endophytic bacteria. They normally colonize above-ground plant parts such as stem, leaves, seeds, fruits. Therefore, fungal endophytes can be easily distinguished from mycorrhizal fungi. However, some fungal endophytes may colonize root tissues as well (Faeth and Fagan 2002). Most of the identified fungal endophytes belong to phylum Ascomycota (González-Menéndez et al. 2018). As mentioned, they have different benefits to host plants. Fungal endophytes can produce and secrete different alkaloid compounds such as pyrrolizidine, ergot and peramine, increasing the host plant resistance against vertebrate and invertebrate herbivores. These alkaloid compound can be toxic or noxious to the herbivore. Some grass species maintain a relationship with fungal endophytes for this reason (Faeth and Fagan 2002). Endophytic fungi can increase plant resistance against some plant pathogens. They can increase host plant tolerance in drought and water stress conditions (Faeth and Fagan 2002). Fungal endophytes are also able to produce

bioactive secondary metabolites that may have importance in the medical research sector (González-Menéndez et al. 2018).

2.3.3 Rhizospheric interaction

The rhizosphere is a zone of soil around the plant root system, which is influenced by the presence of the plant roots. It is rich in various chemicals compounds, including root exudates, simple and complex sugar compounds, growth regulators, various primary and secondary compounds such as amino acids, organic acids, phenolic acids, flavonoids, enzymes, fatty acids, nucleotides, tannins, steroids, terpenoids, alkaloids and vitamin (Ramond et al. 2013; Gopalakrishnan et al. 2011). In addition to chemical changes, the plant can also adjust soil pH, water potential and partial pressure of oxygen (Vessey 2003). This specific area acts as the best place for microbes, including bacteria, fungi (Ramond et al. 2013). As mentioned, the plant can physically and chemically affect the surrounding environment to facilitate rhizosphere bacterial growth (Vessey 2003). Therefore, microbial distribution in the soil is different from the chemical compound availability in soil. As a result, most soil microbes live within a 50 μm radius of plant root systems. The concentration of microbes is even higher within a 10 μm radius (Pii et al. 2015). Among the bacteria living in plant rhizospheres, most have no interactions with the plant called commensals. They acquire their nutritional requirement from plant released exudates and other compounds. Some bacteria may harm the plant. They release toxic compounds as metabolites that can affect plant growth. Some bacteria that can positively affect on plant growth are known as plant growth-promoting rhizobacteria (PGPR). They may directly or indirectly impact plant growth (Pii et al. 2015; Lucy et al. 2004). Most of these rhizospheric bacteria are attached to the plant root surface. However, some can penetrate and colonize the intercellular spaces of the plant root (Vessey 2003).

Plant growth-promoting rhizobacteria impact plant growth in different ways. Plant growth promotion by rhizobacteria may be direct or indirect. One form of indirect plant growth promotion is by protecting the plant from pathogenic microorganisms. Some rhizobacteria can produce fungal cell wall lysing enzyme, which keeps the plant safe from fungal pathogens. Some rhizobacteria can reduce iron availability in the rhizosphere that also limit pathogen growth. Plant growth-promoting rhizobacteria compete with harmful microorganisms for niches in the rhizosphere. A pathogen has less chance to infect the host plant due to this competition (Lucy et al. 2004).

In terms of in direct plant growth benefits, rhizobacteria can increase plant nutrient acquisition (Pii et al. 2015; Lucy et al. 2004). Some rhizobacteria can increase the availability of phosphorus. Some of the bacteria can fix atmospheric nitrogen as a usable form to the plant. Some bacteria can sequestrate iron for the plant using siderophores. Also, they can produce different plant hormones such as auxins, cytokinins and gibberellins (Lucy et al. 2004).

The review paper prepared by Lucy et al. (2004) summarises twenty-five years of research works based on plant growth-promoting rhizobacteria on different crop plants. According to the review paper, they have identified that rhizobacteria can contribute to promoting plant growth in different ways, including increases in germination rate, root growth, yield, leaf area, chlorophyll content, magnesium content, nitrogen content, protein content, hydraulic activity, drought tolerance and biomass (shoot and root weights), and by delaying senescence.

2.4 Possible microbial inoculants that can associate with sorghum

2.4.1 *Penicillium bilaii*

Phosphorous is the second most limiting mineral nutrient for plant growth. However, it becomes a limited mineral form to the plant due to low solubilization potential (Ehteshami et al. 2018). Therefore, phosphorus solubilization is one of the most common activities that microorganisms do for plant growth promotion (Pandey et al., 2008). Among different microbial strains related to the phosphorus cycling fungi, the genus *Penicillium* is a common type (Pandey et al. 2008). *Penicillium bilaii* is a fungus living in soil rhizosphere, originally isolated from soil in Alberta, Canada (Argento 2016). In previous experiments, this organism has shown phosphorous solubilization ability under laboratory conditions. For example, it can solubilize calcium phosphate in an agar medium and rock phosphate in a liquid medium (Gómez-Muñoz et al. 2018). Another study has shown that *P. bilaii* can release both oxalate and citrate acids to increase the acidic level in the medium. Another study has shown that *P bilaii* can increase $NaHCO_3$ and extractable P levels in soil (Gómez-Muñoz et al. 2018).

Previous studies suggested possible mechanisms that *Penicillium bilaii* uses for phosphorous solubilization. One mechanism is that the reaction between the solid phosphorous compounds in soil and the organic anions released by fungus cause an increase phosphorous availability in soil. Organic anions can react with solid phosphorous compound in two different ways. Firstly, organic anions result in an anion containing solid phosphorous compound (Ca, Fe, Al) as chelators. As a result of this reaction, phosphorus releases into the soil. Secondly, the organic anion can result in a ligand exchange reaction to dissolve phosphorus from the solid phase (Gómez-Muñoz et al. 2018).

Another mechanism is that increased acidity in soil due to the release of acidic compounds by fungus can dissolve solid phosphorus compounds. Another mechanism is that enzymes called phosphatases secreted by fungus can dissolve organic phosphorus compounds in the soil (Gómez-Muñoz et al. 2018). However, plant growth promotion by *P. bilai* through phosphorous mobilization is not still fully understood (Gómez-Muñoz et al. 2018).

Different studies have been conducted to identify the influence of *P. bilaii* on different crop plants growth (Argento 2016). For example, Vessey and Heisinger (2001) conducted a study to identify the effects of *P. bilaii* on field-grown pea. According to their results, pea plants grown with *P. bilaii* and without P fertilizer application showed an increase in root length and root dry weight by 48% and 13%, respectively, compared to control plants. Also, shoot P concentration increased by 13%. According to their study, *P bilai* can significantly increase P concentration in the pea plant under P limited conditions.

Another study conducted by Beckie et al. (1997) that studied *P bilaii* on *Medicago sativa* L. (alfaalfa) growth, showed similar results as the other *P. bilaii* related studies. According to their results, annual P credits (P fertilizer replacement value) were 4.6 kg P ha^{-1} and 5.3 kg P ha^{-1} in small and large pot experiments, respectively, due to *P. bilaii* application. The study showed that the plant benefited from *P.bilaii* through phosphorus solubilization. Gomez-Munoz et al. (2018) conducted a study to examine *P bilaii* effects on wheat (*Triticum aestivum* L. cv. Dacke) plant growth. The study showed that *P bilaii* could influence wheat plant root growth and its function, but it depends on soil nutrient content.

2.4.2 *Gluconacetobacter diazotrophicus*

Gluconacetobacter diazotrophicus was initially isolated from stems and roots of sugar cane plants grown in Brazil in 1988. At that time, the organism was named *Saccharobacter*

nitrocaptans (Cavalcante and Dobereiner 1988). After different experiments, including deoxyribonucleic acid (DNA)- ribosomal ribonucleic and DNA-DNA hybridizations, phenotypic and chemotaxonomic analysis, Gillis et al. (1989) identified that this organism belonged to the genus *Aceotobacter*. Therefore, this organism was renamed *Acetobacter diazotrophicus* (Gillis et al. 1989). Later, after the identification of *Gluconacetobacter* as a subgenus of *Acetobacter*, this organism was renamed *Gluconacetobacter diazotrophicus* (Yamada et al. 1997)

According to the first description by Cavalcante and Dobereiner (1988), *Gluconacetobacter diazotrophicus* is a rod shape with rounded ends, gram-negative bacterium having 1-3 lateral flagella (Cavalcante and Dobereiner 1988). This organism has also been identified as a non-rhizobial, endophytic bacterium belonging to class proteobacteria (subclass alpha-proteobacteria) (Bertalan et al. 2009). This bacterium contains a 3.9Mb chromosomal genome and two plasmids with 16.6Kb and 38.8Kb (Sahai et al. 2015). In addition to sugarcane, this bacterium has been isolated from different plants such as Cameron grass, sweet potato, coffee, tea, banana, rice, pineapple, and finger millet (Cocking et al. 2006; Luna et al. 2012).

Gluconacetobacter diazotrophicus helps its host plant through different mechanisms, including nitrogen fixation, phytohormone production, acting against phytopathogens, mineral nutrient solubilization and plant disease resistance induction (Giongo et al. 2010; Luna et al. 2012). Hence, this organism is considered a plant growth-promoting bacterium (PGPB) in the agricultural sector (Luna et al. 2012).

Gluconacetobacter diazotrophicus is a nitrogen-fixing bacterium in the sugarcane plant. Sugarcane plants can obtain up to 80% of their nitrogen requirement through the symbiotic relationship with this bacterium (Dent 2018). This bacterium normally lives in intercellular spaces in the roots and stems of the plant, and they do not form specific structures like nodules

form in legumes with rhizoids. *Gluconacetobacter diazotrophicus* does not produce nitrate reductase enzyme. Hence, they can fix N_2, even in high nitrate concentration (30mM). Therefore, *G. diazotrophicus* is a suitable microbe for the field that are fertilized with nitrogen (Tian et al. 2009). As mentioned, in addition to N_2 fixation, the host plant obtains various benefits from the association with this bacterium. *G. diazotrophicus* can produce plant hormones such as auxins and gibberellins to promote plant growth. Phytohormone production of this bacterium has been confirmed *in vitro* experiments (Santas et al. 2009). Also, this bacterium can inhibit the growth of phytopathogenic bacteria such as *Xanthomonas albilineans* and *Colletotrichum falcatun* by reducing pH through sugar fermentation (Santas et al. 2009).

Various research has been conducted to identify the colonization ability of *G. diazotophicus* in different crop plants and their effects on plant growth promotion. For instance, Tian et al. (2009) studied the colonization ability of different corn genotypes by *G. diazotophicus*. According to their results, some corn varieties have been successfully colonized by the bacterium. This study also showed that the colonization efficiency of *G. diazotophicus* is positively correlated with the plant's sugar content. Another study conducted by Luna et al. (2011) investigated colonization pattern and yield promotion of tomato by *G. diazotophicus*. According to their results, tomato plants were successfully colonized by the bacterium. The fresh weight of the plant and the weight of the fruit were significantly increased compared to the non-inoculated plants. Another study has been conducted by Yoon et al. (2016) studied the colonization ability of different sorghum varieties by *G. diazotophicus*. This study showed the same results as the corn study mentioned above. Some sorghum varieties were successfully colonized by *G. diazotophicus*. Also, colonization efficiency was positively correlated with the sugar content of the sorghum

plant. Hence higher colonization has been observed in sweet sorghum varieties compared to the non-sweet sorghum varieties.

2.4.3 *Gluconacetobacter azotocaptans*

Gluconacetobacter azotocaptans was originally isolated from the rhizosphere and rhizoplane of coffee plants in Mexico in 1997 by Jimenez Salgado. This organism has shared features with the genus *Gluconacetonacter* sp., but morphological, biochemical, genetic and molecular features differ from *Gluconacetonacter diazotophicus*. Hence, this organism was identified as a novel bacterium belonging to the genus *Gluconacetobacter* and named *Gluconacetobacter azotocaptans* by Fuentes-Ramires (2001).

Later, *G. azotocaptans* was isolated from different plants as an endophytic symbiont in roots and stems. For example, this bacterium has been isolated from the internal tissue of sugar-containing plants such as sugarcane, *Pennisetum purpureum*, sweet potato, *Eleusine coracana* and pineapple. In addition to sugar accumulating plants, *G. azotocaptans* has been isolated from wetland rice varieties (Mehnaz et al. 2006)

Gluconacetobacter azotocaptans has not thoroughly been tested as a plant growth promotion bacterium in the research field. Even though Jimenez Salgado and Fuentes-Ramires (2001) have identified and classified this organism, they did not test the potential effects of this organism for plant growth promotion. However, a study has been conducted to identify the potential of *G. azotocaptans* on different plant growth promotion under greenhouse and field conditions (Mehnaz and Lazarovits 2017). During the study, *G. azotocaptans* has been tested with different plant types such as corn, radish, pepper, tomato, and cucumber. According to their results, *G. azotocaptans* involved plant growth promotion mainly through N_2 fixation and indole acetic acid

(IAA) production. Hence, this study has confirmed that *Gluconacetobacter azotocaptans* could be a potential biofertilizer in the agricultural sector (Mehnaz and Lazarovits 2017).

2.4.4 *Azospirillum brasilense* and *Azospirillum lipoferum*

The genus *Azospirillum* belonging to class proteobacteria (subclass alpha-proteobacteria) find as plant growth-promoting rhizobacteria. Bacteria in the genus *Azospirillum* have been identified as capable of nitrogen fixing (Moutia et al. 2010). Bacteria usually live close to the plant root system. Various crops, including cereals, have been reported to have association with members of this genus (Moutia et al. 2010). Bacteria in the genus *Azospirillum* are considered plant growth-promoting bacteria. In the past, researchers believed that the genus *Azospirillum* contributed to the plant growth-promoting mainly through nitrogen fixation, but recent studies have shown that bacteria do not provide a significant amount of fixed nitrogen to the plant. The bacteria use most fixed nitrogen for their requirements. However, the bacteria increase nitrogen availability around the plant through nitrate assimilation (Moutia et al. 2010).

Several mechanisms have been proposed to explain the way of plant growth promotion by *Azospirillum*. A few decades ago, a hypothesis called the additive hypothesis was introduced to address this problem. According to the hypothesis, instead of using a single mechanism, all proposed mechanisms together can be used to describe the plant growth-promoting activity by members of the genus *Azospirillum* (Cassan et al. 2014)

According to the previous studies, the genus *Azospirillum* can produce different plant hormones such as auxins, gibberellins, cytokinin and ethylene (Cassan et al. 2014; Thuler et al. 2013). Bacteria can increase the concentration of IAA and indole -3-butyric acid (IBA) in the root systems. Also, the bacteria cause increases in the respiration rate and enzyme activity related to

the tricarboxylic acid cycle and the glycolysis pathway. These changes affect root morphological and physiological changes, including increasing the density of root hairs, lateral roots and root surface area. As a result of these changes, the root system can absorb more water and more nutrients to increase plant growth (Moutia et al. 2010, Okon et al. 1994).

In this literature review, two bacterial types in genus *Azospirillum* are considered. They are *Azospirillum brasilense* and *Azospirillum lipoferum*. Both are gram-negative, vibrio or spirillum shaped organisms with peritrichous flagella (Okon et al. 1994). *Azospirillum lipoferum* can be usually found in maize roots, whilst *A. brasilense* is common in wheat and rice root systems (Moutia et al. 2010).

Azospirillum brasilense have been identified as plant growth-promoting bacteria mainly through IAA production. Indole acetic acid can change plant root morphology to absorb more water and nutrient to increase plant growth (Spaepen et al. 2008). Indole acetic acid production by *A. brasilense* and its connection to changes in plant root morphology have been studied by Dobbelaere et al. (1999). In the experiment, normal bacteria and mutant bacteria were tested in wheat plants. The bacterium with a mutated gene *ipdC*, which is related to IAA synthesis pathway, was used as the mutant bacterium. The result has clearly shown that the plants inoculated with mutant bacterium had less change in root morphology whilst plants with normal bacterial inoculation had increased root density. The experiment indicates that IAA production of *A. brasilense* has a great impact on plant growth. Different plant varieties, including wheat (Spaepen et al. 2008), strawberry (Pedraza et al. 2010), pearl millet (Tien et al. 1974), *Zea mays* and sorghum (Lin et al. 1983). have been tested with *A. brasilense* to identify the plant growth promoting ability. All the studies have been successfully achieved positive results from *A. brasilense* association.

Azospirillum lipoferum is also well-known plant growth-promoting bacterium, especially in corn growth. This bacterium is commercially available as a biofertilizer under the trade name of AzoGreen-M for corn. Scientists believe that *A. lipoferum* promotes plant growth primarily by producing phytohormones such as auxin, gibberellin and cytokinin (Mehnaz et al. 2006). In addition, *A. lipoferum* and A. *brasilense* both have phosphorus solubilization ability (Rodriguez et al. 2004).

2.5 Sorghum genotypes used in the experiment

Two sorghum cultivars (CFSH 30, CSSH 45) and two sorghum genotypes (10A×131 and 10A×118) are tested with microorganisms in the present experiment. The two sorghum genotypes 10A×131 and 10A×118, have not been registered therefore information about the two genotypes is still proprietary.

2.5.1 CFSH 30 (Canadian Forage Sudan grass Hybrid 30)

Hybrid sorghum multi-cut variety, CFSH 30 has been developed from Sudan-grass parent species. CFSH 30 stands for Canadian Forage Sudan-grass Hybrid 30. This hybrid has been developed as high yielding fine stemmed plants. The multi-cut ability (two or three cuttings) of this hybrid brings a significant yield increase. Hybrid CFSH 30 was developed by AERC (Agriculture Environmental Renewal Canada) with adaptation to the Canadian climate. This hybrid has been recommended for hay, haylage, silage, green chop, or pasture (AERC Inc 2021)

2.5.2 CSSH 45 (Canadian Sweet Sorghum Hybrid 45)

The hybrid CSSH 45 is another commercial hybrid released by Agricultural Environmental Renewal Canada (AERC). CSSH 45 stands for Canadian Sweet Sorghum Hybrid 45. CSSH 45 is a sweet sorghum hybrid for silage with a single cut. Also, this plant can be used as green chop for dairy and beef. This hybrid reaches its optimum harvesting stages within 110 – 120 days (AERC Inc 2021).

2.6 Objective of the study

Previous studies have been conducted between *Sorghum bicolor* genotypes and different microbial inoculants (Mareque et al. 2015). Some studies only focused on the colonization ability of sorghum by different microbial inoculants (Yoon et al. 2016). However, these studies were limited in the number of microbial inoculants tested and were only conducted under greenhouse conditions. Therefore, the objective of the current study is to screen more plant growth-promoting microbial strains for growth enhancing effect on *Sorghum bicolor* cultivars/genotypes under both greenhouse and field conditions.

3. Materials & Methodology

3.1 First greenhouse study

3.1.1 Experimental design

A completely randomized design was used. Five microbial inoculants (*Gluconacetobacter diazotrophicus* PAL5T, *G. azotocaptans* DS1, *Azospirillum lipoferum* N7, *A. brasilense* N8 and *Penicillium bilaii*) were tested with two sorghum cultivars (CSSH 45 and CFSH 30). Two sets of control (With ^{15}N and without ^{15}N) were used for each cultivar. Each treatment and each control were replicated 9 times. One hundred twenty-six experimental units (pots) were used in the study (5 inoculant types + 2 controls × 2 sorghum cultivars × 9 replicates) (Appendix 3).

3.1.2 Preparation of microorganisms

Four bacterial cultures (*Gluconacetobacter diazotrophicus* PAL5T, *G. azotocaptans* DS1, *Azospirillum lipoferum* N7, *A. brasilense* N8), preserved in -80ºC refrigerator in Dr Vessey's Lab at Saint Mary's University, were used in this study. To reactivate the cultures, around 50 µL from each preserved culture were added to the freshly prepared broths. Liquid LGI-P medium (Cavalcante and Dobereiner 1988) (Appendix 1) was used for *G. diazotrophicus* and *G. azotocaptans* whilst *A. lipoferum* and *A. brasilense* were grown in Luria-Bertani (LB) broth (Appendix 2). Inoculated broths were kept in the shaker at 30ºC temperature for 2-4 days. Glycerol stocks of *A. lipoferum* and *A. brasilense* were prepared after two days of growth in the incubator. *G. diazotrophicus* and *G. azotocaptans* glycerol stocks were prepared after four days of growth in the incubator. A volume of 500 µL of 50% autoclaved glycerol solution (50% of glycerol + 50% distilled water) was mixed with 500 µL of bacterial broth solution to prepare

glycerol stocks. Prepared glycerol stocks were kept in -80ºC refrigerator until it was used for the study.

Penicillium bilaii is a commercially available product and, it is available under the commercial name of Jump Start® from Novozymes (Saskatoon, Saskatchewan)

3.1.3 Quantifying microorganisms

Living microorganism count was an important fact to know before applying them as treatments. Therefore, two different methods were conducted to identify the viable microorganism count in each microbial broth. During the study, these counts were used to apply a constant number of microbes to each plant as inoculant treatment.

3.1.3.1 Bacterial quantification

A volume of 20 µL of each bacterial glycerol stock was inoculated to the 50 ml of broth solution (LGI-P medium for two *Gluconacetobacter* strains and LB medium for two *Azospirillum* strains) and kept in the shaker at 30ºC temperature. A dilution series from 10^{-1} to 10^{-8} was prepared from each bacterial culture from day 0 to day 5 using inoculated broths. In the first two days, spread plates were prepared from each bacterium using the higher concentrations in the dilution series (10^{-3} to 10^{-5}). Lower concentrations (10^{-6} to 10^{-8}) were used in the last three days. Two *Gluconacetobacter* sp. were plated on the LGI-P agar medium, and two *Azospirillum* sp. were plated on the LB agar medium. Three replicates were prepared from each concentration. All plates were incubated at 30ºC for few days. The number of bacterial colonies grown in each plate was counted and recorded. The number of colony forming units per 1 mL (cfu/mL) in the

original broth against the number of days was plotted to identify each bacterial colony's growth rate changes with time.

For measuring absorbance values of bacterial cultures, the same broths, which were used for the dilution plate method, were taken. Absorbance values of each bacterial broth were taken (every day) for five days using a UV spectrophotometer (Pharmacia LKB. Novaspec II). Two different absorbance values were taken from each bacterial broth using two reference solution (water and growth medium). The wavelength was set to 700nm for absorbance reading. Absorbance against the number of days was plotted for each bacterium.

The OD value of each bacterium broth equivalent to 10^8 cfu/ml was identified using the above two graphs. Identified OD values were used as an endpoint of growth of each bacterium before applying to the plants (Appendix 2).

3.1.3.2 Fungal spore quantification

Jump Start® is a quantified commercial product. According to the product label, 1g of product contains 10^8 fungal spores.

3.1.4 Sorghum seed germination

Sorghum seeds were obtained from AERC Canada for the study. Required seed amounts from CFSH 30 and CSSH 45 for the first greenhouse study were 100 and 200, respectively. The seed number of each cultivar was decided using the germination rate of each cultivar. Two sets of seeds were placed on separate moisturized filter paper in separate petri plates. Both sets were kept in the dark conditions until 1cm long of radicle growth appeared. Moisture condition was maintained in petri plates by adding distilled water.

3.1.5 Seedling selection and transfer to the greenhouse

As mentioned in section 3.1.3, germinated seeds were grown in petri plates until radicles reached around 1cm long. Seedlings with similar length were selected and transferred to the greenhouse. Seedlings were grown in plastic pots with 7.5 L capacity. Soil obtained from AAFC farm Nappan (from the land used for the field study) was used for the growing media in the greenhouse. Sorghum seedlings were grown in the greenhouse until they reached the two to three-leaf stage. This stage was used for inoculant application (Yoon et al., 2015).

3.1.6 Inoculant preparation and application

Reactivated bacteria in glycerol stocks were used for the inoculant preparation (Section 3.1.1). Two volumes of 50µL of glycerol stocks from the two *Gluconacetobacter* sp. were separately added to 50 mL of two LGI-P broths. Two volumes of 20 µL of glycerol stocks from two *Azospirillum* sp. were separately added to 50ml of two LB broths. All broths were kept in the shaker at 30ºC. The absorbance value of each broth was measured every day until absorbance values were equivalent to 10^8 cfu/mL count (Section 3.1.2). A volume of 18 ml of microbial broth was added to 1782 mL of distilled water to prepare each inoculant solution (Final volume 1800mL). Final bacterial concentration was 10^8 cfu/100mL.

For preparing fungal spore solution, 18g of the Jumpstart® product was mixed with 1800mL of distilled water. The final spore concentration was similar to the bacterial concentrations (10^8 spores/100mL).

There were 18 pots from two cultivars for each microorganism. A volume of 100 mL of prepared inoculant solution was added to each pot. A volume of 100 mL of distilled water was added to each control pot.

3.1.7 Nutrient preparation and application

A modified Knop's mixture was used to supply required nutrients to sorghum plants (Appendix 7). Two sets of nutrient solutions were prepared by changing the ^{14}N:^{15}N ratio. One solution was prepared, including 2M N containing 2% of 1% ^{15}N and 98% of ^{14}N. The other nutrient solution was prepared, including 2M N containing with 100% of ^{14}N. Except for N, other macronutrients and micronutrients concentrations were similar in both solutions.

The nutrient solution with ^{15}N was applied to all pots except one set of control from each cultivar. Those controls were treated with the nutrient solution without ^{15}N. The nutrient application was done two times per week, starting after one week from the inoculant application. In the beginning, each pot was received 100 mL of nutrient at once. The volume was increased up to 200ml with the growth of the sorghum plant (around three weeks after the inoculant application).

3.1.8 Greenhouse growth conditions

The temperature inside the greenhouse was controlled by heating and ventilation (25/18 °C (day/night)).

The sorghum plants received natural sunlight during the daytime. In addition to the natural solar radiation, plants received supplemental with a minimum photosynthetic photon flux density (PPFD) at bench height from the supplemental lighting at 300 $\mu molm^{-2}s^{-1}$. The photoperiod of supplemental lighting was set at 18/6 h (day/night).

Under these conditions, sorghum plants were grown six weeks in the greenhouse after the microbial treatment.

3.1.9 Sampling and sample analysis

At the end of the six weeks of the growth period, different plant measurements, including plant height, length and width of the leaf and number of leaves, were taken. Plant height and leaf length and width measurements were taken using a measuring tape. After collecting all measurements, sorghum plant shoots were separately harvested and was determined the fresh weight of each sample.

After harvesting, shoot samples were stored in an oven at 70^{o}C for a week to remove water from samples. Dry weights of the plant shoot samples were obtained using a standard scale.

Each cultivar had nine replicates from each microbial treatment and each control. Those nione samples were divided into three groups. Samples belong to each new group were mixed and ground to powder level. The weight of 5g from each sample was separated for ^{15}N analysis. The remaining powder of each sample was sent for tissue nutrient analysis to the Nova Scotia Department of Agriculture.

As mentioned above, a small weight (around 5g) from each grounded sample was obtained for ^{15}N analysis. However, CSSH 45 shoot samples of two controls and PAL5T treated CSSH 45 shoot samples were only selected for the analysis based on the dry weight analysis results. The selected samples were grounded again until an obtained fine powder. A weight between 0.1to 0.3mg was measured from each sample using a microbalance. The measured samples were put into the tin capsules. Those capsules were sealed by folding to both horizontal and longitudinal directions. The sealed capsules were placed in the 96 wells plate. Sample preparation was done using the instructions given by the analysis lab at the University of Saskatchewan. Samples were sent to the University of Saskatchewan for ^{15}N analysis.

The fixed nitrogen percentage out of total nitrogen content was calculated using the below equation.

%Ndfa= (1-(^{15}N atom % excess (fixing plant)/^{15}N atom % excess (non-fixing plant))) ×100

%Ndfa - Nitrogen percentage derived from the atmosphere.

^{15}N atom % excess = ^{15}N atom % of tissue – 0.3663

^{15}N atom % excess of fixing plant (PAL5T-treated CSSH 45 plant) and ^{15}N atom % excess of non-fixing plant (Control CSSH 45 plants) data for the above equation were obtained from the ^{15}N analysis results.

3.2 Second greenhouse study

3.2.1 Experimental design

A completely randomized block design was used. Three microbial inoculants (N7, N8, DS1) were tested with three sorghum genotypes (CSSH 45, 10A×118 and 10A×131) One set of control for each genotype was used. Each treatment and controls were replicated ten times. 120 experimental units were used in the study (3 inoculants + one control× 3 sorghum genotypes× 10 replicates) (Appendix 4)

3.2.2 Preparation of microorganisms

Three bacterial cultures (*Gluconacetobacter azotocaptans* DS1, *Azospirillum lipoferum* N7, *A. brasilense* N8) were used in the second study. The bacterial culture preparation method was the same as the first greenhouse study (Section 3.1.1). The same bacterial quantifying data, obtained from the first greenhouse study was used in the second greenhouse study.

3.2.3 Sorghum seed germination

Three sorghum genotypes (CSSH 45, 10A×118 and 10A×131) were used for the second study. Required seeds were obtained from AERC Canada (Kanata, Ontario). During the second study, pre-treatment was done to the seeds before stimulating the germination due to low germination rates of some genotypes and fungal attacks on germinated seeds. In the pre-treatment process, seeds were washed using $HgCl_2$ for 5 minutes. Then, seeds were immediately washed from sterilized distilled water five times. Each seed set was separately placed on moisturized filter papers in separate petri plates. All plates were refrigerated. After 2-3 days, petri dishes were transferred into the growth chamber (The number of days kept in the refrigerator was determined based on the germination rates of each genotype). The genotypes with higher germination rates were kept for a longer period in the refrigerator. This process was done to keep all genotypes at the same growth level. Then germinated seeds were kept in the dark conditions until 1cm long radicle growth appeared.

3.2.4 Seedling selection

The seedling selection and transferring to the greenhouses was the same as the first greenhouse experiment (Section 3.1.4.2)

3.2.5 Bacterial inoculant preparation and application

Inoculant preparation was also same as the first greenhouse study (Section 3.1.4.3. However, the required broth volumes were different. A volume of 30mL of microbial broth was added to 2970 mL of distilled water to prepare each inoculant solution (Final volume 3000mL). Final bacterial concentration was 10^8 cfu/100mL.

There were 10 pots from each genotype for each bacterial inoculant (30 pots for one microbial inoculant application). A volume of 100 ml of prepared bacterial inoculant solution (Section 3.2.4.3) was added to each pot. A volume of 100ml of distilled water was added to each control pot.

3.2.6 Nutrient preparation and application

A modified Knop's mixture was used as the nutrient solution (Appendix 7). Even though two different nutrient solutions were used in the first greenhouse study, one nutrient solution was used in the second greenhouse study. The nutrient solution was prepared, including 2M N containing 2% of 1% ^{15}N and 98% of ^{14}N.

The nutrient solutions were applied two times per week, starting after one week of inoculant application. In the beginning, each pot was received 100mL of nutrient solution at once. The volume was increased up to 200mL with the growth of the sorghum plants (around 3 weeks after the inoculant application).

3.2.7 Greenhouse growth conditions

The temperature inside the greenhouse was controlled by heating and ventilation (25/18 °C (day/night)).

The sorghum plants received natural sunlight during the daytime. In addition to the natural solar radiation, plants received supplemental with a minimum photosynthetic photon flux density (PPFD) at bench height from the supplemental lighting at 300 μmol m^{-2} s^{-1}. The photoperiod of supplemental lighting was set at 18/6 h (day/night).

Under these conditions, sorghum plants were grown six weeks in the greenhouse after the microbial treatment.

3.2.8 Sampling and sample analysis

At the end of the six weeks of the growth period, different plant measurements, including plant height, leaf area and the number of leaves, were taken. Plant height was taken using a measuring tape. Leaf area was measured using LICOR 3000. After collecting all measurements, sorghum plant shoots were separately harvested and measured the fresh weight of each sample.

After harvesting, samples were stored in an oven at 70^{o}C for a week to remove water from samples. Dry weights of the plant shoot samples were obtained using a standard scale.

Each cultivar had ten replicates from each microbial treatment and each control. Each replicate set was divided into three groups (3-3-4). Samples belonging to each new group were mixed and ground to powder level. The weight of 5g from each sample was separated for ^{15}N analysis. The remaining powder from each sample was sent for tissue nutrient analysis to the Nova Scotia Department of Agriculture.

As mentioned above, a small weight (around 5g) from each grounded sample was obtained for N-15 analysis. However, control samples of each cultivar and N8 treated shoot samples of each cultivar were only selected for the analysis based on the dry weight results of the second greenhouse study and the field study results. The selected samples were ground again until an obtained fine powder. A weight between 0.1to 0.3mg was measured from each sample using a microbalance. The measured samples were put into the tin capsules. Those capsules were sealed by folding to both horizontal and longitudinal directions. The sealed capsules were placed in the 96 wells plate. Sample preparation was done using the instructions given by the analysis lab at

the University of Saskatchewan. Samples were sent to the University of Saskatchewan for ^{15}N analysis.

The fixed nitrogen percentage out of total nitrogen content in each genotype was calculated using the below equation.

%Ndfa= (1-(^{15}N atom % excess (fixing plant)/^{15}N atom % excess (non-fixing plant))) ×100

%Ndfa - Nitrogen percentage derived from the atmosphere.

^{15}N atom % excess = ^{15}N atom % of tissue – 0.3663

^{15}N atom % excess of fixing plant (N8-treated plants of each genotype) and ^{15}N atom % excess of non-fixing plant (Control plants of each genotype) data for the above equation were obtained from the ^{15}N analysis results.

3.3 Field Study

3.3.1 Field Characterization

The field was located in Nappan Research Farm, Agriculture & Agri-food Canada, Nappan, B0L1C0 Nova Scotia (45.760097, -64.242015). In the previous growing season, this field was used for corn cultivation. According to the Canada Land Inventory map of Nova Scotia, this land belongs to CLI 3. Before starting the experiment, soil samples were collected and analyzed in the Nova Scotia Department of Agriculture. Macronutrient and micronutrient contents in the soil were obtained from the analysis (Appendix 6). Weather conditions during the experiment time (temperature and precipitation) were obtained from the Environment Canada weather station close to the field site (~50m). The average monthly temperature for the growing season (July to September) were 19.4, 19.3, 14.2^{o}C, respectively. The average monthly precipitation for the growing season (July to September) were 57.9, 49.7, 84.7mm, respectively

3.3.2 Experimental Design

Sorghum genotypes and microorganisms were similar to the second greenhouse study. In addition, two nitrogen levels were also added to the study. Considering all variables and available space in the land, 72 subplots were included in the experimental design (3 genotypes × 3 microbial treatment and 1 control× 2 Nitrogen level× 3 replicates). Each subplot was 2m× 2m ($4m^2$) size. Each subplot contained three rows of sorghum plants (Appendix 5). Assigning each subplot to the different treatments were done using a completely randomized design.

3.3.3 Field preparation

The land was prepared for the study with the help of the staff of AAFC in Nappan. Round up® by Monsanto.Inc (Creve Coeur, Missouri, US) was applied to the land, and it was kept for few weeks to kill grasses and weeds. The land was prepared by turning soil few times in different time intervals using a tilling tractor to remove herbs and grasses. Land preparation was done at the beginning of summer 2020.

Figure 3.1: The image of the sorghum field after preparation for the study (before seeding). Different colour flags represent the borders of each subplot

3.3.4 Seed Application

Sorghum seeds application was done in manual ways (by hand) on 02nd of July 2020. The number of seeds applied per row was decided based on the germination percentage of each genotype. The initial design was planned to grow 14 sorghum plants per row. Therefore, the number of seeds applied per row from each genotype differed as follows (Table 3.1).

Table 3.1: Number of sorghum seeds from each genotype used in the field. Numbers were selected based on the germination rates of each cultivar

Genotype	Germination Percentage (%)	No of Seeds applied per row
CSSH 45	45	30
10A×118	82	16
10A×131	85	16

3.3.5 Nitrogen application

After 2.5 weeks of growth, nitrogen fertilizer was added to the plots (20th of July 2020). The fertilizer application was only done to the plots assigned for nitrogen application (36 subplots were fertilized). A shallow furrow along with each plant row with a 15 cm gap to the row was dug for applying nitrogen fertilizer. Hand shovels were used for this process. A mass of 12g of fertilizer with the ratio of 46 N:0 P:0 K was equally spread throughout each furrow. The fertilizer was purchased from Truro Agri Mart Limited. The applied fertilizer amount is equal to 43.56kg N/ha.

Figure 3.2: Left – The image of a furrow, dug for nitrogen application, it was dug along the plant row with a 15cm gap to the row using a hand shovel. Right – the image of urea application along the furrow. A 12g of urea was evenly dispersed throughout the furrow.

3.3.6 Inoculant preparation & application

The application was done after almost 2.5 weeks from fertilization (07th of August 2020). Inoculant preparation was similar to the first greenhouse study (Section 3.1.4.3), but the final volumes of bacterial broths were different. A volume of 800mL of microbial broth was dissolved in 80L of water to prepare each bacterial inoculant solution (the final volume prepared was more

than the required volume). Each sorghum plant marked for microbial treatment received 100 mL of microbial broth solution. There were 18 subplots for each microbial treatment (756 plants per treatment). Control plants received 100mL of water.

Figure 3.3: The image of inoculant application in the field. Each plant was received 100mL of inoculant solution. The application was done after five weeks from seeding.

3.3.7 Plot maintenance

Sorghum plants were grown under the normal environmental conditions for six weeks after the inoculant application

After the three weeks of the inoculant application, weeding was completed (26th of August 2020). Weeding between subplots was done using a land mower. Weeding inside the plots was done by hands.

Figure 3.4: Left- the image of a plot with sorghum plants before weeding, right – The image of a plot with sorghum plants after weeding. Weeding inside the plot was done by hands.

3.3.8 Sampling and sample analysis

Harvesting was done after six weeks from inoculation (15th of September 2020). Nine sorghum plants from each subplot were randomly selected for measurements and harvesting. Three plants from each row (two plants from both edges and the middle plant) were selected. Plant height, number of leaves per plant and the leaf area were taken as plant measurements. Each selected plant shoot was separately harvested at the end of measurements.

After harvesting, samples were stored in an oven at 70^{o}C for a week to remove water from the samples. Dry weights of the plant shoot samples were obtained using a standard scale.

Each cultivar had three subplots, and nine sorghum plants were separately harvested from each subplot. Samples from each sub plot were mixed and ground to powder level. The prepared samples were sent for tissue nutrient analysis to the Nova Scotia Department of Agriculture.

3.4. Statistical analysis

All data were collected and preprocessed using Microsoft Excel 2020, including calculating the mean of the samples. For statistical analysis and data processing, R (3.6.2 version) software was used.

Since all dependant variables, including above-ground biomass, plant height, tissue nitrogen content collected in the first greenhouse study, displayed normal distribution and homogeneity, a two-way ANOVA test was used for analysis (two independent variables: inoculant types, cultivar type). The Interaction effect of these two variables were also considered. Tukey's HSD post hoc analysis was used to identify the pairs responsible for the significant results of the two-way ANOVA test.

In the second greenhouse study, shoot dry weight, plant N content, and plant height data set displayed a lack of normality and homogeneity. Hence, a Generalized Linear Model (GLM) was used for data analysis. Data were analyzed using the basic ANOVA based on the GLM model. Also, Tukey's post hoc test was used to identify the pairs responsible for the significant results of the ANOVA test. Leaf area, tissue N concentration and plant phosphorus content data sets were analyzed using a two-way ANOVA because both data sets showed normality and homogeneity.

All the dependant variables (above-ground biomass, tissue nitrogen content, height, leaf area) displayed normal distribution and homogeneity of the data in the field study. Therefore, a three-way ANOVA test (three independent variables; cultivar, microbial inoculant, nitrogen application level) including interaction effect was used for analysis. Tukey's post hoc test was used to identify the pairs responsible for the significant results of the three-way ANOVA test. Significance was accepted at 5% in all the analysis.

4.Results

4.1 First greenhouse study

One of this study's main objectives is to identify the growth-promoting ability of microbial treatments on sorghum genotypes bred specifically as biomass feedstock crops. Sorghum growth was measured using different parameters such as dry weight, plant height and tissue nutrient content. At the beginning of the study, development in the two sorghum cultivars was similar. However, at the end of the growing period (60 days after planting), the CFSH 30 cultivar had reached its reproductive stage, while the CSSH 45 cultivar was still within the vegetative growth stage. The appearance of the two cultivars was also different at the end of the growth period. The CSSH 45 was comparatively larger with broad leaves, and the CFSH 30 was taller plants with thin leaves (Figure 4.1).

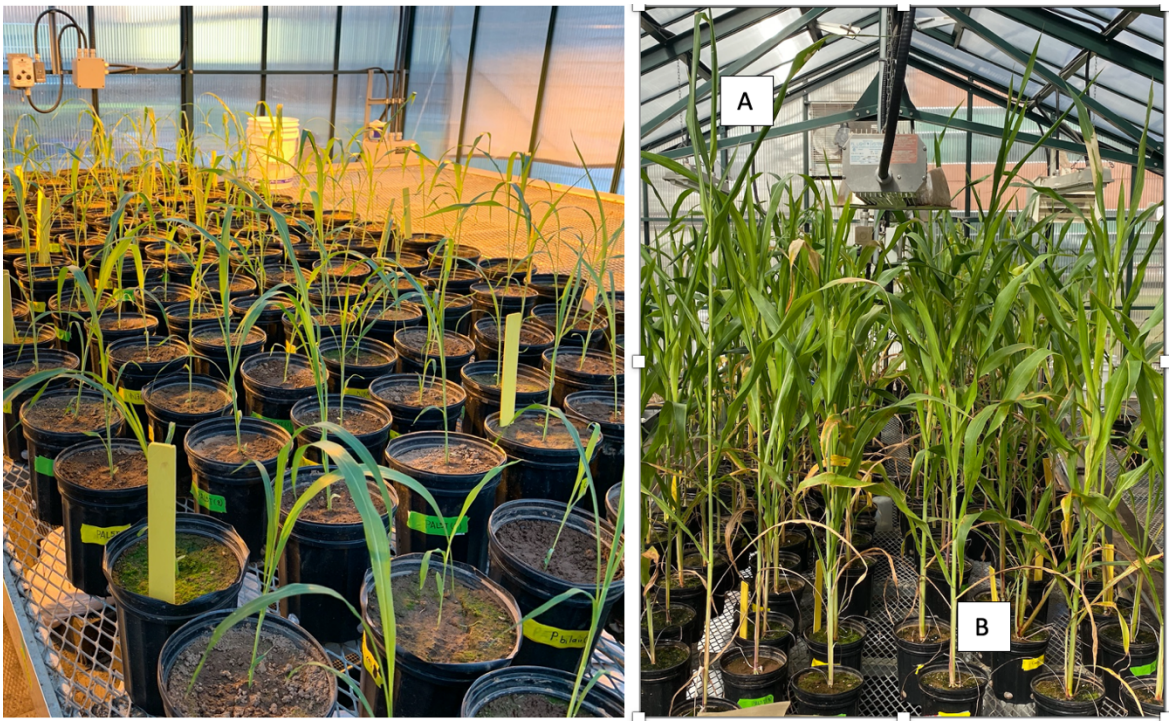

Figure 4.1: **Left:** Sorghum plants just after the inoculant application. Green labels on the pots represent CSSH 45 cultivar, and yellow labels on the pots represent CFSH 30. Both cultivars are similar in appearance. **Right:** Sorghum plants just before harvesting. The plant labelled as A in the image represents CFSH 30, and the plant labelled as B represent CSSH 45. Plant A was a taller plant with thin leaves, while plant B was a wider plant with broad leaves.

4.1.1 Shoot dry weight

The shoot dry weight of the sorghum was analyzed using a two-way ANOVA for identifying the effects of microbial treatments and cultivars. In CSSH 45, the shoot dry weights of microbial treatments were numerically greater than the mean dry weight of the control plants (Figure 4.2). However, the shoot dry weights of PAL5T ($p<0.05$) and DS1($p<0.1$) treated plants were significantly different compared to the control by 19% and 16%, respectively. The shoot dry weights of the other three microbial treatments were not statistically different from the control.

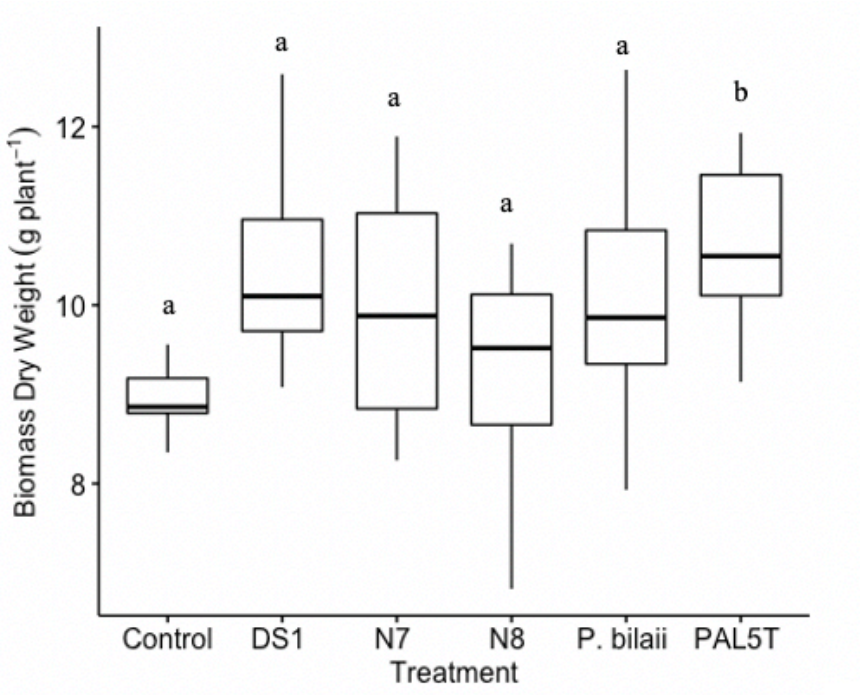

Figure 4.2: Box plot of shoot dry weight (g plant^{-1}) of CSSH 45 with different microbial treatment. DS1=*Gluconacetobacter azotocaptans*, N7=*Azospirillum brasilense*, N8=*Azospirillum lipoferum*, P. bilaii=*Penicillium bilaii*, PAL5T=*Gluconacetobacter diazotrophicus*. Bold horizontal lines represent the median dry weights, and boxes represent the interquartile range (IQR) of shoot dry weight. Different letters indicate significant differences between inoculation treatments at $p<0.05$. NB: DS1 has a significant difference in dry weight compared to the control at $p<0.1$.

In CFSH 30, the shoot dry weights of each microbial treatments were not significantly different from the dry weight of uninoculated control plants (Figure 4.3).

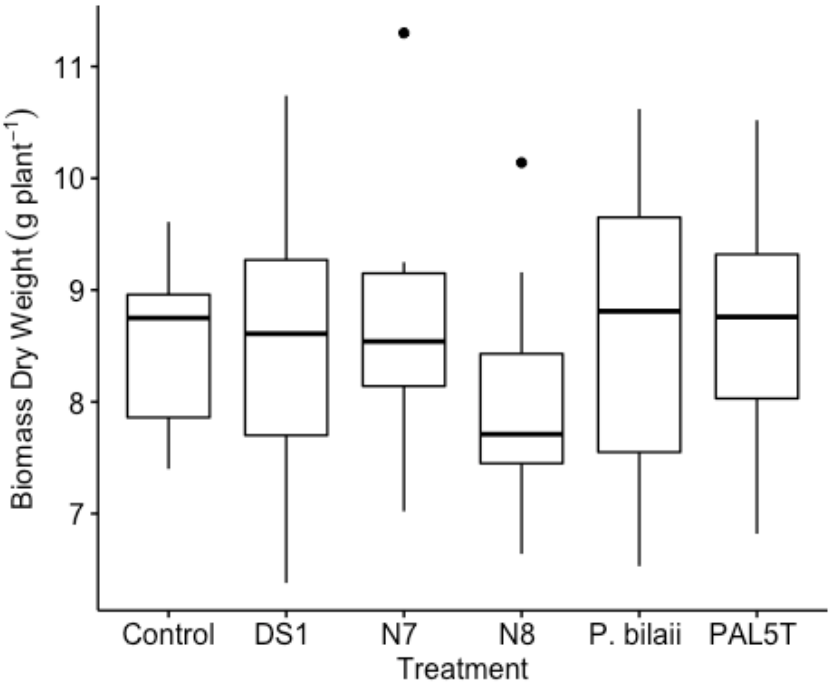

Figure 4.3: Box plot of shoot dry weight (g plant^{-1}) of CFSH 30 with different microbial treatments. DS1=*Gluconacetobacter azotocaptans*, N7=*Azospirillum brasilense*, N8=*Azospirillum lipoferum*, P. bilaii=*Penicillium bilaii*, PAL5T=*Gluconacetobacter diazotrophicus*. Bold horizontal lines represent the median dry weights, and boxes represent the interquartile range (IQR) of shoot dry weight. The two dots above the boxplots of N7 and N8 indicate outliers of the data set.

The two cultivars showed statistical significance differences in shoot dry weights ($p<0.05$). Averaged across all inoculation treatment, cultivar CSSH 45 had 16% higher biomass compared to CFSH 30 (Figure 4.4).

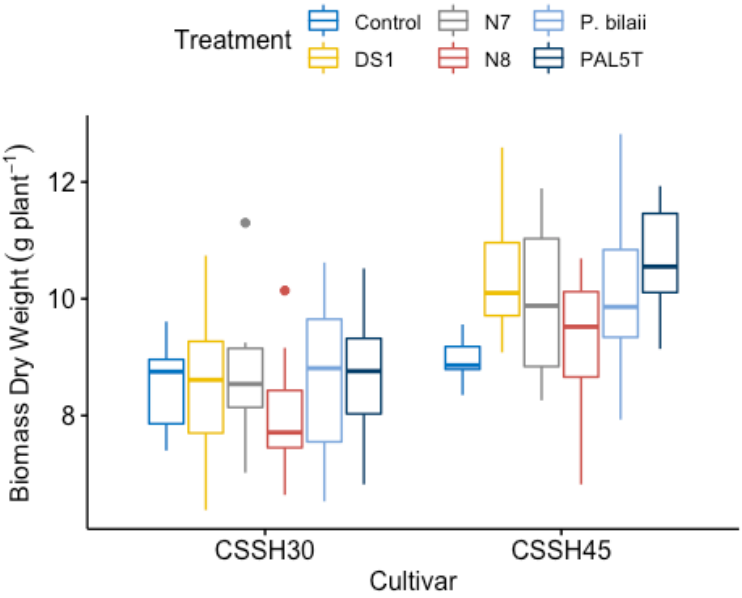

Figure 4.4: The box plot of shoot dry weight (g plant $^{-1}$) of two sorghum cultivars (CSSH 45 and CFSH 30) with different microbial treatments. Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum lipoferum*, Red=*Azospirillum brasilense*, Pale blue=*Penicillium bilaii*, Dark blue=*Gluconacetobacter diazotrophicus*. Bold horizontal lines represent the median dry weights, and boxes represent the interquartile range (IQR) of shoot dry weight. NB: The cultivar CSSH 45 significantly increased the shoot dry weight compared to the CFSH 30 ($p<0.05$). Two dots above the boxplots of N7 and N8 in CFSH 30 indicate outliers of the data set.

4.1.2 Tissue nutrient analysis

Dried shoot tissue samples were sent to Nova Scotia Department of Agriculture for analysis. The macronutrient and micronutrient contents of tissue samples were reported as a percentage of the dry weight of the sample (Appendix 5). According to the literature (e.g., Vessey, 2003), most plant growth-promoting microbes promote plant growth mainly through nitrogen fixation and

phosphorus solubilization. Therefore, only plant N and P contents and tissue N and P concentrations were analyzed statistically.

The effect of microbial treatments and cultivars on the total N content of the plants (g N $plant^{-1}$), tissue N concentration (N%) (N accumulation rate per gram of dry weight) of the dried shoot samples, total P content of the plants (g P $plant^{-1}$) and tissue P concentration (P %) (P accumulation rate per gram of dry weight) of the dried shoot sample were statistically analyzed. The N and P contents of plants were calculated by multiplying the percent of each nutrient (tissue N or P concentration) from the tissue analysis by the shoot dry weight of each plant. According to a two-way ANOVA of both cultivar and inoculation treatments on nitrogen tissue analysis, the plant N content within each cultivar was not significantly different among microbial treatments compared to the control. Also, plant N content between cultivars was not significantly different (Figure 4.5). However, a one-way ANOVA of plant N content within cultivar CSSH 45 solely showed significant differences in the plant N content among microbial treatments ($p<0.05$) (Figure 4.6). The plant N content of N7- inoculated CSSH 45 and the plant N content of PAL5T- inoculated CSSH 45 were significantly greater compared to the uninoculated control by 17% and 27%, respectively.

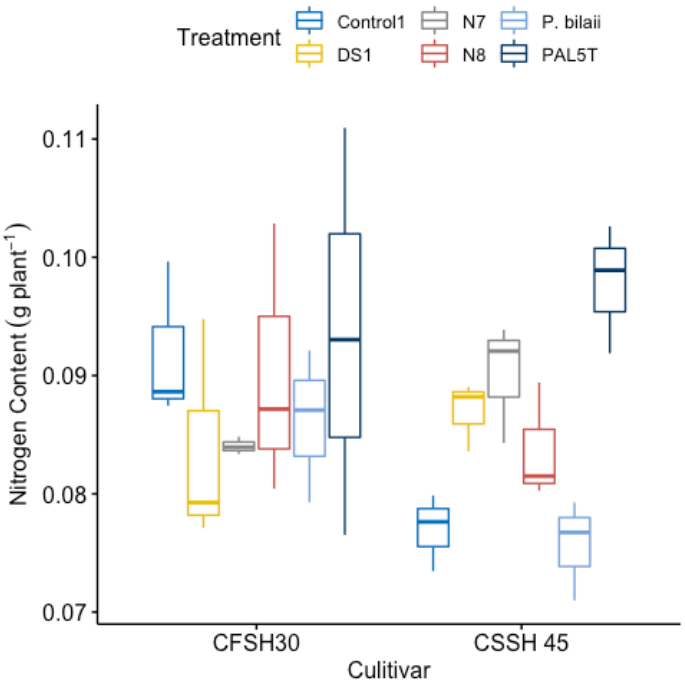

Figure 4.5: The box plot of plant N content (g plant $^{-1}$) of two sorghum cultivars (CSSH 45 and CFSH 30) with different microbial treatments. Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum brasilense*, Red=*Azospirillum lipoferum,* Pale blue=*Penicillium bilaii*, Dark blue=*Gluconacetobacter diazotrophicus*. Bold horizontal lines represent the median plant N content per plant and boxes represent the interquartile range (IQR) of nitrogen content per plant.

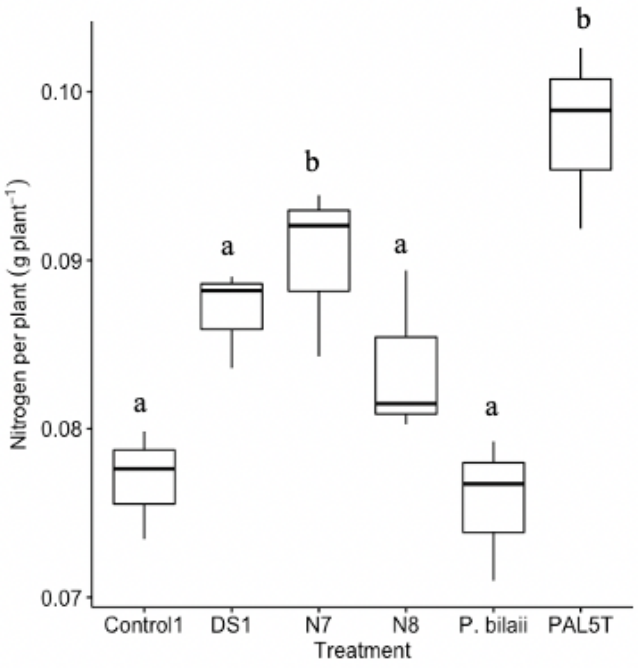

Figure 4.6: The Box plot of plant N content (g plant^{-1}) of CSSH 45 with different microbial treatment. DS1=*Gluconacetobacter azotocaptans*, N7=*Azospirillum brasilense*, N8=*Azospirillum lipoferum*, P. bilaii=*Penicillium bilaii*, PAL5T=*Gluconacetobacter diazotrophicus*. Bold horizontal lines represent the median N content per plant and boxes represent the interquartile range (IQR) of N content per plant. Different letters indicate significant differences between inoculation treatments at $p<0.05$.

The tissue N concentration of the dried shoot tissue samples was also analyzed using a two-way ANOVA. According to the results, none of the microbial treatments was statistically different from uninoculated control. However, the tissue N concentrations of the dried tissue samples of CFSH 30 were significantly greater than the tissue N concentration of dried tissue samples of CSSH 45 by 20.2% (Figure 4.7).

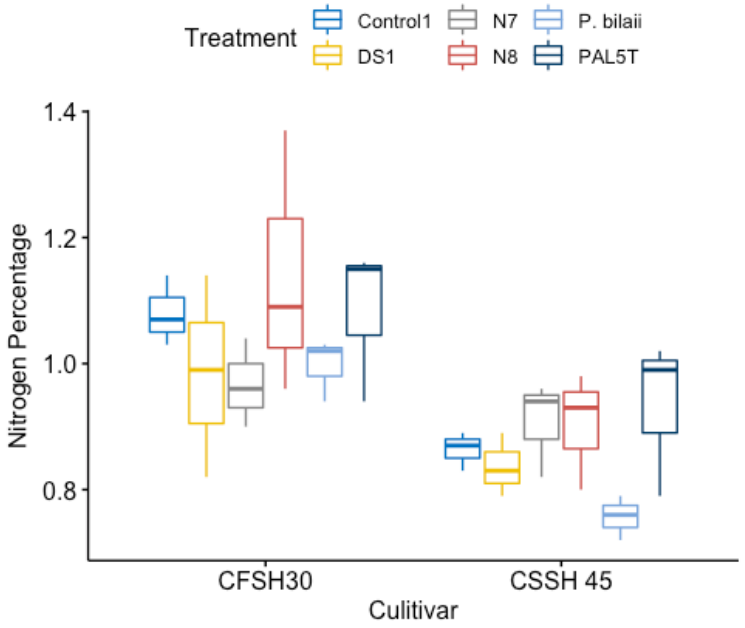

Figure 4.7: The box plot of tissue N concentration of two sorghum cultivars (CSSH 45 and CFSH 30) with different microbial treatments. Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum brasilense*, Red=*Azospirillum lipoferum,* Pale blue=*Penicillium bilaii*, Dark blue=*Gluconacetobacter diazotrophicus*. Bold horizontal lines represent the median tissue N concentration, and boxes represent the interquartile range (IQR) of tissue N concentration. NB: The tissue N concentration of CFSH 30 was significantly greater than CSSH 45 ($p<0.05$).

The plant P contents of each cultivar among microbial treatments showed a significant difference compared to the uninoculated controls ($p<0.05$). Also, the plant P contents between cultivars resulted in a significant difference ($p<0.05$). There was also an interaction between microbial treatment and cultivars ($p<0.05$) (Figure 4.8). Therefore, a post hoc test was conducted, including

interaction terms, to identify pair-wise significant differences. Microbial inoculations DS1, N7, N8, PAL5T and *P.bilaii* resulted in an increase in plant P content of CSSH 45 compared to the uninoculated control by 18, 22, 23, 37, 25%, respectively.

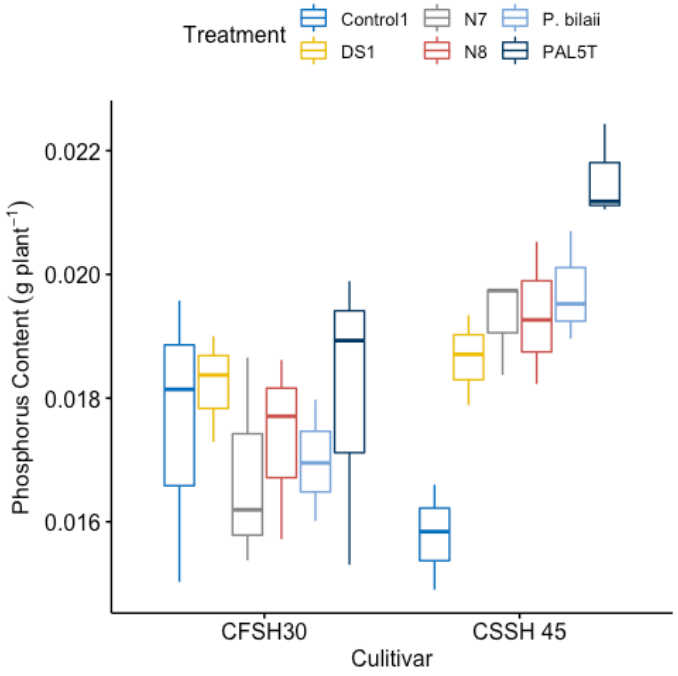

Figure 4.8: The box plot of plant P content (g plant $^{-1}$) of two sorghum cultivars (CSSH 45 and CFSH 30) with different microbial treatments. Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum brasilense*, Red=*Azospirillum lipoferum*, Pale blue=*Penicillium bilaii*, Dark blue=*Gluconacetobacter diazotrophicus*. Bold horizontal lines represent the median P content per plant and boxes represent the interquartile range (IQR) of the P content per plant. NB: Microbial treatments DS1, N7, N8, PAL5T, and *P. bilaii* inoculated CSSH 45 showed significant differences in plant P content compared to the uninoculated control ($p<0.05$).

The tissue P concentrations of dried shoot samples of two cultivars with microbial treatments were not statistically different compared to the uninoculated controls. However, the tissue P concentration of CFSH 30 tissue samples were significantly greater than CSSH 45 samples by 6.5% (Figure 4.9).

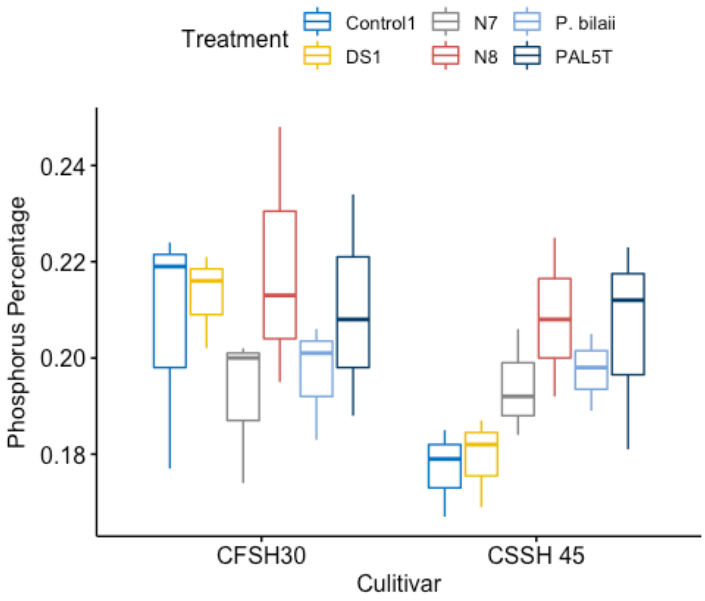

Figure 4.9: The box plot of tissue P concentration of two sorghum cultivars (CSSH 45 and CFSH 30) with different microbial treatments. Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum brasilense*, Red=*Azospirillum lipoferum,* Pale blue=*Penicillium bilaii*, Dark blue=*Gluconacetobacter diazotrophicus*. Bold horizontal lines represent the median tissue P concentration, and boxes represent the interquartile range (IQR) of tissue P concentration. NB: The phosphorus percentage of CFSH 30 was significantly greater than CSSH 45 ($p<0.05$).

4.1.3 ^{15}N Analysis

Shoot samples of PAL5T inoculated CSHH 45 plants, and uninoculated control plants of CSSH 45 were only sent for ^{15}N analysis from the first greenhouse study. According to the results, the mean value of ^{15}N atom % excess of PAL5T inoculated samples (0.4693%) was higher than the mean value of ^{15}N atom % excess of control (non-N fixing sample) (0.4547%) (Table 4.1).

Table 4.1: ^{15}N analysis results of the first greenhouse study

Treatment	Atom% ^{15}N	Mean value (%)
Control – Replicate 1	0.4580	0.4547
Control – Replicate 2	0.4580	
Control – Replicate 3	0.4481	
PAL5T – Replicate 1	0.4680	0.4693
PAL5T – Replicate 2	0.4713	
PAL5T – Replicate 3	0.4685	

The ^{15}N atom % excess values in Table 4.1 were fed into the formula for calculation of the %NDFA (see Material and Methods section 3.1.9). As there was no dilution of the ^{15}N atom % excess in the PAL5T-treated plants, there is no evidence of nitrogen fixation by PAL5T in CSSH 45.

4.1.4 Plant height

Plant height was not statistically different among the microbial treatments. However, as seen in some other parameters, the two cultivars had a significant difference in plant height ($p<0.05$), with CFSH 30 on average being 9.3cm taller than CSSH 45 (Figure 4.10).

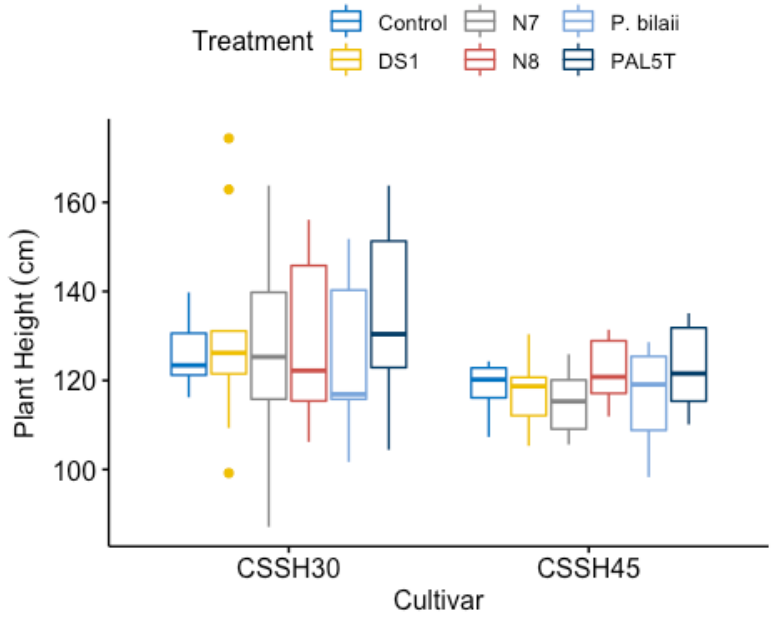

Figure 4.10*:* The box plot of plant height (cm) of two sorghum cultivars (CSSH 45 and CFSH 30) with different microbial treatments. Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum brasilense*, Red=*Azospirillum lipoferum*, Pale blue=*Penicillium bilaii*, Dark blue=*Gluconacetobacter diazotrophicus*. Bold horizontal lines represent the median plant height, and boxes represent the interquartile range (IQR) of plant height. NB: The plant height of CFSH 30 was significantly different compared to CSSH 45 cultivar ($p<0.05$). The three dots above and below the box plot of DS1 in CFSH 30 indicate outliers of the data set.

4.2 Second greenhouse study

Three microbial inoculants (DS1, N8 and N7) and three sorghum genotypes (CSSH 45, 10A×118, 10A×131) were used in the second greenhouse study. Shoot dry weight, plant height and leaf area were measured to identify growth changes due to microbial applications. In addition, tissue nutrient analysis and ^{15}N analysis were conducted on plant tissue samples. During the growth, a clear difference in sorghum plant growth was not observed. The appearance of all sorghum plants growth was similar except for a few plants in the CSSH 45 cultivar. A few CSSH 45 plants were abnormally larger and taller compared to the rest of the plants (not specific to the microbial treatment; see the plant labelled with "A" in Figure 4.11). Visible growth trends among treatments or genotypes were not observed (Figure 4.11).

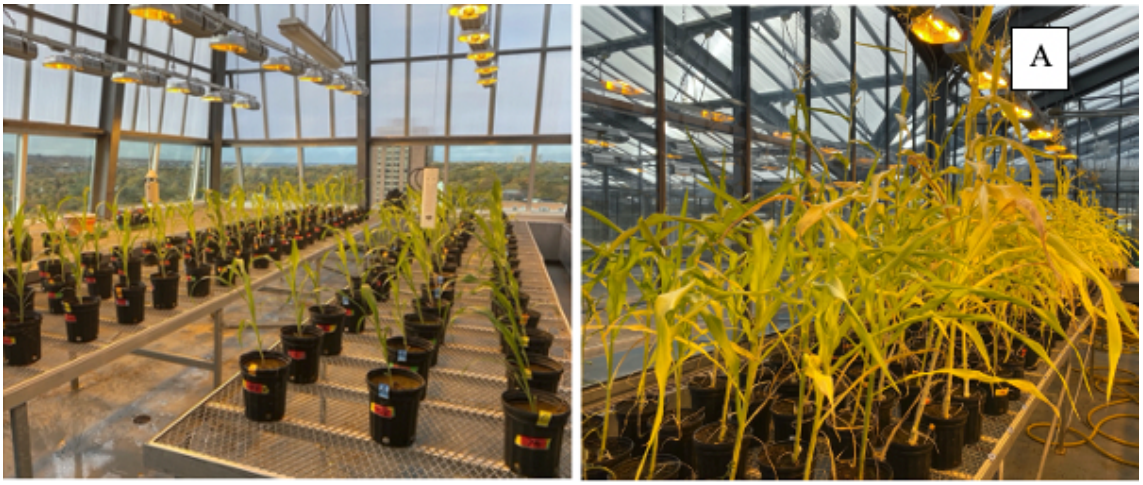

Figure 4.11: Left: The image of sorghum plants in the greenhouse after four weeks of the inoculant application. **Right:** The image of sorghum plants in the greenhouse just before the harvesting. Plant A represents abnormally larger CSSH 45 plants in the control set

4.2.1 Shoot dry weight

Due to the lack of normality of the data set, a standard ANOVA could not be used to analyze the shoot dry weights. Therefore, a generalized linear model (GLM) approach with a gamma distribution function was used to analyze the dry weights. According to the analysis, microbial treatments resulted in significant differences in dry weights ($p<0.05$). There was also a significant difference in shoot dry weight among genotypes ($p<0.05$) (Figure 4.12) and an interaction effect between treatments and genotypes ($p<0.05$).

Tukey's post hoc test was conducted to identify pair-wise significant differences between microbial treatments and genotypes without considering the interaction terms. However, neither microbial pairs nor genotype pairs could be identified from this post hoc analysis. Therefore, a post hoc test was conducted again, including interaction terms, to identify pair-wise significant differences. The second Tukey's post hoc analysis gave acceptable results. According to analysis, DS1 inoculation of CSSH 45 plants and N8 inoculation of CSSH 45 plants resulted in a decrease in shoot dry weight by 32% and 29%, respectively, compared to the uninoculated control plants ($p<0.05$) (Figure 4.12).

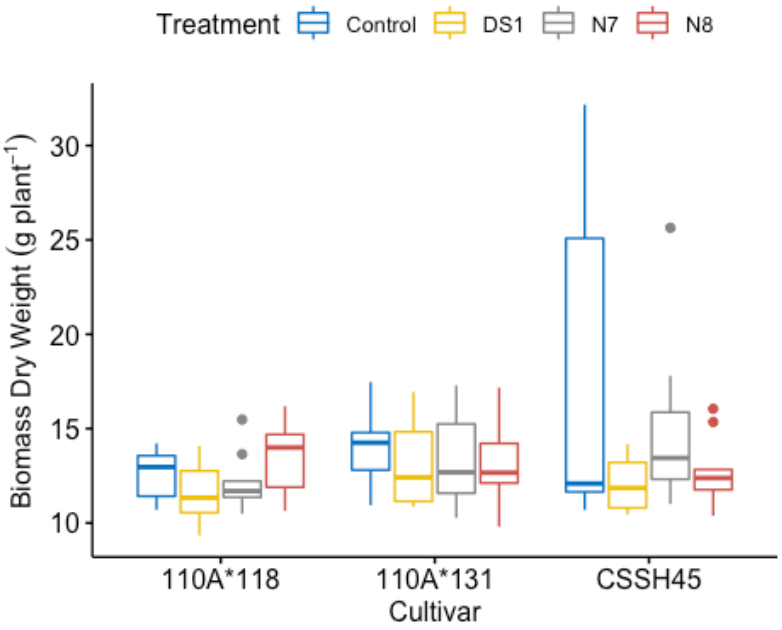

Figure 4.12: The box plot of shoot dry weight (g plant $^{-1}$) of three sorghum genotypes (CSSH 45, 10A×118 and 10A×131) with different microbial treatments. Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum lipoferum*, Red=*Azospirillum brasilense*. The bold horizontal lines represent the median shoot dry weight, and boxes represent the interquartile range (IQR) of shoot dry weight. NB: N8 and DS1 inoculated CSSH 45 showed significant differences compared to the control ($p<0.05$). The two dots above the box plot of N7 in 10A×118 and the three dots above the box plots of N7 and N8 in CSSH 45 indicate outliers.

4.2.2 Tissue nutrient analysis

Dried shoot tissue samples were sent to the Nova Scotia Department of Agriculture for Analysis. The macronutrient and micronutrient contents of tissue samples were reported as a percentage of the dry weight of the sample (Appendix 6). As in the first greenhouse study, only the effects of

microbial treatment and cultivars on the total N content of the plants (g N plant^{-1}), tissue N concentration (N%) of the dried shoot samples, total P content of the plants (g P plant^{-1}) and tissue P concentration (P%) of the dried shoot sample were statistically analyzed

A GLM was used for plant N content analysis due to the lack of normality and homogeneity of the data set. According to the results, plant N content within each genotype was not significantly different among microbial treatments. Also, plant N content among cultivars was not significantly different (Figure 4.13).

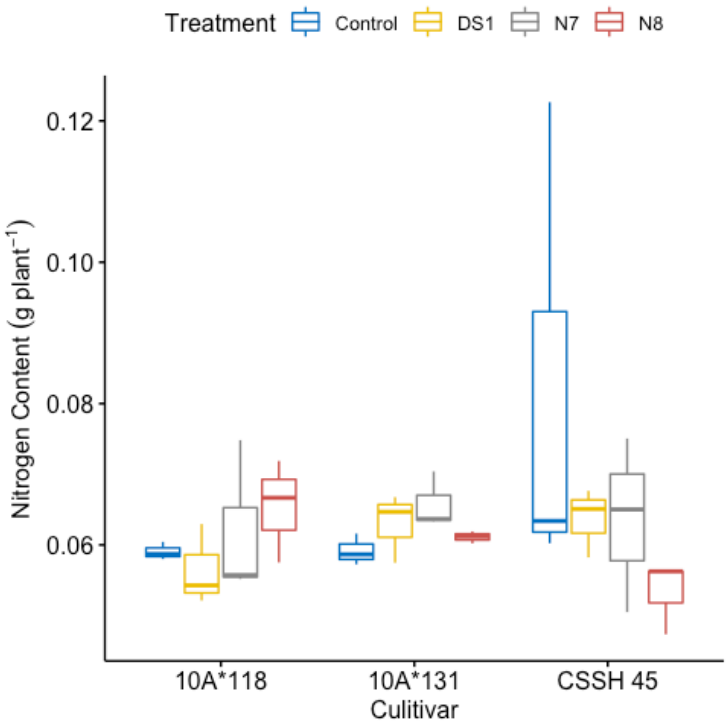

Figure 4.13: The box plot of plant N content (g plant ^{1}) of three sorghum genotypes (CSSH 45, 10A×118 and 10A×131) with different microbial treatments from the second greenhouse study. Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum lipoferum*, Red=*Azospirillum*

brasilense. The bold horizontal lines represent the median N content per plant and boxes represent the interquartile range (IQR) of N content per plant.

A two-way ANOVA test was used to analyze the tissue N concentration of dried shoot samples because the data set met the normality and homogeneity assumptions. According to the result, neither microbial treatments nor genotypes showed significant differences in tissue N concentration of dried shoot samples (Figure 4.14)

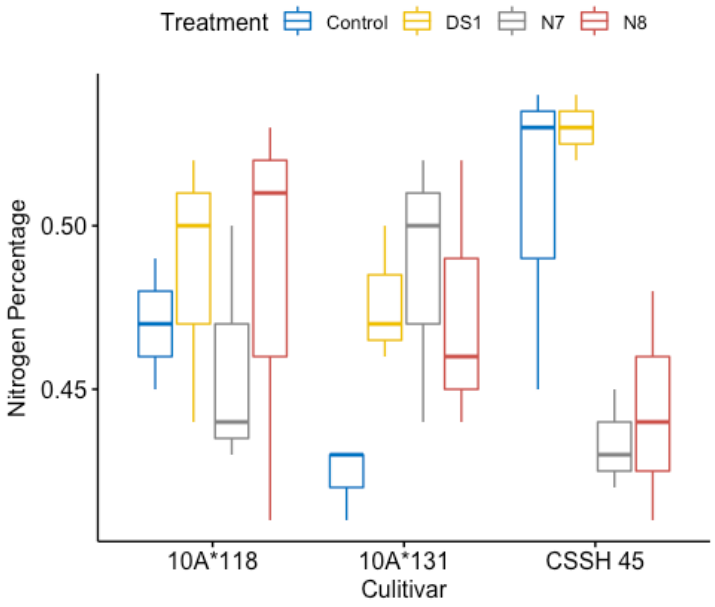

Figure 4.14: The box plot of tissue N concentration of three sorghum genotypes (CSSH 45 10A×118 and 10A×131) with different microbial treatments. Different colours represent different microbial treatments: Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum lipoferum*, Red=*Azospirillum brasilense*. The bold horizontal lines represent the median tissue N concentration, and boxes represent the interquartile range (IQR) of tissue N concentration.

Plant P content data were analyzed using a two-way ANOVA. According to the analysis, the Plant P content of each genotype was not significantly different among microbial treatment compared to the control. However, plant P content among genotypes showed significant differences ($p<0.05$) (Figure 4.12). Tukey's post hoc test showed that the plant P contents of CSSH 45 and 10A×131 were significantly higher compared to 10A×118 by 9 and 14%, respectively (Figure 4.15).

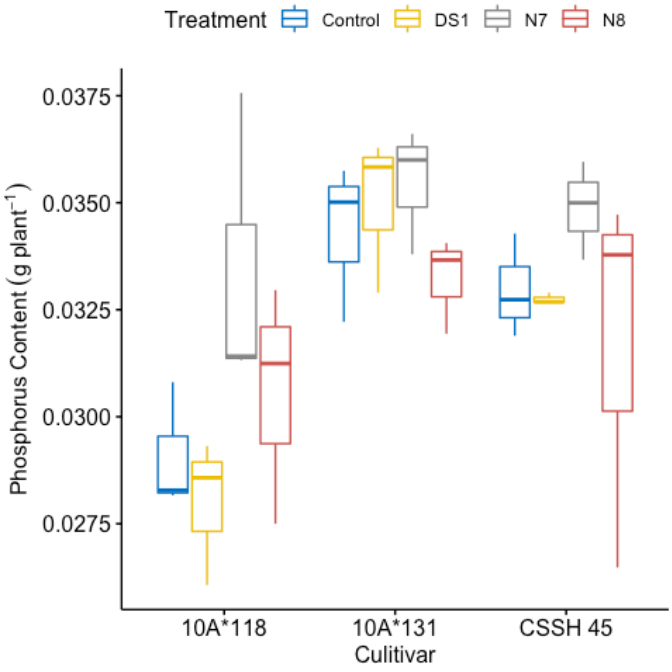

Figure 4.15: The box plot of plant P content (g plant ^{1}) of three sorghum genotypes (CSSH 45, 10A×118 and 10A×131) with different microbial treatments from the second greenhouse study. Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum lipoferum*, Red=*Azospirillum brasilense*. The bold horizontal lines represent the median P content per plant and boxes represent the interquartile range (IQR) of P content per plant. NB: The plant P content of CSSH 45 and 10A×131 were significantly higher than the plant P content of 10A×118 ($p<0.05$).

A Generalized Linear Model was used to analyze the tissue P concentration of dried shoot samples because the data set did not meet the normality and homogeneity assumptions. According to the result, neither microbial treatments nor genotypes showed significant differences in tissue P concentration of dried shoot samples (Figure 4.16)

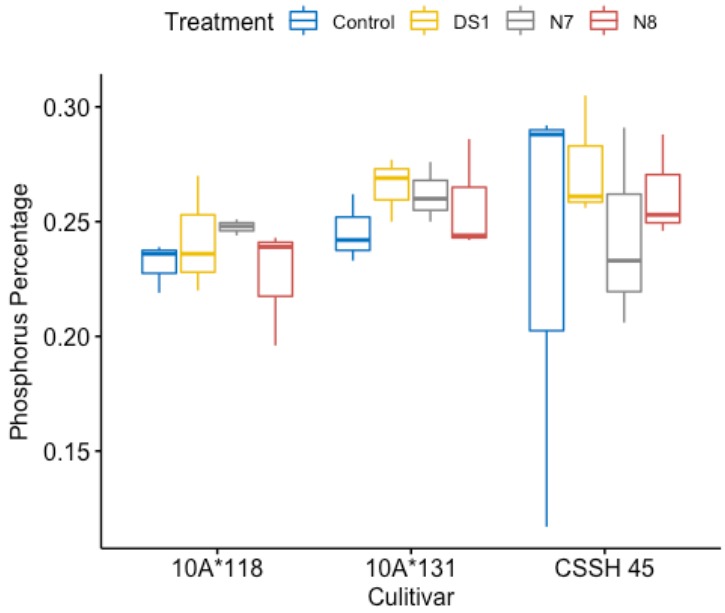

Figure 4.16: The box plot of tissue P concentration of three sorghum genotypes (CSSH 45 10A×118 and 10A×131) with different microbial treatments. Different colours represent different microbial treatments: Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum lipoferum*, Red=*Azospirillum brasilense*. The bold horizontal lines represent the median tissue P concentration, and boxes represent the interquartile range (IQR) of tissue P concentration.

4.2.3 ^{15}N Analysis

Among the microbial inoculants, N8-inoculated CSSH 45, 10A×118 and 10A×131 cultivar's shoot samples and uninoculated control's shoot samples of each cultivar were only used for ^{15}N

analysis from the second greenhouse study. According to the analysis, the mean value of ^{15}N atom % excess of N8 inoculated samples of CSSH 45 and 10A×131were higher than the mean value of ^{15}N atom % excess of controls (non-N fixing sample). The ^{15}N atom % excess of N8 treated 10A×118 were lower than the control. However, the difference between values was small (0.0050) (Table 2).

Table 4.2: ^{15}N analysis results of the second greenhouse study

Cultivar	Treatment	^{15}N Atom %	Mean value (%)
CSSH 45	Control – Replicate 1	0.5292	0.6409
	Control – Replicate 2	0.6890	
	Control – Replicate 3	0.7045	
	N8 – Replicate 1	0.6956	0.6735
	N8 – Replicate 2	0.6493	
	N8 – Replicate 3	0.6757	
10A×118	Control – Replicate 1	0.6793	0.6683
	Control – Replicate 2	0.6746	
	Control – Replicate 3	0.6510	
	N8 – Replicate 1	0.6784	0.6633
	N8 – Replicate 2	0.6462	
	N8 – Replicate 3	0.6652	
10A×131	Control – Replicate 1	0.6535	0.6633
	Control – Replicate 2	0.6725	
	Control – Replicate 3	0.6639	
	N8 – Replicate 1	0.6930	0.6784

	N8 – Replicate 2	0.6971	
	N8 – Replicate 3	0.6639	

The ^{15}N atom % excess values in Table 4.2 were fed into the formula for calculation of the %NDFA (see Material and Methods section 3.2.8). As there was no dilution of the ^{15}N atom % excess in the N8-treated CSSH 45 and 10A×131 plants, there is no evidence of nitrogen fixation by N8 in CSSH 45 and 10×131. However, there was a minute dilution of the ^{15}N atom % excess in the N8-treated 10A×18 (1.66%). Therefore, the N_2 fixation of N8 in 10A×118 is negligible.

4.2.4 Plant height

The generalized linear model (GLM) approach with a gamma distribution function was used for plant height analysis due to the lack of normality of the data set. According to the analysis, none of the plant heights of microbial treatments was significantly different compared to the control. However, there was a significant difference among genotypes in plant height ($p<0.05$). Pair-wise comparisons did not identify significant differences by the normal Tukey's test. However, after including the interaction effect to the pair-wise analysis, N7-inoculated CSSH 45 was identified as being significantly greater in plant heights compared to N7-inoculated 10A×118 by 13.2% ($p<0.05$) (Figure 4.17).

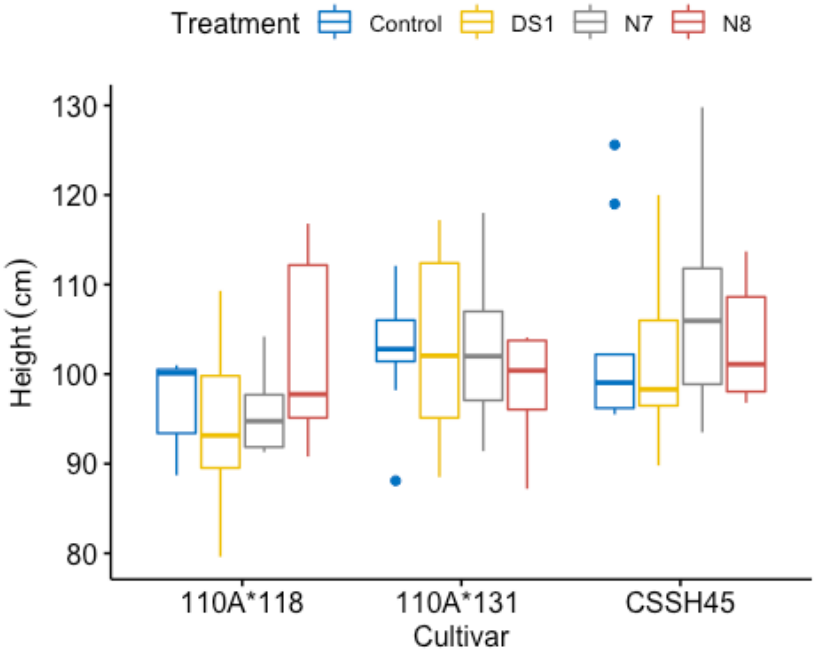

Figure 4.17: The box plot of plant height (cm) of three sorghum genotypes (CSSH 45, 10A×118 and 10A×131) with different microbial treatments. Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum lipoferum*, Red=*Azospirillum brasilense*. The bold horizontal lines represent the median plant height, and boxes represent the interquartile range (IQR) of plant height. NB: A dot below the boxplot of control in cultivar 10A×131 and the two dots above the box plot of control in cultivar CSSH 45 indicate outliers of the data set.

4.2.5 Plant leaf area

The distribution of leaf area data met the normality and homogeneity assumptions. Therefore, a two-way ANOVA test was used for the plant leaf area analysis. According to the analysis, the mean leaf area of each cultivar among microbial treatments was not significantly different to the

leaf area of the control plants. Also, the leaf area among cultivars was not significantly different (Figure 4.18).

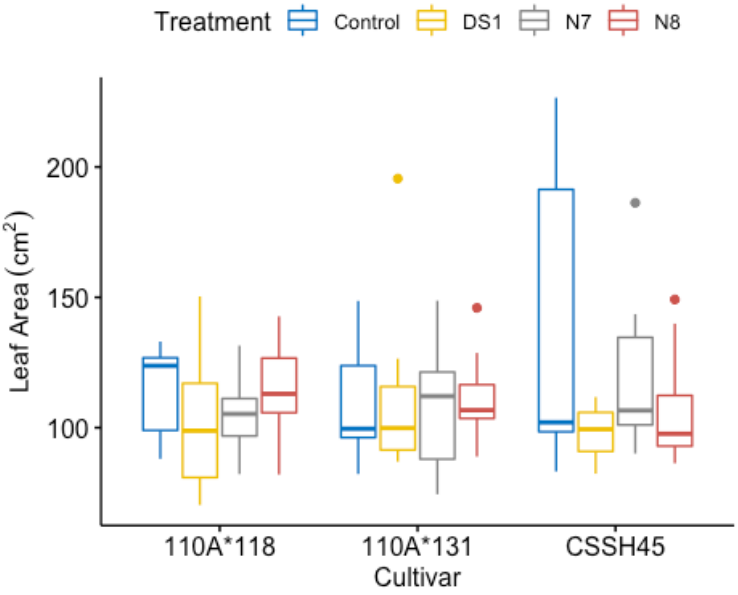

Figure 4.18: The box plot of leaf area (cm^2) of three sorghum genotypes (CSSH 45, 10A×118 and 10A×131) with different microbial treatments from the second greenhouse study. Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum lipoferum*, Red=*Azospirillum brasilense*. The bold horizontal lines represent the median leaf area, and boxes represent the interquartile range (IQR) of the leaf area. NB: The two dots above the box plots of DS1 and N8 in 10A×131 and the two dots above the box plots of N7 and N8 in CSSH 45 indicate outliers of the data set.

4.3 Field study

The microbial inoculants and sorghum genotypes used in the field study were the same as those used in the second greenhouse study. Two nitrogen fertilizer levels (0 and 43.5 kg N ha^{-1}) were used as another independent variable. As dependant variables, shoot dry weight, plant height,

leaf area and tissue nutrient contents were measured. There was a visible difference in plant growth between plants with and without nitrogen before harvesting. Plots with nitrogen were more dense, greenish, and taller than the plots without nitrogen application (Figure 4.19). Statistical analysis results of each parameter also supported these visible differences (see below). All dependant variables were statically analyzed using a three-way ANOVA.

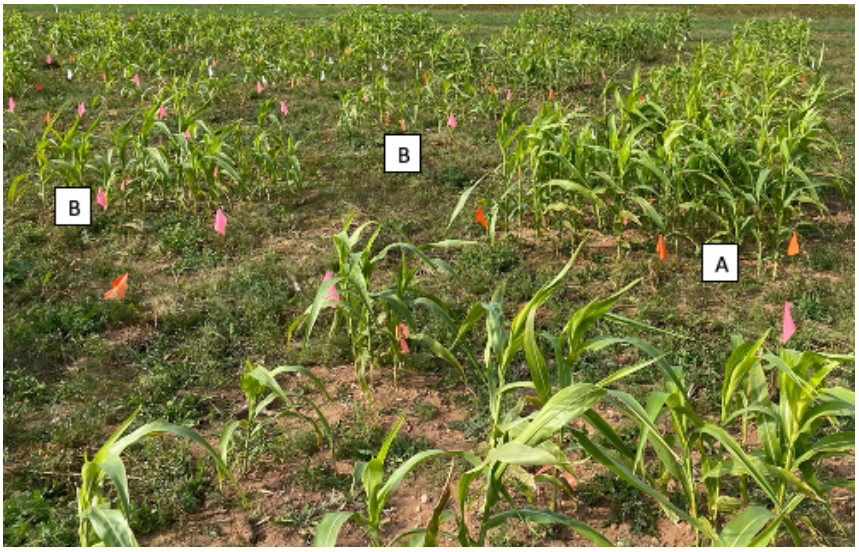

Figure 4.19: Sorghum plant in the field. The flags represent the borders of each plot. Plot A represents sorghum plants with nitrogen fertilizer, and plot B represents sorghum plants without nitrogen fertilizer application. Plants with nitrogen fertilizer were larger than plants without nitrogen fertilizer.

4.3.1 Shoot dry weight

There was a significant difference in shoot dry weights among cultivars ($p<0.05$). Among the genotypes, the shoot dry weight of 10A×131 was significantly greater than the shoot dry weight of 10A×118 by 40% ($p<0.05$). The shoot dry weight of CSSH 45 was not significantly different compared to the dry weights of the other two cultivars. Also, shoot dry weights of each cultivar

were significantly greater in the higher nitrogen treatments by 29.45% ($p<0.05$). There was no significant difference in shoot dry weights among microbial treatments (Figure 4.20). However, some patterns of dry weight distribution among microbial treatments can be identified using the box plots (Figure 4.21). Each genotype with no nitrogen displayed a gradual increase in dry weights in the order of control, DSI, N7 and N8, respectively. This pattern is identical in all genotypes with no nitrogen application. However, this pattern cannot be seen in genotypes with nitrogen application.

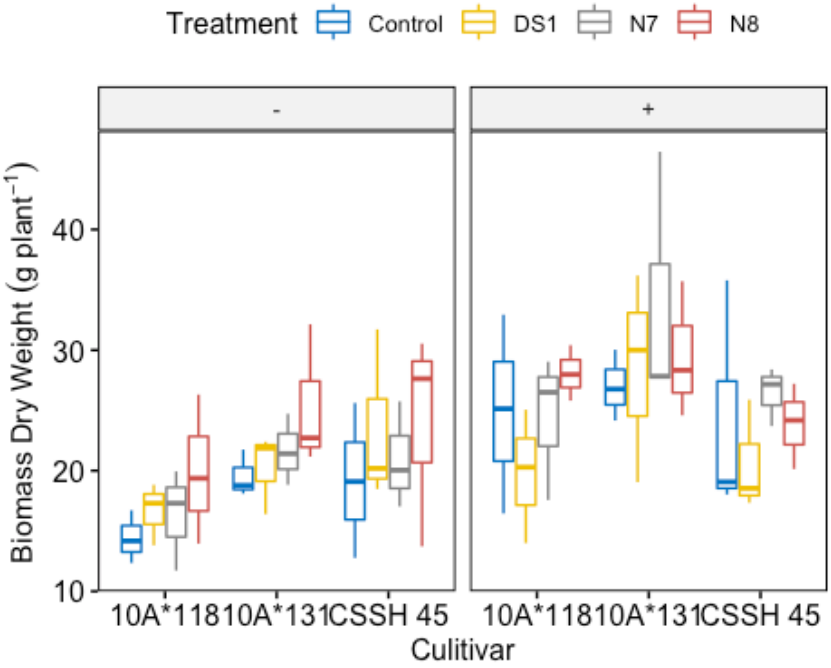

Figure 4.20: The box plot of shoot dry weight (g plant $^{-1}$) of three sorghum genotypes (CSSH 45, 10A×118 and 10A×131) under two different nitrogen levels with different microbial treatments from the field study. Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum lipoferum*, Red=*Azospirillum brasilense*. Positive and negatives signs represent with and without fertilizer application, respectively. The bold horizontal lines represent the median shoot dry weight, and boxes represent the interquartile range (IQR) of Shoot dry weight. NB: The shoot dry weight of 10A×118 and 10A×131 were significantly different ($p<0.05$). The shoot dry weights of each cultivar between two nitrogen levels were also significantly different ($p<0.05$).

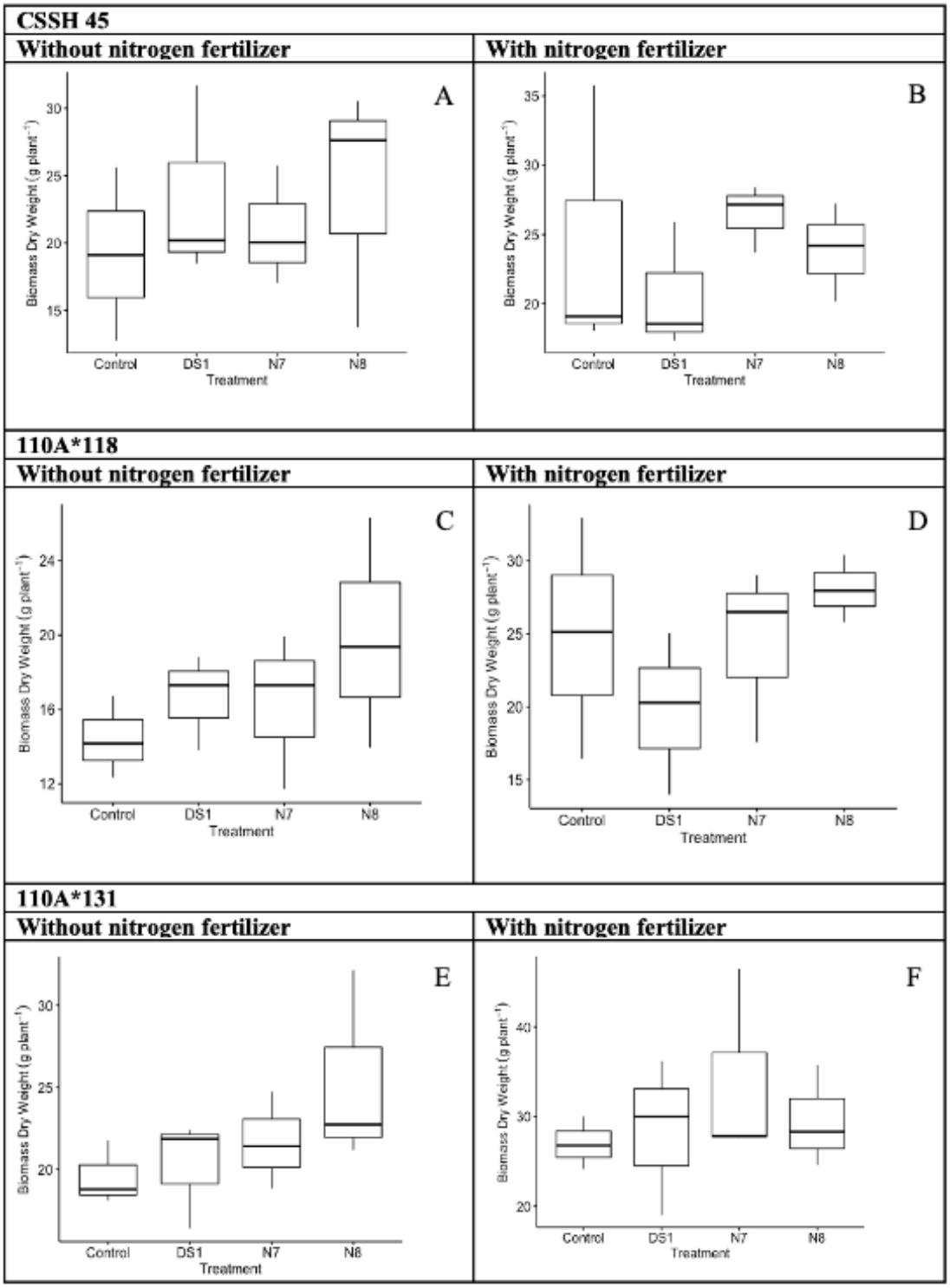

Figure 4.21: The boxplots of shoot dry weight (g plant^{-1}) for three sorghum genotypes under two different nitrogen levels with different microbial treatments from the field study. A: the boxplot of shoot dry weight (g plant^{-1}) of the CSSH 45 with different microbial treatments with no

nitrogen, B: the boxplot of shoot dry weight (g plant^{-1}) of the CSSH 45 with different microbial treatments with nitrogen, C: the boxplot of shoot dry weight (g plant^{-1}) of the 10A:118 with different microbial treatments with no nitrogen, D: the boxplot of shoot dry weight (g plant^{-1}) of the 10A:118 with different microbial treatments with nitrogen, E: the boxplot of shoot dry weight (g plant^{-1}) of the 10A:131 with different microbial treatments with no nitrogen, F: the boxplot of shoot dry weight (g plant^{-1}) of the 10A:131 with different microbial treatments with nitrogen. DS1=*Gluconacetobacter azotocaptans*, N7=*Azospirillum lipoferum,* N8=*Azospirillum brasilense*. The bold horizontal lines represent the median of shoot dry weight, and boxes represent the interquartile range (IQR) of shoot dry weight.

4.3.2 Tissue nutrient analysis

Dried shoot tissue samples were sent to the Nova Scotia Department of Agriculture for Analysis. The macronutrient and micronutrient contents of tissue samples were reported as a percentage of the dry weight of the sample (Appendix 7). As in the two greenhouse studies, the effect of microbial treatments and cultivars on the total N content of the plants (g N plant^{-1}), tissue N concentration of the dried shoot samples, total P content of the plants (g P plant^{-1}) and tissue P concentration of the dried shoot samples were statistically analyzed.

According to the analysis, both microbial treatments and nitrogen fertilizer levels resulted in significant differences in plant N content within each sorghum genotype (Table 4.3). According to a post hoc analysis, N8 inoculated genotypes resulted in an increase of plant N content by 31% compared to the control genotypes ($p<0.05$). Also, genotypes with nitrogen application resulted in a significant increase in plant N content by 26.54% compared to genotypes without nitrogen application ($p<0.05$) (Figure 4.22).

However, microbial treatments did not result in any significant differences in tissue N concentration of dried shoot samples within each genotype compared to the uninoculated controls. Also, neither genotypes nor nitrogen fertilizer levels significantly affected the tissue N concentration of dried shoot samples (Figure 4.23).

Statistical analysis of plant P content among genotypes resulted in a significant difference ($p<0.05$). Post hoc analysis indicated that the plant P content in 10A×131 was significantly greater than the plant P contents of CSSH 45 and 10A×118 by 32.6% and 38.2%, respectively (Figure 4.24). Also, the plant P content of genotypes with nitrogen application was significantly greater compared to the plant P content of genotypes without nitrogen application ($P<0.05$) by 31.3% (Table 4.4). There was no significant difference in the plant P content in genotypes among microbial treatments.

The tissue P concentration of dried samples of each genotype was not significantly different between microbial treatments and uninoculated controls. However, the tissue P concentration among genotypes was significantly different ($p<0.05$). The tissue P concentration of 10A×131 was significantly greater than both genotypes (10A×118 and CSSH 45) by 11.1% and 18.3%, respectively. Also, tissue P concentration of 10A×118 was significantly greater than CSSH 45 by 6.4%. There was no significant difference in tissue P concentration between the two nitrogen levels (Figure 4.25)

Table 4.3: The statistical analysis results of the plant nitrogen content data from the field study. Microbial treatments and nitrogen levels, and cultivars were statistically analyzed. Only significant results are shown in the table.

Variable	p-value	Tukey Post hoc result
Microbial treatment	0.027	(N8-Control) = 0.039
Nitrogen level	0.003	(With N- Without N) = 0.002

Table 4.4: The statistical analysis results of the plant phosphorus content data from the field study. Microbial treatments and nitrogen levels, and cultivars were statistically analyzed. Only significant results are shown in the table.

Variable	p-value	Tukey Post hoc result
Cultivar	0.0003	(10A×131-10A×118) = 0.0006 (CSSH 45-10A×131) = 0.0024
Nitrogen level	0.0024	(With N- Without N) = 0.0024

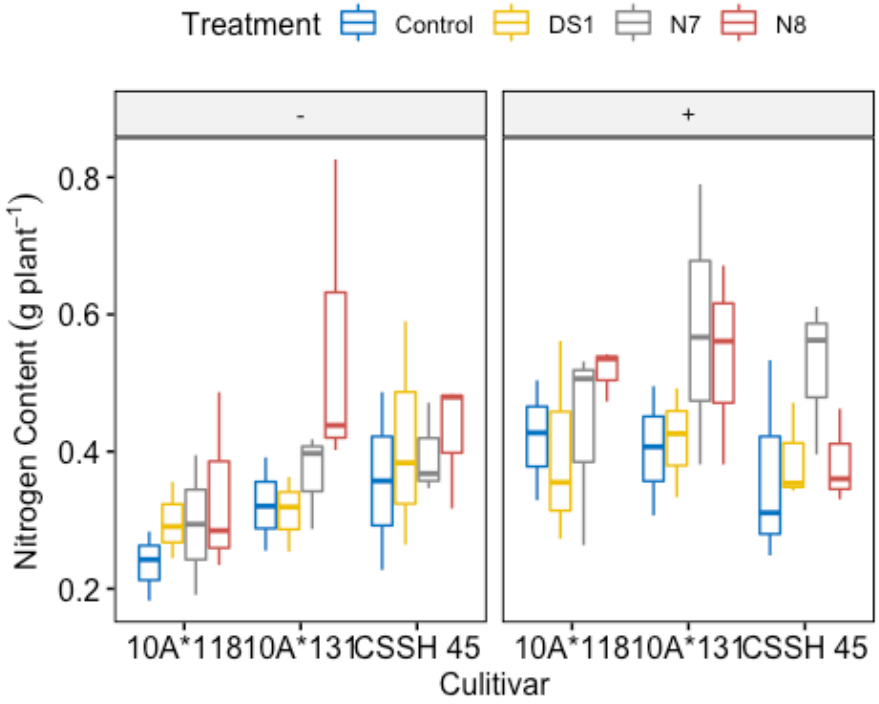

Figure 4.22: The box plot of plant N content (g plant ^{1}) of the three sorghum genotypes (CSSH 45, 10A×118 and 10A×131) under two different nitrogen levels with different microbial treatments from the field study. Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum lipoferum*, Red=*Azospirillum brasilense*. Positive and negatives signs represent with and without fertilizer application, respectively. The bold horizontal lines represent the median N content per plant and boxes represent the interquartile range (IQR) of the N content per plant. NB: The plant nitrogen content in N8 treated sorghum genotypes was significantly greater than the uninoculated control plants ($p<0.05$). The plant nitrogen content in genotypes between two N levels was significantly different ($p<0.05$).

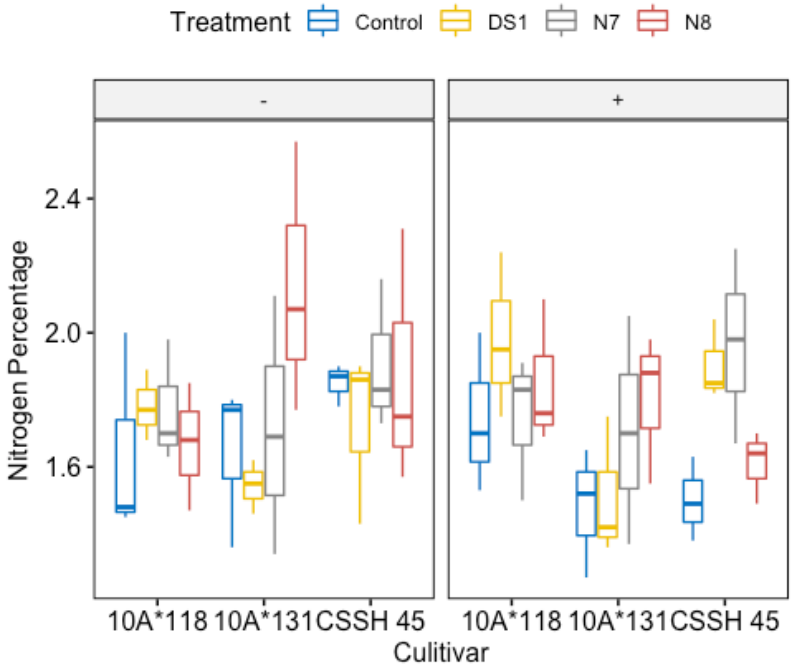

Figure 4.23: The box plot of tissue N concentration of three sorghum genotypes (CSSH 45, 10A×118 and 10A×131) under two different nitrogen levels with different microbial treatments from the field study. Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum lipoferum*, Red=*Azospirillum brasilense*. Positive and negatives signs represent with and without fertilizer application, respectively. The bold horizontal lines represent the median tissue N concentration, and boxes represent the interquartile range (IQR) of tissue N concentration.

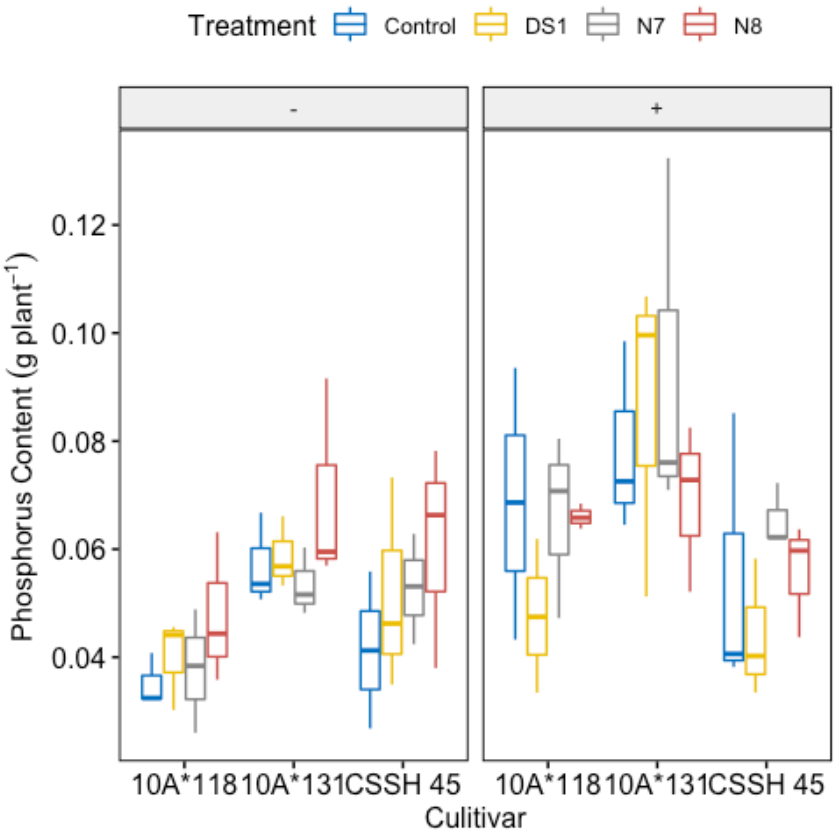

Figure 4.24: The box plot of plant P content (g plant ^{1}) of three sorghum genotypes (CSSH 45, 10A×118 and 10A×131) under two different nitrogen levels with different microbial treatments from the field study. Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum lipoferum*, Red=*Azospirillum brasilense*. Positive and negatives signs represent with and without fertilizer application, respectively. The bold horizontal lines represent the median P content per plant and boxes represent the interquartile range (IQR) of the P content per plant. NB: The plant P contents among genotypes were significantly different ($p<0.05$). The tissue phosphorus contents genotypes with nitrogen application were significantly greater than the plant P content of genotypes without nitrogen application ($p<0.05$).

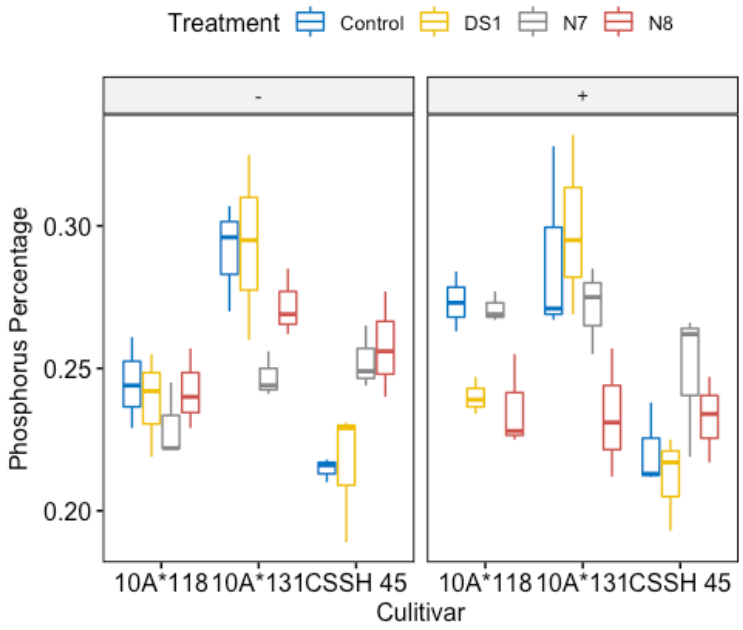

Figure 4.25: The box plot of tissue P concentration of three sorghum genotypes (CSSH 45, 10A×118 and 10A×131) under two different nitrogen levels with different microbial treatments from the field study. Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum lipoferum*, Red=*Azospirillum brasilense*. Positive and negatives signs represent with and without fertilizer application, respectively. The bold horizontal lines represent the median tissue P concentration, and boxes represent the interquartile range (IQR) of tissue P concentration. NB: The tissue P concentration of 10A×131 was significantly greater than CSSH 45 and 10A×118 ($p<0.05$). Also, the tissue phosphorus concentration of 10A×118 was significantly greater than CSHH 45 ($p<0.05$).

4.3.3 Plant height

Plant heights within each genotype among microbial treatments were not significantly different from uninoculated controls. Also, plant height among genotypes was not significantly different.

However, height of plants that received nitrogen fertilizer was on average across genotypes and inoculant treatments, 10.2% taller than that were not fertilized ($p>0.05$) (Figure 4.26).

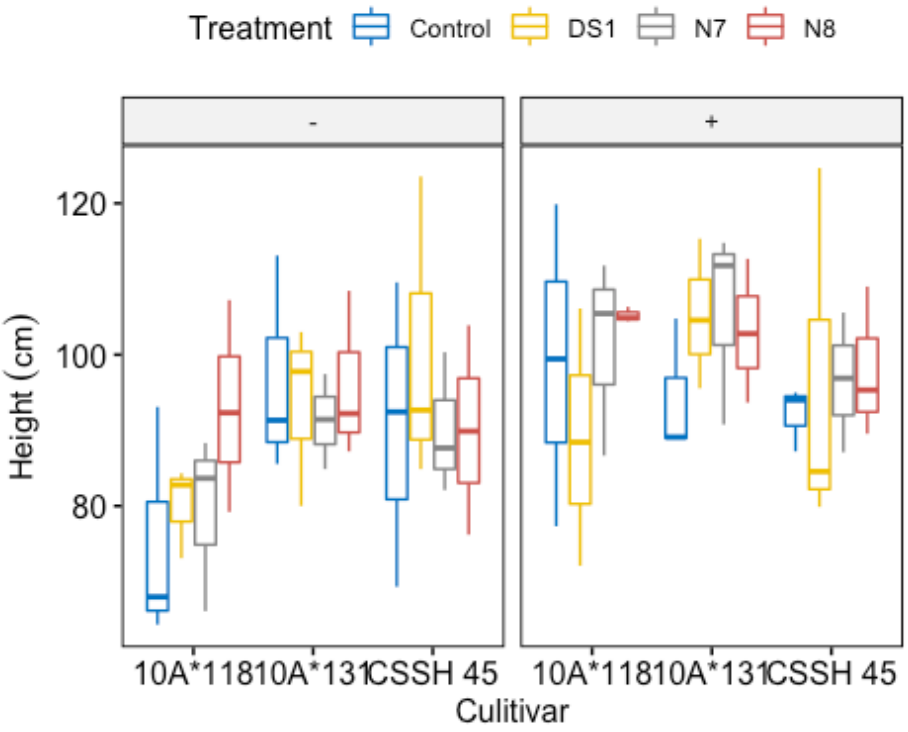

Figure 4.26: The box plot of plant height (cm) of three sorghum genotypes (CSSH 45, 10A×118 and 10A×131) under two different nitrogen levels with different microbial treatments from the field study. Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum lipoferum*, Red=*Azospirillum brasilense*. Positive and negatives signs represent with and without fertilizer application, respectively. The bold horizontal lines represent the median plant height, and boxes represent the interquartile range (IQR) of plant height. NB: Each genotype with nitrogen application resulted in a significant increase in plant height compared to without nitrogen application ($p<0.05$).

4.3.4 Plant leaf area

Leaf area within each genotype among microbial treatments were not significantly different to the uninoculated controls. Also, the leaf area did not differ among genotypes to each other. However, leaf area of plants that received nitrogen fertilizer was on average across gebotypes and inoculant treatments, 29.2% larger than that were not fertilized ($p>0.05$) (Figure 4.27).

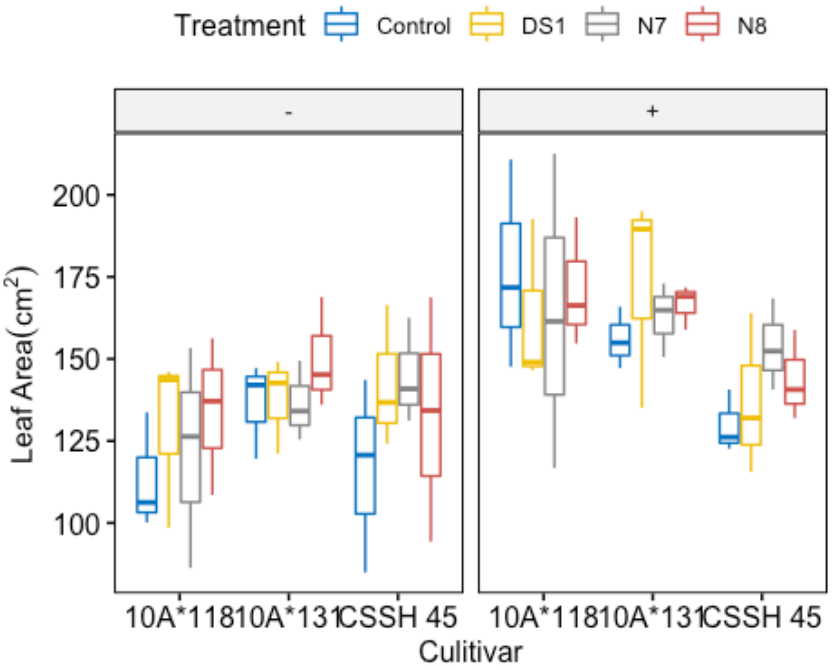

Figure 4.27: The box plot of leaf area (cm^2) of three sorghum genotypes (CSSH 45, 10A×118 and 10A×131) under two different nitrogen levels with different microbial treatments from the field study. Different colours represent different microbial treatments: Blue=Control, Yellow=*Gluconacetobacter azotocaptans*, Grey=*Azospirillum lipoferum*, Red=*Azospirillum brasilense*. Positive and negatives signs represent with and without fertilizer application, respectively. The bold horizontal lines represent the median of the leaf area, and boxes represent the interquartile range (IQR) of the leaf area. NB: Genotypes with nitrogen application resulted in a significant increase in leaf area compared to without nitrogen application ($p<0.05$).

5. Discussion

The main objective of this study was to identify the growth-promoting potential of selected microorganisms on genotypes of sorghum bred for biomass production. Two greenhouse studies and one field study were conducted to evaluate combinations of four sorghum cultivars/genotypes and five microbial inoculants. In general, it was found that some of the evaluated microorganisms may affect the growth parameters of some of the sorghum genotypes. However, inoculation effects on plant dry weight were only significant in one of the greenhouse studies, although some patterns of microbial effects on sorghum biomass were identified in the field study. Shoot dry weight changes with microbial treatments were mainly considered in this study because this study aims to establish sorghum cultivation on marginal lands of Nova Scotia for use as a biofuel feedstock.

Other growth parameters such as leaf area, plant nitrogen and phosphorus contents and tissue nitrogen and phosphorus concentrations, plant height were measured to identify any growth changes due to the microorganisms. Significant changes of some parameters with microbial treatments have been identified. Genotype 10A×131 has performed best between the two new genotypes and greater than the old cultivar (CSSH 45) in the field. As expected, the nitrogen fertilizer application increased most growth parameters, including dry weight, leaf area, plant height, and plant nitrogen and phosphorus contents, compared to no nitrogen fertilizer application.

5.1 First greenhouse study

5.1.1 Shoot dry weight

In the first greenhouse study, PAL5T inoculation of CSSH 45 resulted in a significant increase ($p<0.05$) in shoot dry weight by 19% compared to the uninoculated control (Figure 4.2). Also, DS1-inoculated CSSH 45 resulted in a 16% increase ($p<0.10$) in shoot dry weight compared to the control. A previous study (Yoon et al. 2015) identified that PAL5T could colonize sorghum. The study also identified that higher colonization and a higher number of bacteria were seen in sugar-rich sorghum cultivars. The findings of the current study also support those of Yoon et al. (2015). The significant result in biomass accumulation was only be seen in PLA5T-inoculated CSSH 45, not in PAL5T-inoculated CFSH 30. CSSH 45 is a sweet sorghum cultivar, while the CFSH 30 is a forage sorghum cultivar. *Gluconacetobacter diazotrophicus* (PAL5T) is a nitrogen-fixing bacterium initially isolated from sugar cane plants in Brazil (Cocking et al. 2005). It is a famous bacterium for colonizing sugar-rich plants such as sugarcane, sweet sorghum, sweet potato, cameron grass (Sahai et al. 2015). In addition to nitrogen fixation, this bacterium promotes plant growth through phytohormone production, solubilization of mineral nutrients including, phosphorus and acting against phytopathogen (Sahai et al. 2015; Saravana et al. 2006). Hence, PAL5T becomes an essential plant growth-promoting bacterium for sugar-rich plant growth (Sahai et al. 2015). Based on these previous findings, it can be concluded that the significant effect of shoot dry weight of CSSH 45 was an effect of PAL5T.

Gluconacetobacter azotocaptans (DS1) inoculated CSSH 45 also significantly increased plant shoot dry weight, albeit at only 0.1 probability. Mehnaz et al. (2005) identified that the bacterium could successfully colonize the corn. Since corn and sorghum belong to the same subfamily (Panicoidear) of the Poaceae and have many similarities in genetics, growth, development and

physiology (Schnable 2015), it is reasonable to conclude that DS1 can colonize the sorghum. Also, DS1 has been identified as a plant growth-promoting bacterium in previous studies through plant growth hormone (IAA) production, N fixation, phosphate solubilization and inhibition of fungal pathogens invasion (Mehanz et al. 2005). Based on these findings, it can be concluded that DS1 might affect the growth of CSSH 45.

Even though shoot dry weights of CSSH 45 with the other three microbial inoculants (N7, N8 and *P. bilaii*) were not significantly different compared to the control, mean shoot dry weights of CSSH 45 inoculated with N7, N8, and *P. bilaii* were numerically higher than the mean shoots dry weights of the uninoculated controls (Figure 4.2). None of these microorganisms did affect the shoot dry weight of CFSH 30 (Figure 4.3). However, these three strains have been identified as plant growth-promoting microorganisms in previous studies. In generally, *A. brasilense* and *A.lipoferum* can promote plant growth through plant hormone production and N_2 fixation (Spaepen et al. 2008; Mehnaz et al. 2006). *P bilaii* promotes plant growth mainly via P solubilization (Vessey and Heisinger. 2001). In previous studies, both *A. lipoferum* and *P bilaii* were tested with corn plants. Fulchieri et al. (1993) identified that corn plants with *A. lipoferum* inoculants showed a higher growth rate than uninoculated plants. Gomez-Munoz et al. (2018) showed that *P. bilaii* increased corn plant growth through increasing mineral uptake (mainly P). As mentioned in 5.1.1, corn and sorghum belong to the same subfamily (Panicoidear) of the Poaceae, and both have similarities in genetics, growth, development and physiology. It could be assumed that *A. lipoferum* and *P.bilaii* are possible sorghum plant growth-promoting microorganisms. However, the first greenhouse study results did not support this hypothesis. Also, N8 had been tested with sorghum as a plant growth-promoting bacterium in several previous studies. One of the studies conducted by Pacovsky et al. (1984) showed that N8-treated

sorghum plant increased dry weight by 25% compared to uninoculated control. Another study by Sarig et al. (1990) showed N8-treated sorghum plant significantly increased dry weight, yield and plant leaf area compared to the control plants. Based on these results, it has been confirmed that N8 can successfully colonize sorghum and can promote its growth. As with N7 and *P. bilaii*, N8 did not give significant results in shoot dry weight in this study. Also, previous literature showed that the inoculation response of N8 could be highly variable (Sarig et al. 1990). Therefore, the possible conclusion is that N8 could not successfully colonize the sorghum cultivars in the first greenhouse study.

5.1.2. Other growth parameters

In addition to dry weight, plant height was obtained as growth parameters in the first greenhouse study. Plant leaf area was not measured. The leaf area meter used in the second greenhouse study and the field study was not available during the first greenhouse study. Therefore, plant height was only measured as the other growth parameter. The plant height data were statistically analyzed to identify the changes related to microbial activity. According to the results, none of the microbial treatments with each cultivar showed a significant difference in plant height compared to control (Figure 4.10). Even though PAL5T-treated CSSH 45 was significantly greater in shoot dry weight, PAL5T did not significantly change plant height compared to the control. According to the previous literature, PAL5T can significantly increase sugarcane plant height (Indi et al. 2014). Also, Batian et al. (1998) showed that PAL5T could produce IAA (Indole Acetic Acid) and gibberellins in a chemically defined culture media. Gibberellins is mainly responsible for stem elongation during plant growth and development (Ross and Reid

2010). Even though these facts show that PAL5T can increase plant height, PAL5T did not significantly affect the plant height of any sorghum cultivar in the first greenhouse study.

5.1.3 Plant N and P contents and tissue N and P concentrations

Effects of the microbial inoculants on plant macronutrient and the micronutrient contents in each plant sample were measured. However, due to their relative importance, only N and P contents in plant samples were used for statistical analysis. In previous studies, microbial inoculants tested with sorghum have been identified as plant growth-promoting microbes through increasing N and P availability to the plants. As mentioned in the previous section, PAL5T is a popular N_2 fixing bacteria. DSI, N7 and N8 have also been identified as N_2 fixing bacteria (Mehnaz and Lazarovits 2017; Moutia et al. 2010). Phosphorus solubilization ability of N7, N8 and *P bilaii* has been identified in previous studies. Kucey (1983) identified *P. bilaii* as a phosphorus solubilizing fungus. Rodriguez et al. (2004) found that *Azospirillum brasilense* (N8) and *Azospirillum lipoferum* (N7) can produce gluconic acid when calcium phosphate is available. Gluconic acid reduces pH in the medium and releases soluble phosphate. Therefore, in tissue nutrient analysis, plant N and P contents and tissue N and P concentrations were mainly used for further analysis (statistical analysis) to identify the differences in plant N and P contents and tissue N and P concentrations between treated and control plants. The plant N or P content is a measurement of the total N or P amount per plant (g plant^{-1}). The tissue N or P concentration is a measurement of the accumulation rate of N or P per gram of dry weight (% N). Significant differences in plant N and P contents and tissue N and P concentrations between treated and control plants may indicate that the microbial activities affect the N and P uptake.

In the first greenhouse study results, PAL5T-treated and N7-treated CSSH 45 plants resulted in a significant increase in plant nitrogen content compared to the uninoculated control by 27% and 17%, respectively (Figure 4.6). Based on the shoot dry weight and plant N content results, it can be concluded that PAL5T positively affected cultivar CSSH 45 growths. However, there were no significant differences in tissue N concentration of dried shoot samples of CSHH 45 between microbial treatments and uninoculated control (Figure 4.7). The tissue N concentration results showed that the accumulation rate of nitrogen per gram of dry weight was not different between treatments and control plants. The significant increase in plant nitrogen content of PAL5T-treated plants, therefore, is likely due to the increase in dry weight (i.e., the increased plant nitrogen content of PAL5T-treated plants is an effect of the increase in dry weight, and not cause of the increase in dry weight). It appears that PAL5T stimulates other mechanisms to increase the growth rate of CSSH 45 cultivar other than by an increased in nitrogen uptake per unit weight of plant tissue. Kumarasamy and Santhaguru (2011) reported that PAL5T-inoculated sorghum plants showed the increased growth compared to the uninoculated plants in terms of total biomass, total N content, soluble sugar and chlorophyll content. This study also showed that PAL5T could use different mechanisms to increase sorghum growth, such as N_2 fixation, phytohormone production and improved nutrition uptake. According to the plant N content and tissue N concentration results of the study, it can be concluded that PAL5T likely improved sorghum growth using different mechanisms such as phytohormone production other than increasing N uptake. However, further studies are required to identify and verify the growth-promoting mechanism of PAL5T on CSSH 45.

Azospirillum lipoferum (N7) also increased the plant N content by 17% compared to the control, but the shoot dry weight of N7-treated CSSH 45 plants did not significantly increase compared

to the control as PAL5T. Also, the percent nitrogen in tissues of N7 treated CSSH 45 was numerically greater, but not significantly different compared to the control plants. Hence, the increase in plant N content of N7-treated CSSH 45 plants appears to be the product of slight, but not statistically significant, increases in plant dry weight and tissue N concentrations, that when combined resulted in significant increases in N content.

Also, the plant nitrogen content and tissue nitrogen concentration of CSSH 45 treated DS1 was not significantly different from the uninoculated controls. The mean plant nitrogen content of DS1 (0.87g) is similar to that of the N7 (0.90g) and numerically higher than the control. Also, DS1-treated CSSH 45 was significant in shoot dry weights at 10% level and not significant in plant nitrogen content. Therefore, it can be concluded that DS1-treated CSSH 45 had a higher growth rate compared to uninoculated control. It appears that DS1 may have mechanisms other than increasing N content that resulted in increased plant growth.

The two sorghum cultivars (CSSH 45 and CFSH 30) had significant differences in shoot dry weight, but there was no significant difference in plant nitrogen content and tissue nitrogen concentration between the two cultivars. Generally, plant growth rates are different from cultivar to cultivar based on their genetic differences (Govindaraj et al. 2014). Therefore, it can be concluded that genetic differences between two sorghum cultivars may be the reason for differences in shoot dry weights.

The plant P content of CSSH 45 was significantly different in all microbial treatments compared to the uninoculated control. The PAL5T treatment had the greatest increase at 37% compared to the control. *P. bilaii*, N8, N7 and DS1 treatments were also significantly greater than the control by 25, 23, 22, 18%, respectively (Figure 4.8). However, there is no significant difference in tissue P concentration (P accumulation rate per gram of dry weight) between microbial treatment

and uninoculated control (Figure 4.9). Even though plant P content was significant in all microbial treatment, only PAL5T and DS1 treatments resulted in significant increases in shoot dry weights. The positive effects on shoot dry weight and plant P content in PAL5T-treated CSSH 45 and DS 1-treated CSSH 45 is likely due to an increase in dry weight (i.e., the increased plant P contents of PAL5T-treated plants and DS1-treated plants are an effect of the increase in dry weight, and not cause of the increase in dry weight).

A study conducted by Meenakshisundaram and Santhaguru (2011) reported that PAL5T associated with arbuscular mycorrhizal (*Glomus fasciculatum*) fungi increased *S. bicolor* growth. According to the results of the study, this association has caused to increase the P, soluble sugars, and photosynthetic pigments. Therefore, these changes may increase water and nutrient uptake. In the first greenhouse study, PAL5T treated CSSH 45 was significant in shoot dry weight by 19% and plant P content was significant by 37%. Also, during the harvesting, a clear difference of two root systems (PAL5T treated CSSH 45 and Control CSSH 45) were observed. The root system belonging to PAL5T treated CSSH 45 was dark coloured and bigger while the root system of control CSSH 45 was light coloured and smaller (Figure 5.1). Therefore, this could be another possible reason for the growth enhancing effect of PAL5T in CSSH 45.

Figure 5.1 The image of two root systems of CSSH 45 cultivar from two different treatments from the first greenhouse study. The root system labelled as A in the image represents PAL5T treated CSSH 45, and the root system labelled as B represents Control CSSH 45.

However, N8, N7 and *P. bilaii* treated CSSH 45 were only significant in plant P content, not significant in shoot dry weight and tissue P concentration. Also, the tissue P concentration of N8, N7 and *P.bilaii* treated CSSH 45 was numerically greater, but not significantly different compared to the control plants. The increase in plant P content in N8, N7 and *P. bilaii* treated CSSH 45 plants appears to be the product of slight, but not statistically significant, increases in plant dry weight and tissue P concentrations, that when combined resulted in significant increases in plant P content.

5.1.4 N_2 Fixation using the ^{15}N Isotope Dilution Technique

Due to the expense associated with ^{15}N analysis, PAL5T-treated and uninoculated control samples of CSSH 45 were only used for ^{15}N analysis from the first greenhouse study. Among different microbial treatments, only PAL5T-treated CSSH 45 plants had significant increases in shoot dry weight. Therefore, this tissue sample was used for analysis. The ^{15}N isotope dilution method can be used to identify and measure fixed nitrogen from the atmosphere by microorganisms (Hardarson and Danso 1993). In this method, reference plants (control sorghum plants) and potential nitrogen-fixing plants (microbial treatment applied sorghum plants) receive the same amount of fertilizer with the same $^{15}N/^{14}N$ ratio in the fertilizer. However, plants associate with nitrogen-fixing microbes can obtain N_2 from the atmosphere, which has a much lower ratio of $^{15}N/^{14}N$ (i.e., the atmosphere only contains 0.366% ^{15}N). Therefore, using $^{15}N/^{14}N$ ratios of the plant tissues in the test and reference plants and the equation identified in the materials and methodology, the percentage of nitrogen fixation can be calculated.

According to the result (Table 4.1), the mean ^{15}N atom % excess for PAL5T-treated and control samples were 0.4693% and 0.4547%, respectively. Since the ^{15}N atom % was not diluted in the PAL5T-treated plants compared to the control, this indicates that there was no evidence of N_2 fixation in the PAL5T-treated plants

Based on these results, it can be concluded that nitrogen fixation of PAL5T was not the reason for the significant increase in shoot dry weight in CSSH 45. According to the previous literature, N_2 fixation is not the only mechanism that PAL5T uses for increasing plant growth. As mentioned in section 5.1.3, PAL5T involves increasing plant growth using different mechanisms such as N_2 fixation, phytohormone production and improved nutrient uptake and act against phytopathogen (Kumarasamy and Santhaguru 2011; Saravanan et al. 2008). As mentioned in

section 5.1.2, phytohormones such as IAA and gibberellins synthesis by PAL5T was tested and identified in a chemically defined medium (Batian et al. 1998). Based on these previous findings, it can be concluded that PAL5T involved in increasing the shoot dry weight of CSSH 45 significantly using different mechanisms other than N_2 fixation in the first greenhouse study.

5.2 Second greenhouse study

5.2.1 Shoot dry weights

In the second greenhouse study, inoculation treatments effects on shoot dry weights provided some unexpected results. Shoot dry weights of DSI-inoculated, and N8-inoculated CSSH 45 plants significantly decreased compared to the control plants by 32 and 28%, respectively (Figure 4.12). Also, the dry weight accumulation of the hybrids 11A×118 and 10A×131 was lower than that of CSSH 45. The data did not meet the normal distribution and homogeneity assumptions. Therefore, a Generalized Linear Model (GLM) was used for data analysis. Mean shoot dry weights of two new hybrids (10A×118 and 10A×131) with microbial treatments were not statistically different to the uninoculated controls. The first greenhouse study results for inoculation effect on cultivar CSSH 45 were different in the second greenhouse study. The first greenhouse study indicated some positive trends with microbial treatments, while the second greenhouse results had a negative trend with microbial treatment. An explanation for this may be, although not being identified as statistical outliers (Figure 4.11), several of the CSSH 45 control plants seemed unusually large in the second greenhouse study. An explanation for this may be, as in other sorghum hybrid cultivars (e.g., Elknina et al. 2015), CSSH 45 may be susceptible to genetic reversions; the process in which a hybrid cultivar is known with given distinct characteristics “reverts” back to a different form found in the plant’s parentage. In

addition, three outliers (two from N8 treatment and one from N7 treatment) were identified from the statistical analysis. All of these issues would have led to greater variance in the datasets. Therefore, it may be that these negative trends are the result in unusual growth of the uninoculated CSSH 45 plants. The colonization and growth-promoting ability of two new genotypes (10A×118 and 10A×131) by bacteria (N7, N8, and DS1) are still unclear. Based on the second greenhouse results, microbial treatments did not affect the growth of two new genotypes. However, further studies are required to verify these results.

5.2.2 Other growth parameters

In addition to dry weight, plant leaf area and height were obtained as growth parameters. They were statistically analyzed to identify the growth changes. A Generalized Linear Model was used for plant leaf area and height data analysis because both data sets did not meet the normality and homogeneity assumptions. According to the results, neither plant height (Figure 4.17) nor leaf area (Figure 4.18) data showed significant differences between microbial treatments and the uninoculated controls for each genotype. These results are similar to the findings for treatment effects on shoot dry weight (Section 5.2.1) and may also reflect the unusual growth of some uninoculated CSSH 45 control plants resulting in high variability in the data.

5.2.3 Plant N and P contents and tissue N and P concentrations

The trends in plant tissue nitrogen content distribution in the second greenhouse study (Figure 4.13) were the same as the shoot dry weight data distribution. Again, the data did not meet the normality and homogeneity assumptions. Therefore, a Generalized Linear Model was used for plant nitrogen content analysis. According to the analysis, neither microbial treatments nor

genotypes showed significant differences (Figure 4.13). However, tissue N concentration data met the normality and homogeneity assumptions. Therefore, a two-way ANOVA test was used for tissue N concentration data analysis. According to the results, neither microbial treatment nor sorghum genotypes showed significant differences in tissue N concentration (Figure 4.14). Since both plants shoot dry weight and tissue N concentration results of each genotype with microbial treatments were not significantly different compared to the control, significant results in plant N content between microbial treatments and control cannot be expected. However, previous studies showed that these microbial inoculants helped the increase nutrient uptake (N) in sorghum plants. Lin et al. (1983) showed that N8 increased sorghum and corn plant growth (shoot dry weight) through enhancing mineral uptake, especially NO_3^-, K^+ and PO_4^{-3} uptake. Rai and Gaur. (1982) showed that N7 increased wheat plant growth through enhancing nutrient uptake and N_2 fixation. Even though the information about DS 1 is few, it has been identified as one of the N_2 fixing bacteria in the Acetobacteraceae family (Pedraza 2008). These previous studies showed that the microbial inoculants used in the second greenhouse study involve an increased nitrogen uptake, but none of the inoculants significantly increased nitrogen uptake in sorghum plant in the second greenhouse study. The plant N content results are similar to the findings for treatment effects on shoot dry weight (Section 5.2.1) and may also reflect the unusual growth of some uninoculated CSSH 45 control plants resulting in high variability in the data.

The distribution of plant P content data was different from the distribution of tissue nitrogen content data. The plant P content dataset met normal distribution and homogeneity assumptions. Therefore, a two-way ANOVA test was used for data analysis. Microbial treatments did not significantly affect the plant P content of any genotype (Figure 4.15). A Generalized Linear Model was used for tissue P concentration analysis due to violation of normality and

homogeneity assumptions. According to the results, there were no significant differences in tissue P concentration between microbial treatments and uninoculated control (Figure 4.16). Since there were no significant differences in shoot dry weight and tissue P concentration, significant results in plant P content of each genotype between microbial treatments and uninoculated control cannot be expected. The previous studies showed that these microbial inoculants involve an increased P uptake. Rodriguez et al. (2004) showed the P solubilization ability of N7 and N8 in vitro conditions. Also, the P solubilization ability of DS1 has been identified the previous studies (Mehanz et al. 2005). However, significant changes in plant P content or tissue P concentration were not identified in N7, N8, and DS1 treated sorghum genotypes compared to the uninoculated sorghum genotypes in the second greenhouse study. In contrast, in the first greenhouse study, DS1, N8 and N7 treated CSSH 45 showed increased growth rate to have significant differences in plant P content compared to the uninoculated control. However, this trend cannot be seen in the second greenhouse study results. Therefore, it can be concluded that either these three microorganisms did not increase CSSH 45 growth rate, same as the first greenhouse study or that several of the unusually large CSSH 45 control plants might have affected the final ability to decern these effects. Also, plant P content in CSSH 45 and 10A×131 was significantly higher than 10A×118. Between two new genotypes, 10A×131 have a higher growth rate compared to 10A×118. Therefore, higher plant P content can be expected in 110×131 genotype.

5.2.3 N_2 Fixation using the ^{15}N Isotope Dilution Technique

Because none of the microbial treatments resulted in significant effects on shoot dry weight with any genotypes in the second greenhouse and due to the expense associated with 15N analysis,

only *A. brasilense* (N8) was selected for measurement of N_2 fixation by ^{15}N analysis in the second greenhouse study.

According to the ^{15}N analysis, there is no evidence of N_2 fixation by N8 in CSSH 45 and 10A×13. ^{15}N atom % excess values of both genotypes (fixing plants) was higher than the control (reference plants) ^{15}N atom% excess values (Table 4.2). According to the equation, the nitrogen percentage derived from the atmosphere for N8 was a negative value for both genotypes. However, the ^{15}N atom % excess value of 10A×118 is lower than the control values, suggesting some level of fixed-N in the genotypes. According to the equation, the percentage of nitrogen derived from the atmosphere was only 0.75%. Even though the positive percentage indicates fixed nitrogen from the atmosphere, the fixed N content in the sorghum plant was negligible. Previous studies have identified the N_2 fixing ability of N8 (Moutia et al. 2010). Even though N8 fixes N_2 from the atmosphere, fixed products are used themselves (Moutia et al. 2010). Therefore, several studies related to N8 identified that most of the growth responses were due to some factors other than N input (Pacovsky et al. 1984).

5.3 Field study

5.3.1 Shoot dry weight

Microbial treatments (DS1, N7 and N8) did not result in any significant differences in shoot dry weight of any of the sorghum genotypes (CSSH 45, 10A×118 and 10A×131) compared to the uninoculated controls. However, a pattern was identified in the shoot dry weight of each cultivar with microbial treatments when nitrogen fertilizer is absent (Figure 4.20). The mean shoot dry weights of CSSH 45 with control, DS1, N7, and N8 were 19.16, 23.46, 20.94 and 23.96g, respectively. Mean dry weight of 10A×118 with control, DS1, N7 and N8 were 14.41, 16.65,

16.32, 19.88g, respectively. Mean dry weight of 10A×131with control, DS1, N7 and N8 were 19.54, 20.22, 21.65, 25.34g, respectively. According to the results, N8-treated plants consistently had the numerically highest mean shoot dry weights, and control plants had the numerically lowest value among treatments. The dry weight of CSSH 45 inoculated with DS1 and N7 was intermediate between N8 and the control treatments. This pattern is identical for all genotypes (Figure 4.21). However, genotypes with nitrogen application did not show a specific pattern in shoot dry weights with microbial treatments.

These results suggest that the test inoculants may have the ability to increase sorghum plant shoot dry weight when nitrogen fertilizer is absent. However, the inoculants effects on shoot dry weight were not statistically significant. One of the reasons could be that the replicate number (three replicate per each treatment) may have been too low to differentiate the effect of microbial treatments compared to uninoculated control of each genotype.

In addition, niche competition between the novel and native microorganisms, extreme climatic conditions, environmental and soil pollutants, poor soil conditions, and the inadequate number of microbial strains could be possible factors limiting microbial effects in large scale applications such as field experiments (Nosheen et al. 2021). One or several of these factors may have affected the activity of microbial treatments. Therefore, a set of field studies in different locations with different strain concentrations (>10^8 per 100ml) are required to identify the actual effect of these microbes on the sorghum plant. Also, Hardarson et al. (1991) showed that the plant growth-promoting microbes suppress their activity (e.g., N_2 fixation) when the plant receives a sufficient amount of nutrients (e.g., nitrogen) from chemical fertilizer. This could be the reason that no differences or trends were found in sorghum plants by the microbial treatments in the fertilized plots.

Shoot dry weights of two new hybrid genotypes (10A×118 and 10A×131) were not significantly different compared to the commercially available cultivar CSSH 45 (Figure 4.20). However, shoot dry weights of the two new genotypes were significantly different. The mean dry weight of 10A×131 was 40% higher than 10A×118. The mean dry weight of CSSH 45 is intermediate between the two new genotypes. Therefore, more field studies are required to decide the best genotypes for the marginal lands.

As expected, sorghum plants treated with nitrogen fertilizer resulted in a significant difference in shoot dry weight compared to the plant without nitrogen fertilizer. Nitrogen is an essential macronutrient that directly affects plant growth. Nitrogen is required for the plant to synthesize amino acid, proteins and chlorophyll. Those products mainly involve plant growth and development by affecting the photosynthesis and mineral uptake process (Wang et al. 2020). Also, Almodares et al. (2008) showed that N fertilizer treated sweet sorghum cultivars significantly increased the growth in terms of stem height, stem fresh weight, total fresh weight, total sugar and juice extract compared to no N fertilizer applied control plants. Therefore, a higher growth rate (higher biomass accumulation) of sorghum genotypes can be expected with the nitrogen fertilizer. Even though genotypes with N fertilizer performed the best in growth compared to the genotypes with no N fertilizer application in the field, N fertilizer application is not the best solution for increasing plant growth when considering the harmful effect on the environment. Nitrogen fertilizer causes major environmental problems such as the greenhouse gas effect and eutrophication (Byrens 1990; Khan and Mohammad 2013).

Nitric oxide (N_2O) releases into the atmosphere during biological activities in the soil, such as nitrification and denitrification. Nitric oxide is a greenhouse gas (Byrens 1990). Byrens. (1990) identified that N fertilizer contributes to higher nitric oxide production. Also, higher nitrogen

application rates result in runoff excess nutrient to water bodies. Accumulation of nutrient in the water bodies helps grow algae and cyanobacteria on the water body's surface. This is identified as eutrophication. As a result of eutrophication, water bodies become unsafe for humans and animals (Khan and Mohammad 2013). Hence, developing sorghum cultivar in marginal lands with low nitrogen fertilizers is crucial for minimizing the harmful effect on the environment., Developing biofertilizer, as this research, is a better solution to minimize N fertilizer application. According to the field study results, mean shoot dry weights of DS1 and N8-treated CSSH 45 without N fertilizer showed numerically higher values than the mean dry weights of DS1and N8-treated CSSH 45 with N fertilizer. These results suggest that some microbial sorghum combinations may be more efficient than N fertilizer applications. These combinations will be helpful in future studies to establish environmentally and economically favourable sorghum growth in marginal lands.

5.3.2 Other growth parameters

In addition to dry weight, plant leaf area and height were obtained as growth parameters. According to the plant height and leaf area results, there was no significant difference in plant height and leaf area between microbial treatments and control. Also, plant height or leaf area data did not follow any pattern with microbial treatment, similar to that of the shoot dry weight (Figure 4.26 and 4.27). Therefore, it can be concluded that there is no connection between growth parameters such as plant height and plant leaf area and microbial treatments with or without nitrogen application. However, fertilized plants' leaf area and height were significantly greater than non-fertilized plants by 29.2% and 10.2%, respectively. According to the availability of nitrogen to the plant, plant height and leaf area can vary. When a plant receives more

nutrients, they grow higher with larger and broader leaves than less receiving plants (Yin et al. 2012; Leghari et al. 2016).

5.3.2 Tissue N and P contents and percentages

Many macronutrient and micronutrient contents of tissue samples were determined (Appendix 13), but nitrogen and phosphorus contents of tissue samples were statistically analyzed (Tables 4.3 and 4.4). As explained in the 5.1.2 section, DS1, N8, and N7 have shown plant growth-promoting ability through nitrogen fixation and phosphorus solubilization in previous studies. Therefore, the plant N and P contents and tissue N and P concentrations were statistically analyzed to identify the microbial effect on sorghum plant growth.

Among microbial treatments, only the N8 treatment resulted in a significant increase in plant N content of each cultivar compared to the controls (Figure 4.22). Also, none of the microbial treatments gave significant results with tissue N concentration compared to the control of each cultivar (Figure 4.23). In shoot dry weights analysis, N8 treated sorghum plants were not significantly different compared to the control. However, as mentioned in the previous section, the mean dry weight of N8 is numerically higher than the control and other treatments. Between plant N content and tissue N concentration, only plant N content in N8-treated sorghum plants were significantly greater than the control. The increase in plant N content of N8-treated sorghum genotypes appears to be the product of slight, but not statistically significant, increases in plant dry weight and tissue N concentrations, that when combined resulted in significant increases in N content.

A pattern in shoot dry weight distribution of each genotype with no N fertilizer application were observed (5.3.1). The mean shoot dry weights of N8 treated genotypes were highest while the

mean dry weights of uninoculated controls of each genotypes were lowest in the pattern. This suggests other mechanisms were at work to increase the growth rate and biomass accumulation. Genus *Azospirillum* has been identified as a potential nitrogen-fixing bacterial genus in previous studies. However, nitrogen fixed by N8 is used for their requirements. They can increase the nitrogen availability to the plant through nitrogen assimilation (Moutia et al. 2010). However, several studies related to N8 identified that most of the growth responses were due to some factors other than N input (Pacovsky et al. 1984).

Also, the growth responses initiated by N8 inoculation can be highly variable (Pacovsky et al. (1984). Also, Pacovsky et al. (1984) tested sorghum with N8 and reported increased dry weight and N assimilation by 25%. Spaepen et al. (2008) identified that *Azosprillum brasilense* promotes plant growth mainly through IAA production. They explained that IAA can change plant root morphology to increase water and nutrient uptake. Therefore, based on the previous study results, it can be concluded that N8 likely increased the sorghum growth rate in this study by mechanisms other than an increase in N uptake. In the first greenhouse study, N8 with sorghum cultivars (CSSH 45 and CFSH 30) did not show a significant result in shoot dry weight. However, the mean dry weight of N8-treated CSSH 45 was numerically higher than the uninoculated control in the first greenhouse study. In the field study, N8 showed positive trends with sorghum genotypes (CSSH 45, 10A×118 and 10A×131). Growth responses of N8 have been variable in different conditions, but trends were positive in both studies. These results suggest that further research is warranted to determine if N8 has the potential to promote the growth of sorghum hybrids.

In plant P content and tissue P concentration analysis, none of the microbial treatments of each cultivar significantly affected the plant P content and tissue P concentration (Figure 4.24 and

Figure 4.25). As mentioned in section 5.2.3, the P solubilization ability of N7, N8 and DS1were identified in the previous studies (Rodriguz et al. 2004; Mehanz et al. 2005). However, significant changes in plant P content or tissue P concentration were not identified in N7, N8 and DS1-treated sorghum genotypes in the field study.

According to the tissue nutrient results of both greenhouse studies and the field study, some microbial treated sorghum cultivars/genotypes showed significant differences in plant N and P contents. However, none of microbial treated sorghum cultivars/genotypes showed significant differences in tissue N and P concentrations. When plant increases its growth due to some factors, available mineral nutrients dilute in the plant tissue. This is called as dilution effect (Jarrell and Beverly. 1981). Therefore, this could be a possible reason that some inoculant treated sorghum genotypes showed in significant results in shoot dry weight and plant tissue N and P contents but not in tissue N and P concentration (PAL5T treated CSSH 45 from the first greenhouse study).

The plant P content and tissue P concentration of 10A×131 was significantly higher than both CSSH 45 and 10A×118. Also, the shoot dry weight of 10A×131 was significantly greater than 10A×118 by 40%. In the second greenhouse study, 10A×131 also showed significant results with plant P content. Therefore, it can be concluded that 10A×131 has a higher growth rate and higher nutrient (especially P) absorption rate compared 10A×118.

6. Conclusion

Studies such as the current one, are important to Canada because five provinces already have established renewable fuel mandates. For example, British Colombia and Ontario provinces require to blend 5% of ethanol with gasoline when used for vehicle fuel. However, Canada still buys a large amount of ethanol from the USA (Environment and Climate Canada. 2017). Therefore, Canada needs to develop a continuous and ample ethanol supply to minimize significant annual expense (Environment and Climate Canada. 2017). This research attempted to establish sorghum cultivar in marginal lands of Nova Scotia to use as a biofuel feedstock. Since these lands are marginal and soil is not favourable for plants, microbial treatments were tested to use as biofertilizers to minimize the cost of chemical fertilizer (Especially for N).

According to greenhouse studies, *G. diazotrophicus* PAL5T demonstrated the greatest potential increase in sorghum plant growth. Even though PAL5T is a popular nitrogen-fixing bacteria (Sahai et al., 2015), there was no evidence of N_2 fixation from the ^{15}N isotope method. Therefore, it can be assumed that this bacterium increases sorghum growth (shoot dry weight) using other mechanisms such as phytohormone production (Sahai et al. 2015). However, this bacterium was not used in the field study due to restrictions of using novel microbes in Canadian soil during study time (2020). However, this bacterium has since been registered as a commercial biofertilizer in Canada by NexusBIoAg (https://nexusbioag.com/products/envita). Therefore, to follow up on this research, a field study will be conducted in summer 2021 using this bacterium. The rest of the microbes did not significantly affect sorghum shoot dry weight in the first greenhouse study. DS1 and N8-treated CSSH 45 shoot dry weights were significantly lower than the control plants in the second greenhouse study. According to the first greenhouse study results and previous studies results, DS1 and N8 have not negatively affected sorghum

growth. Even the field study results did not support the second greenhouse study. Therefore, it can be concluded that the second greenhouse study results have deviated from the standard results/trend. A possible reason for this deviation is that several plants in CSSH 45 control group grew unusually large, possibly due to the genetic reversion of these individual plants. Although these data were not identified as outliers in the statistical analysis, they did greatly increase the variance of the control group, which may have affected the ability of the statistical analysis to identify treatments effects.

In the field study, none of the microbial treatments resulted in a significant effect on shoot dry weight. However, a pattern could be identified in shoot dry weight when the nitrogen fertilizer application was absent. Microbial treatments gave numerically higher mean shoot dry weight in all genotypes. Among the treatments, N8 was the best in this pattern. Several factors such as competition between native microbes for niches, soil conditions, climate condition can be affected to the survival of novel microorganism in new environments (Nosheen et al. 2021). Therefore, microorganisms may not be able to give the best results in the field. However, Nonetheless, this pattern may be a positive sign of microbial activity on sorghum growth in these hybrids and warrants further research.

Based on the overall results, a new field study will be designed with more replicates to observe the growth potential ability of these microbes. In summer 2021, a new field study will be conducted using N8 and PAL5T microbial treatments with more replicates.

Between two new hybrids genotypes, 10A×131 had greater growth compared to 10A×118. The same genotypes will be used in the second field study as well. Performances of new genotypes can be verified furthermore using new field study results in summer 2021.

According to the overall study results, microbial treatments may have the potential (mainly N8 and PAL5T) to increase the sorghum plant growth (shoot dry weight). Since N8 effects on plant growth were not significant in the field study and the growth-promoting ability of PAL5T on these sorghum cultivars/genotypes in the field is unknown, more field studies are required to verify the results.

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Appendix 1

LGI – P Medium Recipe

Ingredient	**Required amount per one liter medium**
K_2HPO_4	0.2g
KH_2PO_4	0.6g
$MgSO_4.7H_2O$	0.2g
$CaCl_2.H_2O$	0.02g
$NaMoO_4.2H_2O$	0.002g
$FeCl_3.6H_2O$	0.01g
0.5% Bromothymol blue solution in 0.2M KOH	5ml
Biotin	0.1mg
Pyridoxal HCl	0.2mg
Sucrose	100g
$(NH_4)_2SO_4$	1.32g

Final pH of the medium should be 5.5. pH can be adjusted after autoclaved using 1% acetic acid.

Incubation temperature is 30^{o}C.

Appendix 2

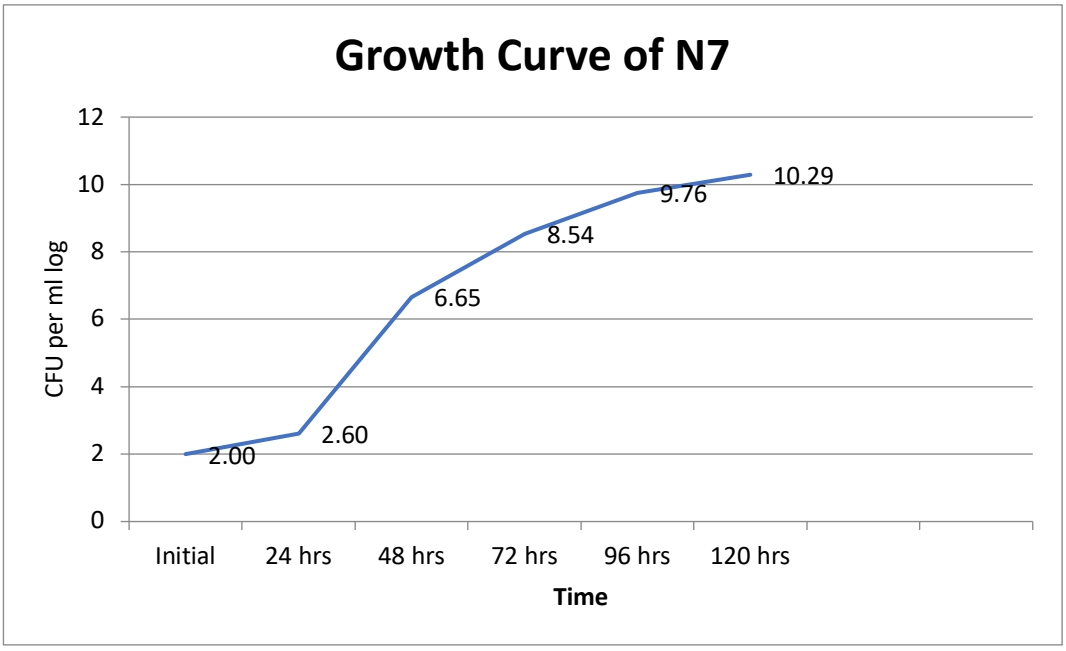
Microbial Quantitation Data

Azospirillum lipoferum (N7)

Colony Forming Unit (CFU) count data and OD values of N7 with time

Time	OD	CFU per ml	CFU per ml log
Initial	0	1×10^{2}	2.000
24 hrs	0.018	4×10^{2}	2.602
48 hrs	0.707	4.5×10^{6}	6.653
72 hrs	0.849	3.5×10^{8}	8.544
96 hrs	0.878	5.7×10^{9}	9.756
120 hrs	0.988	1.95×10^{10}	10.290

A graph of CFU per ml log vs time

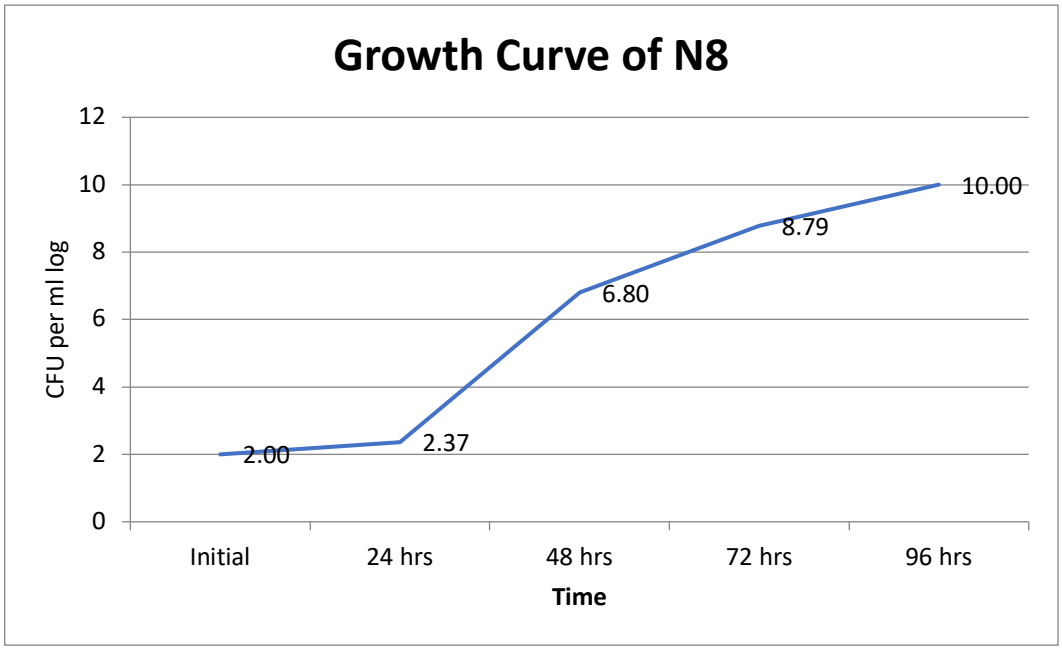


Azospirillum brasilense (N8)

Colony Forming Unit (CFU) count data and OD values of N8 with time

Time	OD	CFU per ml	CFU per ml log
Initial	0	1.00×10^{2}	2.000
24 hrs	0.015	2.33×10^{2}	2.367
48 hrs	0.607	6.03×10^{6}	6.799
72 hrs	0.759	6.10×10^{8}	8.785
96 hrs	0.811	1.01×10^{10}	10.004

A graph of CFU per ml log vs time

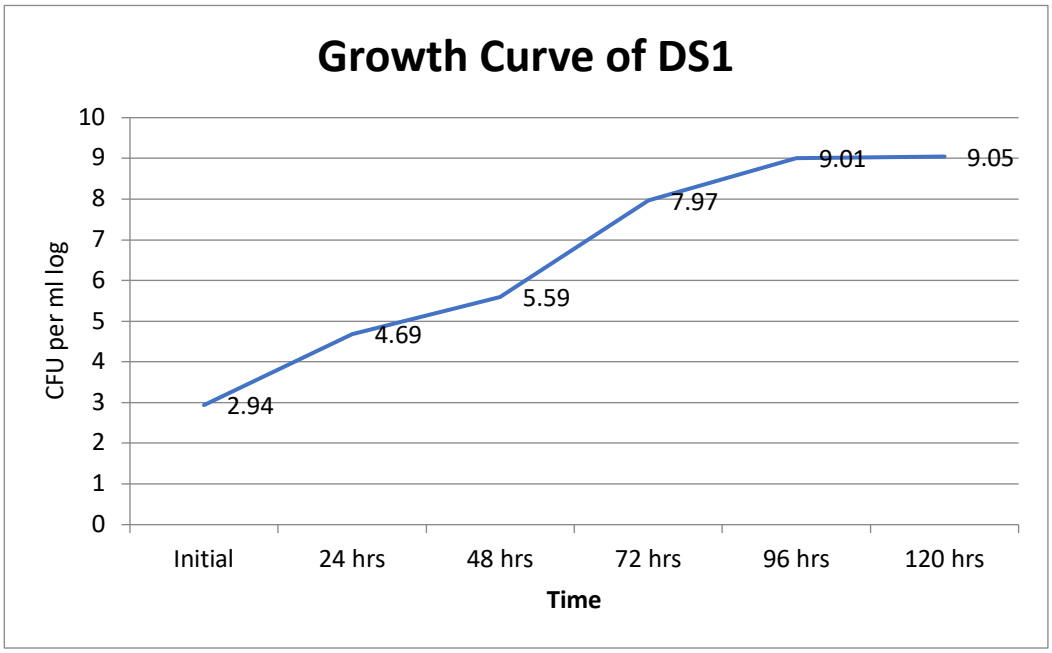


Gluconacetobacter azotocaptans (DS1)

Colony Forming Unit (CFU) count data and OD values of DS1 with time

Time	OD	CFU per ml	CFU per ml log
Initial	0	8.67×10^{2}	2.938
24 hrs	0.023	4.85×10^{4}	4.686
48 hrs	0.102	3.93×10^{5}	5.594
72 hrs	0.48	9.23×10^{7}	7.965
96 hrs	0.513	1.03×10^{9}	9.013
120 hrs	0.5	1.11×10^{9}	9.045

A graph of CFU per ml log vs time

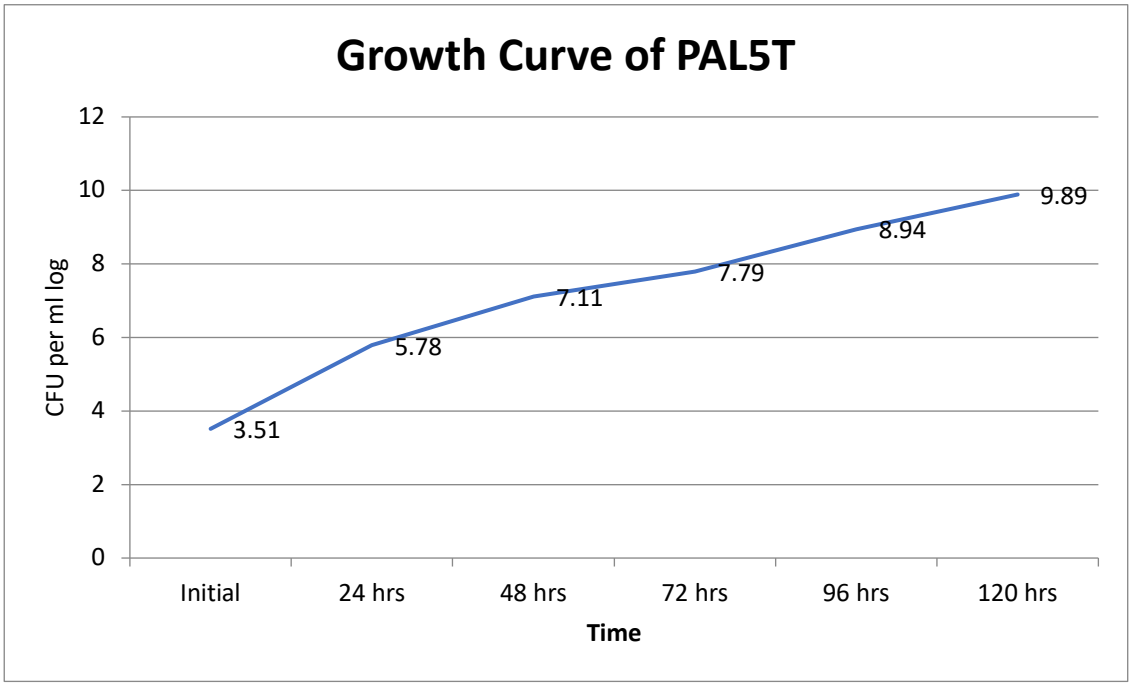


*Gluconacetobacter diazotrophicus (*PAL5T)

Colony Forming Unit (CFU) count data and OD values of PAL5T with time

Time	OD	CFU per ml	CFU per ml log
Initial	0	3.27×10^{3}	3.514
24 hrs	0.023	6.07×10^{5}	5.783
48 hrs	0.102	1.29×10^{7}	7.111
72 hrs	0.48	6.20×10^{7}	7.792
96 hrs	0.513	8.80×10^{8}	8.944
120 hrs	0.5	7.77×10^{9}	9.890

A graph of CFU per ml log vs time



Appendix 3

First greenhouse experimental design

CSSH45	CSSH30														
P. bilaii 7	N7 7	Control2 1	PAL5T 3	N8 6	N7 7	Control2 3	DS1 2	DS1 7	P. bilaii 8	Control2 3	Control1 3	P. bilaii 3	PAL5T 7	Control2 7	PAL5T 6
N7 5	Control1 9	P. bilaii 3	PAL5T 8	Control1 2	N7 9	DS1 5	DS1 3	N8 5	Control2 5	Control2 9	PAL5T 2	DS1 9	P. bilaii 4	Control2 9	P. bilaii 9
Control1 8	P. bilaii 7	PAL5T 3	P. bilaii 2	PAL5T 1	N8 3	N8 7	N7 6	DS1 9	N8 4	PAL5T 8	N7 8	N8 2	DS1 6	Control2 5	N8 1
Control1 5	Control1 6	Control2 1	N7 4	N8 6	Control1 1	P. bilaii 2	P. bilaii 1	PAL5T 6	DS1 8	DS1 7	DS1 5	P. bilaii 6	N8 4	Control1 5	P. bilaii 6
Control1 6	Control2 4	N8 8	Control1 2	N7 3	N8 9	DS1 8	N7 6	Control1 7	Control1 3	Control2 6	Control1 1	PAL5T 4	Control2 7	N7 5	P. bilaii 9
N8 2	N8 8	Control1 4	DS1 2	P. bilaii 4	N8 7	PAL5T 4	PAL5T 1	N8 3	PAL5T 9	DS1 1	PAL5T 2	N7 1	Control1 8	PAL5T 5	N7 8
N7 2	N8 9	N8 5	Control2 6	DS1 4	N8 1	DS1 3	Control2 8	Control1 7	PAL5T 5	PAL5T 7	N7 4	P. bilaii 8	N7 1	DS1 1	
Control2 8	PAL5T 9	P. bilaii 1	DS1 6	N7 3	Control2 2	Control1 9	P. bilaii 5	Control2 4	N7 9	N7 2	DS1 4	P. bilaii 5	Control1 4	Control2 2	

Appendix 4

Second greenhouse experimental design

72	116	32	26	41	7	22	59	92	93	75	17	77	78	11	34	100	115	56	104
52	53	45	88	96	99	43	33	108	10	40	119	82	25	109	24	37	94	44	85
103	9	86	91	6	5	46	65	13	8	36	114	15	120	19	90	29	12	110	118

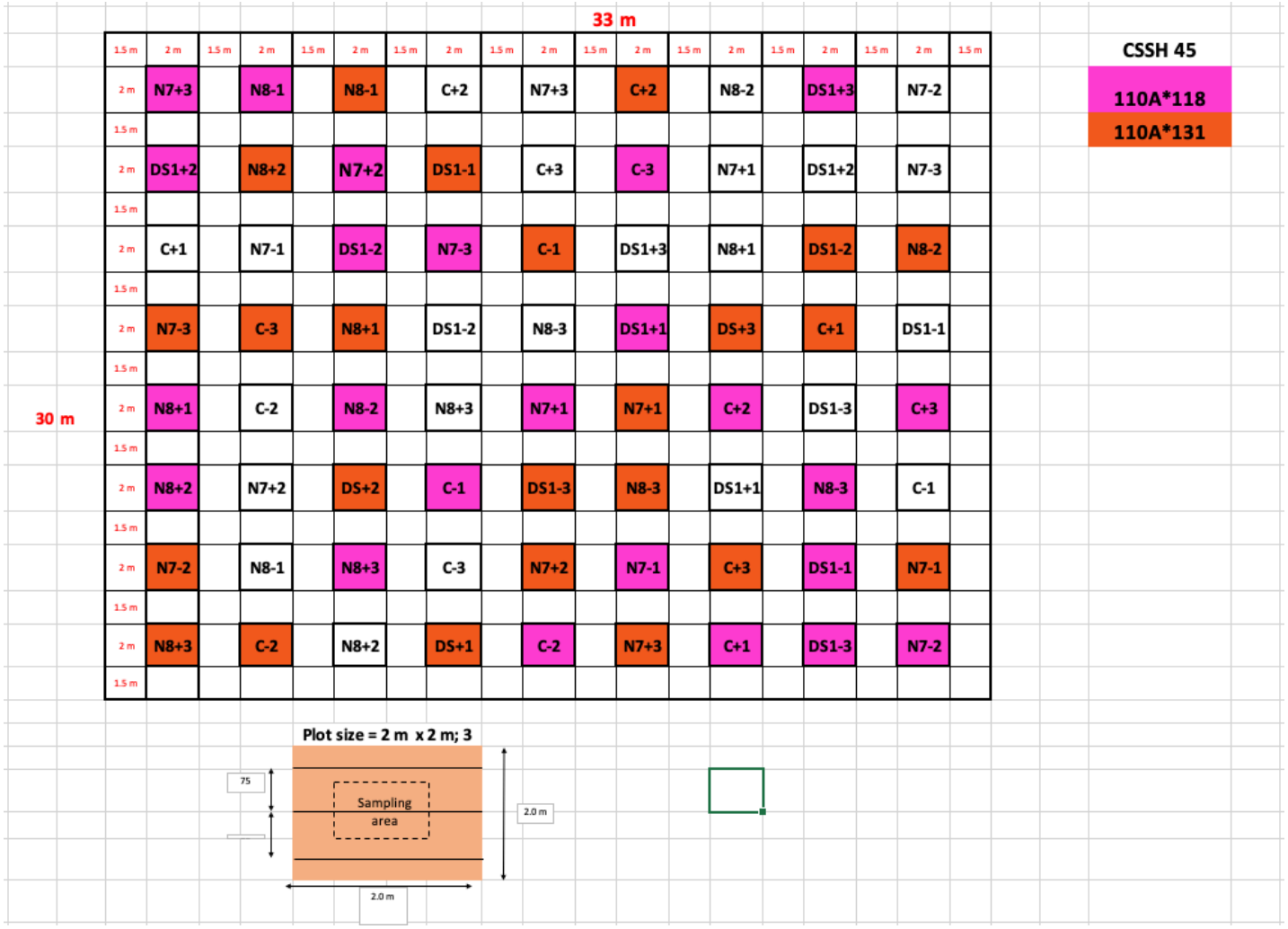
61	39	107	38	95	3	68	27	57	21	105	117	4	98	20	106	1	63	2	55
102	48	69	80	89	79	67	47	72	42	112	5	83	54	70	84	51	31	49	18
76	101	23	87	81	71	30	111	73	64	28	35	113	60	66	58	14	97	62	60

Sorghum genotype distribution	
1-40	CSSH40
41-80	10A*118
81-120	10A*131

Microbial culture distribution			
Control	DS1	N7	N8
1–10	11–20	21-30	31-40
41-50	51-60	61-70	71-80
81-90	91-100	101-110	111-120

Appendix 5

Field study experimental design



Appendix 6

Field Soil Analysis

	Soil Depth	
	0-15cm	**15-30cm**
pH	6.49	5.98
Buffer pH	7.55	7.43
Nitrogen (%)	0.33	0.22
NO_3^- N (ppm)	31.59	6.19
NO_3^- N (kg ha^{-1})	67.76	13.28
Organic Matter (%)	6.1	4.3
P_2O_5 (kg ha^{-1})	222	68
K_2O (kg ha^{-1})	164	82
Calcium (kg ha^{-1})	3700	1729
Magnesium (kg ha^{-1})	127	80
Sodium (kg ha^{-1})	22	<16
Sulfur (kg ha^{-1})	20	19
Aluminium (ppm)	1189	1361
Boron (ppm)	0.57	<0.50
Copper (ppm)	0.9	0.64
Iron (ppm)	234	258
Manganese (ppm)	76	49
Zinc (ppm)	0.89	0.62
CEC (meq $100g^{-1}$)	13.6	9.3
Base Saturation K (%)	1.3	0.9
Base Saturation Ca (%)	68	46.3
Base Saturation Mg (%)	3.9	3.6
Base Saturation Na (%)	0.4	0.3
Base Saturation H (%)	26.5	48.9

Appendix 7

Modified Knox nutrient solution

This nutrient solution mixture was used in the first and second greenhouse studies

Ingredient	Required amount
$Ca(NO_3)_2$	0.656 g/L
KNO_3	0.202 g/L
$K_2H_2PO_4$	0.250 g/L
$MgSO_4$	0.120 g/L
H_3BO_4	2.86 mg/L
$MnCl_2.4H_2O$	1.81 mg/L
$ZnCl_2.4H_2O$	0.22 mg/L
$CuSO_4.5H_2O$	0.08 mg/L
$H_2MoO_4.H_2O$	0.02 mg/L
$FeSO_4.H_2O$	6.95 mg/L

Concentration of macro elements

1. 0.656 g/L $Ca(NO_3)_2$ = 4mM
2. 0.202 g/L KNO_3 = 2mM
3. 0.25g/L $K_2H_2PO_4$ = 1.8mM
4. 0.12g/L $MgSO_4$ = 1mM

Final Concentration of macro elements – 10mM NO_3^{-1}, 1.8mM PO_4^{-3}, 4mM Ca^{+2}, 1mM Mg^{+2}, 1mM $SO4^{-2}$)

Stock solutions

S1. 2M NO_3^{-1} with 1% ^{15}N – 1ml/L

0.8M $Ca(NO_3)_2$
0.38M KNO_3

0.02M ^{15}N- KNO_3
(2Mm NO3/ml, 0.4mM K^{+}/ml, 0.8mM Ca^{+2}/ml)

S1. 2M NO_3^{-1} without 1% ^{15}N -1ml/L

0.8M $Ca(NO_3)_2$
0.4M KNO_3
(2Mm NO3/ml, 0.4mM K^{+}/ml, 0.8mM Ca^{+2}/ml)

S2. 0.9M $K_2H_2PO_4$ (0.9mM K^{+}/ml, 0.9mM PO_4^{-3}/ml) -2ml/L

S3. 0.5M $MgSO_4$ (0.5mM Mg^{+2}/ml, 0.5mM $SO4^{-2}$/ml) -2ml/L

S4. $FeSO_4$-EDTA according to Hoagland (26.1g EDTA + 24.9g $FeSO_4.7H_2O$/l) – 0.5ml/L

S5. Micronutrient mixture according to Hoagland – 1ml/L

S6. 0.5M K_2SO_4 (1mM CA^{+2}/ml) -1.6ml/L

S7. 1M $CaCl_2$ (1mM Ca^{+2}/ml) – 3.2ml/L

Appendix 8

First greenhouse data

Cultivar	Treatment	Measurement				
		Dry weight (g)	height (cm)	Longest leaf length (cm)	Longest leaf width (cm)	Number of leaves
CSSH 45	Control 1	8.86	116.1	72.4	5.4	9
CSSH 45	Control 1	8.35	111.7	65.4	5.1	10
CSSH 45	Control 1	9.56	122.8	71.1	4.5	10
CSSH 45	Control 1	9.31	123.3	71.7	5.7	10
CSSH 45	Control 1	8.79	120.2	72.8	5.1	10
CSSH 45	Control 1	8.45	121.2	73.7	5.4	10
CSSH 45	Control 1	9.18	107.3	67.6	5.5	9
CSSH 45	Control 1	8.92	124.3	69.4	5.1	9
CSSH 45	Control 1	8.82	116.3	79.6	5.3	10
CSSH 45	Control 2	9.14	121.3	70.4	5.1	10
CSSH 45	Control 2	8.55	100.4	64.1	5.2	10
CSSH 45	Control 2	9.70	117.3	67.9	5.2	10
CSSH 45	Control 2	9.33	98.4	66.8	5.4	8
CSSH 45	Control 2	9.21	126.2	87.2	5.1	10
CSSH 45	Control 2	9.05	107.7	68.3	5.6	10
CSSH 45	Control 2	9.26	114.8	71.4	5.2	9
CSSH 45	Control 2	9.17	114.1	74.5	5.5	9
CSSH 45	Control 2	6.04	81.4	65.3	5.1	11
CSSH 45	DS1	9.71	108.1	70.5	5.1	8
CSSH 45	DS1	11.06	118.7	66.1	5.0	12
CSSH 45	DS1	9.24	105.3	67.3	5.2	10
CSSH 45	DS1	10.82	120.5	75.4	5.4	10
CSSH 45	DS1	10.82	120.7	73.6	5.4	10
CSSH 45	DS1	10.10	130.4	84.3	5.3	9
CSSH 45	DS1	9.08	112.1	65.0	5.2	11
CSSH 45	DS1	10.08	116.1	74.0	5.5	9
CSSH 45	DS1	12.59	124.8	74.5	5.1	10
CSSH 45	N7	11.89	125.9	82.8	5.8	9
CSSH 45	N7	10.64	119.8	65.7	4.8	9
CSSH 45	N7	8.31	109.1	63.1	5.2	10

CSSH 45	N7	11.03	120.1	73.7	5.2	8
CSSH 45	N7	9.88	108.3	70.0	5.3	9
CSSH 45	N7	9.05	125.0	81.0	5.1	9
CSSH 45	N7	8.26	105.6	62.2	4.7	10
CSSH 45	N7	11.67	115.3	74.6	5.5	9
CSSH 45	N7	8.84	109.2	70.9	5.3	10
CSSH 45	N8	9.25	111.9	74.4	5.3	9
CSSH 45	N8	10.16	128.9	80.2	5.2	10
CSSH 45	N8	10.69	128.1	69.3	5.1	10
CSSH 45	N8	10.12	131.4	76.5	5.8	9
CSSH 45	N8	8.66	120.8	78.5	5.3	10
CSSH 45	N8	8.59	129.6	83.2	5.8	9
CSSH 45	N8	9.52	112.2	70.3	5.0	9
CSSH 45	N8	6.82	119.6	81.3	5.3	10
CSSH 45	N8	9.95	117.1	67.4	4.9	8
CSSH 45	PAL5T	11.44	116.7	70.8	5.3	8
CSSH 45	PAL5T	11.93	133.4	79.5	5.6	10
CSSH 45	PAL5T	11.52	110.1	70.2	4.6	10
CSSH 45	PAL5T	5.49	76.5	54.1	4.3	11
CSSH 45	PAL5T	10.56	116.4	76.4	5.0	10
CSSH 45	PAL5T	9.56	112.2	75.5	5.7	11
CSSH 45	PAL5T	10.54	126.4	78.1	5.1	9
CSSH 45	PAL5T	9.14	131.3	76.2	4.8	10
CSSH 45	PAL5T	10.29	135.1	71.9	5.3	10
CSSH 45	P. bilaii	9.40	125.4	73.8	4.9	9
CSSH 45	P. bilaii	9.86	121.3	71.6	4.9	10
CSSH 45	P. bilaii	10.84	128.6	74.7	5.4	9
CSSH 45	P. bilaii	10.01	98.3	71.2	6.2	7
CSSH 45	P. bilaii	10.94	108.8	73.4	5.8	8
CSSH 45	P. bilaii	9.34	119.1	78.4	6.1	10
CSSH 45	P. bilaii	7.93	127.3	80.1	5.4	10
CSSH 45	P. bilaii	12.82	117.8	77.2	6.0	9
CSSH 45	P. bilaii	8.83	107.8	73.1	6.0	10
CFSH 30	Control 1	9.61	121.2	61.6	3.8	8
CFSH 30	Control 1	7.86	116.2	60.9	4.5	8
CFSH 30	Control 1	8.75	123.4	68.3	4.5	7
CFSH 30	Control 1	8.96	139.8	83.8	4.2	8
CFSH 30	Control 1	9.11	130.6	76.4	3.9	9
CFSH 30	Control 1	7.40	127.1	65.1	4.2	7

CFSH 30	Control 1	8.93	123.4	65.1	4.2	8
CFSH 30	Control 1	7.52	119.4	58.7	4.3	8
CFSH 30	Control 1	8.40	138.1	71.3	3.9	8
CFSH 30	Control 2	9.31	113.8	64.2	4.7	9
CFSH 30	Control 2	8.94	143.3	72.5	3.8	9
CFSH 30	Control 2	12.36	153.3	74.2	4.5	8
CFSH 30	Control 2	7.63	121.2	58.4	4.2	7
CFSH 30	Control 2	8.52	115.2	65.6	3.6	9
CFSH 30	Control 2	11.05	146.6	80.2	4.1	10
CFSH 30	Control 2	9.86	165.6	68.8	3.5	8
CFSH 30	Control 2	9.60	143.9	67.9	4.1	9
CFSH 30	Control 2	7.91	118 .1	63.2	3.7	7
CFSH 30	DS1	7.30	109.3	76.7	3.7	9
CFSH 30	DS1	10.18	174.4	75.1	3.6	9
CFSH 30	DS1	10.74	162.9	67.7	3.7	10
CFSH 30	DS1	9.03	121.5	61.4	4.2	8
CFSH 30	DS1	8.61	131.1	77.2	4.0	9
CFSH 30	DS1	6.38	99.2	58.8	3.8	9
CFSH 30	DS1	7.97	126.2	64.1	4.3	8
CFSH 30	DS1	9.27	128.3	65.5	4.7	8
CFSH 30	DS1	7.70	123.0	64.8	4.5	8
CFSH 30	N7	7.78	116.8	62.5	4.8	10
CFSH 30	N7	9.25	140.6	65.1	3.5	9
CFSH 30	N7	7.02	139.8	82.9	3.7	9
CFSH 30	N7	8.14	115.3	64.1	4.1	10
CFSH 30	N7	11.30	163.8	77.7	4.3	8
CFSH 30	N7	8.54	125.3	65.5	4.2	9
CFSH 30	N7	9.15	115.8	63.9	3.8	9
CFSH 30	N7	8.46	87.1	59.4	3.5	21
CFSH 30	N7	8.90	129.9	69.3	3.6	9
CFSH 30	N8	6.75	106.2	60.7	3.1	8
CFSH 30	N8	7.68	115.4	62.5	3.7	8
CFSH 30	N8	7.71	122.2	61.2	4.5	9
CFSH 30	N8	9.16	148.7	72.8	4.0	9
CFSH 30	N8	7.94	145.8	69.7	4.0	10
CFSH 30	N8	10.14	116.4	65.9	4.9	9
CFSH 30	N8	6.64	124.9	62.4	4.2	8
CFSH 30	N8	8.43	156.1	79.3	4.4	10
CFSH 30	N8	7.45	115.3	59.4	3.8	7

CFSH 30	PAL5T	9.42	124.2	66.9	4.0	9
CFSH 30	PAL5T	6.82	110.2	58.3	4.2	9
CFSH 30	PAL5T	8.03	122.9	75.7	3.9	9
CFSH 30	PAL5T	9.32	130.4	84.3	5.3	9
CFSH 30	PAL5T	8.85	163.8	83.6	3.6	9
CFSH 30	PAL5T	10.52	133.8	68.3	3.6	8
CFSH 30	PAL5T	8.76	163.7	76.2	4.1	10
CFSH 30	PAL5T	8.59	151.3	72.2	4.0	9
CFSH 30	PAL5T	7.07	104.4	58.4	3.7	7
CFSH 30	P. bilaii	10.62	144.7	64.8	4.2	7
CFSH 30	P. bilaii	9.68	116.9	61.6	4.2	8
CFSH 30	P. bilaii	6.53	109.2	76.6	4.0	11
CFSH 30	P. bilaii	9.33	117.1	60.8	3.9	7
CFSH 30	P. bilaii	8.81	140.3	72.1	3.9	7
CFSH 30	P. bilaii	9.65	151.8	65.9	3.8	9
CFSH 30	P. bilaii	8.26	115.8	69.5	4.6	10
CFSH 30	P. bilaii	7.51	101.7	70.5	3.7	9
CFSH 30	P. bilaii	7.55	115.9	55.4	4.1	8

Appendix 9

Tissue nutrient analysis – First greenhouse study

Cultivar	Microbial Treatment	N%	Ca%	K%	Mg%	P%	Na%	B (ppm)	Cu (ppm)	Fe (ppm)	Mn (ppm)	Zn (ppm)
CSSH 45	Control	0.87	0.554	1.564	0.288	0.167	ND	ND	ND	41.91	37.91	11.36
CSSH 45	Control	0.83	0.608	1.556	0.310	0.179	ND	ND	ND	43.88	37.70	10.24
CSSH 45	Control	0.89	0.603	1.546	0.330	0.180	ND	ND	6.73	44.66	37.00	10.42
CSSH 45	DS1	0.93	0.641	1.666	0.337	0.184	ND	ND	ND	46.18	37.16	10.49
CSSH 45	DS1	0.95	0.564	1.531	0.347	0.175	ND	ND	ND	45.97	40.07	9.06
CSSH 45	DS1	0.92	0.556	1.565	0.290	0.185	ND	ND	ND	41.37	36.35	9.18
CSSH 45	N7	0.89	0.600	1.635	0.318	0.187	ND	ND	ND	41.68	43.04	10.17
CSSH 45	N7	0.83	0.562	1.715	0.303	0.182	ND	ND	ND	40.66	36.25	7.84
CSSH 45	N7	0.79	0.620	1.628	0.303	0.169	ND	ND	ND	34.52	39.05	8.81
CSSH 45	N8	0.82	0.585	1.812	0.321	0.192	0.016	10.46	ND	38.81	38.69	9.12
CSSH 45	N8	0.94	0.593	1.751	0.348	0.184	ND	11.08	ND	41.23	38.89	9.73
CSSH 45	N8	0.96	0.642	1.868	0.324	0.206	ND	11.26	ND	38.86	41.74	9.41
CSSH 45	PAL5T	0.80	0.692	1.856	0.352	0.192	0.016	10.26	ND	41.36	44.04	8.01
CSSH 45	PAL5T	0.98	0.701	1.922	0.416	0.225	ND	10.55	ND	46.31	37.33	8.20
CSSH 45	PAL5T	0.93	0.690	1.909	0.340	0.208	ND	11.22	ND	46.27	44.28	8.33
CSSH 45	P. bilaii	0.79	0.658	1.724	0.330	0.181	ND	10.07	ND	38.00	40.65	9.22

CSSH 45	P. bilaii	1.02	0.644	2.115	0.328	0.223	ND	10.18	ND	39.36	40.94	9.50
CSSH 45	P. bilaii	0.99	0.766	1.925	0.400	0.212	ND	11.40	ND	44.83	44.60	9.26
CFSH 30	Control	0.79	0.595	1.759	0.298	0.189	ND	10.28	ND	37.10	36.91	8.58
CFSH 30	Control	0.76	0.580	1.650	0.312	0.205	ND	ND	ND	38.79	39.22	8.94
CFSH 30	Control	0.72	0.537	1.877	0.314	0.198	ND	ND	ND	41.10	35.93	8.86
CFSH 30	DS1	1.14	0.486	1.776	0.264	0.224	ND	ND	ND	49.86	35.16	12.13
CFSH 30	DS1	1.03	0.549	1.600	0.304	0.177	ND	ND	ND	46.94	38.64	10.99
CFSH 30	DS1	1.07	0.493	1.693	0.271	0.219	ND	ND	ND	44.85	35.76	13.23
CFSH 30	N7	0.84	0.510	1.455	0.246	0.164	ND	ND	ND	32.40	32.79	10.07
CFSH 30	N7	0.96	0.486	1.508	0.290	0.187	0.018	ND	ND	41.31	35.58	14.30
CFSH 30	N7	1.20	0.573	1.621	0.289	0.231	ND	ND	ND	53.63	36.90	14.07
CFSH 30	N8	0.82	0.548	1.737	0.274	0.202	ND	ND	ND	37.70	35.23	14.61
CFSH 30	N8	0.99	0.568	1.820	0.256	0.216	ND	ND	ND	39.25	38.19	12.02
CFSH 30	N8	1.14	0.526	1.725	0.270	0.221	ND	ND	ND	48.99	32.76	12.30
CFSH 30	PAL5T	1.04	0.529	1.666	0.270	0.202	ND	ND	ND	41.71	38.08	12.14
CFSH 30	PAL5T	0.90	0.461	1.615	0.249	0.200	ND	ND	ND	38.67	34.38	11.14
CFSH 30	PAL5T	0.96	0.479	1.532	0.225	0.174	ND	ND	ND	38.11	34.95	10.46
CFSH 30	P. bilaii	1.09	0.539	1.850	0.246	0.213	ND	ND	ND	45.46	36.55	14.80
CFSH 30	P. bilaii	0.96	0.519	1.690	0.272	0.195	ND	ND	ND	41.19	33.68	13.78
CFSH 30	P. bilaii	1.37	0.592	1.886	0.310	0.248	0.021	ND	ND	55.66	40.55	13.81

Appendix 10

Second greenhouse data

Cultivar	Treatment	Measurement type			
		Dry Weight (g)	Height (cm)	Leaf area (cm^2)	Number of leaves
CSSH 45	Control 1	31.64	125.6	203.45	6
CSSH 45	Control 1	27.56	101.9	226.27	5
CSSH 45	Control 1	32.17	119.0	226.61	6
CSSH 45	Control 1	17.66	102.3	155.24	4
CSSH 45	Control 1	10.69	96.2	83.20	4
CSSH 45	Control 1	11.82	96.2	102.90	4
CSSH 45	Control 1	11.59	100.0	99.88	4
CSSH 45	Control 1	12.27	98.1	95.29	5
CSSH 45	Control 1	11.90	95.5	101.19	5
CSSH 45	Control 1	11.05	95.7	97.97	4
CSSH 45	DS1	10.54	97.6	102.14	4
CSSH 45	DS1	13.39	99.0	107.04	5
CSSH 45	DS1	14.17	103.0	111.78	5
CSSH 45	DS1	11.97	96.1	103.50	4
CSSH 45	DS1	12.65	107.0	95.33	6
CSSH 45	DS1	11.74	97.6	82.35	5
CSSH 45	DS1	13.91	110.1	106.65	4
CSSH 45	DS1	10.64	89.8	96.65	4
CSSH 45	DS1	10.45	120.0	89.44	4
CSSH 45	DS1	11.27	96.1	89.19	4
CSSH 45	N7	14.47	111.8	106.42	4
CSSH 45	N7	16.33	111.8	143.01	4
CSSH 45	N7	25.64	109.1	186.27	6
CSSH 45	N7	13.38	102.8	106.81	4
CSSH 45	N7	17.81	126.0	143.56	6
CSSH 45	N7	13.51	129.8	99.51	5
CSSH 45	N7	12.03	96.9	109.49	4
CSSH 45	N7	11.01	93.5	90.08	4
CSSH 45	N7	11.89	100.0	97.13	5
CSSH 45	N7	13.18	98.5	105.69	4
CSSH 45	N8	11.37	96.8	86.34	5

CSSH 45	N8	10.38	97.4	92.85	5
CSSH 45	N8	12.87	104.5	100.88	4
CSSH 45	N8	12.30	112.3	114.58	4
CSSH 45	N8	12.72	101.0	93.16	5
CSSH 45	N8	12.40	98.2	94.42	5
CSSH 45	N8	16.05	113.7	139.90	5
CSSH 45	N8	11.58	98.0	91.31	4
CSSH 45	N8	15.35	110.0	149.17	4
CSSH 45	N8	12.36	101.2	105.73	4
10A×118	Control 1	13.54	101.0	115.44	5
10A×118	Control 1	13.10	100.2	123.52	5
10A×118	Control 1	11.98	97.3	123.99	4
10A×118	Control 1	12.82	100.3	132.02	4
10A×118	Control 1	13.57	101.0	133.03	4
10A×118	Control 1	13.87	100.0	126.74	5
10A×118	Control 1	11.23	92.1	92.51	4
10A×118	Control 1	14.22	100.6	126.85	5
10A×118	Control 1	10.69	90.4	88.05	4
10A×118	Control 1	11.04	88.7	93.50	4
10A×118	DS1	14.08	102.0	115.38	5
10A×118	DS1	12.85	100.0	138.26	4
10A×118	DS1	10.37	89.3	96.96	4
10A×118	DS1	11.13	92.0	85.97	5
10A×118	DS1	9.34	79.6	77.51	4
10A×118	DS1	13.72	109.3	150.37	4
10A×118	DS1	12.49	94.3	117.57	4
10A×118	DS1	11.07	90.2	70.31	5
10A×118	DS1	11.55	99.2	100.67	5
10A×118	DS1	9.95	82.1	79.21	4
10A×118	N7	10.49	104.2	82.20	5
10A×118	N7	11.49	96.8	104.29	4
10A×118	N7	11.25	91.4	98.30	4
10A×118	N7	11.67	91.6	94.74	4
10A×118	N7	11.71	95.2	106.27	4
10A×118	N7	15.48	99.0	131.50	5
10A×118	N7	11.32	92.6	96.33	4
10A×118	N7	13.65	91.3	116.16	5
10A×118	N7	12.09	98.0	111.57	4
10A×118	N7	12.26	94.3	110.00	4

10A×118	N8	14.05	98.3	135.85	4
10A×118	N8	14.77	95.5	128.94	5
10A×118	N8	12.84	95.0	101.14	4
10A×118	N8	10.63	97.2	114.80	4
10A×118	N8	13.95	116.8	111.18	3
10A×118	N8	11.58	90.8	82.02	5
10A×118	N8	15.16	114.6	104.07	5
10A×118	N8	16.19	114.4	142.71	5
10A×118	N8	14.45	105.5	119.91	5
10A×118	N8	11.45	92.0	110.77	4
10A×131	Control 1	14.51	98.2	92.61	6
10A×131	Control 1	17.47	112.1	102.45	7
10A×131	Control 1	13.33	102.1	96.12	5
10A×131	Control 1	14.80	103.3	112.37	5
10A×131	Control 1	13.99	108.6	138.81	4
10A×131	Control 1	10.94	88.1	96.47	4
10A×131	Control 1	16.00	101.2	148.65	4
10A×131	Control 1	12.63	104.0	96.73	5
10A×131	Control 1	12.55	102.3	82.23	5
10A×131	Control 1	14.76	106.7	127.62	5
10A×131	DS1	11.44	96.4	105.40	4
10A×131	DS1	11.05	102.1	86.95	5
10A×131	DS1	15.57	117.2	126.49	6
10A×131	DS1	10.86	88.5	92.66	5
10A×131	DS1	12.87	94.7	91.05	4
10A×131	DS1	14.89	110.0	119.24	5
10A×131	DS1	11.05	94.2	89.26	4
10A×131	DS1	14.64	102.0	104.33	5
10A×131	DS1	16.94	113.2	195.55	4
10A×131	DS1	11.96	114.2	95.44	5
10A×131	N7	11.63	96.1	84.10	5
10A×131	N7	15.28	107.1	119.72	5
10A×131	N7	11.80	107.1	90.08	5
10A×131	N7	10.27	100.0	106.85	4
10A×131	N7	17.29	94.4	140.13	5
10A×131	N7	15.15	107.6	121.87	5
10A×131	N7	10.76	106.7	74.47	5
10A×131	N7	17.11	91.4	148.74	5
10A×131	N7	11.56	118.0	87.23	5

10A×131	N7	13.57	102.0	117.30	5
10A×131	N8	13.48	104.1	105.48	5
10A×131	N8	12.21	103.8	104.34	4
10A×131	N8	12.21	93.2	88.90	4
10A×131	N8	14.46	102.0	117.21	5
10A×131	N8	10.31	103.6	108.00	4
10A×131	N8	12.08	98.8	114.32	5
10A×131	N8	13.12	98.6	103.28	5
10A×131	N8	9.80	87.2	88.98	4
10A×131	N8	17.18	95.2	145.98	5
10A×131	N8	15.24	103.8	128.67	5

Appendix 11

Tissue nutrient analysis – Second greenhouse study

Cultivar	Microbial Treatment	N%	Ca%	K%	Mg%	P%	Na%	B (ppm)	Cu (ppm)	Fe (ppm)	Mn (ppm)	Zn (ppm)
CSSH 45	Control	0.45	0.350	1.052	0.254	0.117	ND	11.80	ND	21.93	132.45	18.21
CSSH 45	Control	0.53	0.411	1.906	0.215	0.288	ND	ND	ND	23.08	111.01	19.29
CSSH 45	Control	0.54	0.411	1.949	0.213	0.292	0.017	10.68	ND	25.68	117.25	20.53
CSSH 45	DS1	0.52	0.466	1.889	0.240	0.261	ND	10.39	ND	23.21	126.71	20.36
CSSH 45	DS1	0.53	0.399	1.889	0.217	0.256	ND	ND	ND	20.16	117.28	18.75
CSSH 45	DS1	0.54	0.472	1.981	0.222	0.305	ND	10.31	ND	21.18	134.12	19.20
CSSH 45	N7	0.43	0.391	10598	0.238	0.206	ND	10.52	ND	23.07	118.69	17.54
CSSH 45	N7	0.45	0.382	1.774	0.223	0.233	ND	ND	ND	20.64	110.58	17.83
CSSH 45	N7	0.42	0.430	1.861	0.231	0.291	ND	10.88	ND	21.04	128.92	20.03
CSSH 45	N8	0.48	0.418	2.017	0.234	0.288	ND	ND	ND	22.74	125.31	20.98
CSSH 45	N8	0.41	0.452	1.744	0.253	0.253	ND	10.02	ND	21.36	123.96	19.88
CSSH 45	N8	0.44	0.440	1.812	0.248	0.246	NDD	10.28	ND	21.83	121.60	19.01
10A×118	Control	0.47	0.457	1.688	0.276	0.219	ND	11.43	ND	24.13	114.35	19.36
10A×118	Control	0.45	0.408	1.929	0.239	0.239	ND	10.92	ND	25.16	113.69	18.26
10A×118	Control	0.49	0.436	1.727	0.239	0.236	ND	10.78	ND	22.55	115.18	19.01
10A×118	DS1	0.52	0.437	1.954	0.240	0.236	ND	11.07	ND	29.02	119.29	20.44

10A×118	DS1	0.44	0.375	1.786	0.228	0.220	ND	ND	ND	20.45	112.02	19.54
10A×118	DS1	0.50	0.400	2.092	0.213	0.270	ND	11.14	ND	22.43	122.92	19.89
10A×118	N7	0.50	0.394	1.885	0.216	0.251	ND	ND	ND	22.24	118.30	19.74
10A×118	N7	0.43	0.419	1.802	0.251	0.244	ND	11.81	ND	23.62	129.14	20.38
10A×118	N7	0.44	0.410	1.784	0.242	0.248	ND	10.74	ND	25.79	122.63	20.23
10A×118	N8	0.51	0.466	1.839	0.264	0.239	ND	11.11	ND	25.10	138.53	20.16
10A×118	N8	0.53	0.409	1.959	0.259	0.243	ND	11.00	ND	23.23	119.37	19.41
10A×118	N8	0.41	0.389	1.703	0.248	0.196	ND	ND	ND	20.88	121.02	17.60
10A×131	Control	0.41	0.422	1.634	0.272	0.223	ND	ND	ND	22.45	118.91	19.68
10A×131	Control	0.43	0.442	1.815	0.257	0.262	ND	11.71	ND	22.38	107.52	22.81
10A×131	Control	0.43	0.354	1.711	0.216	0.242	ND	ND	ND	22.68	102.80	19.52
10A×131	DS1	0.47	0.418	1.962	0.237	0.269	ND	ND	ND	21.99	111.56	18.15
10A×131	DS1	0.50	0.475	1.979	0.243	0.277	ND	15.25	ND	26.48	125.89	21.24
10A×131	DS1	0.46	0.450	1.802	0.269	0.250	0.015	12.41	ND	23.64	124.71	21.18
10A×131	N7	0.52	0.406	1.993	0.242	0.276	ND	10.57	ND	22.49	117.70	17.12
10A×131	N7	0.44	0.429	1.853	0.266	0.250	ND	11.14	ND	21.75	117.91	17.38
10A×131	N7	0.46	0.406	1.802	0.239	0.260	ND	ND	ND	25.44	106.96	19.08
10A×131	N8	0.45	0.305	1.712	0.180	0.244	0.024	ND	ND	20.20	103.94	17.63
10A×131	N8	0.54	0.368	2.182	0.234	0.286	0.018	ND	ND	24.68	109.15	18.54
10A×131	N8	0.42	0.390	1.658	0.257	0.242	0.018	ND	ND	21.05	102.55	16.50

Appendix 12

Field study data

Cultivar	Treatment	Nitrogen	Measurements		
			Dry weight (g)	Height (cm)	Leaf area (cm^2)
CSSH 45	Control	+	19.07	95.00	122.61
CSSH 45	Control	+	35.79	94.00	140.67
CSSH 45	Control	+	18.02	87.22	126.14
CSSH 45	Control	-	25.62	109.56	143.61
CSSH 45	Control	-	19.10	92.44	120.66
CSSH 45	Control	-	12.77	69.33	84.88
CSSH 45	DS1	+	25.89	124.67	163.95
CSSH 45	DS1	+	17.34	79.89	115.61
CSSH 45	DS1	+	18.54	84.56	132.01
CSSH 45	DS1	-	20.19	92.67	124.17
CSSH 45	DS1	-	18.48	84.89	136.72
CSSH 45	DS1	-	31.72	123.56	166.43
CSSH 45	N7	+	23.70	96.89	140.66
CSSH 45	N7	+	28.40	87.11	168.44
CSSH 45	N7	+	27.16	105.56	152.38
CSSH 45	N7	-	17.03	82.11	131.17
CSSH 45	N7	-	25.75	100.33	162.53
CSSH 45	N7	-	20.04	87.67	140.88
CSSH 45	N8	+	20.14	89.56	131.96
CSSH 45	N8	+	24.18	95.33	158.82
CSSH 45	N8	+	27.21	109.00	140.66
CSSH 45	N8	-	27.63	89.89	134.27
CSSH 45	N8	-	30.54	103.89	168.77
CSSH 45	N8	-	13.72	76.22	94.31
10A×118	Control	+	16.45	77.33	147.60
10A×118	Control 1	+	25.14	99.44	171.78
10A×118	Control 1	+	32.94	119.89	210.80
10A×118	Control 1	-	16.72	93.11	133.74
10A×118	Control 1	-	12.34	64.33	100.15
10A×118	Control 1	-	14.17	68.00	106.24
10A×118	DS1	+	20.29	72.11	146.50
10A×118	DS1	+	13.99	88.44	149.00
10A×118	DS1	+	25.06	106.11	192.64

10A×118	DS1	-	18.83	84.33	143.64
10A×118	DS1	-	13.81	73.11	98.53
10A×118	DS1	-	17.30	82.78	146.08
10A×118	N7	+	26.50	105.44	161.44
10A×118	N7	+	17.57	86.67	116.72
10A×118	N7	+	29.04	111.78	212.54
10A×118	N7	-	17.30	83.67	126.33
10A×118	N7	-	19.94	88.33	153.32
10A×118	N7	-	11.72	66.11	86.32
10A×118	N8	+	25.81	104.44	154.69
10A×118	N8	+	27.98	104.89	193.20
10A×118	N8	+	30.41	106.33	166.30
10A×118	N8	-	26.31	92.33	156.29
10A×118	N8	-	13.95	79.22	108.51
10A×118	N8	-	19.37	107.22	137.10
10A×131	Control 1	+	30.03	89.11	165.94
10A×131	Control 1	+	24.16	104.78	154.91
10A×131	Control 1	+	26.77	88.89	147.20
10A×131	Control 1	-	18.10	85.56	147.20
10A×131	Control 1	-	21.75	91.33	119.52
10A×131	Control 1	-	18.78	112.11	142.03
10A×131	DS1	+	30.00	115.33	195.06
10A×131	DS1	+	36.19	104.56	189.57
10A×131	DS1	+	19.05	95.56	135.08
10A×131	DS1	-	16.39	80.00	121.11
10A×131	DS1	-	21.86	97.78	142.65
10A×131	DS1	-	22.40	103.00	149.14
10A×131	N7	+	27.65	111.78	150.60
10A×131	N7	+	46.45	114.78	172.97
10A×131	N7	+	27.83	90.78	164.85
10A×131	N7	-	24.72	97.44	149.42
10A×131	N7	-	21.41	84.89	125.45
10A×131	N7	-	18.83	91.44	134.09
10A×131	N8	+	24.59	93.67	158.93
10A×131	N8	+	28.33	112.67	171.81
10A×131	N8	+	35.71	102.78	169.05
10A×131	N8	-	32.14	108.44	168.90
10A×131	N8	-	21.17	92.22	136.03
10A×131	N8	-	22.72	87.22	145.20

Appendix 13

Tissue nutrient analysis – Field study

Cultivar	Microbial Treatment	Nitrogen	N%	Ca%	K%	Mg%	P%	Na%	B (ppm)	Cu (ppm)	Fe (ppm)	Mn (ppm)	Zn (ppm)
CSSH 45	Control	+	1.63	0.664	0.823	0.468	0.213	ND	ND	7.11	87.87	143.91	37.86
CSSH 45	Control	+	1.49	0.741	0.749	0.492	0.238	ND	ND	7.10	88.49	118.55	36.91
CSSH 45	Control	+	1.38	0.665	0.703	0.431	0.212	ND	ND	7.10	69.07	115.92	33.86
CSSH 45	Control	-	1.90	0.809	0.489	0.474	0.218	0.016	ND	7.19	77.06	169.00	44.64
CSSH 45	Control	-	1.87	0.830	0.526	0.496	0.216	0.017	ND	8.30	81.75	159.09	44.62
CSSH 45	Control	-	1.78	0.715	0.429	0.574	0.210	0.015	ND	7.76	81.09	150.55	45.35
CSSH 45	DS1	+	1.82	0.803	0.470	0.572	0.225	ND	ND	7.38	84.77	124.49	43.15
CSSH 45	DS1	+	2.04	0.746	0.365	0.589	0.193	ND	ND	8.06	97.23	144.31	46.39
CSSH 45	DS1	+	1.85	0.670	0.576	0.483	0.217	ND	ND	6.74	84.34	142.20	36.43
CSSH 45	DS1	-	1.90	0.788	0.457	0.642	0.229	ND	ND	8.01	80.70	104.53	44.70
CSSH 45	DS1	-	1.43	0.548	0.606	0.385	0.189	ND	ND	6.29	65.04	110.77	29.97
CSSH 45	DS1	-	1.86	0.793	0.533	0.534	0.231	ND	ND	7.20	98.79	146.63	34.55
CSSH 45	N7	+	1.67	0.787	0.636	0.519	0.262	ND	ND	7.22	89.38	106.72	31.76
CSSH 45	N7	+	1.98	0.855	0.612	0.494	0.219	ND	ND	7.47	88.48	129.79	36.46
CSSH 45	N7	+	2.25	0.930	0.764	0.591	0.266	ND	ND	9.45	126.15	152.04	47.98
CSSH 45	N7	-	2.16	0.868	0.566	0.501	0.249	ND	ND	8.90	96.62	154.18	43.66

CSSH 45	N7	-	1.83	0.836	0.481	0.506	0.244	ND	ND	7.71	80.34	139.25	40.91
CSSH 45	N7	-	1.73	0.881	0.444	0.552	0.265	ND	ND	7.62	90.12	140.11	37.38
CSSH 45	N8	+	1.64	0.708	0.585	0.432	0.217	ND	ND	6.52	75.12	135.06	37.23
CSSH 45	N8	+	1.49	0.778	0.567	0.439	0.247	0.015	ND	6.38	76.93	116.55	31.29
CSSH 45	N8	+	1.70	0.778	0.610	0.477	0.234	ND	ND	6.78	106.49	120.42	36.46
CSSH 45	N8	-	1.75	0.842	0.627	0.497	0.240	ND	ND	8.31	115.04	126.54	38.51
CSSH 45	N8	-	1.57	0.749	0.733	0.436	0.256	ND	ND	6.69	88.32	105.78	32.50
CSSH 45	N8	-	2.31	0.872	0.639	0.458	0.277	ND	ND	7.54	90.58	159.02	43.85
10A×118	Control	+	2.00	0.880	0.490	0.475	0.263	ND	ND	8.21	86.55	148.61	37.77
10A×118	Control	+	1.70	0.853	0.649	0.489	0.273	ND	ND	6.98	81.96	121.18	33.27
10A×118	Control	+	1.53	0.814	0.561	0.480	0.284	ND	ND	6.04	72.92	122.66	33.28
10A×118	Control	-	1.45	0.660	0.921	0.379	0.244	ND	ND	5.46	68.99	90.54	30.28
10A×118	Control	-	1.48	0.682	0.786	0.432	0.261	ND	ND	5.85	73.30	100.57	30.72
10A×118	Control	-	2.00	0.667	0.686	0.404	0.229	ND	ND	6.91	86.65	128.93	36.72
10A×118	DS1	+	1.75	0.816	0.830	0.401	0.234	ND	ND	6.48	81.87	106.74	33.75
10A×118	DS1	+	1.95	0.863	0.772	0.412	0.239	ND	ND	7.62	94.71	118.64	40.66
10A×118	DS1	+	2.24	0.861	0.624	0.464	0.247	0.018	ND	8.30	111.76	174.93	49.79
10A×118	DS1	-	1.89	0.792	0.617	0.403	0.242	0.016	ND	7.83	71.42	132.83	39.82
10A×118	DS1	-	1.77	0.691	0.521	0.386	0.219	0.018	ND	7.64	69.20	132.19	37.38
10A×118	DS1	-	1.68	0.872	0.591	0.411	0.255	ND	ND	6.55	70.62	115.95	32.19
10A×118	N7	+	1.91	0.798	0.561	0.492	0.267	ND	ND	6.52	83.64	146.30	36.80
10A×118	N7	+	1.50	0.728	0.681	0.410	0.269	0.016	ND	5.73	71.56	97.48	28.54

10A×118	N7	+	1.83	0.848	0.662	0.495	0.277	0.016	ND	7.28	92.77	107.98	34.92
10A×118	N7	-	1.70	0.730	0.407	0.405	0.222	ND	ND	6.35	67.39	126.12	34.41
10A×118	N7	-	1.98	0.833	0.464	0.469	0.245	ND	ND	8.08	87.34	155.33	40.98
10A×118	N7	-	1.63	0.682	0.433	0.407	0.222	ND	ND	6.37	66.70	122.07	32.64
10A×118	N8	+	2.10	0.879	0.437	0.503	0.255	ND	ND	7.33	94.71	163.75	45.39
10A×118	N8	+	1.69	0.851	0.563	0.404	0.228	ND	ND	6.06	71.43	129.66	32.94
10A×118	N8	+	1.76	0.805	0.410	0.474	0.225	ND	ND	6.62	83.52	163.78	39.52
10A×118	N8	-	1.85	0.796	0.386	0.544	0.240	0.015	ND	6.68	79.42	143.36	31.63
10A×118	N8	-	1.68	0.809	0.382	0.469	0.257	0.015	ND	6.11	71.58	161.68	36.32
10A×118	N8	-	1.47	0.664	0.369	0.446	0.229	ND	ND	6.24	63.59	126.43	34.91
10A×131	Control	+	1.65	0.899	0.472	0.605	0.328	ND	ND	6.84	98.72	116.04	38.10
10A×131	Control	+	1.27	0.722	0.623	0.433	0.267	ND	ND	5.70	70.14	111.28	30.21
10A×131	Control	+	1.52	0.787	0.451	0.536	0.271	ND	ND	6.33	80.45	119.91	33.06
10A×131	Control	-	1.77	0.837	0.352	0.538	0.296	ND	ND	6.80	79.15	163.29	38.79
10A×131	Control	-	1.80	0.906	0.466	0.613	0.307	ND	ND	6.67	105.91	142.59	40.43
10A×131	Control	-	1.36	0.785	0.371	0.501	0.270	ND	ND	5.54	65.51	115.00	31.27
10A×131	DS1	+	1.42	0.695	0.903	0.467	0.332	0.016	ND	5.18	87.11	98.82	31.70
10A×131	DS1	+	1.36	0.740	0.865	0.391	0.295	0.017	ND	ND	98.22	93.77	29.19
10A×131	DS1	+	1.75	0.746	0.692	0.497	0.269	ND	ND	6.87	96.37	159.79	36.73
10A×131	DS1	-	1.55	0.882	0.903	0.457	0.325	ND	ND	5.85	84.75	122.96	38.19
10A×131	DS1	-	1.46	0.625	0.594	0.404	0.260	ND	ND	6.11	104.31	168.36	36.29

10A×131	DS1	-	1.62	0.860	0.648	0.544	0.295	0.023	ND	6.60	113.91	144.56	37.54
10A×131	N7	+	2.05	0.753	0.565	0.479	0.275	0.022	ND	6.65	87.69	188.70	42.19
10A×131	N7	+	1.70	0.872	0.622	0.526	0.285	0.019	ND	6.20	114.65	145.62	37.62
10A×131	N7	+	1.37	0.742	0.735	0.466	0.255	ND	ND	5.37	83.05	137.54	35.03
10A×131	N7	-	1.69	0.675	0.630	0.460	0.244	0.017	ND	6.37	81.04	144.56	40.23
10A×131	N7	-	1.34	0.681	0.791	0.405	0.241	0.020	ND	5.36	79.50	99.53	29.40
10A×131	N7	-	2.11	0.710	0.875	0.377	0.256	0.016	ND	6.47	86.47	157.43	43.34
10A×131	N8	+	1.55	0.639	0.691	0.376	0.212	ND	ND	6.32	77.15	162.68	40.41
10A×131	N8	+	1.98	0.889	0.910	0.458	0.257	ND	ND	7.18	121.79	145.62	41.98
10A×131	N8	+	1.88	0.734	0.602	0.396	0.231	ND	ND	5.53	77.70	202.61	43.93
10A×131	N8	-	2.57	0.891	0.699	0.470	0.285	ND	ND	7.31	120.48	238.64	51.93
10A×131	N8	-	2.07	0.975	0.713	0.484	0.269	ND	ND	6.92	131.41	164.70	44.22
10A×131	N8	-	1.77	0.956	0.709	0.480	0.262	ND	ND	6.44	107.65	164.16	46.36

Appendix 14

R codes used for statistical analysis

Two-way ANOVA

This R codes were used to analyze all parameters in the first greenhouse study and some of parameters (data sets met the normality and homogeneity assumptions) in the second greenhouse study.

```
install.packages("tidyverse")
install.packages("ggpubr")
install.packages("rstatix")
install.packages("datarium")
install.packages("readr")
library(tidyverse)
library(ggpubr)
library(rstatix)
library(readr)
```

#Summary of the Data set

```
summary<- Data Set %>% group_by(Treatment, Cultivar) %>%get_summary_stats(Dry.weight, type= "mean_sd")
summary
write_tsv(summary, path = "summary.txt")
install.packages("dplyr")
```

#Data Vizualization

```
library(dplyr)

boxplot<- ggboxplot(Data Set, x="Cultivar", y="Dry.weight", color="Treatment", palette =

"jco", ylab = expression(Biomass  ~ Dry ~ Weight ~ (g ~ plant^{-1})))

boxplot

boxplot
```

#Check for the outliars

```
outliars<-Data Set %>% group_by(Treatment, Culitivar) %>% identify_outliers(Dry.weight)

outliars

write_tsv(outliars, path = "outliars.txt")
```

#Normality Assumption

```
model<- lm(Dry.weight~Treatment*Culitivar, data= Data Set)

ggqqplot(residuals(model))

shapiro.test(residuals(model))
```

#Homogeneity assumption

```
Data Set%>% levene_test(Dry.weight~Treatment*Culitivar)
```

#Two-way ANOVA

```
res.aov<- Data Set %>% anova_test(Dry.weight~Treatment*Culitivar)

res.aov
```

Three-way ANOVA

This R codes were used to analyze all parameters in the in the field study.

```
install.packages("tidyverse")

install.packages("ggpubr")

install.packages("rstatix")

install.packages("datarium")

install.packages("dplyr")

library(tidyverse)

library(ggpubr)

library(rstatix)

library(dplyr)
```

#Summary of the Data set

```
summary<-Data Set %>% group_by(Cultivar, Treatment, Nitrogen)

%>%get_summary_stats(Dry.weight, type= "mean_sd")

summary
```

#Data Vizualization

```
boxplot<- ggboxplot(Data Set, x="Cultivar", y="Dry.weight", color="Treatment", palette =

"jco", facet.by = "Nitrogen", ylab = expression(Biomass  ~ Dry ~ Weight ~ (g ~ plant^{-1})))

boxplot

boxplot
```

#Check for the outliars

```
Data Set %>% group_by(Cultivar,Treatment,Nitrogen) %>% identify_outliers(Dry.weight)
```

#Normality Assumption

```
model<- lm(Dry.weight~Cultivar*Treatment*Nitrogen, data=Data Set)

ggqqplot(residuals(model))

shapiro.test(residuals(model))
```

#Homogeneity assumption

```
Data Set %>% levene_test(Dry.weight~Cultivar*Treatment*Nitrogen)
```

#Three-way ANOVA

```
res.aov<- Data Set %>% anova_test(Dry.weight~Culitivar*Treatment*Nitrogen)

res.aov
```

General Linear Model (GLM)

This model was used to analyze some parameters (Data sets did not meet the homogeneity and normality assumptions) in the second greenhouse study.

```
install.packages("agricolae")
install.packages("lme4")
install.packages("nlme")
install.packages("boot")
install.packages("ggplot2")
install.packages("car")
install.packages("dplyr")
install.packages("ggpubr")
install.packages("FSA")
install.packages("rcompanion")
install.packages("MESS")
install.packages("lattice")
install.packages("lsmeans")
install.packages("multcomp")
install.packages("jtools")
install.packages("emmeans")
install.packages("Matrix")
install.packages("tidyverse")
install.packages("ggpubr")
install.packages("rstatix")
```

```
install.packages("datarium")

install.packages("readr")


## Load appropriate libraries

library(agricolae)

library(lme4)

library(nlme)

library(boot)

library(ggplot2)

library(car)

library(dplyr)

library(ggpubr)

library(FSA)

library(rcompanion)

library(MESS)

library(lattice)

library(FSA)

library(lsmeans)

library(multcomp)

library(jtools)

library(emmeans)

library(tidyverse)

library(rstatix)
```

```
library(readr)

## Set working directory

## Attach observed data (Microsoft Excel .csv file)

GH_2 <- read.table("2021-03-03_GH2_2.csv", header = TRUE, sep = ",")

View (GH_2)

Cultivar <- GH_2$Cultivar

Treatment <- GH_2$Treatment

DW <- GH_2$Dry.weight

## Generate a one-way analysis of variance to generate residuals of observed data

DW_aov <- aov (DW ~ Treatment * Cultivar)

Summary (DW_aov)

## Generate residual and predicted values based on observed values ##

DW$resids <- residuals(DW_aov)

DW$preds <- predict(DW_aov)

DW$sq_preds <- DW$preds^2

## Plot the distribution of the observed data to make a visual interpretation of normality

fig.1 <- hist (GH_2$Dry.weight,

        breaks = 15,

        col = "gray",

        main = "",

        xlab = expression (Biomass  ~ Dry ~ Weight ~ (g ~ plant^{-1})),

        border = F)
```

```
## Plot a normal distribution overlay on the histogram
Lines (seq(0, 80, 1), length(GH_2$Dry.weight) * dnorm(seq(0, 80, 1), mean(GH_2$Dry.weight),
                                    sqrt(var(GH_2$Dry.weight))), col = "blue")
## Plot a Gamma distribution overlay on the histogram
rate <- mean (GH_2$Dry.weight)/var(GH_2$Dry.weight)
shape <- rate * mean(GH_2$Dry.weight)
lines(seq(0, 80, 1), length(GH_2$Dry.weight) * dgamma (seq(0,80,1), shape, rate),
     col = "red")
legend.col <- c("blue", "red")
legend.labels <- c("Normal", "Gamma")
legend("topright",
       title = "",
       legend.labels,
       lwd = 2,
       col = legend.col,
       bty = "n")
## Perform a Shapiro-Wilk test for normality of the residuals
shapiro.test(DW$resids)
## Perform Levene's test for homogeneity of variances
leveneTest(Dry.weight ~ Treatment * Cultivar, data = GH_2)
```

Generate a generalized linear model to assess where the distribution of the observed response (DW) doesn't conform to normality assumptions

```
Model 1 <- (glm(Dry.weight ~ Treatment * Cultivar, family = Gamma(link = "log"),
        data = GH_2)) ## Gamma distributed response variable
```

Complete analysis of deviance for each of the GLMs

```
anova(model 1, test ="F")
```

Complete pairwise comparisons using Tukey contrasts to differentiate between treatment means.

```
ghlt.mod1<- glht(model 1, mcp(Treatment = "Tukey"))
summary(ghlt.mod1)
```

#If interactions present, complete pairwise comparisons using Tukey contrasts to differentiate between treatment means

```
GH_2$tcul<- interaction (GH_2$Treatment, GH_2$Cultivar)
glm.posthoc<- glm(Dry.weight~-1+tcul, family = Gamma(link = "log"), data=GH_2)
model2<- glht(glm.posthoc,mcp(tcul= "Tukey"))
summary(model2)
```