Evaluation of Synergy between PGPR and Seaweed Extracts for growth promotion and Biocontrol of *Rhizoctonia solani* on soybean

by

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Abstract

The necessity to increase crop yield is a constant pressure that farmers are faced with, and chemical fertilizers and pesticides are the most effective inputs for yield. There are growing concerns about the amount of synthetic chemicals applied to agricultural systems, especially regarding fertilizer runoff and unintended consequences of pesticides. Biostimulants are now experiencing an influx of research especially plant growth-promoting rhizobacteria (PGPR). PGPR have exhibited promising ability to promote plant growth via nutrient uptake, nutrient availability, and root growth stimulation via phytohormones. PGPR have also been reported as biocontrol microorganisms that secrete secondary metabolites and antibiotics to inhibit plant pathogen growth and virulence. Seaweed extracts are another category of biostimulants that are gaining traction as agricultural applications due to the newly developed extraction processes that help select for certain growth promotion and biocontrol traits. There are overlapping growth promotion and biocontrol mechanisms between these two biostimulants, which may offer an opportunity for synergy if applied together. The *in vitro* experiments demonstrated the ability of one seaweed extract formulation to act as a conducive environment for PGPR to grow. Through the *in planta* experiments we noticed a trend of biocontrol for *Rhizoctonia solani* across several treatments, but there were no significant differences. The growth promotion experiment showed significant growth differences compared to the control across several root growth parameters. The growth promotion assay revealed that the growth promotional effects of seaweed extracts are not dependent on the microorganisms, and instead most likely relies on the metabolites within the seaweed extracts.

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List of abbreviations

PGPR	Plant-Growth Promoting Rhizobacteria
SE	Seaweed Extract
TSA	Triptic Soy Agar
PDA	Potato Dextrose Agar
TSS	Spizizen’s media

Chapter 1 Literature Review

Introduction:

Farmers seek to maximize their crop yield each year, which means trying to enhance all growing conditions including nutrient availability, protection from pathogens, and protection from abiotic stress. Most farmers achieve this through multiple applications of fertilizers and pesticides; however, the runoff from excess fertilizer is affecting water sources downstream of the farms (Zahoor et al. 2014). Farmers may follow a specific plan of fertilizers and pesticides each year for the best economic outcome, but the amount of fertilizer put into the field is rarely all able to be used by the plant (Wallace et al. 2017). One of the consequences of continually adding fertilizers is runoff of nitrogen and phosphorous that is observed downstream in water systems, which often causes algal blooms that can kill many different forms of marine life (Baker and Richards 2002). There are also other unintended consequences of some pesticides such as neonicotinoids suggested to cause colony decline in bees (Walters and Didham 2016). The amount of organically farmed land increased over 100% worldwide from 2001 to 2011, showing a remarkable trend away from traditional farming (Willer et al. 2011). This positive trend may present the ideal time for using biostimulants to positively impact agriculture and our environment (Biostimulant 2013).

Plant growth-promoting bacteria (PGPR) are beneficial bacteria that live in the rhizosphere and have potential for promoting plant health as well as the ability to protect the host plant from pathogens (Souza et al. 2015). PGPR are able to exhibit biocontrol properties through several mechanisms that may have overlapping outcomes for the plant. These bacteria are able to compete for resources within the rhizosphere and secrete secondary metabolites including, antibiotics, siderophores, and volatile organic compounds (Craigie 2011, Khan et al. 2009). PGPR can help alleviate the stress of pollution caused by agricultural inputs, by increasing nutrient availability and nutrient uptake (Calvo et al. 2014). Adesemoye et al. (2010) reported the ability of PGPR to increase nitrogen uptake under reduced fertilization levels, less than the recommended amount.

Seaweed extracts are another biostimulant with the potential to alter the amount of fertilizers and pesticides that are used in traditional crop production systems. Seaweeds have been used in coastal agricultural systems for hundreds of years, but their true potential is just now being discovered (Craigie et al 2011). There are many beneficial seaweed species, but one of the most studied is a brown seaweed, *Ascophyllum nodosum*, that grows abundantly along cold coastal regions (Khan et al. 2009). Seaweed extracts using the same source of seaweed may affect the plant differently depending on the type of filtration and extraction methods. Esserti et al. (2016) reported that methanol extractions of seaweeds retained higher amounts of bioactive compounds. Seaweed extracts are able to upregulate defense systems and nutrient uptake because of their diverse microbial communities, complex polysaccharides, siderophores, and phytohormones (Khan et al. 2009 and Craigie 2011).

Agricultural Biostimulants:

Plant biostimulants are an emerging area of crop applications that specifically manage plant health and ameliorate abiotic stresses for the crop (Calvo et al. 2014). A common trend in agriculture is to reduce fertilizer and pesticide inputs without reducing yield (Craigie 2011). Biostimulants have been well-researched, and specific biostimulants have been shown to be effective supplements for enhancing growth and health of diverse crops (Biostimulant 2013).

There are two major organizations that represent the biostimulant industries: the European Biostimulant Industry Council (EBIC) and the North America Biostimulant Coalition. These have slightly differing definitions of biostimulants for different reasons (Calvo et al. 2014). The EBIC definition focuses on the ability of biostimulants to "stimulate natural processes to enhance/benefit nutrient uptake, nutrient efficiency, tolerance to abiotic stress, and crop quality. Biostimulants have no direct action against pests, and therefore do not fall within the regulatory framework of pesticides." (European Biostimulants Industry Council 2012). This regulatory definition is in place to emphasize that biostimulants do not act as pesticides by directly affecting pathogens. The North American definition defines biostimulants as "substances, including microorganisms, that are applied to plant, seed, soil or other growing media that may enhance the plant's ability to assimilate applied nutrients, or provide benefits to plant development. Biostimulants are not plant nutrients and therefore may not make any nutrient claims or guarantees" (Biostimulant 2013). The focus of the North American definition is to make sure there is no confusion between a biostimulant and any type of fertilizer. Hence, the European and North American councils both differentiate biostimulants from traditional applications due to their mode of action (Calvo et al. 2014).

The use of plant biostimulants is a major target of agricultural researchers that are interested in increasing vegetable yields without adding copious amounts of fertilizer (Rouphael et al. 2018). Foliar sprays for vegetables such as tomatoes are among the most commonly studied for biostimulants to help boost yield while contributing to the amelioration of abiotic stressors.

Microbial inoculants:

Microbial inoculants can be broken down into two categories: biocontrol agents and biofertilizers. Microbial inoculants are any bacteria or fungi that have been isolated from a natural environment and have exhibited the ability to benefit the plant (Berg 2009). Arbuscular mycorrhizal fungi have shown positive results by increasing root growth, fruit quality, and yield (Bona et al. 2018). These fungi increase the plant's absorption of key elements such as nitrogen and phosphorous, as well as modulate the sugar and vitamin concentration in tomatoes and strawberries (Berta et al. 2014; Bona et al. 2015). The most promising and well-studied microbial inoculants are plant growth-promoting rhizobacteria (PGPR), which form an intimate relationship with the host plant. The focus of microbial inoculants is to increase overall plant health; however, the mode of action and ability of the microbial inoculant to do so is not universal (Calvo et al. 2014). Some of the mechanisms that are used by microbial inoculants to increase plant health are: secretion of secondary metabolites, competition for resources, and secretion of plant hormones and regulators (Boukerma et al. 2017). Microbial inoculants may increase the amount of biologically available phosphorous in the rhizosphere by secreting phosphatases that help break down phosphorous in the soil, allowing it to be taken up by the plant (Richardson et al. 2009). There is a large range of secondary metabolites that are released by different bacterial and fungal species, and the function of these can vary greatly. Siderophores, volatile organic compounds (VOCs) and phytohormones all contribute to

growth promotion in different pathways, but may be differentially regulated based on the specific microbial inoculant that is expressing that mechanism (Sharma et al. 2013). Bacterial strains will secrete these hormones and secondary metabolites at different rates, which can be quantified through lab tests (Zhuang et al. 2007). Some studies have shown that the bacteria isolated from agricultural crops may have a predisposition to perform better on the crops from which they were isolated (Vaikuntapu 2014). The outcome of this demonstrates that some of these PGPR have host specificity and therefore, some PGPR may perform better with different host plants (Vaikuntapu 2014).

Humic Substances—Humic and Fulvic Acids:

The term humic substances has historically included two categories: humic acids and fulvic acids (Calvo et al. 2014). Humic and fulvic acids were first differentiated by molecular weight and the origin of the organic material that it is derived from in the soil (Berbara and Garcia 2014). Humic acids are high molecular weight compounds that are naturally available in most soils. Fulvic acids are commonly grouped with humic acids to describe the overall effects of "humic substances" on plants (Canellas et al. 2015).

Humic acids have been linked to many different plant health characteristics through completely different modes of action (Calvo et al. 2014). Humic substances can be considered the heterogeneous mixtures and aggregations of humins that do not fit into the category of humic acids. Some humic substances are reported to be major components of soil health and gas exchange within the rhizosphere. A diverse and stable microbial community contributes to the health of the rhizosphere by decomposition of organic matter and other compatible nutrients (Dell et al. 2012). Maintaining soil health is also important for cultivating a diverse and healthy

rhizosphere microbial community (Varanini and Pinton 2001). Some humic acids were shown to increase nutrient uptake, yield, and fruit quality in a mandarin orchard (Hayani et al. 2016). The ability of humic acids to increase soil health and promote the growth of plants has been validated in more than a dozen plants, including agriculturally important crops (Calvo et al. 2014). Chen demonstrated that humic acids (HA) increased yield and early uptake of nitrogen with sweet potato, and reported that HA can be combined with nitrogen to form a slow release nitrogen source for plants (Chen et al. 2017).

Fulvic acids can carry out similar activities as humic acids, but can easily be distinguished by their ability to persist in acidic and alkaline solutions (Canellas et al. 2015). Fulvic acids can exhibit similar root growth increases to humic acids; however, the fulvic acids seem less active in their biological processes when compared to humic acids (Lulakis and Petsas 1995). Santos et al. (2014) demonstrated the usefulness of fulvic acids in phytoremediation of lead, Pb, in agricultural soil.

Protein hydrolysates:

Protein hydrolysates are derived from plant or animal tissues and are combined into a mixture of amino acids and peptides (Calvo et al. 2014). While these amino acids contain some nitrogen, it has been established that they enhance plant quality in a separate mechanism from nitrogen fertilizer supplementation. The acquisition of nitrogen by roots is increased, indicating that protein hydrolysates increase nitrogen assimilation and nitrogen use efficiency (Jardin 2015). The exact amino acids implicated in causing this enhanced nutrient uptake have not been identified, but there is strong evidence that suggests protein hydrolysates alter forms of nitrogen within the plant so it is more readily available to the plant. Colla et al. (2017) showed that protein hydrolysates can increase the overall health and yield of tomatoes compared to the non-

treated control. Rouphael and collaborators (2017) reported that biostimulants may be crop specific in their actions. They reported that a legume-derived protein hydrolysate differentially benefits plants and may require independent research to confirm positive effects for individual crops, instead of being able to assume that the protein hydrolysate will have the same effects across many crops.

Seaweed extracts:

Seaweed extracts are aqueous or dry formulations of macroalgae that have undergone one of many different extraction processes. Macroalgae are seaweeds of any color, growing in the ocean either attached to some structure or free floating. Brown, red, and green seaweeds have been studied for their effects on plants, but the largest amount of research has been done on brown seaweeds. This may be due to the overall abundance and ease of harvesting brown seaweeds, rather than an increase in ability to help plants (Sangha et al. 2014). The holdfast of *Ascophyllum nodosum* has the ability to survive for around one hundred years and the harvestable fronds can grow for twenty years (Ugarte et al. 2010). Most commercial manufacturers of seaweed extracts need a bountiful area to keep up with demand, and brown seaweeds can fill that niche if harvested sustainably with minimal damage to the holdfasts (Craigie 2011). Seaweed extracts are an increasingly popular biostimulant for agriculture, because of their ability to increase nutrient uptake, chelate soil, stimulate root growth, and improve yield (Calvo et al. 2014). The causes of these beneficial effects have not yet been pinpointed; however, there are growing theories on specific modes of action for these plant health traits (Shekhar 2012). Some of the suspected beneficial components of seaweed extracts are thought to be metabolites of the seaweeds such as complex polysaccharides, growth

hormones, sterols, and betaines (Khan et al 2009). These categories will be discussed in depth in section three, seaweed extracts.

Plant Growth-Promoting Rhizobacteria (PGPR):

Plant growth-promoting rhizobacteria (PGPR) were first described and characterized in 1978 as factors that help determine the growth and health of the plant (Kloepper and Schroth 1978). These bacteria are mostly made up of two groups: the Gram-negative, *Pseudomonas* and related genera, and the Gram-positive, spore-forming *Bacillus* and related genera. *Pseudomonas* spp. have been used in many studies and have shown promise as complex formulations of multiple PGPRs, but lack the spore forming ability to survive harsh conditions (Shameer et al. 2018). Plant growth-promoting bacteria (PGPB) are able to produce secondary metabolites and increase nutrient availability to plants, but in the absence of a rhizosphere (Bashan et al. 2014). *Bacillus* are more common in the use of commercial agriculture products due to their ability to form spores thereby allowing them to be formulated as seed treatments with a long shelf life together with seed-treatment fungicides and insecticides.

The use of PGPR has grown and has now developed into a well-researched scientific area with widespread use by the agricultural microbiology industry, partly to reduce the inputs of agriculture chemicals (Lugtenburg et al. 2009). Specific strains of PGPR can promote the growth and yield of many different crops by harnessing different modes of action (Souza et al. 2015). The increased implementation of PGPR in agriculture has driven millions of dollars into researching how and why specific strains of these bacteria help the plant and rhizosphere.

Plant growth promoting bacteria (PGPB) may interact directly with the plant by colonizing the roots, in some cases PGPBs enter the plant to survive as an endophyte, or the

bacteria may reside in the intimate soil around the roots. PGPB must maintain high population levels to deliver growth promotional traits to plants, but that is not always maintained due to incompatibility with soil profiles (Bashan et al. 2014). Bashan (2010) reported that *Azospirillum* spp. could maintain high population numbers without interacting with the rhizosphere, and can wait in the soil for future host plants.

PGPR have been shown to help with biocontrol of many different plant pathogens, through several different mechanisms including: competition, parasitism, and release of secondary metabolites (Kloepper et al. 2004). PGPR that harbor any of these traits are classified as having biocontrol properties.

Biocontrol of PGPR:

PGPR have multiple modes of action that can work together to inhibit disease progression and promote the health of the plant. Biocontrol can be broken down into two mechanisms: direct (antagonism) or indirect. With direct biocontrol, the PGPR colonizing the roots or closely associated soil produce an array of secondary metabolites, siderophores, antibiotics, and lytic enzymes that directly inhibit pathogens (Compant et al. 2005). PGPR may also directly inhibit the pathogen's ability to communicate effectively by disrupting quorum sensing mechanisms (Dong et al. 2004). The indirect mode of action is induced systemic resistance (ISR), in which the PGPR act as a plant primer to increase the basal plant defense response. ISR is similar to SAR, but there is no physical response by the plant to the PGPR, as there would be in SAR (Liu et al. 2016).

Biocontrol via Antagonism:

Antagonism is the direct ability of the bacteria to inhibit pathogen growth and proliferation through the active secretion of secondary metabolites or the competition for space and resources (Boukerma et al. 2017). These secreted metabolites may have overlapping effects of growth promotion and disease control, but be differentially regulated in the presence of the pathogen (Walters and Heil 2007). This effect is very noticeable *in vitro* against many fungal and bacterial pathogens; however, the antagonistic activity is not nearly as pronounced in field conditions (Govindasamy et al. 2011). Hernandes-Morales investigated the production of lipopeptides produced by *Bacillus sp.* MAO4 and their effects on fungal pathogens (2018). This research identified the specific lipopeptide, fengycin, causing the antifungal activity *in vitro* (Hernandez-morales 2018). PGPR will produce these secondary metabolites as a normal part of their growth, which helps create a conducive environment for the PGPR and host plant to grow.

Antibiotics:

Antibiotics are an integral aspect of how certain bacteria are able to control pathogen populations. Among PGPR strains, *Pseudomonas* spp. are well known for their ability to produce antibiotic compounds, but *Bacillus* spp. do not have as high of an affinity for producing these molecular compounds (Berendsen et al. 2015). Many versatile antibiotic compounds have come from pseudomonads, but one of the most notable and useful compounds for its broad spectrum antifungal activity is 2,4-diacetylphloroglucinol (DAPG) (Lanteigne et al. 2012). New antibiotics are constantly being researched and discovered, especially within the pseudomonas family. Prasannakumar discovered a novel antibiotic, 'amino(5-(4-methoxyphenyl)-2-methyl-2-(thiophen-2-yl)-2,3-dihydrofuran-3-yl)methanol' [$C_{17}H_{19}NO_3S$] (AMTM), from *Delftia tsuruhatensis* strain WGR-UOM-BT1 that inhibited multiple fungal plant pathogens (2015).

Bacillus spp. may not produce as many antibiotics as pseudomonads, but they are still an extremely important part of the biocontrol antagonism. It was recently discovered that *Bacillus* spp. MA04 produce several different fengycins that can inhibit fungal growth of multiple pathogens (Hernandez-Morales et al. 2018).

Biocontrol Indirect:

Induced systemic resistance, ISR, is the ability of an outside source to reduce the severity of a disease on its host without having a direct interaction with the pathogen (Kloepper et al. 2004). Many different attributes of PGPR can work together to induce systemic resistance, or it can be elicited from a single mechanism (Liu et al. 2016). One of the understood modes of actions is to increase plant host defenses through strengthening the cell wall to form a more firm barrier between the plant and the pathogen (Boukerma 2017). The host plant will have a tradeoff of reduced growth for increased protection, especially when a pathogen attacks (Walters and Heil 2007). PGPR possess many traits that can induce systemic resistance from the PGPR cell structure to the specific metabolites that are produced. For example, the physical bacterial structures most likely to trigger ISR in plants are the outer membrane proteins such as the lipopolysaccharide of the cell wall or the flagella (CMJ 2001).

Volatile Organic Compounds (VOC):

One way that PGPR inhibit disease is by secreting volatile organic compounds that can inhibit mycelial growth and spore germination of pathogens. Li et al. (2015) reviewed eight unique bacilli strains and identified over fifty different volatile organic compounds. Two of the eight strains were *Bacillus amyloliquefaciens* subsp. *plantarum*, while the other six were *Bacillus subtilis* (Li et al. 2015). All eight strains contained a form of ketone, and further investigation

showed that this could be the major driving force in fungal disease suppression, but the combination of multiple VOCs is needed for their overlapping contributions to reducing mycelial growth (Li et al. 2015). Mu et al. (2017) reported that vermicompost can increase biocontrol properties against fungal pathogens, due to the endogenous bacteria secreting VOCs. There is some evidence supporting the involvement of VOCs with inducing systemic resistance and upregulating plant defense genes (Mu et al. 2017). VOCs have been implicated in the upregulation of plant defense genes that affect closing stomata for drought tolerance and strengthening cell wall structures, and these can lead to an induced systemic resistance for drought and salinity stress (Etesami et al. 2018).

Growth Promotion by PGPR:

It is important to maximize the potential growth of a crop by allowing it to have the most conducive conditions to reach its maximum potential. It may be impossible to control the weather, but for now we can focus on creating a healthy rhizosphere for the plant. The biological control activity of PGPR helps indirectly with growth promotion by suppressing some of the surrounding pathogen pressure. PGPR are also able to directly stimulate the growth and nutrient uptake by secreting siderophores for chelating iron, specific acids for phosphate solubilization, and phytohormones to increase root growth (Souza et al. 2015).

Phosphorous

Phosphorous is a costly chemical input that farmers must use in order to maintain proper health of their crops. The downside to this chemical input is that the plant is not able to make use of all of the phosphorous before some of it associates with other compounds in the soil. Phosphorous is abundant in most soil types, but is usually bound to aluminum, iron, or calcium compounds, which make them unavailable to the plant (Khan 2009). PGPR can help solve this

problem of insoluble phosphates in soil by solubilizing them and creating a continuous phosphorous source for the plant in the rhizosphere. PGPR are able to solubilize inorganic phosphorous by secretion of formic, gluconic, oxalic and other acids—depending on the specific PGPR strain (Mehta et al. 2013).

Nitrogen:

Nitrogen is crucial for all life on earth, since it is a main component of the building blocks of life, amino and nucleic acids. Most forms of nitrogen are not readily available for uptake and use by biological life, and instead need to be modified for uptake (Calvo et al. 2014). There are two different groups of bacteria that can readily fix atmospheric nitrogen, N_2: symbiotic and non-symbiotic nitrogen fixing bacteria (Lucas et al. 2004). The symbiotic nitrogen fixing bacteria, *Rhizobium* and *Bradyrhizobium,* are the best characterized because of their ability to induce nodulation on legumes (Singh et al. 2018). The other group is considered non-symbiotic nitrogen fixers, and they are made up of many different PGPR microorganisms Salvo et al 2018).

The ability of PGPR to fix nitrogen is a unique and desired trait among soil microbes, because of the importance of biologically fixed nitrogen for uptake by the plant (Salvo et al. 2018). Nitrogen fixing PGPR strains span many different bacterial genera, including *Azotobacter, Azospirillum, Bacillus,* and *Pseudomonas* (Calvo et al. 2014). One of the best examples of PGPR nitrogen fixation was reported in *Azospirillum* spp. (Bashan 2010). The efficiency of supplying nitrogen may also be in part to the close relationship that *Azospirillum* spp. has in the rhizosphere and endophytic aspect of its association with plant roots. Adesomye et al. (2010) demonstrated that a PGPR complex mixture increased nitrogen uptake for tomato plants at reduced fertilizer levels. Kuan et al. reported a similar increase of nitrogen uptake and

availability to corn under greenhouse conditions with the application of PGPR, *B. pumilus* S1r1 (2016).

Siderophores:

Iron availability can be an important nutrient for many different forms of microbial life in the rhizosphere, and the most common form of iron in soils is the insoluble Fe^{3+} (Souza et al. 2015). Some PGPR actively secrete siderophores, which are low molecular weight molecules that are specialized to bind or chelate to Fe^{3+} and make it into a soluble form for use by biological life (Krewulak and Vogel 2008). This bacterial iron complex is ready for uptake by the plant or by the bacteria that secreted the siderophore. Siderophores can also decrease plant pathogen presence by creating a more suitable environment for the siderophore-producing bacteria and filling a niche of microbial life in the rhizosphere (Loaces *et al.,* 2011).

Phytohormones:

Plants require a complex mixture of phytohormones to communicate with surrounding microorganisms and efficiently maintain hormonal homeostasis, especially under stressed growing conditions (Tsukanova et al 2017). Many diverse hormones including, auxins, cytokinins, ethylene, and gibberellins, can be synthesized by PGPR strains to help cell division, root initiation and root elongation (Calvo et al. 2014). Auxin, indole-3-acetic acid (IAA), is one of the most critical hormones required to increase root growth, which in turn creates more surface area for the plant to absorb nutrients (Rolli et al. 2015). The mechanisms that lead to growth promotion effects are also closely linked to alleviating abiotic stresses, especially drought and salinity stresses that reduce root growth (Etesami 2018).

Seaweed Extracts:

Seaweed has been a useful tool in agriculture for hundreds of years, but in continually changing methods. The original methods of incorporating seaweed into agriculture were called weathering, in which kelp was spread into the field and turned over as a soil amendment (Craigie 2011). The coastal regions saw success and thought to try and make a seaweed formulation that could be used more broadly in inland agriculture as early as 1850 (Craigie 2011). It took another century before Milton improved upon this early research and developed a useful way to make seaweed extracts (1952). This greatly increased the interest in seaweed for agricultural purposes, and these seaweed extracts were immediately put through a gamut of tests involving biocontrol and growth promotion effects across many diseases and crops (Senn 1978).

Seaweeds are macroalgae and are classified based on their pigments: Chlorophyta (green), Phaophyta (brown), and Rhodophyta (red) (Khan et al. 2009). The most commonly researched and commercialized seaweed type is the class Phaophyta and a few of the most common species within this class are *Ascophyllum nodosum, Fucus spp, Laminaria spp, and Sargassum spp.* (Hong et al. 2007). One of the reasons for the popularity of brown seaweeds in research is its availability along the coast. *Ascophyllum nodosum* attaches to rocks along the coast via a holdfast and is then the frond is harvested just above its connection to the rock. The holdfast has been reported to survive in a single place for around one hundred years, while the growing frond may live for up to twenty years (Craigie 2011). Seaweed extracts have been documented as beneficial foliar sprays and soil amendments across many different crops and growth conditions (Senn 1978; Calvo et al. 2014). This research demonstrates that seaweed extracts are effective in increasing root growth, seed germination, and fruit set and that they can enhance yield, and plant health under biotic and abiotic stress (Khan et al. 2009).

Biocontrol

Macro algae harbor diverse microbial assemblages across many different seaweed species. The harsh saltwater environment forces the microbial populations to compete for nutrients and fill a niche on the kelp frond (Flewelling et al. 2013). There is great potential for these diverse communities of bacteria and fungi to produce novel antibiotic compounds (Flewelling et al. 2013). However, seaweed extracts primarily confer disease resistance through plant defense elicitors that cause the host plant to induce a cascade of defense responses, which prime the plant for any future pathogen attacks (Cragie 2011). Plants have developed an elicitor response, so that it can upregulate pathogenesis-related proteins and a cascade of defense mechanisms in the early stages of pathogen colonization (Khan et al. 2009).

Brown, green, and red seaweeds harbor similar overlapping components; however, their biocontrol properties seem to stem from the unique Oligo-polysaccharides, lipids and proteins that resemble attacking pathogens (Coˆte´ et al. 1998). Seaweeds have been shown to have vastly different composition between harvests, but the one component that has stayed most consistent through multiple harvests is the polysaccharide content (Rayorath et al. 2009). Red algae is reported to have a bioactive carrageenan, complex polysaccharide, which acts a strong elicitor for plant defenses (Mercier et al. 2001). Alginates such as, laminarin and sulfated fucan are unique polysaccharides of *Ascophyllum nodosum* and have been reported as the elicitors that increase plant defense properties and increase the amount of superoxide dismutase (SOD) in the host plant (Khan et al. 2009 and Zhang et al. 2003).

Subramanian et al. (2011) delved deeper into the specifics of upregulated defense mechanisms and reported that applications of *A. nodosum* increase the amount of jasmonic acid *in planta*. Esserti et al. (2016) demonstrated that a foliar spray containing *A. nodosum* liquid

seaweed extract to increase reactive oxygen species within tomato. This research also showed that methanolic seaweed extracts retain a larger amount of active compounds, opposed to other more harsh solvents that are used in extraction processes (Esserti et al 2016). The phenolic composition of seaweed extracts is another way by which it can cause the host to increase disease resistance. Phlorotannins are the specific polyphenols within brown seaweeds and have been reported to induce jasmonic acid *in planta* (Arnold et al. 2001). Fan et al. (2010) also reported an increased content of phenolics and antioxidant levels in spinach plants after application of a foliar spray of *A. nodosum*.

Growth Promotion

The growth promotion qualities of seaweed extracts are not thought to be derived from direct nutritional contents, but some micronutrient benefits have been seen after seaweed extract applications (Calvo et al. 2014). The technological advances towards laboratory techniques are unlocking new ways to analyze the seaweed extract contents (MacKinnon et al. 2010). MacKinnon et al. (2010) reported that the amount of betaines in raw *A. nodosum* was similar to the amount in seaweed extracts developed from the same seaweed source. Betaines have been inferred to increase chlorophyll content and help regulate cellular osmotic stress (Zhang et al. 2003).

Alginates are common cell wall components in seaweeds and have been shown to help immobilize and increase growth of PGPB (Yabur et al. 2007). Bashan et al. (2002) hypothesized that alginates can offer nutrient content to the PGPB and supplement its growth, if the PGPB strain was applied onto a microbead of the alginate. One of the problems faced here was the lack of oxygen that was available within the microbead and the possibility that the PGPB is unable to actively leave the alginate and colonize plant roots (Bashan et al. 2002).

Therefore, seaweed extracts that are applied as soil drenches with PGPR may be able to increase the nutrients available to PGPR.

Phytohormones:

Seaweed extracts have been mined for possible plant growth promoting substances and plant growth regulators (Khan et al. 2009). These substances have already been well described and studied with PGPR, but since this source of plant hormones is different—it may have an alternate reaction by the host (Calvo et al. 2014). Many seaweed species were analyzed and showed a consistent presence of cytokinins and auxins (Calvo et al. 2014). Although the plant hormones and regulators have been identified in many different seaweed species, *A. nodosum* maintains a high concentration of both cytokinins and IAA (Stirk et al. 2003). Wally et al. (2013) attributed the increased presence of cytokinin and auxin activity in the host plant to the application of seaweed extracts upregulating the production by the host, and not the physical addition of seaweed extract hormones themselves being taken up by the plant.

Ascophyllum nodosum:

Many different seaweed species have shown promise as biostimulants of agriculture but have not gone through enough rigorous testing to earn commercial support. It is critical to determine the nutrient composition of seaweeds at the time of harvesting and after varying extraction protocols to help determine optimal harvesting and extraction protocols (Sangha et al. 2014). *Ascophyllum nodosum* has been the focal point of many different studies evaluating the ability of the seaweed extract to increase overall plant health and yield (Rouphael et al. 2018; Prasanth et al. 2008; and Rayorath et al. 2009).

Shekhar et al. (2012) did a compositional analysis of brown seaweeds and reported on *A. nodosum* two extraction protocols as well as four commercial extract formulations derived from *A. nodosum*. The six formulations were analyzed using an energy dispersive X-ray microanalysis (Inca Energy, Oxford Instruments, UK) for the composition of twelve macro and microelements (Shekhar et al. 2012). Of the twelve elements evaluated six of them showed significant ranges in percent composition including, Na, Mg, P, S, K, and Cu with ranges of .93-7.44%, .04-1.26%, .02-.23%, .29-2.78%, 2.99-13.22%, and.02-.13% respectively (Shekhar et al. 2012). The major ranges in composition are most heavily based on extraction method, but the time of harvest may be a determining factor. The six formulations were also evaluated by dry matter content, pH, carbon, and nitrogen content, which ranged from 3.73-6.41, 4.28-8.88, 25.84-36.22, and .49-1.98 respectively among all six formulations (Shekhar et al 2012). The complex carbohydrates mannitol, fucoidan, and laminarin were all identified in the seaweed extracts by pyrolysis gas chromatography (Py-GC/MS); however, the peaks in the acidic extracts were much fainter which could mean the extraction process may alter structure of some of the carbohydrates within the seaweed (Shekhar et al. 2012).

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Chapter II Screening PGPR and Seaweed Extract formulations for biological control of *Rhizoctonia solani in vitro* and *in planta* under greenhouse conditions

Abstract

An experiment was designed to evaluate the ability of commercial PGPR strains and commercial seaweed extract formulations to inhibit the growth and virulence of *Rhizoctonia solani in vitro* and under a greenhouse setting. The three commercial strains of PGPR used were, MBI600 (*Bacillus amyloliquefaciens*), GB03 (*Bacillus subtilis*), and FZB24 (*Bacillus subtilis*) (Beri et al. 2018; Han et al. 2014; Sharaf-Eldin et al. 2008). An Auburn strain, AP218 (*Bacillus amyloliquefaciens*), was also used since it has been evaluated as one of the top PGPR biocontrol strains in previous research. The four commercial seaweed extracts were from the same source of seaweed, *Ascophyllum nodosum*, but the extraction methods varied between all four formulations. The four seaweed extract formulations were also gamma irradiated, to evaluate if any beneficial or deleterious effects come from the indigenous microbial assemblages or the nutrient content of the kelp itself. The PGPR strains exhibited different magnitudes of inhibition of *R. solani in vitro*, but they inhibited the mycelial growth on PDA, an agar used for fungal growth. The seaweed extracts were also evaluated *in vitro* for the ability to inhibit mycelial growth of *R. solani*. Only one seaweed extract was able to inhibit mycelial growth due to competition for resources by indigenous fungi. The *in planta* tests showed a trend in increased biocontrol over several treatments, but due to low disease severity there were no statistical significances compared to the control.

Introduction

The chemical inputs, fertilizers and pesticides, which our agricultural systems rely so heavily on are gradually increasing the amount of environmental pollution near farming communities (Zahoor et al. 2014). These chemical inputs are seen as necessities to farmers that rely on maximizing their crops yield each year; however, the pesticides must be put on a stringent application schedule to avoid a buildup of resistance by pathogens and pests (Wallace et al. 2017). *Rhizoctonia solani* has many different anastomosis groups and causes seedling disease and dampening off on many crops including, soybean (Ajayi et al. 2018). Ajayi et al. (2017) analyzed the most pathogenic isolates of *R. solani* and identified that soybeans are susceptible to the most virulent isolates. These isolates were also found to infect corn, which is sometimes used in crop rotations with soybean; however, this provides evidence that it is not a suitable rotation for the avoidance of pathogen pressure from *R. solani*. The amount of organically farmed land increased over 100% worldwide from 2001 to 2011, showing a remarkable trend away from traditional farming (Willer et al. 2011). This offers an opportune time for biostimulants to shift the dynamic of inputs for agriculture.

Plant growth-promoting bacteria (PGPR) are beneficial bacteria that live in the rhizosphere and have potential for promoting plant health or protecting the host plant from pathogens (Souza et al. 2015). There are a plethora of mechanisms that PGPRs can use to exhibit biocontrol activities including: competition, parasitism, and the release of diverse secondary metabolites (Kloepper et al. 2004). Biocontrol can be divided into two categories: direct (antagonism) or indirect (induced systemic resistance). PGPR exhibiting antagonism inhibit pathogen growth *in vitro* via the secretion of secondary metabolites, which could include various antibiotics, siderophores, or volatile organic compounds (Liu et al. 2016). The indirect

method requires the PGPR to elicit a basal immune response in its host plant, so that it can upregulate pathogenesis-related proteins, cell wall strengthening, and increased antimicrobial compounds within host tissue (Boukerma et al. 2017). Commercial PGPR strains are often reported to harbor multiple growth promoting and biocontrol abilities, usually involving multiple crops and multiple diseases.

The most commonly researched and commercialized seaweed type is the class Phaophyta, and a few of the most common species within this class are *Ascophyllum nodosum, Fucus spp, Laminaria spp, and Sargassum spp.* (Hong et al. 2007). There are diverse microbial assemblages, of fungi and bacteria that remain in many of the commercial seaweed extracts (Flewelling et al. 2013). These fungi and bacteria can contribute to biocontrol through competition of resources or secretion of antibiotics and secondary metabolites (Rayorath et al 2009). Seaweed extracts have often been reported as elicitors of host plant defense systems through induced systemic resistance (Khan et al. 2009). The biocontrol properties seem to stem from the unique complex polysaccharides, lipids and proteins that resemble components of attacking pathogens (Coˆte´ et al. 1998). Seaweeds have been shown to have vastly different composition between harvests, but the component that has stayed most consistent in *A. nodosum* through multiple harvests is the polysaccharide content (Rayorath et al. 2009). Laminarin and sulfated fucan are the two polysaccharides often found in *A. nodosum*, and are thought to be the major elicitors of upregulated defense mechanisms by the host (Khan et al. 2009).). Abkhoo and Sabbagh (2016) reported an increase in host defense expression after applications of commercial extracts containing *A. nodosum* to reduce dampening off on cucumber by *Phytopthora melonis*.

PGPR and seaweed extracts have overlapping biocontrol traits such as antagonism, production of antibiotics, and induction of systemic resistance (Khan et al. 2009 and Calvo et al. 2014). These two categories of biostimulants have potential to produce synergistic effects on the overall plant health in the presence of a pathogen. PGPR may induce systemic resistance after the host interacts with the lipopolysaccharide outer membrane or flagella (CMJ 2001). Extracts of *Ascophyllum nodosum* have been reported to induce systemic resistance and upregulated defenses after soil drenches or foliar applications, especially if the *A. nodosum* was extracted using methanol (Esserti et al. 2016).

Materials and Methods

Assessment of microbial life within seaweed extracts:

Full strength tryptic-soy-agar (TSA) was used as a growth medium for two different concentrations, one and five percent, of the seaweed extracts. The gamma irradiated seaweed extracts were also streaked onto TSA plates to ensure that the seaweed extracts were completely sterilized by gamma irradiation.

Seaweed Extracts:

Ocean Organics provided four seaweed extracts that were made using different extraction protocols, but had the same seaweed source. The four SE formulations included Kelp Flowable, SeaClear, ZR100c, and a Digestate. The extraction protocols are all proprietary, but some main differences were provided by Ocean Organics when we received the extracts. Kelp Flowable was considered a high solids extract. Digestate was also a high solids extract but digested. SeaClear was from a clarified process. ZR100C was also clarified but went through an extra decanting process.

Gamma irradiating seaweed extracts:

Dr. Max Cichon, current faculty and member of Auburn University's radiation safety committee, coordinated the sterilization of seaweed extracts via gamma irradiation. Over a seven day period, four Pyrex 500ml jars with plastic lids containing a seaweed extract were exposed to ~ 4.0 Mrad of direct gamma in pairs due to space constraints. The maximum flux of gamma was 29.85 R/second. The source was calibrated on January 23, 2018, using a Standard Imaging Max 4000 dose meter/electrometer and its accompanying ion chamber. The seaweed extracts received 2,230 minutes of continuous gamma for each exposure (Figure 2) .

In vitro screening for inhibition of *Rhizoctonia solani* on PDA:

Rhizoctonia solani was grown on potato dextrose agar, PDA, for three days (or until the mycelia reached the edge of the plate) and then 4.0 mm plugs were taken from the edge of actively growing mycelium, and placed in the center of a new PDA plate. The new PDA plate had four sterilized discs placed equidistant from the center and each other. The treatments were then inoculated on sterilized discs after allowing the *R. solani* to grow for 24 hours. The four PGPR strains were grown on TSA for 24 hours then bacterial suspensions were made with water. The bacterial suspensions were adjusted to 10^6-10^7 CFU/ml, using McFarland standards. The seaweed extracts were inoculated using the same method, except the suspensions were simply one percent, five percent, and gamma irradiated for all four formulations. The four PGPR strains used included MBI600 (*Bacillus amyloliquefaciens*), GB03 (*Bacillus subtilis*), and FZB24 (*Bacillus subtilis*), and an in house strain labeled AP218 (*Bacillus amyloliquefaciens*). All treatments were tested in triplicate to ensure consistency.

In planta screening for inhibition of *Rhizoctonia solani* in potting mix under greenhouse conditions:

Sunshine mix was placed into 4.5-inch pots and tamped down to make the potting mix settle to the bottom of the pot. A 2.5 cm deep hole was placed in the center of the pot into which a single untreated soybean seed was placed. One ml of PGPR suspension was applied, approximately (10^6-10^7 CFU/ml), onto the seed and then covered with potting mix. When seaweed extracts were applied the seeds were covered first, and then 10 ml of the seaweed extract formulations were applied. After the soybeans grew for two weeks a 4.0 mm agar plug of actively growing *R. solani* mycelium was applied to the crown of the soybean, and covered with potting mix. One week after pathogen challenge, the disease severity was rated using a 0-5 scale where: 0 = Healthy, 1 = <10% dark brown lesion at crown, 2 = 25% of the crown girdled with necrotic lesion, 3 = 50% of the crown girdled with necrotic lesion, 4 = 75% of the crown girdled with necrotic lesion, 5 = 100% of the crown girdled with necrotic lesion. The treatment list included a water control, PGPR alone, gamma irradiated seaweed extracts at 5% alone, and PGPR combined with the individual seaweed extracts. The tests were set up in a randomized complete block design with ten treatments and ten replicates, then the tests were replicated.

Statistical Analysis:

An analysis was performed using Dunnett's method of comparing all treatments to the control; however, there were no significant differences, so an alternate analysis was done (Tukey's HSD). There were also no significant differences for disease reduction among treatments at the 95% confidence interval using ANOVA with Tukey's HSD in R, statistical software.

Results

Seaweed Extract Characteristics:

Kelp Flowable, SeaClear, ZR100C, and Digestate are seaweed extracts from the same source of *Ascophyllum nodosum* but using varying proprietary extraction methods developed by Ocean Organics. Kelp Flowable, SeaClear, ZR100C, and Digestate were individually mixed with water to make a 5% solution and tested for pH using litmus strips and reported pHs of 6, 4, 2, and 8 respectively. Kelp Flowable is a murky dark green liquid that has the largest amount of physical kelp frond fragments still in the extract, which corresponds to it also containing the most microbial life, bacterial and fungal, of the four extracts. SeaClear and ZR100C are light brown liquids that exhibited similar traits in pH, but SeaClear had much more bacterial growth on TSA. SeaClear appears to be dominated by a few bacterial species upon visual inspection. The extraction methods were probably very similar, but ZR100C underwent an additional proprietary process to eliminate more microbial life than the other extracts. Digestate is a black thick liquid that smells strongly of sulfur and exhibited microbial life similar to SeaClear on TSA; however, visually it looks like Digestate has a more diverse microbial assemblage based on observing their colony sizes and speed of growth. The microbial population within the seaweed extracts ranged from 10^2, 10^4, 10^5, and 10^7 cfu/ml corresponding to ZR100C, SeaClear, Digestate, and Kelp Flowable respectively (Figure 3).

In vitro:

All PGPR strains inhibited the growth of *R. solani*, but the amount of antifungal activity varied among the bacteria. Strains MBI600 and AP 218 exhibited the strongest inhibition of *R. solani* with an average of 11 mm of space between the pathogen and the PGPR after the mycelia

grow to over the control disc. The bacteria strains FZB24 and GB03 had lower levels of antagonism, averaging 9 mm and 6 mm respectively. Seaweed extracts were evaluated using the same method for detection of antifungal activity. Seaweed extract treatments were evaluated using a 1% and 5% solution for each of the four SE formulations. The only seaweed extract to inhibit *R. solani* was Kelp Flowable, and it inhibited the pathogen equally at the 1% and 5% treatments (Figure 1). The inhibition observed was likely due to an assortment of microbial life growing and competing for space and nutrients on the PDA plate.

In planta:

The overall disease pressure was low, averaging below a two out of five on the severity of infection (Figures 4, 5, 6, and 7). This is a limiting factor when trying to find significant differences within this assay. The test involving AP218 showed some positive trends towards protection, but still nothing statistically significant. Dry weights of shoots and roots were weighed, but there were no significant differences.

Discussion

Results *in planta*:

The seaweed extracts were all applied at the same time and concentrations even though their extraction protocols were all different. The mechanism of each extraction interacting with the plant may differ according to the concentration applied (Craigie 2011). A pretrial was done in potting mix to evaluate if any negative effects on germination could be observed between a 1% and 5% concentration of the seaweed extracts, and none were observed. The 5% concentration was chosen, so that it could have an increased chance of inducing systemic resistance while the PGPR colonized the roots to act as antagonists. The seaweed extracts may

inhibit growth at high concentrations but provide a long-term boost to upregulated defense genes and accumulation of antioxidants in the host plant (Zhang et al. 2003). The different extraction methods will most likely not all work at the same concentrations or under the same modes of action, so evaluating the dose responses of seaweed extracts individually may be beneficial (Craigie 2011). The seaweed extracts could have been applied after the pathogen to offer an alternate mode of action, but to date, the literature suggest that seaweed extracts inhibit pathogens by induced resistance instead of antagonism (Zhang et al 2003).

Results *in vitro*:

Kelp Flowable was the only seaweed extract able to offer any physical competition/antagonism to *R. solani in vitro*, so it was the only extract included in the *in planta* treatment list that was not sterilized by gamma irradiation. However, there were still no differences in host plant protection when challenged with *R. solani*. The isolation of bacteria and fungi from Kelp Flowable is a good future prospect for identifying candidate bacteria with potential for competition and or antagonism against plant pathogens.

Future work:

The main focus of biocontrol via seaweed extracts is induced systemic resistance, so longer range experiments evaluating ISR is a promising avenue for seaweed extracts to be included in agriculture. Experiments comparing dose responses of seaweed extracts differing by application method, soil drench vs. foliar spray, is another area of interest that may help elucidate the mode of action for biocontrol (Esserti et al. 2016).

There are many possibilities for future work towards achieving synergy between biostimulants, especially seaweed extracts and PGPR. The advancement of technology has

unlocked new capabilities for high through put isolation and screening of bacteria for common PGPR traits (Bashan 2002). Isolation of possible PGPR candidates directly from seaweed extracts is an area that could increase the possibility for synergy between PGPR and seaweed extracts (Vaikuntapu 2014). The isolation of bacteria and fungi from these extracts can be directly screened for plant pathogens using *in vitro* assays for control of plant pathogens. This could reveal new mechanisms for control of plant pathogens or possibly the discovery of novel antibiotics for the control of plant pathogens (Khan et al. 2009).

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Figure 1. Inhibition of *rhizoctonia solani in vitro*

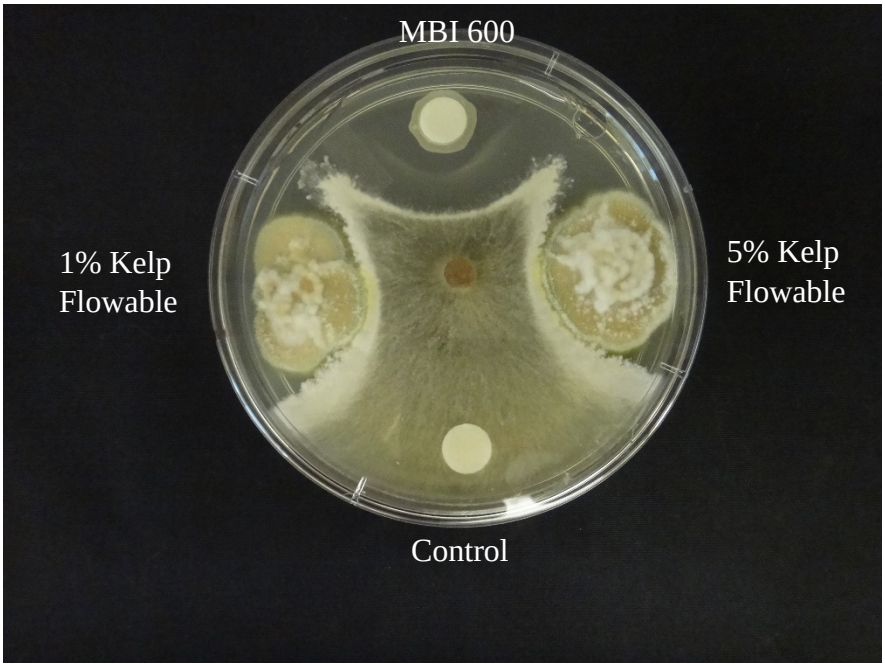


Figure 2. Gamma instrument calibration for sterilization of seaweed extracts

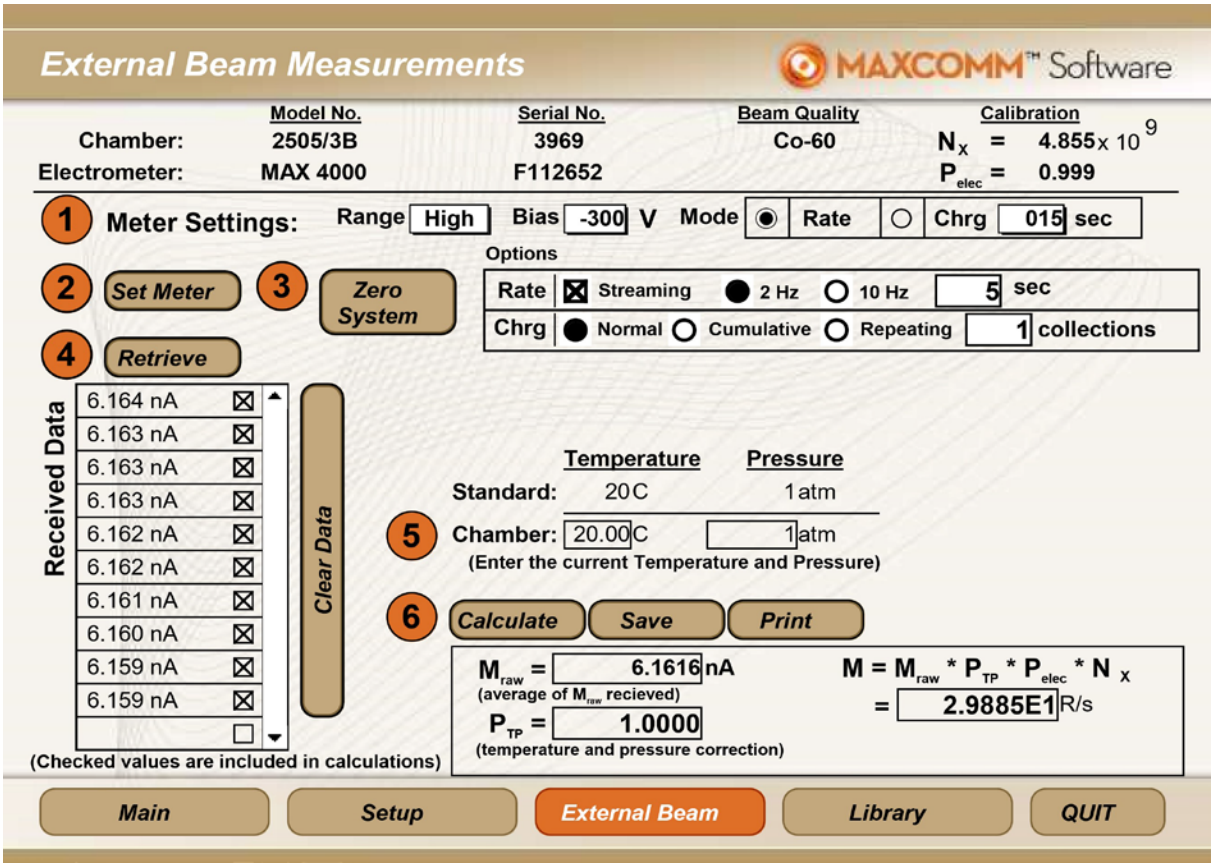


Figure 3. Microbial populations of different seaweed extract formulations

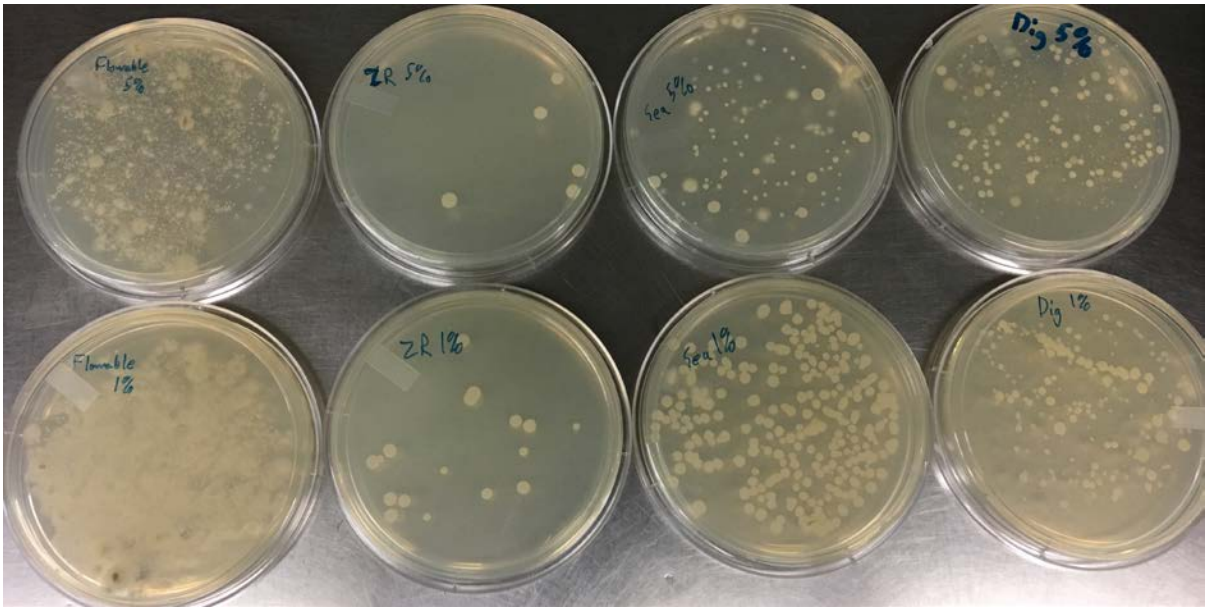


Figure 4: Disease rating data for *in planta* experiment with GB03

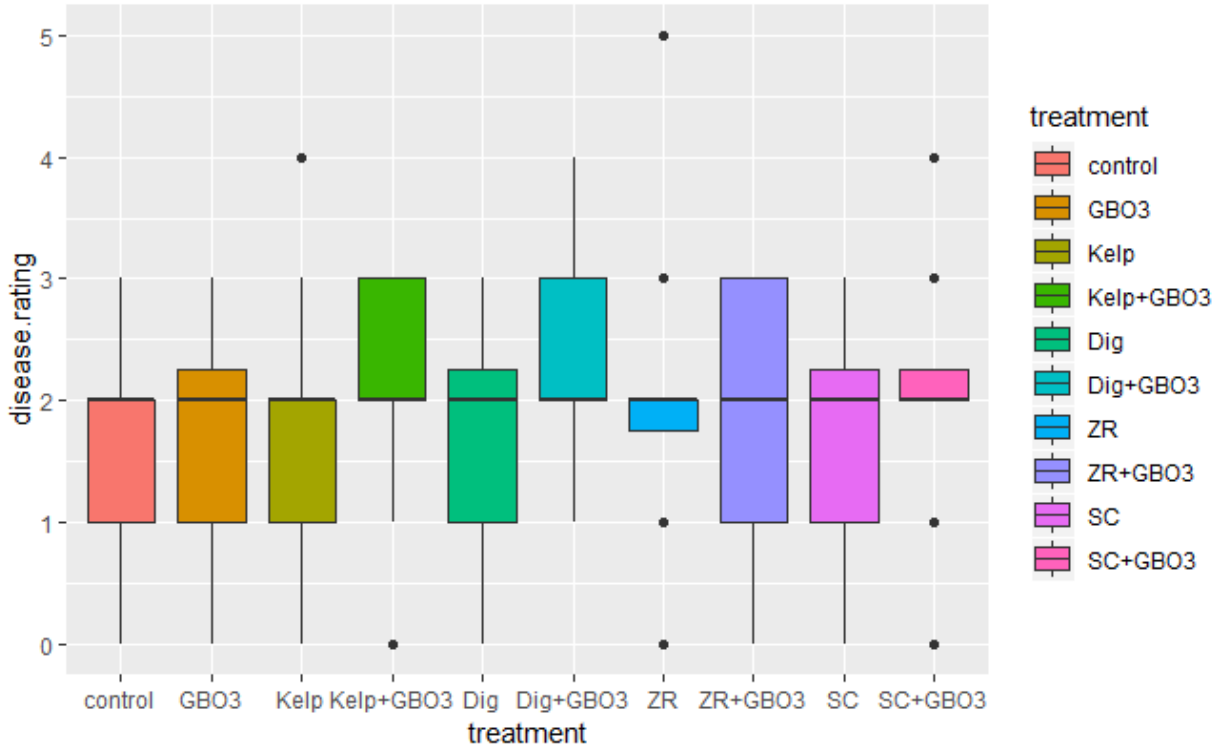


Figure 5: Disease rating data for *in planta* experiment with MBI600

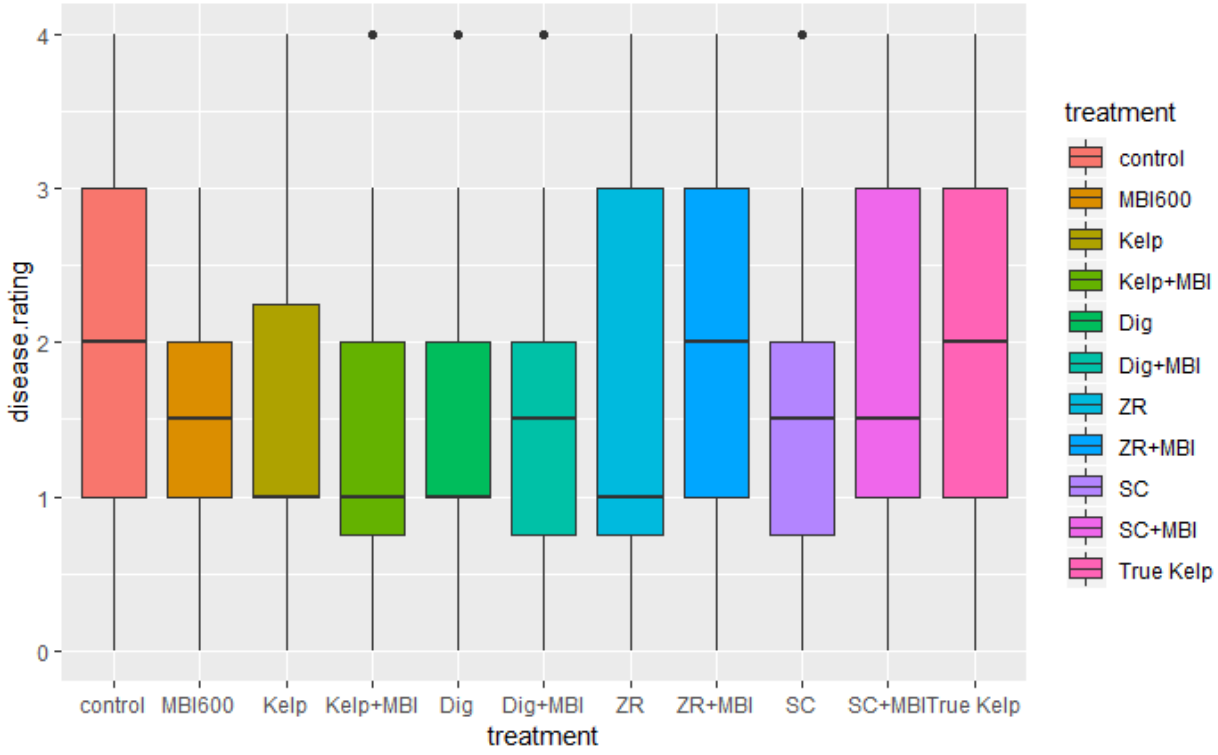


Figure 6: Disease rating data for *in planta* experiment with FZB24

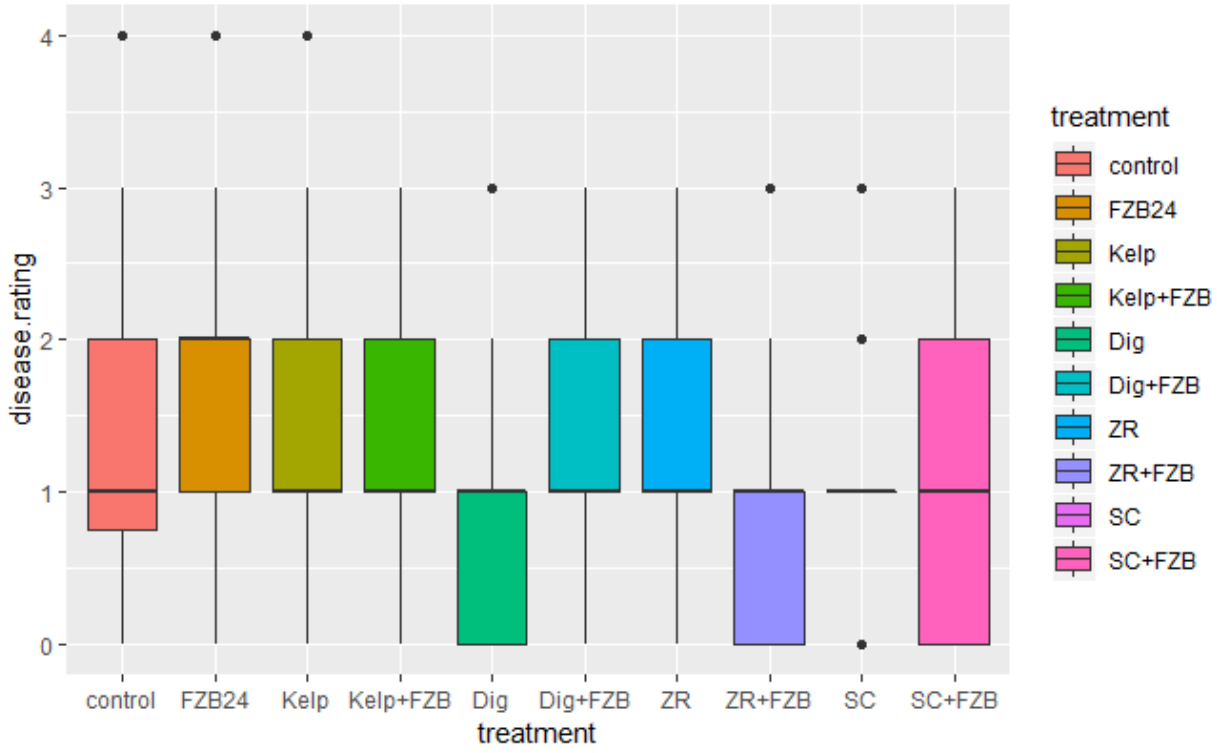
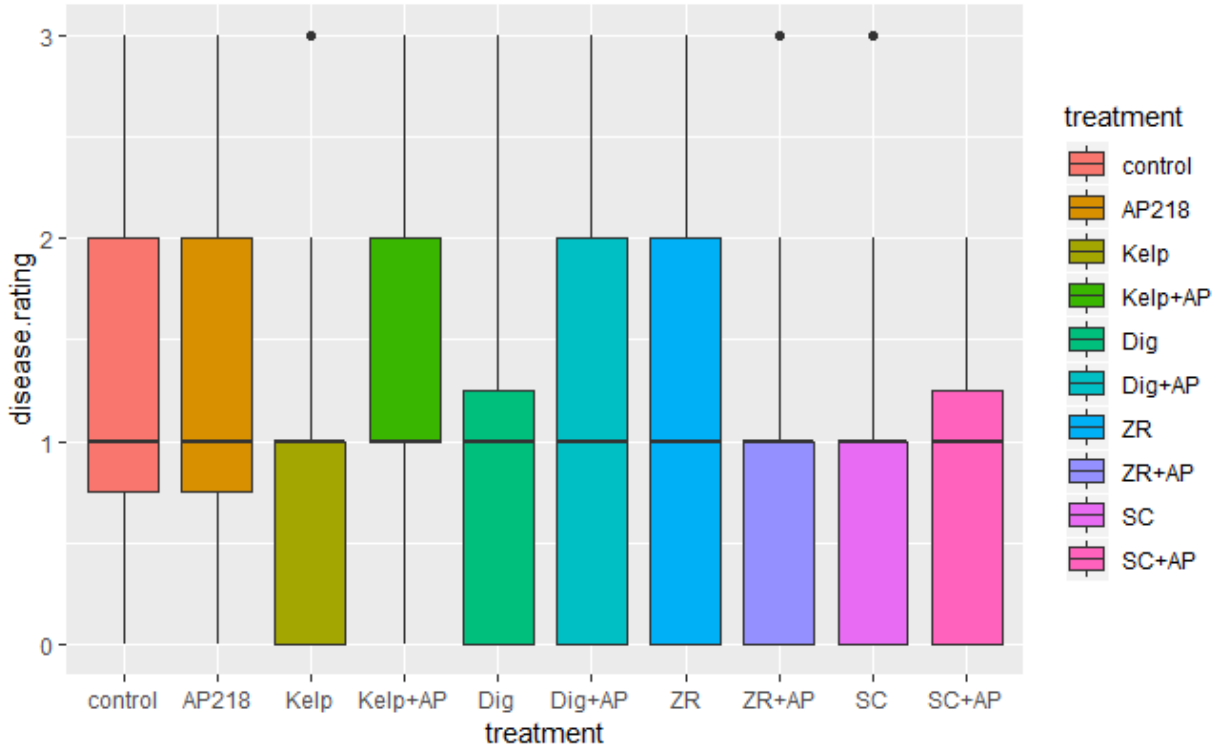


Figure 7: Disease rating data for *in planta* experiment with AP218



Chapter III Early Growth Promotion Assay for Seaweed Extracts with and without gamma irradiation and evaluation of synergy *in vitro* of PGPR and Seaweed Extracts

Abstract

The use of chemical fertilizers in agriculture are necessary for optimal growth of crops, but often the amount of nutrients put into the soil are not taken up by the plant. The use of biostimulants like seaweed extracts and PGPR have been reported to promote root growth as well as nutrient uptake. Together these can be used to help alleviate the environmental stresses caused by over application of fertilizers on crops. Seaweed extracts have been reported to increase colonization of some symbiotic, nitrogen-fixing, organisms like *Bradyrhizobium japonicum* on soybean. Seaweed extracts might also increase the growth of PGPR strains by providing alginate as a carbon source. Here we evaluate the ability of four different gamma irradiated seaweed extract formulations to promote the growth of four different PGPR strains *in vitro* with Spizizen's liquid medium. An early growth promotion assay in conetainers using pasteurized soil was used to evaluate differences between raw and gamma irradiated seaweed extracts. The plant growth parameters recorded included plant height, dry shoot weigh, dry root weight, root surface area, root volume, and average root diameter. The first test showed increases in root parameters for all treatments with gamma irradiated kelp being significantly significant. The second test showed increases across all treatments compared to the control for height, dry shoot weight, and dry root weight, but no statistical significance was observed. The

treatment Digestate was significantly significant across all root growth parameters compared to the control in the second test.

Introduction

Seaweed extracts have been used in agriculture for hundreds of years as amendments to soil with the understanding that they could increase the long-term health of the plant, even if the initial growth of the plant is inhibited (Khan et al. 2009). The early applications were incorporated by harvesting kelp fronds then placing them in soil and continually turning them over in the soil, which is called weathering (Craigie 2011). This weathering could result in adding beneficial microorganisms, phytohormones, or unique polysaccharides to farm land. The addition of physical seaweed can have inhibitory effects on plant growth initially, but this may be a tradeoff associated with induced systemic resistance and priming of the plants defense system for better long-term growth (Flewelling et al. 2013).

Seaweed extracts as biostimulants in agriculture depend heavily on the extraction process, the concentration, and the growth stage of the plant (Craigie 2011). Extraction processes can vary by temperature, pressure, enzymes, filtration, and cell burst technologies (Calvo et al. 2014). Most extraction protocols remain proprietary to each individual company that believes they have achieved the most efficient extraction method to retain the positive effects of their seaweed extract solutions. The amount of endogenous bacteria and fungi remaining in the extract is one of the varying aspects between extraction procedures, and these microorganisms may be able to act as PGPR (Rayorath et al. 2008). The endogenous bacteria of seaweeds could release beneficial secondary metabolites and antifungal compounds that inhibit plant pathogen growth.

Bashan et al. (2014) reported that alginates can provide a nutrient source as well as a way of immobilizing *Azospirillum* spp., so that it can proliferate and be more biologically active in soil. Yabur et al. (2007) and Bashan et al. (2002) reported that alginates in the form of

microbeads created a conducive medium for *Azospirillum* spp. to grow and release secondary metabolites responsible for plant growth promotion or phytoremediation of contaminated soils.

Phytohormones are an important and well-researched aspect of biostimulants, especially PGPR (Boukerma et al. 2017). PGPR and seaweed extracts share some plant hormones like auxins and cytokinins, which can benefit the host plant by catalyzing cell division, root initiation, and root elongation (Calvo et al 2014). MacKinnon et al. (2010) reported that *A. nodosum* contains γ-amino butyric acid betaine which helps increase chlorophyll content in the leaves. The enhanced growth from a foliar spray and the increased chlorophyll content may have resulted from delayed chlorophyll degradation (MacKinnon et al. 2010). El-Alsayed et al. (2018) reported that seaweed extracts increased soluble phosphorous in soil and increased several plant growth promoting parameters, including height, shoot dry weight, and number of leaves in dahlia plants. Another aspect of growth promotion by seaweed extracts is decreasing the amount of abiotic and biotic stresses to the host plant like pathogens, drought, and salinity (Tsukanova et al 2017).

Materials and Methods

In vitro

A variety of tests were carried out to establish if any of the seaweed extracts were able to produce a conducive environment for PGPR growth. The most promising series of experiments involved a modified version of Spizizen's minimal salts medium (TSS) with the replacement of glucose, the only carbon source in the media, with a 5% addition of seaweed extract. The recipe for the media used in this experiment is K_2HPO_4 [1.4%], KH_2PO_4 [0.6%], $(NH_4)_2SO_4$ [0.2%], Trisodium citrate dihydrate [0.1%], $MgSO_4$* $7H_2O$ [0.02%], Seaweed Extract [5%], 100

microliters of PGPR at approximately 10^2 (Spizizen 1958). Once the TSS solution was thoroughly homogenized, it was vacuum filtered to ensure that the starting materials were sterile. The final solution was 5mL and was placed in a 15 mL centrifuge tube on a shaking incubator at 28 degrees Celsius and 150 rpm. Bacterial suspensions of three commercial PGPR strains including MBI600, GBO3, and FZB24 with the addition of one in house PGPR strain, AP218 were made using an OD 600nm of 1.0 then using serial dilutions to achieve consistent bacterial counts. The rationale was that there would be countable colonies over the span of a two days, and a growth curve was drawn to determine if the PGPR used any of the seaweed extract formulations as a carbon source.

In planta:

An early growth promotion assay was performed in conetainers using soil supplied from E.V. Smith Agricultural Research Station. The soil was known to contain pathogens, so a pasteurization process was carried out for three days to eliminate the majority of the plant pathogens. The soil was then placed into containers with approximately 650 grams of soil per conetainer. A single untreated soybean seed was planted 2.5cm deep, covered with soil and then treated with the appropriate seaweed extract. Each greenhouse experiment consisted of 10 replications. The treatment list included a water control, Kelp Flowable, ZR100C, SeaClear, Digestate, and a gamma irradiated version of all four seaweed extracts. All seaweed extracts were applied at a concentration of 5% to be consistent between the previous tests in potting mix. The parameters selected for evaluation of early growth promotion were height, dry shoot weight, dry root weight, and root scan via WinRHIZO scanner. The supplemental lighting for the greenhouse was started between the two tests, so there are some distinct differences in overall

plant growth. Therefore, the extra light and heat that the second test experienced may have influenced alternate growth characteristics.

Statistical Analysis:

The statistics were done separately for each experiment using a Tukey HSD test using R software. No statistical differences were seen between the seaweed extracts and their gamma irradiated counter parts in the first experiment; however, gamma irradiated kelp showed a positive trend in outperforming its counterpart in all measured parameters. The gamma irradiated kelp increased root surface area, root volume and average root diameter compared to the control.

Results

In vitro:

Three of the four seaweed extracts created an environment too harsh for the proliferation and survival of PGPR in solution with TSS. However, SeaClear seaweed extract increased growth of all PGPR strains. The results showed stark contrasts between being conducive to the PGPR and not allowing any growth at all as can be seen in Figure 4. The amount of growth over 24 hours increased exponentially. This increased growth suggests that the PGPR strains were able to use something in the extracts as a carbon source. SeaClear resembles ZR100C in many properties, but the low pH of ZR100C may have been the reason it was not able to promote growth.

In planta:

The parameters measured for early growth promotion effects included plant height, dry shoot weigh, dry root weight, root surface area, root volume, and average root diameter. The second test was slightly different than the first due to the supplemental lighting being turned on for the seasonal changes in amount of natural daylight. The SeaClear treatments caused inhibitory effects on seedling germination and could not be included in the analysis due to half the seeds not germinating. All seaweed extract treatments showed positive trends towards growth promotion across height, dry shoot weigh, and dry root weigh. If there was another replication then some statistical significances may be seen. The Digestate seaweed extract showed significant differences compared to the control across all WinRHIZO measurements, average root diameter, root volume, and root surface area.

Discussion

In vitro:

The *in vitro* tests showed that at least one of the formulations of seaweed extracts, SeaClear, promoted the growth of multiple PGPR strains. Modifications to this test may help elucidate how SeaClear increased the growth of PGPR strains. Bashan et al. (2002) reported that processed forms of algae provided a nutrient source for *Azospirillum* spp. to increase the ability of PGPB to survive in soil. Different concentrations of the seaweed extract in the TSS solution could be tested to determine if there is a minimum concentration that is able to help the PGPR grow.

In planta:

The second test showed more growth promotion effects of the seaweed extracts, so another test done under the same conditions could help confirm the those results. Since SeaClear showed inhibitory germination effects in soil there needs to be an alternate test on dose response, so that germination inhibition is not a problem. Once an optimal concentration is achieved this should be tested alongside PGPR to see if it can enhance colonization by the PGPR. The plants in the second test may have experienced conditions similar to drought stress because of the increased heat directly impacting the soil. Therefore, some of these growth promotional effects may be in response to alleviating abiotic stress. A drought stress test could help focus on the differences made by seaweed extracts and their gamma irradiated counterparts.

Future Directions:

Long-term studies should be done to evaluate chlorophyll content after the application of seaweed extracts via foliar and soil drenches, because the betaines have been reported to decrease chlorophyll degradation (MacKinnon et al. 2010). The seaweed extract formulations should be individually tested in a dose response study to evaluate the concentration of seaweed extract most beneficial to the plants chlorophyll content. Many studies including the one from El-Alsayed et al. (2018) used rates as low as 0.5%. The isolation of microorganisms from seaweed extracts is important for the discovery of new PGPR strains that contain qualities *in vitro* such as phosphate solubilization, nitrogen fixation, and siderophore production (Flewelling et al 2013). A culture independent method for future work evaluating if PGPR can use complex carbohydrates within seaweed extracts is to use a stable isotope C-13.

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Figure 8. *In vitro* test using TSS and MBI600

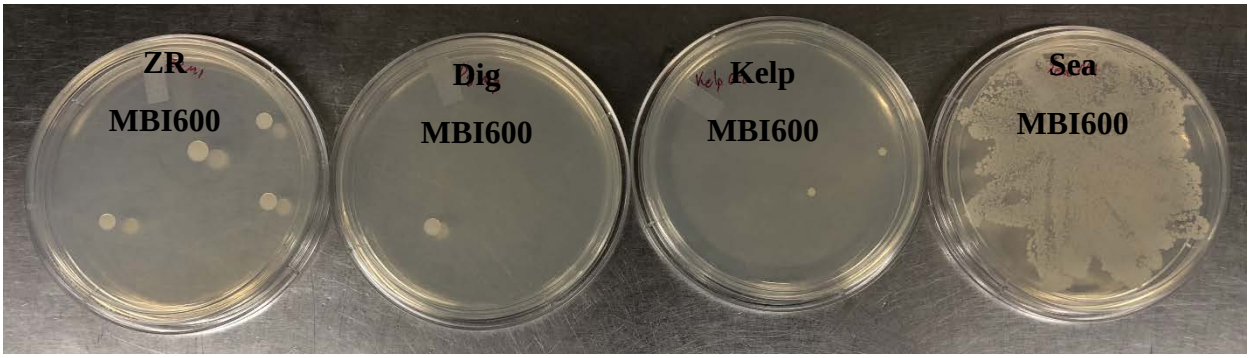

Figure 9: Root Surface Area Test 1

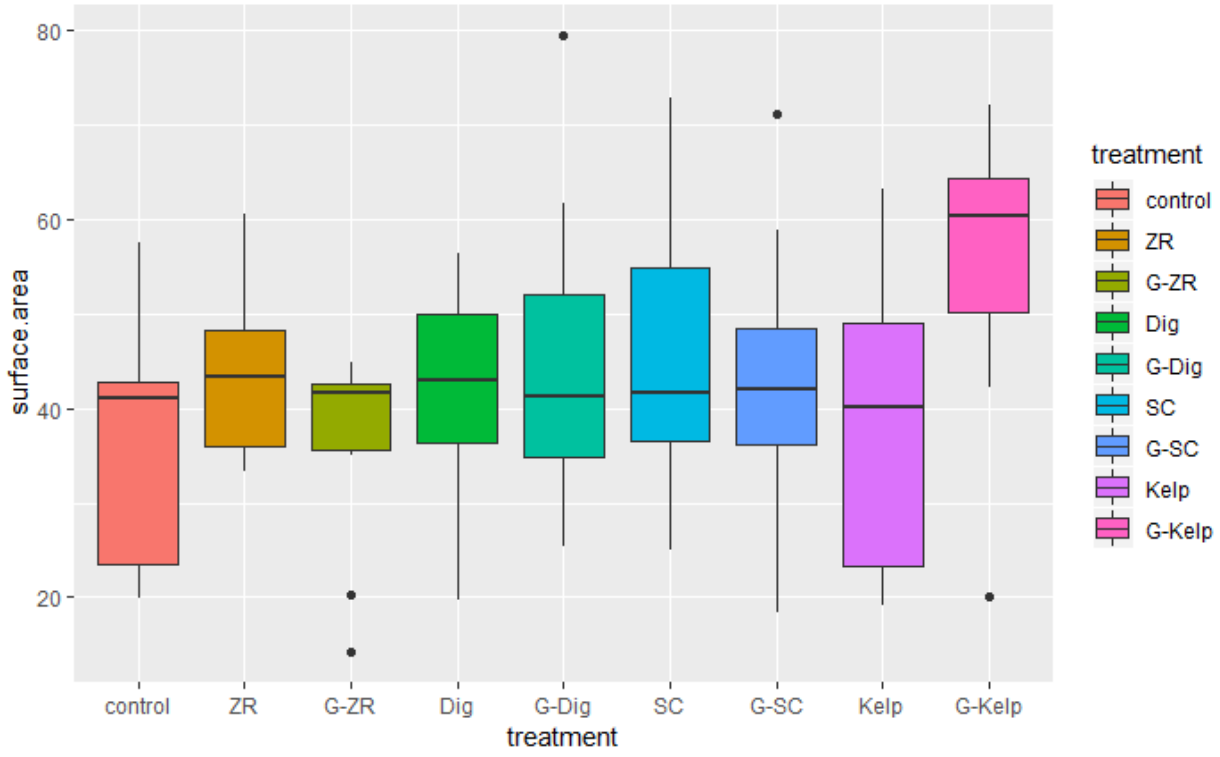

Table 1: Root Surface Area Test 1

```
Tukey multiple comparisons of means
    95% family-wise confidence level
    factor levels have been ordered

Fit: aov(formula = surface.area ~ factor(treatment), data =
SE.growth.trial.1)

$`factor(treatment)`
                   diff         lwr      upr     p adj
G-ZR-control    0.35963 -19.1781671 19.89743 1.0000000
Kelp-control    2.93636 -16.6014371 22.47416 0.9999195
Dig-control     5.26739 -14.2704071 24.80519 0.9944201
G-SC-control    7.25184 -12.2859571 26.78964 0.9578881
ZR-control      7.39886 -12.1389371 26.93666 0.9527303
SC-control      9.07355 -10.4642471 28.61135 0.8613997
G-Dig-control   9.18385 -10.3539471 28.72165 0.8532039
G-Kelp-control 19.34766  -0.1901371 38.88546 0.0543332
```

Figure 10: Root Volume Test 1

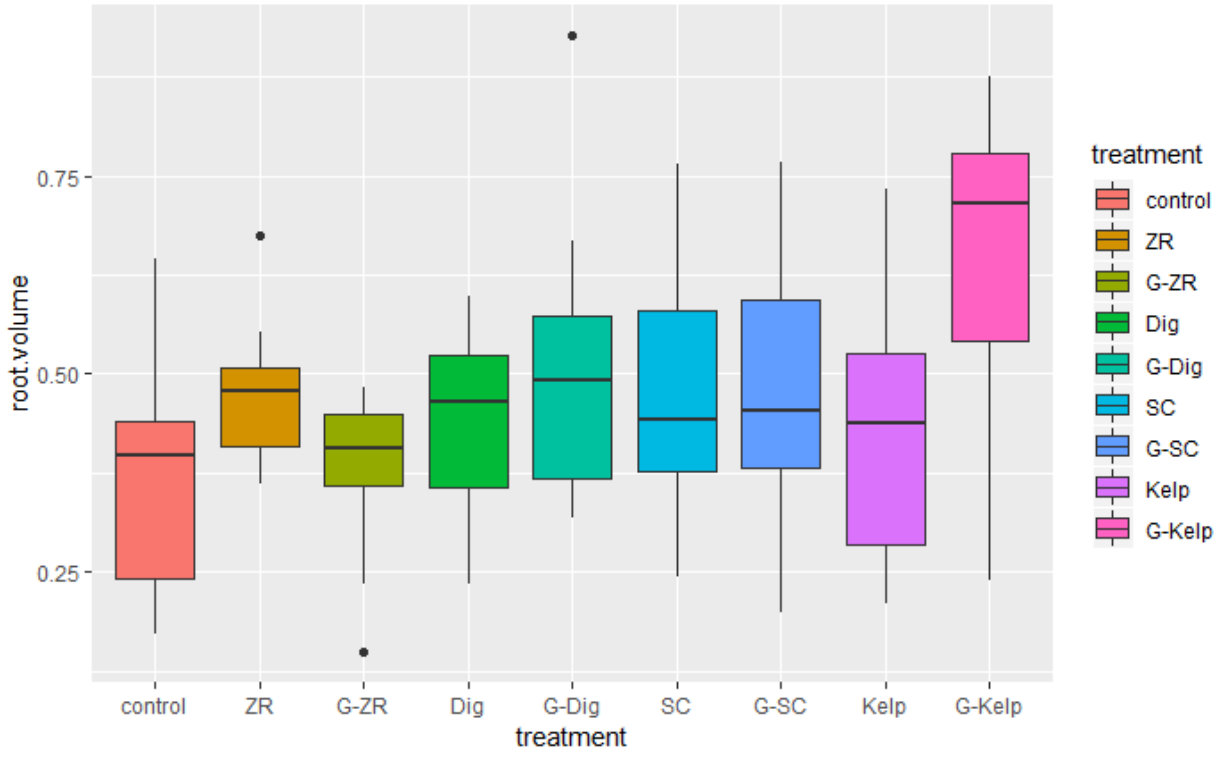

Table 2: Root Volume Test 1

```
Tukey multiple comparisons of means
    95% family-wise confidence level
    factor levels have been ordered

Fit: aov(formula = root.volume ~ factor(treatment), data = SE.growth.trial.1)

$`factor(treatment)`
                 diff         lwr       upr     p adj
Dig-control    0.0589 -0.16666815 0.2844681 0.9955104
Kelp-control   0.0653 -0.16026815 0.2908681 0.9910230
SC-control     0.0862 -0.13936815 0.3117681 0.9502218
ZR-control     0.0950 -0.13056815 0.3205681 0.9152298
G-SC-control   0.0985 -0.12706815 0.3240681 0.8977616
G-Dig-control  0.1320 -0.09356815 0.3575681 0.6391182
G-Kelp-control 0.2676  0.04203185 0.4931681 0.0086310
```

Figure 11: Average Root Diameter Test 1

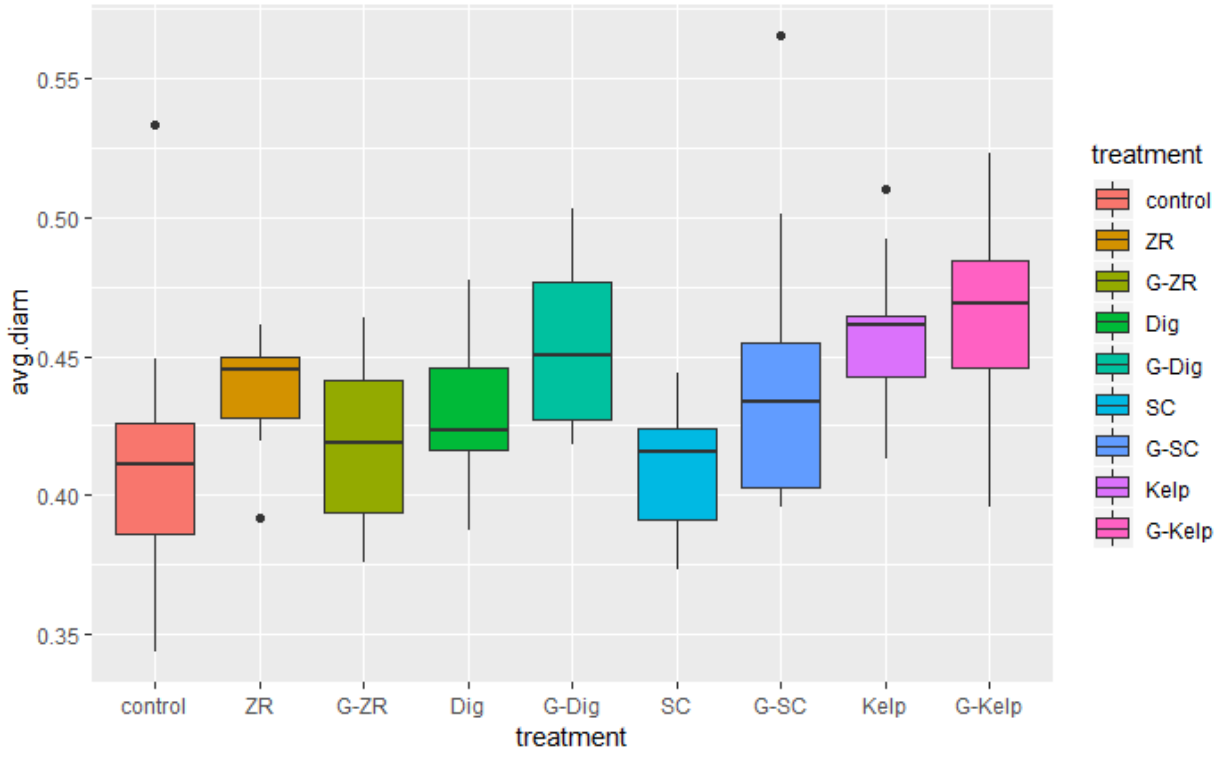

Table 3: Average Root Diameter Test 1

```
  Tukey multiple comparisons of means
    95% family-wise confidence level
    factor levels have been ordered

Fit: aov(formula = avg.diam ~ factor(treatment), data = SE.growth.trial.1)

$`factor(treatment)`
                    diff           lwr        upr     p adj
G-ZR-control     0.00331 -0.0476058234 0.05422582 0.9999999
Dig-control      0.01416 -0.0367558234 0.06507582 0.9931230
ZR-control       0.02277 -0.0281458234 0.07368582 0.8847241
G-SC-control     0.02856 -0.0223558234 0.07947582 0.6899012
G-Dig-control    0.03841 -0.0125058234 0.08932582 0.2958794
Kelp-control     0.04299 -0.0079258234 0.09390582 0.1675282
G-Kelp-control 0.05070 -0.0002158234 0.10161582 0.0518492
```

Figure 12: Dry Root Weight Test 2

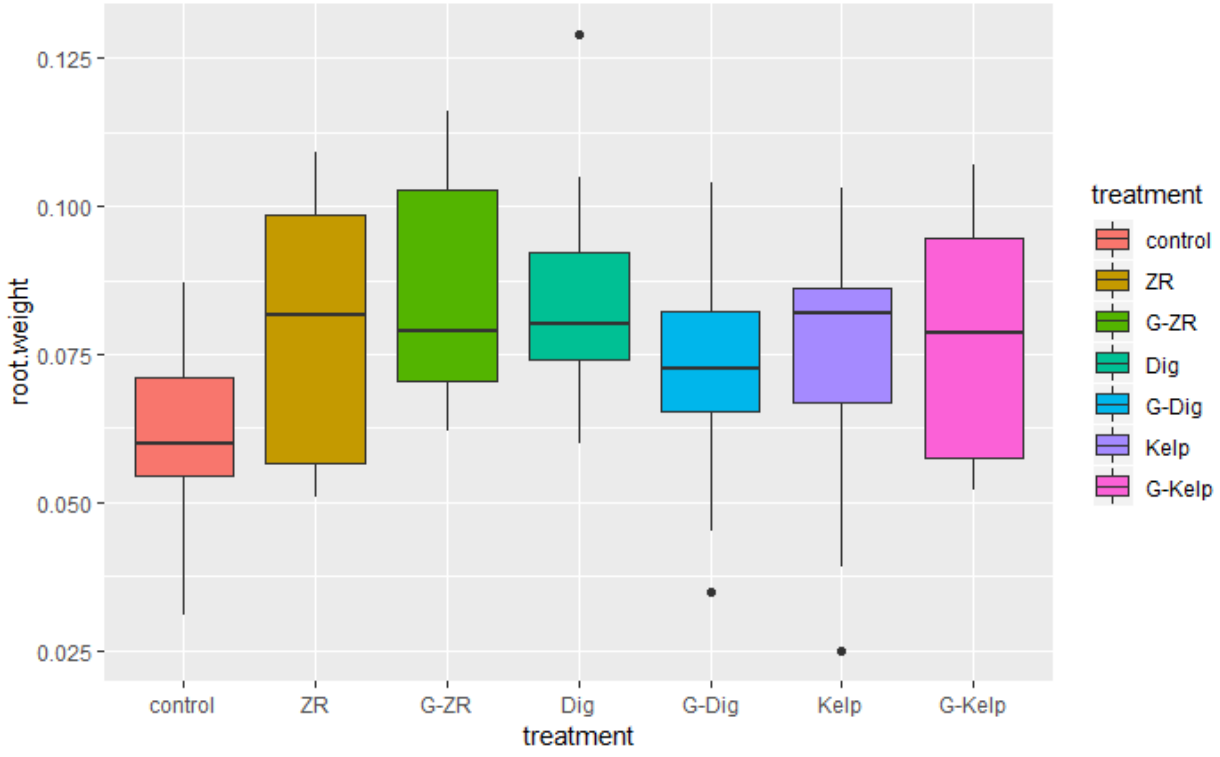

Figure 13: Shoot Weight Test 2

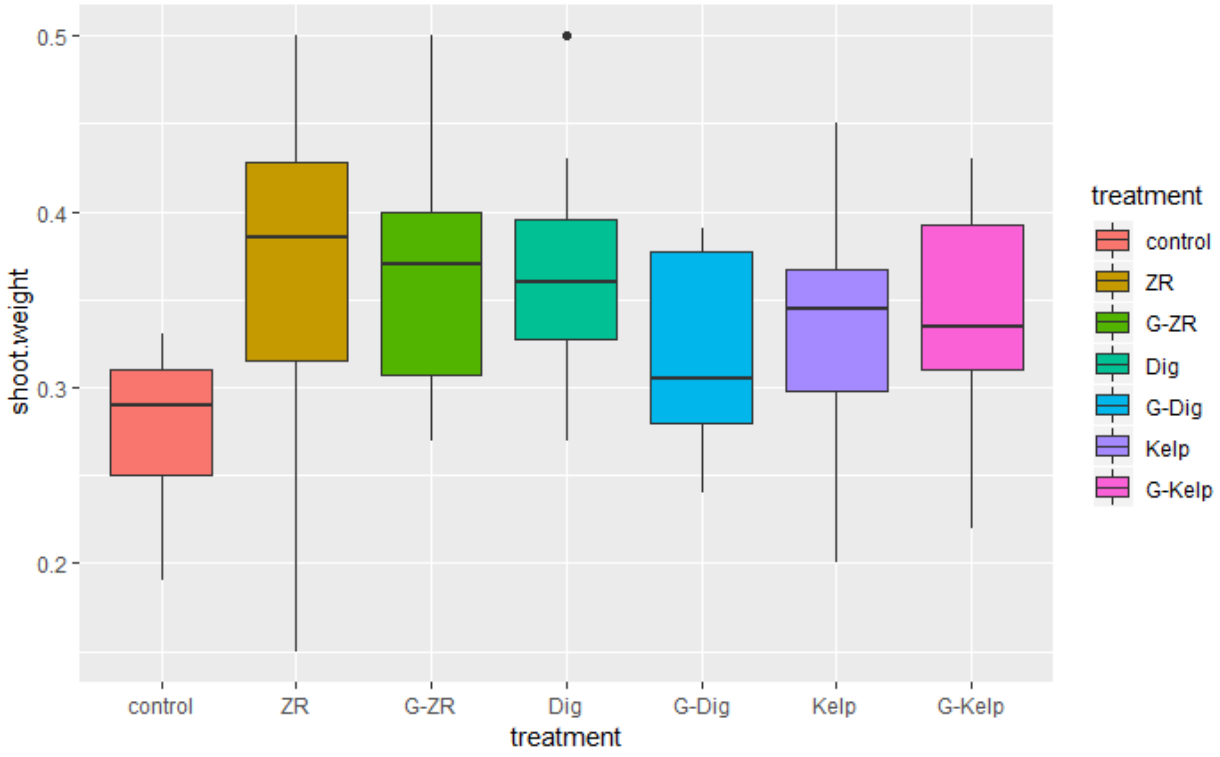

Figure 14: Plant Height Test 2

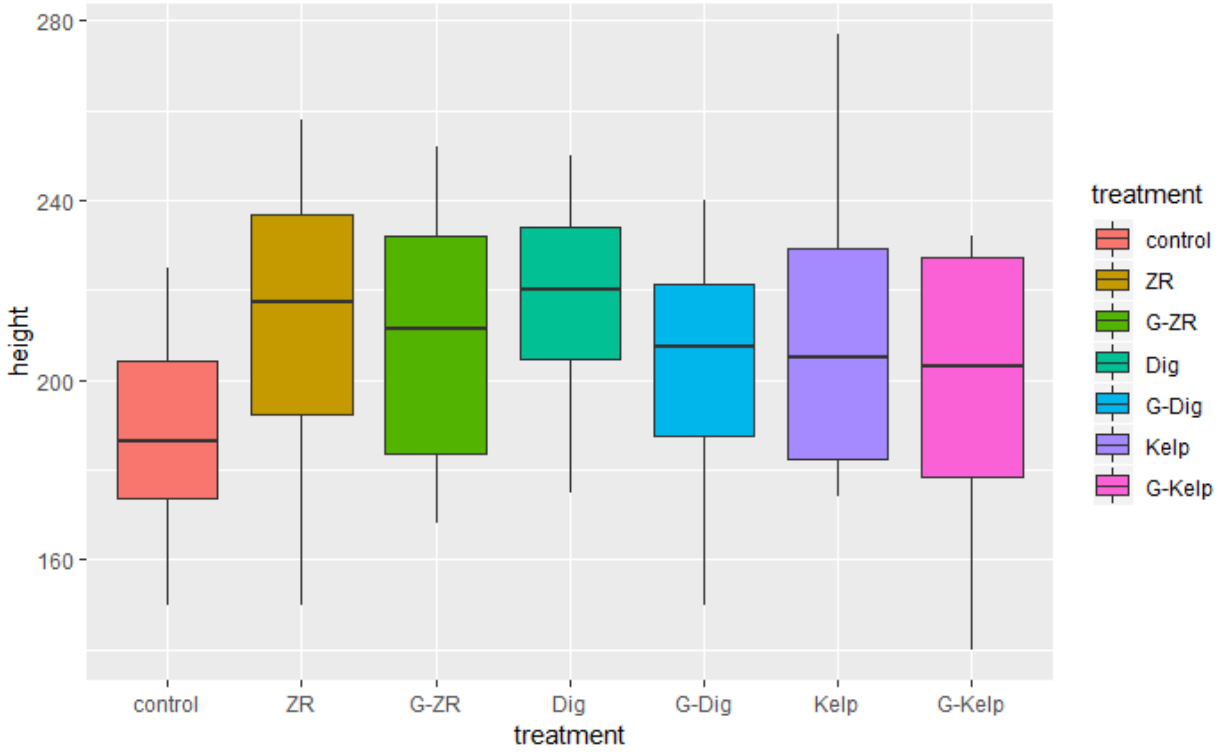

Figure 15: Root Surface Area Test 2

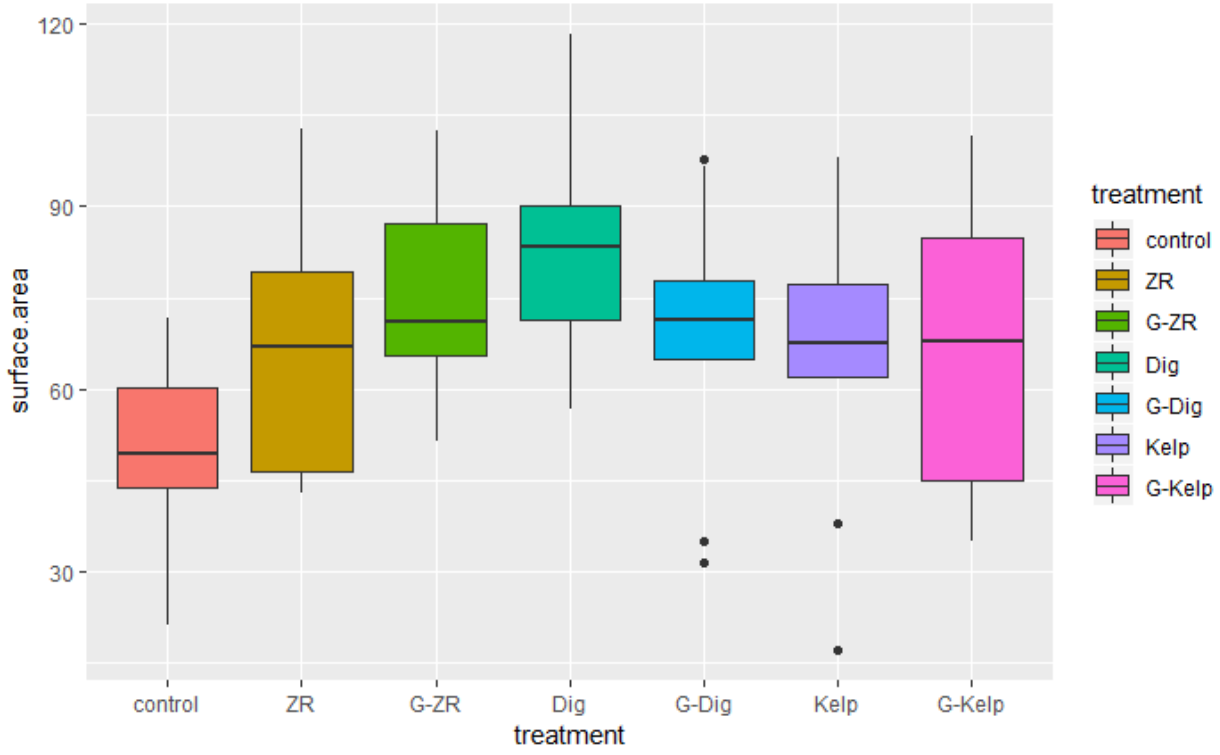

Table 4: Tukey HSD of Root Surface Area Test 2

```
Tukey multiple comparisons of means
    95% family-wise confidence level
    factor levels have been ordered

Fit: aov(formula = surface.area ~ factor(treatment), data =
SE.growth.trial.2)

$`factor(treatment)`
                   diff        lwr      upr     p adj
Kelp-control   13.23404 -14.680689 41.14877 0.7759040
G-Kelp-control 14.67737 -13.237359 42.59210 0.6819157
ZR-control     16.58779 -11.326939 44.50252 0.5466308
G-Dig-control  17.75654 -10.158189 45.67127 0.4641437
G-ZR-control   24.58612  -3.328609 52.50085 0.1198072
Dig-control    32.03658   4.121851 59.95131 0.0145060
```

Figure 16: Root Volume Test 2

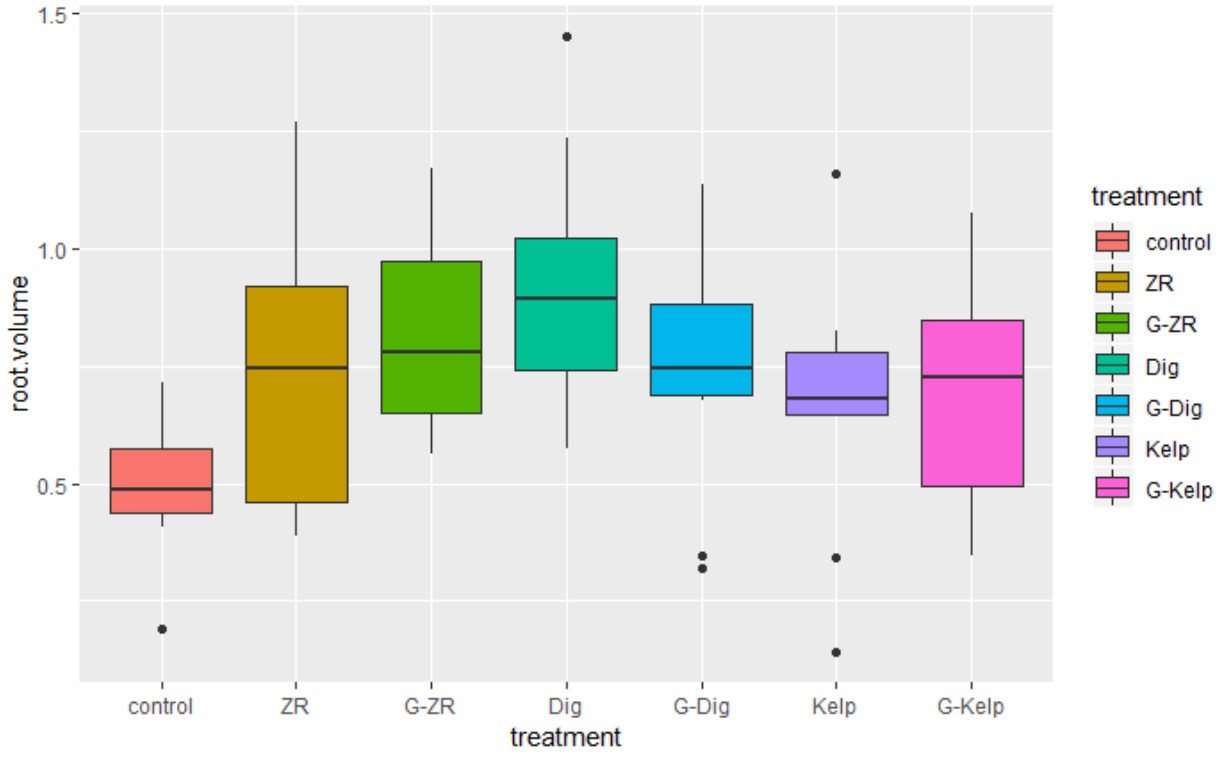

Table 5: Tukey HSD of Root Volume Test 2

```
95% family-wise confidence level
    factor levels have been ordered

Fit: aov(formula = root.volume ~ factor(treatment), data = SE.growth.trial.2)

$`factor(treatment)`
                  diff         lwr       upr     p adj
Kelp-control    0.1719 -0.17422661 0.5180266 0.7363244
G-Kelp-control 0.1938 -0.15232661 0.5399266 0.6151523
ZR-control      0.2596 -0.08652661 0.6057266 0.2678207
G-Dig-control  0.2606 -0.08552661 0.6067266 0.2636076
G-ZR-control    0.3265 -0.01962661 0.6726266 0.0769297
Dig-control     0.4286  0.08247339 0.7747266 0.0063171
```

Figure 17: Average Root Diameter Test 2

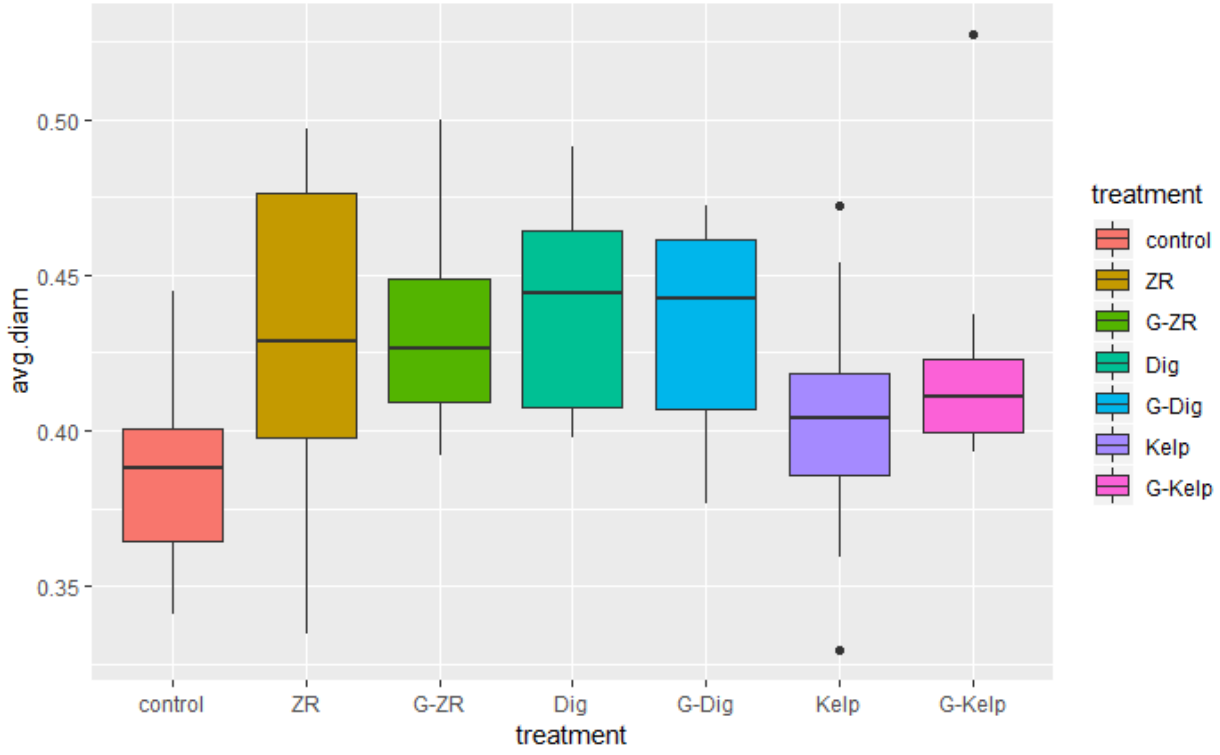

Table 6: Tukey HSD of Average Root Diameter Test 2

```
Tukey multiple comparisons of means
    95% family-wise confidence level
    factor levels have been ordered

Fit: aov(formula = avg.diam ~ factor(treatment), data = SE.growth.trial.2)

$`factor(treatment)`
                   diff           lwr        upr     p adj
Kelp-control    0.01808 -0.034277126 0.07043713 0.9394891
G-Kelp-control 0.03717 -0.015187126 0.08952713 0.3305546
ZR-control      0.04591 -0.006447126 0.09826713 0.1230139
G-ZR-control    0.04642 -0.005937126 0.09877713 0.1151210
G-Dig-control   0.04767 -0.004687126 0.10002713 0.0974897
Dig-control     0.05390  0.001542874 0.10625713 0.0395614
```