Brews and blooms: Development of a novel seaweed biostimulant supporting sustainable primary industry practices

A thesis

submitted partial fulfilment

of the requirements for the degree

of

Doctor of Philosophy in Ecology and Biodiversity

at

The University of Waikato

by

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THE UNIVERSITY OF
WAIKATO
Te Whare Wānanga o Waikato

2024

“Be the kindest and most loving despite the stress you go through.

Keep on pushing, I am proud of you”

- Jessica Blas (age 11)

Abstract

With a growing global population and increasing needs for environmentally friendly food production, novel technologies and products are required to increase food production sustainably. In this regard, seaweed biostimulants are an innovative tool to improve crop growth and yield. However, seaweed biomass is typically obtained through wild harvest or collection of beach cast, with challenges around the consistency of quality and reliability of supply. Furthermore, common manufacturing methods can have issues with high chemical input, loss of potentially important compounds, and cost. Therefore, this thesis aims to develop a seaweed biostimulant utilising the cultivated seaweed *Ulva stenophylloides* and a novel low-input fermentation method. This research covers optimisation of fermentation parameters and detailed compositional characterisation of the resulting products (Chapter 2), and the quantification of the effect of selected ferments on growth and yield in plants in hydroponics systems (Chapter 3) and in potted soil (Chapter 4).

The fermentation parameters: biomass loading, sucrose input, and incubation temperature were optimised. The initial sucrose input had the largest impact on fermentation progression and success, and was the main driver in resulting glucose content, total dissolved solids, pH, and pellicle yield. Excluding treatments with low biomass loadings, treatments with low and medium sucrose inputs reached completion and treatments with high sucrose inputs did not. As indicated by residual glucose, high biomass loadings provided valuable nutrients for microbial growth and high incubation temperatures were optimal for microbial growth. A range of nutrients were extracted from the seaweed biomass, but concentrations were insufficient as a sole nutrient source for plants based on chemical analyses. High biomass loadings were the main driver in the yield of protein and the sulfated polysaccharide ulvan, which both may modulate gene expression and induce metabolic changes in plants. High incubation temperature was the main driver for the yield of the auxin phenylacetic acid and indicates the production of other possible plant growth stimulating microbial biochemicals at high temperatures.

Of the four ferments selected for further testing by ferment progression and chemical composition, ferment four (high biomass loading, mid-range sucrose loading, and high incubation temperature) was the best performing biostimulant tested on mung bean seedlings in hydroponics; at a 1 % (v/v) dose, plant fresh and dry weight were increased by 15 and 16 %, respectively, and the most root growth was generated. An auxin-like effect was not detected at any biostimulant dose. At a 1 % dose, an interactive effect with fertiliser was demonstrated in tomato seedling root number. A 2 % dose of biostimulant had adverse impacts on root growth over multiple root growth assays. When applied to potted tomato plants, inconclusive results were obtained in growth and yield due to sub-optimal application volumes and growing conditions that require further method development and retesting.

Overall, this thesis advances knowledge in seaweed biostimulant manufacturing utilising *U. stenophylloides* with a fermentation production method. These results warrant further investigation into refinement of plant assays and growing conditions, the composition of the ferments through metabolomics and microbial identification, and the biostimulant effects on soil.

Acknowledgements

Firstly, I would like to thank my supervisors Dr Christopher Glasson, Dr Marie Magnusson, and Dr Mike Clearwater. Thank you for choosing me for the opportunity to do this research. Chris, you have kept me on my toes with your attentiveness to details and helped a lot with my writing and chemistry skills. Marie, you have been a tremendous help, thank you for all your valuable input. Mike, you have been a wonderful addition to the team, thank you for adding some balance, and your skills and knowledge to do with plants has been really valuable, it has been a pleasure working with you.

Huge thank you to all the people who helped me in the lab and with my plant assays, it most definitely took a team to complete; Mark Stantiall, Ariane Brandenburg, Logan Forsythe, Julia Rambacher, Nethmie Jayasooriya, Alisa Mihaila, Zoe Battershill, Bethany Hughes, Jacob Nepper-Davidson, Ashton Budd, Cindy Hartmann, Caitlin Thomson, Emma Donald, Eden Mutton, and Kiri Reihana. There were some really long and hard days in there that I could not have gotten through without you, your time and effort is much appreciated. Ari and Nethmie, thank you for taking your time (nice and slow) to help this baby scientist with her chemistry skills. Julia, those long hot days in the farm when you had so much of your own work to do were amazingly helpful. Ari and Logan, I would not have been able to complete this work without your assistance, you guys are superstars. Also, a special mention to the other staff at the Coastal Marine Field Station for your time and assistance.

I would like to thank the Entrepreneurial Universities Macroalgal Biotechnologies Program, University of Waikato, and the Tertiary Education Commission New Zealand for the financial support through my scholarship, this research would not have been possible without this help.

To my family and friends, I am so incredibly lucky to have you in my life. You have always cheered me on and have been a great deal of strength through the hard parts of this journey. Special shout out to my mumma Kerry Jenkins, you took on a lot to make sure I succeeded here: taking the time to look after my babies, turning off the

lights, the dorky waves, the long walks with the stroller, and the yummy meals and treats, thank you from the bottom of my heart, I love you very much - forever and always. To my brother Ben, I hope I am making you proud. I miss you.

To my soon to be husband Mark Stantiall and my babies, what a ride this has been! Mark, I cannot thank you enough - you have been so patient and my rock through all of this. There are no words to describe what you mean to me and how much your endless support has helped me along this journey, from listening to me vent, keeping my spirits up and motivation going, the sleep ins, all the help with the kids, running of the house, and help with the lab work, I really appreciate what you have done to make this possible. If we can make it through this, we can most certainly make it through anything! Marrying you will be the easiest decision of my life. My babies Jessica and Zachary, you both give me so much strength and determination, you give me purpose, and I cannot wait to be present with you and spend so much more time together. I will always love you – my amazing family.

Statement of contributions

This research was part of the Entrepreneurial Universities Macroalgal Biotechnologies Program, jointly funded by the University of Waikato and the New Zealand Tertiary Education Commission.

Principle and editorial guidance were provided by my supervisors from the University of Waikato; Christopher Glasson (chief supervisor), Marie Magnusson (co-supervisor), and Mike Clearwater (co-supervisor). Additional technical guidance was provided by Rebecca Lawton. Laboratory assistance was provided by Mark Stantiall, Ariane Brandenburg, Logan Forsythe, Julia Rambacher, Nethmie Jayasooriya, Alisa Mihaila, Zoe Battershill, Jacob Nepper-Davidson, Ashton Budd, Cindy Hartmann, Caitlin Thomson, Emma Donald, Eden Mutton, and Kiri Reihana.

Elemental analysis of ferment samples was conducted commercially by OEA Labs Ltd, United Kingdom, mineral content of ferment samples was determined by Dannielle Blackwell at the University of Waikato, Hamilton, New Zealand, and the leaf nutrient profiles were conducted commercially by Hill Labs, Hamilton, New Zealand.

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Chapter 1

General Introduction

1.1 Agriculture

The ability to feed the population worldwide relies on a robust, healthy, and productive primary industry sector. The population of Earth is projected to rapidly increase to over 9 billion people sometime between 2040 – 2050 (Tilman *et al.*, 2001; Vance, 2001; Rouphael & Colla, 2018). This swift population increase requires significantly greater quantities of food to ensure adequate nutrition for the growing amount of people. However, increased food production has led to the conversion of natural ecosystems into agriculture, loss of species biodiversity, greater use of fertilisers and pesticides, and has impacted water, soil and air quality (Vance, 2001). Thus, with a growing global population and increasing demand for food sources and food security coupled with environmental degradation, there is more pressure on modern agriculture to generate food in a way that increases productivity sustainably (Vance, 2001; Ghaderiardakani *et al.*, 2019).

When farming first began, functioning of agriculture relied on natural processes; sun, rain, plants, animals, soil, and human labour (Doran *et al.*, 1996). However, from the 1960s, the Green Revolution occurred (Davies, 2003; Evenson & Gollin, 2003). Also referred to as the Third Agricultural Revolution, the Green Revolution rapidly advanced farming methods by developing high yielding crops, chemical fertilisers, mechanisation, and irrigation (Matson *et al.*, 1997; Davies, 2003; Evenson & Gollin, 2003). In comparison to traditional farming, these new technologies greatly increased food production and employment, and reduced food cost, the amount of land required for food production, and labour input (Davies, 2003).

1.1.1 Fertilisers

To sustain high productivity, modern agriculture has increasingly relied on inputs of synthetic fertilisers to meet plant nutrient requirements (Vance, 2001; Halpern *et al.*,

2015). Fertilisers are designed to deliver essential plant nutrients, nitrogen (N), phosphorus (P) and potassium (K), in a bioavailable form to quickly enhance crop performance (Vance, 2001; Dawson & Hilton, 2011). The addition of fertilisers has allowed for farming practices to intensify and greatly increase crop production and yield to feed more people using less space than traditional farming methods (Tilman *et al.*, 2001; Trewavas, 2001; Vance, 2001). However, the nutrient efficiency of fertiliser is low and environmental costs of crop benefits are high. Nutrient efficiency for fertiliser application of N is around 50% (Kopittke *et al.*, 2019; Norton & Ouyang, 2019), P around 10-30% (Veneklaas *et al.*, 2012) and K around 40% (Baligar & Fageria, 2015), with the remaining nutrients accumulating in soil, leaching, degassing, or entering the surrounding environment as runoff. Consequently, N and P runoff from land can lead to detrimental environmental effects such as eutrophication of waterways, habitat destruction, loss of ecosystem services, extinction of species, and soil erosion (Matson *et al.*, 1997; Tilman *et al.*, 2001). So, while fertiliser use has allowed intensive farming practices to supplement nutrient levels for optimal crop growth and production inexpensively, there have been negative impacts on soil, waterways, crop productivity, quality, and overall environmental health (Halpern *et al.*, 2015).

Several measures are being taken to reduce the harmful effects of fertilisers, including the timing of fertilisation and the application of slow-release fertilisers (Norton & Ouyang, 2019). Additionally, new and innovative agricultural practices are being developed, for example, crop rotations or intercropping (Yang *et al.*, 2020). These measures are designed to address system-wide health, which will, in turn, improve and sustain overall productivity. Enhancements to current farming practices can lead to better environmental outcomes in terms of sustainability, less chemical use, healthy ecosystems, resource use, improved soil biology, plant health and growth.

1.1.2 A holistic approach

Globally, there is growing interest in a more holistic, sustainable approach to agriculture (Doran *et al.*, 1996; Plenchette *et al.*, 2005; Bulgari *et al.*, 2015). Scientific knowledge of ecosystem processes, modern technology, and societal partnerships will need to be challenged and embraced to develop the tools necessary to meet this

vision (Doran *et al.*, 1996). It is understood what makes a plant grow well; sunlight, water, carbon dioxide, and nutrients (Kumar *et al.*, 2016). However, overall system health is more than just the current agricultural NPK model (Kumar *et al.*, 2016). Plants and soil require the proper nourishment to ensure the best growth and yield. To improve system health, the focus needs to include soil health too, and the effects of phytohormones, trace elements, and complex sugars (Bulgari *et al.*, 2015). By focussing on single components, e.g. crop productivity, parameters that will improve ecosystem-wide health and sustainability for long term gain are overlooked.

To address the need for products that enhance system wellness, biostimulant use has become increasingly popular in modern farming practices as a new and innovative tool in environmentally sustainable food production (Bulgari *et al.*, 2015). When applied in small quantities, biostimulants promote plant growth and system-wide health (du Jardin, 2015). However, it is important to understand how plants respond to their environment, what makes a healthy plant, and what makes healthy soil, and then use that knowledge to produce products that encompass overall system wellness for a long-term benefit. Therefore, the following sections will provide a brief context and background on plant and soil health before discussing general biostimulants (section 1.6) and seaweed biostimulants (section 1.7).

1.2 Plant disease and stress

Plants inhabit complex environments with a wide range of fluctuating biotic and abiotic influences (Van Dam, 2009; Khan *et al.*, 2014). These environmental influences can be beneficial to crops, such as pollinators, some soil microbes, or sunlight, which help plants reproduce and grow; however, some interactions may be damaging and limit growth and development, such as herbivores, salinity, nutrient deficiency, or drought (Van Dam, 2009; Broekgaarden *et al.*, 2015).

In addition to natural environmental fluctuations, periods of stress on agricultural systems will become more frequent and intense due to the impact of climate change; including extreme weather conditions such as drought, increased temperatures, storms, flooding, and unpredictable rainfall patterns (Trewavas, 2001; Ghaderiardakani

et al., 2019). Additionally, plant disease has the potential to cause massive crop failures and loss. For example; late blight, caused by the plant fungus *Phytophthora infestans*, ravaged potato crops causing full crop failures in Northern Europe from mid-1800 and current crop losses still range between 9-44% (region dependent) (Arora *et al.*, 2014); rice blast, caused by fungus *Magnaporthe oryzae*, is a devastating disease on rice crops and can reduce yield by 10-35 % (Li *et al.*, 2019); and citrus huanglongbing, also known as citrus greening disease, caused by bacteria *Candidatus Liberibacter* spp., is a severe citrus disease affecting whole tree health, particularly in oranges and mandarins, and can reduce yield by 30-100 % (Dala-Paula *et al.*, 2019). These diseases have spread over extensive geographical areas and risk food security (Skamnioti & Gurr, 2009; Arora *et al.*, 2014). Therefore, crops must have resilient systems to adapt to periods of stress or disease.

1.3 Plant phytohormones and immunity

To survive and prevent damage, plants have physiological, biochemical and molecular responses to adapt to a range of stressors (Van Dam, 2009; Atkinson & Urwin, 2012). However, once defence mechanisms are initiated, resources are generally diverted from growth and reproduction to stress and immune responses (Herms & Mattson, 1992; Shao *et al.*, 2008). Therefore, understanding plant phytohormones and immunity will allow for novel ideas to support plant health for more resilient crops and greater yield.

In response to internal and external signals, plants can synthesise a range of phytohormones that regulate growth, and coordinate development, reproduction, and response to stress (Table 1.1) (Gray, 2004; Denancé *et al.*, 2013; Westfall *et al.*, 2013; Sezgin & Kahya, 2018).

Table 1.1: Summary of main plant phytohormones and their effects. Modified from: Westfall et al. (2013)

Phytohormone	**Main effect**	**Specific effects**
Auxin	Growth promotion	Cell elongation, apical dominance, tropisms, branching, lateral root growth
Brassinosteroids	Growth promotion	Cell division, stem elongation, root growth, photomorphogenesis (light-regulated development), reproduction, leaf senescence, stress response
Cytokinins	Growth promotion	Cell division, shoot formation, delaying senescence
Gibberellins	Growth promotion	Stem elongation, root growth, seed germination, floral development, fruit growth
Strigolactones	Growth promotion	Branching, leaf senescence, root development, plant-microbe interactions
Abscisic acid	Growth inhibition	Stomatal closure, seed maturation, germination, storage, desiccation tolerance, root and shoot growth, leaf senescence
Ethylene	Growth inhibition	Flowering and fruit ripening, stress response, seed germination
Jasmonic acid	Stress response	Stress response, root growth inhibition, necrotrophic pathogen response and induced systemic resistance (ISR)
Salicylic acid	Stress response	Stress response, biotrophic pathogen response and systemic acquired resistance (SAR)

The major signalling pathways regulating plant defence responses are salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) (Glazebrook, 2005; Koornneef & Pieterse, 2008; Van Dam, 2009; Denancé *et al.*, 2013; Broekgaarden *et al.*, 2015). However, other phytohormones (auxin, abscisic acid, cytokinins, gibberellins, and brassinosteroids) are also important immune response regulators (Denancé *et al.*, 2013). As with growth and development, there is considerable cross-talk between the signalling pathways for a coordinated and targeted immune response (Glazebrook, 2005; Browse, 2009; Broekgaarden *et al.*, 2015).

1.4 Soil health and quality

Soil and plants have a symbiotic relationship; therefore, to have strong, resilient, and healthy plants, it is essential to have fertile, healthy soil (Dawson & Hilton, 2011; Rinot *et al.*, 2019). Plant growth is supported by nutrients, root oxygen, and water accessed from the soil and microbiome in the rhizosphere, and soil also provides a medium to physically anchor roots (Doran et al., 1996). Furthermore, healthy, good quality soils provide many other ecosystem services beneficial to human health and wellbeing (Lal, 2016).

Like water, soil is an essential factor in the functioning of Earth's biosphere (Doran *et al.*, 1996). Because soil is a non-renewable resource (within a human lifespan) (Doran *et al.*, 1996; Yang *et al.*, 2020), it needs to be looked after to maintain functionality and sustainable systems to support plant and animal life. This life supporting property of soil has been demonstrated with archaeological links between several previous civilisations collapse and soil erosion and degradation (Olson, 1981). Unfortunately, lessons from the past have not been heeded. Still, due to the impact of human activities, soil depletion and degradation are currently occurring far more rapidly than fertile soil creation (Yang *et al.*, 2020). In fact, soil loss due to erosion is estimated to be around 20-30 Gt/year, and soil degradation is estimated at a global cost of $400 billion USD/year (Kopittke *et al.*, 2019).

These trends in soil erosion and degradation also apply to New Zealand (NZ) soils. Before humans arrived in NZ, the land was predominately covered in native forest (Schipper *et al.*, 2017). Over time, the natural landscape has been deforested for pasture, and intensification of agricultural production has been achieved through changes to farm management and the addition of N and P fertilisers (Schipper *et al.*, 2017). Deforestation has led to high rates of soil erosion and sedimentation of waterways (Dymond *et al.*, 2012; Fernandez, 2017), and high fertiliser use has led to nutrient excess and environmental damage (Snelder *et al.*, 2018). With soil resources having a high economic value (17 % of NZ's gross domestic product) (Martin *et al.*, 2017), and with the risk of environmental harm, it is crucial to have effective soil management and understanding of soil processes. However, soils are complex systems

that are comprised of interactions between physical, chemical, biological, and ecological properties (Lal, 2016; Rinot *et al.*, 2019). These properties have a dynamic and complex relationship directly influencing soil health as a vital living system (including a diverse microbial community) and soil quality as a functional system supporting plant and animal productivity (Lal, 2016).

1.5 Soil microbes

The physical, chemical, and nutrient properties of soil are strongly influenced by a diverse microbiome (Matson *et al.*, 1997; Miransari, 2013). One gram of soil can contain thousands of different microbial species with vital ecosystem function roles (Doran *et al.*, 1996; Fierer, 2017). For example, bacterial mucilage affects soil structure and aggregation by binding soil particles, reducing erosion risk (Watt *et al.*, 1993). Mycorrhizal fungi play a major functional role through their plant-root symbiotic relationship with 80 % of terrestrial plant families (Pivato *et al.*, 2007; Davison *et al.*, 2015; Lekberg & Waller, 2016), including most important agricultural crop species (Pivato *et al.*, 2007). Mycorrhizal fungi increase the reach of plant roots with their hyphae creating a crucial link further into the soil, improving uptake of water and nutrients (Jansa *et al.*, 2013). Mycorrhizal fungi also provide other benefits to plants, including stress resilience (Davison *et al.*, 2015; Lekberg & Waller, 2016), phytohormone production, and protection from root pathogens (Remy *et al.*, 1994).

Soil microbes also have a major impact on nutrient cycling. For example, microbial fixation of atmospheric N is regulated through ammonification; this converts the unavailable pool of N_2 gas into bioavailable forms of ammonium (NH_4^+), and through nitrification to nitrate (NO_3^-) (Fageria & Baligar, 2005). Of particular importance is the symbiotic relationship between N-fixing bacteria and the roots of legumes, where atmospheric N is enzymatically fixed by bacteria within plant root nodules (Gage, 2004). Additionally, microbial transformation of P in soil is essential to global P cycling and P availability (Richardson & Simpson, 2011). Microbes can be beneficial to plants through solubilisation and mineralisation of soil P to bioavailable forms of hydrogen phosphate (HPO_4^{2-}) and dihydrogen phosphate ($H_2PO_4^-$); although, microbes are also involved in the immobilisation of P (Richardson & Simpson, 2011).

1.5.1 Soil microbes in agriculture

The functioning of natural soil biology can suffer major changes under intensive farming (Matson *et al.*, 1997). Soil harm can be caused by farm management techniques, such as intensive tillage, heavy machinery, and pesticide and fertiliser use (Yang *et al.*, 2020) and subsequent changes to soil O_2, soil pH, and organic carbon (Fierer, 2017).

Conventional tillage has been used to improve crop yield; however, this negatively affects soil quality, the microbial community, and often results in erosion and runoff into the environment (Mohammadi, 2011). Compared to no tillage systems, conventional tillage decreases microbial biomass and soil carbon, resulting in poor nutrient content, deteriorated soil structure, and reduced water retention (Mohammadi, 2011; Wang *et al.*, 2017). Overuse of heavy machinery can lead to soil compaction; this reduces microbial activity, pore space, aeration and infiltration capability, and increases erosion and runoff (Upadhyaya, 1992). Tillage and heavy machinery affect the oxygen depth and distribution within soils and, in turn, key microbial functioning and processes. For example, in upper, oxygenated portions of the soil aerobic respiration can occur; below this point, anaerobic processes occur (Young & Ritz, 2000). This is important for microbial processes such as denitrification, which needs anaerobic conditions to take place (Sexstone *et al.*, 1985; Young & Ritz, 2000). Additionally, changes to land use may also have an impact on soil organic carbon. Results drawn from meta-analyses conclude that conversion of native vegetation to agricultural cropping reduces soil carbon by around an average of 30-43% (Guo & Gifford, 2002; Murty *et al.*, 2002; Kopittke *et al.*, 2017). Reduced soil organic carbon decreases the main energy source for microbes, water retention, mineralisation of plant nutrients, and capacity for decomposition (Lal, 2016; Kopittke *et al.*, 2019).

As demonstrated, plant and soil health are complex and dynamic topics, and agricultural harm has a major impact on the functioning of the immediate plant-soil system and surrounding ecosystems. However, due to increasing global population and the necessity of food supply and security, agriculture is an essential part of human

survival. Therefore, it is vital that novel technologies and products, such as biostimulants, are developed to reduce system-wide harm for sustainable, environmentally friendly food production.

1.6 Biostimulants

Biostimulants aim to enhance crop quality and yield through delivering a range of bioactive ingredients to improve system-wide health, such as soil health, nutrient uptake efficiency, and stress resilience (du Jardin, 2015). The use of biostimulants is important for reduced reliance on chemical fertilisers and pesticides, to increase organic matter and microbial activity in soils, and to decrease erosion and nutrient loss (Corte *et al.*, 2014). Biostimulant benefits extend further than just enhanced ecosystem health. With a rapidly growing global market, biostimulants have major economic potential. Growing at an annual rate of 11.46 % (Peter *et al.*, 2020), biostimulant worth is projected to increase from the 2018 market value of $2 billion (Rouphael & Colla, 2018) to reach $4.9 billion USD by 2025 (Peter *et al.*, 2020). This rapid increase in value is associated with the availability of novel products to address specific agricultural needs, the desire to use synthetic agrichemicals more effectively and efficiently, and increasingly deteriorating growing conditions (Rouphael & Colla, 2018). Additionally, policy is being driven towards waste reduction and circular bioeconomies (Ubando *et al.*, 2020). The biostimulant market contains a diverse range of substances and can be divided into several categories, with the major groups being humic substances, protein hydrolysates, microorganisms, and seaweed extracts (du Jardin, 2015; Halpern *et al.*, 2015).

1.6.1 Humic substances

Humic substances are a natural, vital component of soil as part of soil organic matter (SOM), resulting from the decomposition of plants, animals, and microbial residues (Rose *et al.*, 2014). Farm management practices, such as tillage and heavy machinery, reduce the natural humic material of soils (Piccolo & Mbagwu, 1997; Novotny *et al.*, 1999). Consequently, soil amendments containing humic substances derived from organic waste are sometimes applied to maintain soil productivity (Piccolo & Mbagwu,

1997). The biostimulant effect of humic substances is induced by hormone-like activity and enhanced interactions between organic matter, soil microbes and plant-root systems (Rose *et al.*, 2014). This improves soil structure and fertility, stress resilience, pH buffering capacity, cation exchange, water retention, and nutrient uptake (Rose *et al.*, 2014).

1.6.2 Protein hydrolysates

Protein hydrolysates are derived from amino acids and peptides extracted through thermal, chemical, and enzymatic hydrolysis of plant or animal products (Colla *et al.*, 2014; Corte *et al.*, 2014; du Jardin, 2015). The biostimulant effects of protein hydrolysates on plant and root growth is achieved through hormone-like activity and improved N uptake and metabolism (Schiavon *et al.*, 2008; Ertani *et al.*, 2013; Colla *et al.*, 2014). Nitrogen reduction and assimilation are enhanced through an increase of key enzymatic activity; specifically, nitrate reductase, nitrite reductase, glutamine synthetase, glutamate synthase, and aspartate aminotransferase, and C metabolism is improved through an increase in malate dehydrogenase, isocitrate dehydrogenase, and citrate synthase enzymatic activity (Schiavon *et al.*, 2008). Nutrient uptake and plant growth are supported by improvements to root growth and structure (Colla *et al.*, 2014) and increased microbial biomass (du Jardin, 2015). Stress resilience and growth promotion are enhanced by small peptides and amino acids acting as hormone precursors to induce phytohormone-like activity (Schiavon *et al.*, 2008).

1.6.3 Microorganisms

As summarised in the soil microbe section of this review (section 1.5), microbial processes in soil have a major impact on plant health and have an interconnected relationship with the structural and chemical properties of soil. Therefore, developing biostimulants as microbial inoculants has the potential to benefit soil and plant health. Inoculation with beneficial fungi and bacteria can result in biostimulant effects. For example, mycorrhizal fungi can improve the rate of mycorrhizae development (Sorensen *et al.*, 2008) and protect plants against pathogen-related disease (Sharma *et al.*, 1992; Albuquerque da Silva Campos, 2020) and improve resilience during salt

stress (Colla *et al.*, 2008), high alkalinity (Rouphael *et al.*, 2010) and drought (Sánchez-Blanco *et al.*, 2004). Additionally, due to the increased root reach of mycorrhizae hyphae, uptake and assimilation of P and other less mobile nutrients are enhanced, stimulating plant growth (Sorensen *et al.*, 2008). Fungal inoculation with *Trichoderma* suppresses disease from plant pathogens through antimicrobial and antifungal properties (Handelsman & Stabb, 1996; Kashyap *et al.*, 2017), improves the yield and nutrient content of fruit (Nzanza *et al.*, 2012), and increases phytohormone activity, resulting in enhanced growth (Hoyos-Carvajal *et al.*, 2009) and resilience to stress (Kashyap *et al.*, 2017). Bacterial inoculation with plant growth-promoting rhizobacteria share similar benefits to fungal inoculation with improvements to crop growth, yield and quality (Mia *et al.*, 2005; Mena-Violante & Olalde-Portugal, 2007), nutrient uptake (Mia *et al.*, 2005), disease suppression, and stress resilience (Zehnder *et al.*, 2001; Backer *et al.*, 2018). However, effects of microbial inoculants vary depending on the microbial and plant species or strain utilised, with some combinations of microbes and plants showing greater effectiveness than others (Plenchette *et al.*, 2005; Mena-Violante & Olalde-Portugal, 2007; Sorensen *et al.*, 2008; Hoyos-Carvajal *et al.*, 2009; Colla *et al.*, 2015).

1.7 Seaweed biostimulants

Within the seaweed industry, one of the most exciting developments is seaweed biostimulant products. Seaweeds, a type of macroalgae, are ubiquitous in coastal marine environments (Battacharyya *et al.*, 2015). As primary producers, seaweeds utilise photosynthetic energy and provide a vital food source to a range of marine life (Battacharyya *et al.*, 2015). Seaweeds also provide a complex habitat and shelter and contribute to several important ecosystem processes and services (Battacharyya *et al.*, 2015; Kraan, 2020). Types of macroalgae are generally classified into three broad groups based on their pigmentation; Ochrophyta (brown), Rhodophyta (red), and Chlorophyta (green) (Khan *et al.*, 2009). Each of these seaweed types, often right down to species, are different in their structure, composition, and exploitable qualities, making them unique in their benefits and uses as a human resource.

Humans have long been exploiting seaweeds for their benefits, with evidence of their use for food and medicine dating back at least 14,000 years (Dillehay *et al.*, 2008). Today, global seaweed resources are a multi-billion dollar industry with various applications being developed and investigated to meet growing demand. Currently, the diverse uses of seaweed include; food (Ortiz *et al.*, 2006; Yaich *et al.*, 2011), food products (Jensen, 1993), cosmetics, medicine, animal feed, fertilisers, bioplastics (Kraan, 2020), dyes, textiles (Janarthanan & Senthil Kumar, 2018), biochar (Roberts & de Nys, 2016), bioremediation of waterways (Lawton *et al.*, 2013), hydrocolloids (Khalil *et al.*, 2018), biostimulants (Khan *et al.*, 2009), and biofuels (Yanagisawa *et al.*, 2011; Hong *et al.*, 2014). In NZ, there are more than 900 known species of seaweed (Nelson *et al.*, 2019), and only a few are being utilised for their commercial potential (Table 1.2) (White & White, 2020). This suggests that the NZ seaweed industry is underutilised and at the early stages of development, which means there is enormous potential to create high-quality and diverse products to meet local and international demand. Additionally, very few of these species are cultured or farmed, with collection of seaweed biomass typically obtained through wild harvest, beach cast, or import. Developing seaweed products from cultivated species will help meet the goals of the NZ Government Aquaculture Strategy, which projects an increase of the NZ aquaculture industry from $600million in 2018 to $3billion NZD in value by 2035 (Nash, 2020).

Table 1.2: Seaweed species targeted for commercial products in New Zealand. Modified from: White and White (2020).

Seaweed type	Scientific name	Common name	Uses
Red (Rhodophyta)	*Pterocladia spp.*	Agar weed	Food products
	Gracilaria chilensis	Gracilaria weed, Sea moss	Food products
	Gigartina spp.		Food products
	Pyropia spp.	Nori, Karengo, Parengo, Reporepo	Food products
Brown (Ochrophyta)	*Macrocystis pyrifera*	Bladder kelp	Health products, biostimulant
	Ecklonia radiata	Brown kelp	Biostimulant, animal health
	Durvillaea spp.	Bull kelp, Rimurapa, Kōauau	Fertiliser, food products
	Lessonia variegata		Food products
	Undaria pinnatifida	Wakame	Food, cosmetic, and health products, biostimulant
Green (Chlorophyta)	*Ulva* spp.	Sea lettuce	Compost

Seaweeds are rich in bioactive compounds, making them a particularly attractive candidate for biostimulant production (Battacharyya *et al.*, 2015). In fact, seaweeds have been used as fertilisers to condition soil for centuries (Temple & Bomke, 1988). Currently, seaweed biostimulants makeup 37% of the rapidly growing biostimulant market (Peter *et al.*, 2020) and is dominated by products manufactured from brown algae (Sharma *et al.*, 2014; du Jardin, 2015). This pattern is also occurring in NZ, with the growing industry based on brown kelp species; *Ecklonia radiata*, *Undaria pinnatifida,* and *Macrocystis pyrifera* (White & White, 2020). Globally, *Ascophyllum nodosum,* a brown seaweed, is the most studied and utilised seaweed in biostimulant production (Khan *et al.*, 2009; Craigie, 2011; Hayyawi *et al.*, 2020), and a variety of the biggest international seaweed biostimulant brands also use brown seaweeds, such as *M*. *pyrifera, E. maxima, Fucus* spp*., Laminaria* spp*.* and *Durvillea* spp. (Khan *et al.*, 2009; Sharma *et al.*, 2014). Brown seaweeds are easily studied and utilised due to having great abundance and large size, rather than being targeted specifically for their

biochemical components (Stirk & Van Staden, 1997; Battacharyya *et al.*, 2015). Additionally, most of the seaweed biomass for biostimulant manufacturing is collected through wild harvest. This is due to the tendency of brown seaweed to grow in monospecific stands and the ease of shoreline harvesting after storms (Mac Monagail *et al.*, 2017). However, there are issues with the supply, reliability, and consistency of biomass and the impact of wild harvesting or collection of beach cast on the environment (Mac Monagail *et al.*, 2017). Therefore, other seaweed species and aquaculture of these must be considered and developed for a consistent and sustainable product and business model.

1.7.1 *Ulva* spp.

A current candidate for the production of a seaweed biostimulants are *Ulva* spp. Species of *Ulva,* generally known as sea lettuce, are a common and widespread green macroalgae found worldwide (Nelson, 2020). Species of *Ulva* are tolerant to a wide range of environmental conditions including, temperature, salinity, and nutrient availability (Nelson, 2020). Nineteen species of *Ulva* have been identified in NZ, and some of these form nuisance blooms due to their rapid and prolific growth (Heesch *et al.*, 2009; Nelson *et al.*, 2015). Currently, when bloom quantities of *Ulva* accumulate on beaches in Tauranga, NZ, removal of biomass is undertaken and used in commercial compost applied in organic orchids (White & White, 2020). However, the chemical characteristics of *Ulva* make it an attractive and promising genus for producing high-quality and valuable products such as biostimulants. *Ulva* spp. contain essential nutrients and vitamins, phytohormones, polysaccharides, and other compounds (Castellanos-Barriga *et al.*, 2017). Yet, the chemical composition of *Ulva* species varies depending on biotic and environmental conditions (Lahaye & Jegou, 1993; Yaich *et al.*, 2011). Also, depending on factors such as location and season, wild algae stocks may have high heavy metal content (Kaimoussi *et al.*, 2004; Yaich *et al.*, 2011).

Ulva species are particularly suitable for aquaculture in land-based systems. In aquaculture, *Ulva*'s rapid growth and broad environmental tolerance is a benefit which could be harnessed to produce a monoculture suitable for the development of a high-quality biostimulant. Aquaculture can control for deviations in chemical composition

and environmental variables and provide a reliable, consistent, and continuous source of biomass material that is renewable and sustainable (Mac Monagail *et al.*, 2017). Additionally, aquaculture has less impact on wild stocks and natural ecosystems (Mac Monagail *et al.*, 2017), has the potential for bioremediation of nutrient-rich wastewater (Lawton *et al.*, 2013; Kraan, 2020), and requires less processing to remove unwanted organisms and inorganic substances (Yaich *et al.*, 2011).

1.7.2 Extraction methods

An important part of the seaweed biostimulant manufacturing process is the method of extraction. Due to the complex cell wall of seaweeds, different extraction methods have been developed to maximise the yield of bioactive compounds (Godlewska *et al.*, 2016). Extracted bioactives from seaweed include; macro and micro nutrients, polysaccharides, phytohormones, amino acids, fatty acids, vitamins, polyphenols, and osmolytes (Khan *et al.*, 2009; Battacharyya *et al.*, 2015; Hayyawi *et al.*, 2020). To extract these components, seaweed biostimulants are generally processed through chemical extraction (acid or alkali hydrolysis), or aqueous extractions using heat and/or pressure (Arioli *et al.*, 2015). Other methods of extraction include; enzyme, ultrasound, and microwave assisted extraction, and supercritical fluid extraction (Michalak & Chojnacka, 2014; Godlewska *et al.*, 2016). These processes liquefy and extract seaweeds bioactive components into a concentrated liquid solution or a dried preparation for ease of use, storage and distribution. However, not all extracts are the same, and bioactives differ depending on the seaweed species utilised, season of production and harvest, and extraction method (Arioli *et al.*, 2015). Additionally, chemical extractions and other hydrothermal methods have several limitations, such as high chemical input, loss of potentially important compounds, and cost (Shobharani *et al.*, 2013; Boukhari *et al.*, 2020). This study will produce a seaweed biostimulant from cultivated *Ulva stenophylloides* using a more environmentally friendly fermentation extraction method to overcome these issues.

1.8 Benefits of seaweed biostimulants in agriculture

Due to being such a complex mix, the precise mechanisms and mode of action of seaweed bioactive molecules and synergistic effects remain hard to pinpoint; however, there is demonstrated improvements to agricultural systems following applications with seaweed biostimulants; particularly plant growth and stress resilience, and soil health (Khan *et al.*, 2009; Arioli *et al.*, 2015).

1.8.1 Plant growth promotion

Seaweed biostimulants have been reported to enhance the growth, yield, and quality of many important fruit and vegetable crops. For example, a kelp extract produced from *Durvillaea potatorum* and *Ascophyllum nodosum* increased leaf area by up to 70 %, stem diameter by 65 %, and biomass by 145 % in broccoli (Mattner *et al.*, 2013) and improved root density by 38 % and fruit yield by 8 % in strawberry (Mattner *et al.*, 2018). A seaweed extract produced from *Sargassum horneri* increased yield by 4.6 to 6.9 % and fruit hardness by 10.2 to 19.8 % in tomato (Yao *et al.*, 2020). Application of an *Ecklonia maxima* extract increased height by 35 %, pod number by 45 %, and seed number by 43 % in soybean (Kocira *et al.*, 2019). An *A. nodosum* extract increased fruit weight by 23 % and fruit length by 16 % in kiwifruit (Chouliaras *et al.*, 1995). Another extract produced from *A. nodosum* increased yield by 120.8 % in onion (Dogra & Mandradia, 2012). An extract produced from *E. maxima* increased yield by 51.5 % in spinach (Rouphael *et al.*, 2018).

Initially, plant responses to seaweed biostimulants were attributed to the nutrient content of the extract, improvements to soil texture, and soil water holding capacity (Craigie, 2011). However, the effects on plant growth cannot be explained by the nutrient content of seaweed biostimulants alone (du Jardin, 2015). While seaweed extracts contain nutrients that can enhance plant nutrition and play a critical role in plant development, these are typically not present in high enough concentrations to meet nutritional requirements for plant growth (du Jardin, 2015; Yakhin *et al.*, 2017). In general, observed benefits to growth and nutrient uptake are associated with changes to physiological and biochemical processes; for example, improvements to

photosynthesis (Yao *et al.*, 2020), modulation of phytohormones (Khan *et al.*, 2009), uptake and utilisation of nutrients and water (Mattner *et al.*, 2018), enzymatic regulation (Cluzet *et al.*, 2004), and root architecture (Mattner *et al.*, 2018). Regulation of these plant growth processes are thought to be induced by content of polysaccharides, phytohormones, vitamins, amino acids, and osmolytes contained in seaweed extracts, but it is not fully understood exactly how these components work together to elicit the observed effects (Khan *et al.*, 2009; Sharma *et al.*, 2014). However, key components, such as polysaccharides and phytohormones, have been analysed separately for effects.

Seaweeds contain large amounts of cell wall polysaccharides, some of which are not found in terrestrial plants (Alves *et al.*, 2013). For example, brown seaweeds contain laminarian, alginate, and fucoidan (Khan *et al.*, 2009; Hong *et al.*, 2014), red seaweeds contain agar, mannan, and carrageenan (Hong *et al.*, 2014), and green seaweeds contain ulvan (Alves *et al.*, 2013; Kidgell *et al.*, 2019). These polysaccharides have an essential role within algae for a range of biological functions and can be applied to various novel technologies, including biostimulants (Alves *et al.*, 2013). Algal polysaccharides have been reported to improve plant growth by enhancing nitrogen assimilation, photosynthesis, cell division, basal metabolism, increasing fatty acid content, and protection from pathogens (González *et al.*, 2013).

As introduced in section 1.3, plant phytohormones and immunity, the growth and development of plants are regulated by phytohormones. Many types of plant growth regulating phytohormones have been identified in seaweed extracts, such as auxins, gibberellins, cytokinins, abscisic acid, and ethylene (Panda *et al.*, 2012). Studies suggest that plant phytohormones in seaweed extracts are beneficial as plant growth regulators, particularly cytokinin for shoot growth and auxin for root development (Werner *et al.*, 2001; Khan *et al.*, 2009). In plants, cytokinin promotes cell division, shoot formation, root growth, and delays senescence (Westfall *et al.*, 2013). Biostimulant effects on crop yield such as earlier flowering, more flowers, larger fruit, and improved quality, are thought to be due to the presence of cytokinin in seaweed extracts (Panda *et al.*, 2012; Ali *et al.*, 2021). Auxin also plays a significant role in plant

development. Auxins key roles involve cell elongation, apical dominance, tropisms, branching, and lateral roots (Westfall *et al.*, 2013). Biostimulant effects on shoot growth and root stimulation, including enhancement of lateral root growth and total volume of root system leading to increased surface area for nutrient and water uptake, has been attributed to auxin-like activity in seaweed extracts (Battacharyya *et al.*, 2015). Also, cytokinins and auxins enhance chlorophyll content in leaves, improving photosynthesis and therefore, growth (Blunden *et al.*, 1996).

1.8.2 Plant defence against stress, pests, and disease

As explained in the plant stress and phytohormone sections of this chapter (sections 1.2 & 1.3), plants are exposed to a variety of continuous and variable stressors. Stress response involves signalling pathways via plant phytohormones to connect environmental influences to cellular responses and are crucial to the survival of plants through periods of stress (Westfall *et al.*, 2013). Additionally, once defence mechanisms are initiated, resources are diverted from growth and reproduction to stress and immune responses compromising crop yield (Herms & Mattson, 1992; Shao et al., 2008). Therefore, for agricultural crops to maximise productivity, it is essential to develop products that enhance resilience to stress. Seaweed biostimulants contain bioactives that can mitigate stress from abiotic and biotic sources. However, once again, it is not fully understood how each of the bioactive components induces positive responses and their synergistic mode of action (Ali *et al.*, 2021).

1.8.2.1 Abiotic stress

Major issues with crop productivity can arise from abiotic stress, particularly drought, salinity, and temperature (Bulgari *et al.*, 2019). Abiotic stress can cause extreme water loss in plants and results in damage to cell membranes, DNA, lipids, carbohydrates, and proteins due to osmotic stress, ion imbalance, and oxidative toxicity (Khan *et al.*, 2009; Patel *et al.*, 2018). Finding solutions for these stressors is essential due to the increasing frequency and intensity of abiotic stress resulting from climate change (Trewavas, 2001; Ghaderiardakani *et al.*, 2019). By 2050, 50 % of arable lands are predicted to be affected by high saline and drought environments (Ali *et al.*, 2021).

Drought stress can result in minor to complete crop losses depending on the stage of plant development and severity and duration of stress (Farooq *et al.*, 2009). Additionally, temperature stress also occurs during cold periods, and most crop plants are stressed when temperatures drop below 10 °C (Karabudak *et al.*, 2014). Seaweed biostimulants can relieve damage and improve response to abiotic stressors through enhancements to root morphology, metabolism, water regulation, and accumulation of polysaccharides and osmolytes (Ali *et al.*, 2021).

The imbalance of water and damage to photosynthetic pigments in response to ionic and osmotic stress can harm plants when faced with abiotic stress. Application of seaweed biostimulants can improve water regulation, regulate gene expression, and reduce harm to photosynthetic processes in response to stress. Under drought stress, the application of *A. nodosum* seaweed extract on spinach resulted in increased leaf growth and photosynthetic rate induced by improved leaf water relations (Xu & Leskovar, 2015). In another study, application of an *A. nodosum* seaweed extract under drought stress in soybean demonstrated improvements to gene expression and plant physiology that resulted in greater relative water content, stomatal conductance, antioxidant activity, and upregulation of stress-related genes (Shukla *et al.*, 2017). Under drought and high salinity conditions, application of *Kappaphycus alvarezii* seaweed extract on durum wheat resulted in higher water content, upregulation of defence-related genes, enhancement of chlorophyll content, prevention of damaging ROS production, improvement of ionic balance, and maintenance of cell turgor (Patel *et al.*, 2018). Polysaccharides extracted from *Lessonia nigrescens* alleviated salt stress in wheat through increased chlorophyll content, improving antioxidant activity and regulating intracellular ions (Zou *et al.*, 2019).

Algae contain betaines, an essential osmolyte composed of nitrogenous molecules that alleviates osmotic stress in plants during periods of drought or increased salinity (Panda *et al.*, 2012). By buffering against osmotic changes, betaines ensure that turgor and water movement within the leaf is maintained; in turn, this helps keep stomata open and continue taking up CO_2 for photosynthesis, improving growth and response during stress (Estaji *et al.*, 2019). Betaines also reduce the degradation of chlorophyll,

leading to improved leaf chlorophyll in plants after biostimulant application (Blunden *et al.*, 1996). Without this increased resistance to chlorophyll damage, photosynthesis is potentially impaired (Estaji *et al.*, 2019). Betaines can also protect against low temperatures. For example, glycine betaine increased fatty acid content in tomato, providing protection at low temperatures through alteration of the lipid content of the cell membrane (Karabudak *et al.*, 2014). Cell membranes are typically the first site of harm during low temperatures, and increased cell membrane fatty acid content has an association with alleviation of photoinhibition and protection in chill resistant plants (Liu *et al.*, 2008). Therefore, betaines are potentially an essential component of seaweed biostimulants capabilities in resilience to abiotic stressors.

1.8.2.2 Biotic stress

After applying seaweed biostimulants, plants can become more resistant to fungal, viral, and bacterial pathogens, and attack from nematodes and insects. For example, *A. nodosum* seaweed extract upregulated defence genes, increased enzymatic defence activity, and phytoalexins leading to reduced fungal infections of *Alternaria* and *Botrytis* in carrot by 57 % and 53.5 %, respectively (Jayaraj *et al.*, 2008). Three extracts produced from brown seaweeds *Cystoseira myriophylloides, Laminaria digitata*, and *Fucus spiralis,* all significantly reduced disease severity through induced resistance in tomato against the fungal pathogen *Verticillium dahlia* and the bacterial pathogen *Agrobacterium tumefaciens* (Esserti *et al.*, 2017). A sodium alginate extract significantly reduced the number of lesions in tobacco plants caused by *Tobamovirus* sp. (the tobacco mosaic virus) by blocking the virus at the plant cell membrane surface (Sano, 1999). Seaweed extracts have been shown to play a major role in reducing nematode invasion and egg recovery in tomatoes (Wu *et al.*, 1997) and *Arabidopsis thaliana* (Wu *et al.*, 1998). A *Padina pavonica* brown seaweed extract demonstrated nymphicidal and ovicidal properties, causing more than 85 % mortality of *Dysdercus cingulatus* (red cotton stainer) nymphs and eggs after 48-72 hours exposure (Sahayaraj & Kalidas, 2011). Therefore, seaweed extracts can act as a natural pesticide to protect plants against a wide range of biotic harm.

Along with other seaweed bioactives such as fatty acids, carotenoids (Hamed *et al.*, 2018), and betaines (Wu *et al.*, 1998) which have antipest and antimicrobial properties, polysaccharides have been shown to be an important component of seaweed biostimulants that acts as a protectant to biotic stress. The unique polysaccharides in algal extracts can act as elicitors and activate salicylic acid, jasmonic acid, and/or ethylene stress signalling pathways (Jaulneau *et al.*, 2010; Vera *et al.*, 2011). Activation of these hormonal stress pathways build resilience to biotic stress by increasing expression of genes involved in pathogen defence (Mercier *et al.*, 2001; Cluzet *et al.*, 2004; Vera *et al.*, 2011). For example, in tobacco, carrageenan activated salicylic acid, jasmonic acid, and/or ethylene signalling pathways and induced defence genes (Mercier *et al.*, 2001). Laminarian has been demonstrated to induce antifungal properties in alfalfa (Kobayashi *et al.*, 1993). Tobacco treated with fucan (a brown algae polysaccharide) activates the salicylic acid signalling pathway and accumulates defence response phytoalexins and pathogenesis-related proteins, and also primes the systemic acquired resistance response against the tobacco mosaic virus (Klarzynski *et al.*, 2003). Ulvan, extracted from *Ulva* spp., induces an effect similar to treatment with methyl jasmonate, eliciting a jasmonic acid response (Jaulneau *et al.*, 2010). Additionally, ulvan upregulates a range of defence-related genes; including genes related to enzymes involved in phytoalexin and phenylpropanoid biosynthesis, several pathogenesis-related genes, and cell wall proteins, which, in turn, protected against anthracnose fungus in barrel clover *Medicago truncatula* (Cluzet *et al.*, 2004). Importantly, upregulation of immune response, in this case, did not sacrifice or reduce primary metabolism-related genes, therefore allowing the plant to have pest protection and continue to grow simultaneously (Cluzet *et al.*, 2004).

Development of pest control from organic sources can reduce environmental harm due to effects at lower doses, reduced risk to non-target organisms, and higher biodegradability than synthetic pesticides (Hamed *et al.*, 2018). Therefore, seaweed extracts have an important role as an environmentally friendly way to reduce chemical pesticide use and control disease in agriculture.

1.8.3 Soil health

Maintaining fertile, healthy soil and reducing erosion remains a challenge to agriculture worldwide (section 1.4). In addition to promoting the growth of plants, seaweed biostimulants also affect soil conditions which improve soil microbiome health, soil structure and chemistry, water retention, resilience to abiotic and biotic stress, plant growth and seed germination, and root structure (Khan *et al.*, 2009; du Jardin, 2015). Due to the previous sections discussing the effects of biostimulants on plant growth and stress, this section will focus on soil microbes, structure and chemistry, and water retention.

Seaweed biostimulants can enhance the growth of beneficial microbes, which greatly promotes plant growth (Kuwada *et al.*, 2006; Alam *et al.*, 2014; Renaut *et al.*, 2019). Application of extracts from red and green algae stimulated hyphal growth of mycorrhizal fungi in vitro and enhanced root colonisation in papaya and passionfruit (Kuwada *et al.*, 2006). The growth and development of mycorrhizal fungi and their hyphal network are particularly important for increasing the reach and surface area of plant roots for uptake of nutrients and water (Jansa *et al.*, 2013). The reach of roots and root development also allows the root system to interact with other soil microbes to impact further on nutrient uptake and availability, which results in plant growth promotion (Richardson *et al.*, 2009). Applying an *A. nodosum* seaweed extract in a greenhouse trial on pepper and an *A. nodosum* extract with added hen manure on tomato plants resulted in increased root, shoot, and fruit biomass (Renaut *et al.*, 2019). Additionally, microbial diversity and composition differed in comparison to the controls, suggesting that application with the seaweed extract helped shape the microbial communities (Renaut *et al.*, 2019). Similarly, an *A. nodosum* extract improved yield in carrot and increased soil microbial growth, respiration, and functional activities. Results also showed a strong relationship between the increase of microbial activity and the increase in yield, showing the related benefit of the biostimulant application (Alam *et al.*, 2014). Other plant growth-promoting effects induced by soil microbes include nitrogen fixation, production of phytohormones, and resilience towards stress (Kurepin *et al.*, 2014). Therefore, seaweed biostimulants can

have a prebiotic effect by providing bioactives that enhance beneficial bacteria's growth and diversity.

In addition to the enhanced microbial activity commonly reported in response to seaweed biostimulant treatments, the extraction process intended for this research will add a microbial consortium through a fermentation process similar to kombucha production. Bacteria and yeast involved in this method of fermentation can convert tea and sugar into a liquid medium with bioactive ingredients. The fermentation process produces a drink for human consumption with health benefits from the extracted constituents of the tea and microbes in the liquid phase of the drink (Villarreal‐Soto *et al.*, 2018). Reported benefits of kombucha consumption include antibacterial and antifungal properties (Battikh *et al.*, 2012), antidiabetic effects in rats (Srihari *et al.*, 2013), treatment for gastric ulcers in mice (Banerjee *et al.*, 2010), and reducing cholesterol in mice (Yang *et al.*, 2009). Therefore, a kombucha fermentation method with seaweed in place of tea may induce more profound microbial benefits to plants and soil through a probiotic effect of added microbes within the extract.

The structure, water holding capacity, nutrient availability, and nutrient retention capacity of soil is essential to crop productivity and erosion reduction. Soil structure, including capillary action and water movements, is essential to root development, nutrient movement, and soil microbes. The gelling and chelating properties of algal polysaccharides in combination with metallic compounds can not only absorb moisture but also retain moisture within the soil (Khan *et al.*, 2009). The action of algal polysaccharides leads to improved soil aeration and capillary action within soil pore space, enhancing plant growth, root growth, and growth of soil microbes (Kumari *et al.*, 2013). Amendment with chopped pieces of *Laminaria digitata* increased pore volume, improved aggregate stability, increased microbe biomass and respiration (Haslam & Hopkins, 1996). Seaweed biostimulants can also affect soil pH (Kumari *et al.*, 2013), affecting nutrient availability and mobility (Miransari, 2013). The optimal pH for most agricultural soils is typically around 6 – 6.5 (Miller, 2016); however, intensive cropping can reduce pH, nutrient availability and inhibit root growth and productivity in plants (Zhao *et al.*, 2014). Application of a *Sargassum johnstonii* seaweed

biostimulant improved soil conditions by raising the pH to within the optimal range. This biostimulant application also resulted in higher porosity, higher water holding capacity, and increased the concentration of essential nutrients, leading to boosted growth and yield in tomato (Kumari *et al.*, 2013).

1.9 Thesis aims and objectives

Seaweed biostimulants can complement and reduce reliance on the usage of synthetic agrichemicals and support maximum crop quality and yield (Khan *et al.*, 2009). Currently, the seaweed biostimulant market is dominated by manufacturing using brown seaweeds (Sharma et al., 2014). However, internationally and in NZ, brown seaweed biomass for biostimulant manufacturing are typically obtained through wild harvest or the collection of beach cast, which has issues with supply, reliability, and consistency of biomass and the impact on the environment (Mac Monagail *et al.*, 2017). Furthermore, most international brands utilise an acid or alkali chemical extraction method (Ali *et al.*, 2021). Chemical extractions also have several limitations, such as high chemical input, loss of potentially important compounds, and cost (Shobharani *et al.*, 2013). To address these issues, this research will utilise a more environmentally friendly fermentation extraction method using the cultivated green seaweed *Ulva stenophylloides*. Due to *U. stenophylloides* being a novel seaweed biomass source, a reliable and optimal fermentation process needs to be developed. Additionally, one of the important effects of biostimulants is the promotion of plant growth. Definitions of biostimulants typically focus on the effects of biostimulant application rather than the product's chemical composition (du Jardin, 2015). Therefore, effects-based plant assays are essential in determining the efficacy of biostimulants.

1.9.1 Significance of topic and thesis aims

The overall objective of this research is to develop a fermented seaweed biostimulant from *Ulva stenophylloides* that enhances plant productivity in agricultural cropping systems. This involved optimising the fermentation process to achieve successful, efficient fermentation to obtain desirable plant growth bioactives, screening and

evaluating promising ferments in hydroponics for enhanced root growth, and investigation of effects in pot trials to determine the effect of biostimulants on productivity and yield.

Chapter 2: Optimisation of ferment parameters

The seaweed *U. stenophylloides* used in this research is a novel choice of biomass to produce a seaweed biostimulant and the fermentation process is untested. Because fermentation works with living organisms that respond to the conditions and parameters of their environment, optimisation of fermentation conditions and understanding the final chemical composition is the necessary first step to produce a seaweed biostimulant.

This chapter aims to develop an optimal fermentation process to produce an *Ulva* biostimulant and answer the following questions:

- What are the optimal biomass loadings, sucrose inputs, and incubation temperature ratios?
- What effect does this have on ferment progression and success?
- What effect does this have on the composition of the end-products?

Chapter 3: Evaluation of the seaweed biostimulant on mung bean and tomato seedling root growth in hydroponics

The efficacy of a product is determined by evidence collected from plant growth assays demonstrating biostimulant effects. Having the best composition is of no use if it does not translate into plant growth. Therefore, the next step in developing a novel biostimulant is to test efficacy when applied to plants.

This chapter aims to assess the most promising four fermented *Ulva* biostimulant from the previous chapter in hydroponics using mung bean and tomato to determine the best performer to use in subsequent assays and to answer the following questions:

- What effect do the four best biostimulants have on root growth in mung bean?
- What is the ideal dose of biostimulant?
- Does the best performing biostimulant have an auxin-like effect on root growth?
- Does the best performing biostimulant have an interactive effect when used in combination with fertiliser on root growth?

Chapter 4: Evaluation of the best performing seaweed biostimulant on tomato productivity and yield in soil

Many of the effects that lead to enhanced growth occur in the soil or between plant soil interactions, and with the addition of microbes and microbial metabolites within the fermented biostimulant, there is the potential for effects that may further enhance soil and plant health due to the manufacturing method. Therefore, the next step in this research was to test tomato productivity and yield in potted plants.

This chapter aims to assess the most promising fermented *Ulva* biostimulant in a greenhouse experiment using tomato in pots alone and in combination with a conventional fertiliser and to answer the following questions:

- What effects does the biostimulant and fertiliser treatments have on growth, yield, and compositional parameters?
- Are there any interactive effects between the biostimulant and fertiliser on any of those growth and yield parameters?

Overall, it is hypothesised that altering the fermentation parameters will give rise to biostimulants with unique chemical profiles that will have a positive impact on the growth and productivity of plants in hydroponics and soil.

Chapter 2

Optimisation of ferment parameters to produce a novel green seaweed biostimulant from *Ulva stenophylloides*

2.1 Abstract

Seaweed biostimulants represent a valuable tool to improve sustainability in agriculture, while also promoting plant productivity and health. The green seaweed *Ulva stenophylloides* and a novel fermentation extraction method were selected to produce a seaweed biostimulant. Due to using a novel seaweed biomass and manufacturing technique, methods must be developed and optimised. Therefore, using a multifactorial design, this study investigated the impact of the following three fermentation parameters: seaweed biomass loading (10, 25, 40 g L^{-1}), initial sucrose inputs (10, 30, 60 g L^{-1}), and incubation temperature (20, 25, 30 °C) on fermentation progression, measures of successful fermentation, and chemical composition of 27 ferments. The initial sucrose input had the largest impact on fermentation progression and success, and was the main driver in resulting glucose content, total dissolved solids, pH, and pellicle yield. Excluding treatments with low biomass loadings, treatments with low and medium sucrose inputs reached completion and treatments with high sucrose inputs did not. As indicated by residual glucose, high biomass loadings provided valuable nutrients for microbial growth and high incubation temperatures were optimal for microbial growth. A range of micro and macronutrients were extracted from the seaweed biomass with concentrations up to 51 ± 5.1 mg L^{-1} N, 14.3 ± 1.0 mg L^{-1} P, and 567 ± 14.1 mg L^{-1} K, but these were insufficient as a nutrient source for plants at practical applications. High biomass loadings were the main driver in the yield of protein and ulvan, which were found at concentrations up to 504.2 ± 21.9 µg mL^{-1} and 8.1 ± 0.4 mg mL^{-1}, respectively. At practical application level, protein and ulvan content may modulate gene expression and induce metabolic changes in

plants, leading to increased growth. High incubation temperature was the main driver in the yield of the auxin phenylacetic acid, which was found at concentrations up to 2.75 ± 0.61 μg mL^{-1}. These results indicate that the production of other microbial biochemicals occurs at high temperatures. At practical application level, there may be insufficient content to induce an effect on plants, but it has not been tested as part of a seaweed biostimulant and the interactions with other chemical components are unknown. This study identified four promising ferments, with a mix of mid to high biomass loadings, low to mid sucrose loadings, and high incubation temperature, but the biostimulant effects are unknown without adequate testing on plants.

2.2 Introduction

Seaweed biostimulants have major potential for sustainable crop production. Biostimulants can improve plant growth, crop productivity and quality, enhance resistance to abiotic and biotic stressors, improve soil health and structure, and can complement and reduce reliance on synthetic fertilisers and pesticides (Corte *et al.*, 2014; Arioli *et al.*, 2015). When applied as a foliar spray, in hydroponic solutions, or as a soil drench at low concentrations, seaweed biostimulants can regulate and stimulate plant physiology and development, particularly; seed germination, root development, plant growth, nutrient efficiency and uptake, reproduction, and stress responses (Khan *et al.*, 2009; Craigie, 2011; Battacharyya *et al.*, 2015; du Jardin, 2015). The observed effects of seaweed biostimulants on cropping systems may be due to a complex mix of bioactive compounds, such as phytohormones or phytohormone-like compounds (auxin, cytokinin, ethylene, gibberellin, abscisic acid, amongst others), polysaccharides, proteins, phenolics, and vitamins and minerals (macro and micro nutrients) (Khan *et al.*, 2009; Battacharyya *et al.*, 2015). Seaweed species selection and manufacturing methods determine the yield and composition of bioactives extracted from the seaweed (Arioli *et al.*, 2015), which then, in turn, affects the potential impact on crops. Thus, the choice of seaweed species utilised, and the selection and optimisation of production methods can substantially influence the final product and are paramount in developing a biostimulant.

The green seaweed species selected for this research, *U. stenophylloides* (synonymous with *Ulva* sp. B (Lawton *et al.*, 2021) (WELT A027378; sp 1 sensu (Heesch *et al.*, 2009)) (GenBank accession number: MW250819.1) (Lawton *et al.*, 2021) is a highly productive cultivar local to the Bay of Plenty region, New Zealand (Lawton *et al.*, 2021). Although most previous studies have focussed on brown seaweeds (Khan *et al.*, 2009; du Jardin, 2015), *Ulva* spp. have a unique biochemical profile including sulfated polysaccharides (particularly, ulvan) (Shefer *et al.*, 2022), proteins (Pan-utai *et al.*, 2023), phytohormones (Gupta *et al.*, 2011), and nutrients (Hernández-Herrera *et al.*, 2014) which have potential for biostimulant manufacturing. The suitability of *Ulva* as a feedstock biomass for biostimulant production has been demonstrated in several plant trials. For example, a biostimulant produced from *U. lactuca* improved seed germination, shoot and root growth, chlorophyll concentration, and plant protein content in mung bean (Castellanos-Barriga *et al.*, 2017) and similar results with the same seaweed species were also demonstrated with tomato seed germination and growth assays (Hernández-Herrera *et al.*, 2014). *Ulva* products can also act as a plant protectant. A biostimulant produced from *U. armoricana* elicited plant defence responses against three powdery mildew pathogens when applied to common beans, grapes, and cucumber (Jaulneau *et al.*, 2011), an extract of *U. ohnoi* induced defence related genes in grapes (Zarraonaindia *et al.*, 2023), and an extract of *U. lactuca* improved tolerance to saline conditions in lettuce (Aloui *et al.*, 2023). Additionally, unlike most other seaweed biostimulants that source biomass from wild stocks or beach cast (Mac Monagail *et al.*, 2017), *U. stenophylloides* can be cultivated in land-based aquaculture systems (Lawton *et al.*, 2021), which reduces exploitation of wild stocks and provides a reliable, on-demand, sustainable biomass source.

Seaweed biostimulants are traditionally manufactured using thermal extraction methods with either acid or alkali hydrolysis to break down the rigid cell wall of seaweeds to facilitate the extraction of biomolecules (Craigie, 2011; Arioli *et al.*, 2015; Ali *et al.*, 2021). However, novel extraction techniques such as microwave-assisted extraction, pressurised liquid extraction, ultrasound-assisted extraction, and supercritical fluid extraction are also effective (Larios-Cruz *et al.*, 2019). Enzymes and fermentation have emerged as other potential biotransformative extraction methods

that enhance the release of bioactive compounds from the seaweed biomass more sustainably (Larios-Cruz *et al.*, 2019; Pérez-Alva *et al.*, 2022), with fermentation having the added benefit of generating novel compounds through the synthesis of microbial primary and secondary metabolites and breakdown of extracted compounds due to enzyme and acid excretion (Ryu & Kim, 2013; Pérez-Alva *et al.*, 2022). The method of extraction selected for this research is a form of acetic acid fermentation, using a symbiotic culture of acetic acid bacteria (AAB) and yeast, similar to kombucha fermentation.

Fermentation utilising a symbiotic culture of bacteria and yeast has several benefits to offer to the production of seaweed biostimulants. During the first step, a hot aqueous extract targets biomolecules from the biomass, drawing them into a liquid phase (Jaulneau *et al.*, 2011). During the second step, yeast and bacteria produce metabolites, including antimicrobials, antioxidants, antibiotics, plant growth factors, and immunomodulators (Jayabalan *et al.*, 2014; Chakravorty *et al.*, 2016; Sudhanshu *et al.*, 2019; Coelho *et al.*, 2020). Yeast and bacteria can also produce proteins and phytohormones during fermentation (Sreeramulu *et al.*, 2000; Jayabalan *et al.*, 2007; Otieno *et al.*, 2015; Shi *et al.*, 2017). These bioactives are known to improve plant productivity, soil health, and soil conditions (Khan *et al.*, 2009; Calvo *et al.*, 2014; Otieno *et al.*, 2015; Shi *et al.*, 2017). Additionally, because fermentation adds microbes to the final product, there is potential for a probiotic effect that could stimulate the soil microbiome. Furthermore, fermentation is widely used to preserve food products and beverages (Chakravorty *et al.*, 2019; Sudhanshu *et al.*, 2019) and will have an important role in improving the stability of the biostimulant and therefore the shelf life of the product. Having metabolites produced from microbes during fermentation, plus other plant growth and soil benefiting bioactives extracted from algae (polysaccharides, proteins, phytohormones, and other nutrients) within a novel seaweed biostimulant suggests there is high potential for creating a stabilised product with a unique biochemistry to improve crop productivity and system-wide health utilising this extraction method.

Many parameters can determine the success of fermentation: temperature, time, starter culture, biomass selection and loading, and availability of fermentable sugars (Villarreal‐Soto *et al.*, 2018; Bortolomedi *et al.*, 2022) and these parameters differ between kombucha manufacturers (Appendix 7.1). As a result of parameter selection, fermented products have unique properties and chemical compositions; therefore, optimising brewing parameters can enhance the product's content of bioactive compounds (Neffe-Skocińska *et al.*, 2017; Bortolomedi *et al.*, 2022) and subsequent effects on plant-soil systems. Furthermore, only a few studies have recently been undertaken involving this fermentation method with algae as a substrate, and these studies have been for beverage production (Aung & Eun, 2021; Permatasari *et al.*, 2021; Aung & Eun, 2022; Aung *et al.*, 2022; Golovkina *et al.*, 2023; Healy *et al.*, 2023). Therefore, this study aims to evaluate and select fermentation parameters for optimal biostimulant production utilising *U. stenophylloides* as a novel fermentable substrate. Specifically, to determine what the impact of changing the initial biomass content, sucrose input, or fermenting temperature has on the chemical composition of the final fermented biostimulant product. The chemical profile of biostimulant treatments was then used to identify candidates for the plant growth assays in chapter 3.

2.3 Methods

2.3.1 *Ulva stenophylloides* cultivation

U. stenophylloides (Figure 2.1) was cultivated in a recirculating aquaculture system (RAS) in the Facility for Aquaculture Research of Macroalgae (FARM) at the University of Waikato Coastal Marine Field Station, Tauranga, New Zealand. Briefly, *U. stenophylloides* was stocked at 0.5 g fresh weight biomass L^{-1} in 4 m^3 high-rate sea water algal ponds that were part of the 40 m^3 (approximately) RAS. The temperature of the RAS was set to 20 °C, with ambient light conditions. Nutrients were maintained at approximately 3 mg NO_3-N L^{-1} by adding Cell-Hi F2P nutrients (Varicon Aqua Solutions UK) as required. Biomass was harvested weekly and restocked to 0.5 g L^{-1}. The excess biomass from multiple harvests during December 2020 was oven dried (Alphatech UF750 (Memmert, DE)) at 60 °C for 24 hours and milled to <0.5 mm fine

particles (Pulverisette 15 cutting mill, Fritsch GmbH, Germany). Milled biomass was stored in a snap-lock bag with a silica gel sachet prior to homogenising for consistency across all treatments, and this biomass was then used to produce ferments as described below.

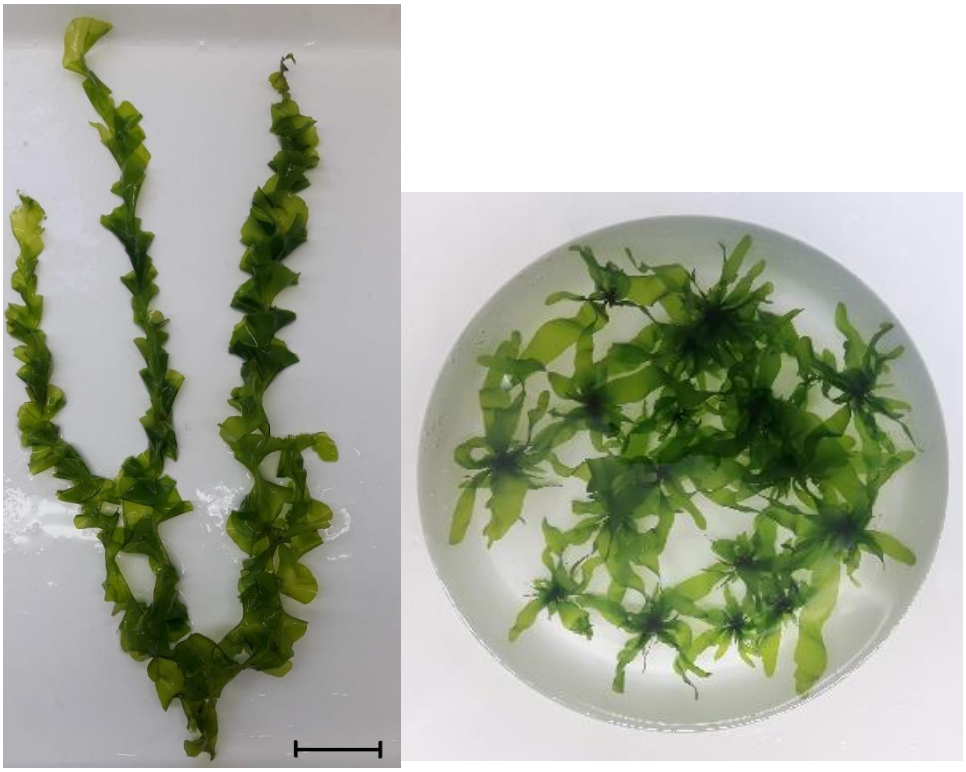


Figure 2.1: Photos of *Ulva stenophylloides*. Left: wild collected, right: cultivated. Scale bar 5 cm.

2.3.2 Starter culture

Dried, milled *U. stenophylloides* (20 g L^{-1}) was extracted in distilled water at 100 °C for 45 mins prior to the addition of sucrose (60 g L^{-1}) and heating at 100 °C for a further 15 mins. Residual seaweed biomass was separated from the liquid using a polyester filter bag with a 63 µm mesh and discarded. The extract was poured into sterilised (autoclaved at 121 °C for 60 mins) 1 L glass jars and cooled to room temperature. Then, commercially available unflavoured and unpasteurised kombucha (15 % v/v) was added to each jar and subsequently covered with a paper towel, secured with a rubber band. Starter cultures were fermented in the dark at 25 °C using a Panasonic MLR-352 culture cabinet. To adapt the microbial consortium to the seaweed biomass, this process was repeated fortnightly for six generations. Subsequent fermentations were conducted as described above, except 150 mL (15 % v/v) from the previous seaweed

brew plus the fresh whole pellicle was used to inoculate the batch, rather than adding commercial kombucha.

2.3.3 Fermentation

To select the optimal fermentation parameters, a fully crossed multifactorial design was utilised with 27 treatments: three seaweed biomass loadings (10, 25, 40 g L^{-1} dry weight), three sucrose additions (10, 35, 60 g L^{-1}), and three incubation temperatures (20, 25, 30 °C) (Figure 2.2). These parameters were selected based on three levels (low, medium and high) determined by preliminary experimental findings and literature involving kombucha fermentation (Appendix 7.1). Furthermore, in preliminary studies (not presented), biomass loading was found to have a limit, where extracts consisting of 50 g L^{-1} of seaweed solidified upon cooling to room temperature to form a gel that was unsuitable for biostimulant manufacturing. Ferments were performed in 500 mL glass jars in triplicate.

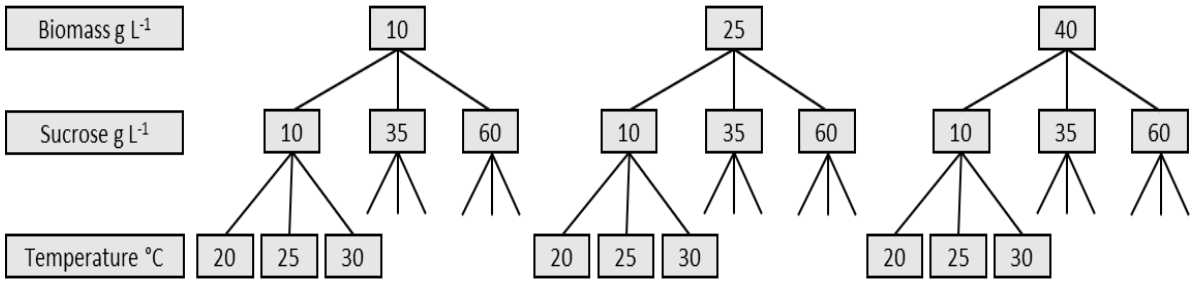


Figure 2.2: Experimental design showing treatments for selecting optimal fermentation conditions to produce a seaweed biostimulant from *U. stenophylloide*s (n=27). Biomass = dried, milled seaweed biomass. Sucrose = additions at the initiation of fermentation. Temperature = incubation temperature for the duration of fermentation.

The following fermentation methods were modified from kombucha fermentation (Chen & Liu, 2000; Raspor & Goranovič, 2008; Jayabalan *et al.*, 2014; Coelho *et al.*, 2020; Júnior *et al.*, 2022) (Figure 2.3). Seaweed biomass loading across different treatments was controlled by using aliquots of three bulk seaweed extracts produced at each loading (either 10, 25, or 40 g L^{-1} dry weight). To do this, dried milled seaweed biomass (150, 375 or 600 g for biomass loadings 10, 25, and 40 g^{-1}, respectively) was

extracted in distilled water (15 L) at 100 °C for 60 minutes. Distilled water (at 100 °C) was then added (by weight) to account for evaporation. The residual seaweed biomass and extract were separated by filtration through a polyester filter bag with a 63 µm mesh. Seaweed extract (either 340 mL, 320 mL, or 300 mL for biomass loadings 10, 25, and 40 g^{-1}, respectively. Volumes differ due to taking into account the extractant that is absorbed into the dry starting biomass) was added to sterilised (autoclaved at 121 °C for 60 mins) 500 mL treatment jars containing sucrose (at either 10, 35, or 60 g L^{-1}). Then wet residual seaweed biomass produced from each bulk seaweed extract was distributed between treatment jars according to treatment biomass loadings (either, 12.8 g, 38 g, 64.4 g, for biomass loadings 10, 25, and 40 g^{-1}, respectively. Weight of biomass added to treatments jars adjusted by taking into account the extractant that is absorbed into the dry starting biomass). Treatments were inoculated with starter culture (10 % v/v) and pellicle (25 ± 1 g L^{-1}) at room temperature and jar openings covered with a paper towel secured with rubber bands. Fermentation was monitored weekly by sampling for pH and fortnightly sampling to measure glucose concentration. To enable sample collection without disturbing pellicle formation, a 110 mm length of 18 mm PVC electrical tape was placed along the top of the paper towel, and a 50 x 1.1 mm needle was inserted vertically through the tape at the centre of the jar opening. This setup had the additional advantage of providing an access point for topping up with more distilled water to account for evaporation. Treatments were then fermented in the dark by incubating at either 20, 25, or 30 °C using Panasonic MLR-352 culture cabinets for 28 days.

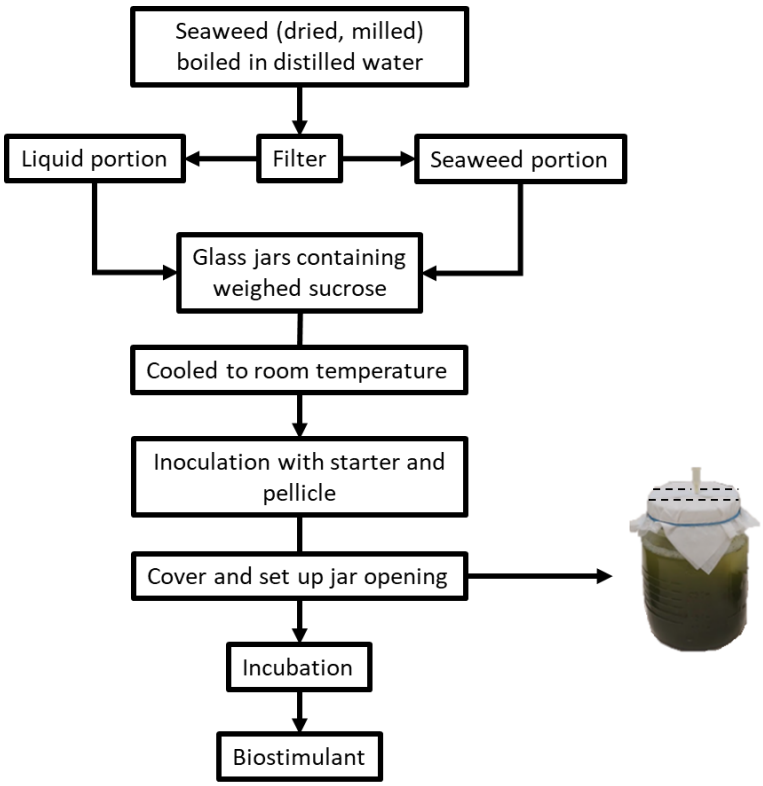


Figure 2.3: Fermented seaweed biostimulant production methods. Jar set up: paper towel secured with a rubber band. Dotted lines indicate the location of tape across the top of the jar. Needle inserted vertically into the centre of each jar.

The last pH readings were taken on day 28 prior to harvesting the ferments by first removing the pellicle and then separation of fermented extract from the residual seaweed biomass by filtration through a 100 µm mesh filter. The residual seaweed biomass from each treatment was frozen in snap lock bags and the pellicle was frozen prior to freeze-drying (Lyovapor L-200, Buchi). The fermented extract from each treatment was divided into three aliquots for subsequent testing; approximately 100 - 150 mL was freeze-dried for elemental and ash analysis and determination of the contents of ulvan, phytohormones, and minerals, 4 mL was frozen for analysis of glucose and protein content, and approximately 100 -150 mL was frozen for plant growth assays. Freeze-dried samples (obtained from the liquid portion) were milled using a kitchen blender (Magic Bullet, MB1001). After freeze drying, several samples (n=12, all three replicates of treatments; biomass 10 g L^{-1} sucrose 35 g L^{-1} temperature 20 °C, biomass 10 g L^{-1} sucrose 60 g L^{-1} temperature 20 °C, biomass 10 g L^{-1} sucrose 60

g L^{-1} temperature 25 °C, biomass 10 g L^{-1} sucrose 60 g L^{-1} temperature 30 °C) were too wet to mill, these were assumed to be well homogenised and were analysed without further processing.

2.3.4 Chemical analyses

To select potential biostimulant candidates, characterisation of biostimulant processing and end-product composition of each ferment was considered by measuring pH and determining the content of; minerals and ash, glucose, total dissolved solids, pellicle, protein, polysaccharide (ulvan), and phytohormones.

2.3.4.1 Fermentation progress and measures of success

The progression of the ferments was monitored by measuring glucose concentration (as a proxy-measure for sucrose utilisation which is metabolised to glucose and fructose (Ojo & de Smidt, 2023)) and pH, and to determine degree of processing success quantity of total dissolved solids, and the pellicle yield were measured.

Samples (3 mL) for glucose analysis were deproteinised by ultrafiltration using Amicon Ultra 15 mL NMWC 10 kDa centrifugal filters (centrifuged for 40 min @ 3900 rcf). The permeate was analysed for glucose using a glucose oxidase/peroxidase assay kit, following the standard protocol (Sigma-Aldrich: GAGO20) at dilutions ranging between 1 and 200.

Ferment pH readings were recorded on days 0, 7, 14, 28. To do this samples (4 mL) from each treatment were obtained using a sterilised syringe attached to the needle in the top of the jar (Figure 2.3) and transferred to a 15 mL centrifuge tube where the pH was measured using a HANNA Instruments Foodcare pH tester. Following pH measurements, samples from day 14 and 28 were stored at -20 °C for glucose and protein assays.

Pellicles harvested on day 28 were freeze-dried and weighed. Total dissolved solids (TDS) of the final ferments (day 28) were determined by differences between the sample's liquid weight and freeze-dried weight.

2.3.4.2 Composition analysis

The composition of the biostimulants produced and unfermented seaweed extracts were assessed as follows. Results determined from freeze-dried ferments were adjusted to account for total dissolved solids in the final fermented product.

Minerals and ash

Elemental analysis (carbon, hydrogen, and nitrogen) and ash content were determined on freeze-dried and milled ferment samples by OEA labs, UK following standard procedures. The mineral content of ferments were determined by Inductively Coupled Plasma Mass Spectrometry (ICP-MS). Sample digests were prepared by a two-step digest where approximately 200 mg (± 0.1 mg) of freeze-dried and milled sample were pre-digested overnight at room temperature in 1 mL of nitric acid (65 %) and 0.4 mL of hydrogen peroxide (30 %). Samples were then heated at 80 °C for one hour with tube caps loosened and cooled for half an hour. Once cooled, the solution was topped up to 50 mL with distilled water and 15 mL of this solution was filtered through a 0.45 µm filter and analysed at the University of Waikato, Hamilton, New Zealand.

Bioactives

The content of protein in ferment samples was determined using a ninhydrin assay adapted from Zhu *et al.* (2009). Briefly, ferments, blanks, or standard samples (700 µL) were hydrolysed in 13.5 M sodium hydroxide (2100 µL) at 120 °C for 20 minutes. Hydrolysates (400 µL) were then neutralised with glacial acetic acid (500 µL). Neutralised hydrolysates (250 µL) were reacted with ninhydrin reagent (250 µL, prepared as per Starcher (2001)) at 100 °C for 10 minutes. Reacted samples (200 µL) were transferred to a 96-microwell plate, and the absorbance of the solution read at 575 nm on a BMG Labtech SPECTROstar Nano. The assay was calibrated using bovine serum albumin (BSA) at concentrations of 15, 30, 60, 125, 250 µg mL^{-1}. Samples that gave absorption readings outside of this calibration range were diluted and re-analysed.

The content of uronic acid was used as a proxy-measure for ulvan using a uronic acid to ulvan conversion factor of 2.68 (Kidgell *et al.*, 2019). Uronic acid content of the

freeze-dried ferments was determined by a colorimetric assay adapted from Blumenkrantz and Asboe-Hansen (1973). Briefly, in an ice bath 2.4 mL 12.5 mM sodium borate solution in concentrated sulfuric acid was added to 400 µL of sample solution (0.45 mg mL^{-1} in RO water). The mixture was vortexed and heated at 100 ºC for 5 min in a water bath. On cooling to room temperature, 40 µL of 0.15 % *m*-phenylphenol in 0.5 % sodium hydroxide was added and the solution vortexed for 20 sec. The absorbance of the solution was read at 520 nm on a BMG Labtech SPECTROstar Nano within five minutes of adding the *m*-phenylphenol solution. The assay was calibrated using glucuronic acid as the standard at concentrations of 2.5, 25, 50, 75, 100 µg mL^{-1}. Standard blanks used distilled water in place of glucuronic acid, and sample blanks used 40 µL of 0.5 % sodium hydroxide in place of the *m*-phenylphenol solution.

The content of phytohormones in ferments were analysed using gas chromatography mass spectrometry (GC-MS) following a methyl chloroformate derivatisation adapted from Rawlinson *et al.* (2015). Briefly, methanol (147 µL) and pyridine (34 µL) were added to a solution of sample (~20 mg) and the internal standard cinnamic acid (20 µL of 20 µg mL^{-1}) in 1% sodium hydroxide solution (200 µL) and, mixed by vortex for 30 sec. Methyl chloroformate (20 µL) was then added and mixed by vortex for 30 sec. The latter step was then repeated. Chloroform (400 µL) and 50mM sodium bicarbonate (400 µL) were added at and the mixture vortexed between. Phase separation was achieved by centrifugation (1 min at 16.1 g) after which the organic bottom layer was transferred to a new 1.5 mL centrifuge tube and dried over anhydrous sodium sulphate. Derivatised sample solution (120 µL) was transferred to a 2 mL GC-MS vial. Samples were then measured using GC-MS with an electron ionisation (EI) source (Nexis GC-2030 Shimadzu gas chromatograph with a single quadrupole mass spectrometer (GCMS-QP2020 NX)) fitted with an SH-I-5Sil MS column (30 mm x 0.25 mm x 0.25 µm). Linear velocity was selected for the flow control mode, with the pressure set at 9.0 psi. Helium was used as the carrier gas (0.96 mL min^{-1}). A 2 µL injection volume was used in spitless injection mode. The oven temperature was programmed at 80 °C and held for one minute and then increased at a rate of 10 °C min^{-1} to 320 °C and held for two minutes. To identify the analytes, the MS was run in scan mode. Phytohormones were identified using the GC-MS solution NIST 17 library

(similarity index > 85 %). A calibration standard using phenylacetic acid in place of samples was prepared using methods as above (at concentrations of 1, 2, 5, 10, and 20 μg mL^{-1}).

2.4 Data analysis

A three factor permutational analysis of variance (PERMANOVA) was used to compare the effect of biomass loading, sucrose loading, and incubation temperature (fixed factors) on processing results (glucose, pH, pellicle yield, total dissolved solids) and bioactive yield (protein, polysaccharide, and phytohormone). The proportion of total variation in the processing results and bioactive yield associated with each factor (biomass loading, sucrose loading, and incubation temperature) was calculated using eta-squared (%) η^2 (SS_{factor} / SS_{total} × 100; SS_{factor} = sum of squares for each factor and SS_{total} = total sum of squares) (Richardson, 2011). All statistical analyses were conducted in Primer v7 (Primer-E., UK) using Euclidean distance resemblance matrices, 9,999 unrestricted permutations of raw data, and Type III sum of squares (Anderson, 2008). Pairwise a posteriori comparisons were carried out if a significant effect (p = > 0.05) was detected. All data is reported as mean ± standard error (SE).

2.5 Results

2.5.1 Fermentation progress and measures of success

2.5.1.1 Glucose

Glucose was recorded at the mid-point (day 14) of incubation and again at the end (day 28) of the incubation period (Figure 2.4). At the mid-point of fermentation (Figure 2.4a), glucose was present in all treatments, excluding treatments with low initial sucrose inputs, in these samples very small amounts or no glucose were detected. The more initial sucrose that was added the more glucose was detected.

There were significant interactions between all factors on the amount of glucose detected at the end of the fermentation period (Table 2.1). However, the interaction that explained most of the variation was between biomass loading and sucrose inputs (η^2 = 17.3 %). This was an inverse relationship with higher biomass loading and lower

initial sucrose inputs resulting in lower final glucose concentrations. The main driver in variation in remaining glucose was the initial sucrose input (η^2 = 44.5 %). At the end of the fermentation period (Figure 2.4b), glucose was absent or present in trace amounts in all treatments with low to medium initial sucrose inputs, but still present in all treatments with high initial sucrose inputs. Biomass loading explained 16.3 % of the variation between treatments, with the more biomass added to the ferment the lower the amount of glucose was in the final product. Incubation temperature explained 6.9 % of the variation, with significantly less glucose in 25 and 30 °C treatments compared to the 20 °C treatments and no difference detected between 25 and 30 °C treatments.

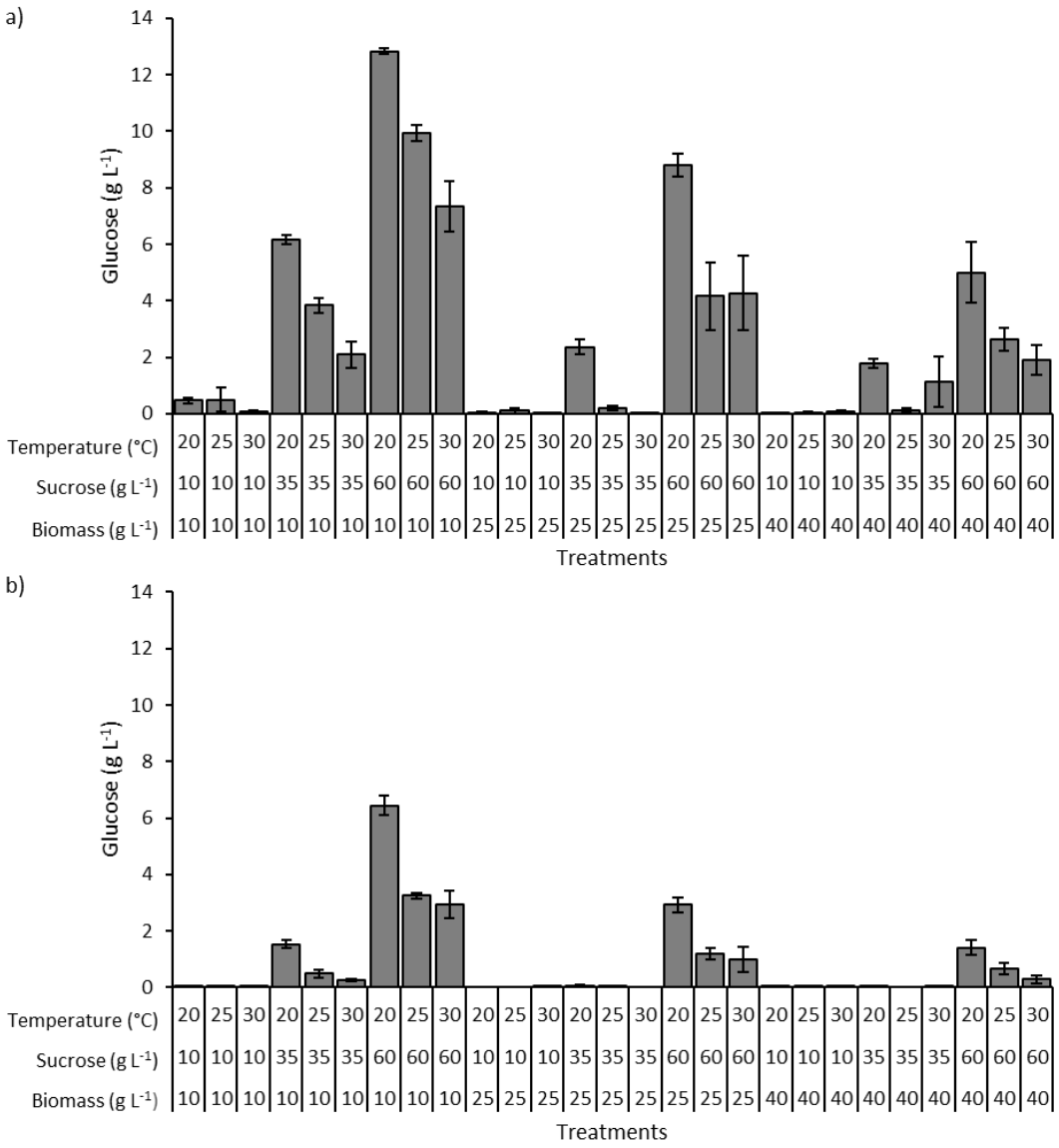


Figure 2.4: Glucose concentrations in *U. stenophylloides* ferments. a) mid-point of the fermentation period (day 14), b) endpoint of the fermentation period (day 28). Average ± SE.

Table 2.1: Results of Permutational Analysis of Variance (PERMANOVA) for the effects of fermentation parameters on processing variables. Significant results (p = < 0.05) are presented in bold, and the eta-squared value (η^2, % of variance) used as a measure of proportion of variance. TDS – total dissolved solids.

Source	***df***	**Glucose**			**TDS**			**pH**			**Pellicle**		
		F	*p*	η^2	F	*p*	η^2	F	*p*	η^2	F	*p*	η^2
Biomass	2	153.68	**0.0001**	16.25	73.14	**0.0001**	7.36	85.18	**0.001**	22.69	87.20	**0.001**	39.22
Sucrose	2	420.51	**0.0001**	44.47	794.66	**0.0001**	79.99	205.56	**0.001**	54.77	82.87	**0.001**	37.27
Temperature	2	65.10	**0.0001**	6.88	38.27	**0.0001**	3.85	42.35	**0.001**	11.28	7.86	**0.001**	3.53
Biomass x Sucrose	4	81.71	**0.0001**	17.28	15.46	**0.0001**	3.11	0.27	0.894	0.15	5.12	**0.002**	4.61
Biomass x Temperature	4	12.24	**0.0001**	2.59	2.51	0.0515	0.51	1.54	0.202	0.82	1.84	0.136	1.66
Sucrose x Temperature	4	37.58	**0.0001**	7.95	10.45	**0.0001**	2.10	3.88	**0.005**	2.07	0.24	0.920	0.21
Biomass x Sucrose x Temperature	8	4.07	**0.0018**	1.72	0.87	0.5407	0.35	0.96	0.471	1.03	0.75	0.642	1.36
Residuals	54												

2.5.1.2 Total dissolved solids

Total dissolved solids (TDS) ranged between 0.8 ± 0.02 % (treatment: 10 g L^{-1} biomass loading, 10 g L^{-1} sucrose input, and 30 °C incubation temperature) and 5.3 ± 0.3 % (treatment: 10 g L^{-1} biomass loading, 60 g L^{-1} sucrose input, and 20 °C incubation temperature) (Figure 2.5). There was a significant interaction between initial sucrose input and incubation temperature on the proportion of TDS (Table 2.1), with greater sucrose inputs and lower temperatures resulting in the highest content of TDS. However, this interaction explained only 2.1 % of the variation between treatments (Table 2.1). The main effects can be explained by sucrose inputs (η^2 = 80.0 %), with higher loadings leading to higher contents of TDS.

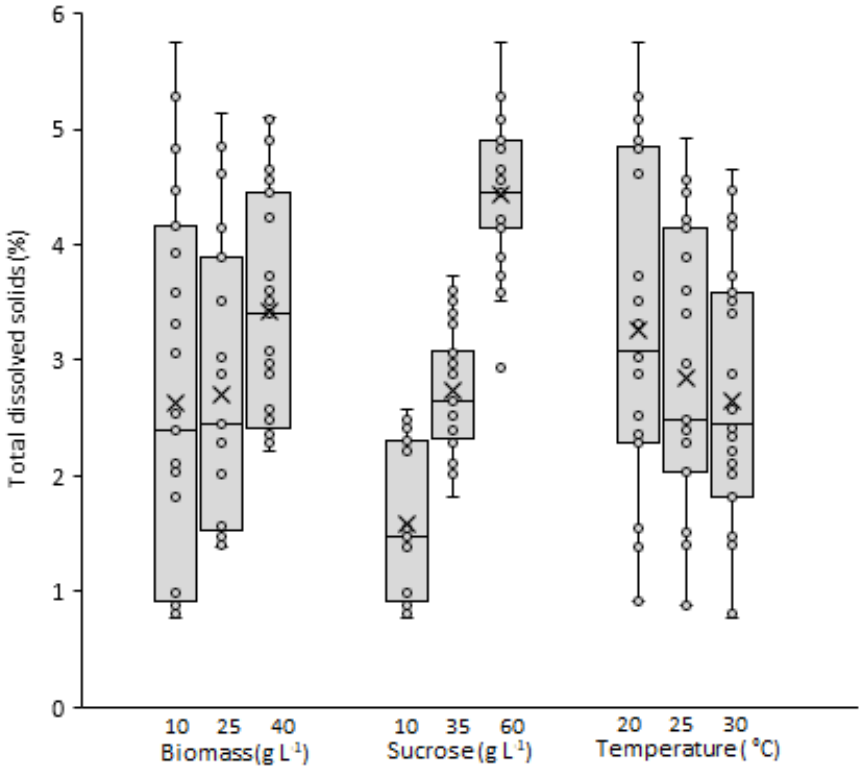


Figure 2.5: Total dissolved solids in *U. stenophylloides* ferments. Biomass = dried seaweed inputs, sucrose = initial sucrose inputs, temperature = incubation temperature.

2.5.1.3 pH

All ferments started at a similar pH of 3.7 ± 0.02 and either decreased or increased over the 4-week fermentation period, depending on the treatment. This deviation resulted in a final pH that was significantly different between treatments and ranged between a minimum of 2.3 ± 0.04 (treatment: 10 g L^{-1} biomass loading, 60 g L^{-1} sucrose input, and 20 °C incubation temperature) and a maximum of 5.0 ± 0.5 (treatment: 40 g L^{-1} biomass loading, 10 g L^{-1} sucrose input, and 30 °C incubation temperature) (Table 2.1, Figure 2.6). There was a significant interaction between sucrose inputs and incubation temperature; however, this only explained 2.1 % of the variation. Sucrose input was the main driver of the differences, explaining approximately 54.8 % of the variation, with the highest sucrose input resulting in the lowest pH. Conversely, lower biomass loading, or temperature led to a lower pH. These factors explained a further 22.7 % and 11.3 % of the variation, respectively.

2.5.1.4 Pellicle

Pellicle yield ranged between 0.3 ± 0.01 g (treatment: 10 g L^{-1} biomass loading, 10 g L^{-1} sucrose input, and 20 °C incubation temperature) and 3.1 ± 0.1 g (treatment: 40 g L^{-1} biomass loading, 60 g L^{-1} sucrose input, and 30 °C incubation temperature) (Figure 2.7). There was a significant interaction between biomass loading and initial sucrose input on the yield of pellicle (Table 2.1), with increasing loadings of both resulting in increased yields. However, this interaction explained less than 5 % of the variation between treatments (Table 2.1), with the main effects of biomass loading and sucrose input explaining 39.2 % and 37.3 %, respectively. As biomass loading, sucrose loading, and incubation temperature increased, the yield of pellicle also increased (Figure 2.7); however, there was no difference in yield between mid to high incubation temperatures ($p=0.17$).

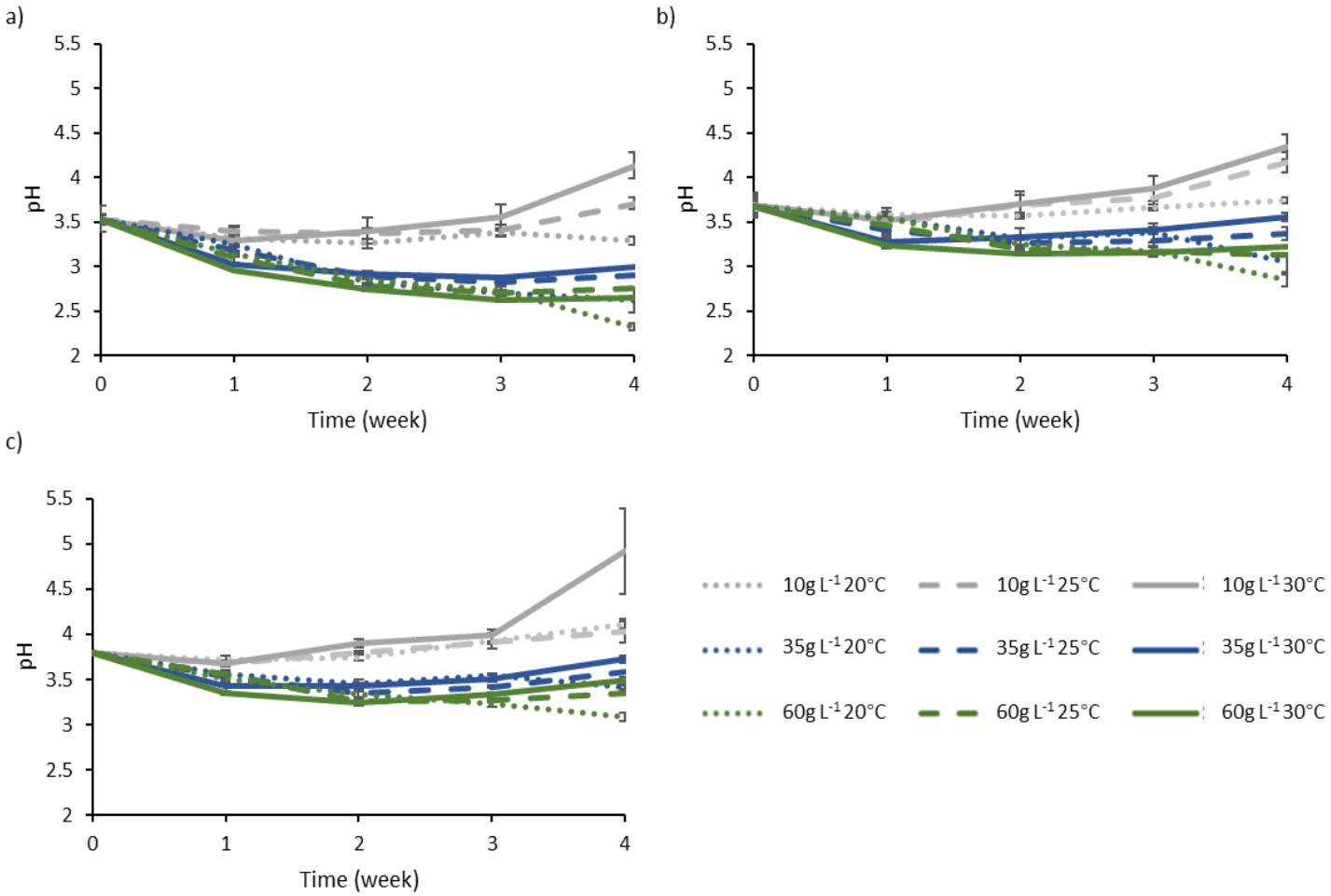


Figure 2.6: Weekly measurements of pH during the incubation period of *U. stenophylloides* ferments. Colour representing sucrose levels (g L^{-1}), line type representing incubation temperature (°C). Seaweed biomass loadings: a) 10 g L^{-1}, b) 25 g L^{-1}, c) 40 g L^{-1}. Average ± SE.

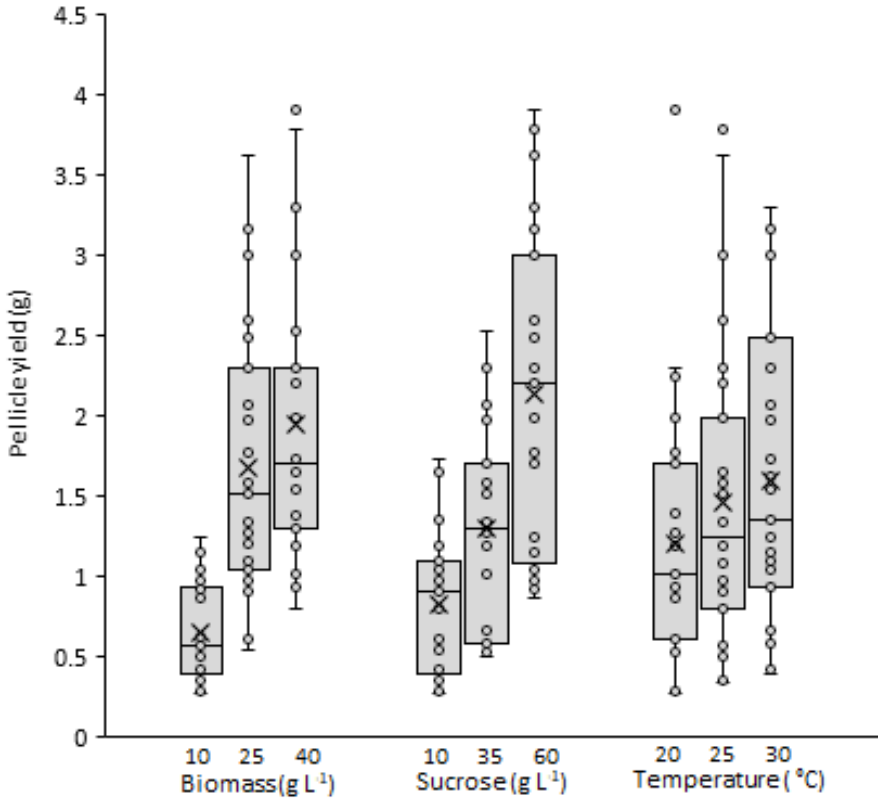


Figure 2.7: Pellicle yield resulting from *U. stenophylloides* ferments. Biomass = dried seaweed inputs, sucrose = initial sucrose inputs, temperature = incubation temperature.

2.5.2 Composition analysis

2.5.2.1 Minerals and ash

A range of macro and micro-nutrients were found in all treatments (Table 2.2, Appendix 7.2). High biomass loadings had almost double the nutrients than the medium biomass loadings. The most abundant elements in high biomass loading treatments were sulfur, sodium, magnesium, potassium, and calcium (Table 2.2, Appendix 7.2). Cadmium, mercury, titanium, lead, and uranium content were also analysed; however, these were either not detected, below quantitation limit or were detected in trace amounts. Specifically, cadmium was not detected in 96.3 % of samples, and only trace amounts in 3.7 % of samples; mercury was not detected in 100 % of samples; titanium was not detected in 97.5 % of samples, and only trace amounts in 2.5 % of samples; lead was not detected in 44.4 % of samples, below

quantitation limit in 42.0 % of samples, and only trace amounts in 13.6 % of samples; and uranium was not detected in 97.5 % of samples, and only trace amounts in 2.5 % of samples.

Table 2.2: Elemental analysis of four selected *U. stenophylloides* fermentation products. Average ± SE.

Biomass loading (g L^{-1})			25	25	40	40
Initial sucrose loading (g L^{-1})			10	35	10	35
Incubation temperature (°C)			30	30	30	30
Macronutrients		**Unit**				
Ash		%	0.38 ± 0.02	0.38 ± 0.03	0.63 ± 0.03	0.65 ± 0.02
Carbon	C	g L^{-1}	2.9 ± 0.1	3.6 ± 1.4	4.6 ± 0.2	5.8 ± 1.5
Nitrogen	N	mg L^{-1}	30 ± 1.0	23 ± 4.9	51 ± 5.1	45 ± 8.5
Phosphorus	P	mg L^{-1}	6.8 ± 0.1	7.7 ± 0.4	14.3 ± 1.0	13.7 ± 0.3
Potassium	K	mg L^{-1}	356.9 ± 9.7	373.9 ± 13.7	566.7 ± 14.1	557.9 ± 7.9
Calcium	Ca	mg L^{-1}	125.8 ± 6.0	157.6 ± 6.2	215.7 ± 10.6	239.3 ± 3.1
Magnesium	Mg	mg L^{-1}	310.1 ± 7.0	319.2 ± 10.9	578.3 ± 30.1	480.2 ± 19.2
Sulfur	S	mg L^{-1}	714.5 ± 20.0	734.8 ± 19.5	1256.7 ± 38.8	1184.1 ± 15.6
Micronutrients						
Copper	Cu	µg L^{-1}	51.1 ± 0.9	50.9 ± 2.2	81.5 ± 9.6	84.8 ± 3.9
Manganese	Mn	µg L^{-1}	116.2 ± 1.8	115.7 ± 3.9	198.6 ± 13.6	194.2 ± 6.7
Iron	Fe	µg L^{-1}	912.0 ± 8.7	1127 ± 48.9	1466.0 ± 9.8	1554.9 ± 17.9
Nickel	Ni	µg L^{-1}	7.0 ± 0.9	6.4 ± 0.8	7.3 ± 0.2	7.5 ± 0.1
Zinc	Zn	µg L^{-1}	654.1 ± 67.9	656.4 ± 245.1	650.3 ± 223.9	466.8 ± 194.9
Cobalt	Co	µg L^{-1}	19.1 ± 0.3	21.8 ± 1.0	32.7 ± 0.5	33.7 ± 0.4
Non-essential element						
Sodium	Na	mg L^{-1}	436.6 ± 14.0	442.4 ± 10.2	843.6 ± 49.3	681.2 ± 4.8
Strontium	Sr	µg L^{-1}	920.5 ± 57.8	1005.8 ± 61.0	1434.2 ± 130.5	1554.9 ± 93.8
Barium	Ba	µg L^{-1}	11.9 ± 0.7	12.3 ± 1.2	15.8 ± 0.4	21.5 ± 1.2
Selenium	Se	µg L^{-1}	4.5 ± 0.2	2.8 ± 0.5	5.9 ± 0.6	5.8 ± 0.0
Aluminium	Al	µg L^{-1}	116.2 ± 4.2	216.6 ± 33.2	149.0 ± 21.9	226.8 ± 12.6
Arsenic	As	µg L^{-1}	6.4 ± 0.2	6.9 ± 0.8	10.8 ± 0.6	11.7 ± 0.1
Chromium	Cr	µg L^{-1}	9.5 ± 1.0	11.8 ± 1.3	18.4 ± 1.0	14.3 ± 1.1

Results shown here are of the four selected biostimulants moving forward to the next chapter. For results for the rest of the treatments refer to Appendix 7.2

2.5.2.2 Free amino acids and protein

Protein yield varied between 115.1 ± 6.4 µg mL^{-1} (treatment: 10 g L^{-1} biomass loading, 10 g L^{-1} sucrose input, and 20 °C incubation temperature) and 504.2 ± 21.9 µg mL^{-1} (treatment: 40 g L^{-1} biomass loading, 60 g L^{-1} sucrose input, and 30 °C incubation temperature) (Figure 2.8). As biomass loading, and incubation temperature increased, the yield of protein also increased (Table 2.3); however, there was no significant difference in yield between mid to high incubation temperatures. The main driver in variation was biomass loading (η^2 = 88.2 %), with the more biomass added to the ferment, the more protein was present in the final fermented product (Table 2.3). Unfermented samples followed a similar pattern of protein with 10 g L^{-1} biomass loading yielding 116.1 ± 7.6 µg mL^{-1}, 25 g L^{-1} biomass loading yielding 258.2 ± 16.3 µg mL^{-1}, and 40 g L^{-1} biomass loading yielding 347.6 ± 15.0 µg mL^{-1}.

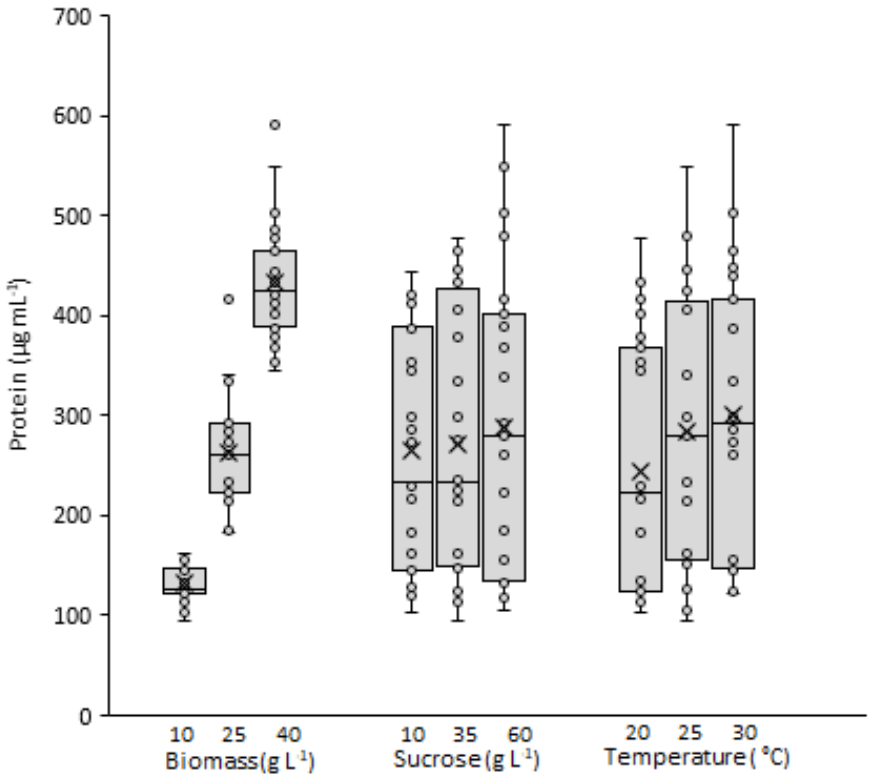


Figure 2.8: Protein yield in *U. stenophylloides* ferments. Biomass = dried seaweed inputs, sucrose = initial sucrose inputs, temperature = incubation temperature.

Table 2.3: Results of Permutational Analysis of Variance (PERMANOVA) for the effects of fermentation parameters on product bioactive content. Significant results (p = < 0.05) are in bold, and the eta-squared value (η^2, % of variance) used as a measure of proportion of variance.

Source	***df***	**Protein**			**Ulvan**			**Phenylacetic acid**		
		F	*p*	η^2	F	*p*	η^2	F	*p*	η^2
Biomass	2	469.27	**0.0001**	88.20	473.98	**0.0001**	84.99	16.62	**0.0001**	17.87
Sucrose	2	2.96	0.06	0.56	35.75	**0.0001**	6.41	2.38	0.10	2.56
Temperature	2	16.68	**0.0001**	3.14	8.76	**0.0008**	1.57	29.44	**0.0001**	31.66
Biomass x Sucrose	4	2.43	0.06	0.91	1.82	0.15	0.65	1.63	0.18	3.51
Biomass x Temperature	4	2.55	0.05	0.96	0.89	0.48	0.32	3.03	**0.02**	6.51
Sucrose x Temperature	4	1.19	0.33	0.45	1.62	0.18	0.58	1.50	0.22	3.23
Biomass x Sucrose x Temperature	8	0.94	0.49	0.71	0.88	0.54	0.63	1.31	0.25	5.63
Residuals	54									

2.5.2.3 Ulvan

Ulvan yield ranged between 1.8 ± 0.1 mg mL^{-1} (treatment: 10 g L^{-1} biomass loading, 10 g L^{-1} sucrose input, and 30 °C incubation temperature) and 8.1 ± 0.4 mg mL^{-1} (treatment: 40 g L^{-1} biomass loading, 60 g L^{-1} sucrose input, and 20 °C incubation temperature) (Figure 2.9). Most of the variation was explained by biomass loading (η^2 = 85.0 %), with the more biomass added to the brew, the more ulvan was present in the final fermented product (Table 2.3). Unfermented samples followed a similar pattern of ulvan with 10 g L^{-1} biomass loading yielding 2.4 ± 0.3 mg mL^{-1}, 25 g L^{-1} biomass loading yielding 4.6 ± 0.4 mg mL^{-1}, 40 g L^{-1} biomass loading yielding 6.3 ± 0.1 mg mL^{-1}. As sucrose loading increased, the yield of ulvan also increased. Conversely, the lowest incubation temperature resulted in more ulvan than higher temperatures. There was no significant difference in ulvan results between mid to high incubation temperatures.

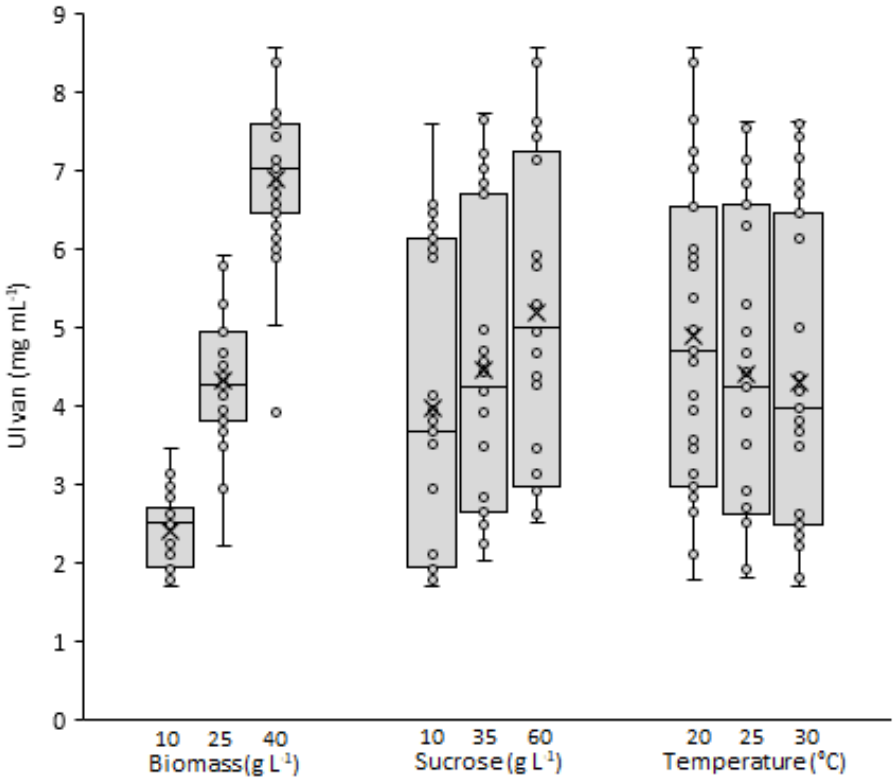


Figure 2.9: Ulvan yield in *U. stenophylloides* ferments. Biomass = dried seaweed inputs, sucrose = initial sucrose inputs, temperature = incubation temperature.

2.5.2.4 Phytohormones (phenylacetic acid)

Phytohormone analysis identified one phytohormone, the auxin phenylacetic acid. Phenylacetic acid varied between 0.4 ± 0.1 µg mL^{-1} (treatment: 10 g L^{-1} biomass loading, 10 g L^{-1} sucrose input, and 20 °C incubation temperature) and 2.8 ± 0.6 µg mL^{-1} (treatment: 25 g L^{-1} biomass loading, 35 g L^{-1} sucrose input, and 30 °C incubation temperature) (Figure 2.10). There was a significant interaction between biomass loading and incubation temperature on the yield of phenylacetic acid, however, this interaction explained less than 7 % of the variation between treatments (Table 2.3). Most of the variation was explained by incubation temperature (η^2 = 31.7 %, Table 2.3), with the highest temperature leading to the most phenylacetic acid yield. Biomass loading had a significant impact on phenylacetic acid yield and explained 17.9 % of the variation (Table 2.3), treatments with the lowest biomass loading had significantly less phenylacetic acid than mid to high biomass loadings. Unfermented samples had small amounts of phenylacetic acid present; 10 g L^{-1} had a peak present

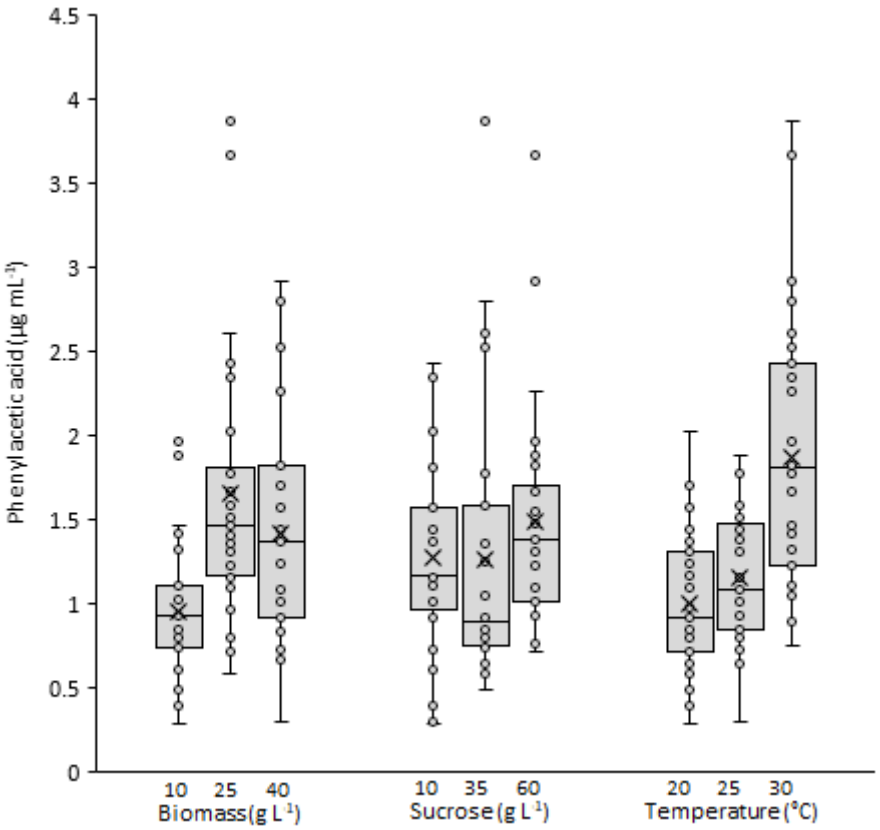


Figure 2.10: Phenylacetic acid yield in *U. stenophylloides* ferments. Biomass = dried seaweed inputs, sucrose = initial sucrose inputs, temperature = incubation temperature.

but when adjusted for total dissolved solids the amount was well below 0, 25 g L^{-1} had 0.04 ± 0.01 µg mL^{-1}, 40 g L^{-1} had 0.07 ± 0.02 µg mL^{-1}.

2.6 Discussion

This study investigated the effect of fermentation parameters on the chemical properties of a novel fermented seaweed biostimulant utilising *U. stenophylloides* as the feedstock biomass. Because this is a novel choice of seaweed biomass, novel extraction method, and because fermentation works with living organisms that respond to the conditions and parameters of their environment, optimisation of fermentation conditions was necessary as a first step to achieve successful, efficient fermentation to obtain desirable plant growth bioactives. A fully crossed multifactorial design evaluated three key parameters: biomass loading, sucrose input, and incubation temperature. These fermentation parameters significantly influenced fermentation rates and chemical profiles of the final 27 ferments.

2.6.1 Fermentation progress and measures of success

Successful kombucha fermentations require a source of fermentable sugars (e.g. sucrose). During yeast fermentations, sucrose is hydrolysed by yeast invertase into fructose and glucose, and these sugars are metabolised to ethanol and carbon dioxide (Kaewkod *et al.*, 2019; Antolak *et al.*, 2021; Ojo & de Smidt, 2023). Glucose is also converted to bacterial cellulose by acetic acid bacteria (De Filippis *et al.*, 2018; Villarreal‐Soto *et al.*, 2018). In this study, glucose was used as a proxy measure of fermentation progress. Except for treatments with low biomass addition, over the 28-day fermentation, the glucose content in treatments with low and medium loadings of sucrose indicated that these fermentations reached completion. Residual glucose remained in all fermentation treatments with high sucrose input contributing to high total dissolved solids in these samples (see below), indicating these fermentations did not reach completion. There were significant negative interactions between sucrose loading and biomass loading, and sucrose loading and fermentation temperature on glucose content in the final fermented products. These results meant that while the main driver of variation in glucose content between treatments was the initial sucrose

input, higher biomass loadings and fermentation temperatures led to significantly lower glucose content in the final fermented products. The effect of biomass loading on the content of glucose in fermented products suggests that the *Ulva* biomass provides valuable nutrients (e.g., nitrogen, carbon and minerals) that enhance microbial growth, demonstrating its suitability for fermentation. The effect of incubation temperature on fermentation rate indicates that the optimum temperature for the growth of the microbes in the inoculant is toward the high end of the fermentation temperatures tested and those considered optimal for comparable fermentation methods. Typically kombucha is fermented between 20 – 30 °C (Neffe-Skocińska *et al.*, 2017; De Filippis *et al.*, 2018; Antolak *et al.*, 2021), with optimal growth for acetic acid bacteria occurring between 25 – 30 °C, and yeasts *Brettanomyces bruxellensis*, *Zygosaccharomyces rouxii*, and *Saccharomyces cerevisiae* optimal temperatures occurring at 22-32, 30, and 32 °C, respectively (Antolak *et al.*, 2021).

The production of organic acids during this method of fermentation causes the pH to decrease and, therefore, is a valuable tool for monitoring fermentation progress (Villarreal‐Soto *et al.*, 2018; Kaewkod *et al.*, 2019; Ojo & de Smidt, 2023). Organic acid production in this method of fermentation mainly occurs through acetic acid bacteria (primarily species from *Acetobacter, Gluconacetobacter*, and *Komagataeibacter*) oxidising ethanol to acetic acid and metabolising glucose to gluconic acid and glucuronic acid (Antolak *et al.*, 2021; Ojo & de Smidt, 2023). In this study, there was an interaction between sucrose inputs and incubation temperature on the pH of the final fermented products. However, the main driver of variation was the initial sucrose input. Presumably, higher inputs of sucrose lead to higher concentrations of ethanol and, subsequently, higher concentrations of organic acids, contributing to lower pH. However, the relationship between sucrose and the production of ethanol and organic acids is not that straightforward. For example, acetic acid can promote further production of ethanol from yeast, which then stimulates the growth of acetic acid bacteria resulting in further accumulation of acetic acid (Chen & Liu, 2000; Dufresne & Farnworth, 2000) and pH stabilises despite organic acids and titratable acidity continuing to increase due to buffering effects of

fermentation itself and the fermentable substrate (Liang & Xu, 2001; Cvetković *et al.*, 2008). When the level of sucrose input was fixed, pH increased with increasing treatment temperature, giving rise to the interaction mentioned above effect. The effect of temperature is consistent with the decomposition of carbonic acid, produced from the fermentation byproduct carbon dioxide, at higher temperatures (Reddy & Balasubramanian, 2014). There was also a main effect of biomass loading on pH, with high biomass loading contributing to higher pH and vice versa. This effect is consistent with the seaweed biomass contributing to a buffering effect on pH. Components of the fermentable substrate (e.g., the seaweed biomass) can act as a buffer, leading to resistance to pH change. For example, polyphenolic oxidation products (thearubigins and theaflavins) in black and green tea lead to a buffering effect in kombucha fermentation (Liang & Xu, 2001). Importantly, treatments with low initial sucrose inputs consumed most added sugar by the midpoint of the incubation period and had a higher pH compared to treatments with higher initial sucrose inputs. The early depletion of glucose paired with higher pH in treatments with low sucrose inputs limits microbial growth and fermentation products which may impact on biostimulant activity and stability.

Acetic acid bacteria (primarily *Komagataeibacter xylinus*) convert glucose and other carbon sources (including ethanol, sucrose, and glycerol) to uridine diphospho-glucose (UDPGlc) and subsequently bacterial cellulose, contributing to the cellulosic pellicle of kombucha fermentations that floats at the top of the liquid portion of the brew (Villarreal‐Soto *et al.*, 2018). Therefore, the development of the pellicle as fermentation time advances is an indication of a successfully progressing fermentation (Aung & Eun, 2021). In this study, the highest pellicle yield was associated with treatments that had higher biomass loadings, sucrose inputs, and incubation temperatures. The main drivers of these differences were biomass and sucrose inputs, with higher levels of both leading to higher pellicle yields and vice versa. The apparent synergy between biomass loading and sucrose input likely results from increased availability of nutrients (e.g., nitrogen, carbon and minerals) for microbial growth combined with cellulose building blocks (i.e., glucose). While not specifically determined, it can be assumed that the rate of pellicle formation is also temperature

dependent; therefore, since temperature had a significant effect on overall pellicle yield, higher temperatures will likely reduce production time and risk of contamination as the product is less prone to contamination and mould growth when there is a well-developed pellicle (Jarrell et al., 2022). Optimum temperatures for bacterial cellulose production have also been demonstrated to be in the range of 28 – 30 °C (Campano *et al.*, 2016), indicating that the ideal fermentation temperature for the production of biostimulants from *Ulva* with the inoculant used in this study is at the higher end of the range tested.

Treatments with high glucose remaining also had the highest total dissolved solids due to the remaining sugars. Residual sugars should be avoided as fermentation will likely continue producing carbon dioxide, metabolites, and bioactives, causing issues with packaging, product specifications, and application rates. Whilst it is acknowledged that a more extended fermentation period for treatments with high sucrose inputs would likely result in complete fermentations, treatments with residual glucose were not tested in plant growth assays (see Chapter 3 and Chapter 4).

2.6.2 Bioactives

Whilst optimal plant growth is reliant on the availability of macro and micronutrients, the application of additives, such as biostimulants, are used to facilitate plant physiological responses, leading to increased growth, yield, and stress resilience (Khan *et al.*, 2009; du Jardin, 2015). Although there is some debate over which components of seaweed biostimulants specifically induce plant growth, seaweed biostimulants have been demonstrated to contain a range of biochemicals and minerals, some of which individually influence plant growth (Shefer *et al.*, 2022; Domingo *et al.*, 2023). For example, an extract of ulvan from *U. lactuca* applied to plant growth media increased plant growth in *Arabidopsis thaliana,* including plant weight by 62 % and root length by 174 % (Shefer *et al.*, 2022) and a protein hydrolysate-rich extract from *Chondrus crispus* applied to potted tomatoes increased plant dry weight and shoot height by up to 20 % (Domingo *et al.*, 2023). In the current study, microbial metabolic processes during the fermentation of *U. stenophylloide*s extracts are expected to contribute to the chemical profiles of the biostimulant. To decipher the effect of

seaweed biomass loading, sucrose loading, and incubation temperature on biostimulant end-product quality, ferments were characterised by determining contents of macro and micronutrients, free amino acids and protein, ulvan, and phytohormones.

Plants require a range of macro and micronutrients for optimal growth and development (McGrath *et al.*, 2014; Kumar *et al.*, 2016). The nutrient requirement of plants is a complex and challenging issue, and requirements change depending on the plant species or cultivar, the developmental stage of the plant, soil properties and condition, and local climate (McGrath *et al.*, 2014). For example, optimised macronutrient concentrations for field grown mung beans in chernozem soil were found to be 34.4 – 42.6 kg ha^{-1} N (as urea), 7.7 – 9.5 kg ha^{-1} P (as P_2O_5), and 44.2 – 55.9 kg ha^{-1} K (as K_2O) (Yin *et al.*, 2018) and micronutrient uptake for high yields in alfalfa, corn and, soybean range between 0.11 – 0.33 kg ha^{-1} B, 0.07 – 0.11 kg ha^{-1} Cu, 1.9 – 2.13 kg ha^{-1} Fe, 0.3 – 0.7 kg ha^{-1} Mn, 0.2 – 0.3 kg ha^{-1} Zn (Martens & Westermann, 1991). Added biomass was the main driver contributing to higher levels of macro and micronutrients in biostimulant end-products, which were found to contain macronutrient concentrations up to 51 ± 5.1 mg L^{-1} N, 14.3 ± 1.0 mg L^{-1} P, and 567 ± 14.1 mg L^{-1} K, and micronutrients concentrations up to 0.08 ± 0.01 mg L^{-1} Cu, 1.6 ± 0.02 mg L^{-1} Fe, and 0.2 ± 0.01 mg L^{-1} Mn. For practical application of biostimulants (i.e., 1-2% solutions, 10,000 L ha^{-1}), these levels are considered insufficient to meet the nutrient requirement for optimum plant growth in soils (du Jardin, 2015; Yakhin et al., 2017). Therefore, the selection of ferments to assess the efficacy of biostimulants in plant growth assays will be based on other constituents.

Applying seaweed proteins can enhance plant growth and provide resilience to stress (Domingo et al., 2023). In this regard, plants can utilise protein as a nitrogen source (Paungfoo-Lonhienne *et al.*, 2008). For example, when axenically grown *A. thaliana* were treated with 6.0 mg mL^{-1} of the protein bovine serum albumin (BSA) as the sole nitrogen source, plant dry weight and nitrogen content significantly increased compared to treatments without nitrogen (Paungfoo-Lonhienne *et al.*, 2008). In the current study, higher biomass loading was the main driver contributing to higher levels

of protein content in biostimulant end-products, which were found to contain concentrations of up to 504.2 ± 21.9 µg mL^{-1} of protein. For practical application of biostimulants (i.e., 1-2% solutions, 10,000 L ha^{-1}), these levels are considered insufficient to contribute significantly to the required nutrients for plant growth (Yin *et al.*, 2018). However, plant growth regulating proteins able to modulate the immune response in plants have been identified in *U. lactuca* extracts and have demonstrated a reduction in disease severity by ~40 % at concentrations as low as 0.02 mg mL^{-1} (Přerovská *et al.*, 2022). In fact, plant growth-regulating peptides have activity at 10^{-7} – 10^{-9} M and may also be produced by bacteria and fungi (Zhang *et al.*, 2023). The potential for peptides and/or proteins able to modulate gene expression and induce metabolic changes in plants leading to biostimulant effects is a possibility and therefore protein content within the extracts produced in this study may be used as an indicator of the presence such bioactive molecules.

Polysaccharide extracts can enhance plant root and shoot growth (Hernández-Herrera *et al.*, 2016; Shefer *et al.*, 2022) and prime plants to respond to periods of stress (Zuo *et al.*, 2021; Sujeeth *et al.*, 2022). For example, when applied as a foliar spray, 10 mg mL^{-1} of an ulvan extract produced from *U. fasciata* induced an elicitor effect against the fungus *Zymoseptoria tritici* in wheat by upregulating defence pathways leading to reduced disease severity and fungal sporulation by 45 and 50 %, respectively (de Borba *et al.*, 2021). Furthermore, the addition of 0.07 mg mL^{-1} of ulvan extract to plant media increased root length by 174 %, root dry weight by 83 %, shoot dry weight by 54 % and total plant dry weight by 62 % compared to control treatments (Shefer *et al.*, 2022). In this study, ulvan represents the predominant known bioactive component of the biostimulant end-products and higher biomass loading was the main driver contributing to higher levels of ulvan, which were found to contain concentrations of up to 8.1 ± 0.4 mg mL^{-1} of ulvan. For practical application of these biostimulants (i.e., 1-2 % solutions, 10,000 L ha^{-1}), these levels are equivalent to those levels demonstrating biostimulant effects in previous studies (Hernández-Herrera *et al.*, 2016; Shefer *et al.*, 2022). The potential for the ulvan content to modulate gene expression, induce an elicitor effect, and/or promote a growth response through a biostimulant effect is a possibility and is central to the selection of biostimulants tested in plant growth assays.

Previously detected phytohormones in seaweeds include auxins, abscisic acid, gibberellins, cytokinins, ethylene, brassinosteroids, salicylic acid, and jasmonic acid (Stirk & Van Staden, 2014). Of these phytohormones, only phenylacetic acid (PAA) was detected and was present in all treatments. PAA is an auxin and plays a role in plant growth regulation by enhancing cell elongation and lateral root formation (Sugawara *et al.*, 2015; Cook, 2019) and has antimicrobial properties (Cook, 2019). In comparison to indole-3-acetic acid (IAA) (the most studied natural auxin in plants), PAA requires higher concentrations (10 to 20 fold) to result in biological activity (Sugawara *et al.*, 2015). Naturally occurring in algae (Korasick *et al.*, 2013; Cook, 2019), PAA was present in low amounts in the unfermented extracts of *U. stenophylloides*. Furthermore, PAA can also be synthesised by bacteria, fungi, and terrestrial plants (Cook, 2019). In this regard, PAA has previously been detected in kombucha beverages; however, the concentrations are not reported in these studies (Vázquez-Cabral *et al.*, 2014; Yu-cheng Sui *et al.*, 2018; Yao *et al.*, 2023; Cheng *et al.*, 2024). The highest yield of PAA (up to 3.87 µg mL^{-1}) occurred in treatments with the highest incubation temperature and mid to high biomass loadings. This is consistent with increased microbial growth rates and metabolism at higher incubation temperatures, promoting the accumulation of organic acids (Aung & Eun, 2022). Experimentally, exogenous application of PAA at 0.5 mM resulted in increased formation of lateral roots and upregulation of genes associated with growth and defence in tobacco; furthermore, activity resulting in reduced disease resistance was reported in concentrations as low as 0.01 mM (Sumayo *et al.*, 2018). The average yield in the highest biomass loading from this study is equivalent to 0.02 mM PAA. For practical application of these biostimulants (i.e., 1-2% solutions, 10,000 L ha^{-1}), these levels are unlikely to be sufficient to contribute to plant growth. However, based on available literature, it is unknown what the effects of PAA will be as part of a seaweed biostimulant and how the other components will interact together on plant growth. Furthermore, the PAA results indicate that there is increased microbial activity at a higher incubation temperature and therefore likely to be indicative of increased production of other microbial biochemicals.

2.7 Conclusion

This is the first reported research to investigate this method of fermentation using *U. stenophylloides* for the manufacture of a biostimulant. This study demonstrates that by manipulating fermentation parameters, extracts can be produced with unique chemical profiles. Initial biomass loading, sucrose input, and incubation temperature are critical parameters in the successful fermentation of *U. stenophylloides*.

Processing results revealed that the initial sucrose inputs were essential drivers of the fermentation process. Treatments with high sucrose inputs were eliminated from selection due to issues with remaining sugars in the final product. Chemical analysis found that treatments with high biomass loading had the highest amounts of ulvan, protein, and minerals. Ulvan and protein are essential elements for biostimulant effects on plants, and minerals are important for fermentation, so treatments with low biomass loadings were eliminated from selection. Treatments at high incubation temperatures resulted in the highest phenylacetic acid yield; although this is likely at insufficient levels for a response in plants, it is indicative of production of other microbial biochemicals that may influence plant growth; this result was used to select the incubation temperature for selected treatments moving forward to plant assays. Therefore, the four ferments moving forward to testing on plants are:

1) 25 g L^{-1} biomass loading, 10 g L^{-1} sucrose input, 30 °C incubation temperature.
2) 25 g L^{-1} biomass loading, 35 g L^{-1} sucrose input, 30 °C incubation temperature.
3) 40 g L^{-1} biomass loading, 10 g L^{-1} sucrose input, 30 °C incubation temperature.
4) 40 g L^{-1} biomass loading, 35 g L^{-1} sucrose input, 30 °C incubation temperature.

Further research into the impact of applying these extracts on plants will determine if there is a biostimulant effect resulting in increased plant growth.

Chapter 3

Impact of *Ulva stenophylloides* seaweed biostimulant on root growth in mung bean and tomato seedlings in hydroponics

3.1 Abstract

The green seaweed *Ulva stenophylloides* was utilised to produce four biostimulants using a novel fermentation method. Although these ferments were previously demonstrated to have promising composition as biostimulants, their effect on plants had not yet been studied. Therefore, this study quantified the impact of applying the biostimulants to mung bean and tomato seedlings in hydroponics to determine any effect on root growth, potential auxin-like effects, and interactive fertiliser effects. Ferment four was the best performing biostimulant tested; at a 1 % (v/v) dose, mung bean fresh and dry weight were increased by 15 and 16 %, respectively, and the most root growth was generated, this biostimulant was therefore tested in further assays. An auxin-like effect was not detected at any biostimulant dose. At a 1 % dose, an interactive effect with fertiliser was demonstrated in tomato seedling root number. A 2 % dose of biostimulant had adverse impacts on root growth over all three root growth assays. This study demonstrates that a 1 % dose the selected *U. stenophylloides* seaweed biostimulant can improve seedling growth in hydroponics with variable results; therefore, this requires further testing in soil over a longer period to determine effects on biomass productivity and yield.

3.2 Introduction

Seaweed biostimulants are products that, when applied in small amounts to plant or soil, stimulate the physiological processes of plants, resulting in improved growth, yield, and development (du Jardin, 2015). As a result, these products are of great interest in sustainable agriculture. Recently, the effects of fermentation parameters on

biostimulant compositional characteristics were evaluated utilising the green seaweed *Ulva stenophylloides* as a novel fermentable substrate (Chapter 2, this thesis). From the 27 biostimulants that were produced and chemically characterised, four were selected for further testing based on their fermentation progression and their content of potential plant growth enhancing bioactives such as protein, polysaccharide (ulvan), and a phytohormone (phenylacetic acid) (section 2.5). Additionally, due to fermentation, these biostimulants contain a mixture of microbes that may induce crop benefits through a probiotic effect and microbial biochemicals that may enhance plant productivity. While the content of bioactive components can indicate quality, there is a drive for effects-based evidence of efficacy (Rouphael & Colla, 2018; Ricci *et al.*, 2019; Boukhari *et al.*, 2020). For example, the EU Fertilising Products Regulations outline that for manufacturers to bring their biostimulants to the market, they need to provide evidence of their product's beneficial biostimulant function on plants (Ricci *et al.*, 2019). Therefore, while the composition of the selected biostimulants appears promising, the true efficacy of a product is determined by evidence collected from plant growth assays demonstrating biostimulant effects.

There are several ways to quantify the effects of seaweed biostimulants on plant growth. These include seed germination trials on a plated growth medium or filter paper in a growth cabinet (Hernández-Herrera *et al.*, 2014; Ghaderiardakani *et al.*, 2019), growth of seedlings in hydroponic systems (Crouch & Van Staden, 1991; Neveux *et al.*, 2020), or greenhouse based pot trials (Hernández-Herrera *et al.*, 2014; Castellanos-Barriga *et al.*, 2017) as starting points to screen for plant growth promoting effects. These controlled environment methods are typically used to identify the target formulations and dilutions before conducting field trials with less environmental control and higher resource requirements. Germination trials are a relatively quick and easy method to assess impacts on seed germination and early plant growth (Hernández-Herrera *et al.*, 2014); however, they do not provide information on seedling growth, root development, and interactions with soil. Conversely, greenhouse pot trials provide information on plant-soil interactions (Hussain *et al.*, 2021) but are typically restricted to fewer treatments due to space requirements and longer time frames to collect harvest and yield data. Growth and

development of young seedlings in hydroponics do not provide data on the effects on final yields or plant-soil interactions; however, they offer an efficient method for concurrently screening multiple biostimulants and concentrations (Neveux *et al.*, 2020). This approach enables control over environmental factors, precise measurements of plant shoot and root systems because there is no damage to the plant tissues, and a direct view of root growth and structure to monitor growth and development.

Longer roots or a more complex root architecture allow for greater access to nutrients and water from the soil for growth and development (Battacharyya *et al.*, 2015). Deeper or further root reach can also lead to plants being better equipped to deal with periods of environmental stress (Chen *et al.*, 2023). Several studies have demonstrated the positive effects of seaweed biostimulants as an effective tool to enhance plant root growth. For example, at the optimal concentration, root growth was enhanced in mung bean with extracts produced from *U. lactuca* (Castellanos-Barriga *et al.*, 2017), *Ecklonia maxima* (Crouch & Van Staden, 1991), and the freshwater alga *Oedogonium intermedium* (Neveux *et al.*, 2020). Root density was increased with an extract from *Duvillaea potatorum* and *Ascophyllum nodosum* in strawberry (Mattner *et al.*, 2018), root biomass was enhanced with two commercial seaweed biostimulant products (Maxicrop® and Seasol®) in lettuce (Yusuf *et al.*, 2019), and root growth was promoted with an extract from *Laminaria japonica* during drought stress in sugarcane (Chen *et al.*, 2023).

This study aims to compare the biostimulant effect of four novel fermented biostimulants produced from *U. stenophylloides*, selected based on their previously characterised favourable biochemical profile (Chapter 2, this thesis). Because of the importance of a complex root system in providing the plant with water and nutrients for productivity, a connection to soil microbes, and resilience to environmental stress (Lombardi *et al.*, 2021), and the ability to precisely test multiple treatments and dilutions on root growth in a controlled environment a hydroponic root assay will be used with mung beans (*Vigna radiata*) (root growth assay one). The best performing biostimulant will be further tested in two additional hydroponic root assays using

mung bean (root growth assay two) and tomatoes (*Solanum lycopersicum*) (root growth assay three).

3.3 Methods

3.3.1 Seaweed cultivation and biostimulant preparation

U. stenophylloides was cultivated in a recirculating aquaculture system (RAS) in the Facility for Aquaculture Research of Macroalgae (FARM) at the University of Waikato Coastal Marine Field Station, Tauranga, New Zealand. The biostimulants were fermented using four selected combinations of biomass loading, sucrose input, and incubation temperature (Table 3.1). For further details, refer to section 2.3.1 for seaweed cultivation and sections 2.3.2 and 2.3.3 for biostimulant preparation. These four biostimulants were selected because mid to high biomass loadings resulted in high amounts of potential growth promoting protein and ulvan in the biostimulant, high sucrose inputs were excluded due to issues with remaining sugars (continued fermentation) in the final fermented product, and high incubation temperatures were selected as these resulted in higher amounts of the plant growth promoting auxin phenylacetic acid and potentially other microbial biochemicals in the product. Details of each ferment's chemical characteristics can be found in Table 3.1.

Table 3.1: Chemical characteristics of the four selected *U. stenophylloides* seaweed biostimulants from Chapter 2, this thesis. Average ± SE.

Fermentation parameters	**Unit**	**Ferment 1**	**Ferment 2**	**Ferment 3**	**Ferment 4**
Biomass loading	**g L^{-1}**	25	25	40	40
Initial sucrose loading	**g L^{-1}**	10	35	10	35
Incubation temperature	**°C**	30	30	30	30
Processing parameters					
Glucose	mg L^{-1}	3.5 ± 2.4	0	0.1 ± 1.5	0.7 ± 2.7
Total dissolved solids	%	1.5 ± 0.04	2.3 ± 0.13	2.4 ± 0.10	3.0 ± 0.23
pH	pH	4.3 ± 0.02	3.6 ± 0.05	4.9 ± 0.5	3.7 ± 0.04
Pellicle	g	1.1 ± 0.02	1.9 ± 0.1	1.3 ± 0.2	2.1 ± 0.3
Bioactives					
Protein	µg mL^{-1}	325 ± 46	303 ± 17	407 ± 19	451 ± 8
Ulvan	mg mL^{-1}	3.2 ± 0.5	3.9 ± 0.2	6.7 ± 0.4	6.2 ± 0.6
Phenylacetic acid	µg mL^{-1}	2.2 ± 0.2	2.8 ± 0.6	1.5 ± 0.3	2.2 ± 0.5

3.3.2 Root growth assays

Three assays were performed in hydroponic solutions to determine the biostimulant effect on root growth. Root growth assay one tested the impact of four *U. stenophylloides* biostimulants, tested at two concentrations, on mung bean (*Vigna radiata*) seedlings to select one biostimulant for subsequent assays. Root growth assay two evaluated the selected biostimulant at a range of concentrations on mung bean seedlings for an auxin-like effect using indole-3-butyric acid (IBA) as a standard, while root assay three used tomato (*Solanum lycopersicum*) seedlings to evaluate the selected biostimulant at a range of concentrations, alone and in combination with a commercially available NPK fertiliser (two-part Egmont hydroponic nutrients).

A pH range between 5.5 and 7 is ideal for most vegetable growth; this ensures the bioavailability of essential plant nutrients and optimal growth and development (Liu & Hanlon, 2012). Here, pH in un-adjusted treatment solutions ranged between 4.22 and 6.35. Therefore, to make sure pH levels were within the ideal range and to standardise treatments within a specific range, pH was buffered with 10 mM 2-(N-morpholino)ethanesulfonic acid (MES) and adjusted to a pH of 6.5 (tomato) or 7.2 (mung bean) using 2M sodium hydroxide. As the concentration of biostimulant and nutrient solution was increased, pH decreased slightly in treatment solutions. pH was buffered in biostimulant treatments for root growth assay two (mung bean) and all treatments for root growth assay three (tomato), including controls for both assays. Root growth assay one (mung bean) treatments were not pH buffered.

3.3.2.1 Root growth assay one: The effect of four *U. stenophylloides* biostimulants on mung bean seedlings

A mung bean (*Vigna radiata*) rooting assay was performed using the four best biostimulant candidates (Table 3.1) based on fermentation processing and chemical composition from the previous chapter. Each biostimulant was tested in hydroponic solutions at two concentrations (1 % and 2 % v/v) on mung bean seedlings (n=15). The results from this assay were used to determine the best biostimulant to test in

subsequent assays. The following methods were adapted from Crouch and Van Staden (1991).

Seed germination and seedling growth

Mung bean seeds were surface sterilised with a 4 % sodium hypochlorite solution (v/v) for five minutes, then triple rinsed in distilled water. Seeds were planted in seedling trays (one seed per cell) containing moist vermiculite. Seedling trays comprised six cells (cell size 37 mm x 47 mm x 55 mm) and sat in a drip tray with a base level of 5 mm dechlorinated tap water; this was maintained throughout the germination and seedling growth period. Seeds were germinated under controlled conditions in a culture cabinet (Panasonic MLR-352) set at 30 °C, 12:12 light/dark cycle, at an irradiance of 45 µmol photons m^2 s^{-1}. Seedling trays were rotated (back to front and moved shelves) daily. Three days after planting, the temperature was reduced to 27 °C, while other settings remained the same. The following day, seedlings were moved to a grow tent (Eclipse Elite grow tent 220 cm x 120 cm x 200 cm) under fluorescent lights (two 4Seasons Helios 450X LED Grow Lights) for a further two days. Light and temperature conditions in the grow tent for the duration of root growth assay one and the two days prior were as follows: 12:12 light/dark cycle: 6,771 ± 45.7 lux and 26 ± 0.07 °C during the light cycle and 0 lux and 22.5 ± 0.05 °C during the dark cycle.

Mung bean assay

Six days after planting, cotyledons were removed from the stem, and seedlings were measured and tagged with a small piece of PVC tape 4 cm below the leaves. Seedlings were selected if they reached between 12 and 14.5 cm in height and had developed their first two true leaves. Seedlings were cut at the substrate using sterilised scissors and transferred to 50 mL centrifuge tubes containing either treatment solution or dechlorinated tap water controls (Figure 3.1). Five cuttings were placed through pre-perforated parafilm in each tube (n=15 plants per treatment, n=3 tubes per treatment). The cuttings were pulse treated in solution for six hours. After six hours, the cuttings were transferred to a layout of randomised test tubes (one plant per tube, tube size 30 mm x 150 mm, Figure 3.1) containing 80 mL of dechlorinated tap water for

ten days in the grow tent (light and temperature conditions above). At the end of the assay, seedling cuttings were removed from the test tubes, and roots were dried gently with a paper towel. Roots were photographed, and root number and total root length were measured using ImageJ and SmartRoot software (Lobet *et al.*, 2011). Plant fresh weight and dry weight (freeze-dried Lyovapor L-200, Buchi) were recorded with a three decimal place balance.

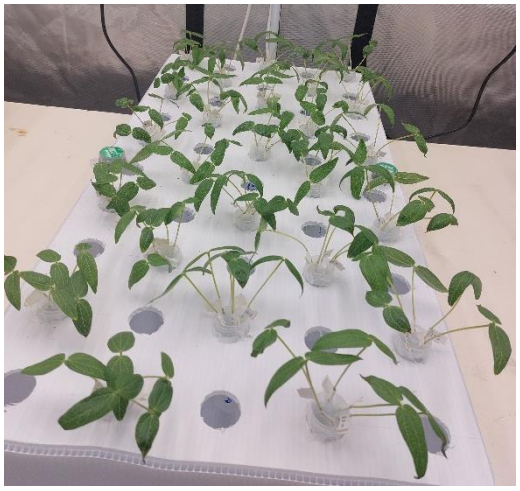
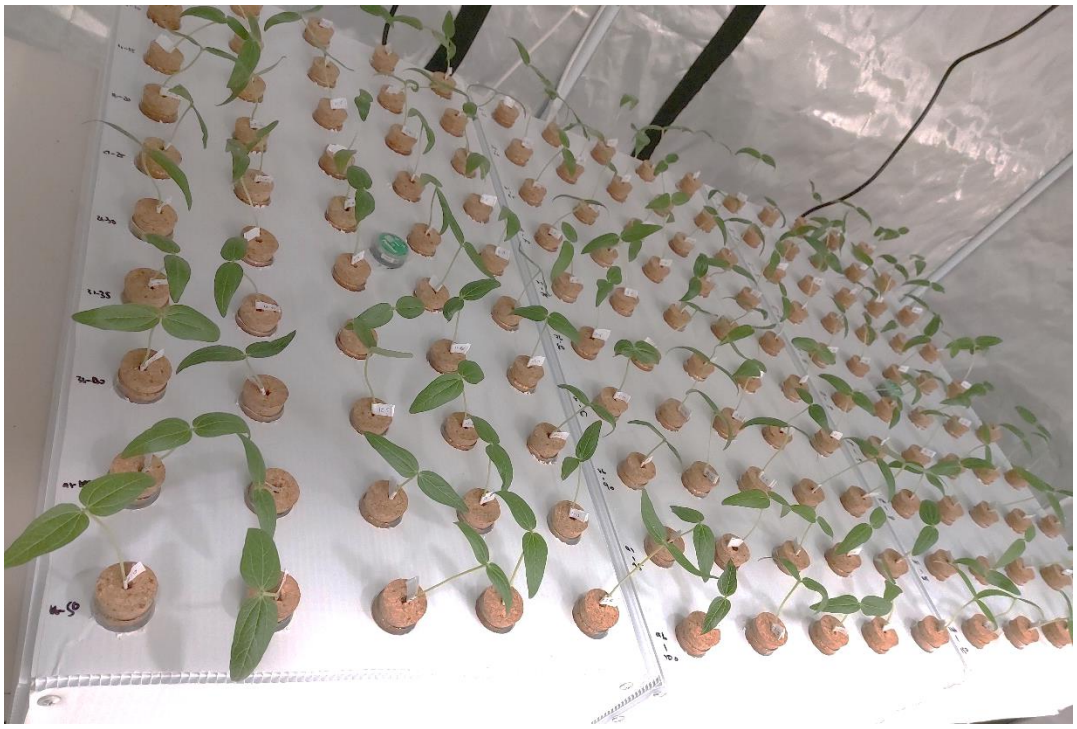


Figure 3.1: Experimental setup for root growth assays. Left: pulse treatment of mung bean seedlings (for root growth assay one and two), five cuttings placed through pre-perforated parafilm into 50 mL centrifuge tubes. Below: layout of mung bean seedlings in randomised test tubes for the duration of the ten day root growth assay, similar set up used for all three root growth assays.



3.3.2.2 Root growth assay two: Mung bean seedlings and auxin-like activity

Auxin-like activity of ferment four (the best performing biostimulant selected from root growth assay one, section 3.5.1) was determined in a mung bean cutting root assay developed by Crouch and Van Staden (1991) and modified by Neveux *et al.* (2020), using indole-3-butyric acid (IBA) as a standard. Due to space limitations, the biostimulant assay and IBA assay were run separately. Seed germination and seedling establishment followed the same methods as root growth assay one (section 3.3.2.1). The mung bean assay itself was similar to section 3.3.2.1, and was modified as follows: plants were tagged 7 cm from the substrate, and due to the seedling size at the start of the assay, smaller plants were selected (between 10 and 14 cm). Pulse treatment and the assay itself remained unchanged. Light and temperature conditions for the duration of root growth assay two and the two days prior were as follows: for the biostimulant assay; 12:12 light/dark cycle: 6,484 ± 26.3 lux and 25.1 ± 0.06 °C during the light cycle, and 0 lux and 21.8 ± 0.06 °C during the dark cycle, and for the IBA standard; 12:12 light/dark cycle: 6,509 ± 16.5 lux and 25.4 ± 0.05 °C during the light cycle, and 0 lux and 22.2 ± 0.04 °C during the dark cycle. The *U. stenophylloides* biostimulant (n=25 per treatment) was tested at five treatment concentrations of 0 (control, dechlorinated tap water), 0.25, 0.5, 1, or 2 % (v/v), and IBA (n=30 per treatment) was tested at five treatment concentrations of 0 (control, dechlorinated tap water), 10^{-6}, 10^{-5}, 10^{-4}, or 10^{-3} M. All biostimulant treatments and their control were pH buffered with MES described as above, IBA treatments and their control were run unbuffered. Root growth assay two (mung bean) was also run unbuffered (results for root growth assay two without pH buffering are presented in (Appendix 7.3). At the end of the assay, plant fresh and dry weights were recorded, and root number and total root length were measured, as described above.

3.3.2.3 Root growth assay three: Tomato seedlings and fertiliser effects

A tomato seedling root growth assay modified from Hernández-Herrera *et al.* (2014) and Neveux *et al.* (2020) assessed any interactive effects between the biostimulant and a conventional fertiliser (ferment four: best performing biostimulant selected from root growth assay one, section 3.5.1). Tomato seeds (*Solanum lycopersicum* Tommy

Toe Heirloom) were surface sterilised with a 4 % sodium hypochlorite solution (v/v) for five minutes, then triple rinsed in distilled water. Seeds were planted in seedling trays containing moist vermiculite and germinated under controlled conditions in a culture cabinet (Panasonic MLR-352) set at 28 °C, 12:12 light/dark cycle, at an irradiance of 45 µmol photons m^2 s^{-1}. Seedling trays comprised six cells (cell size 37 mm x 47 mm x 55 mm) and were rotated (back to front and moved shelves) daily. Seedling trays sat in a drip tray with a base level of 5 mm dechlorinated tap water, which was maintained for six days, after which, all water was poured off and replaced with a nutrient solution (two-part Egmont hydroponic nutrients at a quarter of the manufacturer's directions, 0.625 mL L^{-1} for each part). The following day, seedlings were transferred to a grow tent (Eclipse Elite grow tent 220 cm x 120 cm x 200 cm) under fluorescent lights (two 4Seasons Helios 450X LED Grow Lights). Conditions in the grow tent during the seedling growth and the assay period were as follows: 12:12 light/dark cycle: 14,253 ± 18.4 lux and 28.6 ± 0.06 °C during the light cycle, and 0 lux and 21.8 ± 0.06 °C during the dark cycle. After 21 days from planting, 100 seedlings (average seedling weight 1.55 ± 0.02 g) were selected, removed from the substrate, and the roots were rinsed clean in distilled water. All lateral roots were removed with a sterilised scalpel blade, leaving the main tap root, and the seedling fresh weight was recorded. The seedlings (n= 10 per treatment) were then transferred to a layout of fully randomised glass test tubes (one plant per tube, tube size 30 mm x 150 mm) containing treatment solutions and controls (all MES pH buffered). The *U. stenophylloides* biostimulant was tested using a full factorial design over four concentrations of 0.1, 0.5, 1, and 2 % (v/v), and another four treatments contained the same concentrations of biostimulant plus a dose of fertiliser (two-part Egmont hydroponic nutrients at the manufacturer's directions, 2.5 mL L^{-1} each part), an additional two treatments served as controls; fertiliser in dechlorinated tap water without biostimulant was used as a positive control (0 % + F), and dechlorinated tap water was used as a negative control (0 %). The tomato seedlings were kept in solution in the grow tent under fluorescent lights for ten days (light and temperature conditions as above). Seedlings were removed from the test tubes, and roots were dried gently with a paper towel. Roots were photographed, and root number and total root length were measured using ImageJ

and SmartRoot software (Lobet *et al.*, 2011). Plant fresh weight and freeze-dried dry weight were recorded with a three decimal place balance.

3.4 Data analysis

Due to space constraints, root growth assay two was run in two separate blocks. Data was normalised between runs by calculating the mean for controls on each block (block mean) and calculating the mean for the controls on both blocks together (grand mean) and using the following equation: normalised data = (response*grand mean)/block mean.

For root growth assay one and two, one factor permutational analyses of variance (PERMANOVA) were used to compare the effect of treatments (fixed factor) on mung bean growth (fresh and dry weight) and root growth (root number and total root length). For root growth assay three a two factor PERMANOVA was used to compare the effect of the biostimulant treatments (fixed factor) and determine a fertiliser effect (fixed factor) on tomato seedling growth (fresh and dry weight) and root growth (root number and total root length). The proportion of total variation in root growth assay three was calculated using eta-squared (%) η^2 (SS_{factor} / SS_{total} × 100; SS_{factor} = sum of squares for each factor and SS_{total} = total sum of squares) (Richardson, 2011). All statistical analyses were conducted in Primer v7 (Primer-E., UK) using Euclidean distance resemblance matrices, 9,999 unrestricted permutations of raw data, and Type III sum of squares (Anderson, 2008). Pairwise a posteriori comparisons were carried out if a significant difference (p = < 0.05) was detected. All data is reported as mean ± standard error (SE).

3.5 Results

3.5.1 Root growth assay one: The effect of four *U. stenophylloides* biostimulants on mung bean seedlings

Mung bean seedlings were exposed to four *U. stenophylloides* ferments at two dilutions and subsequently compared to one another (Figure 3.2). Seedlings fresh weight, dry weight, root number and total root length were measured. For each factor

measured, ferment one (2 % dose) consistently resulted in the lowest end of the range and ferment four (1 % dose) resulted in the highest (Figure 3.2, Figure 3.3, Figure 3.4).

Mung bean fresh weight significantly differed between treatments, ranging from 538.5 ± 28.3 mg (ferment one, 2 % dose) to 706.5 ± 25.9 mg (ferment four, 1 % dose) (Figure 3.3a, Table 3.2). Compared to the control (616.9 ± 24.9 mg), a 2 % dose of ferment one resulted in a reduction of fresh weight by 13 % (p = 0.047) and a 1 % dose of ferment four resulted in a 15 % higher fresh weight (p = 0.02). All other ferments were similar in fresh weight to the control (p = > 0.05). There were also notable differences within ferment and dilution groups (Figure 3.3a); all ferments at a 1 % dose resulted in a higher fresh weight than the same ferment at a 2 % dose, although this difference was only significant in ferment one (p = 0.007) and four (p = 0.002), with a 2 % dose of ferment one resulting in a 21 % lower fresh weight compared with a 1 % dose, and this was similar to ferment four, with a 20 % lower weight.

Mung bean dry weight significantly differed between ferments, ranging from 93.5 ± 3.8 mg (ferment one, 2 % dose) to 113.7 ± 2.7 mg (ferment four, 1 % dose) (Figure 3.3b, Table 3.2). Differing from fresh weight, only ferment four (1 % dose) resulted in a significantly higher dry weight (by 16 %) than the control (98.01 ± 3.9 mg) (p = 0.003). A 1 % dose of ferment one also resulted in a significantly higher dry weight (by 12 %) than the control (p = 0.048), while other ferments were a similar to the control (p = > 0.05). As with fresh weight, all ferments at a 1 % dose had a higher dry weight than the same ferment at a 2 % dose (Figure 3.3b); similarly, this difference was only significant in ferment one (p = 0.009) and four (p = 0.02), which had a 15 % and an 11 % lower dry weight at a 2 % dose compared to dry weight at a 1 % dose, respectively.

Root number ranged from 11.7 ± 1.3 (ferment one, 2 % dose) to 18.8 ± 2.0 (ferment four, 1 % dose), but these results were not significantly different to the control (16.7 ± 2.1) or other ferments (Figure 3.4a, Table 3.2). However, differences were detected in total root length between ferments (Table 3.2). Total root length ranged from 11.0 ± 6.0 cm (ferment one, 2 % dose) to 55.8 ± 9.8 cm (ferment four, 1 % dose) (Figure 3.4b), but only ferment one (2 % dose) resulted in significantly inhibited root length (by 72 %) compared to the control (39.7 ± 9.7 cm) (p = 0.02). Following the same pattern as plant fresh weight and dry weight, ferments at a 1 % dose had longer roots than the same ferment at a 2 % dose (Figure 3.4b); however, once again this was only significant in ferment one (p = 0.01) and four (p = 0.005), with a 2 % dose of ferment one inhibiting total root length by 74 % compared with a 1 % dose, and this was similar for ferment four, with a 69 % inhibition.

Figure 3.2: Photos of mung bean seedlings from root growth assay one at day 10 (termination of experiment). Seedlings have been exposed to four selected *U. stenophylloides* biostimulants at 1 % (top row) and 2 % (bottom row) (v/v) concentrations. Control (0 %) far left. Scale bar 1 cm.

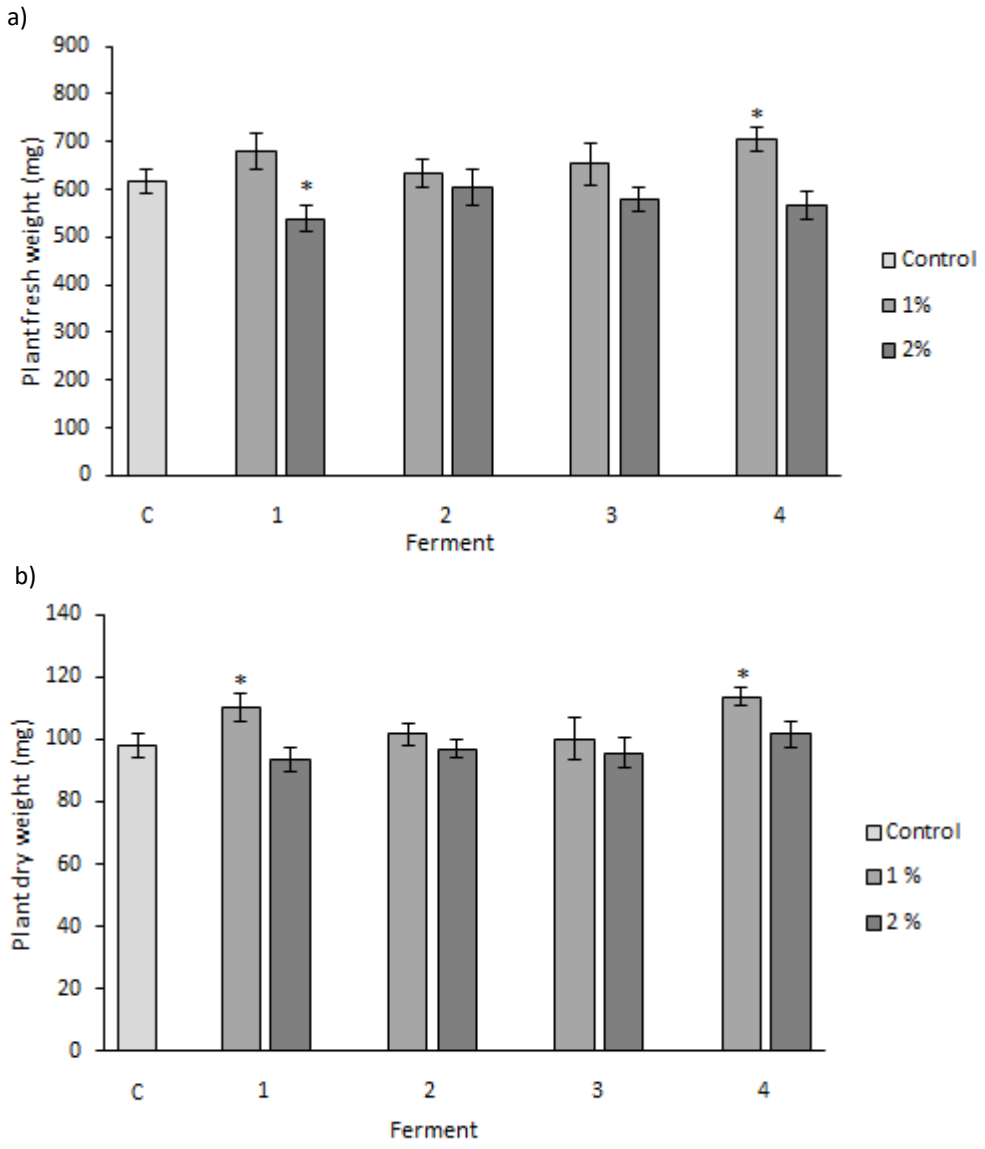


Figure 3.3: Root growth assay one: effect of four selected *U. stenophylloides* biostimulants on mung bean a) fresh weight (mg), and b) dry weight (mg). C = control (dechlorinated tap water); 1 = ferment one, 25 g L^{-1} seaweed biomass, 10 g L^{-1} sucrose loading, 30 °C incubation temperature; 2 = ferment two, 25 g L^{-1} seaweed biomass, 35 g L^{-1} sucrose loading, 30 °C incubation temperature; 3 = ferment three, 40 g L^{-1} seaweed biomass, 10 g L^{-1} sucrose loading, 30 °C incubation temperature; 4 = ferment four, 40 g L^{-1} seaweed biomass, 35 g L^{-1} sucrose loading, 30 °C incubation temperature. Each ferment tested at 1 % and 2 % (v/v) concentrations. * significantly different to the control. n= 15. Average ± SE.

Table 3.2: Results of Permutational Analysis of Variance (PERMANOVA) showing the effects of four *U. stenophylloides* biostimulants on mung bean seedlings. Significant results (p = < 0.05) are in bold

Source	***df***	**Fresh weight**		**Dry weight**		**Root number**		**Root length**	
		F	*p*	F	*p*	F	*p*	F	*p*
Treatments	8	2.86	**0.006**	2.41	**0.016**	1.74	0.094	2.33	**0.022**
Residuals	126								

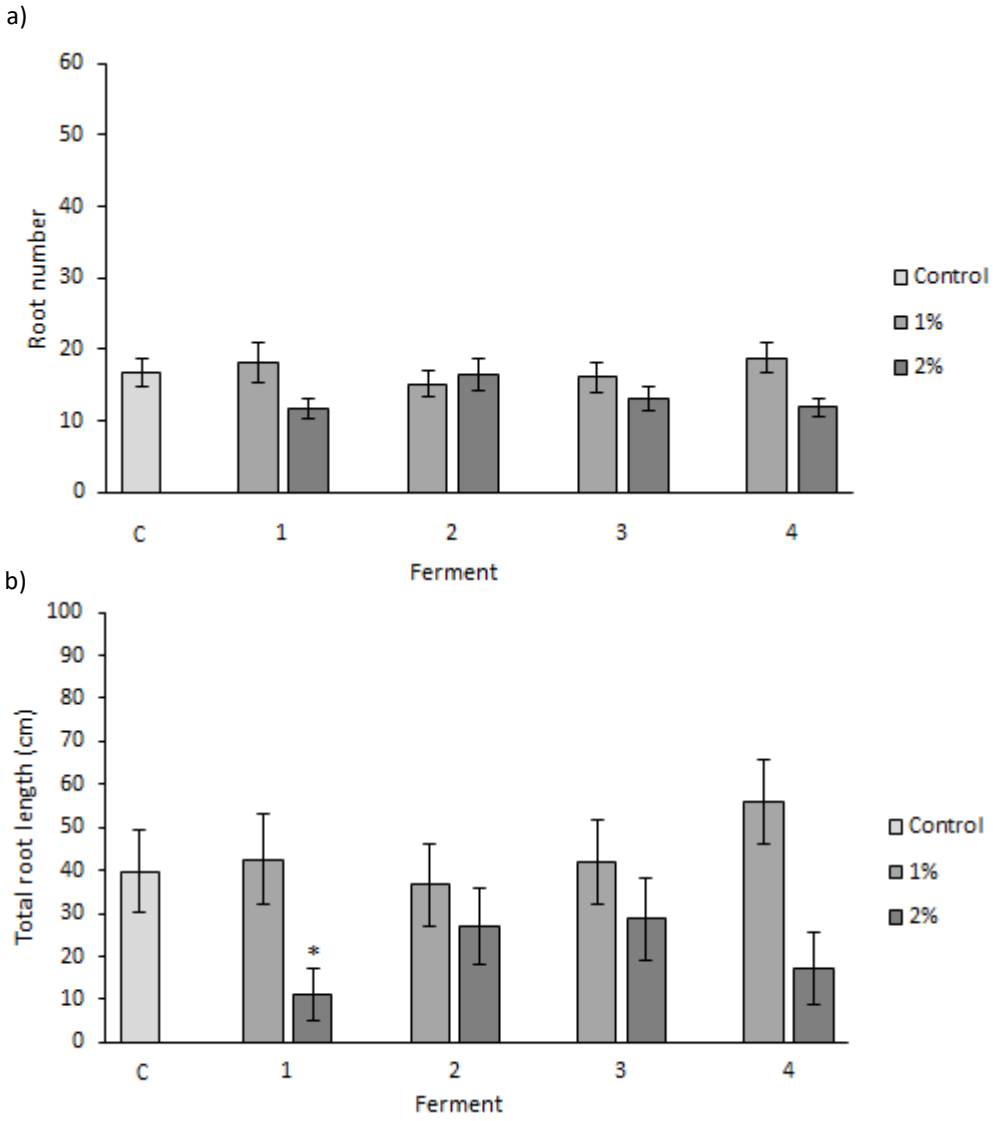


Figure 3.4: Root growth assay one: effect of four selected *U. stenophylloides* biostimulants on mung bean a) root number, and b) total root length (cm). C = control (dechlorinated tap water); 1 = ferment one, 25 g L^{-1} seaweed biomass, 10 g L^{-1} sucrose loading, 30 °C incubation temperature; 2 = ferment two, 25 g L^{-1} seaweed biomass, 35 g L^{-1} sucrose loading, 30 °C incubation temperature; 3 = ferment three, 40 g L^{-1} seaweed biomass, 10 g L^{-1} sucrose loading, 30 °C incubation temperature; 4 = ferment four, 40 g L^{-1} seaweed biomass, 35 g L^{-1} sucrose loading, 30 °C incubation temperature. Each ferment tested at 1 % and 2 % (v/v) concentrations. * significantly different to the control. n=15. Average ± SE.

3.5.2 Root growth assay two: Mung bean seedlings and auxin-like activity

Mung bean seedlings were exposed to a series of dilutions of the selected *U. stenophylloides* biostimulant (ferment four) and then compared to a series of concentrations of indole-3-butyric acid (IBA) to determine the presence of any auxin-like effect (Figure 3.5).

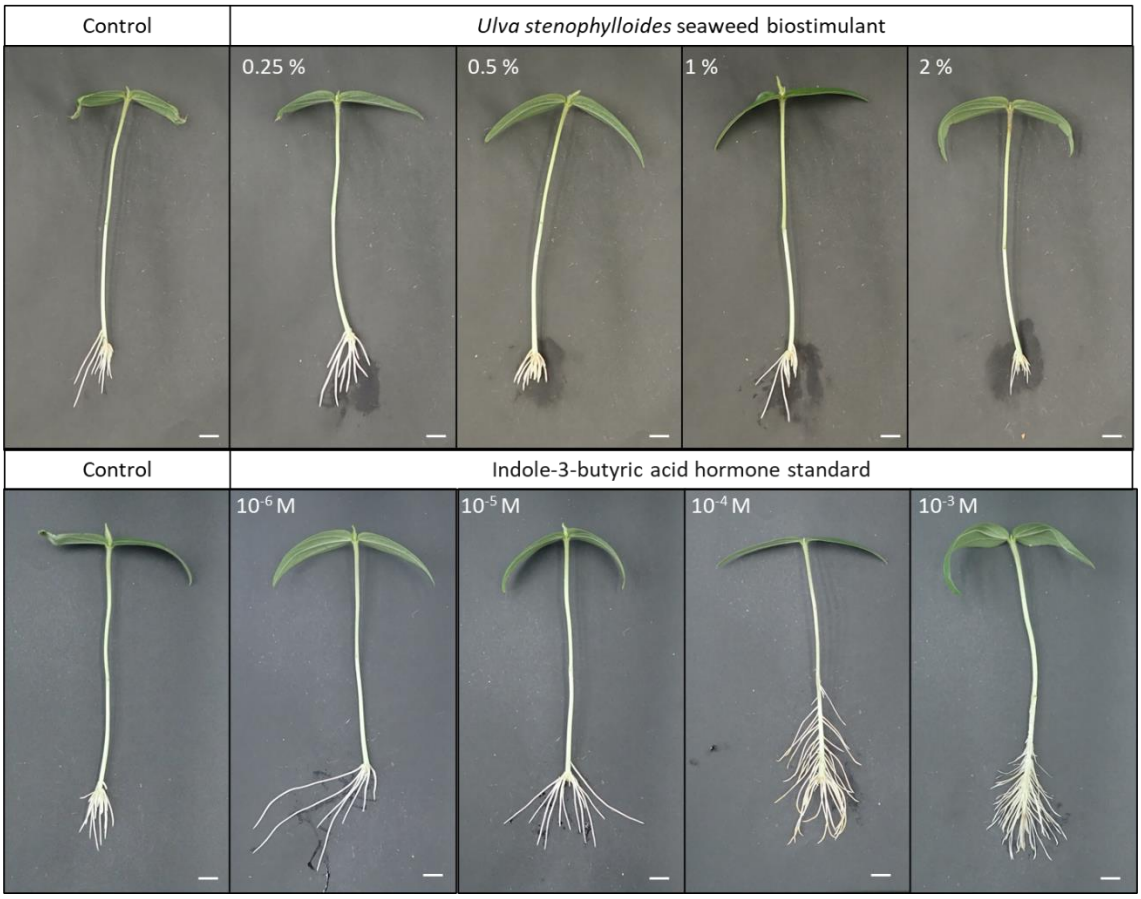


Figure 3.5: Photos of mung bean seedlings from root growth assay two at day 10 (termination of experiment). Seedlings have been exposed to a selected *U. stenophylloides* biostimulant (top row) and compared to indole-3-butyric acid (IBA) auxin (bottom row) as a standard. Control (0 %, dechlorinated tap water) far left. Scale bar 1 cm.

The fresh weight of mung bean seedlings from the biostimulant treatments ranged from 625.2 ± 30.7 mg (2 % dose) to 679.8 ± 27.8 mg (0.25 % dose) (Figure 3.6a), and dry weight ranged from 110.5 ± 4.2 mg (0.5 % dose) to 119.1 ±4.3 mg (0.25 % dose)

(Figure 3.6b). However, neither variable significantly differed from the control (fresh weight 622.2 ± 29.0 mg, dry weight 103.4 ± 4.2 mg) or between treatments (Table 3.3). Conversely, the IBA standard significantly affected the fresh and dry weight of mung bean seedlings (Table 3.3). Fresh weight ranged from 615.9 ± 28.1 mg (10^{-6} M concentration) to 775.4 ± 22.1 mg (10^{-4} M concentration) (Figure 3.6a), and dry weight ranged from 69.1 ± 2.2 mg (10^{-3} M concentration) to 107.9 ± 3.6 mg (10^{-6} M concentration) (Figure 3.6b). The two highest concentrations of IBA, 10^{-4} and 10^{-3}, resulted in a 25 % and 21 % significantly higher fresh weight than the control (622.2 ± 29.5 mg), respectively. Conversely, the two highest concentrations of IBA, 10^{-4} and 10^{-3}, resulted in a 12 % and 33 % significantly lower dry weight than the control (103.4 ± 3.4 mg), respectively.

Mung bean root number ranged from 12.5 ± 1.4 (2 % dose) to 15.9 ± 1.8 (1 % dose) (Figure 3.7a), and root length ranged from 21.0 ± 5.5 (2 % dose) to 34.1 ± 7.9 (1 % dose) (Figure 3.7b) in biostimulant treatments. As with the fresh weight and dry weight of the plants, these did not significantly differ from the control (root number 12.5 ±1.5, root length 28.1 ± 6.3) (Table 3.3). However, IBA did have a significant impact on root number and root length (Table 3.3). Root number in IBA treated mung bean seedlings ranged from 11.1 ± 1.2 (10^{-6} M concentration) to 52.5 ± 2.8 (10^{-3} M concentration) (Figure 3.7a). Root length in IBA treated mung bean seedlings ranged from 24.4 ± 5.7 cm (10^{-6} M concentration) to 89.6 ± 5.7 cm (10^{-3} M concentration) (Figure 3.7b). The two highest concentrations of IBA, 10^{-4} and 10^{-3}, had a significantly greater root number (by 271 % and 318 %, respectively) and root length (by 208 % and 218 %, respectively) than the control (root number 12.5 ± 1.1, root length 28.1 ± 5.5 cm).

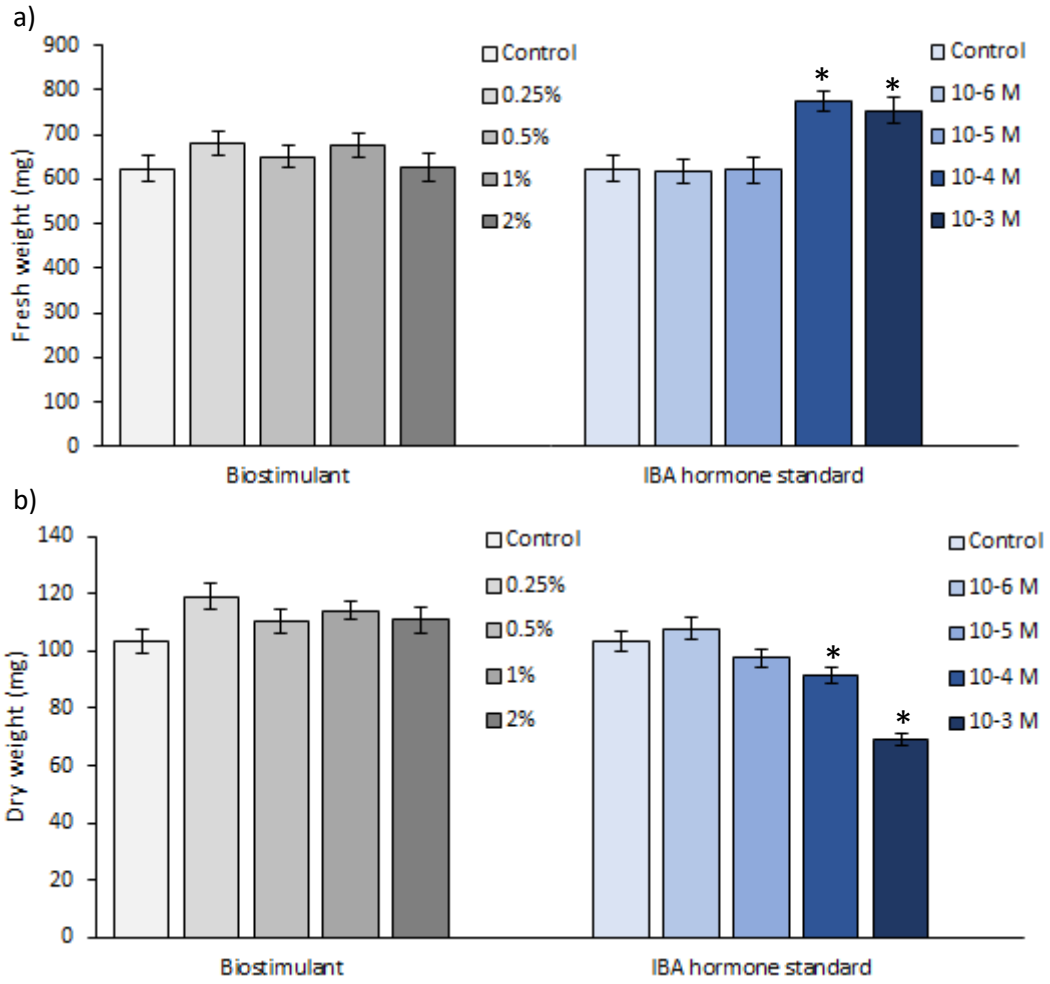


Figure 3.6: Root growth assay two: effect of selected *U. stenophylloides* biostimulant in comparison to indole-3-butyric acid (IBA) auxin standard on mung bean a) fresh weight (mg), and b) dry weight (mg). * significantly different to control. Biostimulant n=25, hormone standard n= 30. Average ± SE.

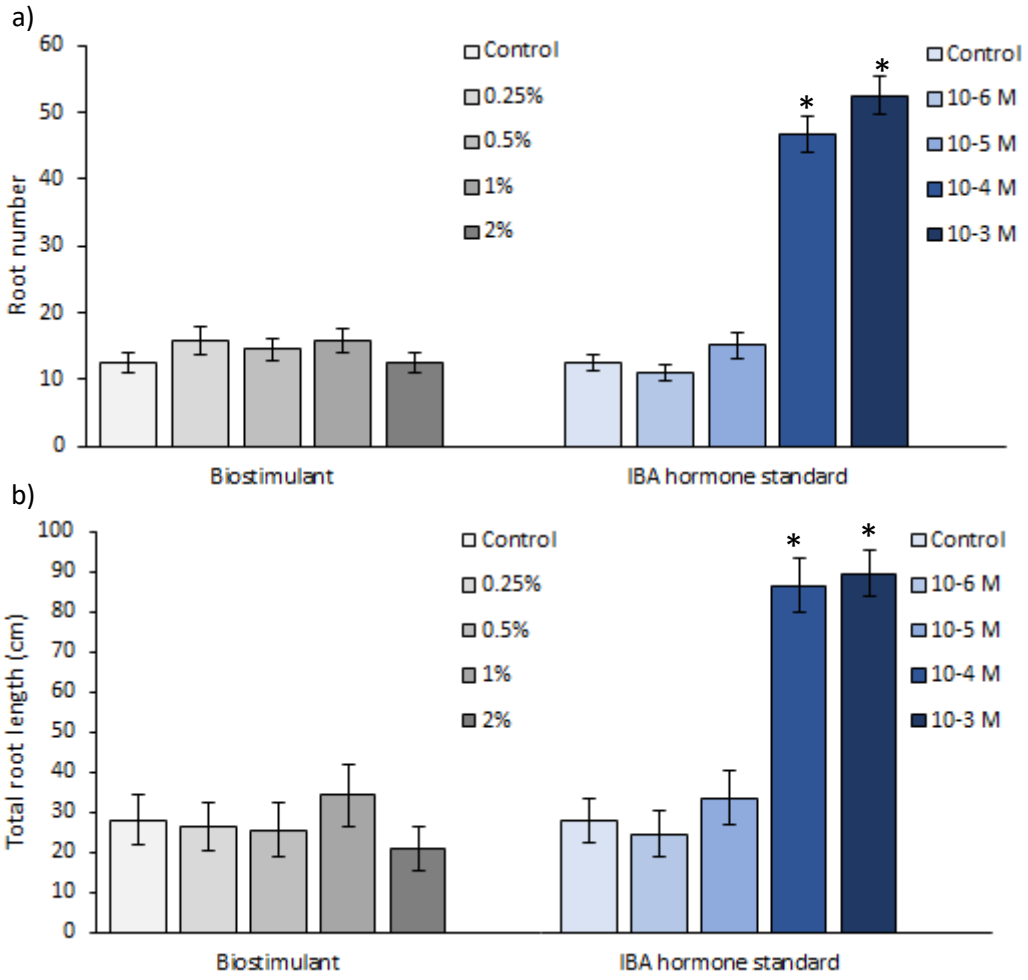


Figure 3.7: Root growth assay two: effect of selected *U. stenophylloides* biostimulant in comparison to indole-3-butyric acid (IBA) auxin standard on mung bean a) root number, and b) total root length (cm). * significantly different to the control. Biostimulant n=25, hormone standard n= 30. Average ± SE.

Table 3.3: Results of Permutational Analysis of Variance (PERMANOVA) showing the effects of *U. stenophylloides* biostimulant and indole-3-butyric acid (IBA) auxin standard on mung bean seedlings. Significant results (p = < 0.05) are in bold.

Source	df	Fresh weight		Dry weight		Root number		Root length	
		F	*p*	F	*p*	F	*p*	F	*p*
Biostimulant	4	0.97	0.43	1.95	0.11	0.96	0.43	0.53	0.71
Residuals	120								
IBA	4	8.53	**0.0001**	24.44	**0.0001**	92.59	**0.0001**	28.82	**0.0001**
Residuals	145								

3.5.3 Root growth assay three: Tomato seedlings and fertiliser effects

Tomato seedlings were exposed to a series of dilutions of the selected *U. stenophylloides* biostimulant (ferment four) and then compared to the same series of dilutions of biostimulant in combination with an NPK fertiliser (two-part Egmont hydroponic nutrients) to determine any interactions between biostimulant and conventional fertiliser (Figure 3.8).

Seedlings only exposed to the biostimulant were significantly different to seedlings exposed to the biostimulant in combination with fertiliser or fertiliser alone (Table 3.4). All treatments in combination with fertiliser had a significantly higher fresh weight and dry weight than all seedlings only exposed to the biostimulant (p = < 0.05) (Table 3.5, Table 3.6, Figure 3.9). Seedlings exposed to a 0 % and 0.1 % biostimulant dose had a similar root number compared to all other treatments in combination with fertiliser, excluding the 1 % dose plus fertiliser, which had a significantly greater number of roots, and the 0 % plus fertiliser dose, which had a significantly greater number of roots than just the 0.1% biostimulant dose (Table 3.7, Figure 3.10a). All other biostimulant treatments combined with fertiliser had a significantly higher root number than all other biostimulant treatments (0.5, 1 and 2 % doses) (p = < 0.05). Root length was longer in seedlings in combination with fertiliser compared to seedlings only exposed to the biostimulant (p = < 0.05), excluding the 0.5 % dose plus fertiliser compared to the 1 % biostimulant dose which did not significantly differ, and all biostimulant treatments above 0.1 % compared to the 2 % dose plus fertiliser, which also had similar root lengths (p = > 0.05) (Table 3.8, Figure 3.10b).

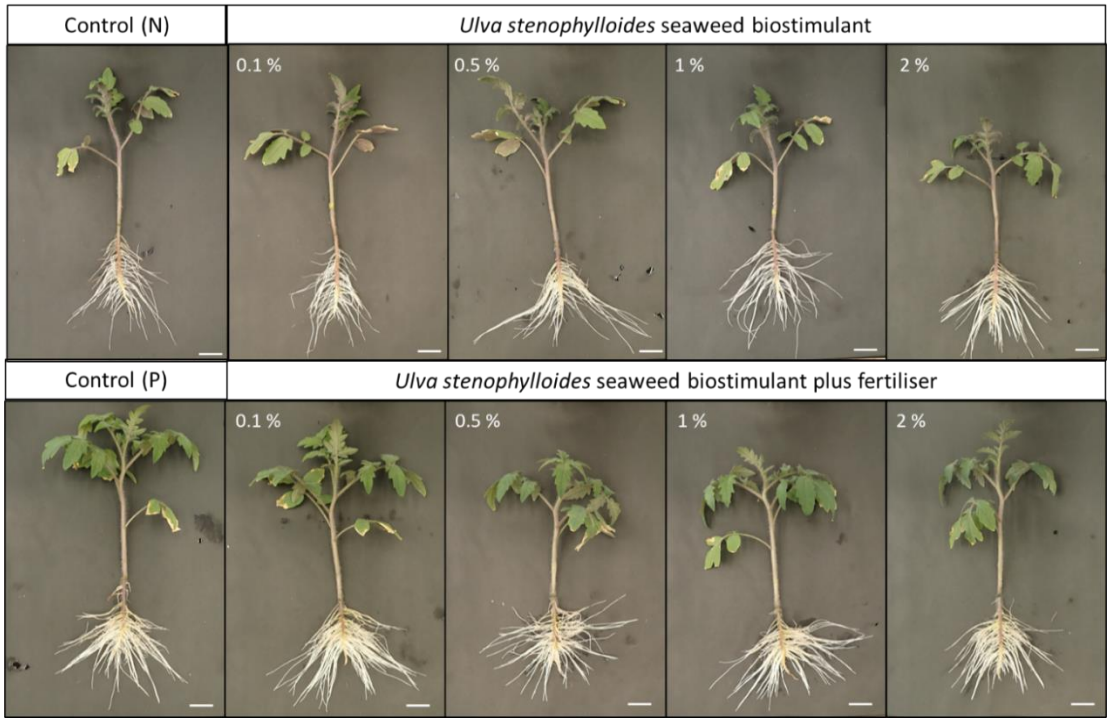


Figure 3.8: Photos of tomato seedlings from root growth assay three at day 10 (termination of experiment). Seedlings have been exposed to a selected *U. stenophylloides* biostimulant (top row) and in combination with fertiliser (bottom row) as a standard. 0 % (negative control) top left, 0 % plus fertiliser (positive control) bottom left. Scale bar 2 cm.

Table 3.4: Results of Permutational Analysis of Variance (PERMANOVA) showing the effects of *U. stenophylloides* biostimulant and fertiliser on tomato seedlings. Significant results (p = < 0.05) are in bold.

Source	***df***	**Fresh weight**		**Dry weight**		**Root number**		**Root length**	
		F	*p*	F	*p*	F	*p*	F	*p*
Biostimulant	4	3.03	**0.02**	3.99	**0.01**	2.44	0.05	2.21	0.07
Fertiliser	1	448.97	**0.0001**	152.91	**0.0001**	65.17	**0.0001**	80.97	**0.0001**
Biostimulant x fertiliser	4	2.17	0.08	1.08	0.37	4.06	**0.005**	3.07	**0.02**
Residuals	90								

Table 3.5: Percent increase in fresh weight of tomato seedlings exposed to biostimulant dose plus fertiliser compared to biostimulant alone. Biostimulant dose = % v/v, + F = added fertiliser.

	0 %	**0.1%**	**0.5%**	**1%**	**2%**
0 % + F	90	81	75	77	87
0.1 % + F	91	82	75	78	88
0.5 % + F	85	76	70	72	82
1 % + F	87	79	72	75	85
2 % + F	60	53	47	49	58

Table 3.6: Percent increase in dry weight of tomato seedlings exposed to biostimulant dose plus fertiliser compared to biostimulant alone. Biostimulant dose = % v/v, + F = added fertiliser.

	0 %	**0.1%**	**0.5%**	**1%**	**2%**
0 % + F	45	42	43	40	56
0.1 % + F	52	49	50	47	64
0.5 % + F	39	37	37	35	50
1 % + F	43	41	41	39	54
2 % + F	23	21	21	19	32

Table 3.7: Percent increase in root number of tomato seedlings exposed to biostimulant dose plus fertiliser compared to biostimulant alone. Biostimulant dose = % v/v, + F = added fertiliser. * not significantly different.

	0 %	**0.1%**	**0.5%**	**1%**	**2%**
0 % + F	11*	11	29	36	37
0.1 % + F	10*	10*	28	34	35
0.5 % + F	10*	11*	28	35	36
1 % + F	22	23	42	49	51
2 % + F	7*	7*	25	31	32

Table 3.8: Percent increase in total root length of tomato seedlings exposed to biostimulant dose plus fertiliser compared to biostimulant alone. Biostimulant dose = % v/v, + F = added fertiliser. * not significantly different.

	0 %	**0.1%**	**0.5%**	**1%**	**2%**
0 % + F	62	61	48	27	45
0.1 % + F	68	66	53	32	50
0.5 % + F	51	50	38	19*	35
1 % + F	62	60	47	27	45
2 % + F	32	30	20*	3*	18*

There were significant differences between fresh and dry weight treatments and a fertiliser effect detected (Table 3.4), these differences were largely driven by the addition of fertiliser (η^2 = 88 % and 72 %, respectively). Fresh weight and dry weight of seedlings only exposed to the biostimulant ranged from 2324.2 ± 95.7 mg (2 % dose) to 2493.0 ± 99.7 mg (0.5 % dose) and 278.3 ± 10.2 mg (2 % dose) to 309.6 ± 9.8 mg (1 % dose), respectively (Figure 3.9). However, neither response differed from the 0 % dose (fresh weight 2289.7 ± 113.4 mg, dry weight 300.1 ± 13.8 mg) or between treatments, excluding the 1 % dose, which was 11 % higher in dry weight than the 2 % dose. When in combination with fertiliser, the fresh weight of seedlings ranged from 3668.1 ± 153.9 mg (2 % dose plus fertiliser) to 4362.0 ± 111.4 mg (0.1 % dose plus fertiliser), and dry weight ranged from 368.3 ± 16.0 mg (2 % dose plus fertiliser) to 456.7 ± 10.8 mg (0.1 % dose plus fertiliser) (Figure 3.9a). Fresh weight and dry weight differed significantly between treatments plus fertiliser. For both fresh and dry weight, treatments at a 2 % biostimulant dose plus fertiliser weighed significantly less than the 0 % plus fertiliser dose (fresh weight 16 % less than 0 % plus fertiliser 4355.3 ± 193.6 mg, dry weight 15 % less than the 0 % plus fertiliser dose 434.7 ± 22.4 mg) (p = 0.01, p = 0.03, respectively). The fresh weight of the 2 % biostimulant dose plus fertiliser treatment also weighed significantly less (13-16 %) than all other treatments plus fertiliser (p = < 0.05). This was also the pattern of the 2 % biostimulant dose plus fertiliser treatment for dry weight, (19 % less than 0.1 % biostimulant dose plus fertiliser and 14 % less than 1 % biostimulant dose plus fertiliser) excluding the treatment at 0.5 % biostimulant plus fertiliser which did not significantly differ.

Root number for seedlings only exposed to the biostimulant ranged from 37 ± 2.0 (2 % dilution) to 45.4 ± 1.6 (0.1 % dilution) (Figure 3.10a). There was a significant interaction between biostimulant treatment and fertiliser addition on the number of roots the seedlings produced (Table 3.4) where a 1 % dose of biostimulant in combination with fertiliser enhanced root number compared to only 0 % fertiliser and the 1 % biostimulant alone; explaining 11 % of the variation. However, most of the variation was driven by the addition of fertiliser (η^2 = 48 %). In treatments with only biostimulant, with increasing concentration of biostimulant, root number decreased, although this was only significantly less at 0.5 % (14 % less than the 0 % dose and 0.1 %

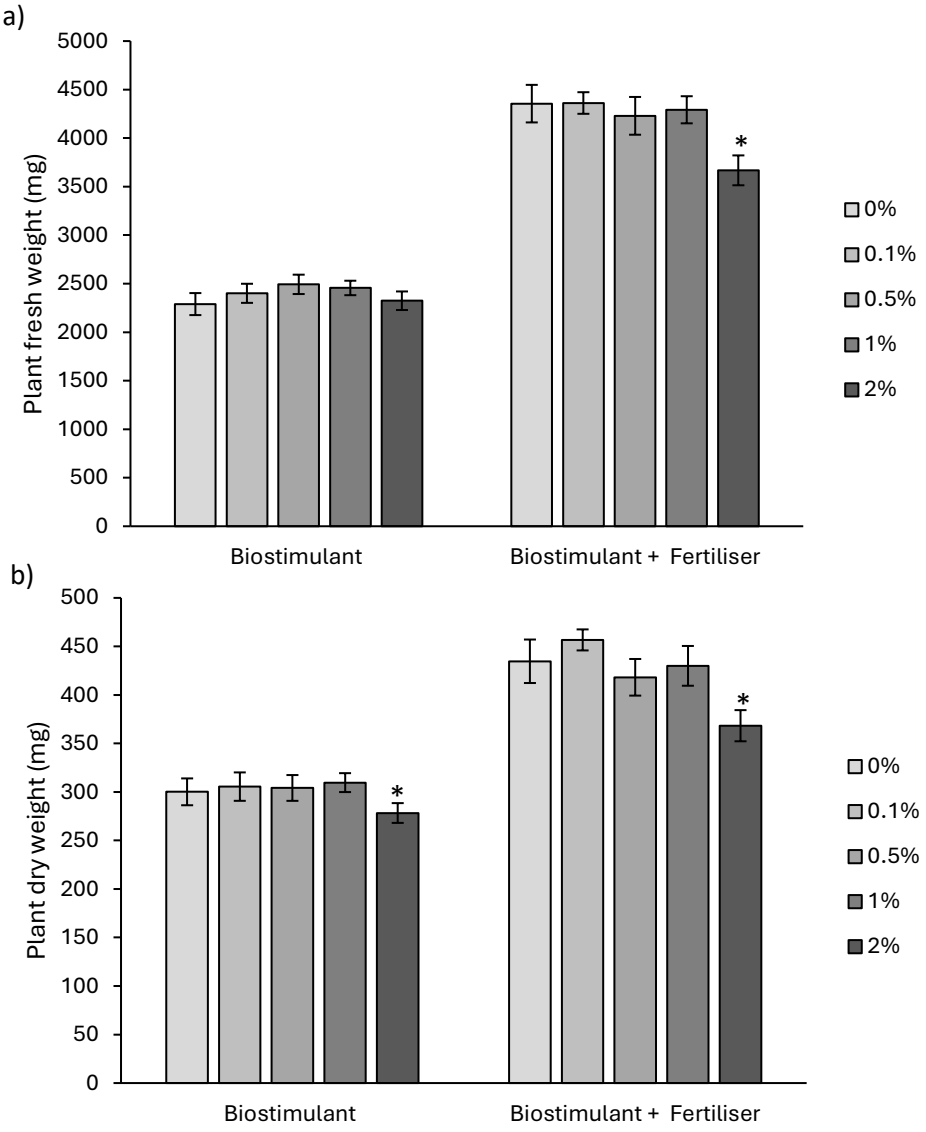


Figure 3.9: Root growth assay three: effect of selected *U. stenophylloides* biostimulant alone and in combination with fertiliser on tomato a) fresh weight (mg), and b) dry weight (mg). * significantly different to the control. Biostimulant dose = % v/v. n=10. Average ± SE.

dose), 1 % (18 % less than the 0 % dose and 0.1 % dose), and 2 % (19 % less than the 0 % dose and 0.1 % dose) treatments in comparison to the 0 % dose (45.6 ± 1.7) and the 0.1 % dose (p = < 0.05). When in combination with fertiliser, root number ranged from 48.7 ± 1.9 (2 % dose plus fertiliser) to 55.7 ± 1.3 (1 % dose plus fertiliser) (Figure 3.10a), and root length ranged from 147.7 ± 9.6 cm (2 % dose pus fertiliser) to 188.6 ± 9.5 cm (0.1 % dose plus fertiliser) (Figure 3.10b). There was a significant interaction between biostimulant treatment and fertiliser addition on the seedlings root length (Table 3.4); however, this only explained 7 % of the variation. Most of the variation was driven by the addition of fertiliser (η^2 = 56 %). Root length for seedlings only exposed to the biostimulant ranged from 113.5 ± 4.4 cm (0.1 % dose) to 143.1 ± 7.0 cm (1 % dose), this significantly differed between treatments (Figure 3.10b). In contrast to root number, root length increased with higher concentrations of biostimulant until decreasing slightly at the 2 % dose. However, significant differences were only detected between the 1 % dose and the 0 % dose (112.3 ± 3.3 cm) (p = 0.001) and the 0.1 % dose (p = 0.003), which was 27 % and 26 % longer in root length, respectively. Root number was significantly higher in the 1 % biostimulant dose plus fertiliser compared to the 0 % dose plus fertiliser (50.6 ± 1.7) and the 2 % biostimulant dose plus fertiliser (10 % and 14 %, respectively). Root length was significantly inhibited by the 2 % biostimulant dose plus fertiliser compared to the 0 % dose plus fertiliser (182.3 ± 12.5 cm), 0.1 % biostimulant dose plus fertiliser, and 1 % biostimulant dose plus fertiliser (19 %, 22 %, and 19 % inhibition, respectively).

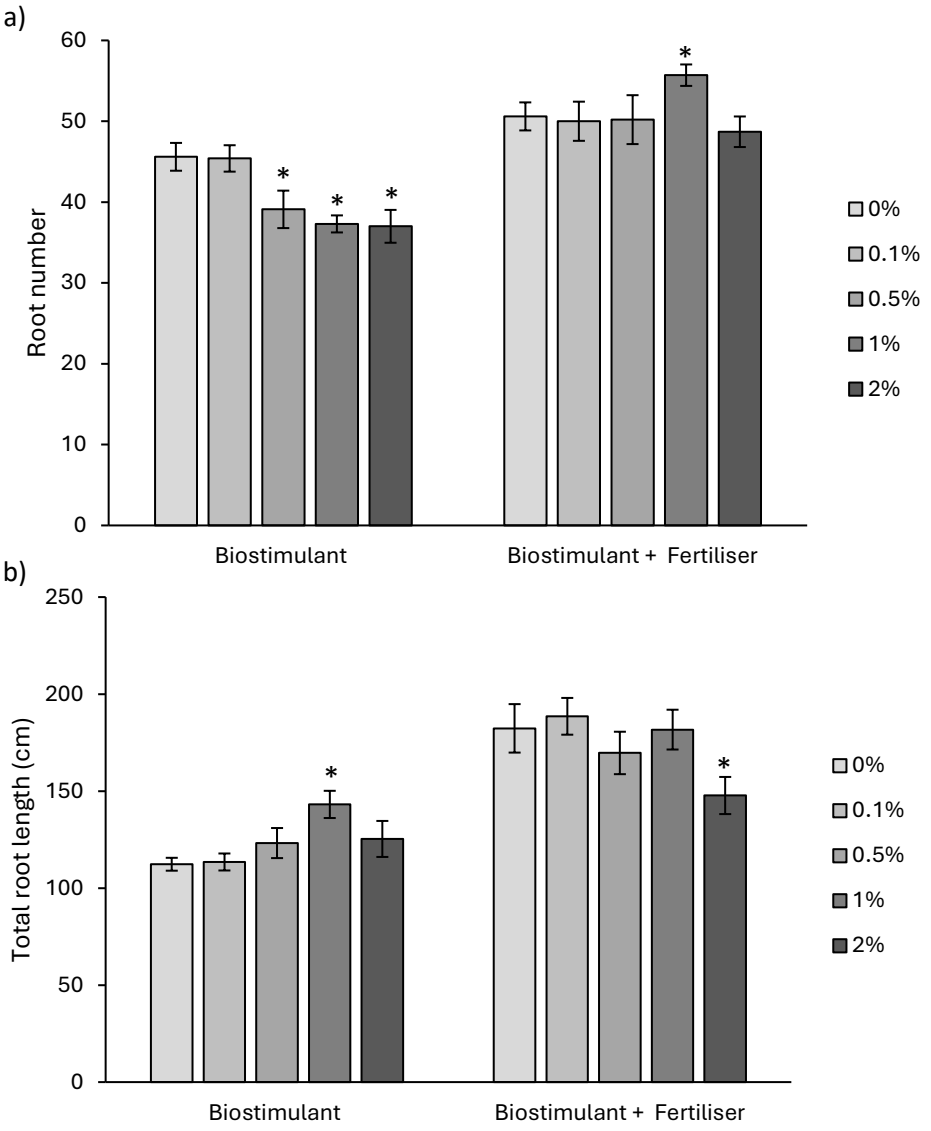


Figure 3.10: Root growth assay three: effect of selected *U. stenophylloides* biostimulant alone and in combination with fertiliser on tomato a) root number, and b) total root length (cm). * significantly different to the control. Biostimulant dose = % v/v. n=10. Average ± SE.

3.6 Discussion

This study investigated the effects of novel *U. stenophylloides* seaweed biostimulants on the growth of seedlings in hydroponics over three root growth assays. The first assay was used to select the best performing biostimulant from four promising candidates from Chapter 2 (this thesis) using mung bean seedlings. The second assay was used to determine if the selected biostimulant impacts root growth compared to a hormone standard using mung bean seedlings. The third assay was used to determine

if the selected biostimulant impacts root growth compared to a fertiliser and any interactions between them using tomato seedlings.

3.6.1 Root growth assay one: The effect of four *U. stenophylloides* biostimulants on mung bean seedlings

For root growth assay one, the effects of four selected *U. stenophylloides* biostimulants were evaluated on mung beans in hydroponics at two doses to determine the best performing ferment to use in further plant growth assays. There were clear differences in effects between the four products. Ferment two and three did not differ from the control in any of the root growth parameters measured at either dose, demonstrating that these ferments do not have anything in their composition that substantially impacted the growth of these plants either positively or negatively in this assay. These products were, therefore, excluded from further testing. However, ferment one (at a 2 % dose) and ferment four (at a 1 % dose) had significant effects on plant growth. Ferment one (at a 2 % dose) was consistently the worst performing ferment and had the lowest response to all growth parameters measured in this study, whereas ferment four (at a 1 % dose) was consistently the best performing ferment in all growth parameters.

Based on the composition of the four ferments tested in this study, it was expected that there would be the most similarity in root growth between ferment one and two, and ferment three and four, due to the yield of bioactives (lower in the former pair, higher in the latter pair of ferments), or between ferment one and three, and ferment two and four, due to the pH level in the final products (higher in the former pair, lower in the latter pair of ferments). However, this was not the case. This suggests the differences observed may have been from a combination of low bioactives paired with high pH in ferment one, leading to poorer root growth, and high bioactives paired with low pH in ferment four, resulting in improved root growth. There may also be an impact of microbial metabolites produced during fermentation and other compounds extracted from the seaweed biomass (not measured due to time restraints) that could be influencing differences in root growth. Ferment one may not have fermented completely (as indicated by higher pH). Lower pH in comparable fermentation

production is caused by the production of organic acids during successful fermentation (Villarreal‐Soto *et al.*, 2018; Ojo & de Smidt, 2023). The presence of these acids or other metabolites may have led to improved root growth in ferment four. For instance, gluconic acid can transform phosphate to soluble forms which increases the availability for plant growth (Otieno *et al.*, 2015) and phenolic compounds can promote plant growth and resilience to stress (Aina *et al.*, 2022). However, this would need further testing to confirm. Furthermore, the mode of action based on single components of seaweed biostimulants is challenging to determine due to the complexity of the composition, often with synergistic and antagonistic interactions between individual components (Boukhari *et al.*, 2020; Ali *et al.*, 2021). Fermentation adds another layer of complexity to the mode of action with added microbes and microbial production of metabolites. Therefore, teasing out which individual component has an impact on root growth is difficult to pinpoint accurately and would need further targeted studies at metabolomic and genetic level to conclusively understand.

The impact of the biostimulant dose was apparent in plant weight and root length, where a 2 % dose had an inhibitory effect compared to a 1 % dose. These results highlight the importance of selecting the correct dilution of a biostimulant to influence plant growth without causing an adverse effect. It is well noted in the literature that biostimulants only need to be applied in small amounts to have a physiological impact on plants (Khan *et al.*, 2009; Craigie, 2011; du Jardin, 2015). For example, a biostimulant produced from *Ecklonia maxima* using a cell burst process extraction technique (further details of biomass loading or manufacture methods not included) improved root growth of mung bean seedlings in hydroponics when added at concentrations below 10 % and adversely impacted root growth at concentrations over 10 % (Crouch & Van Staden, 1991) and a biostimulant produced from an aqueous extract of *Sargassum johnstonii* (100 g L^{-1}) resulted in increased mung bean root growth in concentrations less than 0.8 %, a decrease in root growth in concentrations greater than 0.8 %, and no rooting in plants exposed to concentrations of 10 % (Kumari *et al.*, 2011). Both studies suggested that the reduction in root growth resulted from hormone content inhibiting growth at higher concentrations. A biostimulant produced from *O. intermedium* (50 g L^{-1}) improved root growth at 0.2 – 2 %, but at 10 %, root

inhibition occurred due to increased electro-conductivity (Neveux *et al.*, 2020). Ghaderiardakani *et al.* (2019) applied an aqueous extract produced from *U. intestinalis* (100 g L^{-1}) to *Arabidopsis thaliana*. They found a biphasic effect in primary root elongation, where low concentrations (between 0.03–0.08 %) induced a stimulatory effect, and higher concentrations (greater than 0.3 %) induced an inhibitory effect. They determined this could be due to activation of abscisic acid signalling or aluminium toxicity. Therefore, numerous factors may inhibit root growth at higher concentrations.

Collectively, these findings suggest that ferment four is the best performing biostimulant for root growth in this study and therefore this ferment was selected for further testing in the subsequent two rooting assays. Furthermore, a 2 % dose caused root inhibition, which suggests lower doses should be tested to determine an optimal effect. Additionally, there was much variation in the results regarding the impact on roots that may have masked potential differences. Therefore, replication was increased from 15 to 25-30 for the subsequent root growth assay.

3.6.2 Root growth assay two: Mung bean seedlings and auxin-like activity

Root growth assay two was used to determine the effects of the selected *U. stenophylloides* biostimulant (ferment four) from the previous assay on mung bean seedlings in hydroponics compared to indole-3-butyric acid (IBA) for an auxin-like effect. However, no auxin-like effect was detected in this study.

In root growth assay two, no effects in plant weight or root growth were detected between the biostimulant at any dose or compared with the control. However, the seedlings treated with IBA resulted in increased root growth, confirming the assay was working. The results of the biostimulant treatments in this study contrast with other research which found auxin-like effects in their biostimulants. For example, Crouch and Van Staden (1991) found that when mung bean seedlings were pulse-treated with IBA and their seaweed biostimulant (extracted from *E. maxima* using a cell burst process extraction technique, biomass to solvent ratio not reported) rooting results both

responded in a linear manner, Kumari *et al.* (2011) found that their biostimulant (an aqueous extract of *S. johnstonii* at 100 g L^{-1}) at a 0.8 % dilution had an auxin-like activity between 10^{-6} and 10^{-5} M IBA on mung bean seedlings, and Neveux *et al.* (2020) found that their acid extract at a 10 % dilution and their alkali extract at a 2 and 10 % dilution (both produced from *O. intermedium* at 50 g L^{-1}) had an auxin-like activity equal to 5.10^{-5} M IBA on mung bean seedlings. In comparison to this study, Crouch and Van Staden (1991) and Kumari *et al.* (2011) pulse treated their seedlings in IBA for a further two hours and terminated their assay two days earlier, resulting in relatively decreased root lengths at higher concentrations, whereas, Neveux *et al.* (2020) had the same assay conditions leading to a similar result in root number with IBA treated seedlings to this study.

The lack of root growth in seedlings treated with biostimulant was unexpected due to differences detected in root growth assay one. Overall, root growth assay two produced less above ground biomass and less root growth than root growth assay one. Although the initial plant size was slightly smaller in growth assay two, the final plant weight (fresh and dry) at a 2 % dose was very similar between assays; however, the plant weight at a 1 % dose is lower in assay two, particularly in fresh weight. Compared to root growth assay one, root length and root number were similar in the 2 % dose but fewer and shorter in the controls and the 1 % dose. Differences between the two assays could be due to the MES pH buffering. Root growth assay one was not pH buffered, and root growth assay two was pH buffered; however, root growth assay two was also run unbuffered, and the results (presented in Appendix 7.3) did not significantly differ between unbuffered and buffered runs of the assay.

3.6.3 Root growth assay three: Tomato seedlings and fertiliser effects

Root growth assay three was used to determine the effects of the selected *U. stenophylloides* biostimulant from root growth assay one (ferment four) on tomato seedlings in hydroponics compared to a commercially available NPK fertiliser for an interactive fertiliser effect.

The application of fertiliser provides plants with essential nutrients that improve plant growth, and the application of biostimulants can help enhance the uptake of nutrients (Halpern *et al.*, 2015). Therefore, it is expected that the two of these components may complement each other to improve plant productivity when used in combination. This was demonstrated by a 1 % dose of only biostimulant significantly reducing root number and the same dose plus added fertiliser significantly increasing the number of roots. Increasing concentrations of biostimulant in combination with fertiliser had an adverse effect on the weight of the seedlings, but this effect was only significant in the 2 % dilution plus fertiliser. This is a similar trend to the prior two root growth assays, where the 2 % dose causes adverse effects to the seedling, and, in this case, the pattern in declining weight with increasing biostimulant concentration indicates that there may be an unfavourable interaction between the composition of the biostimulant and the fertiliser causing hindered outcomes on biomass productivity of the seedling.

Robust root systems are advantageous to plant health and productivity by providing access to nutrients and water from the soil, a link to beneficial soil microbes, and can provide resilience to periods of stress (Lombardi *et al.*, 2021). Root growth was stimulated in tomato seedlings with biostimulants produced from aqueous extracts of *U. lactuca* and *Padina gymnospora* (Hernández-Herrera *et al.*, 2014), acid and alkali extracts of *O. intermedium* (Neveux *et al.*, 2020), an aqueous extract of *U. intestinalis* (Karthik & Jayasri, 2023), and an extract of *E. maxima* (using a cell burst technique) (Finnie & van Staden, 1985). In this study, adding fertiliser increased the number of roots and the total root length compared to most biostimulant treatments. Interestingly, increasing biostimulant concentrations (in the absence of fertiliser) resulted in reduced number of roots but increased the total length of roots until the 2 % dose, where root length once again decreased. This suggests that, although not significant, the biostimulant stimulates the elongation of roots at the expense of overall root quantity, but this effect begins to be limited at a 2 % dilution. However, this effect in root number or total root length was not detected in fertilised treatments.

Although the results show that fertilised treatments show a similar root number and length, there were limitations in the software used to measure the roots; this could have also impacted the results for other seedlings with a high number of roots. Additionally, the outcome of this assay was likely affected by the initial size of the tomato seedlings, which may have been too large. In this assay (and the IBA treated seedlings in root assay two), particularly the treatments with added fertiliser, many roots were formed, a high degree of root matting, root overlapping, and a more elaborate root architecture. Therefore, some of the roots could not be separated easily for photographs and could not be accurately identified and measured. The software is very manual and relies on the roots being spread wide and not touching and, in this case, it was not possible with the large number of roots generated. If this study were to be repeated, it is recommended that the assay be started with smaller plants and replication increased to accurately detect differences between treatments.

3.7 Conclusion

This study aimed to test the effect of applying these seaweed biostimulants on root growth to seedlings in hydroponics to determine the best fermentation parameters and further test the best performer in two subsequent assays to determine an auxin-like effect and a fertiliser effect. Root growth assay one determined through improved plant weight and root growth that ferment four was the best biostimulant to move forward to the subsequent root assays. In contrast to root growth assay one, root assay two did not detect any differences in plant weight or root growth at any biostimulant concentration and determined that no auxin-like effect was stimulated by applying the seaweed biostimulant at any dose tested. In root growth assay three, more roots were produced at a 1 % dose of biostimulant in combination with fertiliser. Across all the root growth assays, a 1 % dose of biostimulant was collectively the best, and a 2 % dose adversely impacted growth. The results of this study have identified a fermented biostimulant produced from *U. stenophylloides* with the potential for increasing root growth and root number in hydroponics. Future research is needed to investigate the effects of this biostimulant in pot trials with plants in soil, this will

determine if the effects detected here translate to a more complex production system and increases in productivity or yield.

Chapter 4

Effect of *Ulva stenophylloides* seaweed biostimulant on growth and yield of tomato in greenhouse pot trials

4.1 Abstract

A promising seaweed biostimulant was produced from *Ulva stenophylloides* utilising a novel fermentation extraction method. Previous work identified potential plant growth enhancing bioactive components and variable results were demonstrated on root growth in hydroponics. However, the impact on plant growth and yield may be subject to interactions with soil or similar growing medium. This research investigated the impact of biostimulant application to potted tomato plants, alone and in combination with a commercial conventional fertiliser and found no significant effects at a treatment level on the leaf mineral content, biomass fractions, chlorophyll content, growth allocation parameters, relative growth in height, plant height, basal diameter, number of flowers, number of fruit, quantity of fruit per plant, total yield of fruit, or fruit diameter. Conversely, a fertiliser effect from the conventional fertiliser did increase plant weight (total, leaf, root, stem), plant height and basal diameter, demonstrating that some biomass productivity was stimulated from the addition of fertiliser. However, these results are inconclusive due to issues with application volume and growing conditions that require further method development and retesting.

4.2 Introduction

Seaweed biostimulants have gained substantial interest in sustainable agriculture for improving crop productivity, plant development, and stress response (Khan *et al.*, 2009). The green seaweed *Ulva stenophylloides* has been evaluated as a potential biostimulant candidate using a novel fermentation manufacturing method (Chapter 2,

this thesis). Results show that this method produced promising biostimulant candidates containing a range of potential plant growth-enhancing bioactives (section 2.5). When mung bean (*Vigna radiata*) and tomato (*Solanum lycopersicum*) seedlings were exposed to the best candidate at a range of concentrations in hydroponics, results were variable between three rooting assays (Chapter 3, this thesis) and require further testing in soil for a longer period to confirm positive effects. Overall, a 1 % dose of the best performing *U. stenophylloides* biostimulant stimulated the most growth and a 2 % dose had adverse impacts on growth (section 3.5). Plants in hydroponics offered a quick and easy method to screen biostimulants for seedling and root development, but do not incorporate the effects of plant-soil interactions or evaluate the biostimulants impact on yield.

Application of seaweed biostimulants can improve the biological, physical, and chemical aspects of soil to support soil health, and enhance plant growth (Khan *et al.*, 2009; Tariq *et al.*, 2022). For example, soil biology was improved by in vitro application of extracts from red seaweed (*Gracilaria verrucosa, Gelidium amansii* and *Eucheuma cottonii*) and freshwater green microalga (*Chlorella pyrenoidosa*). These algal extracts stimulated hyphal growth of arbuscular mycorrhizal fungi and enhanced root colonisation in papaya and passionfruit, promoting plant growth (Kuwada *et al.*, 2006). Soil biology was also improved (total bacterial count and increased the number of bacterial families associated with soil health) when an extract produced from brown algae *Durvillaea potatorum* and *Ascophyllum nodosum* (fortified with calcium) was applied to tomato in soil (seed raising mix) (Hussain *et al.*, 2021). Soil microbes are vital to the connection between plant and soil, extending the rhizosphere and contributing towards the uptake of nutrients and water, and resilience to stress, which enhances plant growth (Jansa *et al.*, 2013). Physical aspects of soil can be improved by the biostimulants polysaccharide content, leading to the stimulation of root and plant growth and enhanced microbial activity. Through a combination of gelling and chelating properties, polysaccharides can improve water-holding capacity by absorbing water, swelling, and retaining moisture; these properties lead to improved soil aeration and capillary action within pores (Khan *et al.*, 2009). Seaweed biostimulants can also enhance nutrient uptake and availability. Mechanisms for this include

enhanced nutrient cycling by microbes, alterations to soil enzymatic activity catalysing degradation of organic matter releasing nutrients (Sible *et al.*, 2021), enhanced interactions with arbuscular mycorrhizal fungi, changes to the soil structure, increased root reach, and chelating effects on micronutrients that make them more available (Halpern *et al.*, 2015).

With the addition of microbes and microbial metabolites within the fermented biostimulant, there is the potential for effects that may further enhance soil and plant health due to the manufacturing method. In particular, bacterial production of organic acids (primarily acetic, gluconic, and glucuronic acids) are antimicrobial against pathogenic microorganisms (Sreeramulu *et al.*, 2000; Chakravorty *et al.*, 2016; Freitas *et al.*, 2022) and when applied to soils, organic acids can improve nutrient availability and plant growth (Calvo *et al.*, 2014; Otieno *et al.*, 2015). Phenolic content can also be increased during fermentation; this is due to enzymatic breakdown of larger compounds into smaller molecules by bacteria and yeast; these molecules can act as defence chemicals and have antibiotic and antioxidant activity (Chakravorty *et al.*, 2016; Larios-Cruz *et al.*, 2019; Jafari *et al.*, 2020). When applied to plants, phenolic compounds extracted from macroalgae improve growth and response to stress (Aina *et al.*, 2022). Additionally, using a similar fermentation method (for beverage production, with ultrasound-assisted extraction) utilising the red seaweed laver (*Porphyra dentata*) resulted in high polyphenols, organic acids, and antioxidants (Aung & Eun, 2021). High polyphenols and antioxidants were also found in kombucha made with green seaweed seagrapes (*Caulerpa racemosa*) (Permatasari *et al.*, 2021). Furthermore, by using a fermentation process, a microbial consortium is added to the biostimulant (Villarreal‐Soto *et al.*, 2018), which may have a probiotic effect in soils.

Many of the effects that lead to enhanced plant growth occur in the soil, and the manufacturing method used in this research may further boost this result; therefore, to thoroughly test the effects of *U. stenophylloides* as a biostimulant the investigation should include experiments with plants rooted in soil or a similar growing medium. This chapter aims to evaluate the effect of the best fermented biostimulant produced from *U. stenophylloides* (as determined by Chapter 3, this thesis) in a greenhouse

experiment using tomato seedlings (*Solanum lycopersicum*) in pots, alone and in combination with a nitrogen, phosphorus, and potassium (NPK) fertiliser. Tomato has been selected for this study because of its use as a model species in plant assays and its global importance as a field and greenhouse crop (Hernández-Herrera *et al.*, 2014; Schwarz *et al.*, 2014).

4.3 Methods

4.3.1 Seaweed cultivation and biostimulant preparation

U. stenophylloides was cultivated in a recirculating aquaculture system (RAS) in the Facility for Aquaculture Research of Macroalgae (FARM) at the University of Waikato Coastal Marine Field Station, Tauranga, New Zealand (section 2.3.1). The biostimulant used in this chapter was prepared at the following fermentation parameters: 40 g L^{-1} seaweed biomass loading, 35 g L^{-1} sucrose input, and 30 °C incubation temperature. These fermentation parameters were selected from fermentation processing and chemical composition results (determined in Chapter 2, this thesis) and root growth results (Chapter 3, this thesis). For further details, refer to section 2.3.1 for seaweed cultivation, and sections 2.3.2 and 2.3.3 for biostimulant preparation.

4.3.2 Seedling establishment

Tomato seeds (*S. lycopersicum* Tommy Toe Heirloom) were surface sterilised with 4 % sodium hypochlorite (v/v) for five minutes, then triple rinsed in distilled water. Single seeds were then planted in pre-prepared peat pellets (Egmont Easigrow pellets soaked in warm water until fully expanded) and germinated under controlled conditions in a culture cabinet (Panasonic MLR-352) set at 30 °C, 12:12 light/dark cycle, at an irradiance of 45 µmol photons m^2 s^{-1}. Peat pellets were watered as required with dechlorinated water and were rotated daily. After seven days, seedlings were shifted to a grow tent (Eclipse Elite grow tent 220 cm x 120 cm x 200 cm under two 4Seasons Helios 450X LED Grow Lights) and maintained under a 12:12 light/dark cycle. The light was gradually increased over the next week to prepare for transfer to a greenhouse (Appendix 7.4). After 15 days, seedlings (n=40) were re-potted in large pots (15 L pots with a 300 mm diameter) filled with potting mix (Number 8 Potting Mix, blend of fine

bark, gypsum and three-month slow-release fertiliser). Pots with seedlings were relocated to a greenhouse (FARM at the University of Waikato Coastal Marine Field Station) and were acclimatised for seven days after transplanting (DAT) at ambient conditions before applying treatments. During acclimation, plants were watered three times with dechlorinated tap water to field capacity.

4.3.3 Greenhouse tomato growth assay

The following methods were modified from Hernández-Herrera *et al.* (2014) and the experiment was carried out during late winter to spring (August to November 2023). The tomato seedlings were randomly assigned to treatment groups in a fully randomised design and set out in four rows (Figure 4.1). Eight treatment groups with five replicates were selected for testing using a full factorial design; the biostimulant was tested at concentrations of 0.2, 0.4, and 1 % (v/v), and to determine if there is any interactive effects in combination with fertiliser, the same concentrations were tested in combination with fertiliser (Nitrosol Biological Fertiliser Tomato, at manufacturer's directions of 5 mL L^{-1}), dechlorinated tap water was used as a negative control (0 %), and a fertiliser with no added biostimulant was used as a positive control (0 % + F). To standardise pH for optimal plant growth and development, all treatments (including the controls) were pH buffered with 2-(N-morpholino)ethanesulfonic acid (MES). MES was made up at a 10 mM concentration with dechlorinated water and adjusted to a pH of 6.82 ± 0.02 using 2 M sodium hydroxide. As the concentration of biostimulant and nutrient solution increased, the pH level decreased (min 5.77) (full details in Appendix 7.5). Biostimulants were applied weekly, and fertiliser was applied fortnightly at 50 mL to the soil surrounding each plant until the commencement of flowering (28 DAT); at this stage, the treatment volume was increased to 100 mL per plant. Flowering was chosen as a critical point in the development of vegetative and propagative growth to increase biostimulant application. Plants were watered with dechlorinated water three times weekly to field capacity.

Weekly measurements were taken of plant growth parameters: stem height (from the substrate to the stem apex using a tape measure), and basal diameter (Craftright 150 mm digital calliper), and yield components (number of flowers, and number of fruit (over 5 mm diameter)). Tomato plants were supported by a string hanging from a structure above the plants (Figure 4.1). New growth was attached to the string weekly with a loose twisty tie, and plants were maintained to a single primary shoot by removing lateral shoots. At 35 DAT, lower leaves were removed from each plant to prevent contact with the substrate and the dry weight of the removed leaves was recorded. At 49 DAT, all plants had signs of possible early blight or leaf spot on lower leaves; to prevent spread, these were removed, and the dry weight of the removed leaves was recorded. Potted plants were grown in the greenhouse for a total of 90 DAT at ambient conditions. Due to equipment issues, the temperature was not logged, and an estimated temperature range was calculated from the daily local temperature high (Table 4.1). At the end of the assay, final weekly measurements were taken of growth and yield parameters (stem height, basal diameter, number of flowers, and number of fruit) and non-destructive leaf chlorophyll content was measured using a chlorophyll meter (SPAD502, Konica Minolta, Singapore) at five points on each plant. After this, plants were divided into four components: fruit, leaves, stem, and roots. Each component was weighed separately (fresh and dried). Fruit diameter was also measured with a digital calliper (Craftright 150 mm digital calliper). Leaf area was measured using a leaf area meter (LI3100, Licor, Nebraska) before oven drying at 65 °C for 48 hours, and the stem, roots, and fruit were freeze-dried (Lyovapor L-200, Buchi) and dry weights were recorded with a three decimal place balance. A basic plant nutrient profile of nitrogen, phosphorus, potassium sulfur, calcium, magnesium, sodium, iron, manganese, zinc, copper, and boron were determined from dried and milled (using a kitchen blender - Magic Bullet, MB1001) leaves by Hill Labs, Hamilton, New Zealand. Nitrogen was measured by dumas combustion and all other nutrients followed a nitric acid/hydrogen peroxide digestion followed by inductively coupled plasma - optical emission spectrometry (ICP-OES).

Figure 4.1: Experimental setup for greenhouse-based pot trials. Left: pots with tomato seedlings set out in four rows on the day of transplant. Right: tomato plants supported by a string hanging from a structure above the plants.

Table 4.1: Actual and estimated temperature conditions during experimental timeframe. DAT = days after transplanting, Actual = mean weekly air temperature sourced from NIWA, station Tauranga Aero 1615, Estimated = the estimated weekly maximum greenhouse temperature.

DAT	Actual	Estimated
7	15 ± 0.5	25-30
14	15 ± 0.5	25-30
21	16 ± 0.5	26-31
28	16 ± 0.4	26-31
35	17 ± 0.4	27-32
42	18 ± 0.4	28-33
49	19 ± 0.9	29-34
56	17 ± 0.6	27-32
63	19 ± 0.7	29-34
70	19 ± 0.4	29-34
77	21 ± 0.5	31-36
84	18 ± 0.8	28-33
90	20 ± 0.9	30-35

4.4 Data analysis

Two factor permutational analyses of variance (PERMANOVA) were used to compare the effect of biostimulants (fixed factor) and fertiliser (fixed factor) on leaf mineral

content, plant growth (total plant, leaf, root, stem, and fruit weight), chlorophyll content, biomass allocation parameters (specific leaf area, leaf area ratio, leaf mass ratio, root mass ratio, root shoot ratio), and tomato fruit parameters (fruit quantity, yield per plant, fruit diameter). The proportion of total variation attributed to each factor in significant results were calculated using eta-squared (%) η^2 (SS_{factor} / SS_{total} × 100; SS_{factor} = sum of squares for each factor and SS_{total} = total sum of squares) (Richardson, 2011). Three factor repeated measures PERMANOVA were used to compare the effect of treatments over time (fixed factor) with plant replicate (random factor) nested within dilution on relative growth in height, plant height, basal diameter, number of flowers, and number of fruit. All analyses were conducted in Primer v7 (Primer-E., UK) using Euclidean distance resemblance matrices, 9,999 unrestricted permutations of raw data, and Type III sum of squares (Anderson, 2008). Pairwise a posteriori comparisons were carried out for repeated measures PERMANOVA if a significant difference (p = < 0.05) was detected, with the significance level adjusted for multiple comparisons using a Bonferroni correction.

The following growth-related biomass allocation parameters were calculated as; specific leaf area (SLA) equals leaf area divided by dry weight, leaf area ratio (LAR) equals leaf area divided by total plant weight, leaf mass ratio (LMR) equals leaf weight divided by total plant weight, root mass ratio (RMR) equals root weight divided by total plant weight, root shoot ratio (RSR) equals root weight divided by shoot weight, relative growth in height (RGH) equals natural log of height at time 2 - natural log of height at time 1, divided by the change in time (Lambers & Oliveira, 2019).

All data are reported as mean ± standard error (SE).

4.5 Results

4.5.1 Growth and yield

Relative growth in height ranged between 0.0044 ± 0.001 mm mm-1 d-1 (negative control, 90 days after planting (DAT)) to 0.065 ± 0.002 mm mm-1 d-1 (positive control, 35 DAT). The largest relative growth in height occurred in seedlings at the beginning of the experiment and reduced every week from 49 DAT to the lowest growth rate at the

end of the growth trial (Figure 4.2, Table 4.2). Although relative growth in height significantly differed weekly, differences between biostimulant treatments were not significant over time (Table 4.2), or at the termination of the experiment (Table 4.3).

Plant height ranged from 56.6 ± 2.6 mm to 67.2 ± 2.1 mm at the beginning of the experiment, and from 952.4 ± 44.4 mm (1 % biostimulant) to 1143.2 ± 71.1 mm (positive control) at the end of the experiment (Figure 4.3). There was a significant interaction between time and biostimulant treatment effects (Table 4.2), but pairwise, a posteriori comparisons did not identify any differences between individual treatments using Bonferroni corrected significance values. However, plants did grow significantly taller over time (Table 4.2). At the termination of the experiment, although there was no effect of the biostimulant treatment, there was a fertiliser effect detected, where plants that had been treated with fertiliser grew taller than plants that were given only biostimulant (Table 4.3).

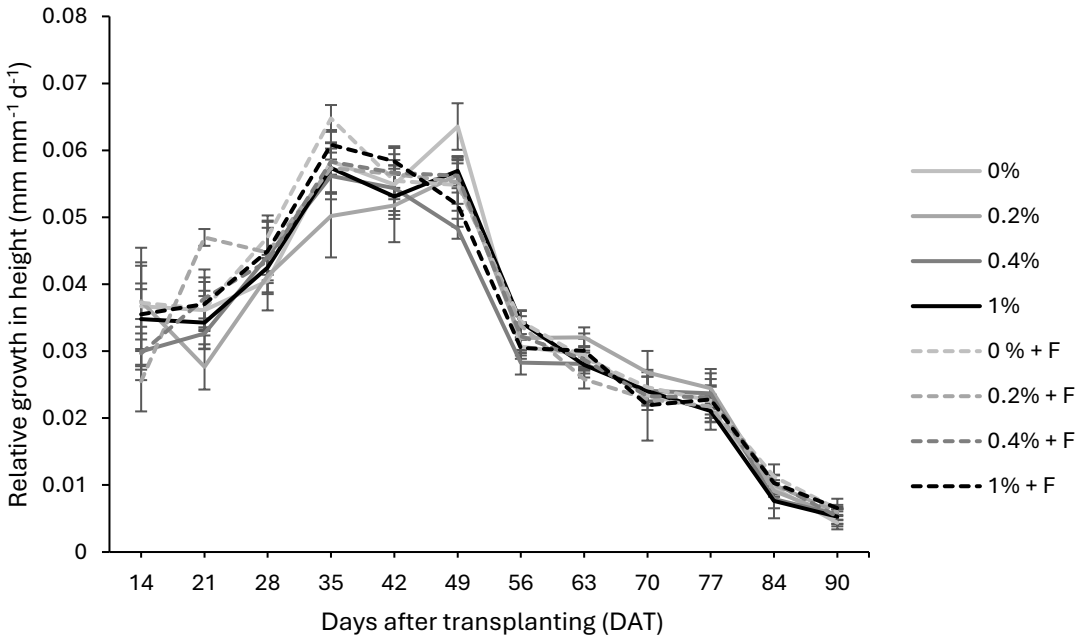


Figure 4.2: Relative growth of plant height over experimental period of tomato plants exposed to *U. stenophylloides* seaweed biostimulant, alone and in combination with fertiliser. Treatments are biostimulant % (v/v), F = treatments containing fertiliser. n=5. Average ± SE.

Basal diameter ranged between 2.31 ± 0.07 mm to 2.6 ± 0.12 mm at the beginning of the experiment to 9.3 ± 0.49 mm (0.2 % biostimulant) to 10.2 ± 0.12 mm (0.2 % biostimulant plus fertiliser) at the end of the experiment (Figure 4.4). Differences in basal diameter between biostimulant treatments did not significantly differ over time; however, basal diameter did grow thicker over time (Table 4.2). As with plant height, at the termination of the experiment, although there was no significant biostimulant effect there was a fertiliser effect, where plants that had been treated with fertiliser grew a thicker basal diameter than plants that received only biostimulant (Table 4.3).

Table 4.2: Results of repeated measures Permutational Analysis of Variance (PERMANOVA) for the effects of biostimulant and fertiliser on relative growth in height (RGH), plant height, and basal diameter over time. Significant results (p = < 0.05) are in bold.

Source	***df***	**RGH**		**Height**		**Basal diameter**	
		F	*p*	F	*p*	F	*p*
Time	12	251.34	**0.0001**	2492.40	**0.0001**	2028.40	**0.0001**
Treatments	7	1.33	0.28	1.54	0.18	0.96	0.47
Plant (Treatment)	32	0.98	0.50	14.42	**0.0001**	31.17	**0.0001**
Time x Treatment	84	0.93	0.64	1.44	**0.01**	0.82	0.85
Residuals	384						

Table 4.3: Results of Permutational Analysis of Variance (PERMANOVA) for the effects of biostimulant and fertiliser on relative growth in height (RGH), plant height, and basal diameter at the termination of the experiment (90 DAT).

Source	***df***	**RGH**		**Height**		**Basal diameter**	
		F	*p*	F	*p*	F	*p*
Biostimulant	3	0.10	0.96	1.01	0.40	0.26	0.86
Fertiliser	1	1.32	0.26	8.91	**0.004**	5.31	**0.03**
Biostimulant x fertiliser	3	0.42	0.75	0.39	0.76	0.22	0.88
Residuals	32						

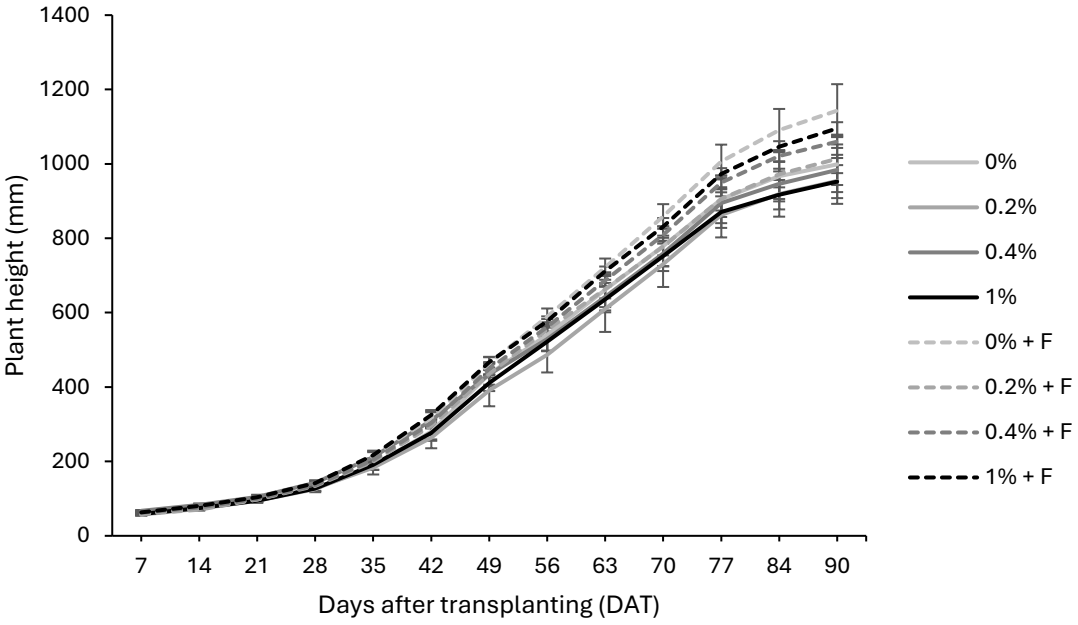


Figure 4.3: Plant height over experimental period of tomato plants exposed to *U. stenophylloides* seaweed biostimulant, alone and in combination with fertiliser. Treatments are biostimulant % (v/v), F = treatments containing fertiliser. n=5. Average ± SE.

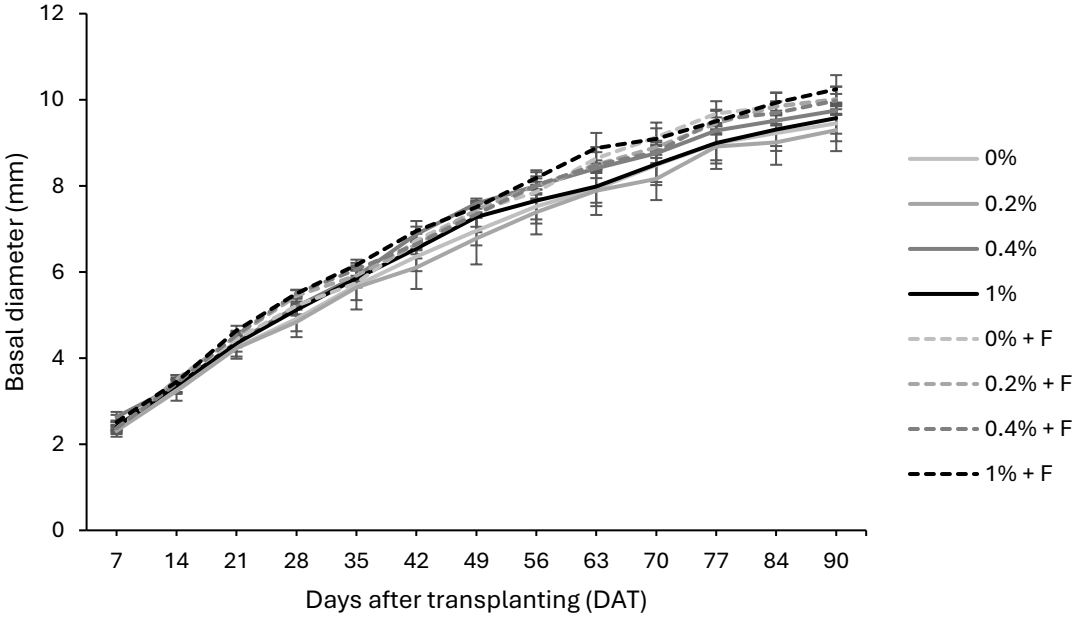


Figure 4.4: Basal diameter over experimental period of tomato plants exposed to *U. stenophylloides* seaweed biostimulant, alone and in combination with fertiliser. Treatments are biostimulant % (v/v), F = treatments containing fertiliser. n=5. Average ± SE.

Flowers emerged 28 DAT and were present on most plants and significantly more flowers developed over the experimental period (Table 4.4). Flower number per plant ranged from 4.8 ± 1.3 (0.2 % biostimulant) to 6.6 ± 0.2 (1 % biostimulant plus fertiliser) on 28 DAT and ranged from 27.6 ± 3.1 (0.2 % biostimulant) to 33.2 ± 2.4 (positive control) on 90 DAT (Figure 4.5). However, differences in flower number between biostimulant treatments were not significant over time (Table 4.4). Tomato fruit were present from 56 DAT and significantly more fruit developed over the experimental period (Table 4.4). Fruit number per plant ranged from 2.8 ± 0.7 (1 % biostimulant) to 4.0 ± 0.4 (1 % biostimulant plus fertiliser) on 56 DAT and ranged from 7.8 ± 1.7 (0.2 % biostimulant) to 10.8 ± 0.8 (0.4 % biostimulant plus fertiliser) on 90 DAT (Figure 4.6). However, differences in the number of fruit between biostimulant treatments were not significant over time (Table 4.4).

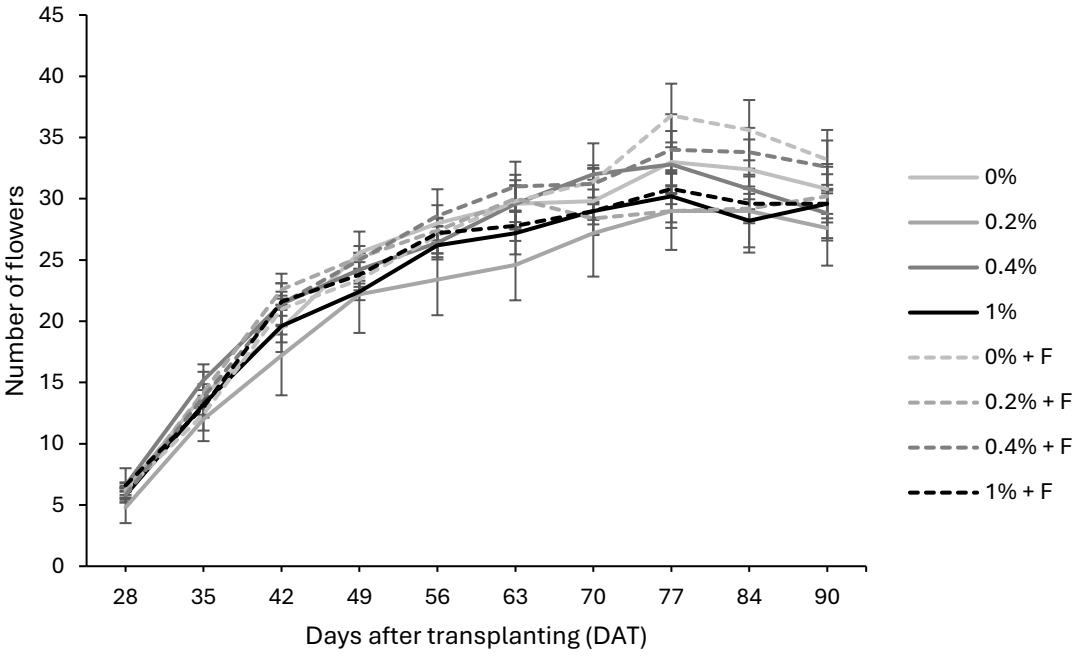


Figure 4.5: Number of flowers per plant over experimental period of tomato plants exposed to *U. stenophylloides* seaweed biostimulant, alone and in combination with fertiliser. Treatments are biostimulant % (v/v), F = treatments containing fertiliser. n=5. Average ± SE.

Table 4.4: Results of repeated measures Permutational Analysis of Variance (PERMANOVA) for the effects of biostimulant and fertiliser on flowering and fruit over time.

Source	***df***	**Flowers**		**Fruit**	
		F	*p*	F	*p*
Time	12	396.88	**0.0001**	146.68	**0.0001**
Treatment	7	0.89	0.54	0.55	0.80
Plant (Treatment)	32	13.86	**0.0001**	22.05	**0.0001**
Time x Treatment	84	1.12	0.27	1.12	0.32
Residuals	384				

At the termination of the experiment (90 DAT), the number of flowers were not subject to a treatment effect or a fertiliser effect (Table 4.5). Fruit quantity per plant at 90 DAT ranged from 7.8 ± 0.75 (0.2 % biostimulant) to 10.8 ± 0.8 (0.4 % biostimulant plus fertiliser) (Figure 4.7a). There was a slight upward trend with increasing biostimulant in the treatments with added fertiliser. However, there were no treatment effects, or fertiliser effects at the end of the experiment (Table 4.5). Fruit yield per plant (total fruit fresh weight) ranged from 66.24 ± 13.92 g (0.2 % biostimulant) to 95.86 ± 8.32 g (0.2 % biostimulant plus fertiliser) (Figure 4.7b). However, once again there were no significant differences between biostimulant treatments and controls or a fertiliser effect (Table 4.5). Fruit diameter ranged between 23.54 ± 1.04 mm (0.4 % biostimulant plus fertiliser) and 28.57 ± 0.93 mm (1 % biostimulant) (Figure 4.7c), with no significant effects of biostimulant or fertiliser (Table 4.5).

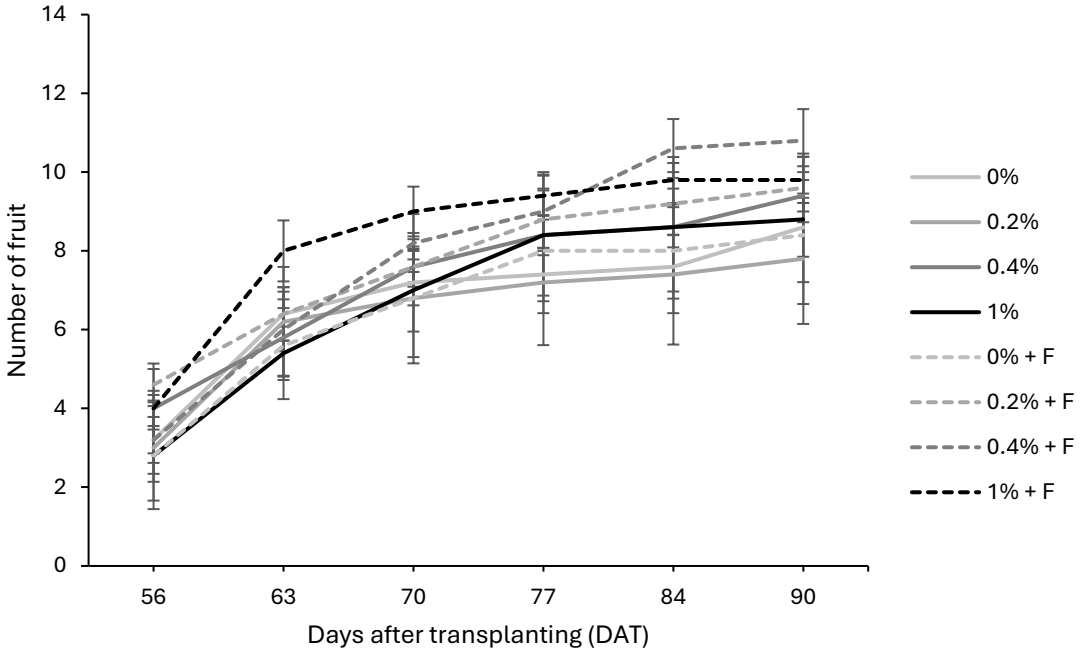


Figure 4.6: Number of tomatoes per plant over experimental period of tomato plants exposed to *U. stenophylloides* seaweed biostimulant, alone and in combination with fertiliser. Treatments are biostimulant % (v/v), F = treatments containing fertiliser. n=5. Average ± SE.

Table 4.5: Results of Permutational Analysis of Variance (PERMANOVA) for the effects of biostimulant and fertiliser on tomato yield parameters at the termination of the experiment (90 DAT).

Source	***df***	**Flowers**		**Fruit quantity**		**Yield**		***df***	**Fruit diameter**	
		F	*p*	F	*p*	F	*p*		F	*p*
Biostimulant	3	0.69	0.57	0.77	0.53	0.33	0.80	3	0.59	0.62
Fertiliser	1	1.81	0.19	1.48	0.23	2.68	0.11	1	0.004	0.94
Biostimulant x fertiliser	3	0.24	0.87	0.28	0.84	1.04	0.39	3	0.73	0.53
Residuals	32							358		

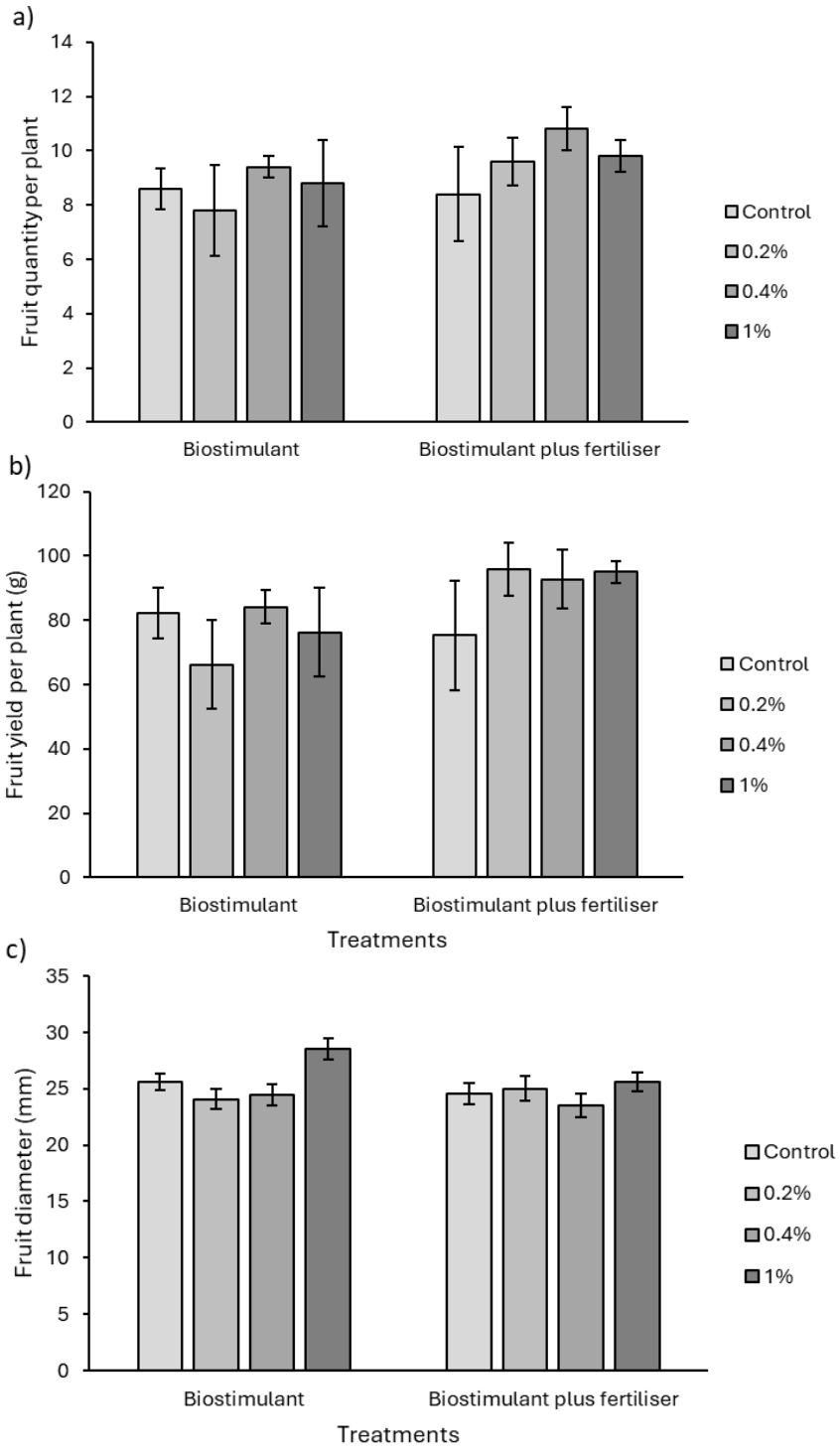


Figure 4.7: Yield parameters at 90 DAT for tomato plants exposed to *U. stenophylloides* seaweed biostimulant, alone and in combination with fertiliser. Treatments are biostimulant % (v/v). a) quantity of fruit per plant, b) total fruit yield per plant (fresh weight), c) average fruit diameter per plant. n=5. Average ± SE.

4.5.2 Plant fractions and biomass allocation

Tomato plants were divided into four fractions; leaf, root, stem, and fruit and were dried for analysis (Table 4.6). No significant differences were detected between the treatments or controls in any plant fractions or total plant weight (Table 4.7). A fertiliser effect was detected where collectively fertilised plants had increased plant weight (η^2 = 33 %), leaf (η^2 = 33 %), root (η^2 = 20 %), and stem (η^2 = 24 %) compared with plants with only added biostimulant; however, no fertiliser effect was detected in fruit weight (Table 4.7). Chlorophyll content (Table 4.6) was also not significantly different between treatments or controls and had no obvious trends or fertiliser effect (Table 4.7).

Table 4.6: Dry weight fractions and chlorophyll content of tomato plants exposed to *U. stenophylloides* seaweed biostimulant, alone and in combination with fertiliser. Treatments are biostimulant % (v/v), F = treatments containing fertiliser. n=5. Average ± SE.

Treatment	Plant weight (g)	Leaf (g)	Root (g)	Stem (g)	Fruit (g)	Chlorophyll (SPAD)
0	34.3 ± 4.0	9.8 ± 1.2	7.9 ± 1.4	8.0 ± 0.9	8.6 ± 0.8	33.0 ± 0.6
0.2	31.4 ± 5.6	10.3 ± 1.5	6.3 ± 1.6	7.5 ± 1.2	7.2 ± 1.4	32.4 ± 1.0
0.4	35.2 ± 1.4	11.4 ± 0.3	6.7 ± 0.6	8.1 ± 0.5	9.0 ± 0.5	33.0 ± 0.9
1	33.8 ± 3.8	11.0 ± 1.4	6.7 ± 0.7	7.9 ± 0.8	8.2 ± 1.3	32.9 ± 0.9
0 + F	41.5 ± 1.2	14.2 ± 1.1	9.6 ± 1.4	9.2 ± 1.2	8.4 ± 1.6	32.7 ± 1.4
0.2 + F	40.0 ± 4.1	12.5 ± 1.3	8.5 ± 1.4	8.8 ± 0.7	10.1 ± 0.9	32.7 ± 0.7
0.4 + F	42.6 ± 2.9	13.8 ± 1.0	9.1 ± 0.6	9.6 ± 0.5	10.1 ± 0.9	32.5 ± 0.5
1 + F	39.1 ± 2.2	12.3 ± 1.2	7.6 ± 0.8	9.2 ± 0.2	9.9 ± 0.4	32.2 ± 0.4

Table 4.7: Results of Permutational Analysis of Variance (PERMANOVA) for the effects of biostimulant and fertiliser on plant fractions and chlorophyll content. Significant results (p = < 0.05) are in bold.

Source	*df*	Plant weight		Leaf		Root		Stem		Fruit		Chlorophyll	
		F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
Biostimulant	3	0.36	0.78	0.37	0.78	0.80	0.50	0.25	0.86	0.39	0.77	0.07	0.97
Fertiliser	1	8.50	**0.01**	9.36	**0.005**	5.01	**0.04**	5.71	**0.02**	3.41	0.07	0.25	0.62
Biostimulant x fertiliser	3	0.08	0.97	0.62	0.61	0.19	0.90	0.02	1.00	0.68	0.57	0.12	0.94
Residuals	32												

The final dry fractions of plant tissues were then used to estimate growth-related biomass allocation. Specific leaf area (SLA), leaf area ratio (LAR), leaf mass ratio (LMR), root mass ratio (RMR), and root shoot ratio (RSR) were calculated (Table 4.8). There was a significant interactive effect between biostimulant and fertiliser on the LMR (Table 4.9), where the negative control had a lower LMR than the positive control, when biostimulant was added this effect reversed and biostimulant treatments resulted in higher LMR than treatments with added fertiliser. This interaction explained 44 % of the variation in leaf mass ratio. No significant differences were detected between the treatments or controls in any biomass allocation parameters (Table 4.9), and no fertiliser effects were observed (Table 4.9).

Table 4.8: Biomass allocation of tomato plants exposed to *U. stenophylloides* seaweed biostimulant, alone and in combination with fertiliser. Treatments are biostimulant % (v/v), F = treatments containing fertiliser. n=5. Average ± SE.

Treatment	Specific leaf area ($cm^2\ g^{-1}$)	Leaf area ratio	Leaf mass ratio	Root mass ratio	Root shoot ratio
0	73 ± 6.6	21 ± 0.8	0.29 ± 0.01	0.23 ± 0.02	0.29 ± 0.03
0.2	64 ± 1.4	22 ± 0.9	0.34 ± 0.01	0.19 ± 0.02	0.24 ± 0.03
0.4	64 ± 1.0	22 ± 0.7	0.33 ± 0.01	0.19 ± 0.01	0.24 ± 0.02
1	60 ± 7.2	20 ± 1.9	0.33 ± 0.01	0.20 ± 0.02	0.26 ± 0.04
0 + F	63 ± 1.6	22 ± 0.7	0.34 ± 0.02	0.23 ± 0.03	0.31 ± 0.05
0.2 + F	66 ± 1.2	21 ± 0.4	0.31 ± 0.01	0.21 ± 0.02	0.27 ± 0.03
0.4 + F	64 ± 0.9	21 ± 0.5	0.32 ± 0.01	0.21 ± 0.01	0.27 ± 0.01
1 + F	70 ± 7.0	21 ± 0.4	0.31 ± 0.02	0.19 ± 0.01	0.24 ± 0.02

Table 4.9: Results of Permutational Analysis of Variance (PERMANOVA) for the effects of biostimulant and fertiliser on biomass allocation parameters. SLA = specific leaf area, LAR = leaf area ratio, LMR = leaf mass ratio, RMR = root mass ratio, RSR = root shoot ratio. Significant results (p = < 0.05) are in bold.

Source	*df*	SLA		LAR		LMR		RMR		RSR	
		F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
Biostimulant	3	0.29	0.84	0.23	0.90	0.32	0.81	1.12	0.35	1.22	0.32
Fertiliser	1	0.04	0.87	0.04	0.86	0.37	0.56	0.34	0.57	0.39	0.54
Biostimulant x fertiliser	3	1.81	0.16	0.80	0.54	4.47	**0.01**	0.43	0.73	0.35	0.80
Residuals	32										

4.5.3 Leaf nutrient content

A range of essential plant minerals were present in the dry leaf material of all tomato plants in this study (Table 4.10). Primary macronutrients, nitrogen, phosphorus, and potassium and secondary macronutrients, calcium, sulfur, and magnesium did not significantly differ between treatments or negative and positive controls (Table 4.11). The content of micronutrients, copper, manganese, iron, zinc, and sodium also did not significantly differ between treatments or negative and positive controls (Table 4.11). There was no fertiliser effect detected in any of the plant nutrients analysed in this study (Table 4.11). However, there was a significant interactive effect between biostimulant and fertiliser on the leaf content of boron (Table 4.11), where increasing concentration of biostimulant in combination with fertiliser resulted in higher boron leaf content and increasing concentrations of biostimulant alone resulted in lower boron leaf content. This interaction explained 32 % of the variation in leaf boron content.

Table 4.10: Mineral content of dry leaf material from tomato plants exposed to *U. stenophylloides* seaweed biostimulant, alone and in combination with fertiliser. Treatments are biostimulant % (v/v), F = treatments containing fertiliser. n=5. Average ± SE.

Minerals		**Unit**	**Treatments**							
			0%	**0.2%**	**0.4%**	**1%**	**0% +F**	**0.2% + F**	**0.4% + F**	**1% + F**
Nitrogen	N	g kg^{-1}	6.8 ± 0.6	6.4 ± 0.2	6.2 ± 0.2	6.0 ± 0.3	6.0 ± 0.0	6.0 ± 0.0	6.4 ± 0.2	6.8 ± 0.8
Phosphorus	P	g kg^{-1}	2.3 ± 0.2	2.4 ± 0.1	2.0 ± 0.05	2.4 ± 0.1	2.4 ± 0.2	2.5 ± 0.2	2.4 ± 0.1	2.5 ± 0.2
Potassium	K	g kg^{-1}	11.2 ± 1.2	11.6 ± 0.2	10.4 ± 0.4	11.4 ± 0.2	11.6 ± 0.7	10.8 ± 0.6	11.6 ± 0.2	12.2 ± 1.5
Calcium	Ca	g kg^{-1}	37.2 ± 3.8	32.1 ± 0.9	32.6 ± 0.8	31.3 ± 0.6	28.9 ± 2.1	32.0 ± 0.5	30.8 ± 0.7	33.8 ± 3.5
Magnesium	Mg	g kg^{-1}	3.9 ± 0.4	3.7 ± 0.1	3.5 ± 0.2	3.7 ± 0.1	3.7 ± 0.1	4.0 ± 0.2	3.7 ± 0.1	3.8 ± 0.2
Sulfur	S	g kg^{-1}	9.3 ± 0.7	8.4 ± 0.1	8.9 ± 0.1	8.4 ± 0.1	7.8 ± 0.6	8.7 ± 0.3	8.1 ± 0.3	8.9 ± 0.9
Copper	Cu	mg kg^{-1}	2.2 ± 0.2	2.0 ± 0.0	2.2 ± 0.2	1.8 ± 0.2	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.3
Manganese	Mn	mg kg^{-1}	145.8 ± 8.7	138.6 ± 2.6	146.8 ± 7.6	132.0 ± 6.5	125.0 ± 11.8	126.4 ± 6.4	134.4 ± 7.0	147.2 ± 16.0
Iron	Fe	mg kg^{-1}	55.8 ± 12.0	33.4 ± 1.9	44.0 ± 6.7	44.4 ± 3.9	36.6 ± 4.4	47.6 ± 3.5	43.4 ± 6.6	37.4 ± 3.9
Zinc	Zn	mg kg^{-1}	13.8 ± 0.9	15.0 ± 1.1	11.8 ± 1.1	15.0 ± 1.4	14.8 ± 1.6	16.8 ± 2.3	17.0 ± 1.1	13.8 ± 1.5
Boron	B	mg kg^{-1}	56.4 ± 4.1	48.8 ± 2.4	52.0 ± 1.3	47.4 ± 1.1	50.8 ± 3.5	54.0 ± 2.3	50.8 ± 1.9	59.2 ± 5.0
Sodium	Na	mg kg^{-1}	276.0 ± 57.3	234.0 ± 11.2	246.0 ± 51.4	276.0 ± 22.9	226.0 ± 23.2	278.0 ± 27.5	286.0 ± 47.7	244.0 ±24.0

Table 4.11: Results of Permutational Analysis of Variance (PERMANOVA) for the effects of biostimulant and fertiliser on leaf mineral content. Significant results (p = < 0.05) are in bold.

Source	***df***	**Nitrogen**		**Phosphorus**		**Potassium**		**Calcium**		**Magnesium**		**Sulfur**		**Copper**		**Manganese**		**Iron**		**Zinc**		**Boron**		**Sodium**	
		F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*	F	*p*
Biostimulant	3	0.12	0.97	1.10	0.36	0.38	0.80	0.16	0.96	0.67	0.61	0.05	0.99	0.67	0.78	0.34	0.80	0.38	0.78	0.57	0.64	0.32	0.84	0.06	0.98
Fertiliser	1	0.03	0.90	3.39	0.07	0.52	0.52	1.80	0.22	0.47	0.51	1.43	0.25	0.18	0.94	1.37	0.26	0.53	0.48	2.79	0.11	1.45	0.25	0.0004	1.00
Biostimulant x fertiliser	3	1.58	0.21	0.77	0.53	0.61	0.64	2.51	0.06	0.55	0.66	1.99	0.13	0.67	0.77	1.48	0.24	2.59	0.07	1.70	0.19	3.22	**0.03**	0.88	0.47
Residuals	32																								

4.6 Discussion

This study investigated the effect of a novel *U. stenophylloides* seaweed biostimulant on growth and yield of tomato in pots alone and in combination with fertiliser. There were some effects of fertiliser, with added fertiliser increasing plant weight (total, leaf, root, and stem), plant height, and basal diameter. There was also an interactive effect leading to increased leaf content of boron when increasing biostimulant concentrations were combined with fertiliser, and leaf mass ratio increased with increasing concentrations of biostimulant alone. However, biostimulant treatment alone had no significant effects on leaf nutrient content, biomass fractions, chlorophyll content, growth allocation parameters, and plant growth and yield. A fertiliser effect demonstrates that the fertiliser increased biomass productivity collectively, but not enough to cause differences between individual treatments or to any yield parameters. The lack of effects at a treatment level may be due to several reasons: the choice of test crop, the volume of product used, the growing conditions, including the effect of disease, or the product may not have biostimulant properties.

The application volume may have a significant impact on the response of the plant and the overall effectiveness of the product. When not enough of the product is applied, there may not be enough to cause a physiological response within the plant or significantly impact on the soil. Conversely, when too much of the product is applied, it can negatively impact growth. The study the methods were modified from for this research (Hernández-Herrera *et al.*, 2014) used a product dilution (at a higher biomass loading of 100 g L^{-1}) at a weekly application volume of 50 mL per pot on potted tomato seedlings (size of pots not noted). Their results showed that biostimulants produced from *U. lactuca*, and *Padina gymnospora* improved tomato shoot length, root length, and weight. Similarly, Crouch and Van Staden (1992) tested the effects of a seaweed biostimulant produced from *Ecklonia maxima* (biomass loading not reported) in two experiments on potted tomato (125 mm pots); one at an application rate of 50 mL per pot weekly, which improved the growth of seedlings, and another at a weekly application rate of 100 mL per pot, which resulted in earlier ripening of fruit and improved fruit weight and quantity. Furthermore, Kumari *et al.* (2011) tested a

Sargassum johnstonii seaweed biostimulant (at a higher biomass loading of 100 g L^{-1}) at a range of dilutions between 0.1 and 10 % on potted tomatoes (380 mm pots) at an application rate of 100 mL per 15 days which resulted in increased vegetative growth, reproductive parameters, and biochemical constituents. In contrast, Hussain *et al.* (2021) utilised a biostimulant produced from *Durvillaea potatorum* and *Ascophyllum nodosum* (biomass loading not reported) on potted tomato plants (pot size 400 mm) at an application rate and frequency which was much higher (250 mL per pot, twice daily, dilution 0.25 %). Their results demonstrated an improvement in growth, productivity, and soil biology. In this study, pot size was 300 mm, biomass loading was either 25 or 40 g L^{-1}, and the application rate was 50 mL weekly until the initiation of flowering, where the application rate was increased to 100 mL weekly. Results in the present study showed no differences at a treatment level, but the application of fertiliser increased overall biomass production, and an interactive effect reduced leaf mass ratio when treated in combination with fertiliser. Further effects to a treatment level may have been masked by the slow-release fertiliser present in the potting mix. Additionally, pot size in this study was similar to Kumari *et al.* (2011) and Hussain *et al.* (2021), but was notably smaller than Crouch and Van Staden (1992). Results were apparent at the higher application rate and frequency in large pots (Hussain *et al.*, 2021). Furthermore, the seaweed biomass loading in this study was much lower than Hernández-Herrera *et al.* (2014) and Kumari *et al.* (2011), which could result in lower extracted bioactive components, requiring a higher application volume for a response in plants. Collectively, these differences suggest that the present study could be repeated with adjustment of the application volume for the amount of soil present for an effect on the plants. Overall, the present results indicate that further investigation into the application volume of the product needs to be completed, and ideally in the absence of slow-release fertiliser in the potting mix to more clearly separate the effects of biostimulant product and mineral fertiliser addition on plant growth.

Leaf nutrient levels can be used to identify nutrient deficiency or toxicity (Campbell & Plank, 2000), or in the case of this study, the effectiveness of the treatments applied to the soil. If nutrient availability and uptake is too low, the yield and growth of tomato can be negatively influenced (Reid & Morton, 2020). Therefore, providing adequate

nutrients is important to ensure proper growth. A basic plant nutrient profile of essential macro and micro nutrients were analysed for this study and found an interactive effect with increasing content of boron when the biostimulant was combined with fertiliser. Boron is an essential micronutrient and was within sufficient levels in all treatments (Reid & Morton, 2020), therefore, increased boron may not make much difference in overall growth in this study. In other leaf nutrients there were no treatment effects or fertiliser effects detected; however, the majority of nutrients in the leaves were below an ideal level. Leaf nutrient content were deficient or low in nitrogen, phosphorus, potassium, magnesium, iron, zinc, and copper (Campbell, 2000; Reid & Morton, 2020). Interestingly, the plants that were treated with NPK fertiliser were also deficient in nitrogen, phosphorus, and potassium in leaf tissues. These results further add to the conclusion that the application volume was too low for a measured response in plant productivity between treatments and suggests that the slow-release fertilisers in the potting mix may not have had a strong influence on available NPK. However, the uptake of nutrients may have also been impacted due to stressful growing conditions (further detailed below).

When the ambient temperature is outside of the optimal range for a plant, there may be irreversible negative impacts on growth and development (Wahid *et al.*, 2007). Excessive heat and cold temperatures can cause adverse effects on photosynthesis, water relations, nutrient use and efficiency, growth, reproductive processes, and metabolic processes (Van Ploeg & Heuvelink, 2005; Wahid *et al.*, 2007); however, the extent of damage is dependent on the absolute temperature, duration, the rate of temperature change, and variations in the plant itself (Van Ploeg & Heuvelink, 2005; Wahid *et al.*, 2007). For example, Camejo *et al.* (2005) found that reductions in net photosynthetic rate occurred when the tomato genotype Campbell-28 was exposed to heat stress (45 °C for two hours). Sato *et al.* (2006) found that during moderately increased temperature stress (32 °C day/ 26 °C night), fruit set failed due to disruptions in sugar metabolism and proline translocation during male reproductive development. Rajametov *et al.* (2021) found that when they analysed the response of 35 tomato accessions to low temperatures (10 °C), most varieties had significantly reduced leaf length, leaf width, number of fruits, and fruit yield. Furthermore, no tomato growth is

expected when the temperature is below 12 °C or above 35 °C (Criddle *et al.*, 1997). In this study, the estimated upper temperature in the greenhouse was typically between 25-35 °C. Therefore, the air temperature was potentially outside an ideal range, implying there were periods where the heat may have caused damage to the plants or had an impact on the ability of the plants to produce biomass or produce flowers and fruit. However, this research would need to be repeated with better monitoring of air temperature to confirm the actual temperature range.

Plant disease can also have a significant impact on the health and productivity of plants. At the midpoint of the assay (49 DAT), there was possible early blight or Septoria leaf spot detected (based on visual inspection) on the lower leaves of all plants in the study. Early blight (caused by the fungal pathogen *Alternaria solani*) and Septoria leaf spot (caused by the fungus *Septoria lycopersici*) both present as lesions on the leaf and stem and can reduce photosynthetic area, reduce yield, decrease fruit quality, and defoliate plants (Narendra Babu *et al.*, 2015; Mattos *et al.*, 2020). A fungicide typically treats both diseases (Narendra Babu *et al.*, 2015; Mattos *et al.*, 2020). In this study, to avoid losing any potential effects of the biostimulant, the affected leaves were removed as an alternative to applying a chemical treatment. Therefore, there may still have been remnant effects of disease on the plants, which could have impacted their growth and productivity in this study.

4.7 Conclusion

This research investigated the effects of an *U. stenophylloides* fermented seaweed biostimulant on the growth and yield of potted tomato plants. Results were inconclusive due to issues that require further method development and retesting. To detect differences between treatments, changes could include increased application volume of the product, using a soil or potting mix that does not have added slow-release fertiliser, improving the growing conditions, and increasing replication.

General discussion

5.2 Main research findings

The main aim of this thesis was to develop a seaweed biostimulant utilising the novel seaweed biomass source *Ulva stenophylloides* and a novel fermentation extraction method. This research developed and optimised a fermentation method using a symbiotic culture of bacteria and yeast for the production of a seaweed biostimulant, measured product parameters to assess fermentation progress, chemically characterised biostimulant end-products, and assessed biostimulant activity in both hydroponic and pot-based plant cultivation experiments.

The extraction phase of developing a seaweed biostimulant is one of the most important steps. The goal of this step, rather than targeting a specific compound, is to extract high yields of a range of plant growth-promoting bioactive substances (Boukhari *et al.*, 2020). The biostimulant production method selected for this study used a hot aqueous extract to draw target molecules from the seaweed into a liquid phase (Jaulneau *et al.*, 2011), followed by synergistic fermentation with yeast and bacteria to generate novel compounds through microbial metabolism (Jayabalan *et al.*, 2014). Fermentation organisms also contribute a probiotic effect on plant-soil systems, and microbial metabolites stabilise the product (Sudhanshu *et al.*, 2019).

Altering levels of biomass loading, initial sucrose input, and incubation temperature significantly influenced the fermentation rates and chemical profiles of the final 27 ferments (Chapter 2). Although there were significant interactions within all fermentation parameters, the initial sucrose input had the largest impact on fermentation progression and success and was the main driver in the resulting glucose content, total dissolved solids, pH, and pellicle yield of the final ferments. Excluding treatments with low biomass loadings, treatments with low and medium sucrose inputs reached completion and treatments with high sucrose inputs did not. Higher biomass loadings provided valuable nutrients for microbial growth (Sudhanshu *et al.*, 2019), and higher incubation temperatures were optimal for microbial growth, which were at the higher end of temperatures suitable for comparable fermentation

methods (Neffe-Skocińska *et al.*, 2017; Antolak *et al.*, 2021). High biomass loadings were a critical driver in the increased yield of nutrients, protein, and ulvan, and high incubation temperature was the main driver in the increased yield of phenylacetic acid. At a practical application level, nutrient quantities were deemed too low to elicit a response in plant growth (du Jardin, 2015; Yakhin *et al.*, 2017); however, protein and ulvan content may potentially enhance plant growth through modulation of gene expression and metabolic changes (Přerovská *et al.*, 2022; Shefer *et al.*, 2022; Zhang *et al.*, 2023). Although phenylacetic acid levels were likely too low for a response in plants (Sumayo et al., 2018), greater concentrations at high temperatures indicated increased microbial activity, which may increase the production of other beneficial microbial biochemicals. Overall, these findings demonstrate that through fermentation of *U. stenophylloides*, unique biostimulants can be produced with varying chemical profiles. However, practically testing all final 27 ferments on plants would be logistically difficult and result in small differences between treatments due to small incremental changes and combinations between parameters. Therefore, four biostimulant candidates were selected (Table 5.1), these were based on fermentation progression and their content of potential plant growth enhancing bioactives (protein, ulvan, and phenylacetic acid).

Table 5.1: Four biostimulant candidates selected from Chapter 2

Fermentation parameters	**Unit**	**Ferment 1**	**Ferment 2**	**Ferment 3**	**Ferment 4**
Biomass loading	**g L^{-1}**	25	25	40	40
Initial sucrose loading	**g L^{-1}**	10	35	10	35
Incubation temperature	**°C**	30	30	30	30
Processing parameters					
Glucose	mg L^{-1}	3.5 ± 2.4	0	0.1 ± 1.5	0.7 ± 2.7
Total dissolved solids	%	1.5 ± 0.04	2.3 ± 0.13	2.4 ± 0.10	3.0 ± 0.23
pH	pH	4.3 ± 0.02	3.6 ± 0.05	4.9 ± 0.5	3.7 ± 0.04
Pellicle	g	1.1 ± 0.02	1.9 ± 0.1	1.3 ± 0.2	2.1 ± 0.3
Bioactives					
Protein	µg mL^{-1}	325 ± 46	303 ± 17	407 ± 19	451 ± 8
Ulvan	mg mL^{-1}	3.2 ± 0.5	3.9 ± 0.2	6.7 ± 0.4	6.2 ± 0.6
Phenylacetic acid	µg mL^{-1}	2.2 ± 0.2	2.8 ± 0.6	1.5 ± 0.3	2.2 ± 0.5

Although the four biostimulant candidates produced in Chapter 2 had promising biochemical profiles, there must be evidence that the product will actually enhance

growth when applied to plants. Robust root systems are advantageous to plant health and productivity by providing access to nutrients and water from the soil, a link to beneficial soil microbes, and can provide resilience to periods of stress (Lombardi *et al.*, 2021). Seedlings in hydroponics offer an efficient way to screen biostimulant candidates and application rates for root growth-promoting effects (Neveux *et al.*, 2020). Therefore, root growth assays in hydroponics were used to determine biostimulant effects (Chapter 3). Root growth assay one evaluated the four biostimulants selected from Chapter 2 at two concentrations and identified the best biostimulant for mung bean root growth. Ferment one was consistently the worst performing biostimulant tested, ferment two and three did not significantly impact root growth either positively or negatively, and ferment four was consistently the best performing biostimulant tested. At a 1 % (v/v) dose, ferment four significantly increased seedling weight and had the best root growth response. These results provided evidence of a biostimulant effect on seedling growth and therefore ferment four was used for all subsequent testing (root growth assays two and three, and pot trials in Chapter 3).

Root growth assay two used mung bean seedlings to evaluate ferment four at a series of concentrations compared to indole-3-butyric acid (IBA) to determine an auxin-like effect. In contrast to root growth assay one, there were no biostimulant effects on root or seedling growth at any of the doses tested. Additionally, no auxin-like effects were detected through biostimulant application. This contrasts with other studies that found auxin-like effects from their biostimulants (Crouch & Van Staden, 1991; Kumari *et al.*, 2011; Neveux *et al.*, 2020). The lack of biostimulant effects highlights variable results from applying the *U. stenophylloides* biostimulant.

Root growth assay three used tomato seedlings to evaluate ferment four at a series of concentrations compared to a conventional fertiliser and determined any interactive effects. A 1 % dose of ferment four in combination with fertiliser enhanced root number compared to only fertiliser and the 1 % biostimulant alone. This result demonstrates that at a 1 % dose, the selected biostimulant can interact with fertiliser to improve the number of roots when used in combination. This means that, at the

correct dose, there is a potential for the biostimulant and fertiliser to complement each other, which could result in reduced fertiliser use. Additionally, tomato seedlings could have been too large at the beginning of the assay, resulting in far too many roots to measure accurately with the software used, therefore, retesting with smaller plants may pick up further fertiliser effects.

Over the three root growth assays, doses of ferment four at 2 % had an adverse effect on seedling weight and root growth. It is noted in the literature that seaweed biostimulants only need to be applied in small doses (Khan *et al.*, 2009; Craigie, 2011), and other studies have reported adverse impacts on plant growth at low concentrations (Kumari *et al.*, 2011; Neveux *et al.*, 2020). These results highlight the importance of selecting the correct dilution of a biostimulant to influence plant growth without causing an inhibitory effect. Collectively, the results of this chapter identified that a biostimulant (ferment four) produced from *U. stenophylloides* has the potential to increase seedling growth in hydroponics and also identified the concentration where adverse effects to seedling and root growth occurred. However, variable results were found over the three assays.

A biostimulant effect from applying ferment four was detected in hydroponic root growth assays (Chapter 3); however, these were only short assays at the seedling stage, and further research was required to determine if the effects detected there could be translated to a more complex production system resulting in increased productivity or yield. Many of the effects that lead to enhanced plant growth occur in the soil (Jansa *et al.*, 2013; Halpern *et al.*, 2015). In addition to the bioactive components extracted from the seaweed, the manufacturing method used in this research may further boost plant growth through the addition of microbes and microbial biochemicals. Therefore, to thoroughly test the impact of *U. stenophylloides* as a biostimulant, the investigation using ferment four proceeded to a greenhouse experiment using potted tomato seedlings alone and in combination with a commercial conventional fertiliser (Chapter 4).

The pot assay was impacted by issues with the application volume of the product and environmental conditions, and as a result, at a treatment level, there were no

significant effects on the leaf mineral content, biomass fractions, chlorophyll content, growth allocation parameters, relative growth in height, plant height, basal diameter, number of flowers, number of fruit, quantity of fruit per plant, total yield of fruit, or fruit diameter. However, fertiliser application did increase some biomass productivity, but this did not lead to increased yield. These results do not directly translate to a failure of the product, and further method development and retesting are required. Leaf growth analysis showed that the plants were overall deficient in required nutrients, which is consistent with the application volume being below the ideal level or could be an impact of the stressful growing conditions. The ideal application volume differs between biostimulants (Kumari *et al.*, 2011; Hernández-Herrera *et al.*, 2014; Hussain *et al.*, 2021), and care needs to be taken when comparing biostimulants as biomass loadings and experimental conditions vary and need to be taken into consideration. Growing conditions also require improvement; air temperature and disease can have adverse effects on plant growth (Criddle *et al.*, 1997; Wahid *et al.*, 2007; Narendra Babu *et al.*, 2015). Air temperature in the greenhouse may have been above ideal conditions for growth and plants exhibited signs of disease. Therefore, future studies must improve growing conditions, e.g. better air flow and disease mitigation, to detect potential differences.

5.3 Future research directions

This thesis significantly contributes to developing sustainable seaweed biostimulants for sustainable agricultural cropping. The following suggestions are designed to further progress this research.

Chapter 2 investigated a novel fermentation manufacturing method for producing a seaweed biostimulant. Because this method had not been optimised, ferment parameters and chemical composition needed to be assessed. Due to the manufacturing method, there was a range of microbes and microbial metabolites within the fermented biostimulant (Antolak *et al.*, 2021), and these constituents could potentially enhance plant growth (Otieno *et al.*, 2015; Aina *et al.*, 2022). Time restraints restricted the overall analysis of the composition of the biostimulant. Therefore, future research should further explore the composition of the ferments

using metabolomic analysis to gain a more in-depth understanding of potential growth promoting end-products, and determine the microbial species present for a more thorough understanding of microbial and biochemical dynamics in the fermented biostimulants. One of the byproducts of this fermentation method is the production of a cellulosic pellicle produced by acetic acid bacteria (Raspor & Goranovič, 2008). Bacterial cellulosic pellicles have physical properties suitable for various applications (food, medical, and textile industries) (Laavanya *et al.*, 2021). Future research could also determine uses for the pellicle to produce further high-value products from the same process, therefore, reducing waste and maximising potential profits and markets.

Chapter 3 investigated biostimulant effects on mung bean and tomato seedlings in hydroponics. This study determined the impacts of biostimulant application on root growth to determine any biostimulant effects. Further exploration into the mechanisms involved in growth could be determined by looking at the expression of growth-related genes involved in the biosynthesis of plant phytohormones; auxin (*IAA)*, cytokinin *(ITP*) and gibberellin (*Ga2Ox*) in plant tissues (Ali *et al.*, 2019).

Chapter 4 investigated potential biostimulant effects, fertiliser effects, and any possible interactions in potted tomato plants. Once the methods and growing conditions have been refined for application volume and growing conditions, this research could be further extended to include the biostimulant effects on soil. Application of seaweed biostimulants can improve the biological, physical, and chemical aspects of soil to support soil health and enhance plant growth (Khan et al., 2009; Tariq et al., 2022), and many of the effects that lead to enhanced plant growth occur in the soil. Understanding of the changes in soil properties (nutrient profiles, moisture, pH, soil organic carbon (SOC), N, C/N ratio, cation exchange capacity (CEC), and electrical conductivity), and the soil microbiome diversity and composition will provide a more thorough understanding of the mechanisms involved in biostimulant effects to growth and productivity in plants.

5.4 Conclusion

This thesis addresses key knowledge gaps in the development of a novel fermented seaweed biostimulant utilising *U. stenophylloides* to support sustainable agricultural cropping practices within three main steps:

1. By manipulating fermentation parameters, seaweed biostimulants were successfully produced with unique chemical profiles.
2. Hydroponic assays identified the best fermentation parameters for biostimulant production with effects-based evidence in seedling root growth and identified the best and upper-limit application concentrations.
3. Greenhouse pot trials were inconclusive due to issues with application volume and growing conditions, and further method development and retesting are required.

Collectively, these findings demonstrate that a seaweed biostimulant can be produced from *U. stenophylloides* with a fermentation manufacturing method. Despite suboptimal conditions during plant assays, effects were detected, and with further refinement, these biostimulant effects may become more evident. This research provides methods and suggestions for further study that may remove some of the confounding variables of the plant assays conducted. To further develop a seaweed biostimulant using the fermentation method in this research, the next steps should be to further refine plant assay methods and growing conditions, gain further understanding of the composition of the ferments through metabolomics and microbial identification, and investigate the biostimulant effects on soil.

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Appendix

Appendix 7.1: Literature for selecting fermentation parameters.

Biomass ($g\ L^{-1}$)	Sugar ($g\ L^{-1}$)	Temperature (°C)	Starter (%)	Pellicle ($g\ L^{-1}$)	Time (days)	Reference
5	50	20-22 (18-26*)	20	24	10-14	Jayabalan *et al.* (2014)
-	50-150	20-30	10-20	-	7-21	Lynch *et al.* (2019)
12	100	24	10	30	18	Jayabalan *et al.* (2007)
-	50	Ambient	-	-	7-56	Raspor and Goranovič (2008)
100	-	28	10-15	-	7-12	Chakravorty *et al.* (2016)
8	100	Ambient	10	Added	14	Jafari *et al.* (2020)
27	100	Ambient	10	-	14	Bhattacharya *et al.* (2013)
50	50	25	-	24	14	Aung and Eun (2021)**
8	80	20-30	10	30	10-20	Gaggìa *et al.* (2019)
6	100	20, 25*, 30	6	50	10	Neffe-Skocińska *et al.* (2017)
10	100	20, 30*	3	20	21	De Filippis *et al.* (2018)
10	20	Ambient	-	10	21	Battikh *et al.* (2012)
6	100	29	-	50	10	Li *et al.* (2022)
10	100	Ambient	10	-	12	Nuryanti (2022)
10	50-80	Ambient	10	-	15	Kaewkod *et al.* (2019)
4.9	100	Ambient (23)	10	Added	10	Marsh *et al.* (2014)
2x tea bags	100	Ambient (24)	20	25	14 and 60	Chen and Liu (2000)
-	100	Ambient (25)	10-15	Added	7-12	Bortolomedi *et al.* (2022)
10	10	25	10	2.5	7	Gamboa-Gómez *et al.* (2016)
-	50*-150	20-30	10	Added	6-10	Kumar and Joshi (2016)
5	50	20	10	-	12	Lopes *et al.* (2021)
10	20	Ambient	10	10	21	Pure and Pure (2016)
5	125	25	-	12.5	14	Sreeramulu *et al.* (2000)
10	60	28	10	-	1.5	Ulusoy and Tamer (2019)
10	60	28	10	-	14	Abuduaibifu and Tamer (2019)

Several different biomass sources as a fermentable substrate are referenced in the literature, typically black or green tea, but other alternatives are also used. The weight of initial biomass added is not always specified. Sugar: initial inputs at the beginning of fermentation, * indicates optimal input. Temperature: incubation during fermentation, * indicates optimal temperature or temperature range. Starter: portion of liquid culture added from a previous kombucha brew. Pellicle: also known as SCOBY (symbiotic culture of bacteria and yeast), portion added from a previous successful brew. Where data says 'added', an amount of pellicle was added to the new brew, but weight or size was not specified. ** indicates studies involving kombucha fermentation using algae.

Appendix 7.2: Elemental analysis of *U. stenophylloides* fermentation products. Average ± SE.

Treatment			**1**	**2**	**3**	**4**
Biomass loading (g L^{-1})			10	10	10	10
Initial sucrose loading (g L^{-1})			10	10	10	35
Incubation temperature (°C)			20	25	30	20
Macronutrients		**Unit**				
Ash		%	0.18 ± 0.01	0.18 ± 0.02	0.17 ± 0.03	0.10 ± 0.02
Carbon	C	g L^{-1}	1.4 ± 0.4	1.7 ± 0.4	1.4 ± 0.4	5.3 ± 0.5
Nitrogen	N	mg L^{-1}	11 ± 2.9	15 ± 3.4	13 ± 3.5	12 ± 2.4
Phosphorus	P	mg L^{-1}	2.7 ± 0.1	2.8 ± 0.1	2.7 ± 0.2	4.2 ± 0.3
Potassium	K	mg L^{-1}	172.1 ± 6.8	167.9 ± 5.5	165.2 ± 6.5	196.6 ± 2.9
Calcium	Ca	mg L^{-1}	69.7 ± 2.9	67.9 ± 1.8	64.8 ± 3.5	82.3 ± 1.9
Magnesium	Mg	mg L^{-1}	143.8 ± 9.8	138.5 ± 9.7	127.7 ± 6.3	181.1 ± 10.8
Sulfur	S	mg L^{-1}	352.0 ± 15.0	342.5 ± 12.7	337.9 ± 13.3	396.7 ± 7.3
Micronutrients						
Copper	Cu	µg L^{-1}	5.4 ± 14.8	49.7 ± 12.7	32.2 ± 2.9	42.5 ± 7.2
Manganese	Mn	µg L^{-1}	42.9 ± 3.2	50.2 ± 1.9	47.1± 4.4	55.0 ± 2.2
Iron	Fe	µg L^{-1}	316.2 ± 23.7	370.7 ± 15.4	348.1 ± 27.5	462.9 ± 34.6
Nickel	Ni	µg L^{-1}	4.5 ± 0.9	3.4 ± 0.7	3.2 ± 0.7	5.9 ± 1.6
Zinc	Zn	µg L^{-1}	113.3 ± 73.0	219.3 ± 160.6	180.1 ± 102.9	259.0 ± 145.6
Cobalt	Co	µg L^{-1}	7.1 ± 0.4	8.1 ± 0.3	8.4 ± 0.5	9.0 ± 0.7
Non essential elements						
Sodium	Na	mg L^{-1}	209.8 ± 8.1	205.9 ± 10.7	202.2 ± 8.1	254.5 ± 8.0
Strontium	Sr	µg L^{-1}	421.1 ± 1.9	414.7 ± 17.9	447.4 ± 31.1	484.2 ± 3.9
Barium	Ba	µg L^{-1}	11.6 ± 2.5	10.4 ± 1.6	11.9 ± 1.4	16.6 ± 2.6
Selenium	Se	µg L^{-1}	1.7 ± 0.1	1.6 ± 0.1	1.6 ± 0.0	1.5 ± 0.5
Aluminium	Al	µg L^{-1}	106.1 ± 4.9	102.1 ± 5.0	93.8 ± 16.2	131.1 ± 11.3
Arsenic	As	µg L^{-1}	3.4 ± 0.2	3.2 ± 0.1	3.5 ± 0.1	5.2 ± 1.1
Chromium	Cr	µg L^{-1}	9.4 ± 2.4	7.1 ± 1.4	5.0 ± 0.7	14.0 ± 3.8

Treatment			5	6	7	8
Biomass loading (g L^{-1})			10	10	10	10
Initial sucrose loading (g L^{-1})			35	35	60	60
Incubation temperature (°C)			25	30	20	25
Macronutrients		**Unit**				
Ash		%	0.32 ± 0.05	0.28 ± 0.06	0.07 ± 0.02	0.08 ± 0.01
Carbon	C	%	0.29 ± 0.07	0.25 ± 0.07	0.72 ± 0.18	0.33 ± 0.04
Hydrogen	H	%	0.06 ± 0.01	0.05 ± 0.02	0.14 ± 0.04	0.06 ± 0.01
Nitrogen	N	%	0.001 ± 0.000	0.001 ± 0.000	0.001 ± 0.000	0.000 ± 0.000
Phosphorus	P	mg L^{-1}	3.8 ± 0.4	4.2 ± 0.1	4.4 ± 0.3	3.8 ± 0.2
Potassium	K	mg L^{-1}	203.4 ± 12.5	203.0 ± 5.2	195.2 ± 5.9	181.2 ± 0.8
Calcium	Ca	mg L^{-1}	87.4 ± 5.8	83.4 ± 2.0	86.4 ± 1.7	77.5 ± 1.4
Magnesium	Mg	mg L^{-1}	166.0 ± 8.2	159.4 ± 6.4	191.8 ± 11.1	148.0 ± 4.5
Sulfur	S	mg L^{-1}	412.1 ± 25.3	411.8 ± 10.0	396.0 ± 13.6	363.6 ± 4.5
Micronutrients						
Copper	Cu	µg L^{-1}	47.1 ± 12.0	43.8 ± 1.8	53.9 ± 10.0	43.2 ± 12.5
Manganese	Mn	µg L^{-1}	63.8 ± 2.9	57.5 ± 1.8	57.1 ± 4.4	62.9 ± 0.9
Iron	Fe	µg L^{-1}	439.9 ± 33.9	398.7 ± 21.0	482.9 ± 48.6	418.4 ± 9.9
Nickel	Ni	µg L^{-1}	3.5 ± 1.0	3.2 ± 0.4	6.1 ± 2.1	9.5 ± 6.4
Zinc	Zn	µg L^{-1}	92.3 ± 4.5	221.5 ± 130.5	252.0 ± 67.7	147.2 ± 38.9
Cobalt	Co	µg L^{-1}	8.3 ± 0.6	8.7 ± 0.2	8.0 ± 0.8	7.0 ± 0.2
Non essential elements						
Sodium	Na	mg L^{-1}	246.8 ± 14.8	246.4 ± 9.7	250.0 ± 9.1	222.7 ± 2.7
Strontium	Sr	µg L^{-1}	527.7 ± 30.9	494.5 ± 8.8	478.6 ± 23.9	468.5 ± 4.0
Barium	Ba	µg L^{-1}	17.8 ± 5.3	13.3 ± 1.7	22.1 ± 6.7	15.4 ± 2.9
Selenium	Se	µg L^{-2}	1.1 ± 0.1	1.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0
Aluminium	Al	µg L^{-1}	159.5 ± 22.8	142.2 ± 8.7	173.1 ± 13.7	172.2 ± 20.6
Arsenic	As	µg L^{-1}	3.8 ± 0.8	3.8 ± 0.2	4.3 ± 0.7	3.1 ± 0.2
Chromium	Cr	µg L^{-1}	10.8 ± 5.9	6.8 ± 1.9	19.1 ± 6.3	11.7 ± 3.3

Treatment			9	10	11	12
Biomass loading (g L^{-1})			10	25	25	25
Initial sucrose loading (g L^{-1})			60	10	10	10
Incubation temperature (°C)			30	20	25	30
Macronutrients		**Unit**				
Ash		%	0.08 ± 0.02	0.38 ± 0.04	0.37 ± 0.01	0.38 ± 0.02
Carbon	C	%	0.62 ± 0.08	0.24 ± 0.09	0.31 ± 0.02	0.29 ± 0.01
Hydrogen	H	%	0.12 ± 0.02	0.05 ± 0.02	0.07 ± 0.00	0.07 ± 0.00
Nitrogen	N	%	0.001 ± 0.000	0.002 ± 0.001	0.003 ± 0.000	0.003 ± 0.000
Phosphorus	P	mg L^{-1}	4.7 ± 0.2	7.4 ± 0.4	7.1 ± 0.4	6.8 ± 0.1
Potassium	K	mg L^{-1}	187.5 ± 4.1	349.1 ± 8.5	335.1 ± 4.7	356.9 ± 9.7
Calcium	Ca	mg L^{-1}	80.0 ± 1.0	135.3 ± 3.1	128.6 ± 1.9	125.8 ± 6.0
Magnesium	Mg	mg L^{-1}	150.4 ± 4.4	292.5 ± 26.4	314.1 ± 11.2	310.1 ± 7.0
Sulfur	S	mg L^{-1}	380.6 ± 7.2	716.2 ± 12.8	685.2 ± 7.3	714.5 ± 20.0
Micronutrients						
Copper	Cu	µg L^{-1}	46.5 ± 0.6	55.0 ± 1.2	51.3 ± 1.0	51.1 ± 0.9
Manganese	Mn	µg L^{-1}	61.2 ± 0.7	100.1 ± 7.3	98.1 ± 3.6	116.2 ± 1.8
Iron	Fe	µg L^{-1}	410.1 ± 6.0	881.4 ± 41.2	882.2 ± 40.9	912.0 ± 8.7
Nickel	Ni	µg L^{-1}	3.9 ± 0.2	6.6 ± 1.0	6.0 ± 0.5	7.0 ± 0.9
Zinc	Zn	µg L^{-1}	123.1 ± 11.3	552.0 ± 71.7	280.5 ± 139.8	654.1 ± 67.9
Cobalt	Co	µg L^{-1}	8.3 ± 0.4	17.7 ± 1.0	17.7 ± 0.8	19.1 ± 0.3
Non essential elements						
Sodium	Na	mg L^{-1}	242.5 ± 6.5	454.7 ± 36.2	446.9 ± 29.4	436.6 ± 14.0
Strontium	Sr	µg L^{-1}	594.7 ± 125.4	844.2 ± 35.3	852.3 ± 23.0	920.5 ± 57.8
Barium	Ba	µg L^{-1}	22.3 ± 3.1	10.9 ± 0.9	14.2 ± 2.9	11.9 ± 0.7
Selenium	Se	µg L^{-2}	0.1 ± 0.0	3.9 ± 0.6	3.2 ± 0.1	4.5 ± 0.2
Aluminium	Al	µg L^{-1}	151.4 ± 6.1	166.6 ± 6.4	143.3 ± 9.0	116.2 ± 4.2
Arsenic	As	µg L^{-1}	4.6 ± 0.6	6.7 ± 0.3	6.0 ± 0.4	6.4 ± 0.2
Chromium	Cr	µg L^{-1}	9.4 ± 1.0	12.2 ± 3.0	10.4 ± 1.3	9.5 ± 1.0

Treatment			13	14	15	16
Biomass loading (g L^{-1})			25	25	25	25
Initial sucrose loading (g L^{-1})			35	35	35	60
Incubation temperature (°C)			20	25	30	20
Macronutrients		**Unit**				
Ash		%	0.43 ± 0.07	0.42 ± 0.04	0.38 ± 0.03	0.49 ± 0.06
Carbon	C	%	0.35 ± 0.08	0.29 ± 0.02	0.36 ± 0.24	0.98 ± 0.57
Hydrogen	H	%	0.08 ± 0.01	0.06 ± 0.00	0.08 ± 0.05	0.20 ± 0.11
Nitrogen	N	%	0.001 ± 0.000	0.002 ± 0.000	0.002 ± 0.001	0.002 ± 0.001
Phosphorus	P	mg L^{-1}	8.0 ± 0.2	8.5 ± 0.1	7.7 ± 0.4	8.4 ± 0.0
Potassium	K	mg L^{-1}	425.0 ± 16.7	411.0 ± 3.2	373.9 ± 13.7	435.7 ± 2.8
Calcium	Ca	mg L^{-1}	178.3 ± 6.9	177.1 ± 1.0	157.6 ± 6.2	185.3 ± 0.5
Magnesium	Mg	mg L^{-1}	337.3 ± 19.7	348.4 ± 13.6	319.2 ± 10.9	396.2 ± 24.2
Sulfur	S	mg L^{-1}	842.0 ± 31.1	815.7 ± 5.0	734.8 ± 19.5	874.3 ± 6.3
Micronutrients						
Copper	Cu	µg L^{-1}	67.3 ± 2.2	58.8 ± 2.2	50.9 ± 2.2	66.4 ± 7.4
Manganese	Mn	µg L^{-1}	72.6 ± 7.2	111.3 ± 9.0	115.7 ± 3.9	68.1 ± 3.5
Iron	Fe	µg L^{-1}	1133.2 ± 36.1	1185.4 ± 11.1	1127.4 ± 48.9	1216.3 ± 23.1
Nickel	Ni	µg L^{-1}	7.0 ± 1.3	6.4 ± 0.5	6.4 ± 0.8	5.4 ± 0.1
Zinc	Zn	µg L^{-1}	726.4 ± 317.9	357.4 ± 145.2	656.4 ± 245.1	1020.3 ± 417.4
Cobalt	Co	µg L^{-1}	21.3 ± 0.6	22.8 ± 0.4	21.8 ± 1.0	21.1 ± 0.4
Non essential elements						
Sodium	Na	mg L^{-1}	499.5 ± 21.7	455.3 ± 6.4	442.4 ± 10.2	535.1 ± 9.9
Strontium	Sr	µg L^{-1}	1114.4 ± 24.3	1040.0 ± 37.3	1005.8 ± 61.0	1089.5 ± 13.2
Barium	Ba	µg L^{-1}	15.2 ± 2.4	14.2 ± 0.9	12.3 ± 1.2	17.6 ± 7.7
Selenium	Se	µg L^{-2}	3.6 ± 0.5	3.7 ± 0.4	2.8 ± 0.5	3.1 ± 0.3
Aluminium	Al	µg L^{-1}	214.5 ± 12.3	204.6 ± 6.9	216.6 ± 33.2	212.6 ± 9.8
Arsenic	As	µg L^{-1}	7.6 ± 0.5	8.4 ± 0.3	6.9 ± 0.8	6.9 ± 0.4
Chromium	Cr	µg L^{-1}	9.6 ± 0.4	9.8 ± 0.1	11.8 ± 1.3	10.1 ± 0.9

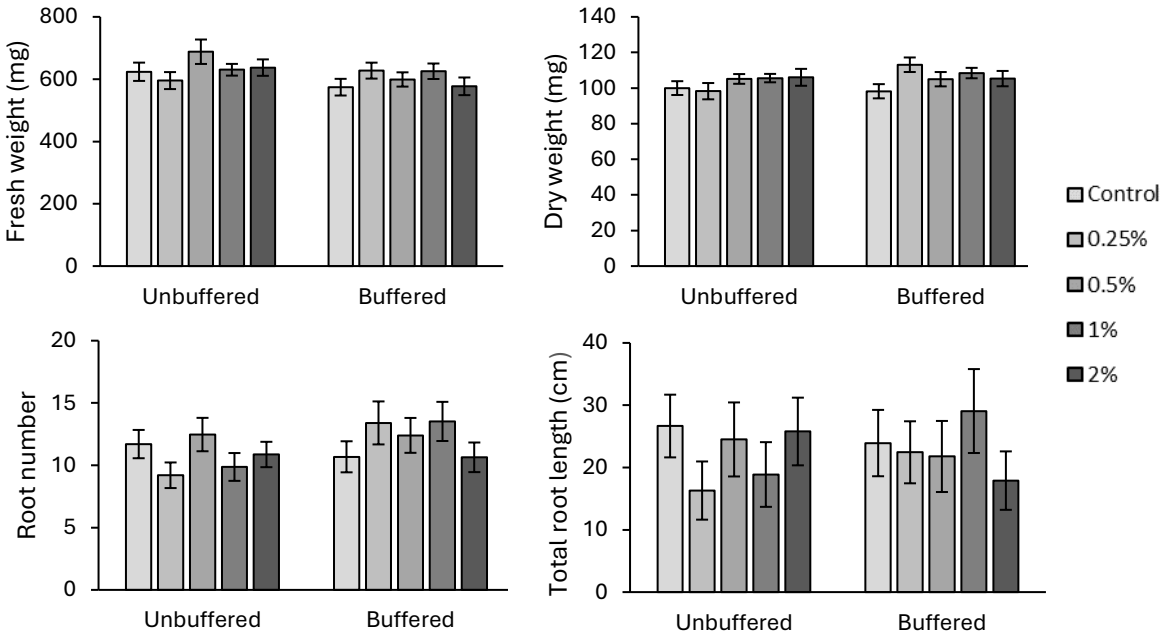
Treatment	17	18	19	20

Biomass loading (g L^{-1})			25	25	40	40
Initial sucrose loading (g L^{-1})			60	60	10	10
Incubation temperature (°C)			25	30	20	25
Macronutrients		**Unit**				
Ash		%	0.56 ± 0.05	0.54 ± 0.10	0.60 ± 0.04	0.63 ± 0.01
Carbon	C	%	0.59 ± 0.09	0.49 ± 0.10	0.36 ± 0.14	0.48 ± 0.04
Hydrogen	H	%	0.12 ± 0.02	0.10 ± 0.02	0.08 ± 0.03	0.11 ± 0.01
Nitrogen	N	%	0.002 ± 0.000	0.002 ± 0.000	0.004 ± 0.002	0.005 ± 0.000
Phosphorus	P	mg L^{-1}	8.8 ± 0.1	9.4 ± 0.5	12.6 ± 0.6	13.2 ± 0.5
Potassium	K	mg L^{-1}	421.9 ± 9.0	434.8 ± 5.4	533.3 ± 20.6	539.9 ± 12.0
Calcium	Ca	mg L^{-1}	178.8 ± 4.2	183.9 ± 4.9	204.2 ± 4.9	205.2 ± 7.8
Magnesium	Mg	mg L^{-1}	367.4 ± 9.8	354.5 ± 24.4	496.0 ± 14.3	534.2 ± 33.4
Sulfur	S	mg L^{-1}	852.5 ± 18.8	855.6 ± 14.7	1149.2 ± 54.3	1182.1 ± 34.5
Micronutrients						
Copper	Cu	µg L^{-1}	54.6 ± 3.6	49.1 ± 1.5	97.1 ± 14.5	94.3 ± 7.6
Manganese	Mn	µg L^{-1}	97.3 ± 8.0	150.4 ± 7.2	172.1 ± 10.6	149.2 ± 10.7
Iron	Fe	µg L^{-1}	1201.4 ± 73.7	1251.8 ± 47.3	1256.9 ± 78.8	1425.6 ± 25.8
Nickel	Ni	µg L^{-1}	5.6 ± 0.4	6.4 ± 0.4	7.2 ± 0.6	8.4 ± 0.8
Zinc	Zn	µg L^{-1}	913.2 ± 363.0	551.7 ± 320.7	672.7 ± 219.9	959.6 ± 91.6
Cobalt	Co	µg L^{-1}	22.2 ± 1.0	24.3 ± 0.8	29.2 ± 1.6	31.3 ± 0.6
Non essential elements						
Sodium	Na	mg L^{-1}	509.8 ± 7.9	509.0 ± 9.4	671.3 ± 16.2	754.1 ± 52.7
Strontium	Sr	µg L^{-1}	1092.6 ± 50.3	1148.3 ± 68.7	1300.9 ± 89.2	1408.9 ± 72.5
Barium	Ba	µg L^{-1}	16.3 ± 4.9	15.0 ± 1.6	15.6 ± 3.1	12.2 ± 0.3
Selenium	Se	µg L^{-2}	2.3 ± 0.7	2.9 ± 0.4	6.1 ± 0.5	6.0 ± 0.2
Aluminium	Al	µg L^{-1}	216.2 ± 4.3	239.5 ± 6.6	159.3 ± 7.3	176.4 ± 20.2
Arsenic	As	µg L^{-1}	7.2 ± 0.1	8.3 ± 0.6	10.5 ± 0.7	10.1 ± 0.3
Chromium	Cr	µg L^{-1}	9.6 ± 0.4	10.7 ± 1.4	13.4 ± 0.8	17.0 ± 1.1

Treatment			21	22	23	24
Biomass loading (g L^{-1})			40	40	40	40
Initial sucrose loading (g L^{-1})			10	35	35	35
Incubation temperature (°C)			30	20	25	30
Macronutrients		**Unit**				
Ash		%	0.63 ± 0.03	0.63 ± 0.05	0.65 ± 0.06	0.65 ± 0.02
Carbon	C	%	0.46 ± 0.04	0.53 ± 0.29	0.78 ± 0.12	0.58 ± 0.26
Hydrogen	H	%	0.11 ± 0.00	0.11 ± 0.06	0.17 ± 0.01	0.13 ± 0.05
Nitrogen	N	%	0.005 ± 0.001	0.004 ± 0.002	0.005 ± 0.000	0.005 ± 0.001
Phosphorus	P	mg L^{-1}	14.3 ± 1.0	15.4 ± 0.4	14.1 ± 0.4	13.7 ± 0.3
Potassium	K	mg L^{-1}	566.7 ± 14.1	606.0 ± 14.5	589.0 ± 15.2	557.9 ± 7.9
Calcium	Ca	mg L^{-1}	215.7 ± 10.6	261.8 ± 3.7	252.0 ± 8.9	239.3 ± 3.1
Magnesium	Mg	mg L^{-1}	578.3 ± 30.1	542.0 ± 50.8	602.3 ± 15.6	480.2 ± 19.2
Sulfur	S	mg L^{-1}	1256.7 ± 38.8	1268.6 ± 23.5	1257.7 ± 41.6	1184.1 ± 15.6
Micronutrients						
Copper	Cu	µg L^{-1}	81.5 ± 9.6	116.8 ± 6.5	79.4 ± 9.2	84.8 ± 3.9
Manganese	Mn	µg L^{-1}	198.6 ± 13.6	225.2 ± 15.2	164.0 ± 4.9	194.2 ± 6.7
Iron	Fe	µg L^{-1}	1466.0 ± 9.8	1581.3 ± 58.9	1610.1 ± 84.9	1554.9 ± 17.9
Nickel	Ni	µg L^{-1}	7.3 ± 0.2	7.4 ± 0.4	8.2 ± 0.7	7.5 ± 0.1
Zinc	Zn	µg L^{-1}	650.3 ± 223.9	824.1 ± 277.5	793.7 ± 285.0	466.8 ± 194.9
Cobalt	Co	µg L^{-1}	32.7 ± 0.5	33.4 ± 0.9	34.1 ± 2.0	33.7 ± 0.4
Non essential elements						
Sodium	Na	mg L^{-1}	843.6 ± 49.3	726.7 ± 26.8	771.7 ± 56.7	681.2 ± 4.8
Strontium	Sr	µg L^{-1}	1434.2 ± 130.5	1557.7 ± 32.6	1581.4 ± 53.8	1554.9 ± 93.8
Barium	Ba	µg L^{-1}	15.8 ± 0.4	16.1 ± 1.1	15.6 ± 0.9	21.5 ± 1.2
Selenium	Se	µg L^{-2}	5.9 ± 0.6	5.5 ± 0.8	5.6 ± 0.4	5.8 ± 0.0
Aluminium	Al	µg L^{-1}	149.0 ± 21.9	221.6 ± 10.2	231.4 ± 25.1	226.8 ± 12.6
Arsenic	As	µg L^{-1}	10.8 ± 0.6	11.7 ± 0.2	11.2 ± 0.7	11.7 ± 0.1
Chromium	Cr	µg L^{-1}	18.4 ± 1.0	15.5 ± 1.7	15.4 ± 1.5	14.3 ± 1.1

Treatment			25	26	27
Biomass loading (g L^{-1})			40	40	40
Initial sucrose loading (g L^{-1})			60	60	60
Incubation temperature (°C)			20	25	30
Macronutrients		**Unit**			
Ash		%	0.68 ± 0.05	0.68 ± 0.02	0.70 ± 0.01
Carbon	C	%	0.90 ± 0.47	0.58 ± 0.04	0.53 ± 0.05
Hydrogen	H	%	0.19 ± 0.09	0.12 ± 0.01	0.12 ± 0.01
Nitrogen	N	%	0.003 ± 0.002	0.003 ± 0.000	0.003 ± 0.001
Phosphorus	P	mg L^{-1}	15.4 ± 0.2	16.0 ± 1.3	16.1 ± 0.9
Potassium	K	mg L^{-1}	671.3 ± 24.8	657.3 ± 24.0	653.3 ± 19.0
Calcium	Ca	mg L^{-1}	296.2 ± 11.2	286.0 ± 13.6	285.0 ± 8.3
Magnesium	Mg	mg L^{-1}	636.9 ± 69.3	583.0 ± 30.3	547.6 ± 28.6
Sulfur	S	mg L^{-1}	1402.8 ± 56.9	1350.4 ± 52.8	1341.7 ± 34.8
Micronutrients					
Copper	Cu	µg L^{-1}	97.0 ± 9.6	98.8 ± 13.0	259.1 ± 172.2
Manganese	Mn	µg L^{-1}	117.1 ± 10.7	198.5 ± 29.8	226.1 ± 10.0
Iron	Fe	µg L^{-1}	1870.0 ± 93.6	1767.8 ± 84.6	1929.6 ± 92.2
Nickel	Ni	µg L^{-1}	9.5 ± 1.3	9.5 ± 1.7	13.3 ± 4.6
Zinc	Zn	µg L^{-1}	1953.2 ± 145.6	310.3 ± 22.6	756.3 ± 255.7
Cobalt	Co	µg L^{-1}	37.7 ± 1.4	37.0 ± 1.4	40.1 ± 1.7
Non essential elements					
Sodium	Na	mg L^{-1}	813.8 ± 40.8	758.3 ± 29.5	777.3 ± 23.9
Strontium	Sr	µg L^{-1}	1816.2 ± 63.7	1668.3 ± 38.9	1784.6 ± 19.8
Barium	Ba	µg L^{-1}	15.8 ± 0.9	26.2 ± 6.7	22.7 ± 7.0
Selenium	Se	µg L^{-2}	5.9 ± 0.4	5.1 ± 0.8	5.6 ± 0.4
Aluminium	Al	µg L^{-1}	293.9 ± 16.1	276.2 ± 15.0	323.4 ± 16.5
Arsenic	As	µg L^{-1}	13.4 ± 0.5	13.1 ± 0.4	13.0 ± 1.0
Chromium	Cr	µg L^{-1}	14.3 ± 1.7	14.3 ± 0.7	28.7 ± 10.8

Appendix 7.3: Mung bean assay 2: not pH buffered compared to pH buffered. Average ± SE. PERMANOVA was used to detect significant differences. All fresh weight, dry weight, root number, and root length treatments did not significantly differ.



Source	df	Fresh weight		Dry weight		Root number		Root length	
		F	*p*	F	*p*	F	*p*	F	*p*
Treatments	9	1.47	0.15	1.40	0.18	1.31	0.23	0.58	0.81
Residuals	265								

Appendix 7.4: Gradual light and temperature increase for tomato seedlings in grow tent in preparation for transfer to greenhouse. Temperature and light presented from light cycle only. Average ± SE.

Day	Temperature (°C)	Light (lux)
1	28 ± 0.6	13554 ± 311
2	29 ± 0.3	14058 ± 29
3	31 ± 0.5	18369 ± 591
4	31 ± 0.4	20444 ± 65
5	32 ± 0.6	22387 ± 810
6	36 ± 0.5	29897 ± 35
7	36 ± 0.5	29722 ± 36
8	36 ± 0.5	29588 ± 44

Appendix 7.5: pH buffered measurements of treatments. Biostimulant measured as percent concentration (v/v). Fertiliser concentration 5 mL L^{-1}. DAT = days after transplanting.

	Biostimulant %				Biostimulant % + Fertiliser			
DAT	**0**	**0.2**	**0.4**	**1**	**0**	**0.2**	**0.4**	**1**
7	6.81	6.79	6.73	6.61	5.93	5.89	5.87	5.77
14	6.81	6.76	6.72	6.6	6.81	6.76	6.72	6.6
21	6.87	6.83	6.78	6.66	6.32	6.27	6.25	6.15
28	6.75	6.63	6.61	6.58	6.74	6.66	6.63	6.58
35	6.74	6.71	6.68	6.55	6.26	6.2	6.19	6.1
42	6.86	6.79	6.74	6.63	6.82	6.79	6.73	6.63
49	6.82	6.78	6.74	6.62	6.31	6.25	6.22	6.14
56	6.97	6.93	6.87	6.69	6.97	6.93	6.87	6.73
63	6.79	6.79	6.77	6.64	6.39	6.32	6.31	6.22
70	6.79	6.8	6.75	6.61	6.8	6.8	6.72	6.56
77	6.76	6.72	6.67	6.57	6.26	6.22	6.21	6.12
84	6.85	6.83	6.81	6.71	6.86	6.85	6.82	6.72