The Effect of Oligosaccharides in an extract of the Brown Seaweed *Ascophyllum nodosum* on Plant Growth and Plant Immune Responses in Soybean (*Glycine max* L.) and Duckweed (*Lemna minor*)

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ABSTRACT

The Effect of Oligosaccharides in extract of Brown Seaweed *Ascophyllum nodosum* on Plant Growth and Plant Immune Responses in Soybean (*Glycine max* L.) and Duckweed (*Lemna minor*)

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Ascophyllum nodosum extract (ANE) is a processed brown seaweed extract used as a biostimulant. The known effects of ANE, when applied to plants in the aforementioned agricultural setting, include amplified plant growth, improved abiotic and biotic stress tolerance, enhanced fruit quality, and productivity. ANE contains partially hydrolyzed carbohydrates, proteins, hormones, and minerals from brown seaweed and its endophytic fungus, *Mycosphaerella ascophylli*. ANE is diluted down to a low concentration (e.g. 3mL/L or 0.3% ANE) and applied to a large area (1to 2 L of ANE per acre). Thus, the bioactive component must be of a relatively high concentration in ANE. Carbohydrates are the major component of brown seaweed's dry mass. During the ANE production process, insoluble carbohydrates are broken down into water-soluble oligosaccharides. Studies have shown that some oligosaccharides (e.g. oligoalginate and oligochitins) can increase plant growth and promote immune responses. Thus, the hypothesis of this study is that one or more of these oligosaccharide components (oligoalginate and/or oligochitin) in ANE is responsible for inducing improved plant growth and enhanced resistance to abiotic and biotic stresses. Soybeans and duckweed were used to test the hypothesis. No significant difference was found between the ANE treatment as a positive control and water treatment as a negative control in chlorophyll and phenolic content. Low light intensity and poor growth condition may be the limiting factors. Also, no significant difference was found between the water and ANE treatment in the number of duckweeds. Growth should be optimized for significant increase in population growth.

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LIST OF ABBREVIATIONS

ANE = Ascophyllum nodosum extract

DMSO = Dimethyl Sulfoxide

Cm = centimeter

g = gram

m = meter

m2 = square meter

mL = milliliter

mM = milliomolar

µL = microliter

µM = micromolar

nM = nanomolar

Na2CO3 = Sodium Carbonate

ppm = parts per million

R1 = Reproductive state 1

R2 = Reproductive state 2

w/v = weight to volume

V1 = Vegetative state 1

V2 = Vegetative state 2

V3 = Vegetative state 3

V4 = Vegetative state 4

ºC = degrees Celsius

1. INTRODUCTION

On a daily basis, many people do not have access to enough food due to rapid population growth, uneven food distribution, and depletion of natural resources. According to the Food and Agriculture Organization of the United Nations, the number of under- or malnourished people increased from 216 million in the 1990s, to approximately 750 million in 2015 (AEDAD, 2015). This striking increase highlights the need to increase global crop yields, which should help abate this statistic from growing further. Chemical fertilizers have propelled farming onto a new modern trajectory. The use of these fertilizers has allowed farmers to produce more crop units with a higher average weight per unit. However, the use of fertilizers alone is not likely to maximize crop yields. Despite being exposed to fertilizers, which stimulate growth, plants often do not reach their genetic potential size due to environmental stresses (Rockstorm & Falkenmark, 2000). Recently, biostimulants have been the subject of intense study due to their capacity to increase plant growth when applied in low concentrations (Khan et al., 2009). Further, it has been observed that by combining chemical fertilizers with biostimulants, synergistic effects can be realized (Wally et al., 2013). Examples of such synergies include significantly faster rates of plant growth, fortified resistance to abiotic and biotic stress, and higher crop yield per plant (Fornes et al., 2002). With regards to the aforementioned biostimulants, extracts of many brown seaweed species (*Ascophyllum nodosum*, *Ecklonia maxia, Kappaphycus alvarezii*, etc) have been shown to act as such biostimulant for crops (Crouch & Staden, 1992; Rathore et al., 2009).

1.1 Importance of *Ascophyllum nodosum* in Agriculture

Ascophyllum nodosum is a brown alga (Phaeophyceae) that grows on rocks in the Northern Atlantic region (Wally et al., 2013). Traditionally, the inhabitants of coastal regions of both Canada and Europe used the brown seaweed species in farmlands to enhance crop growth (Booth, 1969; Sharma et al., 2012). Specifically, farmers directly applied a large amount of fresh or dry brown seaweed to the farmland (Jannin et al., 2013; Ali et al., 2015). Like an organic fertilizer, brown seaweed was broken down by soil microbes, which release small fragments of carbohydrates, proteins, hormones, and various minerals of the seaweed into the soil. Brown seaweed was used only in farmlands near coastal regions due to the high cost of transportation. In the 1950s, Europeans and North Americans developed a seaweed extraction method for liquefying and dry-milling brown seaweed (Milton, 1952). These two forms of seaweed-derived products allowed for much easier transportation and application to agriculture (Jannin et al 2013). Today, a processed brown seaweed product called *Ascophyllum nodosum* extract (ANE) is sold as a commercial biostimulant for both agriculture and horticulture. ANE cab be produced by alkaline extraction, acidification, or low-temperature milling (Jannin et al., 2013). The extraction process in these extreme conditions breaks insoluble seaweed cell walls into smaller organic compounds that are soluble in water. In addition, the process denatures various proteins, hormones, and other minor organic compounds. Thus, other than the soluble carbohydrate fragments, other organic compounds may have little to no effects found in the brown seaweed extract. With foliar spray, a small amount of ANE is diluted and sprayed across fertilized land in a ratio of one to four litres of ANE per acre (Acadian Seaplant, 2015), or as low as less than 0.01% w/v (Crouch & van

Staden, 1993). Over time, usage of brown seaweed *(Ascophyllum nodosum)* has changed from one of a fertilizer to that of a biostimulant, and the application method changed accordingly.

1.2 Effects of *Ascophyllum nodosum* extract in Agriculture

Quality and quantity of crops and fruit enhancement

Recent research on biostimulants has revealed that ANE can improve the quality, quantity, and size of various fruits when applied in small concentration (Acadian Seaplant, 2015). For example, watermelons, cucumbers, peppers, grapes, strawberries, and Mouse-ear cress sprayed with brown seaweed extract exhibited larger size, heavier dry weight, and faster root and shoot growth (Rayorath et al., 2008; Ali et al., 2015). The supernormal growth of ANE treated fruit-bearing plants was compared to both a water-only control sample and a fungicide-treated sample, with the latter two samples producing significantly smaller and lighter fruit (Ali et al., 2015). Studies have also been conducted to examine how plant growth changes with different concentration of ANE. 0.5% ANE was found to result in significantly larger and heavier fruit than 0.2% ANE (Rathore et al., 2009). Moreover, ANE has been shown to increase the thickness and firmness of fruit, with higher concentrations of the seaweed extract being correlated with improved ratings of these two characteristics. For example, greenhouse tomatoes treated with a 0.5% ANE solution were deemed to have the thickest and firmest skin, along with greater size and weight when compared to a 0.2% ANE-treated sample (Ali et al., 2015). In Western countries, tomatoes are assigned a quality grade based on their size, weight,

and overall firmness. Ali et al., (2015) found that in a test of the impacts of the aforementioned biostimulant on tomato quality, higher concentrations of ANE corresponded with better overall ratings. From the test, tomatoes applied with a 0.5% ANE solution, 0.2% ANE solution, and just water were assigned grade A for 55, 38, and 15 tomatoes from 108 tomatoes, respectively. The use of ANE doubled fruit yields when compared to control plant (Ali et al., 2015). Moreover, plants treated with a relatively high concentration of ANE (0.5%) had higher crop productivity and fruit yield per plant (Jannin et al., 2013). When kept at what would be deemed to be a low concentration (1-4mL/L), relatively higher levels of ANE allows the plants to exhibit supernormal growth characteristics in size, weight, and overall quality (such as firmness and thickness) and quantities.

Enhancement of nutrient absorption by roots

ANE has been shown to have positive effects on the roots, leaves, and immune systems of plants. According to many studies, such effects are correlated with improved fruit development. Plant growth and reproduction depend on two key factors: the availability of nutrients in the soil and the absorptive ability of the plant's roots. Roots perform a variety of functions in plants, from absorbing water and essential nutrients (N, P, K, and S) to supporting the plant's overall structure (Zhang et al., 2013). A collection of studies has shown that applying ANE to plants can promote cell differentiation in secondary roots, which in turn leads to higher root biomass and surface area (Rayorath et al., 2008; Mugnai et al., 2008; Roussos et al., 2009; Ali et al., 2015) . For example, in a study by Ali et al., (2015), ANE-treated tomato plants developed roots with significantly higher

biomass than the roots of the control plants. In the same study, it was observed that application of a relatively higher concentration of ANE (0.5%) resulted in the plants developing greater root biomass than plants exposed to a lower concentration dose of ANE (0.2%) (Ali et al. 2015). Larger roots have a wider surface area, which in turn provides more locations for the roots to bind with soil nutrients. However, some researchers proposed that ANE application on plants increased production of vertical roots but the overall root volume reduced after ANE application on plants (Wally et al., 2013). ANE treated plants had larger biomass and higher root growth rate.

The increased nutrient absorption of these larger-rooted ANE-treated plants also allows these specimens to transport more nutrients to the rest of the plant body. Specifically, the stem, leaf, and fruit will all benefit from increased nutrition levels. Thus, plants treated with ANE have higher nutrient uptake and ANE treated plants produce fruit containing higher nutrient content (Ali et al., 2015). For example, ANE treated tomatoes had higher nutrient counts for N (81%), P (8%), K (50%), Ca (570%), Fe (250%), and Zn (33%) than the control tomatoes (Ali et al. 2015). Similar findings in tests involve grapes, carrots, eggplants, and oranges (Mancuso et al., 2006; Alam et al. 2014; Bozorgi et al. 2012; Hegab et al., 2005). In these studies, applying ANE resulted in more nutrients per fruit in grape vines (Mancuso et al, 2006), as well as increased size and growth rates in carrots and eggplants (Alam et al., 2014; Bozorgi et al., 2012). Rathore et al. (2009) observed similar results after applying ANE to soybean plants; N, P, K, and S ion concentration significantly increased in the seeds. ANE has positive effects on soil as well. Alginic acid and polyuronides, found commonly in red, green and brown seaweeds, increase the water retaining capacity and air circulation in soil (Moore 2004). These

augmentations to the soil composition can stimulate roots, promote microbial activity, and increase nutrient accessibility (Mohanty et al., 2013). Overall, ANE increases root size and improves soil quality, which increases plants' uptake of minerals.

Higher Photosynthesis Activity and Chlorophyll Content

Plants treated with ANE have also exhibited higher chlorophyll content and photosynthesis activity. Given chlorophyll's role in producing glucose, which is a plant's primary source of energy, chlorophyll plays an important role in plant growth and reproduction. However, chlorophylls degrade over time. All plants go through a natural process of chlorophyll degradation, which diminishes the amount of chlorophyll in the plant's leaves. This process naturally occurs during aging of leaves, preparation for ripening fruit, and various abiotic and biotic stresses (Rayirath et al., 2009). Given chlorophyll's effects on plant growth, the aforementioned degradation process has been connected to decreased plant growth. Studies have found that ANE treated plants can retain their chlorophyll for longer period of time (Rayirath et al., 2009; Rayorath et al., 2008). Specifically, ANE reduces the chlorophyll degradation rate (Rayirath et al., 2009; Rayorath et al., 2008), increases chlorophyll/chloroplast content, and increases photosynthesis activity (Ali et al., 2015; Jannin et al., 2013). A factor for the reduction in chlorophyll degradation rate may be that ANE reduces the expression of the chlorophyll-degrading gene (AtCLH1 and AtCLH2) for chlorophyllase (Rayirath et al., 2009). For example, ANE treated plants exhibited reduced chlorophyllase enzyme activity in colder temperatures, and the plants had three times the chlorophyll content when compared to water treated plants (Rayirath et al., 2009).

Increased chlorophyll content can also affect photosynthetic activity, and studies have shown to increase the amount of chlorophyll in ANE treated plants (Blunden et al., 1997). Nitrogen is a limiting factor in chlorophyll formation (Ali et al., 2015). As mentioned in section 1.2.2, ANE treated plants develop larger root systems, which allows the plant to absorb greater quantities of nitrogen (Popescu & Popescu, 2014). Therefore, ANE's effects on root growth also translate into the treated plants retaining and increasing chlorophyll content, and ultimately increasing their photosynthetic activity.

Alleviation of abiotic and biotic stresses on plants

Abiotic stresses such as cold, saline, or dry conditions make it difficult for plants to grow, and exposure to abiotic stresses can increase pathogen susceptibility (Jakab et al., 2005). When exposed to these conditions, plants are generally incapable of reaching their full growth potential (as measured by the stems, leaves, roots, fruit, and seeds), cells are damaged, and nutrients leak. Further, these conditions will adversely affect the quality and quantity of both flowers and fruit (Abdel-Aziz et al., 2011). According to Rockstorm & Falkenmark., (2000), half of crop productivity is lost from these negative effects; therefore, it is important to develop a way for plants to increase the resistance to these external difficulties. Plants treated with ANE are more resistant to excessively cold, saline, or dry environments (Nair et al., 2012). Freezing temperatures reduce plant growth by slowing down plant metabolism and disturb osmosis (Nair et al., 2012). Many temperature sensitive crops, such as *Arabidopsis thaliana*, displayed reduced tissue damage from freezing temperatures and improved osmotic adjustment (Rayirath et al., 2009; Nair et al., 2012); and field barley exhibited increased frost resistance (Burchett et

al., 1998). In addition, ANE treated plants have a reduced mortality rate from freezing stresses and recovered faster after the freezing conditions subsided, after which point they produced an increased fruit yield (Rayirath et al., 2009). ANE treated plants also displayed improved chlorophyll protection during these freezing conditions; further, these plants were shown to not be as prone to yellowing as the control plants (Rayirath et al., 2009). Similarly, the negative effects of excessive salinity were not as severe for ANE treated plants – the plants had longer and thicker stems and roots, higher N, K, and P concentration in leaves, roots and stems, and higher numbers of flowers than control plants (Abdel-Aziz et al., 2011). ANE treated plants also exhibit enhanced drought tolerance; these plants were observed to have a higher cytokinin concentration, which increase root biomass, and the plants were found to be in a healthier state than control plants (Zhang et al., 2003). As shown by the above empirical results, ANE treatment has been strongly correlated with increased resistance to abiotic stresses, namely those of temperature, salinity, and moisture extremes.

ANE has also been shown to increase plants' tolerance for biotic stresses. In a manner similar to animals, plants can be infected by pathogens, such as bacteria, viruses, and fungi (Jayaraj et al., 2008). Receptors on the cellular surface can recognize pathogens through pathogen-associated molecular patterns (PAMPs) (Chisholm, et al., 2006). After initial contact with pathogens or pathogenic fragments, plant cell walls will then trigger one of two types of immune responses (Jayaraj et al., 2008). Firstly, local responses occur in the areas that are originally infected (Heil & Bostock, 2002). Various defense chemicals such as reactive oxygen species and phenolics try to kill the pathogen. These oxygen radicals induce an oxidative burst, which leads to the death of the infected cells

(Heil & Bostock, 2002). The aim behind this response is to isolate pathogens within the infected cells to stop the infection from spreading further (Heil & Bostock, 2002). The local response also involves changing the composition of the cell wall. The infected cells increase production of antimicrobial enzymes, such as chitinase and glucanase, to prevent more pathogens from entering (Heil & Bostock, 2002). Secondly, systemic responses occur in distant parts of the plant that are not yet infected. Non-specific pathogen-recognizing and pathogen-attacking enzymes are produced, and once they are produced, they stay in the plant's system for a long time; plant hormone (salicylic and jasmonic acid) cascades also occur to help to kill the pathogens (Heil & Bostock, 2002). In both local and systemic responses, plant cells produce phenolics to kill the pathogens (Heil & Bostock, 2000). Pathogenesis–related (PR) proteins are antimicrobial proteins that are usually absent before pathogen attacks, but their accumulation occurs soon after infection (Heil & Bostock, 2002). ANE-treated plants exhibit increased production of phenolics (Fan et al., 2011), jasmonic acid, salicylic acid (Subramanian et al., 2011), chitinase (Jayaraman et al., 2011), and β-glucanase (Kaku et al., 1997). More specifically, ANE treated cabbages increased phenolic content by approximately two folds higher than the control (2013). Researchers have shown that plants exhibit lower infection rates when chitinase's PR protein production increases (Broglie et al., 1991). The strength of a plant's immune system is highly correlated with its crop yield: one study showed that global crop yields improved by approximately 14% per year in plants that were more resistant to particular diseases (Popp and Hantos, 2011).

Composition of Ascophyllum nodosum extract

To determine the biostimulating components in *Ascophyllum nodosum* extract, studies have focused on identifying the compositions of brown seaweed. *Ascophyllum nodosum* is comprised of 40-70% carbohydrates (alginate, laminarin, fucoidan, and mannitol), 3-10% proteins, 4-8% polyphenols/pigments, and 2-4% phospholipids/glycolipids (Ali et al., 2015). Further aspects of its composition include a low concentration of plant hormones (auxins, cytokinins, and gibberellins), vitamins A and C, and various minerals (calcium, phosphorus, potassium, magnesium, boron, zinc) (Ali et al., 2015). Many known biostimulating components have been found in this brown seaweed, including polysaccharides (alginate, laminarin, fucoidan), vitamins, plant hormones, inorganic substances, and secondary metabolites (Baardseth 1970; Crouch and van Staden 1993; Ryorath et al. 2008; Craigie 2011; Blunden and Gordon 1986; Wang et al. 2003; Kim and Wijesekara 2010). Carbohydrates are the most abundant component in *A. nodosum*. Among these carbohydrates, alginate, a structural compound that makes up the cell walls of brown seaweed, comprises 30% of *A. nodosum*'s dry weight; other components that make up the cell walls, fucose and laminarin, compose 10% and 7% of *A. nodosum*'s dry weight respectively (Kandasamy et al., 2015). Plant growth hormones, such as cytokinins and ABA are found in very small amounts, in 10 ng/g and 5 ng/g, of *A. nodosum* dry weight, respectively (Wally et al., 2013). According to Wally et al., (2013), these concentrations of the plant hormones are not effective to crops grown in field condition since at least 100 nM of each hormonal concentration is required for positive results.

Mycosphaerella ascophylli is an endophytic fungus that grows inside the thallus of *A. nodosum*. This fungus infects brown seaweeds by sporulation and by attaching to the epidermal layer of the brown seaweed (Garbary et al., 1991). The fungus and the brown seaweed have symbiotic relationship (Garbary et al., 2005). The components of this *M. ascophylli* have not been identified in studies, but it is commonly known that chitin is the major component of the fungal cell walls.

1.3 Oligosacchardies found in ANE

Oligosaccharides are smaller fragments of carbohydrates composed of ten or less saccharides. As previously mentioned, ANE is processed via extreme pH treatment or high temperature exposure (section 1.1). During this process, all the carbohydrates are broken down into oligosaccharides and smaller polysaccharides (Jannin et al., 2013). Since the major components of both the brown seaweed and fungus are carbohydrates, it is important to focus on how major oligosaccharides affect plants after treatment. Oligosaccharides of alginate, chitin and its derivative, chitosan have been shown improvement in the plants growth, productivity, and environmental stress tolerance (Zhang et al., 2009).

The effects of alginate-oligosaccharides on plants

Alginate oligosaccharides (AOS) are the major component of brown seaweed and commonly found in the seaweed cell walls. *Ascophyllum nodosum* consists of AOS about 30% of the seaweed's dry weight (Kandasamy et al., 2015). Alginate oligosaccharides (AOS) are composed of α-L-guluronate (G) and β-D-mannuronate (M) (Nagasawa et al., 2000). Alginate oligosacchariteshave been shown to increase crop

yields in many studies (Xu et al., 2003; Zhang et al., 2009). AOS may have an important role in plants as a plant growth regulator. AOS have significant effects on root growth; for example, AOS enhanced cell division in root tips and chromosomal mutation when exposed to cadmium stress (Ma et al., 2010). Application of AOS (10-50mg/L) on Chinese cabbages has been shown to increase root length, volume, and weight; higher nutrients absorption by roots (Zhang et al., 2009). A similar result was shown for root growth in carrots and rice (Xu et al., 2003). Such activities of AOS were more effective than other oligosaccharides (i.e. oligogalacturonic acid) found in seaweeds (Zhang et al., 2009). The effects of alginate have also shown to increase the chlorophyll content and photosynthesis activity in *Botryococcus braunii* (Bailliez et al., 1986). Alginate oligosaccharides show similar effects as ANE, such as an increase in root development, nutrients uptake, chlorophyll content, and photosynthesis activity in plants.

Currently, alginate receptor has not been found in plants. Thus, a clear understanding of the physiological mechanism of AOS is currently limited. However, studies found that application of alginate oligosaccharides have been shown to turn on many genes including the chlorophyll synthase, abiotic stress resistance gene (ex: cold temperature, droughts), which may be influencing the plants growth (Bailliez et al., 1986).

The effects of Chitin-oligosaccharides on Plants

Chitins are rigid and durable structural compounds found in many organisms; such as in the exoskeleton of insects and crustaceans (Terwisscha van Scheltinga et al., 1995). In addition, chitins establish the backbone structures of cell walls in many fungi (including the *M. ascophylli* found in brown seaweed). The main structural components of chitin

can be divided into α- or β- anomers; however, most commonly discussed chitin (found in fungi) consist of a linear polymerization and β-1,4 connectivity like N-acetylchitooligosaccharide (GlcNAc) (Terwisscha van Scheltinga et al., 1995). Smaller chitin fragments are called chitin oligosaccharides (COS).

Plants have a defense system against fungal infection by recognizing and breaking down various fungi components (chitin). Chitin is broken down into smaller chitin fragments (COS) by an enzyme called chitinase located in the cell walls of a leaf (Terwisscha van Scheltinga et al., 1995). These COS stimulate the production of various genes that are responsible for producing secondary defense within plants as mentioned in section 1.2 (Jayaraman et al., 2011; Kaky et al., 2011). Application of COS on plant leaves has also been shown to act as signals for antifungal activity in plants (Andres et al., 2014). Specifically, nanomolar concentrations of COS promoted the production of phytoalexins and the depolarization of membrane potential in rice plants (Yamada et al., 1993; Kikuyama et al., 1997); the expression of chitinase proteins in melons (Roby et al., 1987); and the lignification in wheat (Barber et al., 1989). Different polymers of COS are required to trigger immune responses in different plants (Larroque et al., 2011). Similar effects were shown in chitin derivatives. Chitosan has very small structural differences when compared to chitin. Chitosan is a deacetylated form of chitin with a linear structure, and it comprises β-1,4 glucosamine polymers (Lee et al., 1994; Yamaguchi et al., 2000). Receptors of most plants will respond to a variety of chitosan oligosaccharides while oligochitin must bind to a specific receptor such as chitinase (Kauss et al., 1989). However, a potential drawback to the flexibility of chitosan is that it must be present in much higher concentrations than chitin to induce an immune response

(Shibuya & Minami, 2001). Chitosan in dicot plants, such as soybeans, plays an important role in defense mechanisms. When soybean plant cell receptors detect the presence of chitosan, the plant cell releases electrolytes, the ultra violet radiation absorbing molecules due to chitosan's cationic property (Kohle et al., 1985), and proteins (chitinases) to cut chitosan into smaller oligosaccharides. Chitosan oligosaccharides promote glyceollin (phytoalexin) synthesis and phenolic structural change, which increases defense against disease (Sharp et al., 1984b; Kohle et al., 1985).

1.4 Objectives

Over time, the role of brown seaweed (*Ascophyllum nodosum*) in agriculture has shifted from one of a fertilizer to that of a biostimulant, and its application method has changed accordingly. *Ascophyllum nodosum* extract (ANE) demonstrates biostimulating properties including amplified plant growth, improved resistance to abiotic and biotic stresses, enhanced fruit quality, and augmented plant productivity. Key components of brown seaweed, such as various proteins, plant hormones, and minerals, were thought to promote plant growth, but studies have since proposed that these components are not what make ANE an elicitor for plants. ANE production involves extreme pH or temperature treatments in which the seaweed proteins are most likely denatured (Jannin et al., 2013). Further, the mineral content of brown seaweed, as measured by its dry weight, is less than 1%; this may not be able to provide enough nutrients to ANE treated plants. Given the scarce amounts of these nutrients in ANE, as well as the fact that ANE is applied in very low concentrations (e.g. 3mL per L), it is highly unlikely that the

aforementioned proteins, plant hormones, or minerals are acting as elicitors. Carbohydrates are the major component of brown seaweed's structure, comprising 40-70% of the dry weight. These insoluble carbohydrates are broken down into water-soluble oligosaccharides during the extraction process. Further, many studies proposed that oligosaccharides can promote plant growth, enhance stress tolerance, and increase plant productivity. ANE and oligosaccharides induce similar immune responses in plants. Thus, I predicted that the ANE treated and oligosaccharide treated soybean leaves would have significantly higher chlorophyll and phenolic contents. Further, I predicted that ANE and oligosaccharide treated duckweed would have higher number of frond than water treated duckweed. I hypothesized that one or more of the main oligosaccharide components (oligo-alginate, oligochitin, oligo-β-glucan) in ANE was responsible for inducing the improved plant growth and enhanced resistance to abiotic and biotic stresses found in ANE treated soybeans. The objectives of this research were as follows:

i) To compare the effects of oligosaccharides and ANE on the production of chlorophyll a and b.

ii) To compare the effects of oligosaccharides and ANE on plant phenolics.

iii) To assess the effect of oligosaccharide mixture and ANE on population growth of duckweeds under salt stress.

METHODS

Study Species 1: Soybeans (Glycine max)

Soybeans (*Glycine max*) are rich in protein, and they provide essential vegetative protein source for human and livestock (Rathore et al., 2009). The vegetative stages of soybeans were divided and described by Fehr et al., (1991). For example, V1 represent first set of compound leaves (trifoliate leaves) found at the first node from the bottom; V2 represent second trifoliates found at the second node from bottom. During the vegetative stage (V), each stage takes approximately 5-7 days in optimum condition. Further, soybeans bloom purple flowers; letter R is used to describe bloom (R1,2), pot development (3,4), seed development (5,6), and maturity (7,8) stages (Fehr et al., 1991). In reproductive stage, the plants take longer time to move from a stage to another. For instance, Blooming, seed development, and maturity stage takes about 3, 15, 18 days to develop, respectively (Fehr & Caviness, 1997). Soybeans are economically and agriculturally important crops.

Study species 2: Duckweeds (Lemna minor)

Duckweeds, or *Lemna minor* belong to *Lemnceae* family, are tiny floating aquatic plants found in fresh water in North America, Europe and Asia (Landolt, 1986). The aquatic plant has thin 1.5 mm diameter of frond (commonly known as leaf) with 2 to 3 cm of thin roots (Landolt, 1986). Duckweed is reproduced asexually by budding daughter fronds. At maturation, duckweed consists of four fronds, which breaks off at the tissue-connecting mother and daughter fronds. Duckweeds have rapid population growth rate; they require abundant nitrogen and phosphorus as their nutrients (Maclay, 1976). Duckweed widely used in labs to determine the effect of toxic substances, organic

compounds on aquatic plants due to its sensitivity (Hillman, 1961). Studies have shown that duckweeds can change pH and conductivity of water (Maclay, 1976).

2.1 Soybean Experiment 1

The soybeans used in the experiment were obtained from Annapolis Seeds (Annapolis, Nova Scotia, Canada). These soybeans were soaked for one day in either control or different treatments. The treatments used in this soaking process are as follows: distilled water (the control), a 0.2 % concentration *Ascophyllum nodosum* extract (ANE) solution, a 26.7 ppm oligoalginate solution, a 20 ppm oligochitin solution, and a 25 ppm oligochitosan solution. The ANE was produced through alkaline extraction using potassium hydroxide and neutralized; ANE was provided by Acadian Seaplants Limited (Nova Scotia, Canada), and the oligoalginate and oligochitin powders were supplied by Dr. Dew of Saint Mary's University (Nova Scotia, Canada). These ingredients were dissolved in distilled water to meet the concentrations as mentioned above.

After the 24-hour-long soaking process had been completed, the soybeans were divided into groups of four, with each group consisting of beans soaked in the same solution. Each group was assigned and planted in a pot filled with Promix. Four seeds were planted at a depth of 2 cm in each pot. The soybeans were watered with 100 mL of nutrient solution per pot per day. The nutrient solution was prepared according to the reference of Hoagland & Arnon, (1938). Further, a final modification to the nutrient solution was the addition of an iron solution following the method suggested by McClellan of Guelph University (Ontario, Canada).

A total of 480 soybeans were planted on May, 28th, 2015. These soybean plants were grown outdoor on the Green-roof at Saint Mary's University, Canada for six weeks. The green-roof is located on the top of McNally building and surrounded by other taller building. Thus, these buildings cast shadows around 8 pm although sunlight was available until 9 pm. After six weeks of plant growth, the leaves of the soybean plants were sprayed with 100 mL of either water or several different concentrations of treatment solutions as follows: a 0.3 % ANE, a 40 ppm oligoalginate, a 30 ppm oligochitin , and a 50 ppm oligochitosan solutions. A spray bottle was used for the spraying procedure.

Analysis of chlorophyll, phenolic, and protein content in the control and treated soybean leaves required separating the leaves into samples of several different sizes. Approximately 20 mg were collected for chlorophyll analysis, and 60 mg were obtained for phenolic testing. The second and third trifoliate (a set of compound leaves from the bottom) was chosen for standardizing leaf age. From each treatment group, small pieces of the compound leaves were cut with a circular copper cutter, black rubber, and a pincet. The small pieces of leaf were cut at the leaf blade around the petiole. They were then collected in a 1.5 mL Eppendorf, which was pre-labeled according to treatment received by the leaves in question, the number of hours after treatment, and tube number. Each Eppendorf was also weighed before leaf collection and after collecting the leaves. Subsequently, they were chilled in liquid nitrogen, then stored in -80 ºC. This method was repeated five per treatment. In addition, this process was repeated to create leaf samples 12, 36, 60, 84, and 108 hours from the time the leaves were sprayed with each solution.

Chlorophyll Content Analysis

The procedure for chlorophyll extraction using DMSO was performed as described in Arnon, (1949). Soybean leaves were ground by inserting a micro-centrifuge pestle into the stored Eppendorf. The pestle was attached to a small drill and grounded the leaves for 20 seconds as standardizing the grinding of the soybean leaves. Then, the amount of necessary DMSO was calculated based on the leaf weight in each 1.5 mL Eppendorf tube with 1mL DMSO per 20mg of leaves. The appropriate amount of DMSO was then added to each Eppendorf tube. The leaf residue on the pestle was carefully scraped off using the edge of the Eppendorf tube and inserted back into the Eppendorf to minimize loss of leaf content. The Eppendorf was centrifuged at 1800 rpm for 2 minutes. The supernatant was collected in a glass tube. The same amount of DMSO was added again in each Eppendorf tube and the above process was repeated. The absorbance of the total supernatant was measured at 645 nm and 663 nm by a spectrophotometer. The chlorophyll a, chlorophyll b, and total chlorophyll content were calculated using the equations described by Hiscox and Israelstam, (1979):

$$Let\ x = Absorbance\ at\ 645; y = Absorbance\ at\ 663$$

$$Chla\left(\frac{g}{L}\right) = 0.0127y - 0.00269x$$

$$Chlb\left(\frac{g}{L}\right) = 0.0029y - 0.00468x$$

$$Total\ Chl\left(\frac{mg}{L}\right) = 20.2y + 8.02x$$

$$Chl\ content\left(\frac{mg}{g}FW\right) = \frac{(total\ Chl)(total\ DMSO\ volume\ used)}{(Fresh\ leaf\ weight)}$$

2.2 Soybean Experiment 2

The soybeans from the same company were used for the experiment (Annapolis, Nova Scotia, Canada). The soaking soybean procedure from 2.1 was repeated. Small modifications were made in experiment: the 0.3% concentration ANE solution, 40 ppm oligoalginate solution, and 30 ppm oligochitin solution were used. Moreover, the leaves treated after 12, 24, 36, and 84 hours were collected. Further, the plants were watered in 200mL nutrient solution in five times the concentration used in experiment 1.

A total of 258 soybeans were planted on August, 7th, 2015. These soybean plants were grown inside the greenhouse at Saint Mary's University, Canada for a week. Following this sheltered growth period, the plants were grown outside the greenhouse for three additional weeks. After four weeks of plant growth, the leaves of the soybean plants were sprayed with 200-mL of either control or ANE, oligochitin, or oligoalginate solution in the same concentration as that of the soaking solutions. A regular spray bottle was used for the spraying procedure.

For the leaf collection, 20 mg were collected for chlorophyll testing, 35 mg were obtained for phenolic testing, and 45 mg were collected for protein testing. The second bottom of compound leaves was chosen. The leaves were cut and placed into the Eppendorf and stored in the same way as described in section 3.1. Leaves were collected 12, 36, 60, and 84 hours after spraying.

Chlorophyll Content Analysis

Chlorophyll analysis for this experiment was performed using the procedure as described in Hiscox and Israelstam, (1979). The amount of DMSO necessary was calculated based on the leaf weight in each 1.5 mL Eppendorf tube. Once leaf was weighted, it was applied with 7mL DMSO per 100mg of leaves into the Eppendorf tube. The tube was incubated in a 65 ºC water bath for 5 hours. The absorbance was measured at 645nm and 663nm by a spectrophotometer. The chlorophyll a, chlorophyll b, and total chlorophyll content were calculated using the equations described by Hiscox and Israelstam, (1979).

Phenolic Content Analysis

In this experiment, a gallic acid from Sigma-Aldrich was used to create a standard curve for phenolic content in a plant leaf. A standard curve is prepared following the procedure described in Houshia and Qutit, (2014). A Gallic acid stock was made by dissolving it in 96 % methanol to make a solution with a concentration of 12 $g \cdot L^{-1}$. The Gallic acid standard curve was prepared over the range of approximately 40 ppm to 140 ppm. The solution was prepared bydiluting the gallic acid stock solution with 96% methanol solution. Then 0.2 mL of a dilution was placed in a glass tube, and 1.225 mL of distilled water, 25 µL of Folin Ciocateau reagent (2N), and 0.2 mL of Na2CO3 were added to the tube and vortexed. This solution was poured into a clean cuvette and inserted into the spectrophotometer. The absorption of the solution was read at 725 nm. After measuring all the absorbance at ranges between 40 ppm to 140 ppm of the gallic acid solution, standard curve was plotted on excel.

The procedure for phenolic extraction was performed as described in Jayaraj et al., (2008). The micro centrifuge pestle was inserted into the stored Eppendorf tube and the contents were ground for 1 minute. Soybean leaves were ground in the same way as aforementioned in chlorophyll extraction. However, the leaves were grounded for one minute. The amount of 96% methanolic acid necessary was calculated based on the leaf weight in the Eppendorf tube with the ratio of 0.1 mL methanolic solution per 10 mg soybean leaf. The appropriate amount of the methanol was added to the Eppendorf. The leaf residue on the micro pestle was carefully scrapped off using the edge of the Eppendorf tube and inserted back into the Eppendorf to minimize loss of leaf content. The Eppendorf was incubated at 70 ºC for 5 minutes, then vortexed for five seconds and immediately put back in the incubator. This process was repeated two more times. Followed by incubation, the Eppendorf was centrifuged at 10,000 rpm for 30 seconds. Then, 0.2mL of the supernatant was collected in a new Eppendorf, and the distilled water, Folin Ciocateau reagent (2N), and Na_2CO_3 were added to the Eppendorf. The volume of the solution is mentioned in the gallic acid standard curve procedure as indicated above. The solution was diluted two times with water due to a high concentration. The absorbance of the total supernatant was measured at 725 nm with a spectrophotometer. The standard curve was used as a reference for determining the concentration of a solution of the same compound of unknown concentration.

2.3 Duckweed experiment 1

The experiment was conducted on February 17th, 2016. Duckweeds (*Lemna minor*) were provided by Saint Mary's University, Nova Scotia, Canada. Thirty stock-cultured

duckweed plants were placed in 100mL plastic containers containing 75 mL of different medium solutions. The different solutions used are a control solution with basic nutrients, 0.3% concentration ANE solution, and a 10 ppm oligoalginate + 10ppm oligochitin mixture. The duckweed plants in this experiment were grown in a room at 28 ± 0.5°C under sunlight and fluorescent lighting (16 hours) during the day. Three repetitions of each treatment were done per experiment.

After the duckweed plants had undergone two days of exposure to the initial solutions, NaCl was added to each solution that were labeled "salt treated" only in a concentration of 150 mM. The number of fronds was counted every day for a week to compare population growth rates across the treatments.

To examine the root morphology of the treated duckweed, the roots were stained with neutral red after 48 and 72 hours of salt treatment. The procedure for making neutral red solution was completed in accordance with Stadelmann and Kinzel (1972). The neutral red powder was dissolved in distilled water to make the stock solution at a concentration of 0.1%. Then, the stock solution was diluted down to a 0.005% neutral red solution. One duckweed, with specifically three fronds, was scooped out from each container and soaked in the 0.005% neutral red solution for 5 hours. The stained roots were examined under a dissecting microscope.

2.4 Duckweed Experiment 2

The experiment was conducted on February 23rd, 2016. The excess number of duckweeds were scooped out of the stock-duckweed containers and treated with the solutions as mentioned earlier for one day. For clarification, those solutions are: a basic

nutrient solution, a 0.3% concentration ANE solution, and a 10ppm oligoalginate+10ppm oligochitin solution. Thirty duckweeds with four fronds from each treatment group (which were separated according to which solutions they were exposed to) were then removed from their containers. They were then exposed to either a control or a salt solution at a 100 mM concentration. Fronds in each treatment were counted and recorded. Further, roots were stained with the aforementioned method (section 2.3.1) and observed under a light microscope.

3. RESULTS

3.1 Soybean Experiments 1

Effect of different treatments on Chlorophyll Content of Soybean Leaves.

Chlorophyll contents of soybean leaves were measured from 12 to 108 hours after the soybean leaves were sprayed with various solutions. Soybean leaves treated with the water, ANE, oligochitin, oligochitosan, and oligoalginate solutions exhibited no significant differences in chlorophyll content across solutions for a given length of time after exposure (Fig. 1). Further, none of the previously indicated treatments demonstrated significant increases in chlorophyll content from 12 hours to 108 hours after the leaves were exposed to their respective treatments (Fig. 1).

The graph had slightly symmetrical bimodal shape, which has two peaks of highest chlorophyll content at 36 and 108 hour period. The three repeats of each treatment at a

time point were tested with a normality test and showed no significant difference among the repeats (p-value higher than 0.05). Since the data are normally distributed, ANOVA was used to test the difference among treatments each time point. The chlorophyll content in 12 hours showed the lowest p-value (p=0.058); however, no significant differences were found among all treatments; thus, it failed to reject the null hypothesis.

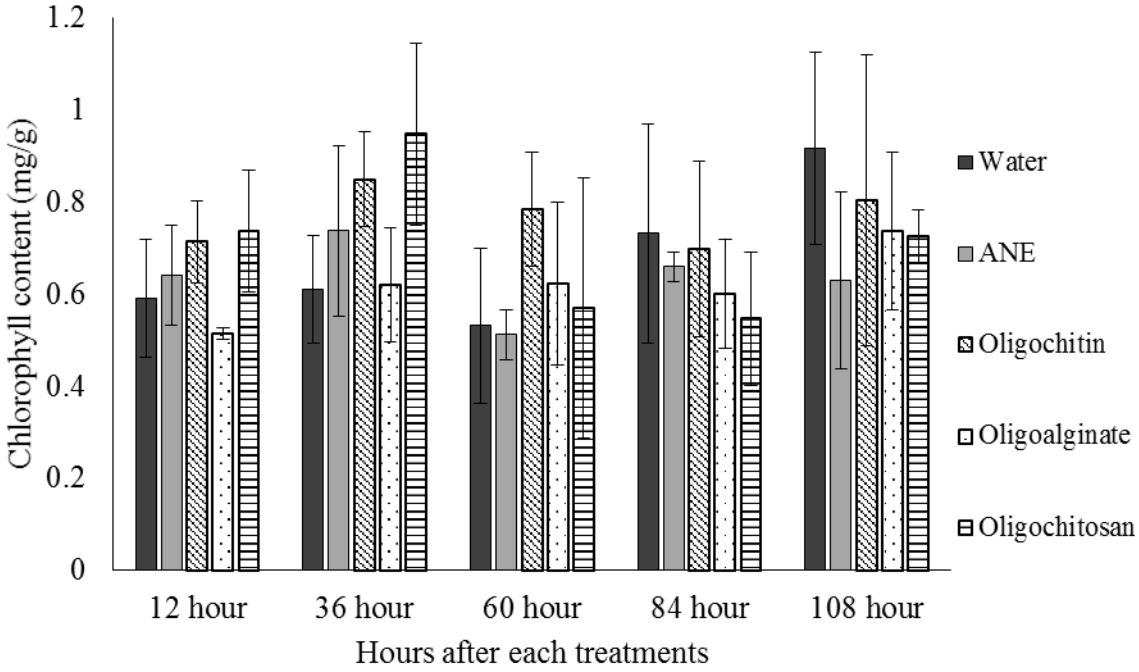

Figure 1. Mean and standard deviation of chlorophyll content found in water, ANE, oligochitin, oligoalginate, and oligochitosan treated soybean leaves at different time point. Soybean leaf collected in Saint Mary's University (Halifax, Nova Scotia, Canada).

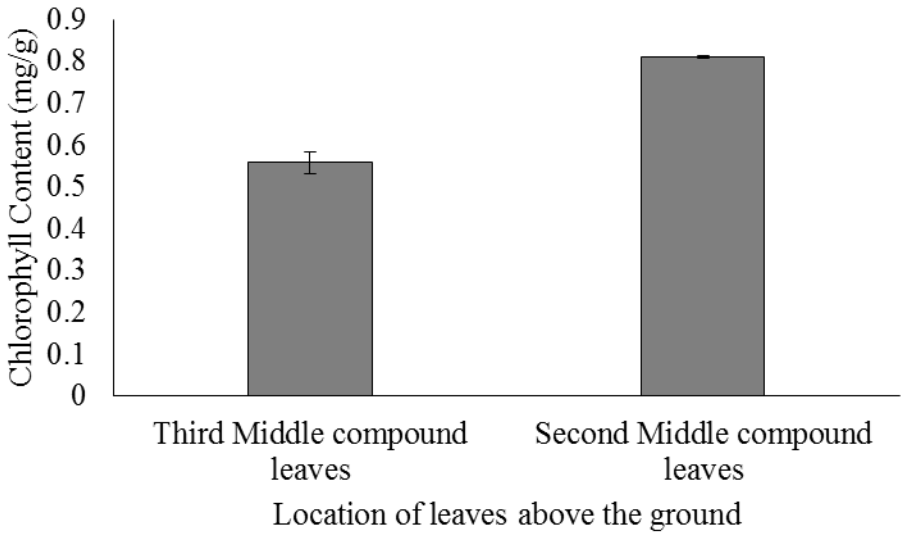

Figure 2. The center of second trifoliate leaflet have more chlorophyll than the third trifoliate leaf. Soybean leaf collected in Saint Mary's University (Halifax, Nova Scotia, Canada).

3.2 Soybean Experiment 2

The Effect of Treatments on Soybean's Chlorophyll and Phenolic Content

The instantaneous light intensity inside the greenhouse was 100 µmol•m•s^{-1}. The outdoor light intensity was 500 µmol•m•s^{-1} on a sunny day. The yellowish-brown trifoliate leaves were found at the first node from the bottom of the plant for all treatments. The compound leaves at the second node from the bottom and the higher nodes from the bottom were relatively healthier and greener. Thus, the compound leaves at the second nodes from the bottom were chosen for chlorophyll analysis.

The ANE treatment was used as positive control while water was used as negative control. The chlorophyll content of the ANE and oligosaccharide treated soybean leaves was not significantly different from the chlorophyll content in the water-treated leaves (Fig. 3). Regardless of the treatment (water, ANE, oligochitin, or oligoalginate), the soybean leaves demonstrated similar chlorophyll levels at each time point (Fig. 3). Normality tests showed that the chlorophyll content increased significantly only in the water treatment from 12 to 84 hours. The ANE treated leaves did not demonstrate significant increases in chlorophyll content from 12 to 84 hours ($p = 0.546$). Analysis of variance (ANOVA) was used to test for differences among treatments at each time point. None of the treatments were significantly different in any of the chlorophyll tests in the experiment (Fig 4); the positive control and negative control showed no significant different. Similar to the chlorophyll content, the phenolic content of the water, ANE, oligochitin, and oligoalginate treated soybean leaves showed insignificant difference among them (Fig. 5). The normality test and ANOVA test was done in the similar

manner as the chlorophyll analysis demonstrated that there is no significant difference among all treatments, and the p-value were higher than 0.05.

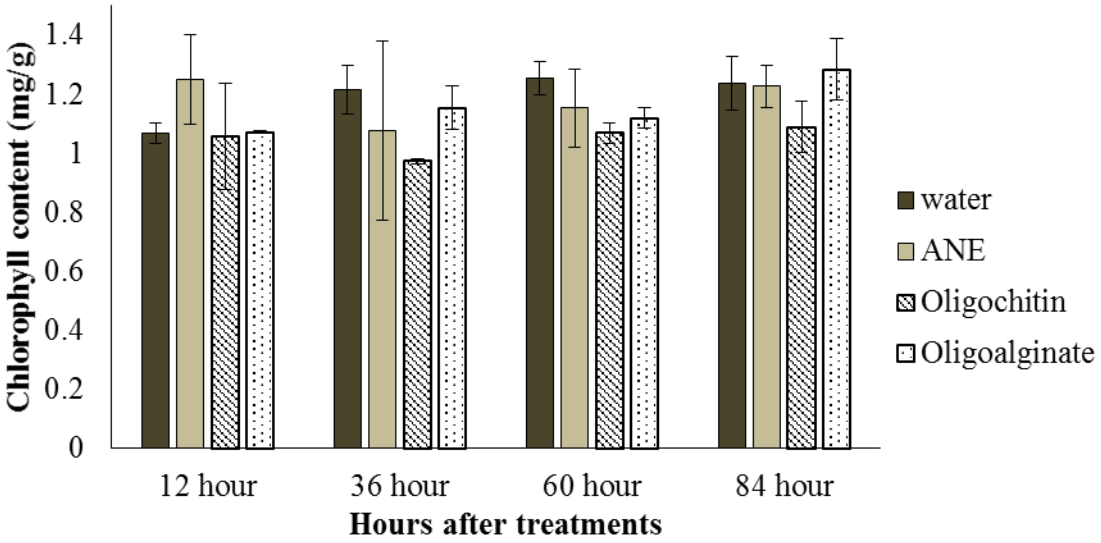

Figure 3. The effects of Water, ANE, oligochitn and oligoalginate on chlorophyll contents of soybean leaves. Soybean leaf was collected in Saint Mary's University (Halifax, Nova Scotia, Canada).

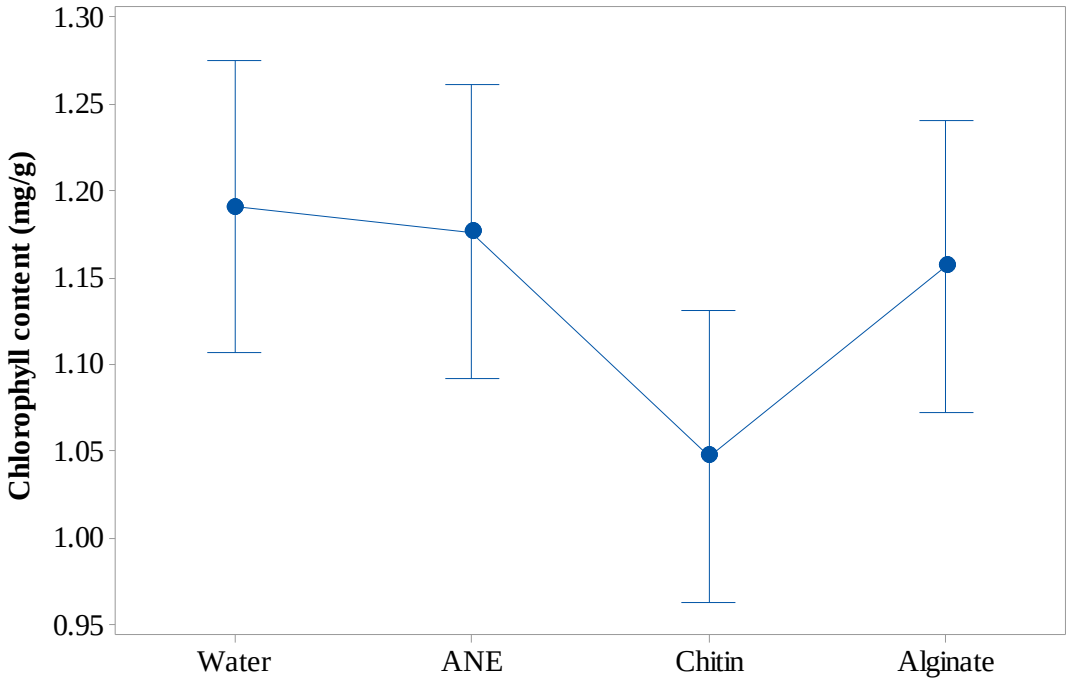

Figure 4. Mean chlorophyll content of each treatment from 12-84 hour found from using ANOVA (p=0.082 F=2.85). Soybean leaf was collected in Saint Mary's University (Halifax, Nova Scotia, Canada).

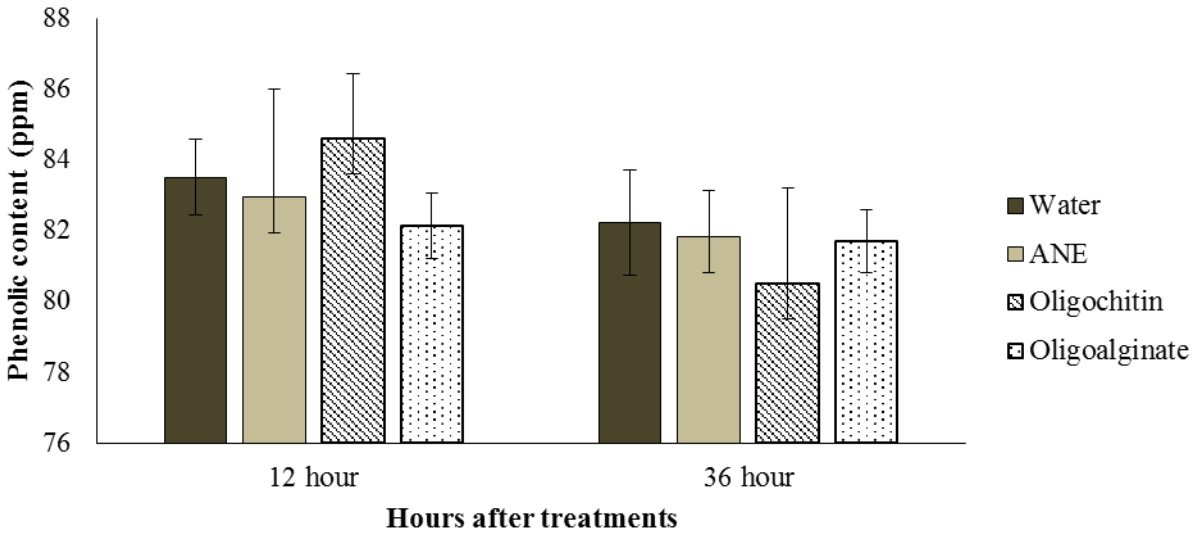

Figure 5. The effects of Water, ANE, oligochitn and oligoalginate treatments on phenolic contents of soybean leaves. Soybean leaves were collected in Saint Mary's University (Halifax, Nova Scotia, Canada).

3.3 Duckweed Experiment 1

Under no salt stress, duckweeds treated in water, 0.2% ANE, and oligosaccharide mixture (10 ppm oligochitin + 10 ppm oligoalginate) solutions had healthy green leaves. However, the duckweed grown under salt stress (150 mM) had light green or white leaves after four days regardless of the treatments listed above. The roots stained in neutral red for each treatment showed different degree of damage. The water treated duckweed had the least damaged surface at the roots. The duckweed grown in the oligosaccharide solution had the surface of the root strand separating from the root body. ANE treated roots were completely covered in the brown seaweed residue; thus, it was not possible to detect the degree of damage. Frond number was highest in water, followed by the oligosaccharide and ANE treatments, in decreasing order, respectively. The same pattern was found regardless of the presence of salt.

Similar to the soybean experiments, the ANE and water treatments were used as the positive and negative control, respectively. The number of duckweed fronds did not differ significantly between the water and ANE treatments. In addition, oligosaccharide treated duckweed leaf number was also not significantly different from water, ANE, and Oligosaccharide treatments mentioned above (Fig. 6). The number of duckweed fronds in water, ANE, and oligosaccharide mixture solution in addition to salt were significantly less than that found in those treatments without salt (Fig. 6). However, the water treated duckweed had significantly higher number of fronds than that of the ANE treated duckweed (Fig. 6). No significant difference was found in number of duckweed fronds between the water and oligosaccharide treatments.

3.4 Duckweed Experiment 2

The same duckweed leaf condition under both salt and no-salt stress were found as described in the section 3.2. The number of duckweed fronds in ANE treatment (positive control) and water treatment (negative control) were not significantly different at each time point (Fig. 7). The oligosaccharide mixture treatment also showed an insignificant result (Fig. 7). The duckweed in each solution with salt showed no significant difference in frond number (Fig. 7).

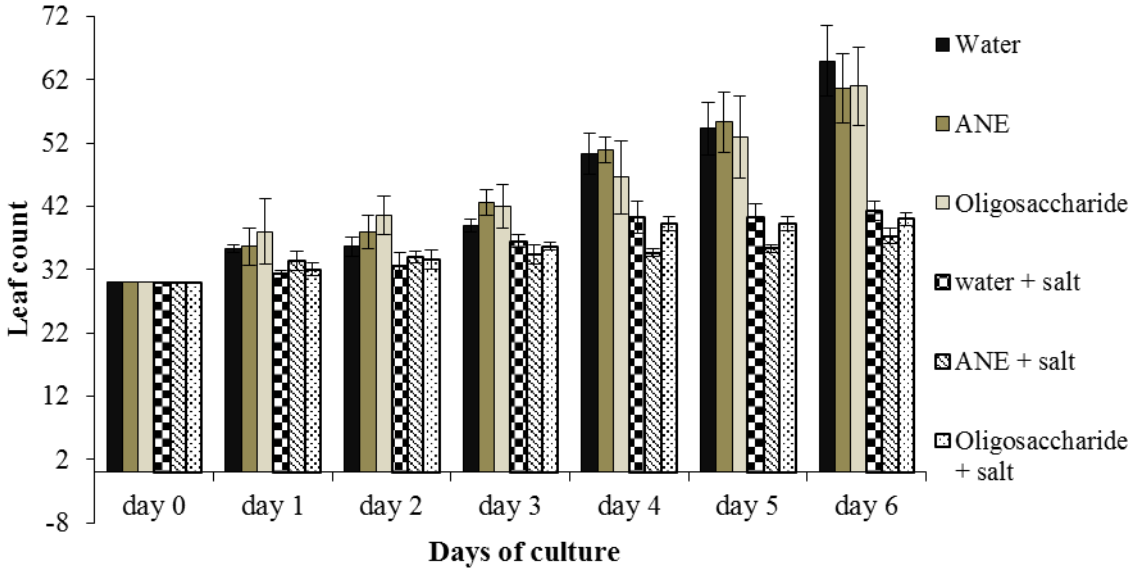

Figure 6. Leaf count of duckweed treated with water, ANE, and oligosaccharides with and without 150 mM salt stress. Duckweed was from Saint Mary's University (Nova Scotia, Canada).

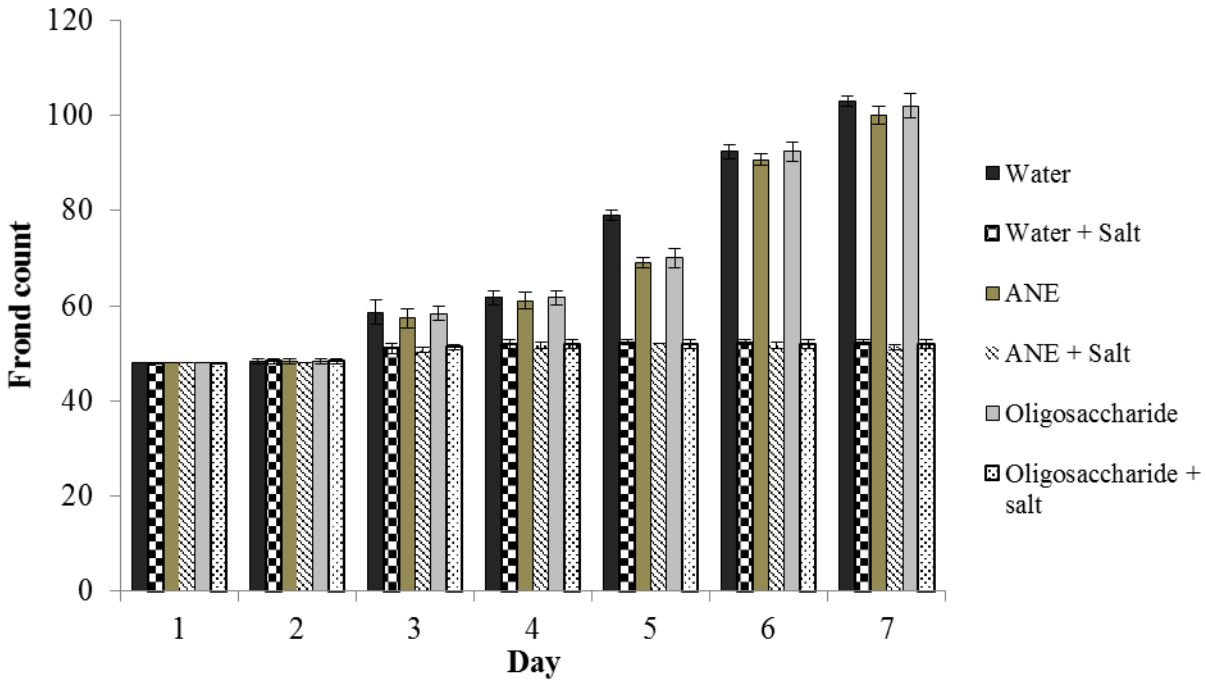

Figure 7. Leaf count of duckweed treated with water, ANE, and oligosaccharides with and without 100 mM salt stress. Duckweed was from Saint Mary's University (Nova Scotia, Canada).

4. DISCUSSION

4.1 Soybean Experiment 1

Previous studies suggest that ANE promotes the production of chlorophyll content in plant leaves. However, in this experiment, the chlorophyll content in the water treatment (the negative control) and the ANE treatment (the positive control) were not significantly different. According to Tandon and Dubey., (2015), ANE significantly increased the chlorophyll content in the soybean leaves in comparison to water treatment. Thus, the plant species was not the cause of the insignificant increase in the chlorophyll content of the ANE treated plants. The negligible difference may be due to the leaf collection procedure. For instance, either second or third trifoliates were selected randomly and examined for the chlorophyll content analysis. The rest of the second and third trifoliate leaflets were used in the phenolic content analysis. However, each trifoliate at a different node on the plant was at a different development stage. Soybean plants produce and unfold a new set of compound leaves for every five to seven days. Thus, the second trifoliate leaves had extra five to seven days for chlorophyll synthesis. Figure 2 shows the second trifoliate had 20 % more observed chlorophyll than the third trifoliate from the bottom. During the leaf collection procedure, it was impossible to distinguish between the green colour of the second and third trifoliates with a naked eye observation. The only time a second trifoliate had a visibly different coloration was when a trifoliate was newly formed. These young leaves had a yellowish green colour rather than a true green colour. The yellowish pigment indicates a lack of chlorophyll content.

Although the leaf had true green colour, the chlorophyll content were below the content literature suggested. According to Marenco and Lopes., (1994), the total chlorophyll

content for soybeans ranged from 1.98 mg/g to 4.34 mg/g from the leaf stage V2 (second trifoliate unfolded) to R4. Further, the leaves were collected at V4 in my experiment, but the observed chlorophyll content for all treatments were below 1.0 mg/g. The lower concentration of chlorophyll content in the result than the value from the literature may be due to use of different method. For instance, Marenco and Lopes., (1994) suggested drying of the soybean leaves at 65 ºC before extracting chlorophyll content. The chlorophyll content in the section 3.2 shows that heat application is much more effective in extracting the chlorophyll and the soybean chlorophyll contents were in the range that the literature suggested (Marenco and Lopes, 1994).

Therefore, the experiment did not show evidence for the hypothesis, as the positive control (ANE treatment) did not show any significant difference from water treatment. Leaf collection should be standardized either using trifoliate leaves at the same developmental stage or extract chlorophyll from all of the different developmental stages and averaged out over a large sample.

4.2 Soybean Experiment 2

The p-value for chlorophyll and phenolic content analysis from ANOVA were larger than 0.05; thus, no significant difference was found. The ANE (positive control) and water treatments (negative control) had no significant difference.

Previous studies showed that application of ANE on plants significantly increased chlorophyll content and phenolic contents (Lola-Luz, et al. 2013; Ali et al., 2015). However, the observed data from section 3.2 showed statistically insignificant result, and the factor may be that the soybean plants were not grown in optimal conditions. For

example, the highest light intensity in the greenhouse was found to be only 20% of the outdoor light intensity, and soybeans require a high intensity of light for optimal growth. Various wavelength of light are essential for photosynthesis. More specifically, blue light are important for stimulating chlorophyll production (Danila and Lucache, 2013) and develop thick and healthy stems (Popp, 1926). After careful observation, it was apparent that the soybeans had long and thin stems, which are a sign of light deficiency (blue rays). If the soybeans were grown under high light intensity, they would have thicker and shorter stems (Popp, 1926). Thus, the insignificant increase in chlorophyll content may be due to this insufficient exposure to sunlight while growing in the greenhouse, which would have translated directly into reduced chlorophyll production.

The soybean plants also suffered from pest infestations, which could have negated the positive growth effects of the plants receiving high-intensity outdoor light after spending a week in the greenhouse. The greenhouse had various pests (like spider mites). Spider mites grow and lay eggs on the back of plant leaves. Infection to spider mites can cause the leaf colour to turn yellowish brown. Many pots holding the soybean plants had cyanobacteria bloom that has green fungus-like-appearance on top of the soil. Cyanobacteria are not directly harmful to plants. In fact, studies have shown that cyanobacteria growing on soil provide nitrogen, vitamin, and growth stimulating hormone for plants, and polysaccharides (Rao & Burns, 1989). However, the group of cyanobacteria can provide a habitat for various pathogenic fungi (Frey-Klett, et al. 2011). Various pathogenic fungi and small insects could significantly damage the plant leaves, reduce photosynthesis activity, and reduce the growth rate (Swoboda & Pedersen, 2009).

ANE aid plants to grow in its full genetic potential as elicitors when the plants were grown under optimum conditions. ANE application on plants also showed to enhance tolerance to some environmental stresses including excess light (Elstner, 1987), but no literature was found for the effect of *Ascophyllum nodosum* extract under low light stress.

4.3 Duckweed Experiment 1 and 2

The effect of ANE and oligosaccharide mixture was insignificant for the duckweed population growth rate. The water and ANE showed insignificantly different number of duckweed fronds. Thus, the observed data did not show any evidence for the hypothesis. This pattern of insignificance was also found under the salt stress.

The no-salt treated duckweed demonstrated significantly faster population growth and greener coloration than the post-salt-treated duckweed. Among the treatments without the salt, the water treated duckweed had greener fronds and healthier roots. The oligosaccharide treated duckweed had slightly lighter green pigmented fronds and slightly damaged roots that had a few detached strands of cells of the surface root. The slightly lower number of fronds may due to the damage in the high concentration oligosaccharide affecting the duckweed roots. High concentration of oligosaccharide causes the reduction in the uptake of nutrients, leaving them more susceptible to various infections. The surface of the ANE treated duckweed root was covered in gel-like residues of the brown seaweed. The lowest number of fronds was found in ANE treated duckweeds, and the residues mentioned above may have influenced the population growth rate.

Different plant species requires a different concentration of ANE and oligosaccharide. The mungbean experiment performed by Chao Wang (a master student) showed that mungbean treated with 0.2 % ANE and a mixture solution of 10 ppm oligoalginate and 10 ppm oligochitin had significantly more numerous and longer branch roots compared to water treated roots. Many previous studies used 0.1-0.5% ANE on various plants such as tomatoes, strawberry, cucumber (Jayaramen et al., 2011; Alam et al., 2013; Ali et al., 2015). However, some species of plants require much less concentration. According to Campbell et al., (1995), frond number of duckweed increased 60 % in comparison to control when the duckweeds were applied with oligosaccharide from grapevine cell such as 357.1 $ng \cdot mL^{-1}$ (0.3571 ppm) instead of 20 ppm. Further, duckweeds grown in a high concentration of gum karaya polysaccharide inhibited growth of duckweed frond number (Foa et al., 1992). Duckweeds require to grow in the water and ANE or other substances are directly affecting the roots; while, the plants that grow in soil does not. Thus, the duckweeds in present study were not grown in optimal concentration of ANE and oligosaccharides.

Under salt stress, duckweed frond number was significantly lower in each treatment than those without salt. Studies have shown that salt stress can cause leaf chlorosis in duckweed. Leaf chlorosis decrease photosynthetic activity in plants (Foa et al., 1992). Foa et al., (1992) suggested that severe leaf chlorosis may be due to oligosaccharide concentration; the study found that duckweed plants had threshold at certain concentration of the oligosaccharides obtained from grapevine cell wall. Further, the sucrose (1% w/v) in their medium may inhibit the population growth, while the glucose had no effect in the same concentration (Foa et al., (1992). Combined with the effects of

high concentration of ANE and its residue covering the roots, the ANE treated duckweeds had the lowest population growth rate.

The second duckweed experiment showed the similar results. The salt treated duckweed suffered leaf chlorosis but not as rapidly as the first experiment. The root morphology was similar in ANE, oligosaccharide treatments without salt. However, the positive and negative controls had no significant difference in frond number, thus our data does not show evidence for the hypothesis. In the salt treatment, the leaf count between ANE, water, and oligosaccharide treated duckweeds were similar unlike duckweed experiment 1. This clearly shows that the ANE residue covering the surface of the root reduced plant growth rate by decreasing the surface area of the roots for nutrient and water uptake.

5. CONCLUSION

Ascophyllum nodosum extract (ANE), or a brown seaweed extract, have shown to act as biostimulant to various plants when applied at low concentration (approximately 0.2 %). The beneficial effects of the application of ANE include increased growth for root, stem, leaf, fruit, enhanced plant immune system, and resulted higher crop yield. The bioactive component of ANE has not been identified. Oligosaccharides hydrolyzed from cell wall of seaweed and endophytic fungus in the seaweed were hypothesized as the effective component. The present study used soybeans and duckweeds to test chlorophyll and phenolic content without stress, and population growth under salt stress. The water treatment as the negative control and ANE treatment as positive control showed no significant difference in chlorophyll and phenolic content in soybean leaves. Similarly,

the duckweeds treated with the positive and negative control showed an insignificant result. The limiting factors for the growth of the soybeans and duckweeds are likely be growing under some stresses such as low light intensity in soybean experiment and too high concentration of ANE in duckweed experiment. The observed data did not show evidence for the hypothesis. Study showed that the optimum growth condition is important to show the biostimulating effect. Moreover, each plant species require different optimum concentration of ANE.

REFERENCES

Abdel Aziz NG., Mahgoub MH., Siam HS. (2011). Growth, Flowering and Chemical Constituents Performance of *Amaranthus tricolor* Plants As Influenced By Seaweed (*Ascophyllum nodosum*) Extract Application Under Salt Stress Conditions. *Journal of Applied Sciences Research*. **7(11)**: 1472-1484.

Agriculture and Economic Development Analysis Division (AEDAD) (2015). The State of Food Insecurity in the World. *Food and Agriculture Organization of the United Nations.*

Alam, M. Z., Braun, G., Norrie, J., & Hodges, D. M. (2014). *Ascophyllum* extract application can promote plant growth and root yield in carrot associated with increased root-zone soil microbial activity. *Canadian Journal of Plant Science.* **94**: 337-348.

Alam, M. Z., Braun, G., Norrie, J., & Hodges, D. M. (2013). Effect of *Ascophyllum* extract application on plant growth, fruit yield and soil microbial communities of strawberry. *Canadian Journal of Plant Science.* **94**: 337-348.

Ali N., Farrell A., Ramsubhag A., Jayaraman J. (2015). The effect of *Ascophyllum nodosum* extract on the growth, yield and fruit quality of tomato grown under tropical conditions. *J App Phycol*. **1**: 1-10. DOI: 10.1007/s10811-015-0608-3.

Andres E., Albesa-Jove D., Biarnes X., Moerschbacher BM., Guerin ME., Planas A. (2014). Structural Basis of Chitin Oligosaccharide Deacetylatioin. *Angew. Chem. Int. Ed.* **53:** 6882-6887.

Baardseth, E. (1970). Synopsis of biological data on knobeed wrack *Ascophyllum nodosum* (Linnaeus) le jolis. *Food and Agriculture Organization Fisheries Synopsis,* **38(1)**: 1-50.

Bailliez C., Largeau C., Berkaloff C., Casadevall E. (1986). Immobilization of *Botryococcus braunii* in alginate: influence on chlorophyll content, photosynthetic activity and degeneration during batch culture. *Applied Microbiology and Biotechnology.* **23(5)**: 361-366.

Barber MS, Bertram RE, Ride JP. 1989. Chitin oligosaccharides elicit lignification in wounded wheat leaves. Physiological and Molecular Plant Pathology **34**: 3-12.

Blunden G, Gordon SM. (1986). Betaines and their sulphono analogues in marine algae. *Prog Phycol Res.* **4**: 39–80.

Blunden G, Jenkins T, Liu Y (1997) Enhanced leaf chlorophyll levels in plants treated with seaweed extract. *J Appl Phycol*. **8**: 535–543.

Booth E. (1969). The manufacture and properties of liquid seaweed extract. *Proc Int Seaweed Symp*. **6**: 665-662.

Bozorgi, H. R. (2012). Effects of foliar spraying with marine plant *Ascophyllum nodosum* extract and nano iron chelate fertilizer on fruit yield and several attributes of eggplant (S*olanum melongena* L.). *Journal of Agricultural and Biological Science,* **7**: 357-362.

Burchett S., Fuller MP., Jellings AJ. (1998). Application of seaweed extract improves winter hardiness of winter barley cv Igri. *Society for Experimental biology.*

Campbell JA., Drake AM., Lee VWK, Strother S. (1995). A Putative Oligosaccharin growth Promoter from *Vitis vinifera* L. Primary Cell Walls. *Annals of Botany*. **75**: 359-363.

Crouch IJ & Van-Staden J (1992). Effect of seaweed concentrate on the establishment and yield of greenhouse tomato plants. *Journal of Applied Phycology*. **4(4)**:291–296

Crouch IJ & Van-Staden J (1993). Evidence for the presence of plant growth regulators in commercial seaweed products. *Plant Growth Regul*. **13**:21–29

Danila E., Lucache DD. (2013). Cost effectiveness of growing plant lighting system. *Journal of Electrical Engineering.* **13(4)**: 224-230

Elstner EF. (1987). Metabolism of activated oxygen species. *Biochem. Plants*. **11**: 253-315

Fan D., Hodge DM., Zhang J., Kirby CW., Ji X., Locke SJ., Critchley AT., Prithiviraj B. (2011). Commercial extrat of the brown seaweed *Ascophyllum nodosum* enhances phenolic antioxidant content of spinach (*Spinacia oleracea)* which protect *Caeneorhabditis elegans* against oxidative and thermal stress. *Food Chemistry*. **124**: 195-202.

Fehr WR., Cavines CE., Burmood DT., Pennington JS. (1971). Stage of development descriptions for soybeans, *Glycine max* (L.). *Merrill Crop Science*. **11**: 929-931.

Fehr WR., Caviness CE. (1997). Stages of soybean development. *Iowa State University Cooperative Extension Service Sepcial report*. **80**: 1-3

Foa LC,, Campbell JA., Lee VWK., Strother S. (1992). Growth inhibition of *Lemna minor* L. by hydrolysates of gum karaya. *Aquatic Botany*. **42**: 287-292.

Fornes F, Sa˜nchez-Perales M, Guardiola JL. (2002). Effect of a seaweed extract on the productivity of ‘de Nules’ Clementine Mandarin and Navelina Orange. *Bot Mar.* **45**: 486–489.

Frey-Klett P., Burlinson P., Deveau A., Barret M., Tarkka M., Sarniguet A. (2011). Bacterial-Fungal Interactions: Hyphens between Agricultural, Clinical, Environmental, and Food Microbiologists. *Microbiol Mol Biol Rev.* **75(4)**: 583-609.

Garbary D.J., Deckert R. and Hubbard, C. 2005. *Ascophyllum* and its symbionts. VII. Three-way interactions among *Ascophyllum nodosum* (Phaeophyceae), *Mycophycias ascophylli* (Ascomycetes) and Vertebrata lanosa (Rhodophyta). *Algae.* **20**: 353-361.

Garbary D.J., Tian Lining and Burke J. 1991. The *Ascophyllum, Polysiphonia, Mycosphaerella* symbiosis. II. Aspects of the ecology and distribution of Polysiphonia in Nova Scotia. *Bot. Mar*. **34**: 391-401.

Hegab, M.Y., A.M.A. Sharawy and S.A.G. El-Saida, 2005. Effect of algae extract and mono potassium phosphate on growth and fruiting of balady orange trees. Bull. Fac. *Agric. Cairo Univ*., **56(1)**: 107-120.

Hiscox JD, Israelstam, GF. (1979). A method for the extraction of chlorophyll from leaf tissue without maceration. canadian journal of botany 57: 1332-1334

Houshia OJ, Qutit A. (2014). Determination of Total Polyphenolic Antioxidants Contents in West-Bank Olive Oil. *Journal of Natural Sciences Research*. **4(15)**: 2224-3186

Jakab G., Ton J., Flors V., Zimmerli L., Mettraux JP., Mauch-Mani B. (2005). Enhancing Arabidopsis Salt and Drought Stress Tolerance by Chemical Priming for Its Abscisic Acid Responses. *Plant Physiol*. **139(1)**: 267-274.

Jannin L., Arkoun M., Etienne P., Laine P. (2013). *Brassica napus* Growth is Promoted by *Ascophyllum nodosum* (L.) Le Jol. Seaweed Extract: Microarray Analysis and Physiological Characterization of N, C, and S Metabolisms. *J Plant Growth Regul.* **32(1)**: 31-52.

Jayaraj J., Wan A., Rahman M., Punja ZK. (2008). Seaweed extract reduces foliar fungal diseases on carrot. *Crop Protection*. **27**: 1360-1366.

Jayaraman J., Norrie J., Pumja ZK. (2011). Commericial extract from the brown seaweed *Ascophyllum nodosum* reduces fungal disease in greenhouse cucumber. *J Appl Phycol.* **23(3)**: 353-361.

Kaku, H., Shibuya, N., Xu, P., Aryan, A.P., and Fincher, G.B. (1997). N-acetylchitooligosaccharides elicit expression of a single (1-3)-b-glucanase gene in suspension-cultured cells from barley (*Hordeum vulgare*). *Physiol. Plant*. **100**: 111–118.

Kandasamy S., Khan W., Kulshreshtha G., Evans F. (2015). The fucose containing polymer (FCP) rich fraction of *Ascophyllum nodosum* (L.) Le Jol. Protects Caenohabditis elegans against *Pseudomonas aeruginosa* by triggering innate immune signalling pathways and suppression of pathogen virulence factors. *Algae*. **30(2)**: 147-161.

Kauss H, Jeblick W, Domard A. (1989). The degree of polymerization and N-acetylation of chitosan determine its ability to elicit callose formation in suspension cells and protoplasts of *Cathalanthus roseus*. *Planta*. **178**: 385-392

Khan W, Rayirath UP., Jithesh MNSS., Rayorath P., Hodges DM., Critchley AT., Craigie JS., Norrie J., Prithiviraj B. (2009) Seaweed extracts as biostimulants of plant growth and development. *J Plant Growth Regul.* **28**: 386–399

Kikuyama M., Kuchitsu K., Shibuya N.. (1997). Membrane depolarization induced by N-acetylchitooligosaccharide elicitor in suspension-cultured rice cells. *Plant and Cell Physiology*. **38**: 902-909.

Kim, S. K., Wijesekara, I. (2010). Development and biological activities of marine-derived bioactive peptides: a review. *J. Funct*. *Foods*. **2**: 1-9.

Larroque M., Ramirez D., Lafitte C., Borderies G., Dumas B., Gaulin E. (2011). Expression and purification of biologically active *Phytophthora parasitica* cellulose binding elicitor lectin in *Pichia pastoris*. *Protein Expression and Purification.* **80(2)**: 217-223

Lola-Luz T., Hennequart F., Gaffney M. (2013). Enhancement of phenolic and flavonoid compounds in cabbage (*Brassica oleraceae)* following application of commercial seaweed extracts on the brown seaweed (*Ascophyllum nodosum*). *Agricultural and Food Science*. **22**: 288-295.

McLay, C.L., (1976). The effect of pH on the population growth of three species of duckweed: *Spirodela oligorrhiza Lemna minor* and *Wolffia arrhiza*. *Freshwater Biol*. **6**: 125–136.

Mancuso S, Azzarello E, Mugnai S, Briand X. (2006). Marine bioactive substance (IPA extract) improve foliar ion uptake and water stress tolerance in potted *Vitis vinifera* plants. *Adv Hortic Sci*. **20**: 156–161.

Marenco RA., Lopes NF. (1994). Leaf Chlorophyll Concentration and Nitrogen Content in Soybean Plants treated with Herbicides. *R. Bras. Fisiol. Veg*. **6(1)**: 7-13.

Milton RF. (1952). Improvements in or relating to horticultural and agricultural fertilizers. *British Patent*. 664989.

Mohanty D, Adhikary SP, Chattopadhyay N. (2013). Seaweed liquid fertiliser (SLF) and its role in agricultural productivity. *Ecosan*. **3**: 147–155.

Moore KK. (2004). Using seaweed compost to grow bedding plants. *BioCycle.* **45**: 43–44.

Mugnai S,, Azzarello E,, Pandolfi C,, Salamagne S,, Briand X,, Mancuso S. (2008). Enhancement of ammonium and potassium root influxes by the application of marine bioactive substances positively affects *Vitis vinifera* plant growth. *J Appl Phycol*. **20**: 177–182.

Nair P., Kandasamy S., Zhang J., Ji Z., Kirby C., Benkel B., Hodges MD., Critchley AT., Hiltz D., Prithiviraj B. (2012). Transcriptional and metabolomics analysis of *Ascophyllum nodosum* mediated freezing tolerance in *Arabidopsis thaliana*. *BMC Genomics*. **13**: 643-666.

Rao DLN., Burns RG. (1990). The effect of surface growth of blue-green algae and bryophytes on some microbiological, biochemical, and physical soil properties. *Biol Fertil Soils*. **9**: 239-244.

Rioux LE., Turgeon SL., Beaulieu M. (2007). Characterization of polysaccharides extracted from brown seaweeds. *Carbohydrate Polymer*. **69**: 530-537.

Roby D., Gadelle A., Toppan A. (1987). Chitin oligosaccharides as elicitors of chitinase activity in melon plants. *Communications*. **143(3)**: 885-892.

Popescu GC ., Popescu M (2014). Effect of the Brown alga *Ascophyllum nodosum* as biofertilizer on vegetative growth in grape vine (*Vitis vinifera* L.). *Current Trend in Natural Science*. **3(6)**: 61-67.

Popp HW. (1926). Effect of light intensity on growth of soybeans and its relation to the autocatalyst theory of growth. *Botanical Gazette*. **82(3)**: 306-319

Rathore SS., Chaudhary DR., Boricha GN., Ghosh A., Bhatt BP., Zodape ST., Patolia JS. (2009). Effect of seaweed extract on the growth, yield and nutrient uptake of soybean (*Glycine max*) under rainfed conditions. *South African Journal of Botany*. **75(2)**: 351-355.

Rayirath P., Benkel B., Hodges DM., Allan-Wojtas P., MacKinnon S., Critchley AT., Prithiviraj B. (2009). Lipophilic components of the brown seaweed, *Ascophyllum nodosum*, enhance freezing tolerance in *Arabidopsis thaliana*. *Planta*. **230**: 135-147. DOI 10.1007/s00325-009-0920-8

Rayorath P., Jithesh MN., Farid A., Khan W., Palanisamy R., Hankins SD., Critchley AT., Prithiviraj B. (2008). Rapid bioassay to evaluate the plant growth promoting activity of *Ascophyllum nodosum* (L.) Le Jol. using a model plant, Arabidopsis thaliana (L.) *Heynh. J Appl Phycol.* **20**: 423–429.

Rockstorm J., Falkenmark M. (2000). Semiarid crop production from a hydrological perspective: gap between potential and actual yields. *Critical Reviews in Plant Sciences*. **19**: 319-346.

Roussos PA., Denaka NK., Damvakaris T. (2009). Strawberry fruit quality attributes after application of plant growth stimulating compounds. *Sci Hortic* (Amsterdam) **119**: 138–146.

Sharma S., Lyons G., McRoberts C. McCall D., Carmichael E., Andrews F., Swan R., McCormach R., Mellon R. (2012). Biostimulant activity of brown seaweed species from Strangford Lough: compositional analyses of polysaccharides and bioassay of extracts using mung bean (*Vigno mungo* L.) and pak choi (*Brassica rapa chinensis* L.). *J Appl Phycol*. **24**: 1081-1091. DOI 10.1007/s10811-011-9737-5.

Shibuya N., Minami E. (2001). Oligosaccharide signalling for defense responses in plant. *Physiological and Molecular Plant Pathology*. **59**: 223-233.

Subramanian S., Sangha JS., Gray BA., Singh RP., Hiltz D., Critchley AT., Prithiviraj B. (2011). Extracts of the marine brown macroalga, *Ascophyllum nodosum,* induce jasmonic acid dependent systemic resistance in *Arabidopsis thaliana* against *Pseudomonas syringae* pv. Tomato DC3000 and *Sclerotinia sclerotiorum. European Journal of Plant Pathology*. **131**: 237-248.

Swoboda C., Pedersen P. (2009). Effect of Fungicide on Soybean Growth and Yield. *Agronomy Journal*. **101(2)**: 352-356.

Tandon S., Dubey A. (2015). Effects of Biozyme (Ascophyllum nodosum) Biostimulant on Growth and Development of Soybean (Glycine max (L)). *Communications in Soil Science and Plant Analysis*. **46(7)**: 845-858.

Terwissacha van Scheltinga AC., Armand S., Kalk KH., Isogai A., Henrissat B., and Dijkstra BW. (1995). Stereochemistry of chitin hydrolysis by a plant chitinase/ lysozyme and x-ray structure of a complex with allosamidin evidence for substrate assisted catalysis. *Biochemistry.* **34(48)**: 15619-15623: DOI: 10.1021/bi00048a003.

Wally, O. S. D., Critchley, A. T., Hiltz, D., Craigie, J. S., Han, X. M., Zaharia, L. I., Prithiviraj, B. (2013). Regulation of phytohormone biosynthesis and accumulation in *arabidopsis* following treatment with commercial extract from the marine macroalga *Ascophyllum nodosum.*. *Journal of Plant Growth Regulation, **32***: 324-339.

Wang W, Vinocur B., Altman A. (2003). Plant responses to drought, salinity and extreme temperatures: towards genetic engineering for stress tolerance. *Planta*. **218**: 1–14.

Yamada A, Shibuya N., Kodama O., Akatsuka T. (1993). Induction of phytoalexin formation in suspension-cultured rice cells by N-acetylchitooligosaccharides. *Bioscience Biotechnology and Biochemistry* **57**: 405-409.

Yamaguchi T., Yamada A., Hong N., Ogawa T., Ishii T., Shibuya N. (2000). Dierences in the recognition of glucan elicitor signals between rice and soybean: b-glucan fragments from the rice blast disease fungus, *Pyricularia oryzae,* that elicit phytoalexin biosynthesis in suspension-cultured rice cells. *The Plant Cell*. **12**: 817-826.

Zhang X., Ervin EH. (2004). Cytokinin-Containing Seaweed and Humic Acid Extracts Associated with Creeping Bentgrass Leaf Cytokinins and Drought Resistance. *Crop Science*. **44(5)**: 1737-1745.

Zhang Y., Liu H., Yin H., Wang W., Zhao X., Du Y. (2013). Nitric oxide mediates alginate oligosaccharides-induced root development in wheat (*Triticum aestivum* L.). *Plant Physiology and Biochemistry*. **71**: 49-56.

Zhang Y., Yin H., Zhao X., Wang W., Du Y., He A., Sun K. (2014). The promoting effects of alginate oligosaccharides on root development in *Oryza sativa* L. mediated by auxin signaling. *Carbohydrate Plymers.* **113**: 446-454