Certification

Oligosaccharides as the effective components of *Ascophyllum nodosum* extract on plant growth and plant resistance against chilling stress in soybean (*Glycine max*) seedlings

By

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ABSTRACT

Oligosaccharides as the effective components of *Ascophyllum nodosum* extract on plant growth and plant resistance against chilling stress in soybean (*Glycine max*) seedlings

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Ascophyllum nodosum extract (ANE) is a biostimulant that is derived from brown algae and has been reported to promote plant growth and resistance against biotic and abiotic stresses. The major dry components of *Ascophyllum nodosum* are polysaccharides that are found in both algae and fungal cell walls. When *Ascophyllum nodosum* is exposed to alkaline extraction, these major polysaccharides are degraded to their corresponding oligo-forms including oligo-alginate, oligo-chitin and oligo-chitosan. In the present study, the effects of ANE and these oligosaccharides on the plant growth and resistance were compared with each other on soybean (*Glycine max*) seedlings under chilling stress. ANE, oligo-alginate and oligo-chitosan treatments induced plant tolerance to chilling stress by decreasing the electrolyte leakage and increasing the recovery rate of the soybean leaves. Oligo-chitosan treatment enhanced plant growth by increasing the chlorophyll content under chilling stress. These results suggest that oligosaccharides might be the effective components of ANE.

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LIST OF ABBREVIATIONS

A.nodosum = *Ascophyllum nodosum*

ABA = Abscisic acid

ANE = *Ascophyllum nodosum* extract

C/N = carbon to nitrogen

cm = centimeter

DMSO = Dimethyl Sulfoxide

EL = electrolyte leakage

F = variation between sample means / variation within the samples

g = gram

IAA = auxin

JA = jasmonic acid

K = potassium

L = liter

m = meter

mg = milligram

mL = milliliter

N = nitrogen

Na_2CO_3 = Sodium Carbonate

ºC = degrees celsius

OD = optical density

P = phosphorus

pH= potential Hydrogen

SWE = seaweed extracts

1. Introduction:

The rapidly growing world population has highlighted the necessity to significantly increase food production in the circumstances of a world with accelerating soil and water shortages as well as climatic stressors. That has generated new interest in the seaweed extracts because of their ability to enhance plant growth qualities through metabolic benefits, triggering disease response pathways and promoting stress tolerance. However, millions metric tons of seaweeds are being harvested yearly from coastal areas for commercial purposes which cause severe impacts on some living organisms and the ecology of these areas. One of these seaweed extracts is the *Ascophyllum nodosum* extract (ANE) which is derived from brown algae and has been reported to promote plant growth and resistance against biotic and abiotic stresses. Many studies have focused on the beneficial effects of applying ANE on different crops; however, its mechanisms are not understood. Due to this lack of information about the mechanism of the *Ascophyllum nodosum* extract (ANE), and in order to reduce the harmful harvesting of seaweeds, this study aims to find the major and effective components in ANE that can promote plant growth and trigger the plant's innate immune responses against various stresses. For a better understanding of the functions of ANE and its mechanisms, this thesis will analyze the effects of the *Ascophyllum nodosum* extract (ANE) and its different oligosaccharides on soybean seedlings under chilling stress.

1.1. Overview of seaweeds

Seaweeds have been used as animal food, soil conditioner and manure. They also have been utilized in the form of liquid extracts, as a growth promoter and crop protectors

against pests and diseases. Historically, the use of seaweeds in agriculture is ancient and dating back to Roman history (Henderson, 2004). It has been widespread wherever there are abundant supplies. In the coastal regions of Iceland, Great Britain, Norway, Ireland and France people still feed their animals with fresh seaweed or a prepared seaweed food. Seaweed is brought directly into the field in coastal regions; alternatively, it is applied as dried and ground seaweed meal. As a soil conditioner, certain red seaweeds are used in acid-humus-rich soils or peat soils as an alternative to lime, because of their extremely high (up to 80%) content of calcium carbonate (Blunden, 1991; Chapman & Chapman, 1980).

Seaweed extracts are made from storm-cast or freshly cut seaweeds. Species of seaweed that are found in the North Atlantic Ocean and used for making extracts are *Ascophyllum nodosum, Laminaria hyperborean, L. digitata*, *Fucus vesiculosus* and *F. serratus.* All these species belong to the brown algae (*Phaeophyceae*), and the most popular and most of the used in agriculture is *Ascophyllum nodosum*. Other species found in the Southern hemisphere, and are used for making extracts are such as the brown algae *Ecklonia maxima* and *Durvillea potatorum* (Verkleij, 1992).

1.2. The beneficial role of seaweeds in agriculture

In recent times, the positive scientific effects of applying seaweed extracts in agriculture have been widely reported and well-reviewed in scientific publications and more broadly in the plant biostimulant literature (Craigie, 2011; Khan et al., 2009; Du Jardin, 2012; Calvo et al., 2014). Seaweed extracts (SWE) are eco-friendly, non-toxic, non-polluting and non-harmful to humans, animals, and ecosystems (Craigie, 2011; Khan et al., 2009). They contain various nutrients, amino acids, vitamins, and plant growth-stimulating

components (Khan et al., 2009; Sharma et al., 2013). Recently, some seaweeds have been used as an alternative to conventional artificial fertilizers (Dhargalkar and Pereira, 2005; Hong et al., 2007; Khan et al., 2009; Zodape et al., 2010). The commercial SWE products can be used in liquid or powder form that work as a soil drench, foliar spray, manure and soil conditioner (Lingakumar et al., 2004, Thirumaran et al., 2009). Seaweed extracts have been found to promote plant growth in many ways, which includes increasing crop yield, enhancing root growth and plant development such as fruit set, leaf development and flowering. Moreover, seaweed extract can improve the ability of plants to climatic stresses and tolerate diseases. In addition, there are benefits that are related to improving soil structure and water-holding capacity (reviewed in Du Jardin, 2012; Khan et al., 2009; Craigie, 2011; Calvo et al., 2014). Although several studies have reported the role of SWE in enhancing plant growth, other studies have found that SWE do not provide benefits (Basher et al., 2012; Tourte et al., 2000). These studies involved an extensive range of SWE derived from many different species, including *Ascophyllum nodosum, Ecklonia maxima, Sargassum, Laminaria, Durvillaea potatorum, Ulva lactuca, Caulerpa sertularioides, Padina gymnospora, Sargassum liebmannii, Sargassum johnstonii,* and extracts produced by a range of different groups. Studies have presented that foliar and drench applications of SWE may cause different effects to crops because of the different application methods used in studies (Crouch and Van Staden, 1992; Hernández-Herrera et al., 2014; Kumari et al., 2011; Spann and Little, 2011).

1.3. What is the *Ascophyllum nodosum* extract (ANE)?

It is widely known that brown algae (Phaeophyta) are one of the most commonly used seaweeds in agriculture (Khan et al., 2009; Craigie, 2011), and among them, *Ascophyllum nodosum* is the most used in agriculture and the most reviewed in recent studies (Baardseth, 1970; Ugarte, 2011; Khan et al., 2009). *Ascophyllum nodosum* extract (ANE) is being produced by exposing *Ascophyllum nodosum* to high temperature and pressure in a process called alkaline extraction (Stirk & Staden, 1997). ANE is also known as rockweed and found in the rocky intertidal shores of Atlantic Canada and Northern Europe (Ugarte, 2011). Annually, approximately 15 million metric tons of seaweed products are collected (FAO, 2006), and a substantial portion is used in the production of plant nutrient supplements as well as bio stimulants to promote plant growth yield and productivity (Crouch and Van Staden, 1992; Craigie, 2011).

1.4. The effects of ANE on plant growth

The use of ANE in agriculture has many beneficial effects on plant growth (Khan et al., 2009). It has been reported in earlier studies that the treated plants with ANE had a better development in the shoot growth (Pise and Sabale, 2010; Crouch, 1990). Application of ANE increased the yield of the grapes and tomatoes, increased the sugar content in melons and sweet corn, increased the sweet pepper's initiation of flower buds, and extended the life of the bloom of Christmas plants (Aitken and Senn, 1965; Dobromilska et al., 2008; Temple and Bomke, 1989). Moreover, it promoted the length, area, total volume and number of roots for strawberry, and the fresh root weight and biomass for carrots (Alam, 2013). Additionally, ANE has been reported to promote the elongation of

roots and leaves for *Arabidopsis thaliana* (Rayirath et al., 2009). Furthermore, ANE has been shown to increase proteins, carbohydrates, free amino acids and polyphenol content (Pise and Sabale, 2010). ANE has also been reported to increase nutrient uptake (Beckett and Staden, 1990) and enhance resistance to biotic and abiotic stresses such as water and salinity stresses (Crouch and Staden, 1993; Nabati et al. 1994., Nabati, 1991). Furthermore, application of the ANE has been shown to increase freezing tolerance of *Arabidopsis thaliana* both inside and outside greenhouse conditions (Rayirath et al., 2009) Additionally, ANE has shown to elicit cytokinin-like activity (Khan et al., 2011). The application of ANE has improved turf quality and delayed senescence in turf grass (Schmidt, 1990). ANE has been found to increase chlorophyll content in beans, tomatoes, barley, wheat and corn (McNeil et al., 1999). Moreover, it has been reported to increase the total phenolic and flavonoid content of spinach, onions, and potatoes (Fan, 2011). In a recent study, Guinan et al. (2013) suggested that while some commercial *A. nodosum* extracts were operative in improving plant growth under abiotic stress conditions, they may be less efficient under biotic conditions and vice versa. Furthermore, ANE plays a key role in inhibiting the fungal disease in cucumbers through the activation of disease-related enzymes in plants such as chitinase, β-glucanase, and peroxidase (Jayaraman, 2011).

1.5. *Ascophyllum nodosum* composition

Ali et al. (2015) have reported that *Ascophyllum nodosum* consists of 40-70% carbohydrates (laminarin, alginate, fucoidan, and mannitol), 3-10% proteins, 4-8% polyphenols/pigments, and 2-4% phospholipids/glycolipids (Table.1). Moreover,

Ascophyllum nodosum also contains a low percentage of vitamins, minerals (potassium, magnesium, calcium, boron, zinc and phosphorus), and various plant hormones (gibberellins, auxins and cytokinins) (Ali et al., 2015). The most abundant components in *A. nodosum* are carbohydrates. These carbohydrates include alginate, fucose and laminarin that make up the cell walls of brown algae and comprise 30%, 10% and 7% of *A. nodosum*'s dry weight, respectively (Kandasamy et al., 2015).

Selosse & Tacon (1998) reported that inside the algal thallus, *Ascophyllum nodosum* is known to have an obligate relationship with the fungus *Mycophycias ascophylli* living between and inside the algal cells. Naturally, *Ascophyllum nodosum* is always found to be infected by *Mycophycias ascophylli* (Garbary et al., 1991). Recently, a study by Van de Reep & Garbary (2015) has presented the importance of this symbiosis in promoting plant growth. Even though the composition of this fungal cell wall is not well understood, fungal cell walls consist of chitin and β-glucan, and some pathogenic fungi have the ability to convert chitin to chitosan by deacetylation (during host invasion) to avoid plant recognition (Sanchez-Vallet et al., 2014).

Table 1: The Composition of *A. nodosum* (Ali et al. 2015).

Composition	Percentages from total dry powder of ANE
Carbohydrates	(40-70%)
Proteins	(3-10%)
Polyphenols and pigments	(4-8%)
Phospholipids and glycolipids	(2-4%)
Hormone and vitamins	(<1%)

1.6. Pre-assumptions about the mechanism of ANE

Earlier studies assumed that the effectiveness of seaweed applications on plants such as *Ascophyllum nodosum* was due to their water content and fertilizer effects (Aitken, 1965). Yet, this assumption is not reasonable due to the positive effects of ANE; for instance, ANE can be effective even at very low concentrations (1-2 L /acre). Zodape (2001) has reported another more recent assumption about the mechanism of ANE, which suggests that the activity of plant hormones in *Ascophyllum nodosum* and ANE may be a key in explaining ANE's effects. This assumption was developed through either the observation of the activity of plant hormones such as IAA, cytokinins, ABA in *Ascophyllum nodosum* and ANE (Brain, 1973; Stirk, 1997) or by detecting the different plant hormones directly (Kingman, 1982). Yet, Wally et al. (2013) stated that the concentrations of plant hormones in ANE are not enough to enhance plant growth or promote the resistance to stresses, so this hypothesis cannot explain the mechanism of ANE. Since the assumptions that suggest insufficient nutrients and plant hormones in ANE have not been able to explain its mechanism, other components in ANE should be studied.

1.7. Oligosaccharides in *A. nodosum* extract (ANE)

As mentioned earlier, the primary components of *Ascophyllum nodosum* are polysaccharides that are found in both seaweed and fungal cell walls. These polysaccharides include laminarin, alginate, fucoidan, chitin and β-glucan. When *Ascophyllum nodosum* is exposed to alkaline extraction under high temperature and pH to make ANE, these major polysaccharides are degraded to their corresponding oligo-forms (Stirk & Staden, 1997). Selected active oligosaccharides have been reported to act as

signal molecules that improve plant growth and development as well as defense reactions by regulating gene expression (Albersheim and Darvilal, 1985). It has also been reported that oligosaccharides have the ability to activate or inhibit plant growth and development (John et al., 1997). This study proposes that the major components of *A. nodosum* extract (ANE) are the oilgo-forms of laminaran, alginate, fucoidan, chitin, chitosan, and β-glucan. For a better understanding of the effects of ANE on plant growth, the effects of these oligosaccharides need to be studied.

1.8. Oligo-chitin and oligo-chitosan

Since *Mycophycias ascophylli* is the symbiotic fungal of the *Ascophyllum nodosum*, and it appears at every developmental stage in all parts of *Ascophyllum nodosum*, the role of the components of this fungus in ANE also need to be identified. One of the major components in all fungal cell walls is chitin (Sanchez-Vallet et al., 2014). It has been reported that chitin and cellulose are the two most abundant polysaccharides on earth (Gooday, 1990). Chitin can be found in different organisms such as animals with chitin-rich tissues include crustaceans, arachnids, and insects. Also it can be sourced from the beaks of cephalopods or by various microbes such as fungal cell walls, membranes, spores, and the spines of diatoms (Gohel et al., 2006; Castro, & Paulín, 2012; Bartnicki-Garcia, & Lippman, 1982).

Chitin and cellulose from plant cell walls have been reported to have sme biochemical similarities. Similar to cellulose, chitin is a polysaccharide that has long-complex-chained linear as shown in Fig.1. Chitin has the ability to provide structural stability by forming mechanical and physical barriers as it has an innate rigidity. Similar to cellulose, the

chitin also has the ability to develop strengthened tissues by combining with other compounds. The differences in length and construction of both polysaccharides from microfibrils depend on the species and cellular location (Gow, & Gooday, 1983). As one of the polymers in the fungal cell walls, chitin content has the highest percentage (22%–40%) of the cell wall among these polymers (Muzzarelli, 1977). Chitin is also known to be supplemented with considerable amounts of calcium, minerals and proteins in invertebrate tissues (Boßelmann et al., 2007). Due to the importance of chitin, its role in ANE should be studied.

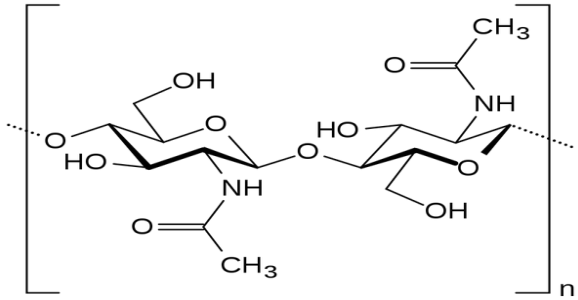


Figure 1: General structure of chitin

Chitosan is a deacetylated form of chitin that is also found in ANE, either in the fungal cell wall or as a result of the ANE extraction process. In addition, it is known to be the major component of the cuticles crustacean such as crabs and shrimps (Kumar et al., 2004). Moreover, chitosan is one of the most important non-toxic marine polysaccharides to humans and it has many biological effects such as anti-cholesterol and anti-bacterial (Chandy & Sharma, 1992). Therefore, the role of chitosan in ANE needs to be addressed. The general structure of chitosan is given in Fig.2.

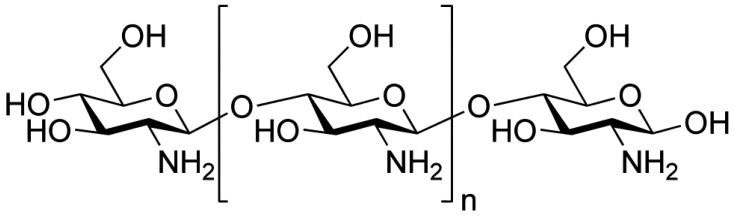


Figure 2: General structure of chitosan

1.9. The role of chitin & chitosan in regulating plant growth, development, nutrition, and tolerance to biotic and abiotic stresses

1.9.1. Plant growth promotion and development

Several studies have reported positive effects in plant growth and pest and disease control for a variety of crops after the application of chitin-based treatments. Substantial improvements in growth have been reported in ornamental plants, such as Gerbera and *Dendrobium* orchids, as well as other crops such as daikon radishes, cabbage, soybean sprouts, sweet basil and grapevine (Russell, 2013).

The effectiveness of chitosan at a very low concentration of 10 mg L^{-1} on orchids was reported by a recent study (Russell, 2013). This suggests that the chitosan's effects were not caused just by just improving nitrogen nutrition or as a carbohydrate energy source, but due to other mechanisms. In a recent study, Nahar et al. (2012) found that the application of chitosan under aseptic conditions has improved the growth of orchids. Chmielewski and others (2007) found that plants that are treated with chitosan in the field had better shoot and root development. Additionally, the storability of vegetables and postharvest fruits was significantly improved after the application of chitosan (El Ghaouth et al. 1991). Plant total weight and rate of germination were also increased after

the chitosan treatment in comparison to a control (Cho and Prinyawiwatkul, 2008). Interestingly, the high concentration of chitosan decreased the shoot length of bean seedlings whereas the length and the number of roots were increased at the low concentration (Sheikha, 2011). Furthermore, the plant flowering time was accelerated by spraying chitosan on freesia corms (Salachna, 2014). Chitosan also increased the diameter and height of stems and leaf area, the photosynthesis net rate and the chlorophyll content of okra (Dzung, 2011; Mondal, 2012) and coffee seedlings (Van, 2013). Other plants such as maize and soybean had better growth development after the application of chitosan (Khan, 2002).

Besides increasing vegetative growth and photosynthesis rate, chitin treatment have also been reported to regulate developmental processes. Limpanavech et al. (2003) found that chitosan has the ability to induce precocious flowering when applied to *Dendrobium* orchids. Utsunomiya and Kinai (1994) also reported that passion fruit had an increase in the flower numbers and precocious flowering when chitosan was used as a soil drench.

At the plant defense mechanisms level, the embryogenesis process in seed formation is thought to regulated by plant chitinase, however; the mechanism of this function is still unknown (Grover, 2012). A variety of crops including maize and wheat treated with chitin treatment were found to have an improvement in the seed germination process (Guan et al., 2009).

1.9.2. Plant resistance to biotic and abiotic stresses

At the level of biotic stress, several studies have reported that oigo-chitin treatment have the ability to enhance resistance to pathogens in plants (Kaku et al., 2006). The

application of oligo-chitin has also been found to promote antifungal activity in plants (Andres et al., 2014). Furthermore, applying chitosan to plants induced defense responses in fruits, such as strawberry, raspberry, grape berry and tomato fruit, and in the leaves of rice (Zhang & Quantick, 1998; Meng, Qin, & Tian, 2010; Romanazzi et al., 2002; Badawy & Rabea, 2009; Agrawal et al., 2002). Choi et al. (2000) revealed a significant role of chitosan treatment in decreasing bud rot in soybeans. It also protected plants from being affected by microorganisms (Pospieszny, Chirkov, & Atabekov, 1991).

At the abiotic stresses level, it has been well studied that chitosan has an antioxidant activity (Russell, 2013). Plant chitinases are also known to be involved in resisting plants from a variety of abiotic stresses (Grover, 2012). Boonlertnirun et al. (2007) found that after treating rice plants with chitosan under drought stress, noticeable effects on the growth of the treated plants were recognized compared to control plants, and a greater effect of chitosan was found when it was applied before exposing the plants to the stressful conditions. This may be due to the ability of chitosan to reduce the transpiration rates and induce stomatal closure in plants (Bittelli et al., 2001). Chitin treatment also has been found to be a successful solution to fix water sources and polluted soil (Russell, 2013). Furthermore, chitosan has been found to remediate other human-made pollutants that can affect water and soil sources such as hydrocarbons (Wang et al., 2007) and dyes (Sanchez-Duarte et al., 2012).

1.9.3. Physiological responses

Bittelli et al. (2001) reported that chitosan treatment reduced the water use of pepper plants by 26% – 43%, with no substantial change in biomass production. Also, the concentration of both abscisic acid (ABA) and jasmonic acid (JA) have been found to be increased after the chitosan treatment (Doares et al., 1995). JA and ABA are well known to be involved in the control of stomatal apertures (Herde et al., 1997). Recent studies found that there was no involvement from JA and ABA signaling in the stomatal closures recorded after chitosan treatment. This happened because JA/ABA mutants still respond to the application of chitosan with stomatal closure (Issak et al., 2013). On the other hand, Khan et al. (2002) reported that chitosan increased transpiration and induced stomatal opening in corn and soybean when used as foliar application.

1.9.4. Plant nutrition

There is high a nitrogen content ranges from 6.1%–8.3% in all chitin-based treatments (Yen & Mau, 2007). While chitin is thermally and chemically stable (Yen & Mau, 2007), it is possible for it to store dry products for enough period of time. Thus, it can be used as an energy and nitrogen source when added to crops. Spiegel et al. (1988) found that there was a significant difference between samples of Chinese cabbages treated with a standard mineral fertilizer and chitin-based products because samples treated with chitin-based treatments grew faster than the other samples. Under cold and dry conditions, the utilization of chitin by microbes was slowest (Yaroslavtsev et al., 2009). Chitin was also considered a beneficial treatment to add organic matter to soils without raising the C: N ratio.

Based on these findings of the effects of chitin, it is clear that chitin and chitosan in ANE may play a major role in promoting plant growth and defense responses of some plants.

1.10. Oligo-alginate

As outlined earlier, ANE derived from brown algae and alginate is considered to be one of the major components of the brown algae cell wall (Vera et al., 2011). The commercial production of alginate began in the early 20th century, but it was first isolated from brown algae in the 1880s. Donati and Paoletti (2009) stated that different groups of brown algae and two kinds of bacteria, *Pseudomonas* and *Azotobacter*, could produce alginate. An estimation of 30,000 metric tons of alginate is being produced yearly (Draget et al., 2005). In some seaweed, especially *Ascophyllum nodosum*, alginate measures up to 30% of the dry form of the plant (Baardseth, 1970) and it is thought to have a similar role to cellulose in plants (Draget et al., 2005; Donati and Paoletti, 2009). Additionally, alginate has been found to regulate plant growth and defense responses in different plants. Therefore, the presence of alginate in ANE and its role in promoting plant growth and defense responses should be considered. The general structure of alginate is given in Fig.3.

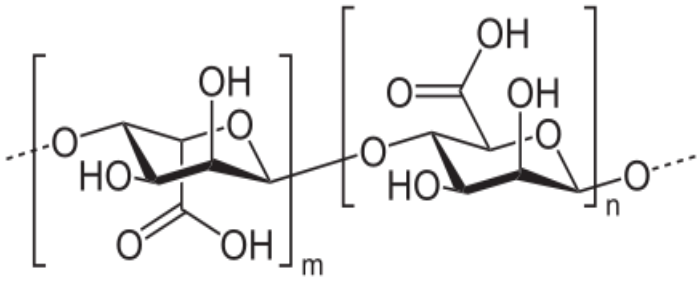


Figure 3: General structure of alginate

1.10.1. The role of oligo-alginate in stimulating plant growth and enhancing resistance against biotic and abiotic stresses

Some alginate-derived treatments have been found to exhibit promotion effects on plant seed germination, root growth and shoot elongation (Natsume et al., 1994; Iwasaki and Matsubara, 2000). In particular, alginate treatment enhanced the growth of peanut and rice plants (Hien et al. 2000). According to Hu et al. (2004), oligo-alginate application enhanced germination through accelerating the metabolic activities and promoting the amylase activity of the corn seeds. In addition, oligo-alginate has been reported to stimulate the growth of roots in lettuce (Iwasaki and Matsubara 2000). At the pre-flowering stage, an increase in the shoot length and dry weight was reported by Aftab et al. (2011) on the *Artemisia annua* plant after the application of alginate. They also found that alginate enhanced the net photosynthetic rate, the flowering and significant increase in the total chlorophyll content. Moreover, the application of oligo-alginate stimulated elongation and formation of rice, wheat and carrot roots (Zhang et al., 2013; Xu et al., 2003). Additionally, the oligo-alginate treatment also has been found to increase the height of tobacco plants (Laporte et al. 2007). In a recent study, oligo-alginates were reported to increase shoot and root length, shoot dry weight, chlorophyll content in opium poppy plants (Khan et al., 2011). Hien et al. (2000) suggested that the foliar spraying of alginate causes an increase in the physiological and biochemical functions of plants thereby an increase in dry matter occurs. Furthermore, Sarfaraz et al. (2011) have reported that alginate increased the weight of fennel tubers, root and shoot length of fennel plants.

Alginate oligosaccharides are also capable of relieving the stress for different plants. For instance, alginate relieved the cadmium caused damage to *Vicia faba* root tips (Ma, L. J. Ma, Zhang, Bu, & Wang, 2010) and recovered the side effects of the drought on the *Triticum aestivum L*. (Liu et al., 2013). Other studies have proven that the application of oligo-alginate alleviated metal stress, and induced the plant defense responses (Hu et al., 2004). Liu et al. (2009) found that alginate application reduced the inhibitory effect of drought stress on tomato seedling growth by reducing the damage to cell membranes. Moreover, alginate increased the electric conductivity of stoma in *Artemisia annua* (Aftab et al., 2011).

Oligo-alginate is a major component of ANE and it is perhaps responsible for promoting plant growth and defense responses.

1.11. Chilling stress

In the present research, chilling stress is going to be used to test the ability of the soybean seedlings to survive after the application of the different treatments. Chilling (cold) stress is considered to be one of the most significant abiotic stresses in agricultural, causes effects to yield and plants' development, and it is divided into freezing (less than 0 $^{\circ}$C) and chilling (less than 20 $^{\circ}$C) (Lang et al., 2005). It is a main factor to determine the phenology and yield potential of crops (Hayashi, 2001), and the natural distribution of plants (Repo et al., 2008). Masaya and White (1991) reported that temperature response curves (minima, maxima, and optima) can be used to determine the biochemical processes. Thus, affects growth and development rates of the plant are affected by temperature. During the life cycle, exposure to low temperature has been found to affect

many subtropical and tropical plant species. For instance, in temperate growing areas, cold temperatures are responsible for approximately 30–40% yield reduction of rice (Andaya and Mackill, 2003; Kaneda and Beachell, 1974). Many important crops such as soybean, cotton, corn and rice do not have the ability to survive under chilling stress (Larcher, 1995). Recently, a number of studies have focused on the cold stress mechanisms in higher plants to find solutions to activate the plant tolerance against stresses in order to have better yield (Iba, 2002). Cold stress has been found to affect several stages in the plant life cycle including reproductive and vegetative stages thereby lowers yield considerably (Nishiyama, 1995). Therefore, cold stress may cause significant social and economic impacts on the humanity main source of food (Thakur et al., 2010).

1.11.1. Fundamental responses of plants during cold stress exposure and acclimation mechanisms (fig. 4):

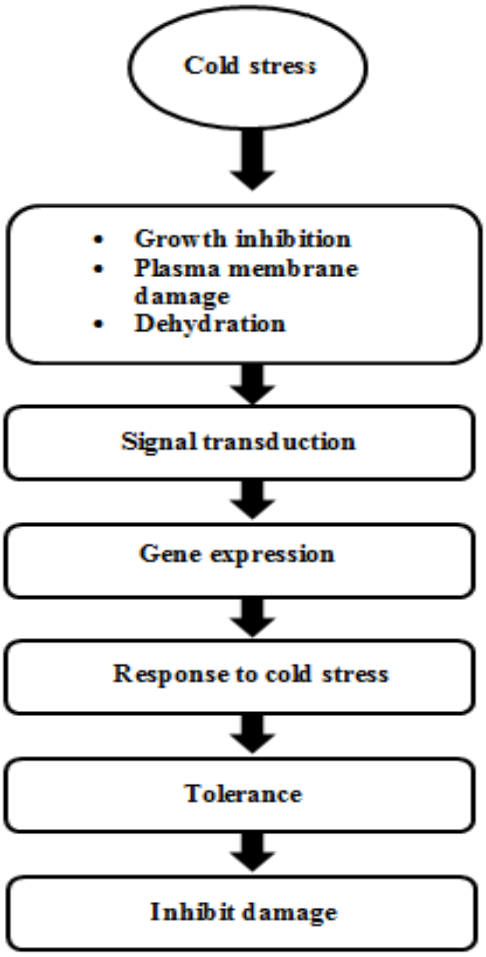


Figure 4: Fundamental responses of plants during cold stress exposure (Yadav, 2010).

In conclusion, the information given above suggests that ANE and its components specifically oligosaccharides, can promote plant growth and induce plant resistance against several biotic and abiotic stresses in different important crops. One of these important plants is soybean because it an important legume and a major source of oil production which provide humans with proteins. In the present study, I will try to understand the mechanism of ANE on soybean (*Glycine max*) seedling by examining and comparing its effects with the effects of its oligosaccharides under chilling stress.

2. Hypothesis

Due to the lack of information about the mechanisms of *Ascophyllum nodosum* extract (ANE), this study proposes that the major oligosaccharides in its components, especially oligo-chitin, oligo-chitosan and oligo-alginate, can work as effective components to promote plant growth and plant resistance against various stresses. For a better understanding of the function of ANE and its mechanisms, I am going to analyze the effects of *Ascophyllum nodosum* extract (ANE) and these different oligosaccharides on soybean seedlings under normal and chilling stress.

3. Objectives

The objectives of the present study were firstly to measure plant recovery and survival ratio after ANE and oligosaccharides treatments were applied to soybean seedlings under chilling stress. My second objective was to compare the chilling damage on soybean leaves (leakage) and chlorophyll content after ANE and oligosaccharides treatments. My last objective was to assess the effects of oligosaccharides and ANE on contents of chlorophyll and phenolic compounds in soybean leaves

4. Materials and methods:

4.1. Preparation of ANE and oligosaccharides

Acadian 100% liquid seaweed concentrate derived from *Ascophyllum nodosum* which contains 0.1% N, 0% P, and 5.0 % K respectively, was diluted to a concentration of 2 mL/L for soybean seeds soaking and 3ml/L for soybean seeds spraying. Oligo-alginate, oligo-chitin, and oligo-chitosan were provided by Dr. Yuguang Du (Institute of Process

Engineering, Chinese Academy of Sciences). Oligo-chitin and oligo-chitosan both possess an average polymerization of 4. 1000 mg/L stock solution was made for all oligosaccharides by dissolving them directly into distilled water.

4.2. Seed soaking

Soybean seeds, cultivar Hidasta, were obtained from the Halifax Seed Company in May 2016. Soybean seeds for all the experiments were soaked at room temperature for 24 hours in May 2016 before planting. Two petrie dishes were prepared for each treatment; each one contained 35 seeds as shown in Fig. 5. Each petri dish consisted of 20 mL of solutions used for soaking the soybean seeds. The concentrations of the six treatments used in this soaking process were 2 mL/L of ANE, 26.7 mg/L of oligo-alginate, 20 mg/L of oligo-chitin and 33.3 mg/L of oligo-chitosan, the mixture treatment (26.7 mg/L of oligo-alginate + 20 mg/L of oligo-chitin + 33.3 mg/L of oligo-chitosan) and distilled water used as the control.

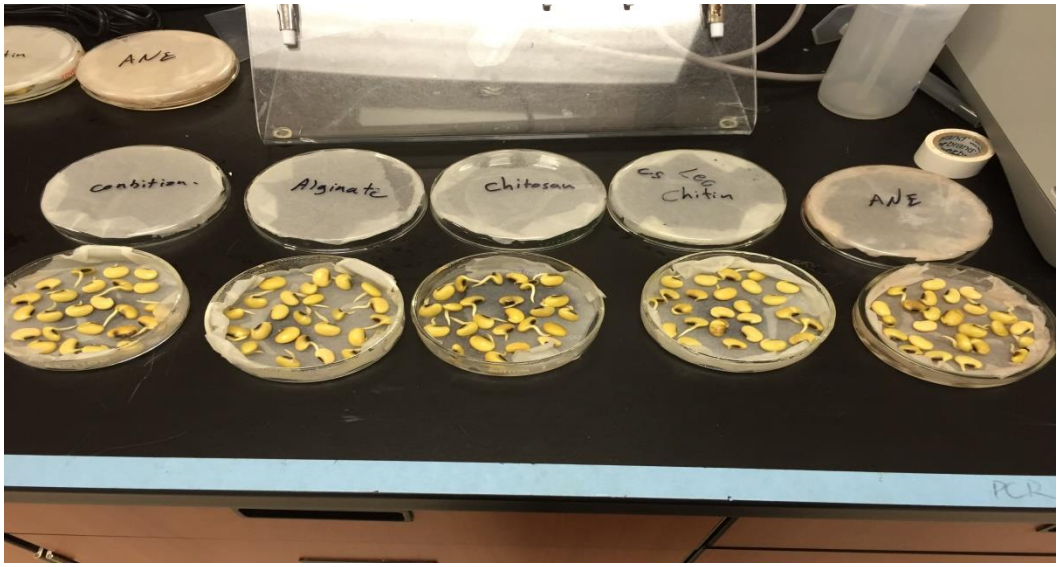


Figure 5. The soaked soybean seeds for all the treatments.

4.3. Planting the seeds

For the chilling stress experiment, soaked seeds were planted in small square pots, and the pots were filled with Promix Lp 15.4 soil, and each pot contained one seed at a depth of 1 cm. For the bio-content experiment, five seeds were planted in 7 inch pots filled with the same soil, at a depth of 2 cm. All these soybean plants were grown in the greenhouse at Saint Mary's University for two weeks (Fig. 6). The soybean plants were watered with 100 mL of a ½ strength Hoagland nutrient solution per pot daily (Hoagland & Arnon, 1938).

Figure 6. The pots after planting the soybean seeds for all the treatments.

4.4. Spraying the soybean leaves

After two weeks of plant growth, when the simple leaves were fully-grown as shown in Fig.7, the leaves of the soybean plants were sprayed with the same treatment in which their seeds were soaked; for example, the soybean seeds that were soaked in ANE were sprayed with ANE. The five treatments were sprayed at these concentrations: 3 mL/L of ANE, 40 mg/L of oligo-alginate, 30 mg/L of oligo-chitin, 50 mg/L of oligo-chitosan and the mixture (40 mg/L of oligo-alginate + 30 mg/L of oligo-chitin + 50 mg/L of oligo-chitosan) at the rate of 100 mL/pot. In addition, distilled water was used for the control.

Figure 7. The soybean plants before spraying the treatments.

4.5. Chilling stress experiments:

After two weeks of growing the soybean seeds for the chilling experiment (15 plants for each treatment) in the greenhouse at Saint Mary's University, the leaves of the soybean plants were sprayed until they became wet with either water or different treatments at the following concentrations: 3 mL/L of ANE, 40 mg/L of oligo-alginate, 30 mg/L of oligo-chitin, 50 mg/L of oligo-chitosan, and the mixture (40 mg/L of oligo-alginate + 30 mg/L of oligo-chitin + 50 mg/L of oligo-chitosan). After 12 hours from spraying the leaves, the plants were transferred into a 4°C growth chamber for 24 hours and then prepared for the electrolyte leakage and the chlorophyll damage analyses.

4.5.1. Recovery and survival ratio:

In September, 2016, the soybean seeds were soaked in 2 mL/L ANE, 33.3 mg/L oligo-chitosan and 26.7 mg/L oligo-alginate. After that, the soybean seeds were planted in small square pots containing soil. Four trays for four treatments (water, ANE, oligo-alginate and oligo-chitosan) were used in this experiment, each tray containing 32 plants. After two weeks the plants were sprayed with different solutions of ANE, oligo-chitosan, and oligo-alginate at a concentration of 3mL/L, 50 mg/L and 40 mg/L respectively, and water was used as a control. After 12 hours, the plants were transferred to the chilling condition at 4 ^{o}C for 24 hours. After 24 hours of chilling, all the leaves had dropped down. The plants were then transferred to the greenhouse for 24 hours to record the recovery ratio after the chilling stress.

To test the survival and recovery ratio for a longer time, the same plants were used; however, the concentrations for the spraying treatments were changed in oligo-chitosan

and oligo-alginate to 75 mg/L and 60 mg/L respectively. All the plants were sprayed with different treatments, then exposed to chilling stress outside the greenhouse for 12 days under 4 -13 ^{o}C. During this period, the survival ratio was recorded at three different times (after three, five, and ten days of chilling). To test the recovery ratio, all the plants were transferred to the greenhouse after 12 days of chilling stress, and the recovery ratio was recorded after one week.

4.5.2. Electrolyte leakage analysis

The electrolyte leakage (EL) was measured as described in Sukumaran (1972) and Ristic (1993). The first leaves from the bottom were used for the analysis. Black rubber, circular copper cutter, and a pincer were used to cut small pieces of the leaves from each treatment group. Six samples (one leaf from each plant) were used for each treatment. The leaf pieces were placed in glass tubes containing 16 mL of deionized water and incubated in a horizontal shaker (150 cycles/minute) for 1 hour at room temperature. Subsequently, the electrical conductivity of the solution (L1) was determined by using a conductivity meter. Then the samples were autoclaved at 120 °C for 15 minutes, and the final electrical conductivity (L2) was obtained after shaking the samples in the horizontal shaker for 1 hour. The EL was calculated as follows: EL (%) = (L1/L2) * 100.

4.5.3. Chlorophyll damage analysis:

DMSO was used to extract the chlorophyll according to Arnon (1949). The amount of DMSO was calculated based on the leaf weight with the ratio of 10 mL DMSO per 100 mg of leaves. The proper amount of DMSO was then added to each Eppendorf and then incubated in a 65 ºC water bath for 5 hours. The absorbance of the DMSO extract was

measured at 645nm and 663nm by a spectrophotometer. Finally, the total chlorophyll content was calculated by the following equations described in Hiscox and Israelstam (1979):

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

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

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

4.6. Bio-content analysis

For the phenolic and chlorophyll content analysis, 35 mg and 20 mg leaf samples were collected respectively. Samples were collected at 12, 36, 60, and 84 hours after spraying the treatments. The simple leaves (leaves from the bottom) were chosen for all the treatments. A stopper borer, black rubber, and a pincer were used to cut small pieces of the leaves from each treatment group. Then, in order to store them, all the leaf pieces were placed in a 1.5 mL Eppendorf. Each Eppendorf was labeled by the tube number, the number of hours after treatment and the treatment received by the leaves then weighed before and after the collection. Afterward, they were chilled in liquid nitrogen, and then stored at -80 ºC. Six samples (one leaf from each plant) were collected from each treatment.

4.6.1. Phenolic content analysis

The procedure described in Jayaraj et al. (2008) was used to determine the phenolic content. The soybean leaf samples were ground for 1 minute by inserting the microcentrifuge pestle into the stored Eppendorfs. The necessary amount of 96% methanol was calculated based on the leaf weight in the Eppendorf with the ratio of 0.1 mL per 10 mg. Then, the Eppendorfs were filled with the appropriate amount of the methanol. After that, the Eppendorfs were incubated for 5 minutes at 70 ºC, then shaken in the vortex machine for five seconds and instantly put back in the incubator. This process was repeated two more times. After the incubation, the Eppendorfs were centrifuged at 10,000 rpm for 30 seconds. Two hundred µL of the supernatant was transformed to test tubes, and 25 µL of Folin Ciocateau reagent (2N), 1.225 mL of distilled water, and 0.2 mL of Na_2CO_3 were added. Then, the tubes were incubated at 25 ºC for 1 hour. The absorbance of the mixture tube was measured at 725 nm with a spectrophotometer. The mixture was diluted with water to a concentration with optical density (OD) 725 lower than 1.0. The total phenolic content was calculated based on a standard curve expressed as gallic acid equivalents (g^{-1}) fresh weight (Appendix 1) (Jayaraj et al., 2008).

4.6.2. Chlorophyll content analysis

The same procedure that was used to measure the total chlorophyll content in the previous chilling experiment was followed to measure the chlorophyll content under the normal condition.

4.7. Statistical analysis

All data were analyzed using Minitab17 software. At first, the data were tested with the normality test. Then, the normal data were analyzed by using a one-way ANOVA test to compare the differences among all the treatments. To determine where the significant differences were, a Tukey's post-hoc test at 0.05 significance level was used. For the non-normal data, the Kruskall Wallis test was used at 0.05 significant level. To determine where the significant differences among the non-normal data were, a Dunn's test was used as post-hoc.

5. Results:

5.1. Effects of ANE and oligosaccharides on the recovery ratio of the soybean plants after 24h chilling:

After the plants were exposed to chilling stress 4 ^{o}C for 24 hours, all the leaves dropped as shown in (Fig.8). Then, the plants were transferred to the greenhouse for a 24-hours-recovery. The results showed that ANE, oligo-chitosan and oligo-alginate treatments had a high percentage of the recovery (75%, 87%, and 81% respectively) after being chilled for 24hours and they are higher than water treatment (28%) (Fig.9) (see Table 2 in Appendix 2 for detailed information). Kruskall Wallis test showed that there is a significant difference between all the treatments compared to the control (P= 0.027, H=9.20).

Figure 8. The drooped soybean leaves after 24h under chilling stress (4 ^{o}C).

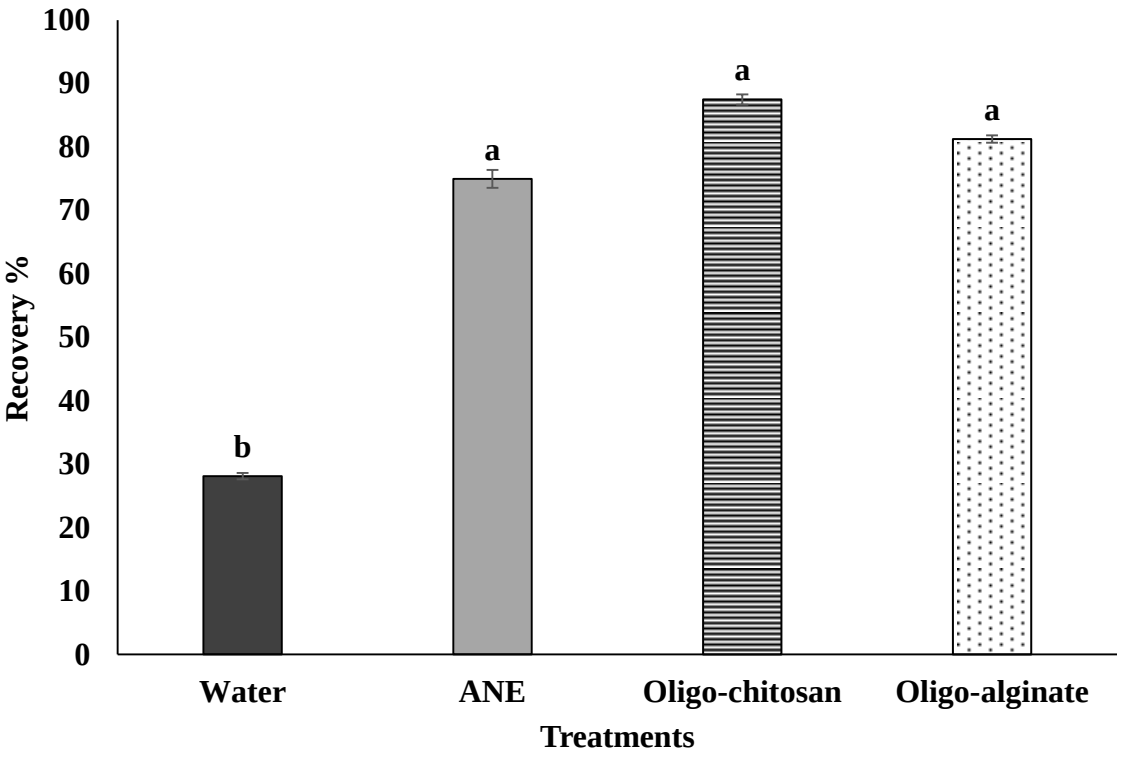


Figure 9. Recovery ratio of the treated soybean plants after 24h of chilling in greenhouse. The values are means of 32 replicates ± standard deviation. Different letters on top of bars indicate significant different at $p \leq 0.05$.

5.2. Survival ratio of the soybean plants:

To measure the survival ratio of soybean plants, the plants were sprayed on October 11th, 2016 with one of ANE (3 mL/L), oligo-chitosan (75 mg/L) or oligo-alginate (60 mg/L). On October 14th, 2016, the plants were exposed to chilling stress outdoor the greenhouse at a temperature of 4 to 13 ^{o}C for a week. After that, the survival rate was recorded at different time points, as follows:

5.2.1. Survival ratio after three days of chilling stress:

After three days of chilling stress, as shown in pictures A, B, C and D (Fig.10), the measured survival ratio of the soybean plants showed that the oligo-chitosan and oligo-alginate treatments had a high percentage of survival (100, 90%, respectively), which was significantly higher than for the water and ANE treatments (59 and 68%) Fig.11 (see Table 3 in Appendix 2 for detailed information). Kruskall Wallis test showed that there was a significant difference between all the treatments compared to the control (P= 0.006, H=11.66).

ANE

Water

Oligo-alginate

Oligo-chitosan

Figure 10. The Survival soybean plants after three days of chilling for all the treatments.

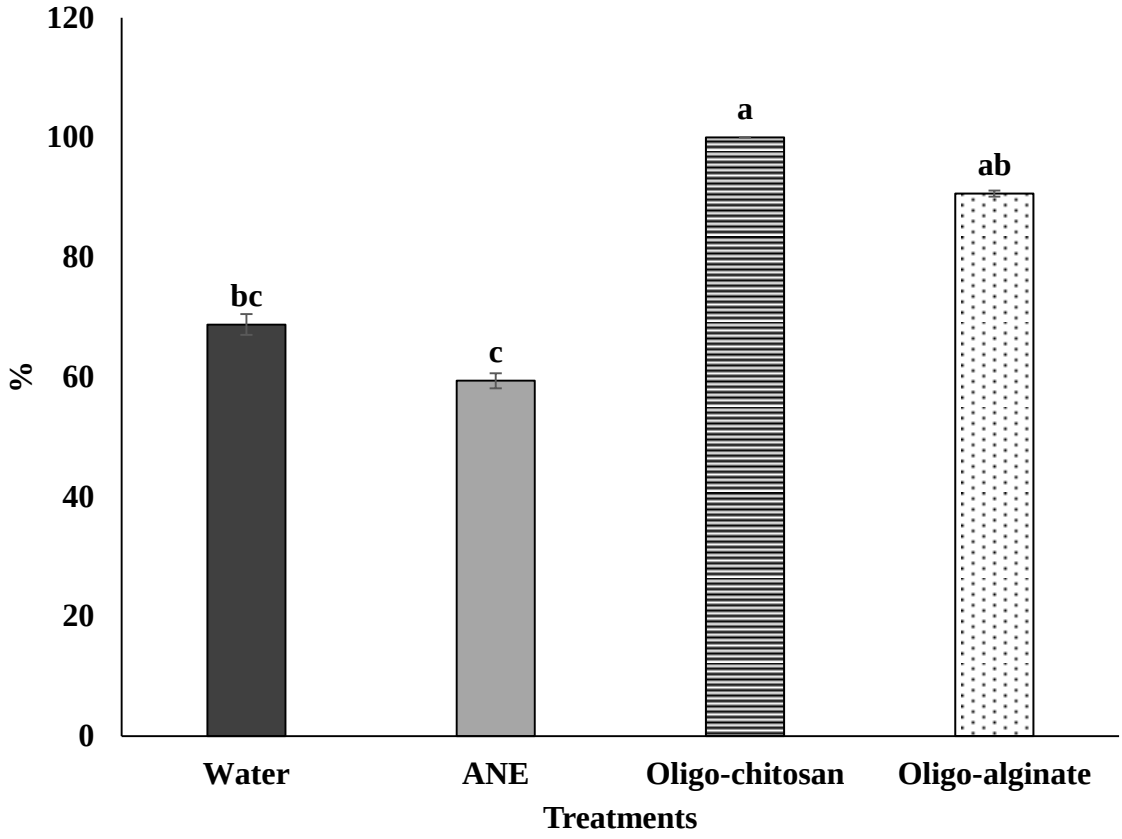


Figure 11. Survival rate of the soybean plants were exposed to chilling stress outdoor the greenhouse for three days. The values are means of 32 replicates ± standard deviation. Different letters on top of bars indicate significant different at $p \leq 0.05$.

5.2.2. Survival ratio after five days of chilling stress:

After five days of chilling stress, as shown in pictures A, B, C and D (Fig.12), the measured survival ratio of the soybean plants showed that the oligo-chitosan and oligo-alginate treatments have a high percentage of survival (93 and 81%, respectively), which was significantly higher than for the water and ANE treatments (50 and 34%) Fig.13 (see Table 4 in Appendix 2 for detailed information). The ANOVA test showed that there are significant differences between all the treatments (P= 0.001, F=11.12).

ANE

Water

Oligo-alginate

Oligo-chitosan

Figure 12. The survival soybean plants after five days of chilling for all the treatments.

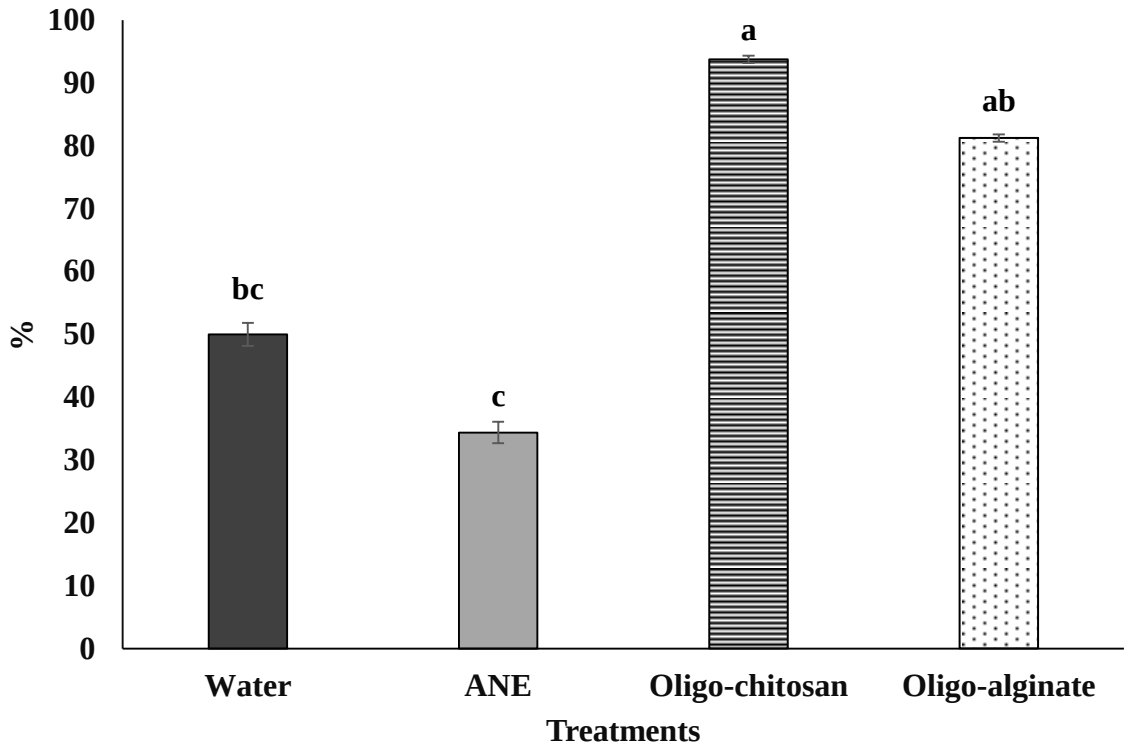


Figure 13. Survival rate of the soybean plants were exposed to chilling stress outdoor the greenhouse for five days. The values are means of 32 replicates ± standard deviation. Different letters on top of bars indicate significant different at $p \leq 0.05$ according to the Tukey's test.

5.2.3. Survival ratio after ten days of chilling stress:

After ten days of chilling stress as shown in pictures A, B, C and D (Fig.14), the measured survival ratio of the soybean plants showed that the oligo-chitosan and oligo-alginate treatments have a high percentage of survival (43 and 65%, respectively), which was higher than for the water and ANE treatments (6 and 3 %) Fig.15 (see Table 5 in Appendix 2 for detailed information). Kruskall Wallis test showed that there is a significant difference between all the treatments (P= 0.008, H= 11.96).

ANE

Water

Oligo-alginate

Oligo-chitosan

Figure 14. The survival soybean plants after ten days of chilling for all the treatments.

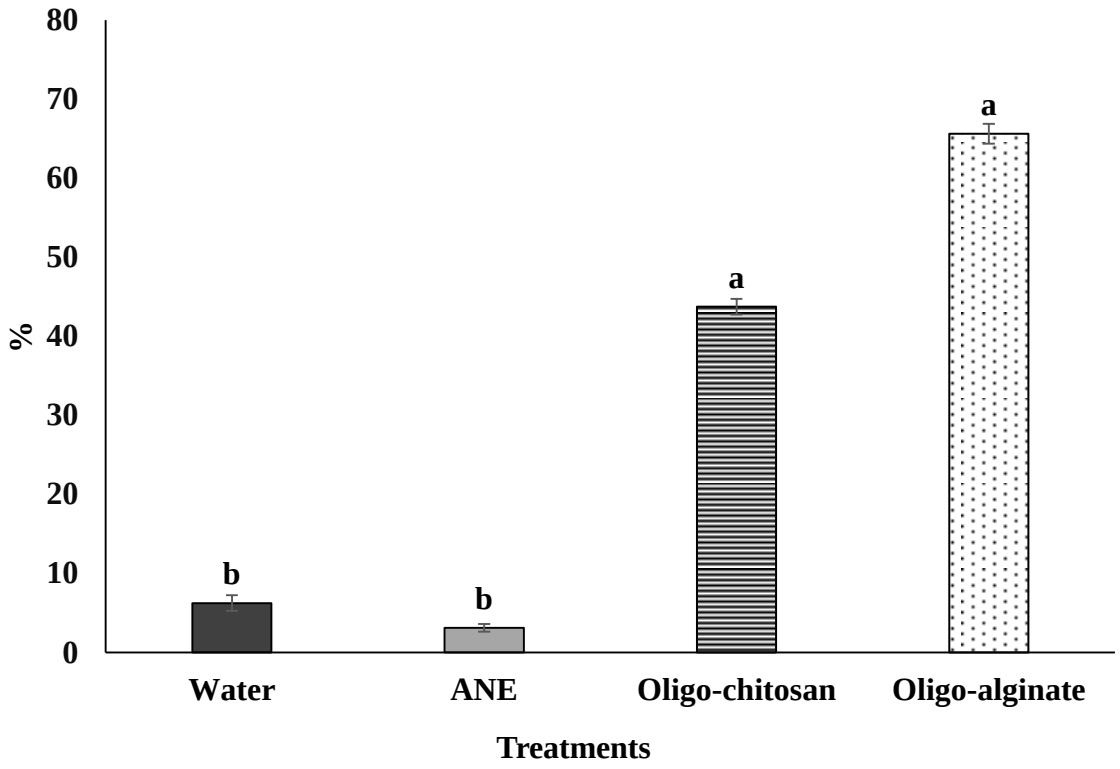


Figure 15. Survival rate of the soybean plants were exposed to chilling stress outdoor the greenhouse for ten days. The values are means of 32 replicates ± standard deviation. Different letters on top of bars indicate significant different at $p \leq 0.05$.

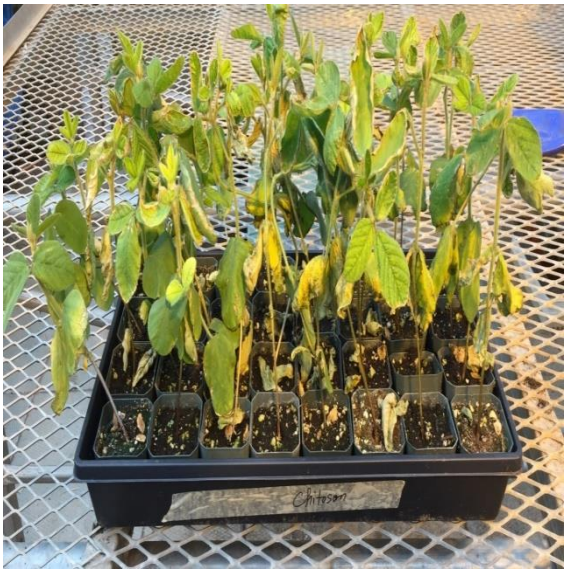
5.3. Recovery ratio of the soybean plants after 12 days of chilling stress:

To measure the recovery ratio, the soybean plants were exposed to chilling stress outside the greenhouse for 12 days, then transferred to the greenhouse for a week to recover as shown in pictures A, B, C, and D (Fig.16). Then, the recovery ratio was measured for the soybean plants. The control soybean plants (Fig.17) were always placed in the greenhouse under normal condition. The findings indicated that the oligo-chitosan and oligo-alginate treatments have a high percentage of recovery (62 and 50%, respectively), which was significantly higher than for the water and ANE treatments (25 and 21%) Fig.18 (see Table 6 in Appendix 2 for detailed information). The ANOVA test showed that there is a significant difference between all the treatments compared to water (P= 0.001, F=11.05).

ANE

Water

Oligo-alginate



Oligo-chitosan

Figure 16. The recovery of the soybean plants from chilling stress for all the treatments after 12 days of chilling stress.

Figure 17. The soybean plants (control) were placed in the greenhouse under normal condition.

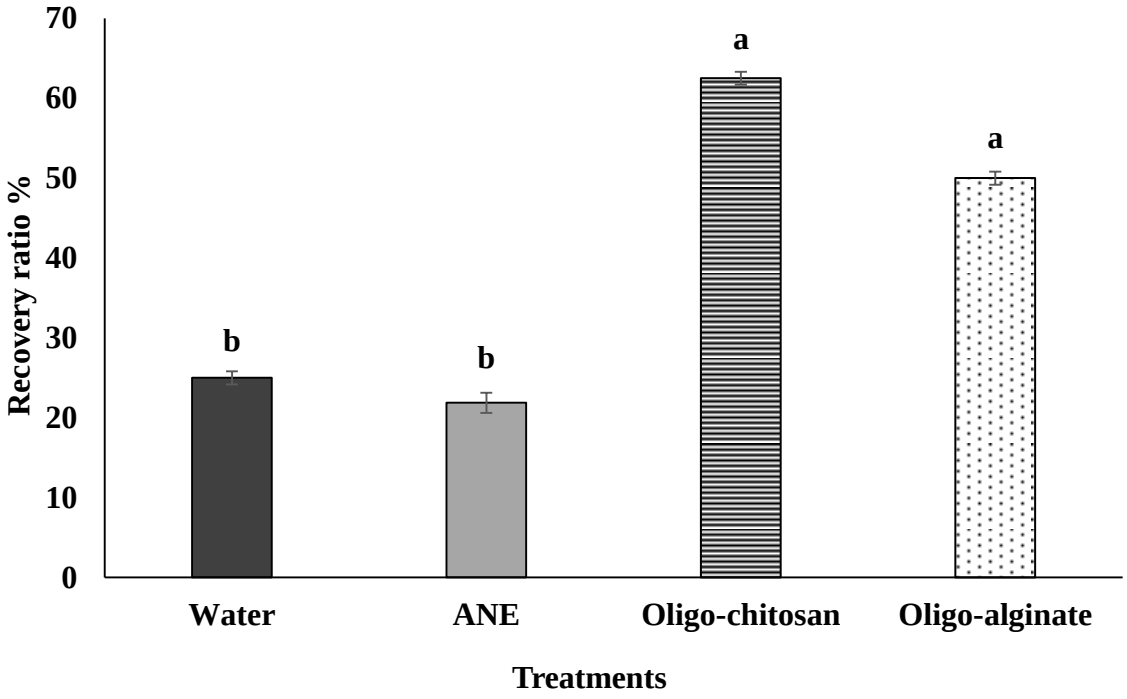


Figure 18. Recovery ratio after the soybean plants were sprayed with different treatments and exposed to chilling stress outdoor the greenhouse for 12 days then placed in the greenhouse for five days. The values are means of 32 replicates ± standard deviation. Different letters on top of bars indicate significant different at $p \leq 0.05$ according to the Tukey's test.

5.4. Effects of ANE and oligosaccharides on leakage after chilling stress:

12 hours after spraying the soybean leaves with different treatments, the ion leakage percentages were measured after a 24-hour-exposure to of either chilling stress at 4 ^{o}C or normal condition. Under the normal condition, the percentage of ion leakage was 17% for the water-treated soybean plants, whereas for ANE-treated plants it was only 13% leakage. The corresponding ion leakage percentages were 14%, 16%, 13% and 14% for oligo-chitin, oligo-chitosan, oligo-alginate, and their mixture treatments respectively (Fig.19) (see Tables 7 and 8 in Appendix 3 for detailed information). ANOVA showed that the ion leakage for ANE and oligosaccharide treated leaves under the normal condition had significant differences in the percentages among the treatments (P=0.019, F=3.24). After chilling stress at 4°C for 24 hours, the percentage of ion leakage in water, oligo-chitin and mixture plants significantly increased to 25.8%, 23%, and 22% respectively. By contrast, the percentage of ion leakage in ANE, oligo-chitosan and oligo-alginate treated plants was 14.6%, 17%, and 16% respectively. The ANOVA showed that the ion leakage for water, ANE, oligo-chitin, oligo-chitosan oligo-alginate and mixture treated plants after chilling had significant differences in the percentages (P= 0.0001, F=8.72). ANOVA also showed a significant increase in ion leakage for the water treatment after chilling compared to the normal condition (P= 0.003, F=15.03). By contrast, no significant differences were found between ANE treatment under the normal and chilling stress (P=0.297, F=1.21). Moreover, oligo-chitosan and oligo-alginate showed non-significant differences in ion leakage under the normal condition and chilling

stress (P=0.492, F=0.51 and P=0.056, F=4.68 respectively). After comparing ANE with oligo-chitosan and oligo-alginate after chilling, no significant differences were found in the ion leakage based on Tukey test. These results indicate that the ion leakage for ANE, oligo-chitosan and oligo-alginate treated leaves decreased after chilling stress; however, the leakage percentage of the water-treated plants increased after chilling stress.

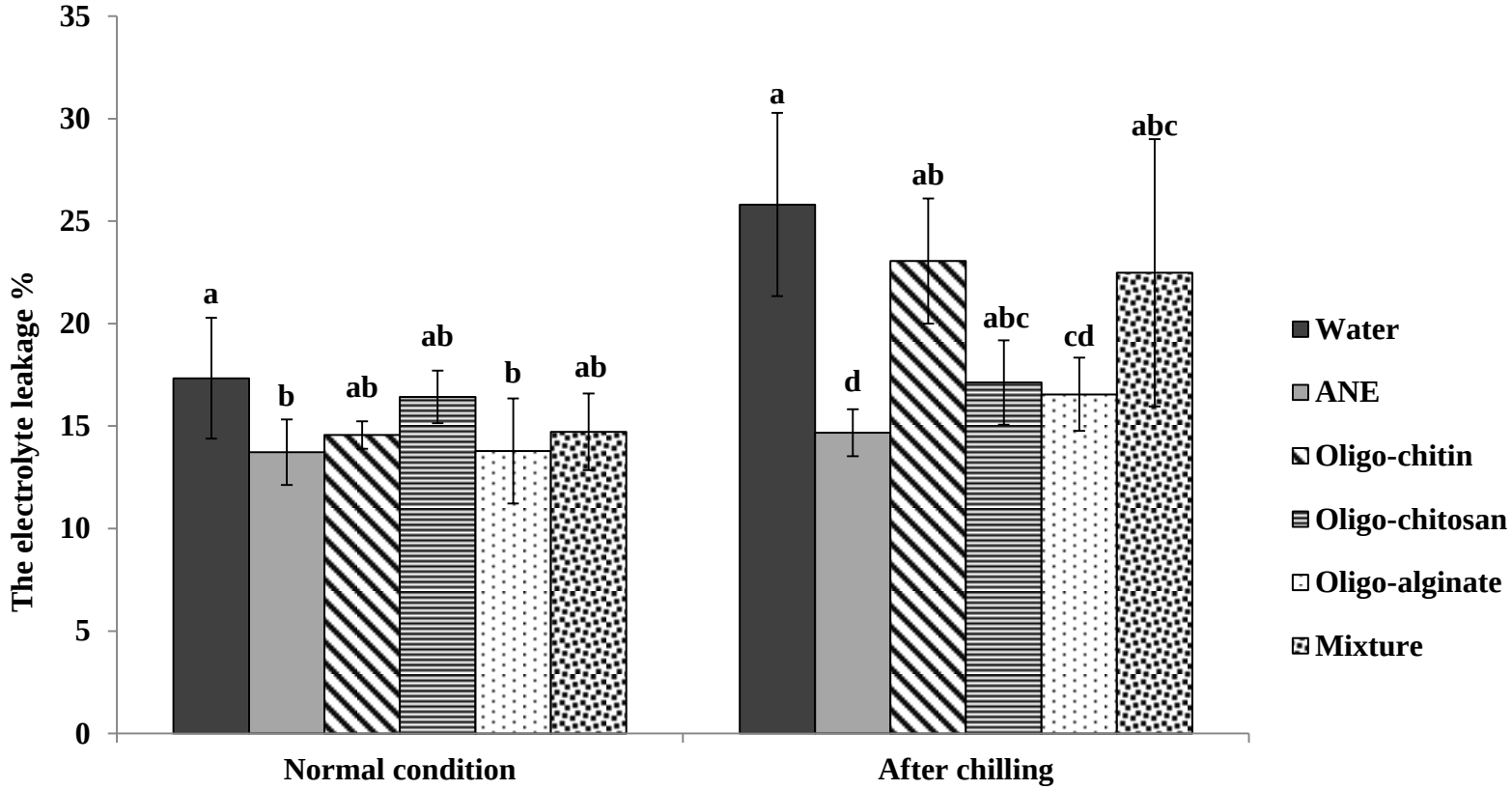


Figure 19. The ion leakage of the soybean leaves after ANE and oligosaccharide treatments under the normal and chilling stress. The values are means of six replicates ± standard deviation. Different letters on top of bars indicate significant different at $p \leq 0.05$ according to the Tukey's test.

5.5. Effects of ANE and oligosaccharides on chlorophyll content after chilling stress:

12 hours after spraying the soybean leaves with different treatments, the chlorophyll contents were measured after the leaves had been exposed to either chilling stress at 4 ^{o}C or normal condition for 24 hours. Under the normal condition, water, ANE, oligo-chitin, oligo-alginate, oligo-chitosan and the mixture treatments showed significant differences in the chlorophyll content after the ANOVA test (P=0.0001, F=7.69). In addition, in the normal condition, ANE increased the chlorophyll content compared to the water as Tukey test showed. Oligosaccharides had no significant effect on chlorophyll content compared to water in the normal condition as Tukey showed. After chilling stress at 4 ^{o}C for 24 hours, chlorophyll content in ANE and water treated soybean leaves had decreased. However, in all four oligosaccharides treatments the chlorophyll content increased (Fig.20) (see Tables 9 and 10 in Appendix 3 for detailed information). Analysis of variance (ANOVA) indicated that the chlorophyll content for ANE and oligosaccharide treated leaves after chilling showed significant differences (P=0.0001, F=19.65). After chilling stress oligo-chitosan treatment significantly increased leaves' chlorophyll content in comparison to control and all the other treatments. Comparing chlorophyll content of water in the normal condition and after chilling showed non-significant differences but seems to be a tendency for it to be significant (P=0.066, F=4.28). Additionally, oligo-chitin, oligo-alginate and mixture treatments in the normal condition and after chilling revealed no significant differences in chlorophyll content (P=0.112, 0.481, and 0.081 and

F=3.03, 0.53 and 3.76) respectively. Significantly, ANE treatment under the normal condition showed higher chlorophyll content than after chilling (P=0.009, F=10.32). Oligo-chitosan treatment showed a significant increase in chlorophyll content after chilling compared to the normal condition (P=0.0001, F=83.49). Therefore, these results indicate that oligo-chitosan promoted the chlorophyll content under chilling stress, thus promote plant growth.

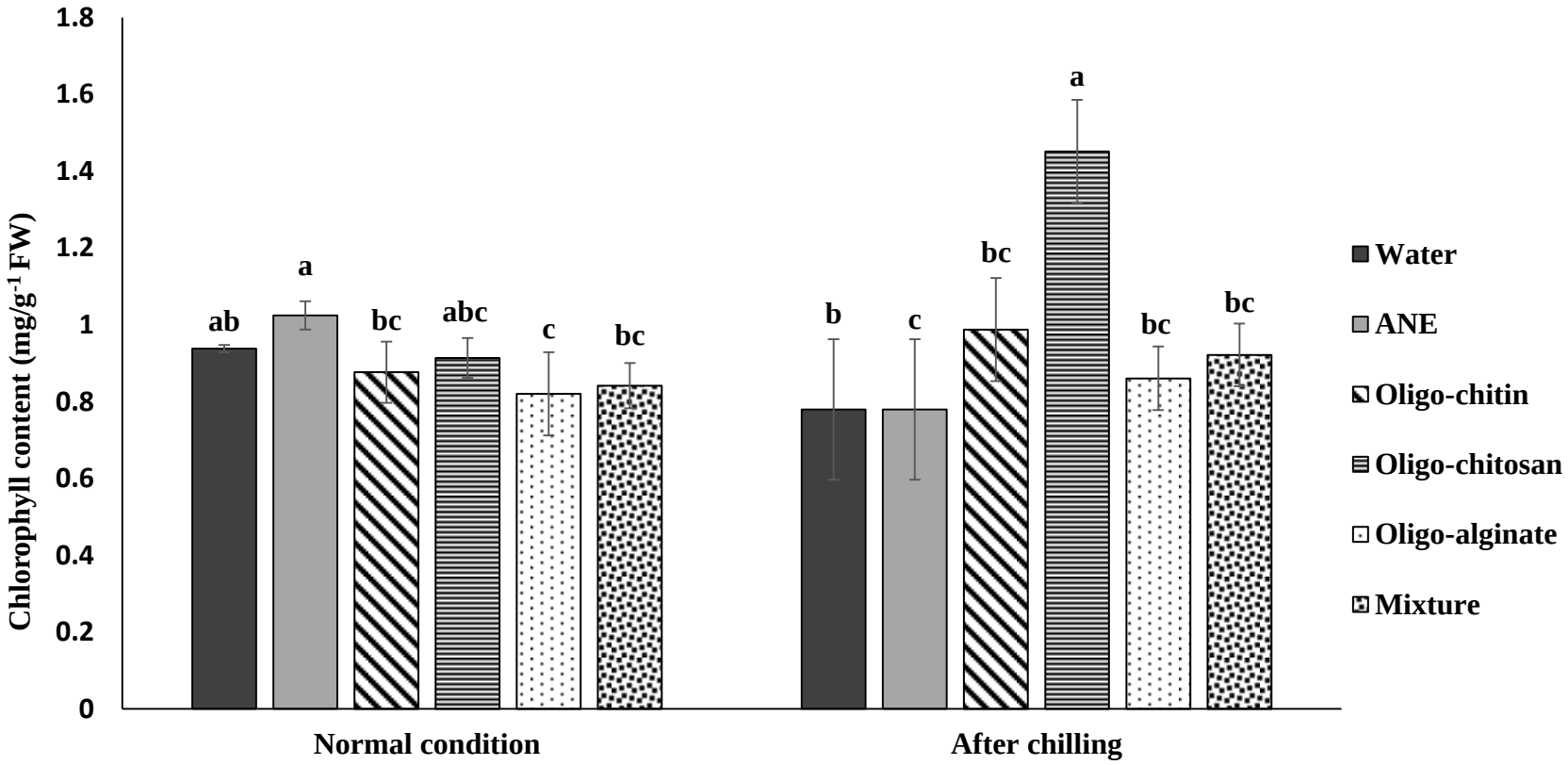


Figure 20. The chlorophyll content of the soybean leaves after ANE and oligosaccharide treatments under normal and chilling stress. The values are means of six replicates ± standard deviation. Different letters on top of bars indicate significant different at $p \leq 0.05$ according to the Tukey's test.

5.6. Effects of ANE and oligosaccharides on phenolic content:

Phenolic contents were measured at 12 to 84 hours after the soybean leaves were sprayed with various solutions. ANE treatment was used as a positive control and water treatment was used as a negative control. At 12 hours, ANE and oligo-chitin treatments showed a similar level of phenolic contents, but were higher when compared to water. However, water showed a high level of phenolic content compared to oligo-chitosan, oligo-alginate and the mixture treatments. Analysis of variance (ANOVA) showed at this time point significant differences between all the treatments (P=0.0001, F=7.28). However, the phenolic contents of ANE and water treated leaves at 12 hours showed non-significant differences after Tukey's test. At 36 hours ANE treatment showed a high level of phenolic content compared to water and other treatments. Analysis of Kruskall Wallis did not show significant differences between the treatments (P =0.297, H=6.09). ANE treatment increased phenolic content at 60 hours compared to water and oligosaccharides treatments. Kruskall Wallis test showed significant differences between the treatments at this time point (P=0.003, H=17.92). In addition, ANE treatment increased phenolic content also at 84 hours compared to the water and the other treatments. The ANOVA test showed significant differences between all the treatments (P=0.0001, F=21.46) at this time point. Total phenolic contents in ANE, oligo-chitin, oligo-chitosan, oligo-alginate and the mixture treated soybean leaves showed significant differences at 12, 60 and 84 hours in comparison to the control (Fig.21) (see Tables 11, 12, 13 and 14 in Appendix 4 for detailed information).

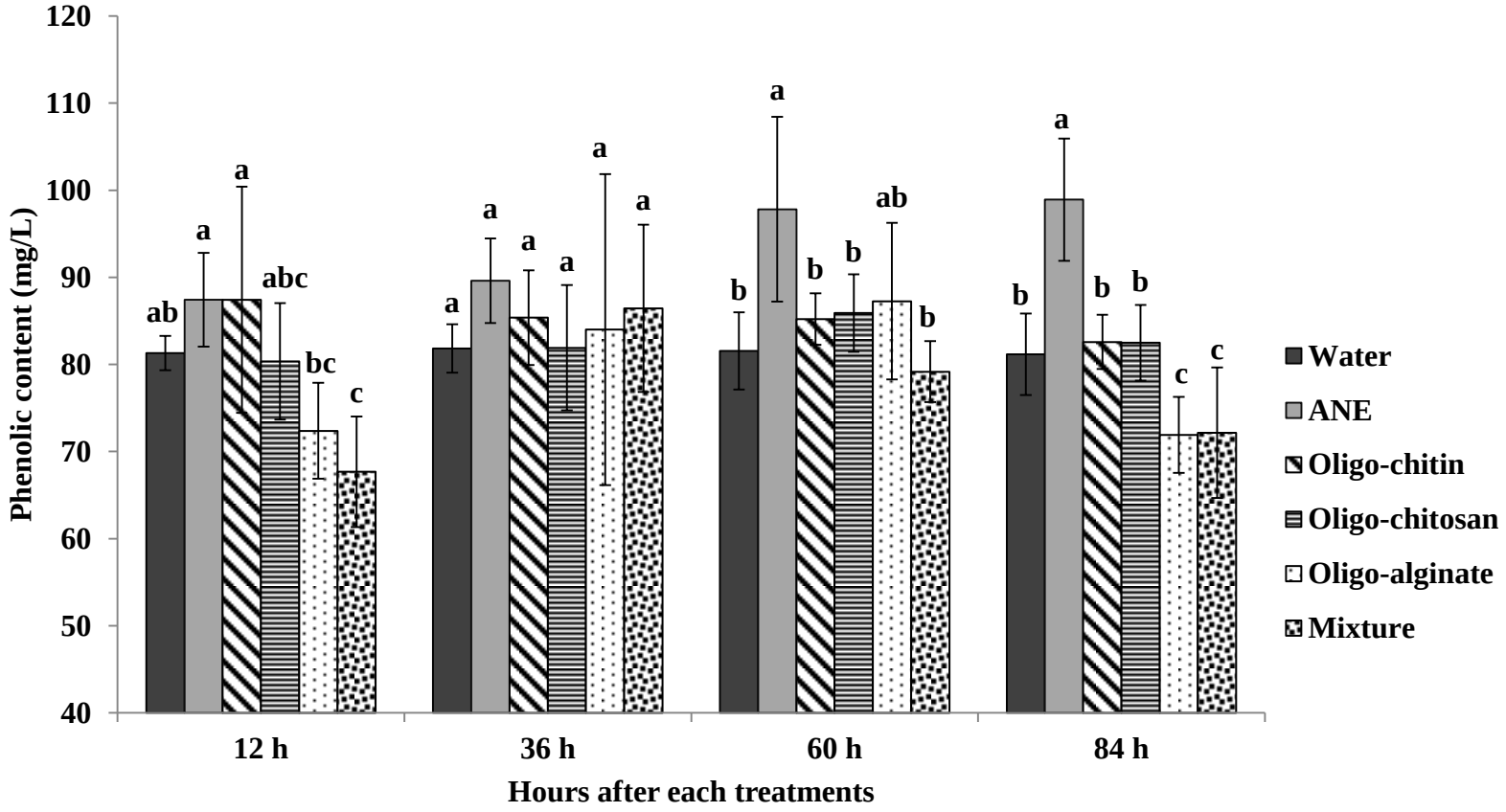


Figure 21. The phenolic content of the soybean leaves after ANE and oligosaccharide treatments at different time points. The values are means of six replicates ± standard deviation. Different letters on top of bars indicate significant different at $p \leq 0.05$ according to the Tukey's test.

5.7. Effects of ANE and oligosaccharides on chlorophyll content:

Chlorophyll contents were measured at 12 to 84 hours after the soybean leaves were sprayed with various solutions. ANE treatment was used as a positive control, and water treatment was used as a negative control. The six replicates of each treatment at a time point were tested with a normality test. The normality test showed that the data of ANE at 12h was not normal while the other treatments were normal. Since the data were not normally distributed, Kruskall Wallis analysis was used to compare the differences between all the treatments. At 12 hours, ANE and oligo-chitosan treatments showed a high level of chlorophyll compared to water and other treatments; however, analysis of Kruskall Wallis showed that the chlorophyll content for ANE and oligosaccharides treated leaves at 12 hours did not show significant differences (P=0.245, H=6.69). At 36 hours, ANE, oligo-alginate and the mixture treatments had a similar level of chlorophyll content, yet all were higher when compared to the water treatment. Additionally, at the same time, the oligo-chitin and oligo-chitosan treatments increased the chlorophyll content compared to the water and other treatments. ANOVA showed non-significant differences between the treatments at this time point but seems to have a tendency to be significant (P=0.057, F=2.44). At 60 hours, no significant differences among the treatments were detected (P=0.218, F=1.50). At 84 hours, the ANE treatment had a significantly higher level of chlorophyll content compared to all other treatments except for water (P=0.002, F=4.92). However, no significant differences were found between ANE and water treatments at this time point. Total chlorophyll content in soybean leaves treated with ANE, oligo-chitin, oligo-chitosan, oligo-alginate and the mixture (oligo-

chitin, oligo-chitosan, and oligo-alginate) showed non-significant differences at 12, 36 and 60 hours (Fig.22) (see Tables 15, 16, 17 and 18 in Appendix 4 for detailed information).

The chlorophyll content measurement was repeated at 36 hours (Fig.23) (see Table 9 in Appendix 3 for detailed information). The results showed that water, ANE, oligo-chitin, oligo-alginate, oligo-chitosan and the mixture treatments had significant differences in the chlorophyll content after the ANOVA test (P= 0.0001, F=7.69). While ANE increased the chlorophyll content compared to the water treatment as Tukey test showed, oligosaccharides had no effect on the chlorophyll content when compared to the water treatment.

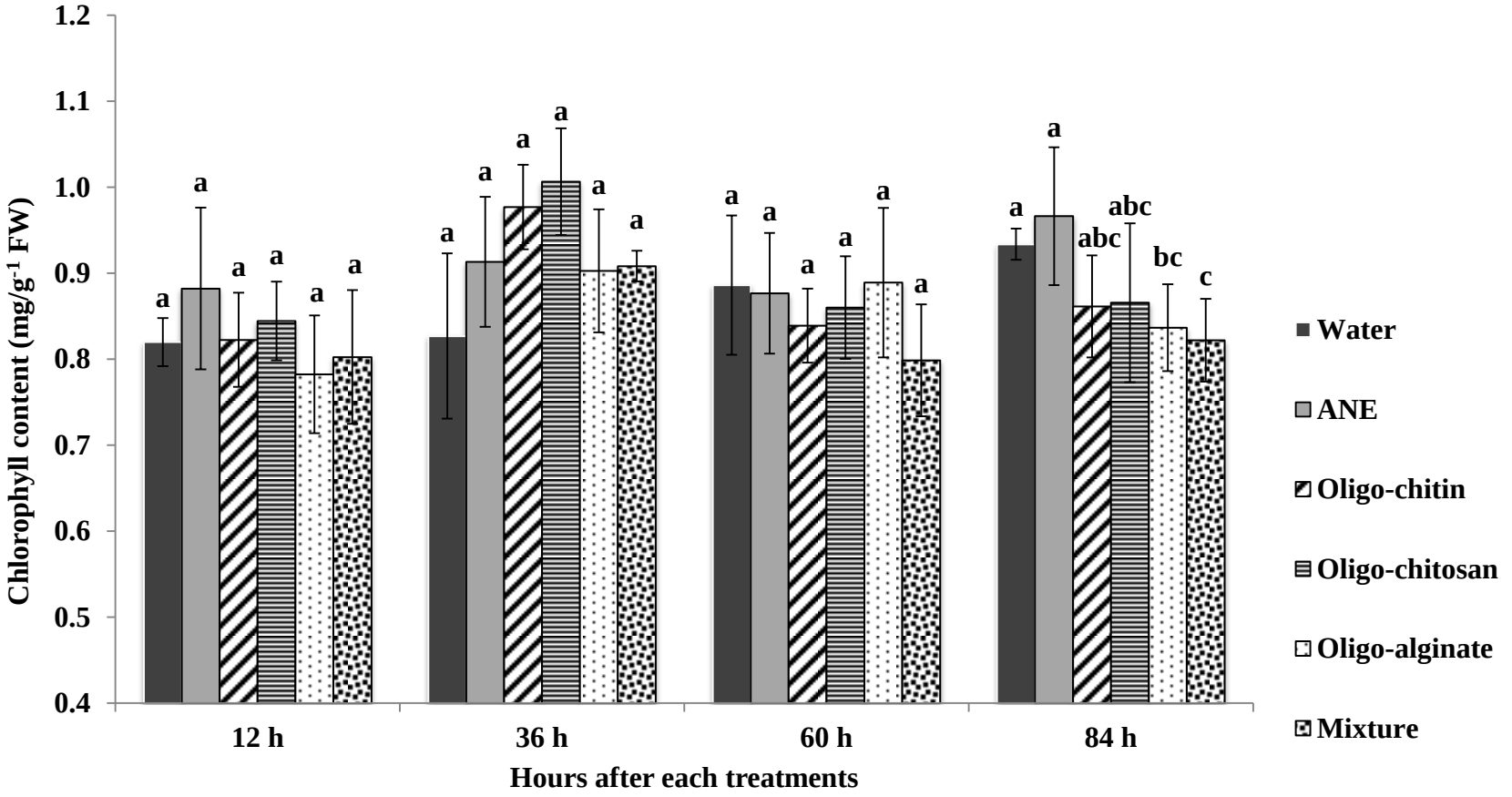


Figure 22. The chlorophyll content of the soybean leaves after ANE and oligosaccharide treatments at different time points. The values are means of six replicates ± standard deviation. Different letters on top of bars indicate significant different at $p \leq 0.05$ according to the Tukey's test.

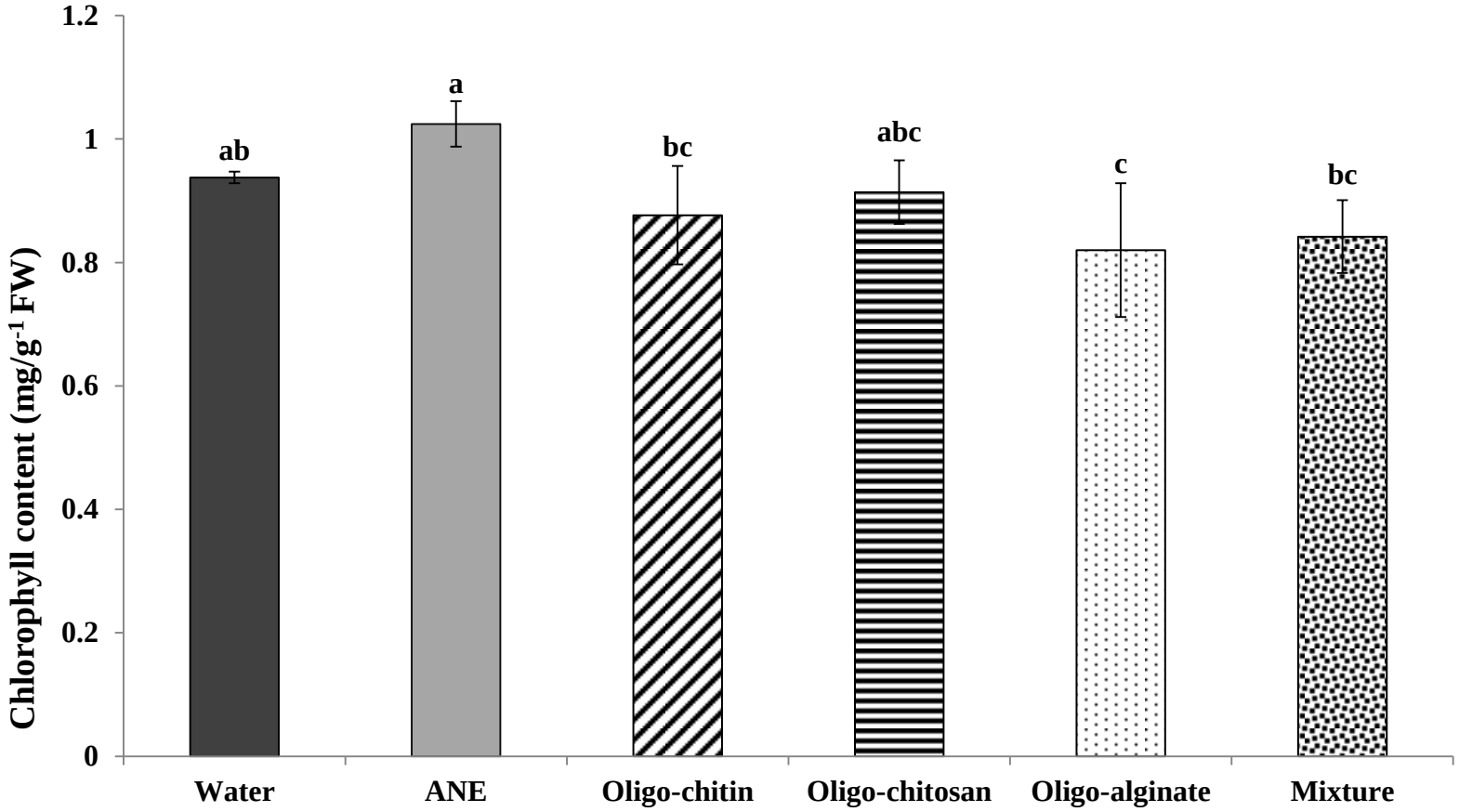


Figure 23. The repetition of measuring the chlorophyll content after ANE and oligosaccharide treatments at 36 hours. The values are means of six replicates ± standard deviation. Different letters on top of bars indicate significant different at $p \leq 0.05$ according to the Tukey's test.

6. Discussion:

As one of the seaweed extracts, *Ascophyllum nodosum* extract (ANE) has been reported to increase crop productivity and plant tolerance to various stresses at low concentartions. Most studies have focused on the effects of ANE on different plants, but there is a lack of information about its mechanisms on plant systems. The present study proposed that ANE's beneficial effects may be related to its bioactive components that hydrolyzed from the cell wall of algae and the entophytic fungus found in seaweed. This study attempted to find the effective components of ANE using soybean plants under chilling stress. The results of this study suggest that ANE and the oligosaccharides (oligo-chitosan and oligo-alginate) treatments have greater effect when the plants are under chilling stress.

6.1. The role of ANE, oligo-chitosan and oligo-alginate treatments on the recovery and survival ratio under chilling stress:

In the present study, ANE, oligo-chitosan and oligo-alginate at a concentration of 3 mL/L, 50 mg/L, and 40 mg/L, respectively, had higher recovery rates compared to the water treatment under chilling stress for 24 hours. In addition, at higher concentrations (75 mg/L and 60 mg/L) oligo-chitosan and oligo-alginate had significantly higher recovery rates after 12 days of chilling stress. However, at 3 mL/L the ANE treatment showed a very low percentage of recovery compared to the other treatments. It seems that higher concentrations of oligo-chitosan and oligo-alginate may have better effects on plants under chilling stress (Dr. Yuguang Du, Institute of process Engineering, Chines Academy of Sciences). In contrast, ANE, as one of the seaweed extracts, is bioactive at low concentrations (Crouch and Van Staden 1992).

During the recovery phase, ANE treatment has been found to induce freezing tolerance and lower the tissue damage in *Arabidopsis* thaliana (Rayirath, Benkel & Prithiviraj, 2009). A study by Zhang et al. (2009) showed that alginate, chitin and chitosan oligosaccharides enhanced the plant's growth and productivity under abiotic stresses.

The survival rate of soybean plants has been measured in this study during ten days of chilling stress. The results at different periods of time showed that oligo-chitosan and oligo-alginate treated plants were less damaged and had a higher tolerance against chilling stress as compared to the water and ANE treatments. In addition, the ANE and water treated plants exhibited a significant amount of damage. These results suggest that oligo-chitosan and oligo-alginate enhanced the plant’s defense mechanisms. Other studies have shown that the application of oligo-alginate promoted the plant growth, alleviated drought and metal stresses, and induced the plant’s defense responses (Hu et al., 2004). Alginate was capable of alleviating the side effects of drought on the *Triticum aestivum* L. (Liu et al., 2013). Oligo-chitosan treatment has also been reported to induce the defense responses in different plants, for instance by increasing the β-1, 3-glucanase and chitinase enzymes activities that are involved in the defense mechanisms (Ma, et al., 2013). These studies and the results of the present study support the hypothesis that oligosaccharides in ANE might be the effective components for enhancing plant resistance against stresses.

6.2. Effects of ANE and oligosaccharides on electrolyte leakage under chilling stress:

A decrease in the electrolyte leakage was recorded in this study after applying ANE treatment to two-weeks-old soybean plants compared to the control at 4 ^{o}C. This result suggests that ANE protects the cell membranes when a plant is exposed to chilling stress at 4 ^{o}C. It has been recorded that ANE treatment reduced the electrolyte leakage in *Arabidopsis* plants compared to water treatment after freezing stress (Rayirath et al., 2009). Chitosan has been found to affect plasma membrane and induce defense responses for cell suspensions (Amborabe, Bonmort, Fleurat-Lessard, & Roblin, 2008). This study also showed that oligo-chitosan treatment reduced the relative permeability of the plasma membranes under low-temperature stress, indicating that oligo-chitosan alleviated the chilling injury of the soybean seedlings compared to water treatment. The same observations have been noticed on maize exposed to cold stress (Li et al., 2004). In this study, oligo-alginate also reduced the damage of cell membranes in soybean leaves after chilling stress. As supportive evidence, alginate-based treatment has the ability to reduce electrolyte leakage of tomato leaves and protect them from damage caused by drought stress (Liu, 2009).

The present study showed that the treatments of ANE, oligo-chitosan and oligo-alginate at the concentrations 3 mL/L, 50 mg/L and 40 mg/L, respectively, can protect the soybean plant from chilling damage. However, chitin and mixture treatments did not show protection under chilling stress. These results suggest that ANE, oligo-chitosan and

oligo-alginate have a similar effect on the electrolyte leakage and this may suggest that oligo-chitosan and oligo-alginate work as effective components in ANE.

6.3. Effects of ANE and oligosaccharides on chlorophyll under chilling stress:

Chlorophyll content was measured 24 hours after chilling stress and compared to plants under normal condition. Oligo-chitosan treatment increased chlorophyll content significantly under chilling stress compared to the control. A similar result was reported by Zhang, Y., Zhang, M., and Yang (2015) suggests that the application of chitosan on guava fruit increased the chlorophyll content under low-temperature stress. As supportive evidence, several studies reported that oligo-chitosan increases the chlorophyll content under different abiotic stresses such as salinity and drought (Ma et al., 2012; Zeng, & Luo, 2012). This result suggests that oligo-chitosan treatment may work as an effective component in the ANE.

Additionally, ANE, oligo-chitin, oligo-alginate and the mixture treatments did not significantly increase the chlorophyll content after chilling stress. This result might be due to different species, concentrations or stress conditions which may have led to different results. Liu (2013) found that spraying oligo-alginate on wheat plants increased chlorophyll content at 96 h under drought stress. However, this study showed no effects on the chlorophyll content after oligo-alginate treatment under chilling stress, and this may be due to different stresses and spraying times because the present study used chilling as a stress and the chlorophyll content was measured 24 hours after the spraying.

6.4. Effects of ANE and oligosaccharides on the bio-contents of the soybean leaves:

Under normal condition, phenolic and chlorophyll contents were measured after the application of ANE, oligo-chitin, olig-chitosan, oligo-alginate and the mixture treatments**:**

6.4.1. Phenolic content:

In this study, an increase in phenolic content after ANE treatment at 60 and 84 hours was recorded. Similarly, recent studies have documented the positive effects of *Ascophyllum nodosum* extracts on the phenolic content of plants. ANE has been found to increase the total phenolic and flavonoid contents of spinach, onions, and potatoes (Fan, 2011; Lola-Luz, 2014). Increases in the production of different phenolics have been found to increase the plant's resistance to pathogen infection (Levine et al., 1994). Observations from the present study suggest that the application of ANE works as a stress because of its bioactive components that derived from fungus cell walls. This stress enhanced the defense system, causing an increase in the phenolic content.

While most studies measured the phenolic content under various stresses, this study measured the phenolic content under the normal condition because the application of oligo-chitin, and oligo-chitosan may act like an attack from a pathogenic fungus due to their deriving from fungal cell walls of *Ascophyllum nodosum*. Likewise, since oligo-alginate is a primary component of the brown algae cell wall, plants receive the application of oligo-alginate as a signal of danger because it induces defence responses.

However, oligosaccharides (oligo-chitin, oligo-chitosan and oligo-alginate) in this study did not increase the phenolic content under the normal condition, and that may be due to the inappropriate concentrations.

6.4.2. Chlorophyll content:

In the present study, the experiment for the chlorophyll content was conducted twice and showed different results. In the first trial, no significant differences were found in the chlorophyll content between all the treatments. However, the second experiment showed that ANE significantly increased the level of chlorophyll content compared to other treatments. The differences in these results may be due to growth condition. In the first experiment, big pots were used for the planting, each pot containing five plants. In the repeated experiment, small square pots were used, and each pot contained one plant only. This resulted in a limited space for root growth that could be stressful for the plant, thus ANE enhanced resistance to stresses by promoting plant growth. Therefore, the levels of the chlorophyll content were higher in the repeated experiment.

Seaweed extracts, regardless of the application methods, have the ability to increase photosynthetic pigments (chlorophyll content) in tomato, corn, *Phaseolus mungo* and *Salvia officinalis* leaves (Lingakumar et al., 2004; Kumari et al., 2011; Kaoaua et al., 2013). An increase in the chlorophyll contents has been found after the application of *Ascophyllum nodosum* extract (ANE) in beans, tomatoes, corn, barley and wheat (Weeraddana et al., 2012).

Oligo-chitin, oligo-chitosan and oligo-alginate treatments have also been recorded to increase plant growth and productivity probably by increasing the total chlorophyll content (Zhang et al., 2009). However, in my study there were no significant effects of oligosaccharides treatments on the chlorophyll and phenolic content in the normal condition. This might be due to the differences between the recommended oligosaccharides concentrations in the present study and the concentrations of these oligosaccharides inside the *Ascophyllum nodosum* extracts.

7. Conclusion:

This study proposed that the oligosaccharides can work as elicitors to promote plant growth and trigger the plant's innate immune responses against various stresses. In the present study, the effects of ANE on plant growth and the effects of these oligosaccharides were compared on the soybean seedlings under chilling stress. The present study found that ANE, oligo-alginate and oligo-chitosan treatments induced plant tolerance to chilling stress by decreasing the electrolyte leakage thereby increasing the recovery rate. In addition, oligo-chitosan treatment has shown the ability to enhance the plant growth by increasing the chlorophyll content under chilling stress. The present study also showed that ANE and oligosaccharide treatments enhanced the tolerance to chilling stress, and inhibited the plant growth under the normal condition. To conclude, oligosaccharide treatments seem to have better effects under stresses, and oligosaccharides might be the effective components in ANE. Therefore, finding these oligosaccharides in other sources may reduce the harmful impact of the extensive harvesting of seaweeds. Future studies should focus on using different concentrations and combinations of oligosaccharides.

8. References

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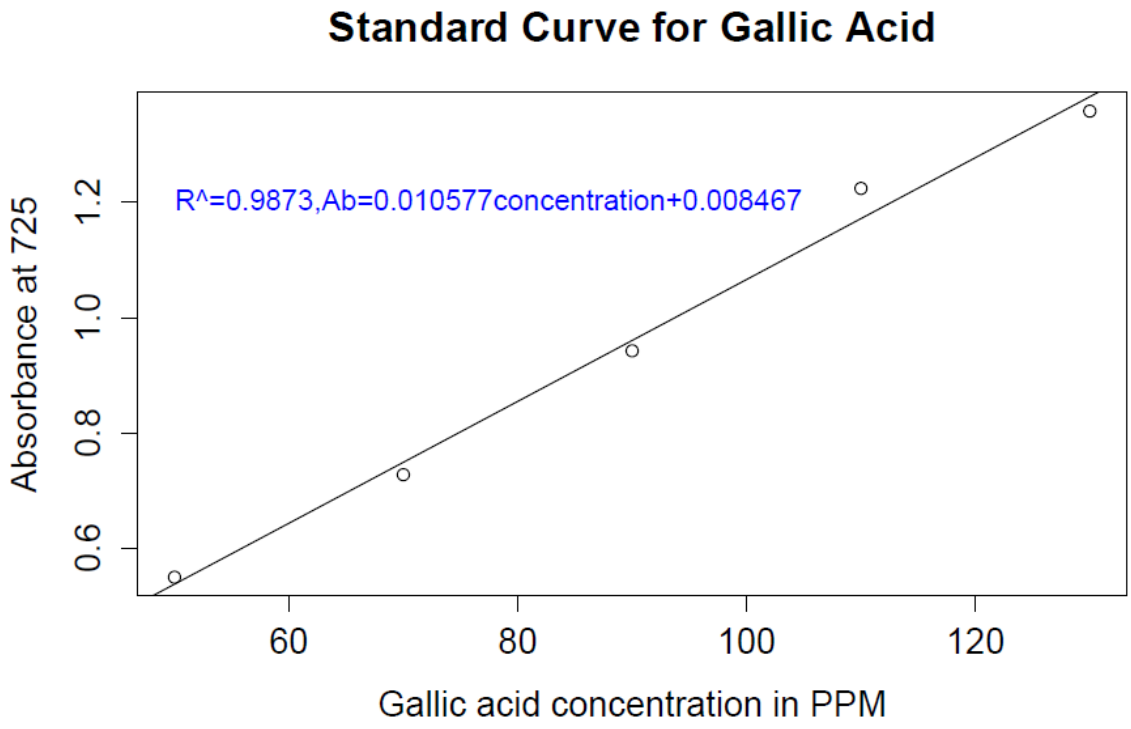
Appendix 1


Standard Curve for Gallic Acid
R^=0.9873,Ab=0.010577concentration+0.008467
Absorbance at 725
0.6
0.8
1.0
1.2
60
80
100
120
Gallic acid concentration in PPM

Appendix 2

Table 2: Kruskal-Wallis Test: Recovery ratio after 24 h chilling stress for all treatments

Treatments	N	Median	Ave Rank	Z
Alginate	4	81.25	10.0	0.73
ANE	4	81.25	9.4	0.42
Chitosan	4	87.50	12.1	1.76
Water	4	25.00	2.5	-2.91
Overall	16		8.5	
H = 9.20 DF = 3 P = 0.027				

Table 3: Kruskal-Wallis Test: Survival ratio after 3 days of chilling stress

Treatments	N	Median	Ave Rank	Z
Alginate	4	87.50	10.6	1.03
ANE	4	62.50	3.6	-2.36
Chitosan	4	100.00	14.0	2.67
Water	4	75.00	5.8	-1.33
Overall	16		8.5	
H = 11.66 DF = 3 P = 0.006				

Table 4: One-way ANOVA: Survival ratio after 5 days of chilling stress

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-value	P-value
Factor	3	9014	3004.6	11.12	0.001
Error	12	3242	270.2		
Total	15	12256			

Means

Factor	N	Mean	StDev	95% Confidence Interval for Mean	
				Lower	Upper
Water	4	50.00	22.8	32.1	67.9
ANE	4	34.4	21.3	16.5	52.3
Chitosan	4	93.75	7.22	75.84	111.66
Alginate	4	81.25	7.22	63.34	99.16

Table 5: Kruskal-Wallis Test: Survival ratio after 10 days of chilling stress

Treatments	N	Median	Ave Rank	Z
Alginate	4	6.25	14.1	2.73
ANE	4	0.00	4.4	-2.00
Chitosan	4	5.00	10.8	1.09
Water	4	0.00	4.8	-1.82
Overall	16		8.5	
H = 11.96 DF = 3 P = 0.008				

Table 6: One-way ANOVA: Recovery rate after 12 days chilling and one week for recovery in greenhouse

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-value	P-value
Factor	3	4639	1546.2	11.05	0.001
Error	12	1680	140.0		
Total	15	6318			

Means

Factor	N	Mean	StDev	95% Confidence Interval for Mean	
				Lower	Upper
Water	4	25.00	10.21	12.11	37.89
ANE	4	21.88	15.73	8.99	34.76
Chitosan	4	62.50	10.21	49.61	75.39
Alginate	4	50.00	10.21	37.11	62.89

Appendix 3

Table 7: One-way ANOVA: The electrolyte leakage before chilling stress

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-value	P-value
Factor	5	64.68	12.936	3.24	0.019
Error	30	119.65	3.988		
Total	35	184.33			

Means

Factor	N	Mean	StDev	95% Confidence Interval for Mean	
				Lower	Upper
Water	6	17.34	2.94	15.67	19.00
ANE	6	13.727	1.759	12.062	15.392
Chitin	6	14.564	0.669	12.899	16.229
Chitosan	6	16.422	1.284	14.757	18.087
Alginate	6	13.79	2.56	12.12	15.45
Mixture	6	14.718	1.876	13.053	16.383

Table 8: One-way ANOVA: The electrolyte leakage after chilling stress

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-value	P-value
Factor	5	586.0	117.20	8.72	0.001
Error	30	403.1	13.44		
Total	35	989.1			

Means

Factor	N	Mean	StDev	95% Confidence Interval for Mean	
				Lower	Upper
Water	6	25.80	4.47	22.75	28.86
ANE	6	14.671	1.149	11.615	17.728
Chitin	6	23.05	3.05	20.00	26.11
Chitosan	6	17.129	2.057	14.073	20.185
Alginate	6	16.551	1.792	13.495	19.607
Mixture	6	22.48	6.52	19.42	25.53

Table 9: One-way ANOVA: Chlorophyll content before chilling stress

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-value	P-value
Factor	5	0.1644	0.032872	7.69	0.001
Error	30	0.1282	0.004274		
Total	35	0.2926			

Means

Factor	N	Mean	StDev	95% Confidence Interval for Mean	
				Lower	Upper
Water	6	0.93772	0.00930	0.88322	0.99223
ANE	6	1.0243	0.0369	0.9698	1.0788
Chitin	6	0.8765	0.0796	0.8220	0.9310
Chitosan	6	0.9139	0.0517	0.8594	0.9684
Alginate	6	0.8200	0.1082	0.7654	0.8745
Mixture	6	0.8417	0.0590	0.7872	0.8962

Table 10: One-way ANOVA: Chlorophyll content after chilling stress

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-value	P-value
Factor	5	1.6835	0.33670	19.65	0.001
Error	30	0.5140	0.01713		
Total	35	2.1976			

Means

Factor	N	Mean	StDev	95% Confidence Interval for Mean	
				Lower	Upper
Water	6	1.0568	0.1407	0.9477	1.1659
ANE	6	0.7797	0.1828	0.6705	0.8888
Chitin	6	0.9873	0.1340	0.8782	1.0964
Chitosan	6	1.4513	0.1344	1.3421	1.5604
Alginate	6	0.8606	0.0828	0.7515	0.9698
Mixture	6	0.9216	0.0818	0.8125	1.0307

Appendix 4

Table 11: One-way ANOVA: Phenolic content at 12h under normal condition

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-value	P-value
Factor	5	1922	384.41	7.28	0.001
Error	30	1584	52.80		
Total	35	3506			

Means

Factor	N	Mean	StDev	95% Confidence Interval for Mean	
				Lower	Upper
Water	6	81.305	1.979	75.246	87.364
ANE	6	87.42	5.39	81.36	93.48
Chitin	6	87.44	12.98	81.38	93.50
Alginate	6	72.37	5.51	66.32	78.43
Chitosan	6	80.35	6.69	74.29	86.40
Mixture	6	67.67	6.35	61.61	73.73

Table 12: Kruskal-Wallis Test: Phenolic content at 36h under normal condition

Treatments	N	Median	Ave Rank	Z
Alginate	6	86.98	21.5	0.76
ANE	6	90.85	25.6	1.80
Chitin	6	85.42	18.8	0.08
Chitosan	6	80.14	12.5	-1.53
Mixture	6	84.76	18.3	-0.06
Water	6	82.83	14.3	-1.06
Overall	36		18.5	
H = 6.09 DF = 5 P = 0.297				

Table 13: Kruskal-Wallis Test: Phenolic content at 60h under normal condition

Treatments	N	Median	Ave Rank	Z
Alginate	6	83.73	19.0	0.13
ANE	6	94.10	31.7	3.35
Chitin	6	84.72	19.3	0.21
Chitosan	6	85.61	20.5	0.51
Mixture	6	79.48	7.3	-2.84
Water	6	84.10	13.2	-1.36
Overall	36		18.5	
H = 17.92 DF = 5 P = 0.003				

Table 14: One-way ANOVA: Phenolic content at 84h under normal condition

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-value	P-value
Factor	5	3122.7	624.54	21.46	0.001
Error	30	873.2	29.11		
Total	35	3995.8			

Means

Factor	N	Mean	StDev	95% Confidence Interval for Mean	
				Lower	Upper
Water	6	88.18	4.67	83.68	92.67
ANE	6	98.92	7.02	94.42	103.41
Chitin	6	82.58	3.11	78.08	87.08
Alginate	6	71.90	4.36	67.40	76.40
Chitosan	6	82.48	4.34	77.99	86.98
Mixture	6	72.17	7.49	67.67	76.67

Table 15: Kruskal-Wallis Test: Chlorophyll content at 12h under normal condition

Treatments	N	Median	Ave Rank	Z
Alginate	6	0.9069	23.3	1.23
ANE	6	0.8906	21.2	0.68
Chitin	6	0.8269	15.3	-0.81
Citosan	6	0.8663	18.3	-0.04
Mixture	6	0.8070	10.7	-2.00
Water	6	0.9099	22.2	0.93
Overall	36		18.5	
H = 6.69 DF = 5 P = 0.245				

Table 16: One-way ANOVA: Chlorophyll content at 36h under normal condition

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-value	P-value
Factor	5	0.05419	0.010839	2.44	0.057
Error	30	0.13333	0.004444		
Total	35	0.18752			

Means

Factor	N	Mean	StDev	95% Confidence Interval for Mean	
				Lower	Upper
Water	6	0.9286	0.0962	0.8730	0.9842
ANE	6	0.9131	0.0756	0.8575	0.9687
Chitin	6	0.9770	0.0491	0.9215	1.0326
Alginate	6	0.9028	0.0716	0.8472	0.9583
Chitosan	6	1.0064	0.0621	0.9509	1.0620
Mixture	6	0.90827	0.01786	0.85268	0.96385

Table 17: One-way ANOVA: Chlorophyll content at 60h under normal condition

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-value	P-value
Factor	5	0.03596	0.007191	1.50	0.218
Error	30	0.14343	0.004781		
Total	35	0.17939			

Means

Factor	N	Mean	StDev	95% Confidence Interval for Mean	
				Lower	Upper
Water	6	0.8861	0.0811	0.8285	0.9438
ANE	6	0.8766	0.0702	0.8190	0.9343
Chitin	6	0.8389	0.0431	0.7813	0.8966
Alginate	6	0.8890	0.0869	0.8314	0.9467
Chitosan	6	0.8599	0.0597	0.8023	0.9176
Mixture	6	0.7986	0.0649	0.7410	0.8563

Table 18: One-way ANOVA: Chlorophyll content at 84h under normal condition

Analysis of Variance

Source	DF	Adj SS	Adj MS	F-value	P-value
Factor	5	0.09700	0.019400	4.92	0.002
Error	30	0.11818	0.003939		
Total	35	0.21518			

Means

Factor	N	Mean	StDev	95% Confidence Interval for Mean	
				Lower	Upper
Water	6	0.93365	0.01796	0.88132	0.98598
ANE	6	0.9664	0.0802	0.9141	1.0188
Chitin	6	0.8614	0.0593	0.8091	0.9137
Alginate	6	0.8365	0.0505	0.7842	0.8888
Chitosan	6	0.8656	0.0922	0.8133	0.9179
Mixture	6	0.8219	0.0481	0.7695	0.8742