INDEXING BIOACTIVITY OF *ASCOPHYLLUM NODOSUM* EXTRACT AGAINST ABIOTIC STRESS USING BY-2 CELLS

By

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Dedicated to……

My caring parents for their understanding and supportive help

My devoted spouse, Hassan, for his unwavering love and support

My loving sister and loved brothers for being with me all the time

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Abstract

The use of active bio-stimulant components of the brown algae *Ascohphyllum nodosum* has triggered much enthusiasm in the research community. For progressive research in this field, establishment of a standardized model system to achieve consistent results while using such components was imperative. Therefore, in this study a cell culture bioassay using *Nicotiana tobacum*. L.cv Bright Yellow 2 (BY-2) cells was developed to identify the protection level that *Ascophyllum nodosum* extract (ANE) offers against abiotic stress. To standardize the experiment, growth parameters of BY-2 suspension cultures under abiotic stresses were measured. The results revealed that ANE was significantly effective on improvement of cells' dry weight and viability under freezing stress. ANE increased the membrane stability, nuclear integrity and decreased ROS formation in the cells during the stress, indicating antioxidant activity of the extract. In addition, gene expression analysis detected differential transcript levels of genes under freezing temperature.

List of Abbreviations and Symbols Used

ANE	*Aschophyllum. nodosum* Extract
BY-2	Bright Yellow 2
FAO	Food and Agriculture Organization
ROS	Reactive Oxygen Species
SOD	Superoxide Dismutase
APX	Ascorbate Peroxidase
POD	Guaiacol Peroxidase
Ψw	Water Potential
ABA	Abscisic Acid
COR	Cold Responsive
LEA	Late embryogenesis abundant
PM	Plasma Membrane
CBF	Core Binding Factor
P5CS	Pyrroline-5-Carboxylate Synthase
GSH	Glutathione Peroxidase
HSP	Heat Shock Protein
RuBisCO	Ribulose-1,5-Bisphosphate Carboxylase/ Oxygenase
H_2O_2	Hydrogen Peroxide
CAT	Catalase
Put	Putrescine
Spd	Spermidine

FAD	Fatty Acid Desaturase
GFP	Green Fluorescent Protein
KH_2PO_4	Potassium Phosphate
HSD	Honestly Significantly Different
PEG	Polyethylene Glycol
NaCl	Sodium Chloride
DAPI	4', 6-Diamidino-2-Phenylindole, Dihydrochloride
PIPES	Piperazine-N,N′-bis (2-ethanesulfonic acid)
EGTA	Ethylene Glycol Tetraacetic Acid
TCA	Trichloroacetic Acid
ME	Mercaptoethanol
BCA	Bicinchoninic Acid
BSA	Bovine Serum Albumin
RNA	Ribonucleic Acid
Cdna	Complementary DNA
RT-PCR	Reverse Transcription Polymerase Chain Reaction
Rpm	Revolutions Per Minute
H2DCF-DA	2,7 Dichlorodihydrofluorescein Diacetate
µM	micro Molar
mM	mili Molar
DNA	Deoxyribonucleic acid
PCD	Programed Cell Death
BADH	Betaine Aldehyde Dehydrogenase

DGD	Digalactosyldiacylglycerol
XyGs	Xyloglucans
FUTs	Fucosyltransferases
AGPs	Arabinogalactan Proteins
MGDG	Monogalactosyldiacylglycerol
GR	Glutathione Reductase
GST	Glutathione S-transferase
ACCase	acetyl-CoA carboxylase
PA	Phosphatidic Acid
PC	Phosphatidylcholine
GolS	Galactinol Synthase

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Chapter 1.0

Introduction

Rapid changes in environmental conditions, manifested by global warming posit serious problem in agriculture. The environmental variability imparts abiotic stresses, such as salinity, cold and drought on plants' physiology (Teixeira et al., 2013). It has been estimated that abiotic stresses lower the yield potential to less than 50%. Among the abiotic stressors, salinity affects approximately half of all irrigated land (Moghadam et al., 2013). Additionally, studies show that about 42% of land on the planet is subjected to the effect of freezing temperature, which is more critical in the Northern Hemisphere (Miura and Furumoto, 2013). On the other hand, increasing global temperature by 1.5-5.8°C will be a serious concern for agriculture in the near future (Hemantaranjan et al., 2014). In general, abiotic stresses influence the morphology, physiology and biochemistry of plant systems, leading to changes in growth and development and finally to decrease or loss of productivity (Dinari et al., 2013).

However, there have been different strategies employed, which diminish stress effects on plants. The use of traditional plant breeding and stress tolerant cultivars, practical methods to avoid stress impact, plant adaptation to stressful situations and biotechnological techniques to produce new tolerant plants could reduce the environmental stresses (Rayirath et al., 2009). Using natural products to improve productivity and decrease effect of stress is a promising practice in modern agriculture, especially in the context of harmful side effects of fertilizers and chemicals on human health and nature.

The marine brown alga *Ascophyllum nodosum* has been known to plant growers for centuries for its positive effects on growth, germination, product shelf life, yield and quality especially under stressful conditions (Nabati et al., 1994; Nabati, 1991). Commercial extracts of *Ascophyllum nodosum* (ANE) contain significant amounts of macro-nutrients, such as potassium and phosphorus, as well as micro-nutrients and plant growth regulators. These extracts are applied at very low rates in the field, suggesting that their beneficial effects are due to the presence of some organic compounds with hormone-like activity, which are able to influence plant growth at very low concentrations (Abetz, 1980; Guiry and Blunden, 1991; Verkleij, 1992).

Thesis Hypothesis and Objective

Extracts made from *Ascophyllum nodosum* have been repeatedly studied on plants, which have shown promising results and triggered much enthusiasm among the research community. However, for progressive research in the field, establishment of a standardized model system, which would conquer the variability of different growth stage and physiological differences of whole plants, while the extracts is being tested for their beneficiary effects at different concentrations, is imperative. It was hypothesized in this project that Tobacco BY-2 cell line can be used in developing robust bioassay systems to index bioactivity of *Ascophyllum nodosum* extracts. In earlier studies, ANE has been shown to protect plant against abiotic stresses (Khan et al., 2009; Rayirath et al., 2009). I, therefore, hypothesized that ANE will protect BY-2 cells under abiotic stresses. The objective of the present study was to identify the level of protection ANE offers against abiotic stresses. Therefore, a cell culture bioassay was developed using *Nicotiana tobacum*. L.cv Bright Yellow 2 (BY-2) cells.

Chapter 2.0

Literature review

2.1 Environmental factors that affect plant growth and productivity

The effects of environmental stresses on plants vary greatly depending on the species and severity of the stress. Plant responses include changes in growth, accumulation of solutes, changes in metabolism, and alternation in gene expression (Mullet and Whitsitt, 1997). In this section, abiotic stresses and their impact on plants as well as plant cells are reviewed.

2.1.1 Salt stress

Salinity affects 930 million hectares of the world's land area and this area is increasing every year (Munns, 2002). The primary effects of salt stress include ionic toxicity and hyperosmolality, causing an increase in ion concentrations to >0.4 M in the cytoplasm that interferes with activity of enzymes by altering secondary and tertiary structure of proteins (Jenks and Hasegawa, 2008). High concentrations of salt decrease water potential and turgor pressure, which generates hyperosmotic shock. The water disequilibrium between the apoplast and symplast, decreases turgor pressure and causes dehydration of the cell (Yokoi et al., 2002).

The secondary effects of salt stress include membrane dysfunction, K^+ acquisition imbalance, biochemical impairment, generation of reactive oxygen species (ROS) and program cell death (PCD) (Jenks and Hasegawa, 2008). Through secondary effects, salinity negatively influences survivability, growth and development.

However, to decrease the effect of salt stress, breeding techniques have been used for producing salt tolerant cultivars. Salinity tolerance is practical, once the genes and characters are defined by physiologists (Munns, 2002). Nevertheless, the mechanisms of salinity tolerance are complicated and many genes and interactions are involved. As a defence mechanism, plants might avoid salinity by completing the growth cycle faster (Yokoi et al., 2002). But, the defense mechanisms are often failed if the salinity is severe (Sawahel and Hassan, 2002). Therefore, mitigating salinity effects, minimizing damages and ameliorating the stress tolerance play key roles in improving plant productivity in saline environments (Huang et al., 2006).

2.1.2 Drought stress

Water availability is one of the key components of plant productivity (Jenks and Hasegawa, 2008). Lack of precipitation leads to decrease in water potential and reduction in turgor pressure, causing loss of productivity. Under drought stress, plant cell division and differentiation change resulting in negative effects on growth and development. Cellular responses to water paucity differ depending on the degree of the stress condition, the duration of stress and plant species (Mullet and Whitsitt, 1997; Shao et al., 2009).

To improve plants' productivity in water limited environments, a complementary approach, considering identification and selection of characteristics associated with drought avoidance and/or tolerance, is required (Mullet and Whitsitt, 1997). Avoiding drought stress, which enables plants to complete life cycle rapidly, is a significant strategy for survival in water limiting conditions. On the other hand, tolerance is the ability of plants to withstand an inadequate environmental condition. There are just few

species able to tolerate severe drought stress. When water availability is limited, plants experience a net loss of water from protoplasm, which leads to dehydration of cells (Jenks and Hasegawa, 2008). Drought avoiding plants attempt to change their stomatal conductance, leaf area, and leaf orientation in order to evade the stress. Tolerant plants, on the other hand, maintain their water potential (Ψw) by osmotic adjustment or elasticity of the cell wall and consequently tolerate the water deficit for a longer period. Both groups of plants mentioned above might experience permanent wilting point, where the water potential diminishes below the threshold level in which re-watering cannot recover the plants (Jenks and Hasegawa, 2008). Desiccation happens when there is a severe water deficit condition and all free water is lost from the protoplast. Desiccation tolerant plants survive the severe low water condition (Jenks and Hasegawa, 2008).

2.1.2.1 Growth is sensitive to water status

For normal growth, the cellular pressure should exceed the cell wall's yield threshold and the water potential from xylem to each growing area such as different organs and cells. On the other hand, the difference between wall's yield threshold and pressure potential is not large; besides, water potential, which helps the water movement inside plants, is small. Therefore, even small changes in water potential of the environment (soil) or a change in amount of water loss from plant organs inhibit the growth (Mullet and Whitsitt, 1997). Depending on organ, cell type and developmental stage of plant cells, cellular responses to drought varies, which result in different degrees of reduction in plant growth (Shao et al., 2009). These differences can possibly be a result of sensitivity of different plant organs to ABA accumulation in response to water deficit

as this hormone increases under water stress conditions in order for plants to tolerate the stress (Mullet and Whitsitt, 1997).

2.1.3 Heat stress

High temperature stress is defined as a temperature causing damage to the growth and development of plants (Hasanuzzaman et al., 2013; Wahid, 2007). Plants experience extensive fluctuation of temperatures during life cycle and at different seasons. For this reason, plants have developed mechanisms to overcome heat stress. As the optimal temperature range of plants is about 10°C below and above the growth optimum temperature, the exposure to the temperatures outside this range might be stressful. The actual upper temperature for temperate plants limits plant growth and distribution in certain areas. Furthermore, depending on duration and severity of the stress, developmental stage of plants and water status, plants respond differently to high temperatures, making it difficult to assess the damage caused by heat stress (Jenks and Hasegawa, 2008).

High temperature affects a large range of molecular functions and protein structures. It also influences metabolic activities. As a result, many proteins are deactivated and denatured by high temperature conditions (Jenks and Hasegawa, 2008).

2.1.4 Cold and freezing stress

Low temperature or cold stress is another important environmental factor that affect plant productivity (Sanghera et al., 2011; Xin and Browse, 2000). It influences cell division, photosynthesis, water transport, survival, growth, and yield of plants. Chilling stress results from temperatures cool enough to cause injury, but not cold enough to

freeze or form ice crystals in the tissues. Chilling occurs at temperatures between 0 to 15°C and freezing results at temperatures less than 0°C. Chilling and freezing stresses are known as cold stress (Hasanuzzaman et al., 2013). Cold temperatures influence a wide range of cellular components and metabolism depending on the intensity and duration of the stress. It has been indicated that at temperatures below 0°C, ice formation is initiated in intercellular spaces since the fluid there has higher freezing point due to its lower solute concentration. As at a certain sub-zero temperature, the chemical potential of ice is less than water, the extracellular ice formation results in water potential reduction outside the cells in order to make cell chemical equilibration. Therefore, water moves from inside the cell, intracellular space, into the intercellular spaces. Dehydration induced by freezing stress leads to severe cells damages and causes degradation of proteins (Hasanuzzaman et al., 2013; Thomashow, 1998).

In the northern hemisphere, sudden freezing is an important environmental stress factor, limiting agricultural productivity. Selecting, breeding and other methods and technologies to find freezing tolerant cultivars are essential to decrease losses and improve plant survival (Guo et al., 2006). Mechanisms of freezing tolerance reduce physical damage caused by freezing stress. Cold acclimation, for instance, stabilizes the membrane against freezing - induced damage (Thomashow, 1998). During this process, lipid composition, unsaturated fatty acids, simple sugars, sterols and cerebrosides of the cell membrane change to stabilize the membrane against freeze-induced damage (Thomashow, 1998). Therefore, to achieve freezing tolerance, exposure to non-freezing temperatures and cold-acclimation, is a practical mechanism (Yamazakia et al., 2009).

2.1.4.1 The role of plasma membrane in freezing tolerance

Plants' plasma membrane is the site for receiving signals of environmental conditions and transducing to other parts of the cells. The membrane systems are the primary site of freezing injury. Membrane fluidity is influenced by the lipid composition which can help prevent the cell damage under freezing (Jan and Andrabi, 2009). However, cellular injury varies depending on the temperature involved and plant freezing tolerance (Thomashow, 1998). Under cold stress in Arabidopsis, the relative proportion of di-unsaturated phosphatidylcholine and phosphatidylethanolamine increased while that of mono-unsaturated phospholipids, free sterols and cerebrosides decreased. Phase transition occurs in unsaturated lipids such as phosphatidylcholine and phosphatidyl glycerol. During this process, the molecules from a highly fluid liquid crystalline phase undergo a shift to a rigid gel phase, which interfere with the membrane permeability. These changes result in ion leakage across the membrane and ultimately cell dysfunction. It has been shown that transgenic tobacco plants expressing Glycerol-3-phosphate acyltransferase (GPAT) from Arabidopsis with decreased level of saturated fatty acids had higher level of trionic lipids and showed higher freezing tolerance in relation to the transgenic lines with higher level of saturated fatty acids (Pandey, 2015).

Therefore, plasma membrane (PM) responds directly to the stress, signals the membrane changes and activates signalling cascades for gene expression regulation, which maintain cellular homeostasis. Consequently, the membrane activity results in a rapid response to alleviate the stress (Barkla and Pantoja, 2011).

2.1.4.2 Role of COR and LEA proteins in freezing tolerance

Plants sense low temperatures by means of cellular changes and rigidification of the membrane that lead to calcium signalling and protein kinases activation by which downstream pathways are triggered to respond the stress (Pandey, 2015). Freezing stress activates a number of cold-inducible genes that encode dehydrins, lipid transfer proteins, late embryogenesis-abundant proteins (LEA) and transcription factors (Rayirath et al., 2009). The cold temperature regulatory pathway or cold-response pathway includes C-repeat Binding Factor (CBF), an active pathway during cold acclimation where COR, (Cold-regulated) genes are involved (Jenks and Hasegawa, 2008). Arabidopsis cold responsive genes such as COR6.6, COR15a, COR78 and homologs of LEA proteins like COR47 play role in freezing tolerance. The COR genes are up-regulated during freezing, resulting in a higher tolerance to freezing stress (Thomashow, 1998).

The CBF pathway, which generally is thought to be ABA- independent, although not completely, includes three CBF proteins, CBF1, CBF2 and CBF3. These proteins are transcriptional activators, are about 90% identical in amino acid sequence, and contain a conserved DNA-binding motif designated the AP2/ERF domain (Jenks and Hasegawa, 2008). It has been indicated that CBF genes are not expressed in plants grown at optimum temperature; but upon transferring to low temperatures, such as 4°C, the transcripts of all CBFs accumulated rapidly. Arabidopsis has two P5CS genes, P5CS1 and P5CS2, encoding pyrroline-5-carboxylate synthase; P5CS2 is a member of CBF regulon pathway (Jenks and Hasegawa, 2008). Low temperature and overexpression of CBF genes increased the level of free proline, contributing to the increase in freezing tolerance (Jenks and Hasegawa, 2008).

2.1.5 Osmotic adjustment and solute accumulation

The accumulation of osmotically active compounds is an important cellular strategy to re-establish turgor pressure. Plant cells adjust turgor pressure through uptake of solutes or breaking down the osmotically active components (Mullet and Whitsitt, 1997; Shao et al., 2009). Osmotic adjustment by accumulation of osmolytes decreases the stress effects through alleviating the secondary effects of the stress. These components are compatible with the enzyme functions even in high concentrations. For instance, in response to drought and/or salt stress, glycine-betaine, proline and polyols accumulate in the cell. Na^+ and Cl^- compartmentalize into vacuole to minimize the ion toxicity of the cell cytoplasm and help the osmotic adjustment during cell expansion through vacuolar volume changes (Yokoi et al., 2002). Amino acids and sugars are the other source of cellular osmoregulation. Stress also induces the expression of genes that encode enzymes involved in betaine and proline biosynthesis (Mullet and Whitsitt, 1997).

2.1.6 Oxidative stress resulting from exposure to abiotic stresses

Abiotic stresses induce the accumulation of reactive oxygen species (ROS), superoxide, hydrogen peroxide, and hydroxyl radicals which are amongst the most potent threat to the survival of cells. For example, both heat and cold stresses increase the accumulation of toxic products, particularly reactive oxygen species (ROS) (Hasanuzzaman et al., 2013; Mittler, 2002; Suzuki and Mittler, 2006; Yin et al., 2008). These molecules activate ROS scavengers as protective mechanisms to decrease the cellular damage (Zhu, 2001). Oxidative stress damages a wide range of biological macromolecules such as nucleic acids, proteins, carbohydrates, and lipids. In optimal

conditions, there is an equilibrium between pro-oxidant species and antioxidant defense machineries like ROS-metabolizing enzymes including catalase, glutathione peroxidase, superoxide dismutases (SODs) and APX (ascorbate peroxidase), and POD (guaiacol peroxidase) (Ashraf and Harris, 2004; Fulda et al., 2010).

In the absence of such equilibrium, oxidative stress occurs and while in most cases it is overcome by the cell's defense system, extreme conditions cause apoptotic or necrotic cell death. Oxidative stress also leads to an increase in expression of certain heat shock proteins (HSPs) which are known as protective proteins against several stresses (Fulda et al., 2010).

In addition, oxidative damage to the cells is caused by reduction in water potential, ion imbalance and cell toxicity. Salinity, for example, produced lipid peroxidation in cell suspension cultures of rice and sunflower (Davenport et al., 2003; Khan and Panda, 2002; Queiros et al., 2007). In high temperature conditions, ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO) enzyme may lead to the production of hydrogen peroxide (H_2O_2) through its oxygenase activites (Hasanuzzaman et al., 2013; Hu et al., 2008; Kim and Portis, 2004). Many studies have shown that ROS scavenging and plant stress tolerance, are directly associated with each other, under extreme temperatures (Hasanuzzaman et al., 2013; Suzuki and Mittler, 2006).

It has been reported that higher activities of catalase (CAT), ascorbate peroxidase (APX), peroxidase (POX) and SOD resulted in chilling tolerance in tomato (Zhao et al., 2009). Additionally, chilling stress reduced the activities of antioxidant enzymes, including SOD, POD, CAT and APX in *Crucos sativus*. The observed changes were

restored by exogenous application of putrescine (Put) and spermidine (Spd), which rendered the plants tolerant to chilling (Hasanuzzaman et al., 2013; Zhao et al., 2009).

2.1.7 The interconnected responses of plants to the environment

The accurate estimate of environmental effect on plant production is not easy, as the impact varies depending on the percentage of the land under stress, type of plant tissues and organs (Wang et al., 2003). Furthermore, due to reduction of arable land, water resources and increasing global warming, the control of environmental factors' effects on plants is an important task (Cramer et al., 2011; Qin et al., 2011).

Plants are complicated organisms and the complexity is even more within cells with multiple organelles, where there are interactions among genomes of different organelles as well as between different cellular regions. Thus, plants' response to the environment is extremely complex. As the earliest reactions to abiotic stress, plants inhibit their growth, reprogram gene expression, change protein synthesis or denaturation and alter metabolism (Cramer et al., 2011). There is an interconnected link among salinity, drought and extreme temperatures, which leads to similar cellular damage in plants. For instance, drought and salt stresses cause osmotic stress which results in ion distribution and cellular homeostasis. Oxidative stress resulting from drought, salinity and heat stress may induce protein denaturation. Therefore, abiotic stresses frequently activate similar signalling pathways and cellular responses including, production of certain proteins and antioxidants, as well as accumulation of compatible solutes (Wang et al., 2003). Figure 2.1 represents a schematic picture of complex response of plants to abiotic stresses.

Under drought stress, a range of physiological, cellular and molecular responses is induced to activate stress tolerance. Drought stress may co-occur with ionic and osmotic stresses. Cold stress may induce osmotic and mechanistic stress. Freezing results in cell dehydration (Amara, 2012; Gong et al., 2013). With decreasing water potential (more negative), and increasing extracellular ion concentration, in plant cells, the availability of water decreases. Therefore, the same responses occur resulting from drought, salinity and cold stress in plants. Drought and salt stress induce abscisic acid (ABA), a growth regulating hormone, leading to activation of down-stream pathways and specific genes. The activated pathways cause arrested growth and reproduction, which finally lead to an adaptive physiological response. Many abiotic stresses cause osmotic imbalance and desiccation of the cells; thus, there is an overlap between expression pattern of certain genes when a plant is under salinity, drought or cold stress. Different stress signals and ABA have common elements in signalling pathways to maintain the cellular homeostasis. Extensive changes in gene expression, activation, suppression and biochemical modulation occur in cells as a result of abiotic stress which lead to survival or death of cells and plants (Amara, 2012; Cramer et al., 2011).

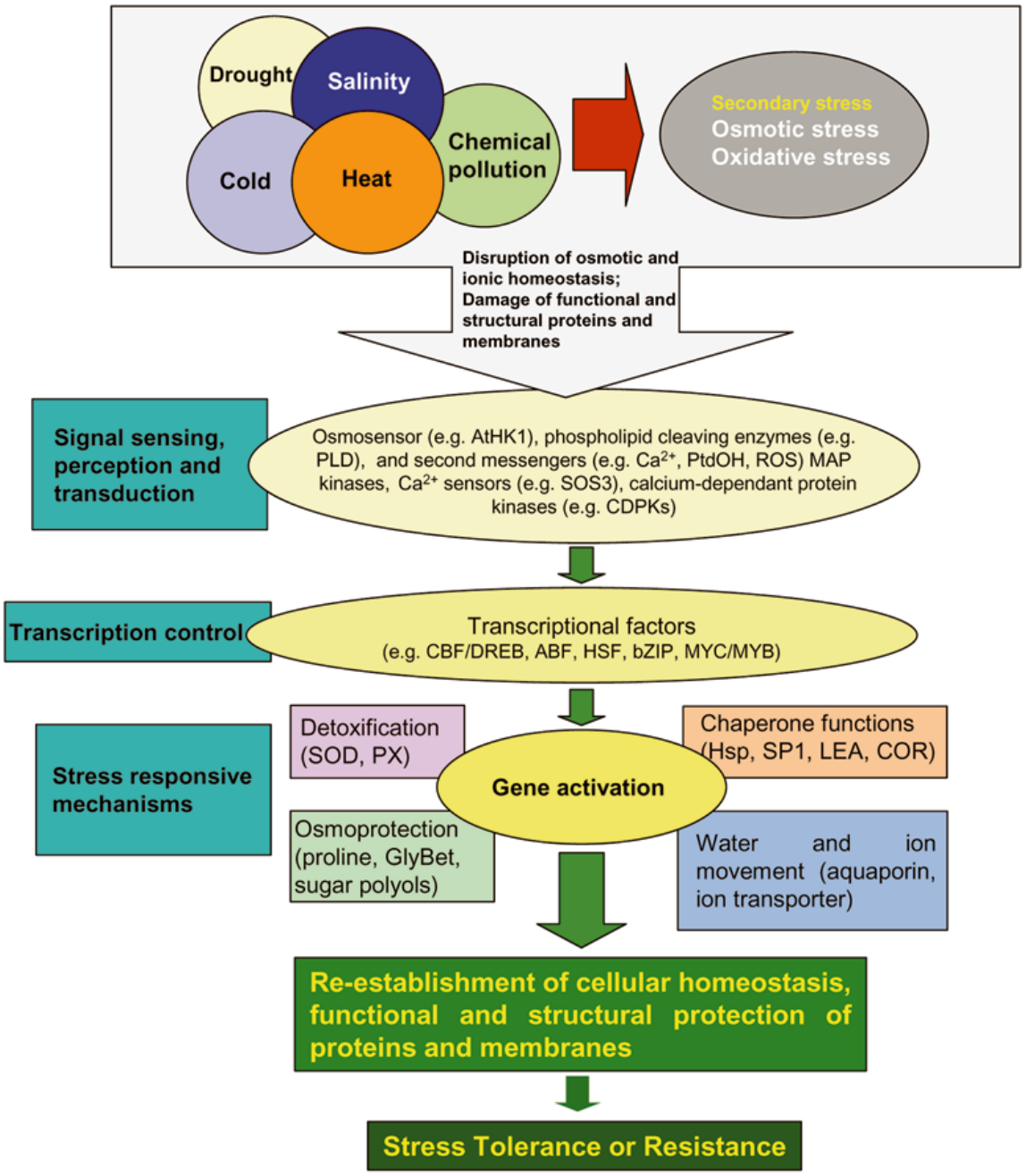


Fig 2.1. Plant response to abiotic stress (Wang et al., 2003)

2.2 Physiological changes of cells resulting from abiotic stress

2.2.1 Alternation in growth of the cell

Cell growth occurs via various processes, such as cell division, enlargement and differentiation; thus, exposure to environmental stresses affects the cell growth. Changes in physiology, morphology and biochemistry result from environmental stresses (Ben-Hayyim and Kochba, 1983; Farooq et al., 2009). Citrus callus, grown in presence of 100 mM NaCl, showed 50% loss in dry weight (Farooq et al., 2009). In another study, potato callus cultured in a medium supplemented with 150 and 200 mM NaCl did not grow (Queiros et al., 2007). On the other hand, salt tolerant potato cell lines survived salt condition, 200 mM NaCl; however, there was reduction in relative growth rate compared to controls (Queiros et al., 2007). Pear suspension cultures grown in 22°C were injured between 50 to 90% after application of heat shock of 42 and 45 ºC for 20 minutes, and were not able to regrow (Wu and Wallner, 1983). Comparing with the wild type of Arabidopsis cell line, fad2 (knockout line) and $FAD3^{+}$ (overexpressed line involved in fatty acid desaturation) lines showed different membrane lipid composition and membrane viscosity when exposed to cold temperature (Matos et al., 2007). Furthermore, the effect of drought stress on tomato cell line indicated that the cells adapted to drought stress lost the drought resistance immediately when transferred to a medium without stress (Handa et al., 1986; Wu and Wallner, 1983). Analysis of *Arabidopsis thaliana* suspension cultures under cold stress showed that decreasing the temperature from 22 ºC to 9 ºC decreased the fresh and dry weights of the cells (Matos et al., 2007). High temperature stress application on pear cells demonstrated that the cell injury and viability

was observed between 50 to 56%, depending on temperature, duration of the heat stress and cell adaptation (Wu and Wallner, 1983).

2.2.2 Cell morphology and structure changes under stress

Environmental factors increase the vulnerability of plant cells to the stress which may affect structure of the cell (McKersie and Lesheim, 2013). Cold and heat stresses, for example, induce structural changes in organelles, such as nuclear shrinking and overall loss of cell shape (Koukalová et al., 1997; Welch and Suhan, 1985). It was concluded that cadmium (Cd^{2+}), a toxic heavy metal, caused nuclear condensation and unusual structures in the nuclei of treated BY-2 cells (Ma et al., 2010). Additionally, organelles such as mitochondria and the Golgi complex, have shown changes in structure under environmental stresses (Welch and Suhan, 1985). BY-2 cells grown at a higher temperature (35°C) were spherical in shape and much shorter in terms of length, due to inhibition of cell expansion (Centomani et al., 2015).

2.2.3 Biochemical changes occurred in cell lines under abiotic stress

The metabolite content of plant cells changes under stressful condition to increase the chance of survival in that condition. Determination of protein content of plants under stress has revealed a possible association between the degree of stress and synthesis of certain proteins (Singh et al., 1985). Under heat, salinity and drought stresses, different polypeptide bands have been detected (Centomani et al., 2015; Singh et al., 1985). In potato callus grown under salt stress, levels of both soluble and insoluble proteins increased (Queiros et al., 2007).

Cell wall structure changes under stress conditions, as stress impedes growth during the cell elongation stage in which auxins play a key role (Nevins et al., 1967; Sakurai et al., 1987). Sugars, as requirements of plant growth and development, are sensed by plant systems through different pathways in which transporters, enzymes and genes are involved (Gupta and Kaur, 2005). Blaschek and Franz showed that cell wall sugar composition changes in response to different media composition, hormones and osmolarity of the medium. It has been indicated that osmotic stress caused reduction of the arabinose/ xylose ratio, increased mannose and decreased galactose in the cell wall of tobacco cells (Blaschek and Franz, 1983). Under optimum conditions, hemicelluloses and cellulose contents of the cell wall increased; however, when applying drought stress, the observed increase was reduced (Sakurai et al., 1987).

2.2.4 *Ascophyllum nodosum* (L.) Le Jol.

Ascophyllum nodosum (L.) Le Jol. is a brown seaweed inhabiting the rocky coastal regions of Atlantic Canada and Northern Europe and is well known for its tolerance to extreme temperatures, salt, and other environmental conditions (Rayorath et al., 2008; Ugarte et al., 2006). *Ascophyllum nodosum* is an economically valuable raw material used as fertilizer. Many companies around the globe harvest this algae to manufacture extracts. The extracts made from *Ascophyllum nodosum* are a combination of macro- and micro-nutrients, betaines, organic acids, alginates, polyphenols, sterols, and plant growth hormones (Abetz, 1980; Blunden, 1991). Seaweed extracts are used as foliar sprays and soil drenches and increase germination, chlorophyll content, root growth, nutrient uptake, yield, shelf life and fruit set; they also improve resistance to

biotic and abiotic stresses (Dixon and Walsh, 2002; Fornes et al., 2002; Nabati et al., 1994). It has been indicated that *Ascophyllum nodosum* extract (ANE) improved freezing tolerance in Arabidopsis. This effect was associated with increased membrane integrity, increased proline content and suppression of chlorophyllase genes (Rayirath et al., 2009). Jithesh and and coworkers showed that treatment of Arabidopsis with *Ascophyllum nodosum* extracts under salt stress condition induced many positive regulators of salt tolerant genes. The beneficial effects of seaweed extracts may be due to the presence of cytokinins (Jithesh et al., 2012). As evidenced by the fact that application of the seaweed extract had similar effects to applying kinetin to plants. Other potential compounds which enhance plant performance have been detected in fresh seaweed, including auxins, gibberellins, polyamines, brassinosteroids, alginic acid, and oligosaccharides (e.g. β-1,3-glucans, alginates and fucans) (Norrie and Hiltz, 1999; Norrie and Keathley, 2005).

2.2.5 *Nicotiana tabacum*. L. cv Bright Yellow 2 (BY-2) cells

BY-2 cells were produced from the induced callus of *Nicotiana tabacum* L. cv. Bright Yellow 2. This cell line grows fast and multiples 80 to 100 times in a week (Nagata et al., 1992).

BY-2 cells might have some specific traits of interest for research as the growth rate of the cells is highest among other known plant cell lines (Nagata et al., 1992; Terui, 1972) (Figure 2.2). BY-2 cells have been studied extensively by many researchers. Furthermore, the cell line is used to test the impact of environmental factors on the physiology of plants (Kovarik et al., 2012). The cells are amenable for microscopic analyses as well as subcellular localization of proteins via green fluorescent protein

(GFP) tagging. Cytoskeleton, organelle-targeted GFP markers and membrane compartments have been visualized in this cell line through live cell imaging (Geelen and Inzé, 2001).

The use of BY-2 cells as a plant model system speeds up the study. In addition, *Ascophyllum nodosum* extract enables the plant cells to tolerate abiotic stresses, so in this project, *Ascophyllum nodosum* extract (ANE), was applied on stress treated BY-2 cells. Thereafter, physiological and biochemical characteristics of the cells were analyzed. The results of this study may be useful towards finding the effectiveness level of *Ascophyllum nodosum* extract in stress reduction and in understanding the mechanisms by which *Ascophyllum nodosum* extract affects the plant system against stress.

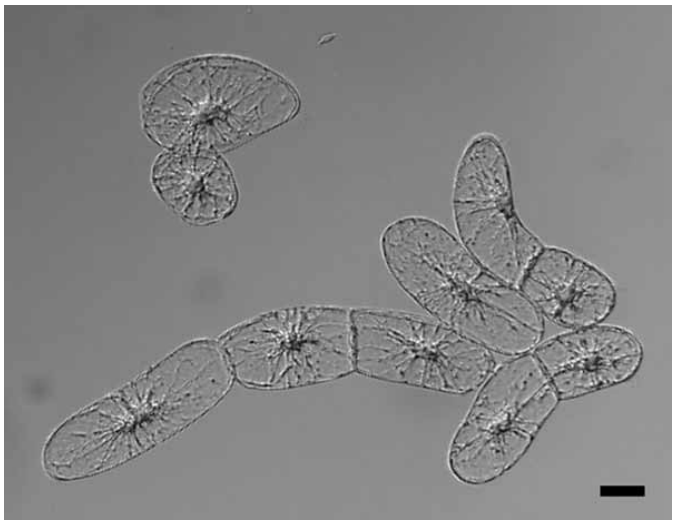


Figure 2.2 BY-2 cells under light microscope (Kumagai-Sano et al., 2007)

Chapter 3.0

Materials and methods

Nicotiana tabacum L. cv. Bright Yellow-2 (BY-2) cell line was kindly donated by Dr. Samuels' plant cell biology laboratory, Department of Botany, University of British Colombia, Canada. The powdered alkaline extract of *Ascophyllum nodosum* (ANE) was provided by Acadian Seaplants Limited, Darmouth, Nova Scotia, Canada. The extract contains organic and inorganic compounds, including alginic acid, fucose polymers, mannitol and amino acids as organic mass as well as nitrogen, phosphorous, potassium, calcium, iron, magnesium, manganese, sodium, sulfur, and zinc (Rayirath et al., 2009).

3.1 Preparation of *Ascophyllum nodosum* extract

An Aqueous solution of the extract of *Ascophyllum nodosum* was prepared as the stock solution by dissolving 0.2 g of powder of the extract in 20 mL of distilled water. The solution was filtered sterilized using 0.2 µm syringe filter (Pall corporation, USA), and stored at 4°C for further use.

3.2 BY-2 cell suspension culture

BY-2 cells were seeded in Linsmaier and Skoog liquid medium (Caisson laboratories, USA), supplemented with 2,4-Dichlorophenoxyacetic acid (2,4-D) 0.2 mg/L, thiamine hydrochloride (1 mg/L), KH_2PO_4 (370 mg/L) and myoinositol (100 mg/L). The cells were grown in 125 mL Erlenmeyer flasks under constant shaking in a shaker incubator (New Brunswick, Canada) at 130 rpm at 27°C in the dark. Cells were

sub-cultured, at a 1:15 dilution, into the fresh medium once a week (Linsmaier and Skoog, 1965; Nagata et al., 1992).

3.3 Determination of *Ascophyllum nodosum* extract (ANE) effect on the growth of suspension cells

Aliquots of the stock suspensions of BY-2 cells, in stationary phase (seventh day of growth) with density of 100,000-120,000 cells/mL, were transferred into 6-well plates (Corning incorporated, USA) containing fresh culture medium, supplemented with different concentrations of filter sterilized *Ascophyllum nodosum* extract (ANE) (0.01, 0.05 and 0.1 mg/ mL), and water served as the control. The plates were incubated in a shaker incubator set at 130 rpm at 27°C in the dark (Nagata et al, 1992). The experiment was carried out in triplicate. Figure 3.1 shows a schematic picture of one independent experiment, where ANE was used at different concentrations. Each independent experiment was repeated three times for each condition (optimum growth and/ or stressful condition). The effect of the ANE on the cell growth was assessed by measuring the cell growth, based on the cell weight; dry weight of the cells was quantified by filtration of 4 mL of the cultured cells through a filter paper (Whatman, 75 mm diameter) of known weight. The cells were dried at 60°C overnight.

Data for the growth were collected at different time points and compared with the controls; the data were analyzed using analysis of variance Tukey's HSD (Honestly Significantly Different) at $\alpha < 0.05$ in Minitab.

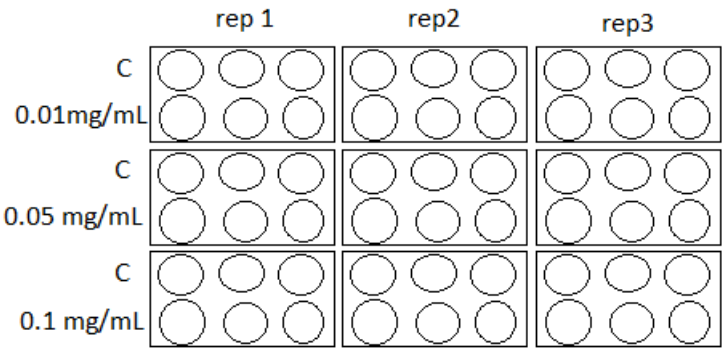


Figure 3.1. The schematic picture of the experiment conducted in 6-well plates. ANE treatment was used at three different concentrations (0.01, 0.05 and 0.1 mg/mL, and C designates "Control"). The figure displays one biological replicate, which contains three technical replicates (rep1, rep 2 & rep 3). Each replicate was included of three control and three treatment wells. There were three biological replicates in the whole experiment.

3.4 Bioactivity of *Ascophyllum nodosum* extract on BY-2 cells in response to abiotic stresses

3.4.1 Salt stress

The cells in stationary phase of growth were sub-cultured in the media supplemented with different concentrations of ANE (0, 0.01, 0.05 and 0.1 mg/mL) and were used for salt stress experiment as in figure 3.1. The culture medium was supplemented with 150 mM NaCl in shaker incubator set at 130 rpm at 27°C in the dark. Growth assessment was carried out using dry weight of the cells, at four sampling time intervals including, 24 h, 48 h, 72 h and 7 days after starting the culture. Each experiment was done in triplicate. Data for the growth were compared with the controls.

3.4.2 Drought stress

As shown in figure 3.1, the suspension culture of the cells was sub-cultured in the media containing ANE (0, 0.01, 0.05 and 0.1 mg/mL). PEG 6000 (8%) was used as a drought stressor. The cells were grown at 27°C in the condition mentioned in section 3.3.

The growth analysis was performed at different time intervals after sub-culturing the cells (24 h, 48 h, 72 h and 7 days old cells); then the cells were harvested and tested for dry weight as described in section 3.3.

3.4.3 Heat stress

BY-2 cells were seeded in the medium as described in section 3.3, where three concentrations of ANE, 0.01, 0.05 and 0.1 mg/mL, were used (Fig. 3.1). The cells grew in optimum temperature condition (27°C), and on the day three, they were transferred into an incubator set at 40°C for 5 h; after applying heat shock the cells were removed and placed in optimum temperature condition and incubated for four more days. The dry weight of the cells was measured at different time interval as described in section 3.3.

3.4.4 Cold and freezing stress

The low temperature stress was conducted on three-day-old BY-2 cells grown in the Linsmaier and Skoog medium supplemented with 0, 0.01, 0.05 and 0.1 mg/mL of ANE (Fig. 3.1). Different low temperatures (0°C, -3°C and -5°C) were used to determine the stress effect on the cells. Shortly, after sub-culturing, the cells were grown at 27°C for 3 days and then transferred to a shaker set at 130 rpm. The shaker was placed inside a low temperature incubator for 24h; the 6-well plates were then returned to the shaker incubator at 27°C, and incubated for three more days (Fig. 3.2). The cells were sampled for growth measurement at time intervals as described in section 3.4.1.

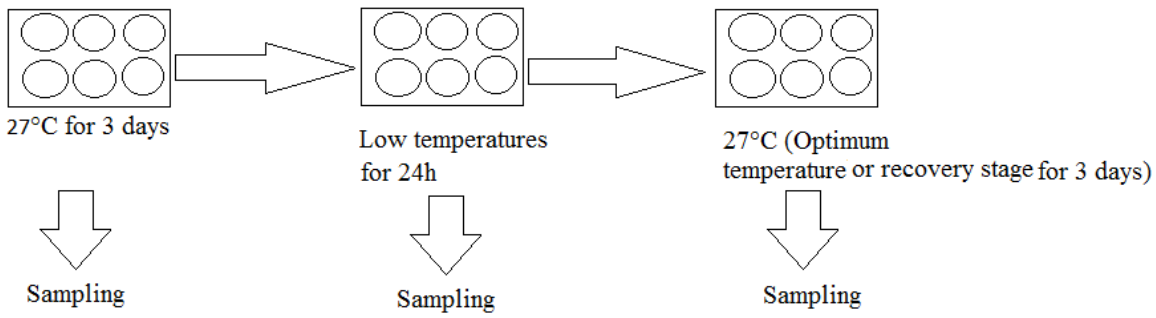


Figure 3.2. A schematic picture of the low temperature experiment. BY-2 cells were sampled before, during and after the stress at different time points.

3.5 Cell morphology screening

3.5.1 Cell viability assay

Cell viability, for the cells described in the previous section, 3.4.4, was measured by a method described by De Pinto with some modifications (De Pinto et al., 1999; De Pinto, 2002). The number of cells was counted with a hemocytometer and the viable BY-2 cells were determined by Trypan blue staining, (3,3'-[(3,3'-dimethyl-4,4'-biphenylylene) bis (azo)] bis (5-amino-4- hydroxyl-2,7-naphthalenedisulfonic acid) tetra sodium salt) using phase-contrast light microscopy (Olympus. 966400, Japan). Briefly, 20 µL of Trypan blue solution (0.4 g/L) was added into 500 µL of the BY-2 cells, in 1.5 mL eppendorf tubes, and centrifuged at 1000 rpm for 2 min. The pellet was destained with distilled water to remove the background stain and was observed under the microscope with 10 X objective lens, as previously described. 500 cells were counted in triplicate for each sample and viable cells were determined (the blue-stained cells were determined as dead cells).

3.5.2 Nile Red Staining

Nile red (5H-benzo[alpha]phenoxazine-5-one, 9-diethylamino) was applied to display possible damages to the plasma membrane (Greenspan et al., 1985). The cells under treatment of 0.1 mg/mL ANE and untreated cells (control) at -5°C were used for this experiment. Based on the staining method the fluorescent dye (Nile red) was prepared as a solution. Briefly, Nile red (Sigma Aldrich, Ontario, Canada) was dissolved in acetone (1mg/mL) and used as a stock solution. The staining solution was diluted with water to final concentrations of 10 or 100 µg/mL. For microscopic observation, the cells were stained and after destaining, by rinsing in Phosphate-buffered saline (PBS), were visualized using a fluorescent microscope (Olumpus DP80, BX63F, Japan) (Greenspan et al, 1985).

3.5.3 Nucleus staining

To analyze BY2 cell nuclei morphology, the nuclear structure was visualized under a fluorescent microscope (Olympus DP80, BX63F, Japan), using DAPI (4', 6-Diamidino-2-Phenylindole, Dihydrochloride) staining (De Pinto et al., 2002). BY-2 cells stressed at -5°C, treated with 0.1 mg/mL of ANE as well as the control cells were fixed in PEM buffer which includes 100 mM PIPES (piperazine-N,N′-bis (2-ethanesulfonic acid), pH 6.8,10 mM EGTA and 10 mM MgSO4 as well as 4% formaldehyde for 30 minutes; the cells were washed in PEM buffer containing 0.2% Triton X-100 and 1µg/mL DAPI (Sigma-Aldrich) and were observed with the microscope.

3.6 Electrolyte leakage assay

Electrolyte leakage was determined based on the method described by Liu et al. (2010). Freezing stress was conducted as described in section 3.4.4 at -5◦C; the cells under treatment of 0.01, 0.05 and 0.1mg/mL ANE and controls were sampled at six time points (0 h, 6 h, 12 h, 24 h, 48 h and 96 h) for electrolyte leakage measurement. The cells in suspension culture were used to record the conductivity (C1); the samples then were boiled for 30 min, and the conductivity was recorded after incubation (C2). The electrolyte leakage was calculated as the relative leakage in percent (C1/ C2×100) (Liu et al., 2010; Zhao et al., 2008). The experiment was conducted in triplicate.

3.7 Quantitative Real Time PCR

The mechanism of ANE on inducing freezing stress tolerance was studied through gene expression experiment. The growth condition of the cells, extract and freezing temperature were as described under freezing stress tolerance assay (3.4.4). The cell samples were collected at different time intervals, including time 0 (before applying the stress), during freezing temperature at 12 h and 24 h and after stress (during recovery stage in which the cells transferred to optimum temperature condition) 2 h and 6 h. The harvested cells were transferred into 1.5 mL microtubes, centrifuged for 2 minutes at 4°C, the media were removed, the cells were then frozen in liquid nitrogen and stored at -80°C until further use. Total RNA was extracted from frozen cells using TRIzol reagent (Invitrogen, Carlsbad, USA) according to the manufacture's instruction. The RNA concentration was measured by using Nanodrop 2000 Spectrophotometer (Thermo Scientific, Ontario, Canada). One microgram of total RNA was treated with DNase using

the Ambion® DNA-free™ kit (Burlington, Ontario, Canada), and first-strand cDNA was synthesized using the Reverse Transcription Kit (Applied Biosystem, Foster, Canada). Quantitative Real-Time PCR (qRT-PCR) was performed to evaluate the fold change in transcripts of selected genes. The data were normalized with elongation factor-1a as the internal standard. qRT-PCR was carried out in a 'Step One *Plus*® Real-Time PCR System' (Applied Biosystems, Streetsville, Ontario, Canada) using GoTaq® qPCR Master Mix (Promega, Madison, USA) adopting the manufacturer's instructions. Data were analyzed using Step One software V2.0 with ΔΔCt mode. The primer sets and the PCR conditions used for gene expression studies are listed in table 3.1.

Table 3.1. Primer sequences and key PCR parameters used in the study

Accession number **Gene name**	**Forward sequence**	**Reverse sequence**	**Product size**
KJ001143.1 Digalactosyldiacylglycerol	GGAAAAGAATGGGGCTC TTC	CACCATGAACATTGCA GACC	140bp
KM205812.1 AP2 TF SlAP2c	CTCAAAGCAAGGAAACC GAG	GGAGCAGTTTGCTTCTC AGG	120bp
KJ683765.1 Galactinol synthase 2	GAGGTACACTGGCGAGG AAG	TTGTCAGCTTCAGCATC CAC	138bp
D10524.1 Glutathione S-transferase	GATGATGCTGCTGTGAAG GA	TGGTGCAAATCAACCA GTGT	122bp
AB498916.1 Fucosyltransferase	AACGTCCCTGCAAATGTA CC	CTGCAGATTCAAAAGC CTCC	132bp
JQ654639.1 BADH1	GAAGACTGCAAGCTTGGT CC	TCTTTAAGTGCTGAGGC CGT	127bp
HM854026.1 P5CS	CTCTAGCAGTCCGAAGTG GG	TCATGCTTGAGCAATTC AGG	172bp
JQ267734.1 Acetyl-CoA carboxylase	GCGTATGGATAGCCACGT TT	GTGCTCTTTTCATGCGT TCA	122bp
AF120093 Elongation factor 1a	TGAGATGCACCACGAAG CTC	CCAACATTGTCACCAG GAAGTG	51bp

3.8 Determination of proline content

Proline content of BY-2 cells stressed at -5°C and treated with 0.1 mg/mL of ANE was determined by using L- Proline (MP Biomedical LLC, Ohio, USA) as the

standard (Fig. 3.3). The cell samples were collected at different time intervals, including time 0 (before applying the stress), during freezing temperature at 12 h and 24 h as well as 2 h and 6 h after stress (during recovery stage). The procedure was done in acidic extracts and quantified spectrophotometrically using acidninhydrin (Sigma Aldrich, Ontario, Canada) reagent (Chen and Li, 2002). In brief, 3 mL of freezing stressed BY-2 cells were harvested and washed with the media thoroughly, centrifuged at 1000 rpm and after removing the media, the fresh weight was recorded and the cells were homogenized in 5 mL of 3% aqueous sulfosalicylic acid. The extract was centrifuged at 10,000 rpm for 4 minutes; 2 mL of the supernatant was mixed with 1 mL acid-ninhydrin and 1ml of glacial acetic acid for 1 hour at 100°C, the reaction was terminated on ice and extracted with 2 ml of toluene followed by vortexing for 30 seconds. The absorbance of the toluene phase was read at 520 nm using a spectrophotometer, and toluene was used as the blank. Proline content was defined as amounts of proline present in 1 g fresh weight of the cells. The proline concentrations of treated cells were compared with controls (the cells without ANE treatment).

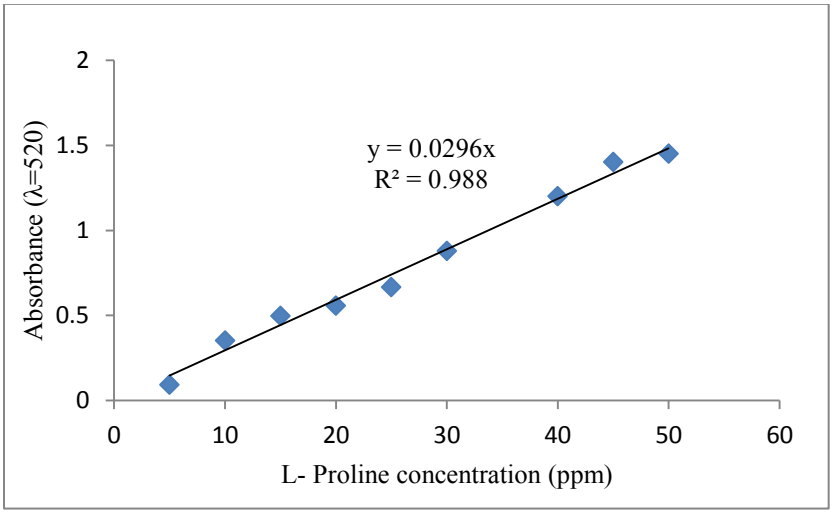


Figure 3.3. The standard Curve for L- Proline

3.9 Determination of reactive oxygen species

The Reactive Oxygen Species (ROS) generation in the suspension culture was visualized using 2,7 dichlorodihydrofluorescein diacetate (H2DCF-DA, 1 μM). The cells used for this assay were treated with 0.1 mg/mL of ANE and incubated at -5°C for 24 h. The dye was added to the culture medium after freezing stress. The cells were incubated at 27°C for 10 minutes and were visualized with the fluorescent microscope (Lyubushkina et al., 2014). Fluorescence intensity of the cells was an indicator of ROS production and program cell death.

Chapter 4.0

Results

4.1 Effect of *Ascophyllum nodosum* extract on BY-2 cells grown in optimum temperature condition

In order to find out whether *A. nodosum* extract affects the cell growth in optimum condition of growth, 27°C, BY2-cells were grown on the media with different concentrations of ANE (0, 0.01, 0.05 and 0.1 mg/mL), and followed by cells' dry weight determination. Treatment with ANE did not increase the dry weight of the cells when compared with cells in control. In fact, the highest concentration of ANE, 0.05 and 0.1 mg/mL, decreased the growth and the difference in growth was statistically significant when compared with the control ; however, the cells treated with 0.01 mg/mL, the lowest ANE concentration, showed statistically similar growth with the cells in control condition (Fig. 4.1).

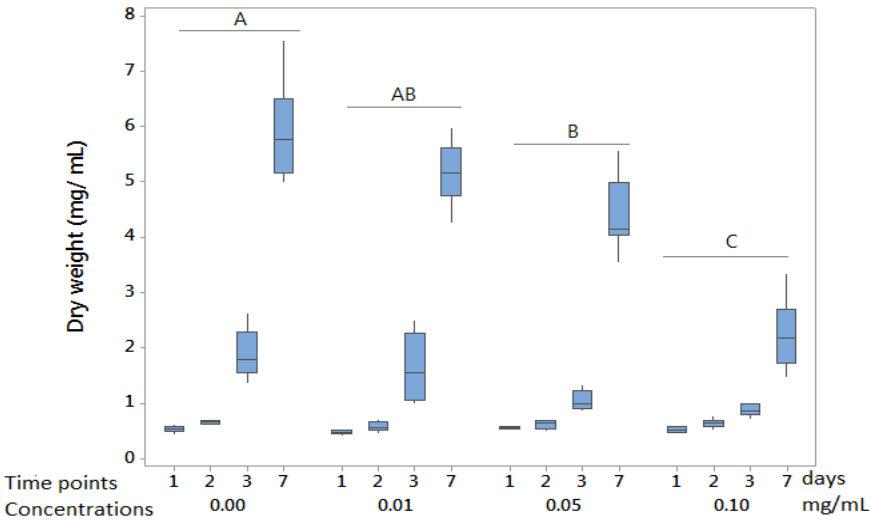


Figure. 4.1. Effect of different concentrations of *Ascophyllum nodosum* extract (ANE) on dry weight of BY-2 cells grown at 27°C, optimum temperature condition; the different letters represent significant difference among the concentrations of ANE at $\alpha < 0.05$.

4.2 *Ascophyllum nodosum* extract's effect on the growth of BY-2 cells in response to salt stress

To investigate the effect of ANE on mediating salt stress, BY-2 cells were treated with 150 mM NaCl, and the cells growth was measured. The results indicated that treatment of ANE, at least, at the concentrations used in this study (0, 0.01, 0.05 and 0.1 mg/mL), did not protect the cells under salt stress when compared with the control. The growth of treated BY-2 cells was statistically similar in all treatments but between the control and 0.1 mg/mL ANE; the highest concentration of ANE inhibited the cell growth as the cells showed minimum dry weight compared with the control and the other two ANE treatments (Fig. 4.2).

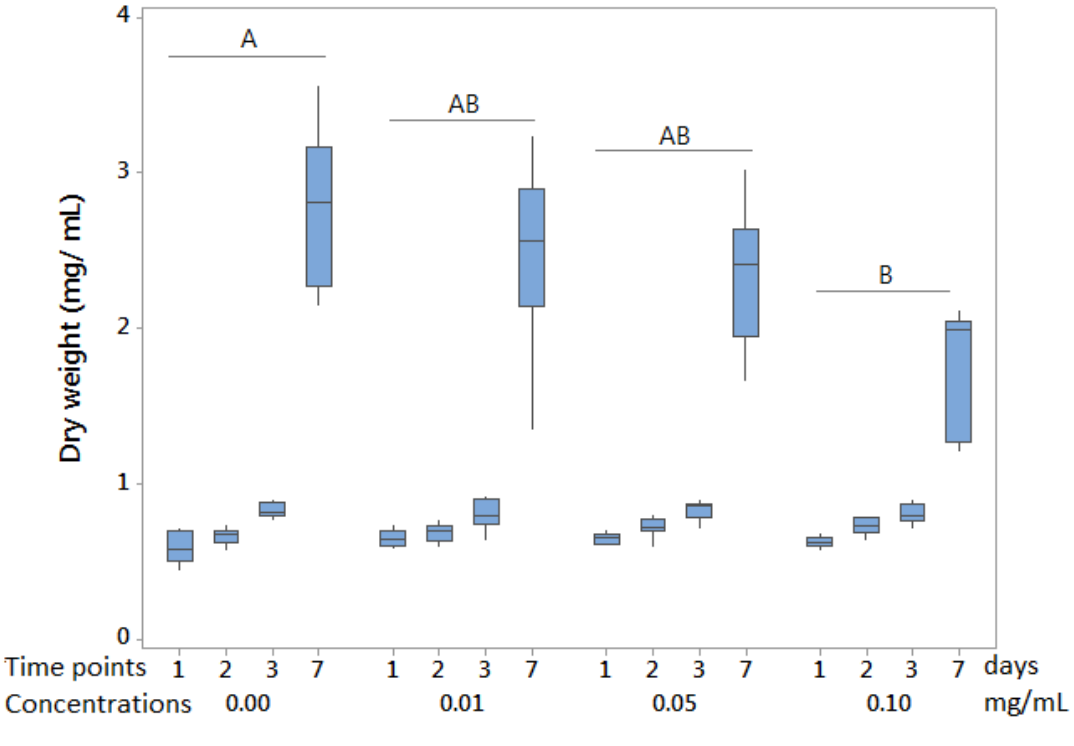


Figure. 4.2. Effect of different concentrations of *Ascophyllum nodosum* extract (ANE) on the dry weight of BY-2 cells under 150 mM NaCl salt stress; the different letters represent significant difference among the concentrations of ANE at $\alpha < 0.05$.

4.3 Effect of *Ascophyllum nodosum* extract on BY-2 cells grown under drought stress

The effect of ANE on drought stress tolerance was determined by BY-2 cells grown in the medium supplemented with 8% of PEG6000. The maximum dry weight resulted from the cells in control condition, without ANE treatment. The cells treated with 0.01 and 0.05 mg/mL of ANE showed similar dry weight to the control. Nonetheless, 0.1 mg/mL ANE led to reduction in dry weight of the cells when compared with the control. In addition, there was not a significant difference in dry weight of cells between the treatment of 0.05 and 0.1 mg/mL ANE (Fig. 4.3).

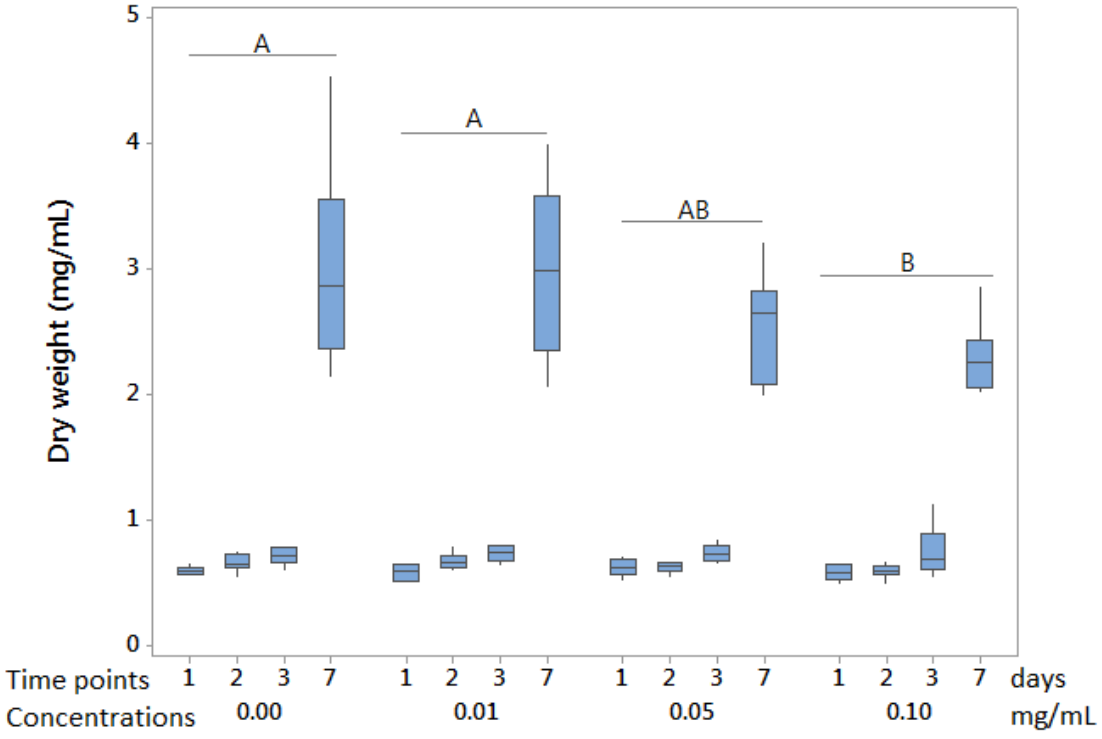


Figure. 4.3. Effect of different concentrations of *Ascophyllum nodosum* extract (ANE) on dry weight of BY-2 cells under drought stress, PEG 8%; the different letters represent significant difference among the concentrations of ANE at $\alpha < 0.05$.

4.4 Effect of *Ascophyllum nodosum* extract on BY-2 cells' growth in response to heat stress

Heat shock (40°C) was applied on three days old cells to identify the influence of ANE on heat stress tolerance. The dry weight of the cells treated with 0.01, 0.05 mg/mL of ANE was not statistically different from the control cells, without ANE. Nevertheless, BY-2 cells treated with 0.1 mg/mL of ANE exhibited less dry weight than other treatments and this difference was significant in comparison with the control (Fig. 4.4).

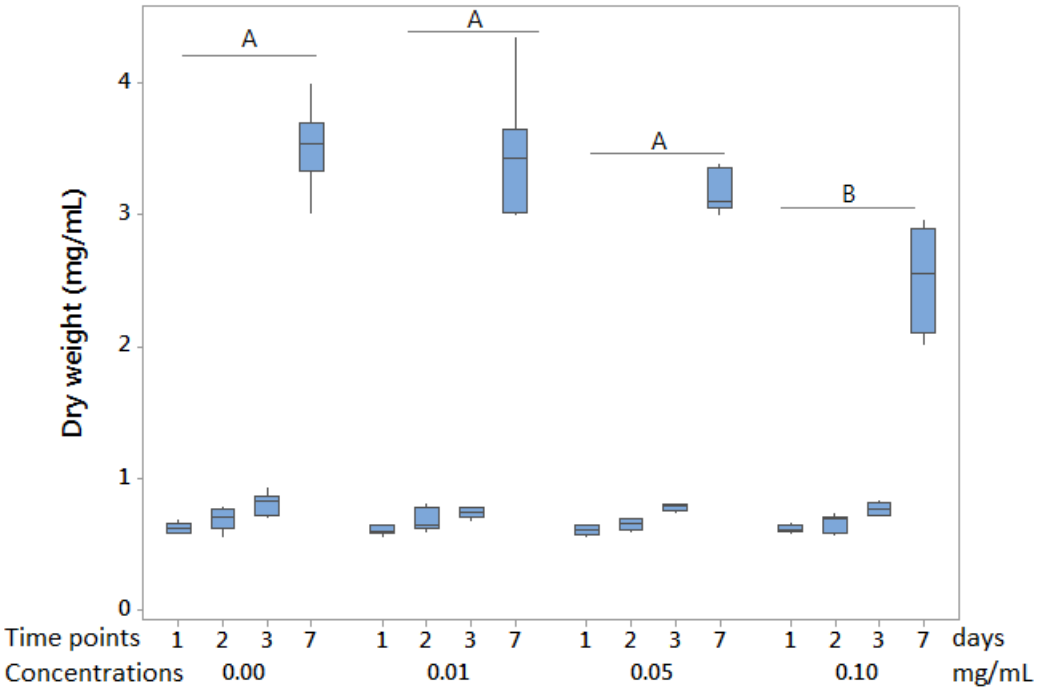


Figure. 4.4. Effect of different concentrations of *Ascophyllum nodosum* extract (ANE) on dry weight of BY-2 cells under heat stress (40 °C for 5 hours); the different letters represent significant difference among the concentrations of ANE at $\alpha < 0.05$.

4.5 Effect of *Ascophyllum nodosum* extract on BY-2 cells under cold and freezing stress

Ascophyllum nodosum extract had a significant, dose-dependent effect on BY-2 cells' growth under freezing stress (-5°C). When the cultures were treated with 0.1 mg/mL ANE, the cells' dry weight was higher comparing with other treatments and the control; however, there was not a significant difference between 0.05 and 0.1 mg/mL ANE treatments in terms of cells dry weight (Fig. 4.5c). ANE increased the dry weight of BY-2 cells exposed to other freezing temperatures (0°C, -3°C); nonetheless, the difference was insignificant statistically (Fig. 4.5 a-b).

Despite the expectation, the highest concentration of ANE (0.1 mg/mL) did not show negative effect on the dry weight of BY-2 cells (Fig. 4. 5 a). The cell growth measurement in treatments at 0°C and -3°C did not show a significant improvement at of ANE treatments (0.01, 0.05 and 0.1 mg/mL) than the control (Fig. 4. 5 b). Lowering the temperature to -5°C, caused the growth increase of the cells at 0.1 mg/mL concentration of ANE, while the cells in control condition and under 0.01 mg/mL ANE treatment showed almost no growth (Fig. 4. 5 c). The cell viability test revealed that ANE imparted tolerance against freezing-induced damage in BY-2 cells (Fig. 4. 6).

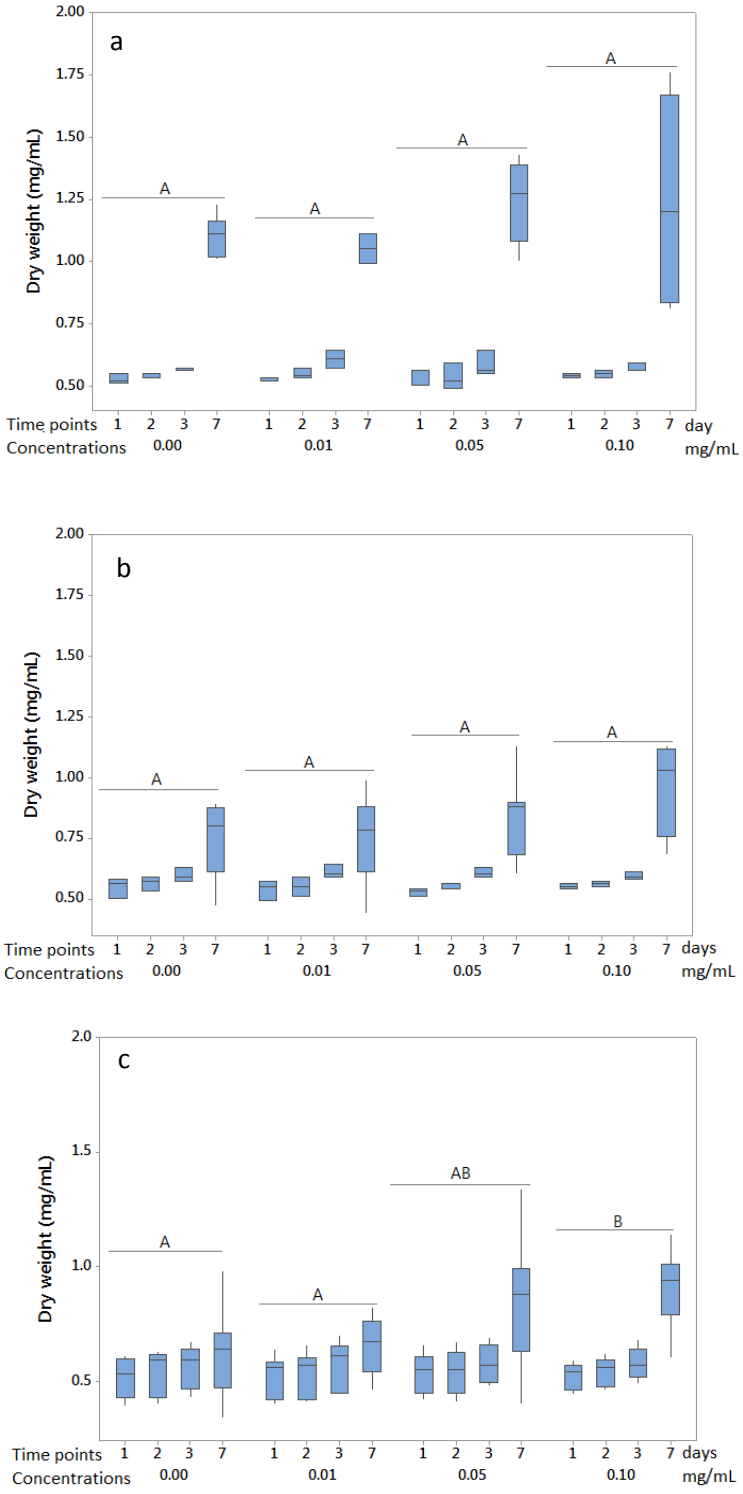


Figure. 4.5. Different concentrations of *Ascophyllum nodosum* extract (ANE) induced freezing stress tolerance on BY-2 cells (a-c). Figures a to c show dry weight of cells stressed at 0°C, -3°C and -5°C respectively. The different letters represent significant difference between the concentrations of ANE at $\alpha < 0.05$.

4.6 Freezing damage quantification by viability test

The cell viability test was conducted using Trypan blue. In all three temperatures used in the present study (0°C, -3°C and -5°C), there was a significant reduction in the cell viability in the day after stress, the day four (Fig. 4.6). The cells in the media treated with different concentrations of ANE are presented in Fig. 4.7. As it is shown, the cells with blue color represent the dead cells. The viability test showed that ANE treatment protected the cells from freezing-induced damages; based on the viability test, freezing stress resulted in high cell damage where there was no ANE treatment (the control) or when ANE was low in concentration (0.01 mg/mL).

The results demonstrated that freezing stress at 0°C reduced the cell viability similarly in the control and cells treated with 0.01 and 0.05 mg/mL ANE; there were about 60% viable cells under the mentioned conditions. On the other hand, in the treatment of 0.1 mg/mL ANE, the cell viability was 50%.

The cell viability was 81% in the cultures supplemented with 0.1 mg/mL ANE when exposed to freezing stress at -3°C; the controls and treatment of 0.01 mg/mL ANE showed less viable cells (30- 40%) (Fig. 4.6).

The result of cell viability test showed that freezing temperature at -5°C resulted in 77% cell viability at higher concentration of ANE (0.1 mg/mL). However, control cells and the cells treated with 0.01 mg/mL ANE displayed only a small number of live cells (< 20%) (Fig. 4.6 and 4.7 c).

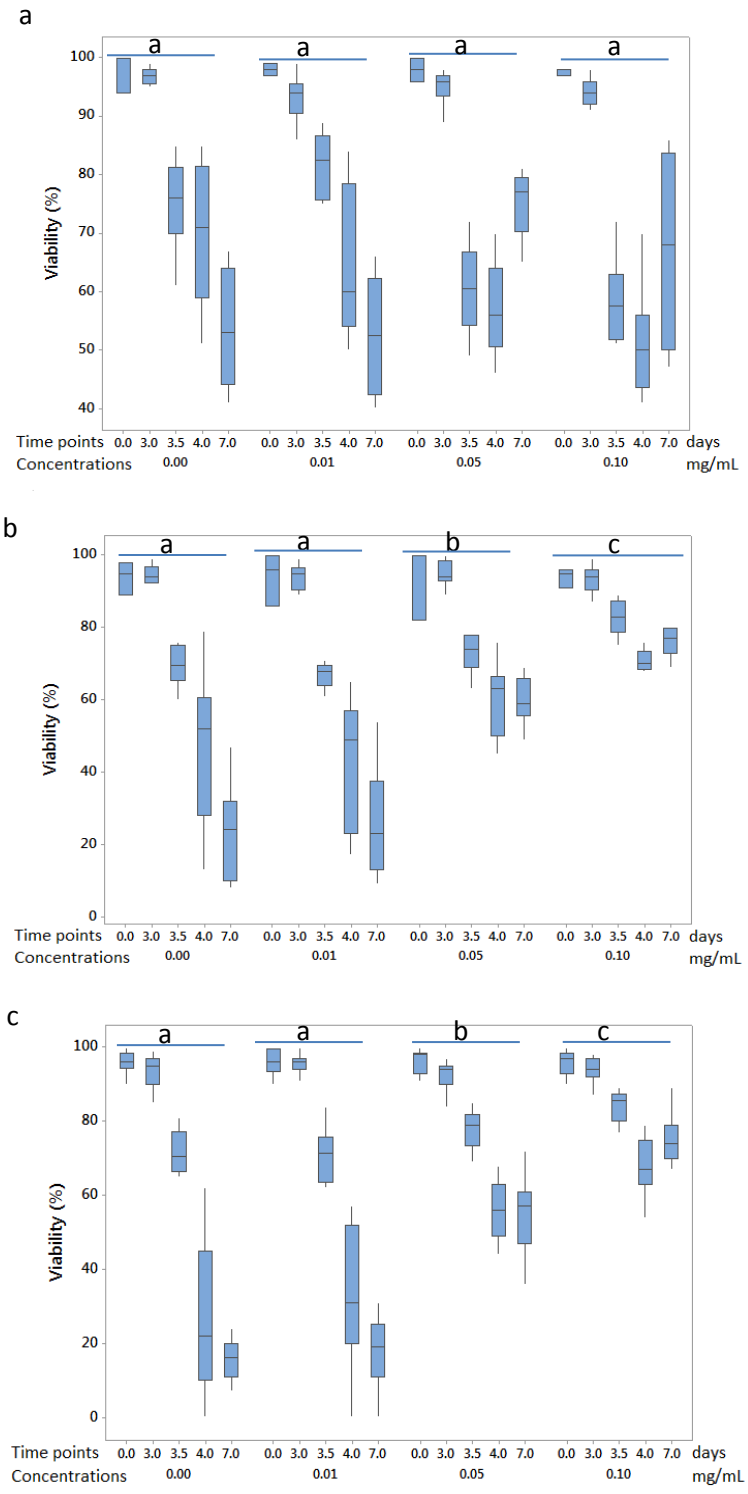


Figure. 4.6. *Ascophyllum nodosum* extract (ANE) effect on BY-2 cell damage induced by freezing stress (a-c), revealed by Trypan blue staining. The viability of cells supplemented with 0, 0.01, 0.05 and 0.1 mg/mL of ANE at 0°C, -3°C and -5°C. different letters show significant difference among ANE concentrations at $\alpha < 0.05$.

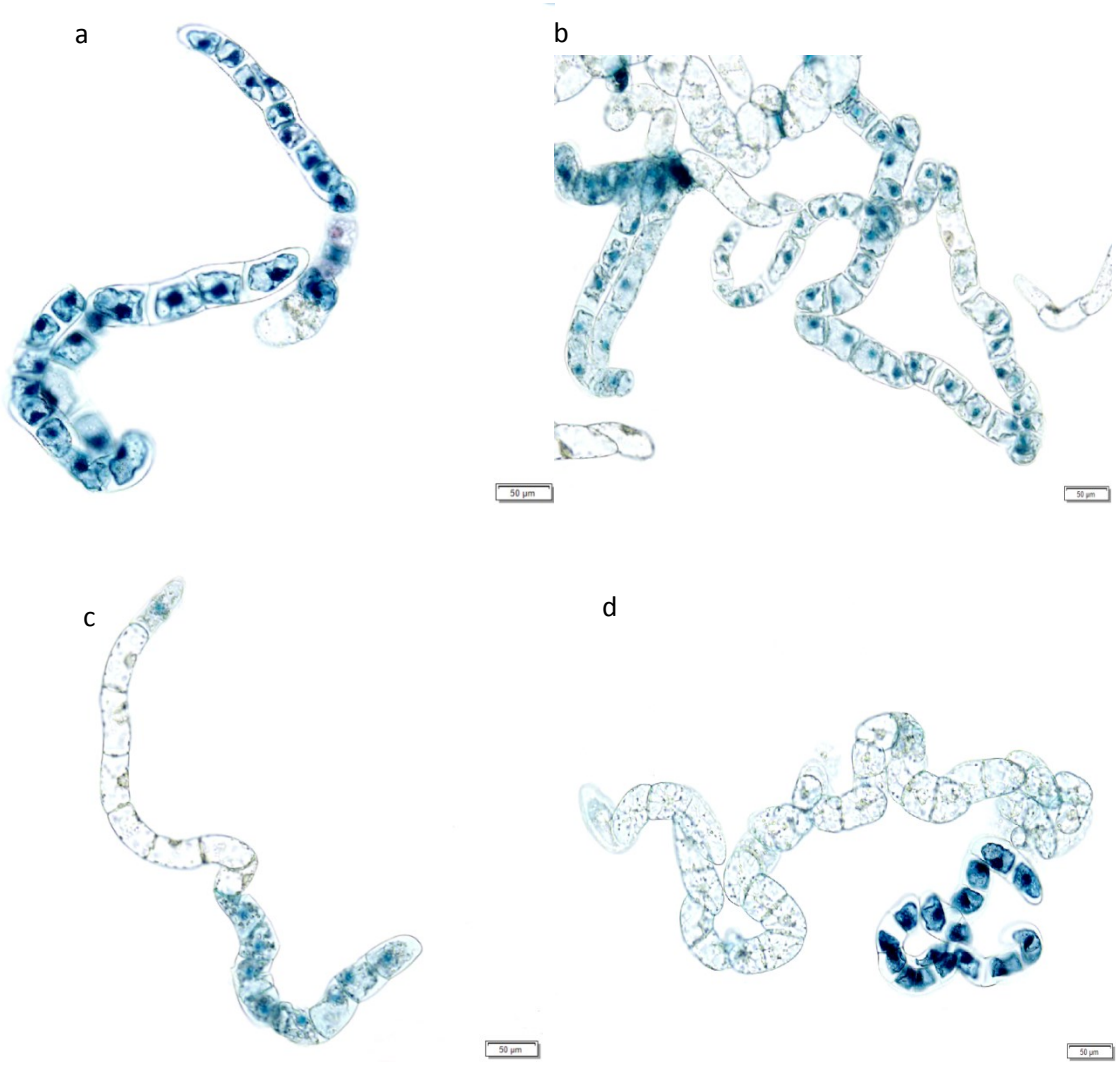


Figure. 4.7. *Ascophyllum nodosum* extract (ANE) effect on BY-2 cell damage induced by freezing stress using by Trypan blue staining. The viability of cells supplemented with 0, 0.01, 0.05 and 0.1 mg/mL, a-d, respectively, of ANE at -5°C. The cells in blue color are dead cells as the stain enters the damaged cells through pores and unhealthy membranes.

4.7 *Ascophyllum nodosum* extract mediated cell membrane integrity under freezing stress

Freezing induces oxidative stress, causing membrane injury and ion leakage to the extracellular space (Zhao et al., 2008). To identify the effect of ANE on imparting freezing-induce damages to the cell membrane, Nile red staining and electrolyte leakage test were conducted.

The BY-2 cells stained with Nile red were observed under fluorescent microscope. It was indicated that ANE treatment caused the cells to maintain the plasma membrane integrity and the cells showed lesser injury and disruption; whereas, the controls exhibited more injury and membrane lipid droplets (Fig. 4.8).

Treatment of BY-2 cells with ANE reduced the freezing-induced ion leakage when compared to the control (Fig. 4.9). In treated cells, the percent of leaked ions varied at different concentrations of *Ascophyllum nodosum* extract. The highest concentration of extract (0.1 mg/mL) was the most protective treatment in terms of reduction in ion leakage. In this treatment, the cells' ion leakage was 5%, 5%, 11%, 23% 32% and 37% at different time intervals (0 h, 6 h, 12 h, 24 h, 48 h and 96 h) respectively. However, the leakage was higher for the respective intervals in other treatments (0.01 mg/mL and 0.05 mg/mL) and the control (without ANE treatment).

Figure. 4.9 also shows that electrolyte leakage increased by increasing the exposure time to freezing stress. The highest percent of leakage was recorded in recovery phase at 96 hours after freezing stress. Interestingly, the ion leakage in the control as well as the cells treated with 0.01 mg/mL of *A. nodosum* extract increased by 65%. The cells

treated with 0.5 mg/mL ANE showed 53% leakage while 37% was observed in treatment of 0.1 mg/mL ANE.

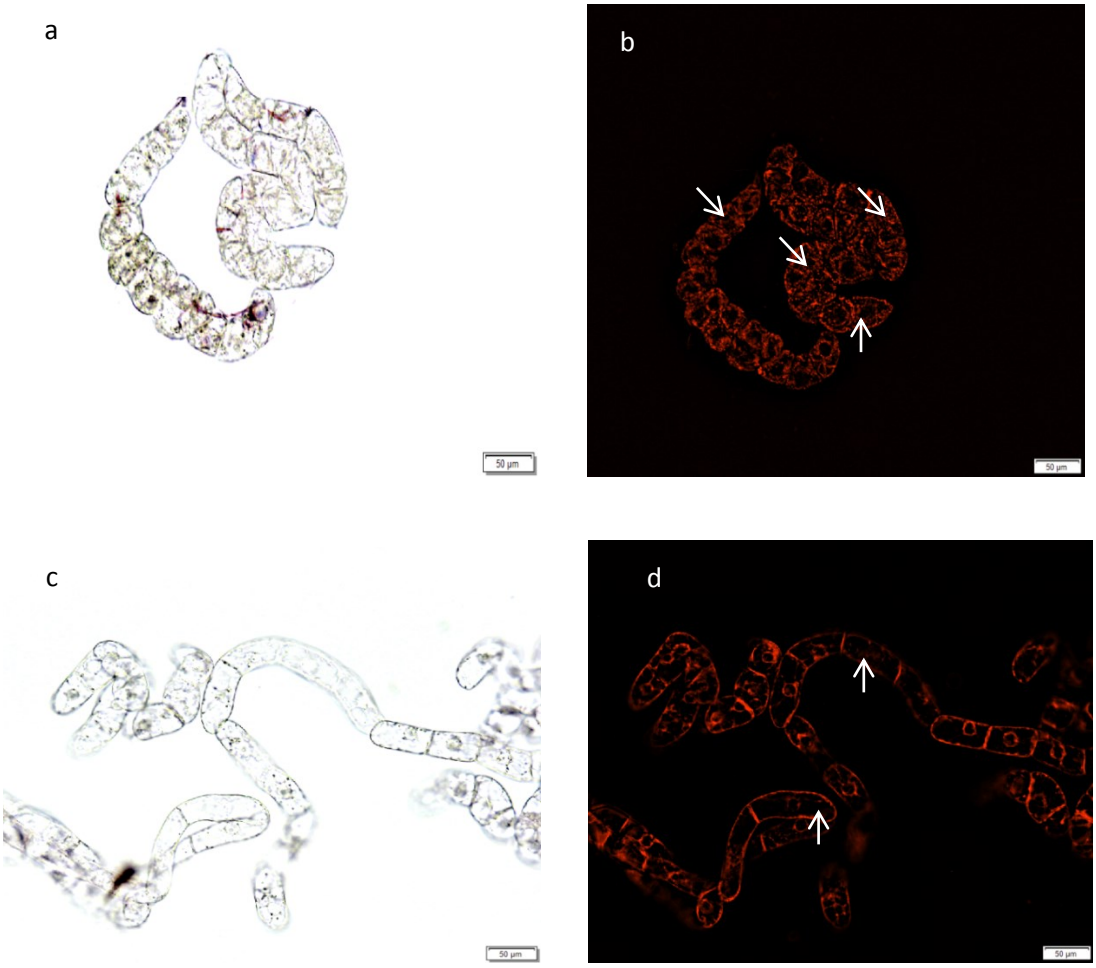


Figure. 4.8. ANE protects the BY-2 cell membrane integrity under freezing stress at -5 °C. Freezing induced membrane damage in cells without ANE treatment after freezing stress, bright field and fluorescent images (a& b). BY-2 cells treated with 0.1 mg/mL ANE after freezing stress (b&c). The arrows show the lipid droplets which are more obvious in the controls.

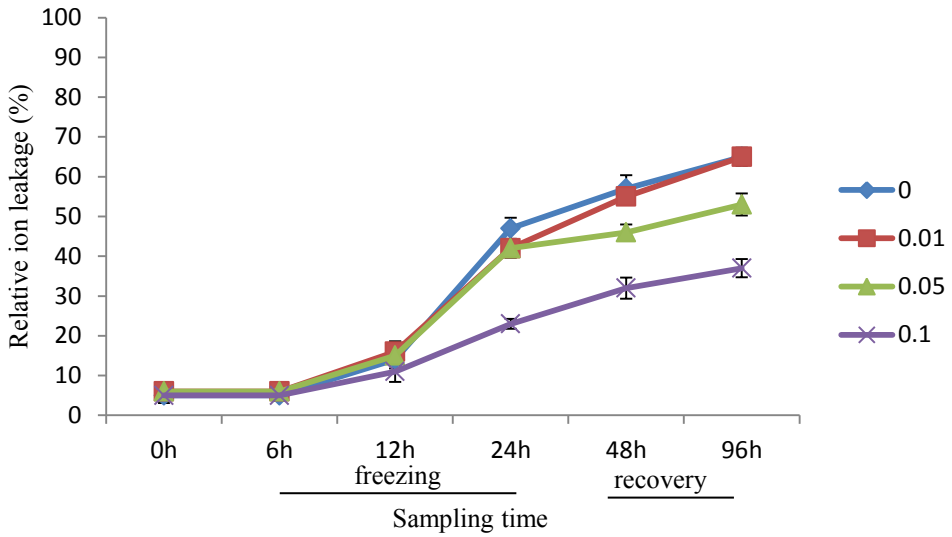


Figure. 4.9. Ion leakage changes in BY-2 suspension cultures treated with *Ascophyllum nodosum* extract. The cells were treated with three concentrations of ANE, 0.01, 0.05 and 0.1 mg/ mL, and the ion leakage was determined at different time points before, during and after freezing stress at -5°C. The bars represent standard error.

4.8 Nucleus staining

The morphology of nuclei was studied by staining the cells with DAPI, which binds to the DNA as a fluorescent dye. Under freezing stress, the nuclei in cells treated with ANE were mostly large, round in shape and showed high fluorescent intensity. In controls, on the other hand, chromatin was more condensed, irregular, sharply circumscribed and concentrated either at the nuclear membrane or on one side of nucleus (Fig. 4.10).

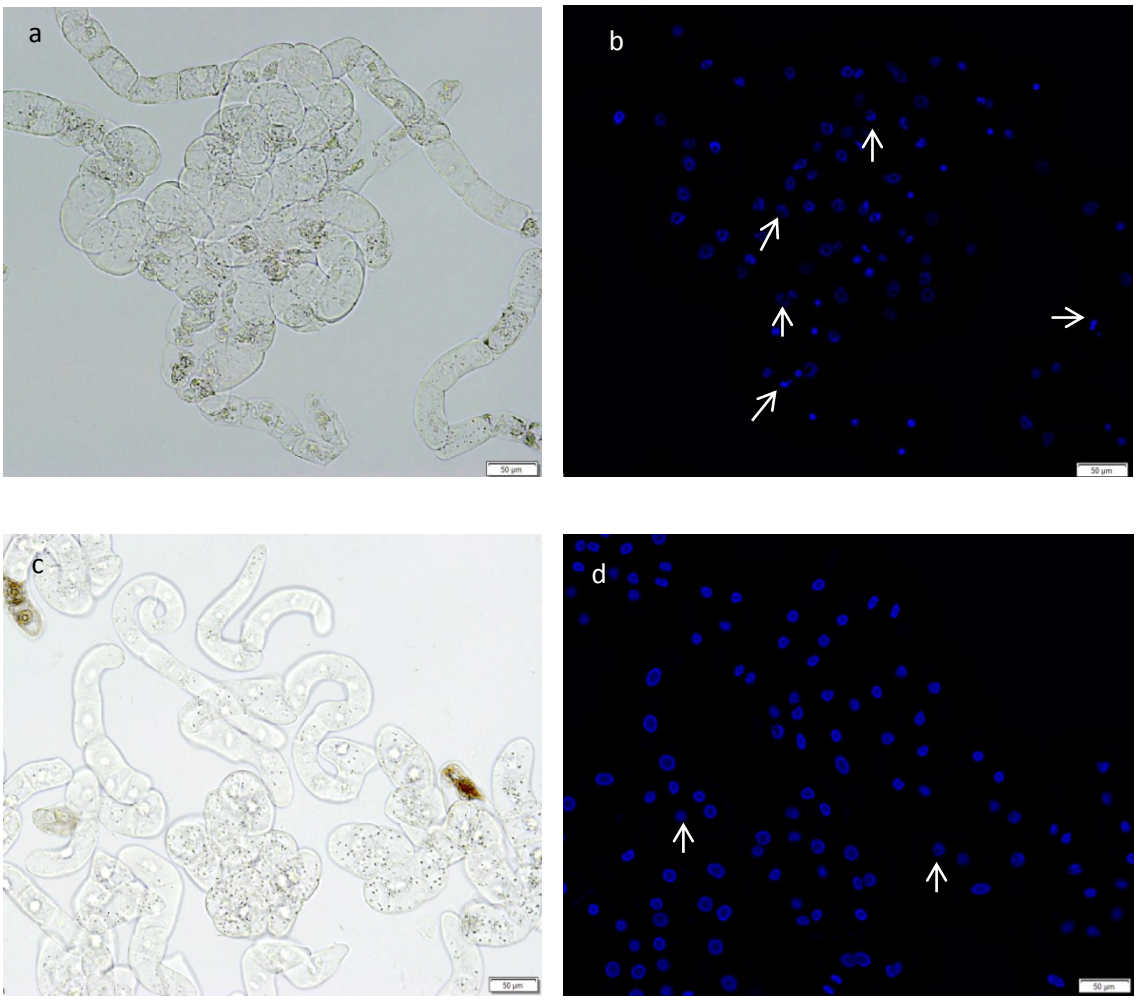


Figure. 4.10. ANE protects the BY-2 cell nuclei under freezing stress, staining by DAPI staining, the cells treated with freezing stress at -5°C. Freezing induced chromatin damage in cells without ANE treatment, bright field and fluorescent images (a& b). BY-2 cells treated with 0.1 mg/mL ANE after freezing stress (c& d). The arrows indicates the damaged nuclei.

4.9 Reactive oxygen species in the cells under freezing stress

Reactive oxygen species (ROS) as signalling molecules are important in program cell death (PCD). A constant increase in ROS affects the activation of PCD, which leads to the cellular death (Lyubushkina et al., 2014). The application of ANE has been reported to increase total phenolics and flavonoids content, total antioxidant activity and

Fe^{2+} chelating ability in spinach leaves, which possibly might reduce oxidative stress and ROS in the mentioned plant (Fan et al., 2011). Therefore, production of ROS in BY-2 cells treated with ANE, in response to freezing stress was tested. The present study indicated that the ROS level increased after 24h of the freezing stress. At that time, the DCF fluorescence intensity in BY-2 cells was mostly higher in control cells comparing with the cells treated with ANE (Fig. 4.11).

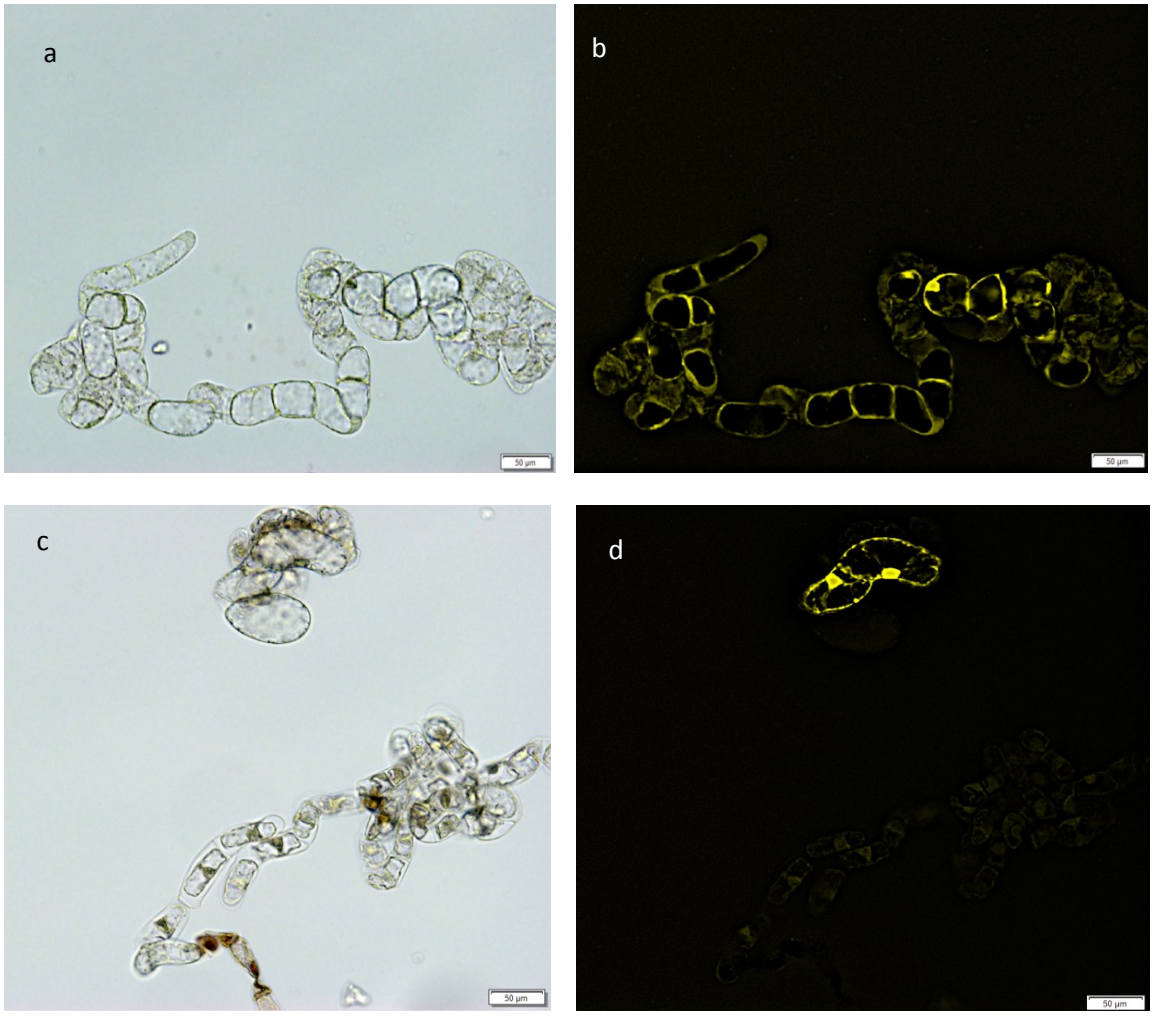


Figure. 4.11. Effect of freezing temperature (-5°C) on ROS production; freezing induced high fluorescent intensity, indicating more ROS production, in control cells than the cells treated with ANE. Bright field and fluorescent images of control cells (a& b) and the cells treated with 0.1 mg/mL ANE (c& d).

4.10 Gene expression analysis of BY-2 cells treated with *Ascophyllum nodosum* extract and freezing stress

Expression analysis of the genes involved in freezing stress response was studied under ANE treatment to determine the mechanism of freezing stress tolerance. To achieve this aim a few genes were selected, then the expression pattern of the genes was quantified. The results of the gene expression analysis are shown in Fig. 4.12.

The expression of Fucosyltransferase gene, involved in cell wall biosynthesis, revealed the highest induction (6.8 folds) in the control during recovery period. In ANE treated cells, the higher induction of Fucosyltransferase gene was observed 2.36-fold in recovery phase. Fucosyltransferase didn't show a considerable change in expression level during freezing stress; however, at this stage the peak induction of the mentioned gene was 2.17-fold in treated cells (Fig. 4.12 a).

The expression level of Betaine Aldehyde Dehydrogenase1 (BADH1), responsible for betaine accumulation under stressful condition displayed an increase during freezing process (2.92 folds), in the treatment of ANE; after freezing period a reduction in the expression level was detected in treated cells. In controls, however, there was not much changes in expression level during freezing stage. However, during recovery condition the BADH1 reached the maximum expression level of 10-fold (Fig. 4.12 b).

The expression pattern of galactinol synthase 2 as the gene associated with raffinose and other sugars synthesis showed that there was no induction before 24 h of freezing stress. The treated cells showed a higher level of expression. The peak induction (5.13 folds) of the gene was seen at 24 h during freezing period. The maximum

expression of galactinol synthase 2 in the control was 2.86-fold increase at recovery phase (Fig. 4.12 c).

The transcript abundant of Glutathione S-transferase which is a major endogenous antioxidant in plants was quantified. The Real-time PCR experiment indicated that Glutathione S-transferase had the highest relative expression (37.1 folds) among the other tested genes. The induction of Glutathione S-transferase gene was higher in control cells in comparison with the cultures supplemented with ANE; BY-2 cells in recovery stage showed a high level of Glutathione S-transferase expression. However, in the presence of ANE, the gene was not induced remarkably; in treated cells, the highest expression of the gene was just a 2.94-fold increase during recovery phase (Fig. 4.12 d).

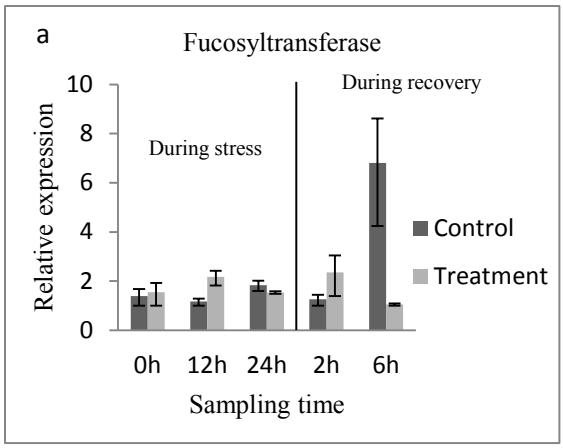
In the control treatment, the transcription factor activating protein 2 (AP2) as the abiotic stress response element had a maximum expression (9.06 folds) during recovery stage. A 2.18-fold induction of this transcription factor was seen in ANE treated cells during freezing stress (Fig. 4.12 e).

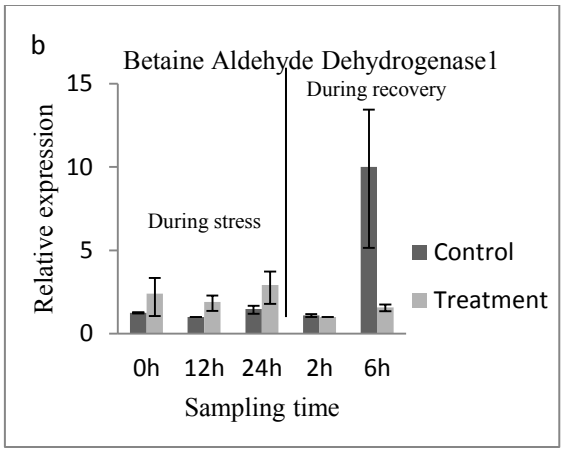
Quantitative Real-time PCR revealed that Pyrroline-5-carboxylate synthase gene, involved in proline biosynthesis, was induced during recovery phase in the control and the cells treated with ANE. In such condition, the highest expression of Pyrroline-5-carboxylate synthase showed 7.03-fold increase in the control and 6.5 in treated cells (Fig. 4.12 f).

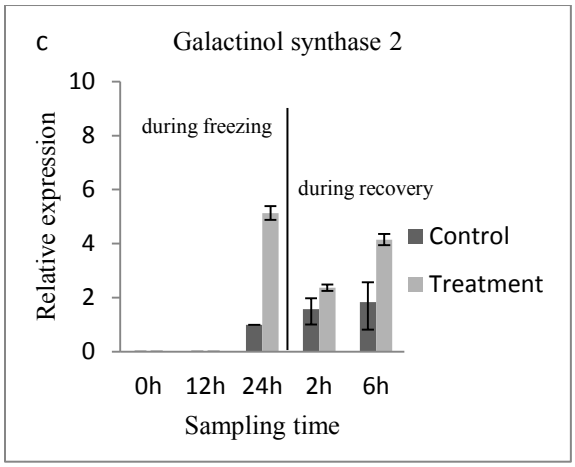
In addition, the expression of Acetyl-CoA carboxylase, a gene involved in fatty acid production was also studied. The results indicated that the peak induction of the gene was 2.17-fold in the control and during recovery phase. The maximum expression of

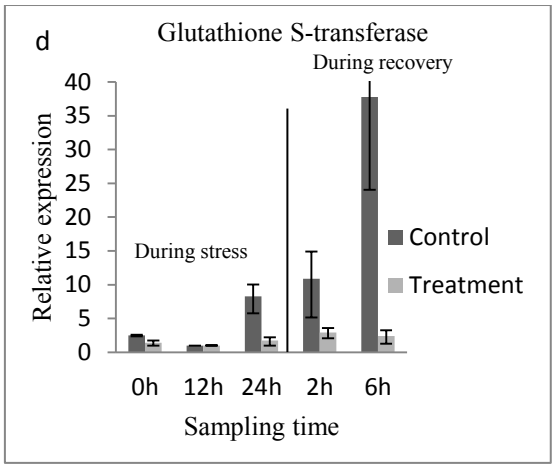
Acetyl-CoA carboxylase in ANE treated cells was observed at 24 h during freezing stress which was 1.71-fold increase (Fig. 4.12. g).

The transcript level of Digalactosyldiacylglycerol (DGD1) gene was also analyzed. This gene plays a role in increasing the lipid content of cell membranes under freezing stress. The expression data indicated that DGD1 did not go through enormous changes under freezing treatment. However, the highest induction of DGD1 was found 4.73-fold more in control cells during recovery. There was a trend of increase induction during time points, in the control, so that the peak of induction occurred 6 h after recovery point. In the ANE treated cells, the maximum expression (3.56 folds) was observed during freezing phase (Fig. 4.12 h).


a
Fucosyltransferase
Relative expression
10
8
6
4
2
0
During stress
During recovery
Control
Treatment
0h
12h
24h
2h
6h
Sampling time


b
Betaine Aldehyde Dehydrogenase1
Relative expression
15
10
5
0
During stress
During recovery
Control
Treatment
0h
12h
24h
2h
6h
Sampling time


c
Galactinol synthase 2
Relative expression
10
8
6
4
2
0
during freezing
during recovery
Control
Treatment
0h
12h
24h
2h
6h
Sampling time


d
Glutathione S-transferase
Relative expression
40
35
30
25
20
15
10
5
0
During stress
During recovery
Control
Treatment
0h
12h
24h
2h
6h
Sampling time

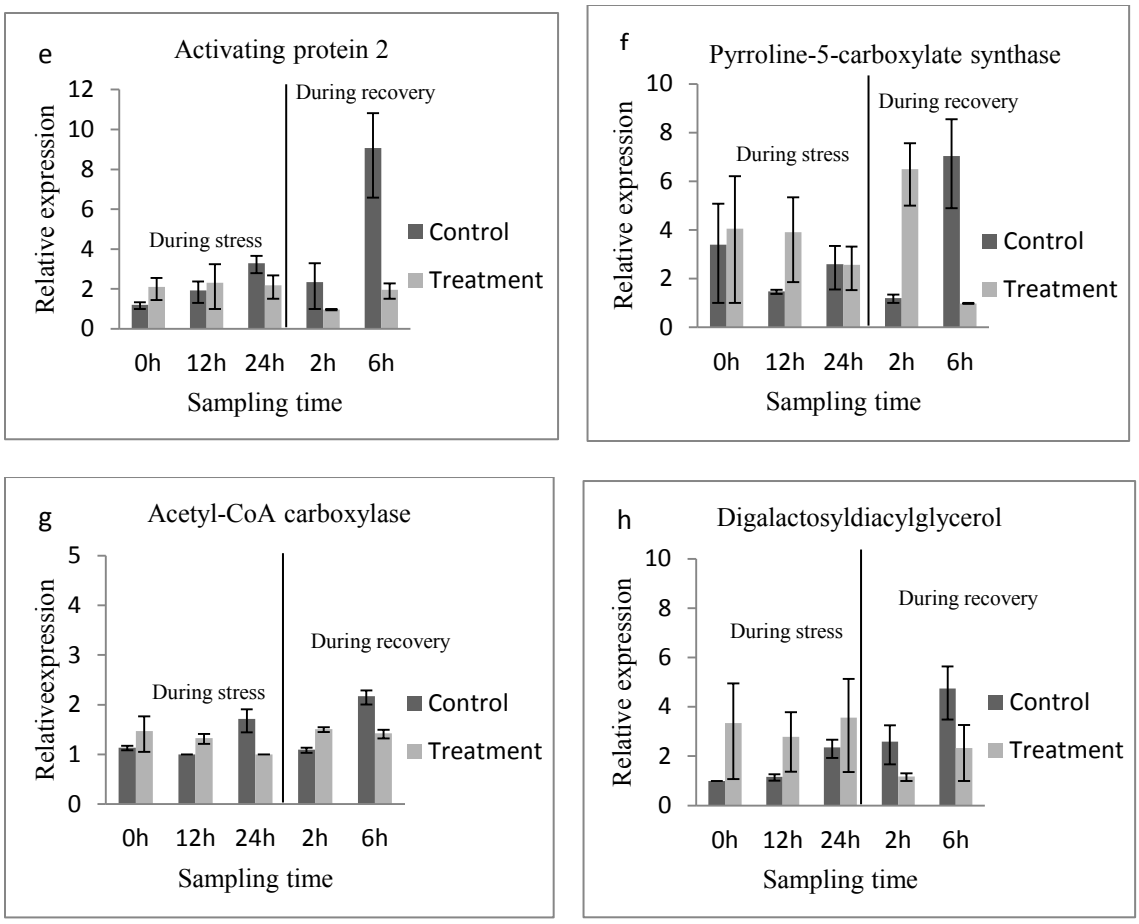


Figure. 4.12. Differential expression patterns of selected genes in ANE treated (0.1 mg/mL) and untreated BY-2 cells under freezing temperature at -5°C, and subsequent recovery stage at 27°C. Bars indicate standard deviation.

4.11 Proline content of the cells treated with *Ascophyllum nodosum* extract in response to freezing stress

Proline content of BY-2 cells treated with ANE exposed to freezing stress was determined (Fig. 4.13). The results demonstrated that ANE treatment, 0.1 mg/ mL, caused the cells to produce higher amount of proline under freezing stress. However, the proline accumulation decreased during recovery stage. Interestingly, the proline content of the cells was higher in treated cells even before starting the stress (70.74 µg/g fresh weight of the cells), showing the effect of ANE on proline production and/or accumulation. Besides, in control cells (without ANE treatment) the highest proline accumulation was seen before freezing stress (43.17 µg/g FW of the cells).

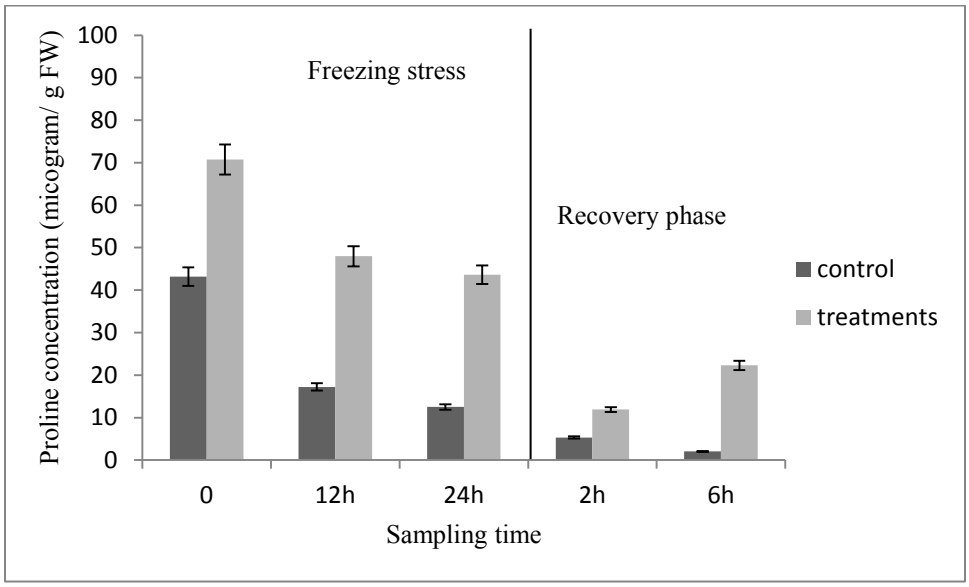


Figure. 4.13. Proline accumulation in BY2 cells supplemented with 0.1 mg/mL of ANE. Sampling times represent before stress (0), during freezing (12 h and 24 h) and after freezing stress during recovery stage (2 h and 6 h). Bars represent standard error.

Chapter 5.0

Discussion

Global changing of the environmental temperature causes a dramatic reduction of agricultural productivity. In addition, extreme temperatures intensify the adverse effects of other environmental factors such as salt and drought stresses that all together influence crop production and quality (Ashraf and Foolad, 2007). In the present study, *Ascophyllum nodosum* extract was tested to determine the effect of extract on BY-2 cells stress tolerance. The results provided valuable information that ANE helps the BY-2 cells to tolerate freezing stress.

5.1 The effect of *Ascophyllum nodosum* extract on the cell growth

Studies have demonstrated that the extracts made from *Ascophyllum nodosum* improve plants' growth and development (Khan et al., 2011; Khan et al., 2009; Rayorath et al., 2007; Rayorath et al., 2008). The extracts contain vitamins, micronutrients, oligosaccharides and compounds with phytohormones-like activity, that promote the growth. It has been suggested that presence of plant growth regulators (e.g. cytokinin) in the extracts may result in the positive effect on the growth (Khan et al., 2011). BY-2 cells treated with *Ascophyllum nodosum* extract showed lower dry weight compared with untreated cells when grown under optimum temperature condition (Fig. 4.1). In optimum growth condition, bioactivity of the extract lowered dry weight of the cells. The other possibility could be related to the concentration of ANE used in the experiment. Interestingly, it has been indicated that fucans, sulfated polysaccharides of the brown algae's cell wall show antiproliferative activity when used to study cancer cells (Haroun-

Bouhedja et al., 2000). Thus, the same effect might cause less growth of the cells in our experiment.

Furthermore, in spite of the expectation, BY-2 cells treated with ANE under drought, salinity and heat stresses did not show any difference in growth as compared to untreated cells (Fig. 4.1-4). However, the exploration of ANE being involved in cell growth has not yet been reported. There is no report on the use of this extract on tobacco plants either. Nonetheless, one possible explanation could be the stress response of the cells, so that the fast growing BY-2 cells could grow and pass the stressful condition without the need to the use of ANE. The complexity of environmental factors and heterogeneity of the plant response towards stress, ANE imparts abiotic stress tolerance in different plant species to a different extent; moreover, the plant cells might behave differently from the whole plant, which makes the experiment condition unpredictable and not comparable with the results obtained from the plant itself. However, it has been reported that ANE improved drought tolerance in tomato plants, promoted growth in *Arabidopsis thaliana* and *Brassica napus*, and improved freezing stress tolerance in Arabidopsis (Jannin et al., 2013; Karunatilleke, 2014; Rayirath et al., 2009; Rayorath et al., 2007). Other studies have shown that the extract of *Ascophyllum nodosum* increased the antioxidant, total phenolic and flavonoids content of spinach, which protected *Caenorhabditis elegans* against oxidative and thermal stress (Battacharyya et al., 2015; Fan et al., 2011). It has also been verified that applying seaweed extract on creeping bentgrass resulted in increasing superoxide dismutase activity, which improved heat stress tolerance (Zhang and Ervin, 2008). In this study, the antioxidant content of the cells was not measured, but gene expression data showed that the relative expression of

the gene involved in antioxidant activity (glutathione S-transferase) was less in ANE treated cells than the controls, Fig. 4.12 d. This finding is supported by a previous study on tomato plants exposed to drought stress, where the plants treated with ANE were found to have a lower antioxidant enzyme activity, but higher tolerance to the stress (Karunatilleke, 2014).

Ascophyllum nodosum extract mediated freezing stress tolerance in BY-2 cells, while the cell viability decreased tremendously in the cultures with no ANE treatment (Fig. 4.6). Dry weight of cells was significantly higher in the cells treated with 0.1 mg/mL ANE at -5°C (Fig. 4.6). Although the exact mechanism by which *Ascophyllum nodosum* imparts stress tolerance is not yet known, previous studies have shown that extracts of *Ascophyllum nodosum* increase accumulation of osmolytes, soluble sugars and proline in treated plants, protecting them against dehydration resulted from freezing stress (Rayirath et al, 2009). Furthermore, as mentioned before, phenolic compounds present in *Ascophyllum nodosum* act as antioxidants and helps in scavanging free radicals and confers protection to cells in stressful conditions. Therefore, it is anticipated that the cells treated with ANE should have higher growth and viability under freezing temperature.

5.2 Microscopic studies of BY-2 cells under freezing stress

Ascophyllum nodosum extract has been reported to increase the cell viability in *Arabidopsis* plants under freezing stress (Rayirath et al., 2009). The ANE reduced cell damage and death of BY-2 cells after freezing treatments (Fig. 4.6 and 4.7). The recovery phase after freezing stress seemed a critical stage in terms of cell viability, where the control cells had the lower survivability rate than the treated cells (Fig. 4.7). Therefore, it

is probable that the antioxidant activity of the seaweed extract protected the cell from cells death and finally resulted in the higher cell viability (Nair et al., 2012; Rayirath et al., 2009).

In addition, ANE maintained the cell membrane integrity (Fig. 4.8). The control cells displayed more numerous spherical bodies than the BY-2 cells treated with ANE. The spherical bodies show intracellular lipid droplets, which are produced as the result of cell membrane damage and/or possibly degradation during freezing stress (Greenspan et al., 1985). The cell membrane stability was also confirmed by the electrolyte leakage test; the electrolyte leakage experiment demonstrated that ANE treatment decreased the ion leakage after freezing stress (Fig. 4.9). Similar results were reported by Rayirath et al.(2009), where the extract of ANE prevented plasma membrane degradation and ion leakage under freezing stress in Arabidopsis (Rayirath et al., 2009).

The extract of *Ascophyllum nodosum* includes phenolic compounds, which are known secondary metabolites in plants, and act as antioxidants. The beneficial effect of these compounds in quenching ROS has been proved (Zhang and Ervin, 2008). Environmental stresses induce ROS production, protein, lipid and DNA degradations, leading to cell death, apoptosis and/or necrosis. It is important for cells to regulate ROS homeostasis, preventing cell damage and death (Fath et al., 2001; Van Breusegem and Dat, 2006). The lower ROS in freezing-exposed BY-2 cells that were treated with ANE verifies the vital role of the extract in ROS scavenging (Fig. 4.11).

On the other hand, cell death is accompanied by the changes in the cell structure. The dehydration resulting from freezing stress might lead to chromatin condensation and nuclear deformation (Van Breusegem and Dat, 2006). Interestingly, *Ascophyllun*

nodosum extract contains osmoprotectants (osmolytes) such as betaine, which prevent the cells from dehydration and suppress nuclear alternation under stress (Banu et al., 2009; Rayirath et al., 2009). The nuclei staining revealed freezing stress damage caused nucleus condensation. The deformation of the nuclear occurred during freezing stress mostly in untreated BY-2 cells. As it is thought that reactive oxygen species might induce apoptosis and affect the nuclei morphology (De Pinto, 2002), observation of BY-2 cells with deformed nuclei is probably due to the same scenario. However, BY-2 cells treated with ANE included more normal shape nuclei whereas untreated cells showed mostly deformed nuclei (Fig. 4.10). Therefore, ANE plays a key role in cell protection against stress either as an osmoprotectant or antioxidant active compound.

5.3 Physiological and biochemical changes of BY-2 cells under application of *Ascophyllum nodosum* extract

It is known that plant membranes undergo a transition phase from a liquid to gel-like stage under low temperatures; during this change, membrane fluidity is reduced, which leads to ion leakage and deactivation of membrane proteins (Iba, 2002; Ward et al., 2003). However, adjustment of membrane fluidity by modulating the level of unsaturated fatty acids is a strategy for freezing stress tolerance (Ward et al., 2003). The use of ANE has been shown to protect the cell membrane from stress injury; Arabidopsis plants treated with ANE showed lower electrolyte leakage under freezing stress whereas higher leakage was observed in control plants without ANE treatment (Rayirath et al., 2009). Furthermore, ANE increased the accumulation of osmoprotectants and unsaturated fatty acid in Arabidopsis plants under freezing stress (Rayirath et al., 2009). Therefore, if

the same process occurs in BY-2 cells, the reduced leakage of ions from treated cells may be a result of a higher degree of the membrane integrity in these cells.

The other effect of ANE on BY-2 cells was an increase in survivability rate under freezing temperatures. ANE also prohibited ROS production, which occurs as a result of ROS formation. The untreated BY-2 cells produced reactive oxygen species, in recovery phase; whereas, ROS production was less in the treated cells with ANE (Fig. 4.11). It has been suggested that cytokinins present in ANE prevent ROS formation and mitigate damage-induced by free radicals through inhibiting xanthine oxidation (Khan et al., 2009). In plants, there are not many reports focusing on PCD during freezing stress; however, it is possible that cold stress induces program cell death in plant cells (D'angeli and Altamura, 2007; Koukalová et al., 1997; Lei et al., 2004; Ning et al., 2002). Therefore, the lower ROS production probably led to the lower PCD and resulted in higher viability of the BY-2 cells under ANE treatment.

When plants face salt stress, proline accumulates in the cytosol and helps substantially in cytoplasmic osmotic adjustment (Leigh et al., 1981). Proline also helps the plant cell by stabilizing subcellular structures such as membranes and proteins, scavenging free radicals, and buffering cellular redox potential under salt stress (Ashraf and Foolad, 2007). In addition, proline (an important osmolyte) may act as a ROS scavenger during and after stress to help the normal growth (Saradhi et al., 1995). Proline is a mediator for osmotic adjustment, membrane and protein stabilizer and inducer of stress related genes; all together it results in low temperature stress tolerance (Rayirath et al., 2009b; Xin Zhanguo and Browse, 1998). Therefore, ANE mediated accumulation of proline, caused the higher cell viability as it decreases ROS production in BY-2 cells

under freezing stress. Our findings are in accordance with the results reported by Rayirath et al. (2009) that ANE treated Arabidopsis plants accumulated more proline than the untreated plants under freezing temperatures. It has been reported that proline content of the cells increased under stress (Türkan et al., 2005). Proline is accumulated under cold stress; this is due to the production of ice crystals in apoplastic spaces and osmotic stress resulted from freezing temperatures (Bohnert et al., 1995; Liu et al., 2013; Wu et al., 2014). The production of proline occurs through the glutamate pathway, in cytosol and plastids of plant cells (Parvaiz and Satyawati, 2008). In this process, Δ-pyrroline-5-carboxylate synthetase (P5CS) reduces L-glutamate to L – glutamate γ – semialdehyde, which is converted to Pyrroline 5 – carboxylate (P5CS) and finally proline is produced (Parvaiz and Satyawati, 2008).

5.4 Effect of *Ascophyllum nodosum* extract on the gene expression under freezing stress

Adaptation of plants to abiotic stresses is a complex process, which guides cells to adapt at molecular, biochemical and physiological levels. The transcriptional machinery associated with stress responses maintains growth, metabolism and development of plants, through an intricate network of transcription factors (TFs) (Agarwal et al., 2013). Transcription factors have the essential role in plant stress response through gene regulation mechanism (Du et al., 2016). It has been demonstrated that ANE treatment increased the expression of transcription factor cbf3 in Arabidopsis plants under freezing temperature (Nair et al., 2012). It was also shown that AP2 transcription factor (plant specific transcription factors, containing AP2/ ERF DNA binding domains) was down regulated in Arabidopsis treated with ANE under freezing (Kizis et al., 2001; Mizoi et al.,

2012; Prasanth, 2009; Rayirath et al., 2009). AP2 transcription factors are known as the genes having a potential of improving cold resistance in plants (Du et al., 2016). The cold temperature response of AP2 transcription factors has been tested at different time points, in *Brasica napus*; the results showed that AP2 subfamily genes were not active within 2 hours of cold stress. However, at 12h after cold stress the transcript reached the maximum level (Du et al., 2016). In our study, the freezing stressed BY-2 cells had the peak of induction in recovery stage (Fig. 12.4 e). It seems that the cells were in a critical condition to survive during recovery phase. Nonetheless, AP2 was less activated in ANE treated cells than the controls, which might highlight the role of ANE as an osmoprotectant compound that let the cells not to experience the stressful condition to the same extent.

Previous studies have reported that exposure to freezing stress stimulates activation of genes associated with abiotic stress in plants under application of *A. nodosum* extract. It has been shown that applying lipophilic component of ANE changed the expression of a number of genes (1.65 %) in Arabidopsis plants under freezing stress (Nair et al., 2012; Rayirath et al., 2009). In the present project, the effect of ANE on genes expression in BY-2 cells, under freezing temperature showed differential expression patterns in the cells treated and untreated with the extract.

Cell wall biosynthesis plays a key role in plant development, signal transduction, biotic and abiotic stress tolerance. Abiotic stresses lead to disruption of cell wall synthesis. Fucosyltransferase functions in cell wall synthesis under stress. Fucosyltransferase was up-regulated under freezing stress in Arapidopsis plants treated with *Ascophyllum nodosum* extract (Prasanth, 2009; Rayirath et al., 2009). In another

study galactoside 2-alpha-L-fucosyltransferase was demethylated (activated) under heat stress in *Brassica napus* (Gao et al., 2014). Under stressful conditions, cross-linking of glycoproteins and phenolic compounds might result in cell wall stiffening (Tenhaken, 2014). The cell wall synthesizes a variety of fucose-containing polymers including xyloglucans (XyGs) and rhamnogalacturonan. Xyloglucan as the major hemicellulose in the primary walls of higher plants contains a fucosyl residue; this residue is added to different acceptors by fucosyltransferase enzyme. Studies have indicated that the increase in expression level of this enzyme was observed during abiotic stress in plants (Perrin et al., 1999; Reiter, 2002). Fucosyltransferases (FUTs) catalyze the fucosylation at different acceptors. Fucosylation is required in Arabidopsis for root cell elongation as a result of auxin activation (Wu et al., 2010). Nonetheless, BY-2 cells and Arabidopsis suspension cultures lack fucosyl residue and are known to contain non-fucosylated arabinogalactan proteins (AGPs) (Wu et al., 2010). In the present study, the expression of this gene was high during post freezing period in ANE untreated BY-2 cells (Fig. 4.10 a). However, there was not much change in up regulation of fucosyltransferase transcript during the stress in ANE treated and untreated cells; the reason for that might possibly be due to lack of fucosyl residue in BY-2 cells. In recovery phase, fucosyltransferase was up-regulated earlier in ANE treated cells than the controls; but the late activation in untreated cells showed a higher expression level. Therefore, it seems that cell wall changes resulting from fucosyltransferase up-regulation were not enough or maybe were late to prevent the membrane leakage and cell damage in the cells without ANE treatment.

On the other hand, one possible strategy by which ANE protects plants against stress is accumulating such compounds like betaine. Interestingly, *Ascophyllum nodosum*

extract contains betaine (Prasanth, 2009). Betaine (glycine betaine, N,N,N-trimethylglycine- an organic compound), a compatible solute, is accumulated in plants under environmental stresses. Two steps have been recognized in betaine synthesis, which are catalyzed by choline mono-oxygenase and betaine aldehyde dehydrogenase (BADH) respectively (Kishitani et al., 1994). The application of ANE showed induced expression of BADH during freezing stress in BY-2 cells. A high induction of BADH during recovery period in untreated cells (without ANE treatment) was triggered probably to overcome the stress by production of betaine (Fig 4.12 b); however, this strategy was not effective (in untreated cells), based on the cell viability and growth tests (Fig. 4.5 and 4.6). It has been revealed that there is a correlation between the level of accumulated betaine and cold stress tolerance in plants such as wheat and barley; therefore, betaine might function as a cryoprotectant while freezing stress is applied (Kishitani et al., 1994). Accumulation of betaine mostly occurs in response to dehydration stress (Ashraf and Foolad, 2007). In some plants such as rice, mustard, Arabidopsis and tobacco, betaine is not produced naturally neither in optimum nor in stressful conditions. Nonetheless, overexpression of the gene responsible for betaine biosynthesis, resulted in accumulation of betaine in the mentioned plants (Ashraf and Foolad, 2007; Rhodes and Hanson, 1993). Exogeneous application of betaine improved freezing tolerance of plants belonging to Solanaceae family (Somersalo et al., 1996). In addition to this, overexpression of BADH1 resulted in increased betaine content and conferred cold tolerance in sweet potato (Fan et al., 2012). In another study on rice plant, using RNA interference technique, down regulation of BADH resulted in decreased

salinity tolerance and increased oxidative stress; however, betaine biosynthesis was not affected (Fan et al., 2012; Tang et al., 2014).

Soluble sugars constitute about 50% of the total osmotic potential in plant cells during salt stress (Cram, 1976). Sugars have also been suggested to play a central role in modulating oxidative stress in plants; they are involved in stress response and act as signalling molecules, resulting in modifications in gene expression patterns (Couée et al., 2006). It has been reported that treatment with lipophilic components of ANE increased sugar alcohols, such as galactinol in Arabidopsis under freezing stress (Prasanth, 2009; Rayirath et al., 2009). Abiotic stresses increased accumulation of the oligosaccharide raffinose in Arabidopsis leaves (Egert et al., 2013). The role of raffinose and galactinol in mediating stress tolerance has been demonstrated (Taji et al., 2002). It has been shown that galactinol synthase is a key enzyme in raffinose biosynthesis. The expression of this enzyme is closely related to environmental stresses (Nishizawa et al., 2008). The expression data in this study demonstrated that there was up-regulation of galactinol synthase gene in presence of freezing stress and ANE treatment in BY-2 cells. The higher transcript accumulation of galactinol synthase gene might be an indicator of ANE influence on raffinose production, leading to stress tolerance (Fig. 4.12 c). Studies have reported that different galactinol synthase genes such as GolS1, GolS2, GolS3 and GolS4 were induced under different abiotic stresses in variety of plants. In coffee (coffee plants), for example, it has been shown that GolS1, GolS2, GolS3 were differentially regulated under water deficit, heat and salinity stresses and increased raffinose content. However, the function of GolS is not clearly understood under cold stress. For example, overexpression of GolS gene in Arabidopsis resulted in increased levels of galactinol and

raffinose, but no effect was observed on freezing tolerance of transgenic lines. Analysis of freezing stress tolerance in transgenic tobacco plants for GolS1, from *Medicago sativa*, showed that overexpressed lines were tolerant to freezing and chilling stresses (Zhuo et al., 2013). Studies also reported that overexpression of GolS2 increased raffinose and galactinol levels in Arabidopsis under optimum condition of growth (Taji et al., 2002). However, plants regulate different molecular pathways to respond to stressful conditions.

In response to abiotic stresses, plants lead to the activation of antioxidant enzymes; ANE caused the up-regulation of such genes (glutathione S-transferase) in Arabidopsis plants exposed to freezing stress (Rayirath, 2009). Glutathione S-transferase as one of the major antioxidants plays a vital role in plant defense mechanisms through scavenging free radicals (Anderson and Davis, 2004). Studies have pointed out that enzymes involved in defensive mechanisms including glutathione S-transferase (GST) and glutathione reductase (GR) show different expression pattern under abiotic and biotic stress treatments (Anderson and Davis, 2004; Seppänen et al., 2000). In our experiment, GST was expressed more strongly in untreated BY-2 cells (without ANE) under freezing stress (Fig. 4.12 d). The low expression of this gene in ANE treated cells, at least in part, might be due to lower oxidative damage, which was confirmed by DCF staining (Fig. 4.10). In another study, the activity of GST enzyme doubled under drought stress in spurge but did not change in response to cold stress. The reason for this difference is that drought stress in spurge causes wilting which increases the expression of glutathione S-transferase, while cold stress did not stimulate the GST expression. In tobacco plants, expression of genes such as glutathione reductase, glutathione S-transferase altered antioxidant metabolism and caused abiotic stress tolerance (Le Martret et al., 2011).

Aside from antioxidant activities, osmotic adjustment is a key strategy in response to abiotic stress; proline as a compatible osmolyte participates in such process and responds to abiotic stresses. Pyrroline-5-carboxylate synthase (P5CS) is involved in proline biosynthesis (Dombrowski et al., 2008). There are two copies of P5CS gene in Arabidopsis, P5CS1 and P5CS2, responsible for stress response and incompatible interactions, respectively. Previously, it has been demonstrated that transcripts of P5CS1 and P5CS2 were not affected by ANE treatment under freezing stress in Arabidopsis, whereas, P5CS1 transcript was down regulated under such conditions by applying lipophilic component of ANE (Rayirath et al., 2009). In another study on tomato plants under drought stress, the transcript level of P5CS was changed at different time points after stress, and re-watering the plants decreased the relative expression of this gene in both ANE treated and untreated plants (Karunatilleke, 2014). Present research, however, showed that P5CS transcript was in peak of induction during recovery phase in both ANE treated and untreated cells, which is not in line with the proline content of BY-2 cells (Fig. 4.12 f and 4.13). It seems that the higher induction of P5CS occurred when there was less proline accumulation in the cells. However, the reason for accumulation of proline specifically under freezing stress is not yet known. In rice, P5CS1 was induced by salinity, and cold stresses; however, the transcript was up-regulated 12h after cold stress (Hur et al., 2004). In another study, cloning P5CS gene from common bean revealed that P5CS was induced after 2 hours of salt and cold stress and 4 day of drought. Additionally, there was the highest proline accumulation at 8 day for drought, 24h for cold and 9h for salt stresses (Turchetto-Zolet et al., 2009). In tomato, it was found that

P5CS gene was strongly induced by salt, drought, and cold stresses (Dombrowski et al., 2008).

Moreover, to overcome the stress and participating in various biological processes, lipids are vital cellular components and have a key role in mentioned conditions. Lipids are used to build cell membranes and involve in cell integrity. It is well known that acetyl-CoA carboxylase (ACCase) catalyzes fatty acid biosynthesis, protecting cell membrane (Sasaki and Nagano, 2004; Xiao and Chye, 2011). ANE has been indicated to significantly affect membrane integrity under freezing stress (Rayirath et al., 2009). One possibility is that the ANE might influence the expression of genes associated with fatty acid biosynthesis. For instance, it was indicated that ANE modified the regulation of Acyl-CoA thioesterase in Arabidopsis plants. Acyl-CoA thioesterase plays an essential role in β-oxidation of fatty acids (Prasanth, 2009). However, it is assumed that the earlier up-regulation of Acetyl-CoA carboxylase in ANE treated cells might be associated to the faster response of the cells due to ANE effect. In this study, the relative expression of ACCase was in agreement with GDG1 expression, staining experiment, and ion leakage data, where BY-2 cells were effectively protected from freezing injury by the use of ANE treatment (Fig. 4.9, 4.10 and 4.12 g). Therefore, taking together all the results, ANE might affecte fatty acid biosynthesis pathway and result in freezing stress tolerance in BY-2 cells.

In addition, membranes as significant systems in terms of receiving stress were found to be the site where ANE is probably affects significantly in response to freezing stress. Digalactosyldiacylglycerol synthase1 (DGD1) is present in photosynthetic membranes of the chloroplast. Under freezing conditions, monogalactosyldiacylglycerol

(MGDG) content of the membranes declines, and is converted to digalactosyldiacylglycerol (DGDG) by DGD enzyme activation (Degenkolbe et al., 2012; Li et al., 2008). This enzyme functions in lipid biosynthesis of the membranes as well as sub-cellular lipid trafficking, growth and development. Digalactosyldiacylglycerol may increase membrane stability under freezing temperatures. In Arabidopsis plants, applying lipophilic components of ANE induced DGD1 expression during freezing temperatures, resulting in higher membrane stability (Nair et al., 2012; Rayirath et al., 2009). It was also indicated that the mutant lines of this gene in Arabidopsis had reductions in digalactosyl lipid content and were stunted. The disruption of lipid biosynthesis in the mutant lines (dgd1), resulted in the disturbance of assembly of other chloroplast lipids (Dörmann et al., 1999). The DGD1 gene showed a higher expression before and during stress but not in recovery phase in ANE treated BY-2 cells, which demonstrates the critical role of ANE for cell protection after stress. Nonetheless, in untreated cells, DGD1 showed a higher induction later during recovery stage that could not protect the cells to survive and/or decrease the stress-induced damages (Fig. 4.12 h).

Chapter 6.0

Conclusion

6.1 Project summary

Low temperature can occur rapidly, influencing plants and products. On the other hand, other stressors including salinity and drought happen more slowly, which make the management of circumstances easier to overcome the stressful conditions. Moreover, extreme temperatures affect other stress factors. Therefore, ability to tolerate environmental stress is a vital approach for plant survival and quality of products.

Ascophyllum nodosum extract has been proven to be effective in increasing germination, nutrient uptake, yield, shelf life and stress tolerance of plants. The mechanism by which this extract is controlled and regulated in plant system is not yet known. The effectiveness of the extract in mediating stress tolerance was tested in this project.

A cell culture bioassay was developed to determine the bioactivity of *Ascophyllum nodosoum* extract on abiotic stress tolerance. BY-2 cells, a fast growing plant model cell line, were used to study the effect of ANE on the cell growth, physiological and biochemical changes in response to drought using PEG 8%, heat stress at 40°C, salinity with 150 mM NaCl, and freezing stresses at 0°C, -3°C and -5°C.

Different stressors were conducted on the cultures supplemented with 0, 0.01, 0.05 and 0.1 mg/mL of ANE, and the cells growth was measured. The results indicated that ANE was not effective at mediating stress tolerance on the BY-2 cells under salinity, drought and heat stresses. In other words, the BY-2 cells that were not treated with the

extract showed more growth in terms of dry weight. Even though the effect of ANE on mediating stress tolerance has been demonstrated in previous studies, it could be concluded that different plants and species might respond differently to ANE treatment. Besides, the plant whole system is much different from the cells in culture. Therefore, it is likely that the outcome is different when using different experimental plant models. However, there are studies where the extracts of brown algae have been used as antiproliferation to control cancer cells. It seems that phenolic contents of ANE have a complicated role in terms of antioxidant activity as well as antiproliferation compound.

Ascophyllum nodosoum extract had a remarkable effect on cell protection under freezing stress. Cell growth screening at different temperatures (0°C, -3°C and -5°C) revealed that the higher concentration of ANE (0.1 mg/mL) resulted in a better growth with regard to cell dry weight. The ANE treated cells showed more than 90% viability after freezing stress, whereas this number was less than 40 or 30% in the control (without ANE treatment). Taken together, all observations, demonstrate that ANE protected BY-2 cells and prevented cell death during post freezing phase. Based on the previous findings, it is possible that antioxidant effect of seaweed extract reduced program cell death and oxidative stress after freezing stress, which was indicated using a fluorescent dye (DCF).

Furthermore, electrolyte leakage test revealed that the extract of *A. nodosoum* dramatically decreased cell membrane damage, resulting in less ion leakage under freezing stress. This result was in agreement with the cell staining experiment with Nile red. In addition, proline content of the cells was increased by ANE treatment.

Finally, the molecular analyses of different genes responsive to low temperature stress displayed that in most cases, the relative expression of genes was lower in the cells

treated with ANE compared to the untreated cells. This can be due to protective effect of ANE on the cells; therefore, the treated cells did not sense the stress severely enough to result in higher gene induction levels.

In conclusion, the results of the present study pointed out that ANE is likely a mediator of freezing tolerance in BY-2 cells.

6.2 Future directions

BY-2 cells were found to be an appropriate system for analyzing *Ascophyllum nodosoum* extract effects in response to freezing stress. The results demonstrated that one of the most important effects of ANE was on the cell membrane. However, to have more concrete evidence on the mechanisms by which ANE confers tolerance further studies are recommended. Microscopic techniques to measure the thickness of the cell membrane using x-ray scattering method may be useful (Mitra et al., 2004). It is expected that ANE affects lipid composition of cell membranes under low temperature; thus, determination of fatty acid composition of membrane in treated cells is suggested to be examined. Another suggestion is using electron microscopic techniques for screening intra-cellular organelle characteristics under use of ANE treatment in the cells exposed to environmental stresses.

Additionally, as the results revealed that ANE protects the cells from freezing damage and increases cell viability, the role of this extract in activation of apoptosis/program cell death should be studied in more detail; molecular analyses such as expression of genes associated with this mechanism as well as cell imaging techniques may support this hypothesis more clearly.

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