

**UNDERSTANDING THE ROLE OF SPHINGOLIPIDS IN
REGULATION OF PROGRAMMED CELL DEATH IN
*CHLAMYDOMONAS REINHARDTII***

Submitted in partial fulfillment of the requirements for the
award of Bachelor of Technology degree in Biotechnology

by

AMBRITHA C (39230001)



**DEPARTMENT OF BIOTECHNOLOGY
SCHOOL OF BIO AND CHEMICAL ENGINEERING**

SATHYABAMA

**INSTITUTE OF SCIENCE AND TECHNOLOGY
(DEEMED TO BE UNIVERSITY)**

Accredited with Grade “A” by NAAC

**JEPPIAR NAGAR, RAJIV GANDHI SALAI, CHENNAI – 600 119
MAY - 2023**



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JEPPIAAR NAGAR, RAJIV GANDHI SALAI, CHENNAI - 600 119
www.sathyabama.ac.in



DEPARTMENT OF BIOTECHNOLOGY

BONAFIDE CERTIFICATE

This is to certify that this Project Report is the bonafide work of **AMBRITHA. C** who carried out the project entitled "**UNDERSTANDING THE ROLE OF SPHINGOLIPIDS IN REGULATION OF PROGRAMMED CELL DEATH IN CHLAMYDOMONAS REINHARDTII**" under my supervision from June 2022 to May 2023.

Internal Guide
(Dr.V.RAMESH KUMAR)

External Guide
(Dr.R.SRINIVASAN)

Head of the Department
(Dr.V.RAMESH KUMAR)

Submitted for Viva voce held on 10/05/2023

10/5/23
Internal Examiner
External Examiner

DECLARATION

I, **AMBRITHA. C (39230001)** hereby declare that the Project Report entitled "**UNDERSTANDING THE ROLE OF SPHINGOLIPIDS IN REGULATION OF PROGRAMMED CELL DEATH IN CHLAMYDOMONAS REINHARDTII**" done by me under the guidance of Dr.R.SRINIVASAN and Dr.V.RAMESH KUMAR is submitted in partial fulfillment of the requirements for the award of Bachelor of Technology degree in Biotechnology.

DATE: 10.5.2023

PLACE: CHENNAI



SIGNATURE OF THE CANDIDATE

ACKNOWLEDGEMENT

I am pleased to acknowledge my sincere thanks to the Board of Management of **SATHYABAMA INSTITUTE OF SCIENCE AND TECHNOLOGY** for their kind encouragement in doing this project and for completing it successfully. I am grateful to them.

I convey my thanks to **Dr. V. Ramesh Kumar, Head of the Department, Dept. of Biotechnology**, for providing me necessary support and details at the right time during the progressive reviews.

I would like to express my sincere and deep sense of gratitude to my project guides **Dr.R.SRINIVASAN and Dr.V.RAMESH KUMAR** for their valuable guidance, suggestions, and constant encouragement that paved way for the successful completion of my project work.

I wish to express my thanks to all Teaching and Non-teaching staff members of the Department of Biotechnology who were helpful in many ways for the completion of the project.

ABSTRACT

Plants exist in an environment of changing abiotic and biotic stresses. The regulation of programmed cell death (PCD) is crucial for the survival and maintenance of multicellular organisms. In *Chlamydomonas reinhardtii*, Sphingolipids are a class of lipids that play a vital role in signal transduction and cell signaling pathways. Sphingolipids have been shown to play an important role in the regulation of PCD in plants. However, the role of sphingolipid in the regulation of PCD in *Chlamydomonas reinhardtii* remains unknown. This study evaluated the role of sphingolipids in regulating programmed cell death (PCD) in *Chlamydomonas reinhardtii* using dihydrosphingosine(DHS) and the function of sphingolipids during stress responses. After the DHS treatments, *Chlamydomonas* cells were evaluated for cell viability and the induction of apoptotic-like markers. DHS treatments were found to trigger apoptotic cell death, with the hallmark of ROS accumulation, lipid peroxidation, and an increase in cytosolic calcium, mitochondrial membrane potential, comet, and caspase activity. These findings reveal that apoptotic cell death affects the lipid peroxidation metabolism of *Chlamydomonas*, indicating that cell death pathways are evolutionarily conserved among eukaryotes.

Keywords: Programmed Cell Death, apoptotic, *Chlamydomonas reinhardtii*, mitochondrial membrane potential, lipid peroxidation.

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ABBREVIATIONS

SLs	-	Sphingolipids
PCD	-	Programmed Cell Death
DHS	-	Dihydrosphingosine
PHS	-	Phytosphingosine
S1P	-	Sphingosine 1-Phosphate
CER	-	Ceramides
FA	-	Fatty Acids
VLCFAs	-	Very-Long-Chain Fatty Acids
GIPC	-	Glycosyl Inositol Phosphoryl Ceramides
GLCCERs	-	Glycosylceramides
TMV	-	Tobacco Mosaic Virus
GSL	-	Glycosphingolipid
SPT	-	Serine Palmitoyl Transferase
KDSR	-	3-ketosphingosine reductase
S1P lyase	-	Sphingosine-1-Phosphate lyase
ACD6	-	Accelerated Cell Death 6
IPCS	-	Inositol Phosphoryl Ceramide Synthase
FB1	-	Fumonisin B1

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CHAPTER – 1

1. INTRODUCTION

1.1. SPHINGOLIPIDS:

Sphingolipids are a class of lipids that are composed of a sphingoid base, long-chain amino alcohol, a fatty acid, and a polar head group. They are important structural components of cell membranes and are involved in a variety of cellular processes, including cell signaling and cell-cell recognition. There are several types of sphingolipids, including:

Ceramides - These are the simplest form of sphingolipids, consisting of a sphingoid base and a fatty acid.

Sphingomyelins - These are sphingolipids that have a phosphorylcholine head group. They are found primarily in the myelin sheath that surrounds nerve cells.

Glycosphingolipids - These are sphingolipids that have a sugar molecule attached to the head group. They are found on the surface of cells and are involved in cell-cell recognition

Gangliosides - These are a type of glycosphingolipid that have a complex sugar chain attached to the head group. They are found primarily in nerve cells and are involved in cell signaling.

Sphingosine-1-phosphate - This is a sphingolipid metabolite that acts as a signaling molecule, regulating a variety of physiological processes, including cell death, cell proliferation and survival, inflammation, and vascular development. Overall, sphingolipids play important roles in maintaining cell structure and function, and their dysregulation has been implicated in a variety of diseases, including cancer, neurodegeneration, and metabolic disorders. Sphingolipids, or lipids with a sphingoid base backbone, are crucial parts of eukaryotic membrane systems and vital signaling molecules that are crucial for regulating plant immunity, regulating cellular homeostasis, and adapting to stress. A group of compounds known as sphingolipids and its intermediates has a very wide range of physical properties. The great var variety of sphingolipid structures may contribute to the numerous biological processes involved in sphingolipids a member of a class of lipids that are vital elements of cellular membranes. According to recent studies, plants have a

major influence over several processes, including Programmed Cell Death (PCD) [1]. Sphingolipids may serve as second messengers in plant cells for a variety of cellular signaling processes, such as stress responses and programmed cell death. Sphingolipids may serve as second messengers in plant cells for a variety of cellular signalings, such as stress responses and programmed cell death (PCD). It is shown that two of the most prevalent free sphingoid Long Chain Base (LCB) in plants, fumonisin B1 and AAL toxin, cause the accumulation of dihydrosphingosine (d18:0, DHS), also known as sphinganine, in plants. Necrotrophic fungi produce mycotoxins that interfere with sphingolipid metabolism and result in cell death symptoms [2]. The structural features of sphingolipids are determined by a fatty acid connected to a sphingoid base acyl chain amide (FA).

This structure, known as ceramide (Cer), can also be changed by adding glycosyl residues and other polar phosphate-containing head groups. The large range of plant structures allows for a wide variety of physical properties of sphingolipids. The sphingolipid's long-chain base (LCB) can be hydroxylated and/or desaturated, and its usual carbon chain length is between 16 and 20. Additionally, modifications can be made to very long-chain fatty acids (VLCFAs), which contain chains that range in length from 16 to 30 carbons [3]. Several Lipid isoforms can be produced by hydroxylating them at the C2 position and desaturating them at the -9 position. The hydroxylation of FA and LCB moieties is what makes sphingolipids unique. These alterations may have a major impact on the subsequent biological activity. Animal, yeast, and plant sphingolipid production and metabolism share many fundamental components, and investigations in both the animal and yeast kingdoms have taught us a lot about plant sphingolipid biology. The cause for this diversity is still not entirely understood, although plant sphingolipids show a lot more structural diversity than sphingolipids from other eukaryotes. One of the primary distinctions between the two kinds of sphingolipids is this. In plants, ceramides with a long-chain base (LCB) esterified to a long-chain fatty acid create complex sphingolipids (long-chain FA or VLCFA). The ceramide can be glucosylated or, as in this case, it can be found in the complex sphingolipid glycosyl inositol phosphoryl ceramide (GIPC), where a phosphodiester bond connects it to an inositol-glucuronic acid unit. R1 can be made up of a hydroxyl, amine, or N-acetyl amine group. There is a relationship between inositol and other saccharides. The four primary groups of plant sphingolipids are ceramides, glycosylceramides (GlcCers), glycosyl

inositolphosphoceramides (GIPCs), and free long-chain bases (LCBs). The sphingolipids that predominate in plant tissue are assumed to be GIPCs, or glycosylated inositol phosphoryl ceramides [4]. The concentrations of the various sphingolipid classes vary depending on the species and the tissue, with GlcCers present at around half the GIPC level and GIPCs being the dominating class in *Arabidopsis* leaves, according to studies on plants [4], [5]. The remaining sphingolipids are primarily composed of ceramides, with tiny amounts of free LCBs and phosphorylated LCBs. The plant's many tissues all contain different amounts of sphingolipids. It has been shown that the sphingolipid profiles of seeds and other plant components differ [6]. In pollen fractions, an abundance of in comparison to the levels of glucosylceramides previously described in leaves. Although they have been found in plant cell cultures, some sophisticated GIPCs are still not present in leaf tissue [7]. These findings raise the problem of the functional significance of alternate data from different tissue types, architectures, and compositions, but no comprehensive defense has yet been offered.

1.1.1. PROGRAMMED CELL DEATH:

A cell dies as a result of internal cellular processes like apoptosis or autophagy, which are referred to collectively as programmed cell death (PCD) or cellular suicide. PCD occurs in a biological process that often provides an advantage throughout the lifecycle of an organism or plant. During the development of both plant and animal tissue, PCD performs essential roles. Now understood to be a crucial cellular mechanism in plants, programmed cell death (PCD) is. Our knowledge of how these mechanisms contribute to plant PCD's defense, development, and stress response would be considerably improved by a deeper understanding of these processes. It is believed that for optimal growth and development, all eukaryotes need programmed cell death (PCD), which is the regulated and planned destruction of cells. It is believed that for optimal growth and development, all eukaryotes need programmed cell death (PCD), which is the regulated and planned destruction of cells. The highly structured, tightly controlled process of programmed cell death (PCD), which is crucial for plant development, defense mechanisms, and architecture, is only now beginning to be understood. However, because PCD usually occurs in a small number of inaccessible cells

buried beneath a large number of nearby healthy cells, studying the mechanism of PCD in whole plants can be difficult. Numerous teams have identified nuclease activity during PCD. In tobacco infected with TMV, for instance, investigation of the nuclease activity after HR-induced PCD revealed at least four different nuclease activities, not all of which resulted in nuclear DNA fragmentation [8]. The scientists hypothesized that the HR may have produced nucleases extracellularly as a defense mechanism, resulting in the destruction of pathogen DNA that had penetrated the body. At least three of these nucleases were discovered to be secreted into the intercellular fluid. It has been possible to examine several physiologically important cell processes in tissue cultures, and in many circumstances, it would be practical to study numerous PCD process components in vitro. Studying PCD in cell cultures may or may not be important, depending on how comparable the process of death in a cultured cell is to the mechanism of PCD in vivo [9]. The three distinct phases of the PCD process in vivo are the induction phase, during which the cell detects a death signal and starts to die, the effector phase, during which the cell commits irrevocably to death and the cell death machinery is activated, and the degradation phase, during which the action of the death machinery results in the morphological and biochemical features that are specific to the cell death process [10]. A cell's ability to activate PCD during the induction phase can be influenced by a variety of extracellular or intracellular stimuli [11]. For example, pathogen attack, abiotic or biotic stress, developmental cues, or cell-cell signaling may all initiate PCD. When the shared PCD machinery is activated and the degradation phase begins, numerous distinctive traits appear that can be utilized to identify cells that are actively dying. These traits include the shrinking of the protoplast and nucleus as well as the activation of endonucleases that cleave DNA. As PCD seems to involve a core mechanism, the death caused in cell culture will essentially resemble a whole-plant developmental or defense-activated cell death. Therefore, analysis of the in vitro death program is directly related to understanding the regulation and mechanism of plant cell death programs throughout growth and development [12]. Cell cultures are a desirable approach for studying the mechanism of PCD for a variety of reasons, like Accessibility: In a homogeneous culture that is quickly dividing, it is reasonably simple to assess quantitatively the growth or death of cells using simple vital stains. Uniformity: It is possible to create collections of quickly dividing, generally homogenous cells using

cell cultures. To reduce complexity, large quantities of cell material can be readily created from them or stored in storage. Dissecting the PCD mechanism can be challenging in whole plants, but it is frequently simpler in cell cultures. In some cases, though, single cells make the process much simpler. To attain maximum cell homogeneity or to investigate social controls and survival.

1.1.2. STRESS RESPONSES:

When a plant senses some sort of stress, it initiates several molecular and cellular processes known as plant stress responses. Abiotic stresses, such as drought or too much sunshine, can also be biotic stresses, like diseases or herbivores. Stress is an outside factor that can have an impact on a plant's growth, development, or output. The phrase "abiotic stress" refers to how non-living components adversely impact living things in a specific environment. The environmental extremes that cause the stresses include heat, drought, salt, extremely low or high temperatures, heavy metals, and others. Abiotic stressors, notably drought and hypersalinity, are the leading causes of crop loss worldwide. From individual cells up to the entire organism, plants will respond to stress. They will start a stress signal that will spread throughout the plant. The signal will equip the additional portion of the plant to withstand the strain. Plants can endure stress in two different ways: through adapting and acclimating. Evolutionary changes that help an organism survive in a certain environment are known as adaptations [13]. (Example: altering already-existing genes). The activation of various responses including complex gene connections and crosstalk with numerous molecular pathways is a characteristic of plant adaptation to abiotic stressors. For a plant to survive poor environmental conditions, it must first become acclimated. For instance, as soil salinity rises, the high-salt environment transforms into an abiotic stress plant because it can upset the ion homeostasis of plant cells. The plant must make osmotic adjustments and regulate the intracellular pH to restore the ion balance in its cells and solve this issue. An unfavorable condition known as "biotic stress" occurs when a plant is unable to maintain its normal growth as a result of interactions with harmful microorganisms, such as herbivore attacks, insect attacks, and disease-causing pathogen attacks (fungi, bacteria, viruses, viroid's, phytoplasma, and nematodes). Plants must continue to protect themselves from the

biotic stressor. Because they are unable to escape from these biotic stresses, plants have developed inducible defense mechanisms [14]. The majority of wild plants are generally in good health, and even when disease does strike, it usually only affects a small number of plants, seldom killing the plant under natural growth conditions. As a result of evolving for a specific mechanism to infiltrate the plant, the majority of diseases only attack a certain portion of the plant: Enzymatic assault, surface layers under mechanical pressure, and natural opening (stomata, lenticels), Disease symptoms include mosaic, necrosis, spotting, wilting, or swollen roots were caused by previously injured tissue. Principal attack methods: Necrotrophy: the death of plant cells, Biotrophy: plant cells continue to function, Cells that are hemibiotrophic are kept alive but eventually die.

CHAPTER – 2

2. REVIEW OF LITERATURE

2.1. SPHINGOLIPIDS IN CELL DEATH:

Sphingolipids are a diverse class of lipids that are involved in a variety of cellular functions, including cell signaling and membrane structure. While sphingolipids contain certain sphingolipid metabolites, such as dihydrosphingosine, phytosphingosine, and ceramide, can induce cell death through a process called apoptosis. Apoptosis is a form of programmed cell death that is necessary for the normal development and maintenance of tissues in the body. Ceramide can activate a signaling pathway that triggers apoptosis in cells, leading to their death. This process is regulated by a variety of factors, including enzymes that metabolize sphingolipids, as well as proteins that regulate the balance between pro- and anti-apoptotic signals. In summary, while sphingolipids themselves are important for many cellular functions, certain sphingolipid metabolites, such as ceramide, can induce cell death through the process of apoptosis. Programmed cell death (PCD), which can happen during plant development or immunity, is regulated by sphingolipids. They belong to a group of bioactive lipids that control numerous critical cellular processes, including apoptosis and autophagy. By concentrating on multiple common regulators, sphingolipid metabolites differentially regulate the induction of apoptosis and autophagy. Sphingolipids (SLs) are now recognized as more than merely the fundamental building blocks of biological membranes. The relative balance between ceramide and sphingosine 1-phosphate (S1P), two SLs with opposing roles in the regulation of cell death and survival, determines the fate of cells in response to specific stimuli. Ceramide in particular interacts with downstream targets to mediate specific cell responses like proliferation, growth arrest, apoptosis, differentiation, or recognition [15]. Ceramide can act as a transducing signal as well as a signal for cell death. After being dispersed by the Golgi, it serves as a promoter, ganglioside precursor, and complex glycosphingolipid (GSL) precursor in several membrane compartments. Ceramide is the prototypical SL that has been investigated the most in terms of cell death induction and stress response. Ceramide can be created by a variety of routes, and since its metabolism

into other GSLs or transformation into survival derivatives, including S1P, modifies its role in mechanisms that cause cell death. Serine palmitoyl transferase (SPT), the rate-limiting enzyme in ceramide biosynthesis, catalyzes the synthesis of 3-ketosphinganine at the endoplasmic reticulum (ER), the site of ceramide de novo synthesis. L-serine and palmitoyl-CoA condense to form SPT at this location. After reducing 3-ketosphinganine to the sphingoid base sphinganine, the (dihydro)ceramide synthase acylates it to create dihydroceramide. The oxidation of dihydroceramide to ceramide is subsequently catalyzed by dihydroceramide desaturase, creating a trans-4,5 double bond. In contrast to the bulk of the other enzymes in the SLs biosynthesis pathway, which typically have one or two isoforms, ceramide synthase genes are present in a wide variety of taxa, including mammals and plants[16]. Even though the origin of this variation in ceramide synthase-expressing genes is unknown, it shows a crucial role for ceramides containing certain fatty acids in cell physiology and death regulation.

2.1.1. ABIOTIC STRESS IN PLANTS:

Abiotic stress is the negative impact of non-living factors on living organisms in a specific environment. A plant's first line of defense against abiotic stress is in its roots. If the soil holding the plant is healthy and biologically diverse, the plant will have a higher chance of surviving stressful conditions. The plant responses to stress are dependent on the tissue or organ affected by the stress. For example, transcriptional responses to stress are tissue or cell-specific in roots and are quite different depending on the stress involved. One of the primary responses to abiotic stress such as high salinity is the disruption of the Na^+/K^+ ratio in the cytoplasm of the plant cell. High concentrations of Na^+ , for example, can decrease the capacity of the plant to take up water and also alter enzyme and transporter functions.[17] Evolved adaptations to efficiently restore cellular ion homeostasis have led to a wide variety of stress-tolerant plants. Facilitation, or the positive interactions between different species of plants, is an intricate web of association in a natural environment. It is how plants work together. In areas of high stress, the level of facilitation is especially high as well. This could be because the plants need a stronger network to survive in a harsher environment, so their interactions between species, such as cross-pollination or mutualistic actions, become more common to cope with the severity of their habitat. Plants also adapt very differently from one another, even from a plant living in the same area [18]. When a group of different plant species was prompted by a variety of different stress signals, such as drought or cold, each plant responded uniquely. Hardly any of the responses were

similar, even though the plants had become accustomed to the same home environment. Serpentine soils (media with low concentrations of nutrients and high concentrations of heavy metals) can be a source of abiotic stress. Initially, the absorption of toxic metal ions is limited by cell membrane exclusion. Ions that are absorbed into tissues are sequestered in cell vacuoles. This sequestration mechanism is facilitated by proteins on the vacuole membrane [19]. An example of plants that adapt to serpentine soil is Metallophytes, or hyperaccumulators, as they are known for their ability to absorb heavy metals using root-to-shoot translocation (which it will absorb into shoots rather than the plant itself). They're also distinguished for their ability to absorb toxic substances from heavy metals. Chemical priming has been proposed to increase tolerance to abiotic stresses in crop plants. In this method, which is analogous to vaccination, stress-inducing chemical agents are introduced to the plant in brief doses so that the plant begins preparing defense mechanisms. Thus, when abiotic stress occurs, the plant has already prepared defense mechanisms that can be activated faster and increase tolerance. Prior exposure to tolerable doses of biotic stresses such as phloem-feeding insect infestation has also been shown to increase tolerance to abiotic stresses in the plant [20].

2.1.1.1. SALT STRESS:

Salt stress has been shown to affect plants physiologically, metabolically, cellularly, and at the molecular level. Around the world, salt has harmed more than 45 million hectares of land, and each year 1.5 million hectares are lost owing to high salinity levels in the soil [21]. ITRAQ-based proteomics research revealed that the enzyme 3- keto sphingosine reductase (KDSR), which catalyzes the second step of the manufacture of phytosphingosine, decreased during the early stage of salt stress in *Carex iridescent*. A worldwide occurrence, soil salinization refers to the buildup of water-soluble salts to levels that adversely impair plant growth. It affects about 831 million hectares of land. In more detail, the phenomena pose a threat to 2.1% of the world's non-irrigated (dry land) agricultural areas and 19.5% of the world's irrigated agricultural land. Because water-soluble salts can change osmotic potential gradients and subsequently hinder many cellular activities, high soil salinity levels can be damaging to plants. High concentrations of water-soluble salts in the soil can lower the osmotic potential of the soil and, as a result, lower the difference in water potential between the soil and the plant's roots, limiting the flow of electrons from H₂O to P680 in Photosystem II's reaction center. For instance, high soil salinity

can inhibit photosynthesis by restricting a plant's ability to absorb water. Many plants have evolved new traits over many generations to fend off the impacts of salt. The hormone ethylene works well in plants to counteract salt. Ethylene is well known for controlling plant development and handling stressful situations. The usage of ethylene signaling in numerous plant growth stages involves multiple key membrane proteins in plants, including ETO2, ERS1, and EIN2. These proteins' mutations have the potential to increase salt sensitivity while also limiting plant growth. Arabidopsis plants that have altered ERS1, ERS2, ETR1, ETR2, and EIN4 proteins have been used to study the effects of salinity. The ethylene precursor ACC is employed to reduce any sensitivity to salt stress, and these proteins are used for ethylene signaling against circumstances like salt stress.

2.1.1.2 TEMPERATURE STRESS:

During the adaptation to cold, sphingolipids serve as both structural components of membranes and signaling molecules. Arabidopsis WT plants accumulate as a result of low temperatures. Although the ratio of total sphingolipids does not increase, the ratio of unsaturated LCBs does. The first evidence of a potential role for sphingolipids in the response to cold was provided by the work by Lynch and Steponkus, in which they separated plasma membrane fractions from the leaves of unacclimatized and acclimated rye seedlings and investigated them for lipid alterations. As a result, it appears that sphingolipids containing unsaturated LCBs are promising candidates for both innate and not induced cold tolerance. They found that glucocerebroside decreased from 16 to 7 mol% of the total lipids in cold-acclimated seedlings. Later, in 1988, Yoshida et al. looked into the thermotropic properties of lipids taken from the plasma membrane and the chilling-sensitive plant *Vigna radiata*. Through interactions with the cell death suppressor AtBI-1, AtSLD1, AtFAH1, AtSBH2 (an LCB C-4 hydroxylase), AtADS2 (acyl lipid desaturase 2), and AtSLD1 (an LCB C-4 hydroxylase), sphingolipid synthesis in response to cold is aided. Recent research has identified LCBK1's function in Arabidopsis freezing tolerance. who investigated the plasma membrane lipid composition of cold-acclimated Arabidopsis genotypes found that cerebral side dropped from 7.3 to 4.3 mol% after cold acclimatization. Functional characterization of the D8 desaturases (AtSLD1 and AtSLD2) in Arabidopsis was done by who also presented genetic

evidence of low-temperature responses to LCB D8 unsaturation. These mutant rosettes contained less GlcCer, according to sphingolipidomic analysis, and a rise in GIPC was observed when GlcCer levels were raised by complementing with an AtSLD1 wild-type copy. Only a small number of studies have discovered proof that sphingolipid metabolism causes heat stress. It has been shown that exogenous LCB-phosphate makes Arabidopsis cell culture more resistant to heat stress.

2.1.1.3 DROUGHT STRESS:

In agriculture, drought stress, which is characterized as a naturally occurring water deficiency, is a major factor in crop losses. This is because water is necessary for numerous critical processes involved in plant growth. Recent years have made it more crucial than ever to find a solution to the drought hardship. Future global warming will almost certainly result in a decrease in precipitation and an increase in drought. To cope with drought stress, plants have developed a variety of mechanisms and adaptations. The closure of stomata is one of the primary strategies used by plants to counteract drought stress. Abscissic acid is a crucial hormone that controls the stomatal opening and shutting (ABA). The binding of ABA to receptors results from its synthesis. This binding then has an impact on how ion channels open, lowering turgor pressure and causing the stomata to close. Recent research has demonstrated how drought-stressed plants' ABA levels rose (2018). They demonstrated that under stressed conditions, plants produced more ABA to hold onto any water they may have in their leaves. Aquaporins are yet another crucial element in managing drought stress and controlling the intake and export of water (AQPs). The membrane proteins known as AQPs are a crucial component of channels. The movement of water and other vital solutes is the primary function of these channels. Many various factors, such as ABA, GA3, pH, and Ca^{2+} , regulate AQPs both transcriptionally and post-transcriptionally. The precise concentrations of AQPs in particular plant sections, such as the roots or leaves, aid in drawing as much water into the plant as possible. [32] Future drought-resistant plant breeding will be made possible by better understanding the processes of AQPs and the hormone ABA. Intriguingly, plants that experience dryness regularly have been shown to develop a kind of "memory". According to a study, plants that had previously experienced drought were able to devise a method to reduce water use

and water loss. They discovered that plants exposed to dry circumstances altered their stomata regulation and what they termed a "hydraulic safety margin" to lessen the plant's sensitivity. Plants were able to function more effectively when there was less available water by altering the management of stomata, which in turn led to transpiration.

2.2. BIOTIC STRESS IN PLANTS:

Due to the significant economic losses to cash crops, biotic stresses are a key area of research in agriculture. Economic choices and practical progress are both impacted by the link between biotic stress and plant output. Plant-stressor coevolution, population dynamics, and nutrient cycling in the ecosystem are all impacted by the effect of biotic damage on crop yield. Horticultural plant health and the ecology of natural ecosystems are likewise affected by biotic stress. Additionally, the host recipient experiences significant alterations. Plants are exposed to a variety of stressors, such as infections, excessive salinity, and drought, which lower plant output or lower the quality of produced goods. Despite the wide variety of biotic stresses, fungi are primarily responsible for most plant illnesses. *Arabidopsis thaliana* is frequently used as a model plant to examine how plants react to various stresses. For several hundred million years, parasites and plants have coevolved. The selection of a diverse spectrum of plant defenses against microbial diseases and herbivorous pests as a result of this co-evolutionary process reduces assault frequency and severity. These defense mechanisms comprise physical and chemical modifications that can either be expressed constitutively or, in many circumstances, only activated in response to an attack. Using high levels of metal ions from the soil, for instance, enables plants to lessen the negative effects of biotic stressors like pathogens and herbivores. At the same time, the distribution of metal ions within the plant is protected by physiological pathways, preventing the infliction of severe metal toxicity. Such induced resistance offers a technique through which the defense expenses are postponed until the defense is advantageous to the plant. Successful diseases and pests have also developed strategies to get beyond their particular host species' inherent and induced resistance. We need a thorough understanding of these interactions at many sizes, from the molecular to the community level, to properly comprehend and regulate plant biotic stress resistance.

2.3 SPHINGOLIPID RESPONSE TO ABIOTIC STRESS:

Plants have evolved a variety of defense mechanisms to withstand cell damage as a result of their sessile nature. Sphingolipids, a vital component of plasma membranes, play an important role in signal transduction and in reducing the effects of abiotic stress on plasma membranes[16]. Abiotic stress has an impact on a plant's growth and development, which eventually leads to vigor loss and death. The plant must deal with the challenge of the changing physical and chemical composition of cell membranes during temperature, salt stress, or hypoxia.

TEMPERATURE STRESS:

When a body adapts to cold temperatures, sphingolipids serve as both signalling molecules and structural components of membranes. In *Arabidopsis* WT plants, low temperatures lead to an accumulation. Unsaturated LCBs are more prevalent at low temperatures, although total sphingolipids are not more prevalent. The earliest evidence of a potential role for sphingolipids in the response to cold was provided by Lynch and Steponkus' study, which separated plasma membrane fractions from the leaves of rye seedlings that had been acclimated and those that had not. They then looked for lipid alterations in the leaves. As a result, it appears that sphingolipids containing unsaturated LCBs are promising candidates for both innate and not induced cold tolerance. They found that glucocerebroside decreased from 16 to 7 mol% of the total lipids in cold-acclimated seedlings [17]. Later, in 1988, Yoshida et al. looked into the thermotropic properties of lipids taken from the plasma membrane and the chilling-sensitive plant *Vigna radiata*. Through interactions with the cell death inhibitor AtBI-1, AtSLD1, AtFAH1, AtSBH2 (an LCB C-4 hydroxylase), AtADS2 (acyl lipid desaturase 2), and AtSLD1 (an LCB C-4 hydroxylase), AtADS2 and AtSLD1 work together to produce sphingolipids in response to cold. A role for LCBK1 in *Arabidopsis* freezing tolerance has been described by recent findings [18]. who investigated the plasma membrane lipid composition of cold-acclimated *Arabidopsis* genotypes found that cerebroside dropped from 7.3 to 4.3 mol% after cold acclimatization. [19] functionally investigated the D8 desaturases (AtSLD1 and AtSLD2) in *Arabidopsis* and presented genetic evidence of low-temperature responses to LCB D8 unsaturation. Sphingolipidomic research showed that these mutant rosettes contained less GlcCer, and complementation with an AtSLD1 wild-

type copy increased GIPC when GlcCer levels were increased [19]. Only a small number of studies have discovered proof that sphingolipid metabolism causes heat stress. Arabidopsis cell culture was shown to be able to withstand heat stress with the aid of exogenous LCB-phosphate [20].

SALT STRESS:

Salt stress has been shown to affect plants physiologically, metabolically, cellularly, and at the molecular level. Around the world, salt has harmed more than 45 million hectares of land, and each year 1.5 million hectares are lost owing to high salinity levels in the soil [21]. ITRAQ-based proteomics research revealed that the enzyme 3- ketosphingosine reductase (KDSR), which catalyzes the second step of the manufacture of phytosphingosine, decreased during the early stage of salt stress in *Carex iridescent* [22]. The authors assumed that KDSR serves as a suppressor of the calcium signal during salt stress based on research done in yeast where 3-ketosphinganine reductase reduced Ca^{2+} sensitivity. Role of sphingolipid signaling Atgint1 mutant seeds exhibited a greater germination rate than WT in response to salt stress, but this difference vanished at higher salt concentrations. Atgint1 is a glucosamine inositol phosphorylceramide transferase that is responsible for the glycosylation of certain GIPCs [23] modulation in the plant response to salt stress. They found that transgenic tobacco with overexpressed *Oryza sativa* sphingosine-1-phosphate lyase (S1P lyase) has lower salt tolerance than wild-type tobacco[24]. When AtACER was overexpressed, plants demonstrated improved tolerance to salinity, demonstrating the role of ceramides in the body's response to salt stress. Plants also displayed enhanced sensitivity to salinity. More specifically, recent research has demonstrated that AtACER controls autophagy brought on by excessive salt stress. The S1P lyase-overexpressing plants also showed reduced expression of a few selective stress-related genes, including NtSAM1, NtTOBLT, and NtLEA5, in response to salt treatment, suggesting that the altered responsiveness of stress-related genes may be responsible for the decreased tolerance in S1P lyase-overexpressing tobacco plants under salt stress[24]. A rice S1P (sphingosine-1-phosphate) lyase gene overexpression in tobacco reduced salt tolerance and altered genes involved in salt stress. The underlying physiological and metabolic mechanisms behind this phenomenon, however, were not clarified by any lipidomic data in these transgenic tobacco lines. In contrast, overexpressing

OsLCBK1 in tobacco plants did not affect the expression of genes associated with salt stress or the tolerance/sensitivity phenotypic in response to salt stress [25], indicating that this enzyme is not involved in rice's responses to salt stress. It's interesting to note found that Arabidopsis acquires salt tolerance thanks to the ceramide-catalyzing enzyme ceramidase (AtACER), which is found in the ER and Golgi. (AtACER loss-of-function mutants had high amounts of ceramide and were more sensitive to salt stress, whereas overexpressed lines were more salt-tolerant [26]. To determine how the LCB content would differ between these two overexpressing plants, no sphingolipidomic investigation has been carried out. The sphingolipid metabolism may be altered, and as a result, the length chain, concentration, and threshold are critical for sphingolipid activity. This sphingolipid signaling pathway included heterotrimeric G-protein, Ca^{2+} mobilization, and ion channel activity modulation. AtLCBK1 was reported to be triggered by low humidity or ABA treatments, which is consistent with this [27]. Additionally, AtACER's role in autophagy in Arabidopsis under nutritional, oxidative, and salt stressors has recently been demonstrated. These investigations suggested that sphingolipid signaling contributes to plant salt stress resistance.

2.4. SPHINGOLIPID RESPONSE TO BIOTIC STRESS:

Plant survival and productivity are seriously threatened by biotic stress brought on by insects and plant diseases. Numerous studies have demonstrated the crucial function that plant sphingolipids play in biotic stress, including protecting against bacterial and fungal infections by instructing the cell to start PCD. When confronted by pathogens, plants have evolved a complex array of defenses. The ability of the plant to recognize its invasion and then translate the various cues into an adaptive response are prerequisites for an effective innate immune response. Before considering the involvement of sphingolipids in PCD, it is crucial to be aware that 2% of random crossings between Arabidopsis wild strains result in F1 offspring that exhibit symptoms of leaf necrosis and are smaller than their parent plants [28]. Sphingolipids are significant molecules at the forefront of pathogen recognition as structural elements of the plasma membrane. Sphingolipids function as mediators in the defense signaling cascade because they have an effect on PCD and the accumulation of various well-known defense chemicals (such as ROS, MAPK, and

hormones). The inbred strains containing the naturally occurring hyperactive allele of the accelerated cell death 6 (ACD6) gene exhibit very identical, but less severe, symptoms [28]. 2003 hypothesize that the accumulation of sphingolipid intermediates may be the cause of the hybrid necrosis seen in F1 progeny [29], [30]. Very recently, sphingolipid pool alterations following exposure to biotic stress were discovered by metabolomic profiling. A reduction in the levels of ceramide N-palmitoylsphinganine was one of the dynamic alterations in sphingolipid metabolism caused by *Xanthomonas campestris* pv. *campestris* infection in *Brassica oleracea* [31]. Additionally, cell death was caused by ceramide levels that were raised as a result of a mutation in the inositol phosphoryl ceramide synthase (IPCS) gene. By inhibiting RPW8-mediated resistance to powdery mildew, the IPCS gene in its functional form has been linked to plant defense. Ceramide phosphatidylinositol levels were identified at higher levels after tomato fruit had been treated with the β -aminobutyric acid elicitor. According to this metabolomic research, biotic stressors may have an impact on how sphingolipids are metabolized. Unexpectedly, mycotoxins from fungi, like AAL and fumonisin B1 (FB1), that block the essential ceramide synthesis enzymes also cause PCD. This is due to the cell accumulating free LCBs, which are also signals for PCD, despite ceramide depletion [33]. In a recent study, it was shown that the long-chain base phosphate lyase (*lcbpl*) mutant of the *Arabidopsis* plant was resistant to the necrotrophic fungus *Botrytis cinerea* but vulnerable to the hemibiotrophic bacterium *Pseudomonas syringae* [32]. It has been proposed that μ signal transduction in response to biotic stress. These metabolites can have either microbial or plant origins or even both, and they function as elicitors to start signal cascades.

CHAPTER – 3

3. AIM:

To regulate the programmed cell death in *Chlamydomonas reinhardtii* using sphingolipid.

3.1. OBJECTIVES:

- To investigate the role of sphingolipids in *C. reinhardtii*
- Action of metabolites like dihydrosphingosine(DHS) and phytosphingosine(PHS)
- Regulation of Programmed Cell Death in *Chlamydomonas*.
- Analysis of cell death in *C. reinhardtii*

CHAPTER – 4

4. MATERIALS AND METHODS

4.1. Algae growth and culture conditions:

The wild-type strain of *C.reinhardtii* CC-124 was obtained from the Chlamydomonas Research Center, University of Minnesota, Minneapolis, MN, USA. The culture was maintained in a tris-acetate phosphate (TAP) medium at 25 °C. The Chlamydomonas culture was inoculated in TAP media and maintained at 20-22 °C for further growth, the culture was then ready by 4-5 days. The inoculated culture was transferred to a falcon tube and centrifuged for 5min at 5000rpm, the pellet is collected and diluted up to 10mL. In a 6-well plate, 2mL of culture is transferred each at the concentration of “0” (control), “0.5µM”, “5µM” and “50µM” of dihydrosphingosine(DHS). Finally, it has been incubated for 1 hour in the shaker to cause cell death. Using various stains different culture treatments have been performed [35]. Cell death was calculated as the percentage of dead cells compared to the total number of cells. The cell counting of each sample was performed from three different randomly chosen microscopic fields containing at least 85–100 cells.

4.2. Cell viability test:

After the initial treatment, incubated sample from each well is transferred to 2mL eppendorf tubes, they are then centrifuged for 5min at 5000rpm two times, and FDA or propidium iodide stain is added to test the cell viability. To the DHS concentration of “0”, “0.5µM”, “5µM” and “50µM”- FDA (Fluorescein diacetate) of concentration “4µL” is added to each Eppendorf tube and incubated for 15min in the shaker. Finally, it has been centrifuged again and diluted into 100µL [36].

4.3. Photosynthetic activity:

The wild-type strain of *C. reinhardtii* CC-124 was obtained from the *Chlamydomonas* Research Center, University of Minnesota, Minneapolis, MN, USA. The culture was maintained in a tris-acetate phosphate (TAP) medium at 25 °C. The Chlamydomonas culture was inoculated in TAP media and maintained at 20-22 °C for further growth, the culture was then ready by 4-5 days. The inoculated

culture was transferred to a falcon tube and centrifuged for 5min at 5000rpm, the pellet is collected and diluted up to 10mL. In a 6-well plate, 2mL of culture is transferred each at the concentration of "0"(control), "0.5µM", "5µM" and "50µM" of dihydrosphingosine(DHS). Finally, it has been incubated for 1 hour in the shaker to cause cell death [37]. The activity is observed in light (Photosystem II) as well as dark phases (non-photosynthetic quenching) using a cuvette in a photon system device.

4.4. ROS and Lipid peroxidation:

Reactive Oxygen Species:

After the initial treatment, incubated sample from each well is transferred to 2mL eppendorf tubes, they are then centrifuged for 5min at 5000rpm two times, and DCFH-DA (dichlorofluorescein diacetate) dye is added to evaluate reactive oxygen species in live cell samples quantitatively [36]. To the DHS concentration of "0", "0.5µM", "5µM" and "50µM"- DCFH-DA dye concentration of "3µL" is added to each Eppendorf tube and incubated for 15min in the shaker. Finally, it has been centrifuged again and diluted into 100µL.

Lipid peroxidation:

After the initial treatment, incubated sample from each well is transferred to 2mL eppendorf tubes, they are then centrifuged for 5min at 5000rpm two times, and C11-bodipy dye is added to evaluate reactive oxygen species in live cell samples quantitatively [36]. To the DHS concentration of "0", "0.5µM", "5µM" and "50µM"- C11-bodipy of concentration "1µL" is added to each Eppendorf tube and incubated for 15min in the shaker. Finally, it has been centrifuged again and diluted into 100µL.

4.5. Antioxidant assay:

After the initial treatment, incubated sample from each well is transferred to 2mL eppendorf tubes, they are then centrifuged for 5min at 5000rpm two times. The supernatant was subsequently used to determine the activities of antioxidant enzymes. The SOD, CAT and APX activities were determined respectively, according to the methods of Beauchamp & Fridovich [38], Nakano & Asada [39] and Aebi [40].

Superoxide dismutase:

Cells were collected by centrifugation at 8,000 x g for 10 min and washed by suspending them in 50 mM K-phosphate (pH 7.8)-0.5 mM EDTA (Buffer A) with subsequent centrifugation at 8,000xg for 10 min at 20°C. Subsequent extraction and purification of SOD were carried out at 0-4°C unless otherwise indicated. Typically, 20 g (fr wt) washed cells were suspended with 60 ml of Buffer A plus 1 mM phenylmethylsulfonyl fluoride, and each 40 ml aliquot was sonicated at 100 W output with three cycles of 1-min sonication followed by a 1-min interval. The sonicate was centrifuged at 144,000 x g for 60 min and the supernatant was saved. The pellet was suspended with 60 ml of Buffer A plus 1 mM phenylmethylsulfonyl fluoride, sonicated with two 1-min bursts, and centrifuged as above. Ammonium sulfate was added to the combined supernatant to 30% saturation, and the resultant precipitate was removed by centrifugation at 10,000 x g for 10 min. To the supernatant, ammonium sulfate was added to 45% saturation, and the resultant precipitate was again removed by centrifugation. To the supernatant, ammonium sulfate was added to 80% saturation, and the resultant precipitate was collected by centrifugation. The precipitate was dissolved in 20 ml of 10 mM K-phosphate (pH 7.8)-0.1 mM EDTA (Buffer B) and dialyzed against 500 ml of Buffer B with one change of buffer. The retentate was centrifuged at 25,000 x g for 10 min, and the supernatant was applied to a DEAE cellulose column (DE23, Whatman, 1 x 50 cm) equilibrated with Buffer B. The applied proteins were successively eluted with 360 ml of a linear gradient of 0-0.1 M KC1 in Buffer B, 40 ml of 0.1 M KC1 in Buffer B, 80 ml of 0.2 M KC1 in Buffer B, and finally 80 ml of 0.4 M KC1 in Buffer B. SODs were eluted in several peaks, and the fractions in the first peak were pooled (Group #1, see Fig. 1) and concentrated with an Amicon PM10 membrane. The concentrate was dialyzed against 20 mM K-phosphate (pH 7.2) and applied to a hydroxyapatite column (1.5x30 cm, Bio-Gel HTP, BioRad) equilibrated with 10 mM K-phosphate (pH 7.2). SOD was eluted with a 200 ml linear gradient of 10- 200 mM K-phosphate (pH 7.2). The peak fractions of SOD activity were combined and concentrated by ultrafiltration with a Centricon-10 (Amicon). The concentrated enzyme was applied to a Superdex 75 column (1 x 30 cm, Pharmacia) equilibrated with Buffer B containing 0.1 M KC1 at 25°C. SOD was eluted with the same buffer. The elution volumes of molecular weight standard proteins were determined under the same conditions.

Ascorbate peroxidase:

AsA (1 mM) was added immediately after breaking the cells to avoid oxidation and inactivation of the enzymes. Cell extract was added to the reaction mix (50 mM phosphate buffer, 0.1 mM AsA, pH 7.0) in a quartz cuvette, and the reaction was initiated by the addition of 0.1 mM H₂O₂. APX activity was increased, which corresponds to the oxidation of AsA.

Catalase:

Cell extract was added to 50 mM phosphate buffer, pH 7.0 with 10 mM H₂O₂. CAT activity was increased as the stress increased which corresponds to the decomposition of H₂O₂

4.6. Mitochondrial membrane potential:

After the initial treatment, incubated sample from each well is transferred to 2mL eppendorf tubes, they are then centrifuged for 5min at 5000rpm two times, and Rh123 is added to monitor the mitochondrial health and membrane damage in the cell[41]. To the DHS concentration of "0", "0.5µM", "5µM" and "50µM"- Rh123 concentration of "3µM" is added to each Eppendorf tube and incubated for 15min in the shaker. Finally, it has been centrifuged again and diluted into 100µL.

4.7. Calcium signaling:

After the initial treatment, incubated sample from each well is transferred to 2mL eppendorf tubes, they are then centrifuged for 5min at 5000rpm two times, and Calcium Green-1 is added in order to regulate the cell death [42]. To the DHS concentration of "0", "0.5µM", "5µM" and "50µM"- Calcium Green-1 concentration of "15µM" is added to each Eppendorf tube and incubated for 15min in the shaker. Finally, it has been centrifuged again and diluted into 100µL.

4.8. Comet assay:

After the initial treatment, incubated sample from each well is transferred to 2mL eppendorf tubes, they are then centrifuged for 5min at 5000rpm two times, and Ethidium bromide is added to measure the extent of DNA damage in cells undergoing different types of cell death. The comet assay can also be used to evaluate the genotoxicity of various compounds or treatments that induce cell death. To the DHS concentration of "0", "0.5µM", "5µM" and "50µM"- Ethidium bromide

concentration of “25 μ l” is added to each Eppendorf tube and incubated for 15min in the shaker. It has been centrifuged and the pellet was washed with 500 μ L S-buffer (50 mM Tris–HCl pH 7.5, 10 mM MgCl₂, 1 M sorbitol), centrifuged again, and re-suspended in S-buffer. Aliquots from the cell suspension containing 10³ cells mL⁻¹ were mixed with low-melting agarose to a final concentration of 0.7 % and were spread as micro-gels on microscopic slides. To set the gels the slides were incubated for 5 min at 4 °C and thereafter submerged in lysis solution (1 M NaCl, 50 mM EDTA, pH 8, 30 mM NaOH, 0.1 % *N-lauroyl sarcosine*) for 1 h at 4 °C; washed three times for 20 min at 4 °C with denaturing solution (30 mM NaOH, 10 mM EDTA, pH 8) and subjected to electrophoresis (0.45 V cm⁻¹) for 10 min at 4 °C in the same denaturing solution. Following electrophoresis, the gels were neutralized for 5 min with dH₂O and dehydrated at room temperature by successive washes in 75 % and 95 % ethanol for 5 min each. After dehydration, the gels were stained with 1 $\times$ SYBR green I and the DNA comets were observed under a fluorescence microscope using a 450–490-nm band-pass filter. One hundred objects per slide were scored and statistically analyzed [43].

4.9. Caspase assay:

After the initial treatment, incubated sample from each well is transferred to 2mL eppendorf tubes, they are then centrifuged for 5min at 5000rpm two times, and NucView® 405 Blue Caspase-3 Dye is added in order to regulate the cell death. To the DHS concentration of “0”, “0.5 μ M”, “5 μ M” and “50 μ M”- NucView® 405 Blue Caspase-3 Dye concentration of “15 μ M” is added to each Eppendorf tube and incubated for 15min in the shaker. Cells were harvested by centrifugation at 1100 $\times$ g for 5 min at 25°C, washed in 1x phosphate buffered saline (PBS), and centrifuged at 5000 $\times$ g for 5 min at 4°C. They were then incubated in chilled lysis buffer (supplied in the kit) on ice for 20 min and centrifuged at 16 000 $\times$ g for 20 min at 4°C to precipitate cellular debris. Clear lysates were used for measuring caspase-3-like activity. Specific activity was expressed as nanomoles of pNA released per mg of protein per min. For determination of the fold increase in activity, the ratios of the caspase-3-like activity of treated and untreated cells from four independent experiments were calculated and means were calculated. Additionally, four independent experiments were performed to show that the caspase inhibitor Ac-DEVDCHO was able to abrogate caspase-3-like activity in DHS-treated cells [44].

CHAPTER – 5

5. RESULT AND DISCUSSION

5.1. CELL VIABILITY TEST:

Chlamydomonas is an unicellular organism and an excellent model system to study the fundamental cellular processes, such as photosynthesis, flagellar movement, and the regulation of gene expression. In earlier studies, the stress response in *Chlamydomonas* demonstrated the common hallmark reactions of ferroptosis, similar to other eukaryotes. Here to investigate cell death in *Chlamydomonas*, cells were subjected to different concentration of dihydrosphingosine (“0”, “0.5 μ M”, “5 μ M” and “50 μ M”) and cell death was observed at regular intervals in 1h. Then it is stained with FDA(Fluorescein diacetate). The results indicate that the addition of dihydrosphingosine(DHS) significantly causes cell death. The cell viability was also assessed by staining the cells with propidium iodide.

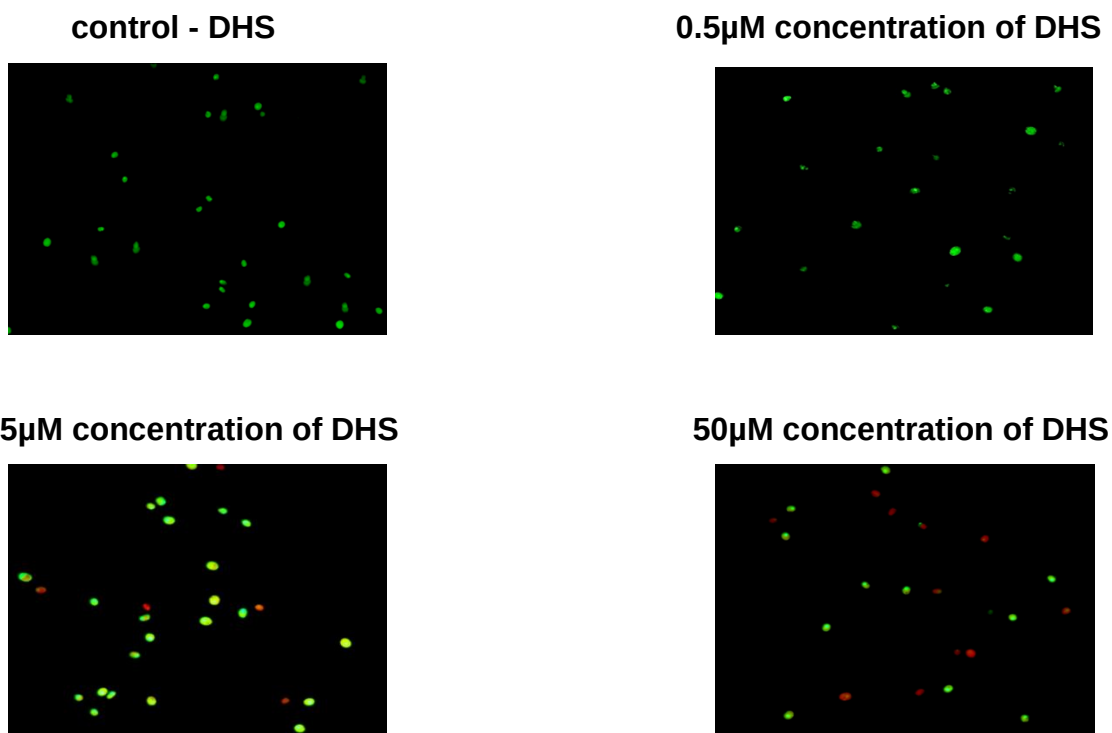


Figure 5.1 – *Chlamydomonas* cells under a fluorescence microscope

(The image represents the dead cells and live cells. The green color represents the live cells and the red color represents the dead cells)

Graphical representation:

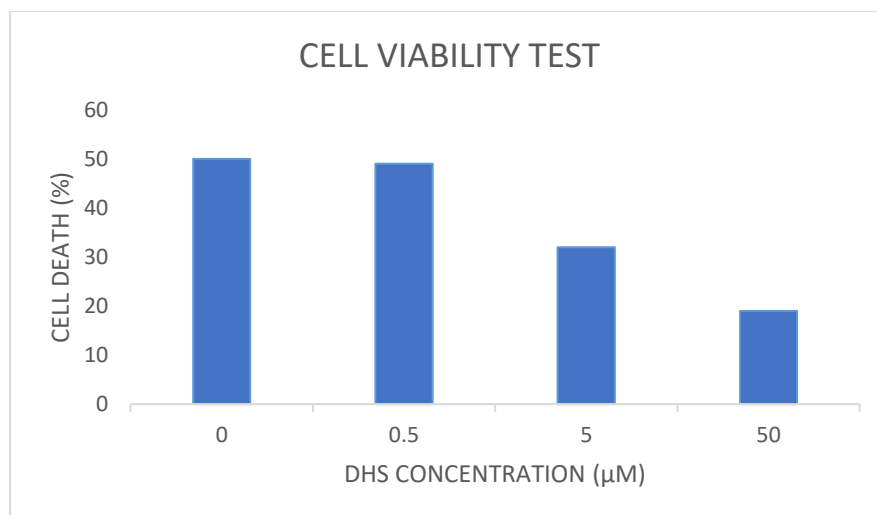


Figure 5.2 – Graphical representation of cell viability test

The graph explains that at the 0 concentration of DHS, there are no dead cells, at 0.5 μM concentration of DHS some cells may be stressed or dead, and at “5 μM ” concentration of DHS more dead cells are seen compared with “0.5 μM ” concentration finally at “50 μM ” of DHS concentration more number of dead cells are analyzed.

5.2. PHOTOSYNTHETIC ACTIVITY:

Photosystem II:

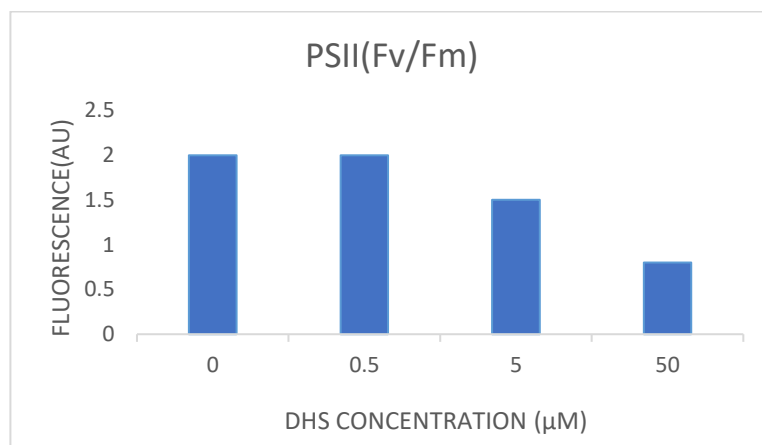


Figure 5.3 – Graphical representation of Photosystem II

The graph represents that at the 0 concentration of DHS, there will be no cell death so the chlorophyll activity is increased, at 0.5 μ M concentration of DHS the cells won't be that much stressed so the chlorophyll activity is decreased by 0.1% and at "5 μ M" concentration of DHS more dead cells are seen compared with "0.5 μ M" concentration so the chlorophyll activity is decreased by 20%. Finally at "50 μ M" of DHS concentration more dead cells are analyzed so the chlorophyll activity is decreased drastically compared to the other three concentrations. PS2(Fv/Fm)-Photosystem II is one such experimental parameter that is done using light energy.

Non-photosynthetic quenching:

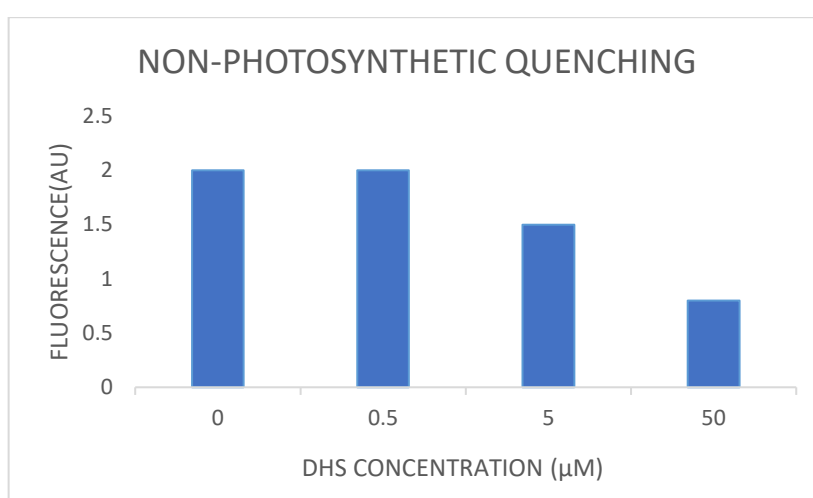


Figure 5.4 – Graphical representation of Non-photosynthetic quenching

Similarly, this graph represents that at the 0 concentration of DHS, there will be no cell death so the chlorophyll activity is increased, at 0.5 μ M concentration of DHS the cells won't be that much stressed so the chlorophyll activity is decreased by 0.1%, and at "5 μ M" concentration of DHS more dead cells are seen compared with "0.5 μ M" concentration so the chlorophyll activity is decreased by 20%. Finally at "50 μ M" of DHS concentration more dead cells are analyzed so the chlorophyll activity is decreased drastically compared to the other three concentrations. Non-photosynthetic quenching is one such experimental parameter that is done in the dark phase.

5.3. ROS AND LIPID PEROXIDATION:

Reactive Oxidative Species treatment:

The cellular level accumulation of ROS in *Chlamydomonas* cells was analyzed using ROS-sensitive fluorescence dye DCFDA. DCF fluorescence signals can be captured with a fluorescence microscope and the intensity at 550nm of the signal reveals the intracellular level of ROS accumulation. The results showed that ROS levels significantly increased as the stress increased. Previous reports revealed a significant increase in lipid production during oxidative stress induced by N-deficit conditions in microalgae.

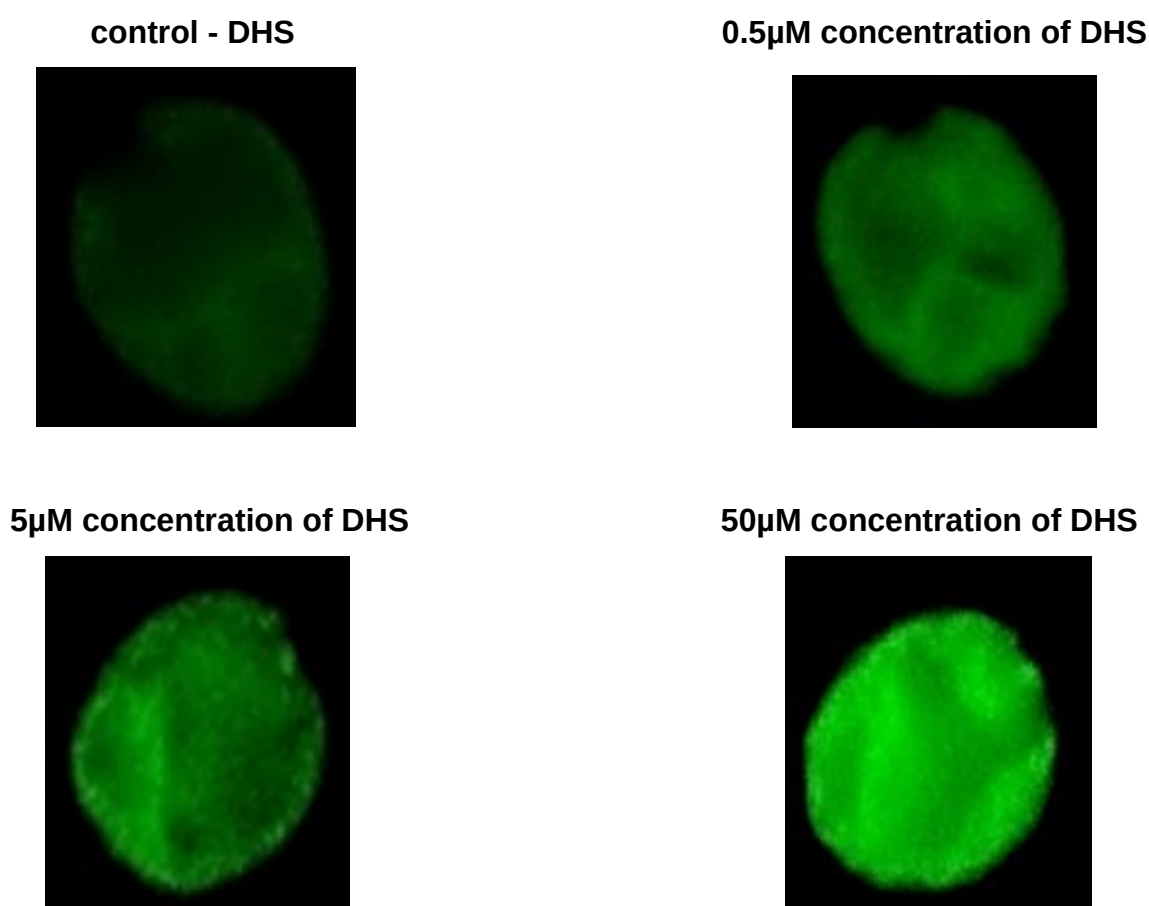


Figure 5.5 – ROS treated *Chlamydomonas* cells

Graphical representation:

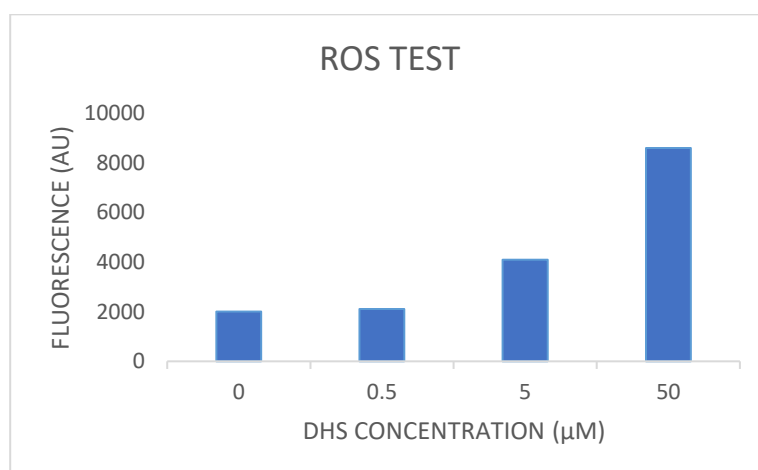


Figure 5.6 – Graphical representation of ROS test

The graph demonstrates that at the 0 concentration of DHS, the stress is less and there are no dead cells, at 0.5μM concentration of DHS some cells may be stressed or dead, and at “5μM” concentration of DHS more cells are stressed and dead compared with “0.5μM” concentration finally at “50μM” of DHS concentration the stress is increased and more number dead cells are seen.

Lipid peroxidation:

Lipid peroxidation and its by-products are common biomarkers for in vivo oxidative stress. Lipid peroxidation was evaluated in the current study using C11-BODIPY. Upon N deficit, the intensity of C11-BODIPY was significantly increased in the wild type than the acds-overexpressing lines. Though, no substantial difference was observed in fluorescence intensity of C11- BODIPY staining and TBARS content between acds-expressing lines and wild type under N-replete conditions. These results suggest that nitrogen stress leads to lipid peroxidation but the effect is counteracted in the acds-expressing transgenic lines that reduce the lipid peroxidation and protect the cells from ROS damage through the enzyme ACC deaminase. Our results accordance with prior findings that heterologous expression of ACC deaminase reduces the ROS accumulation and lipid peroxidation level in *Chlamydomonas* by modulating ethylene during stress conditions.

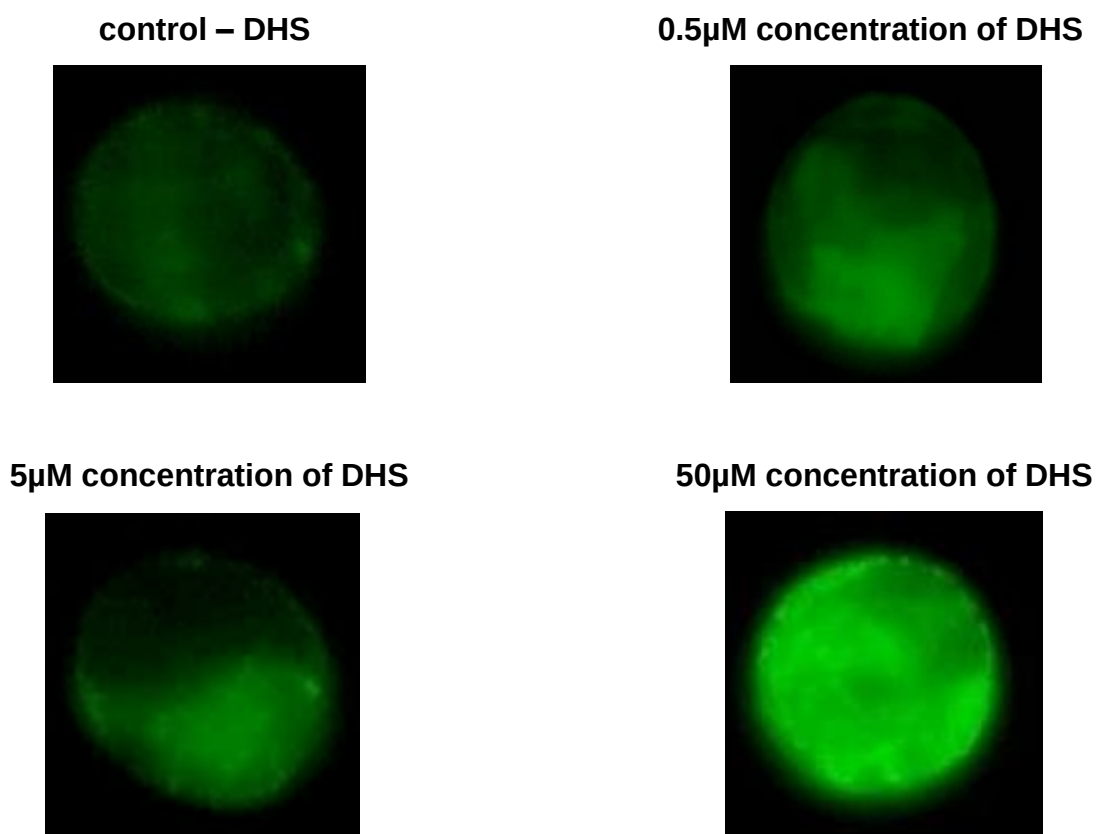


Figure 5.7 – *Chlamydomonas* cells in lipid peroxidation test

Graphical representation:

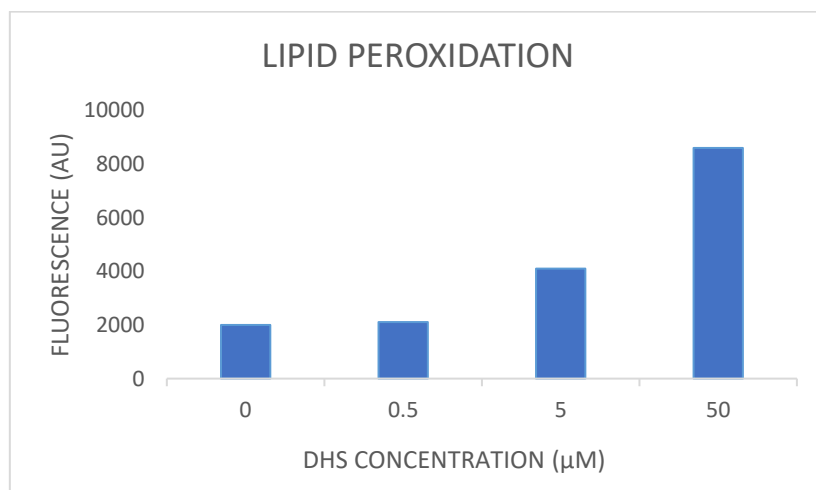


Figure 5.8 – Graphical representation of lipid peroxidation

Similarly, this graph demonstrates that at the 0 concentration of DHS, the stress is less and there are no dead cells, at 0.5 μ M concentration of DHS some cells may be

stressed or dead, and at “5 μ M” concentration of DHS more cells are stressed and dead compared with “0.5 μ M” concentration finally at “50 μ M” of DHS concentration the stress is increased and more number of dead cells are seen.

5.4. ANTIOXIDANT ASSAY:

Under adverse conditions, microalgae usually accumulate ROS, leading to the induction of oxidative stress which in turn damages proteins, DNA, and membrane lipids [45]. To scavenge the ROS, microalgae employ several antioxidant defence mechanisms during stressful conditions. The antioxidant enzymes including APX, CAT and SOD are important factors that help in quenching ROS in microalgae [45]. In the current study, SOD, CAT, and APX activity levels revealed no significant changes were observed in the *Chlamydomonas* sample in stress conditions. However, under stress, *Chlamydomonas* showed a 1.5-fold increase of SOD activity, a 1.6-fold increase in CAT activity, and a 1.4-fold increase in APX activity. In order to control ROS levels and maintain redox homeostasis, microalgae can develop tolerance to abiotic stress by improving the antioxidant enzyme activity. In accordance with the reports emphasizing the importance of antioxidant enzymes in stress conditions, our study reported that dihydrosphingosine increases ROS accumulation and damage during stress by improving antioxidant enzyme activities

Superoxide dismutase:

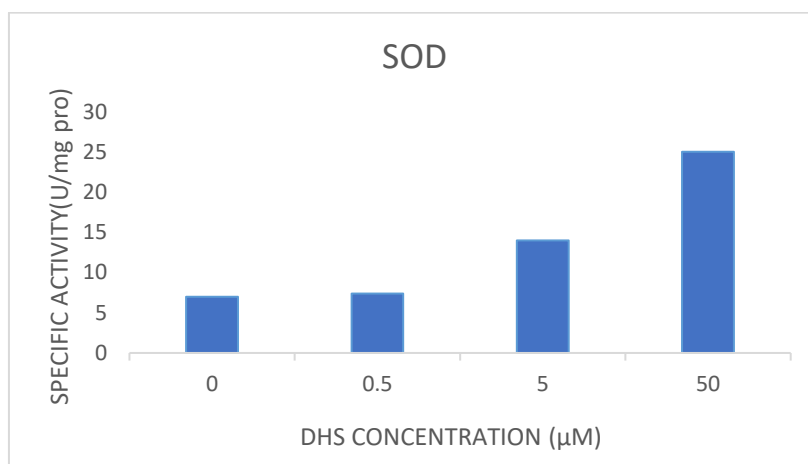


Figure 5.9 – Graphical representation of SOD

Catalase:

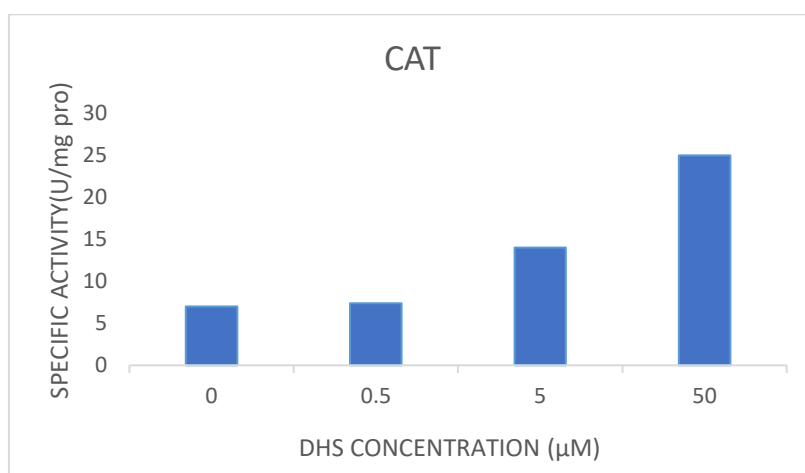


Figure 5.10 – Graphical representation of CAT

Ascorbate peroxidase:

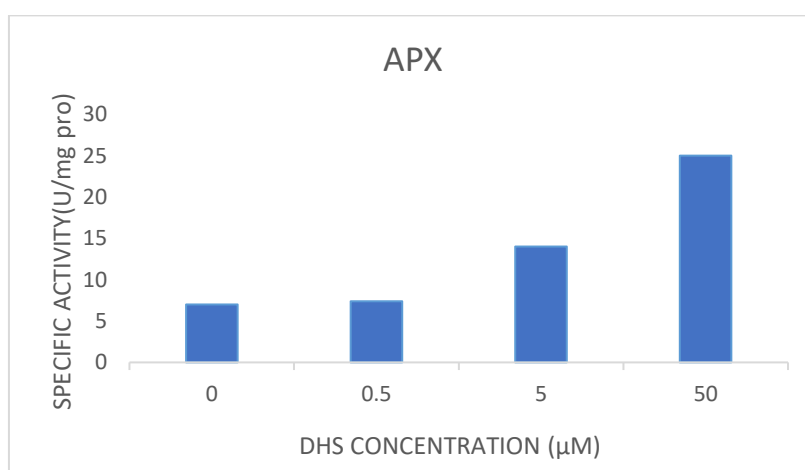


Figure 5.11 – Graphical representation of APX

In these three conditions, SOD, CAT, and APX the graph explains that at the 0 concentration of DHS, the stress is less and there are no dead cells, at 0.5 μM concentration of DHS some cells may be stressed or dead, and at “5 μM ” concentration of DHS more cells are stressed and dead compared with “0.5 μM ” concentration finally at “50 μM ” of DHS concentration the stress is increased and more number dead cells are seen.

5.5. MITOCHONDRIAL MEMBRANE POTENTIAL:

In the programmed cell death mechanism, mitochondrial dysfunction is associated with the membrane potential and the release of pro-apoptotic protein into the cytosol. Recently, researchers have found the important role of mitochondria in ferroptosis-mediated cell death [46]. In this study, Rh123 was used to assess the mitochondrial membrane potential (MMP) of *Chlamydomonas* using fluorescence microscopy (596/538 nm). It exhibits a red fluorescent signal, when the membrane potential is high, whereas when it remains as a monomer, a green fluorescent signal is observed, denoting a low membrane potential. In cells treated with RSL3 and heat shock (50 °C), the Rh123 signal shifted from red to green, suggesting a decrease in the MMP. At the 0 concentration of DHS, the membrane is not damaged which indicates red color, and at the higher concentration of “50µM” DHS, the membrane is fully damaged which indicates green color.

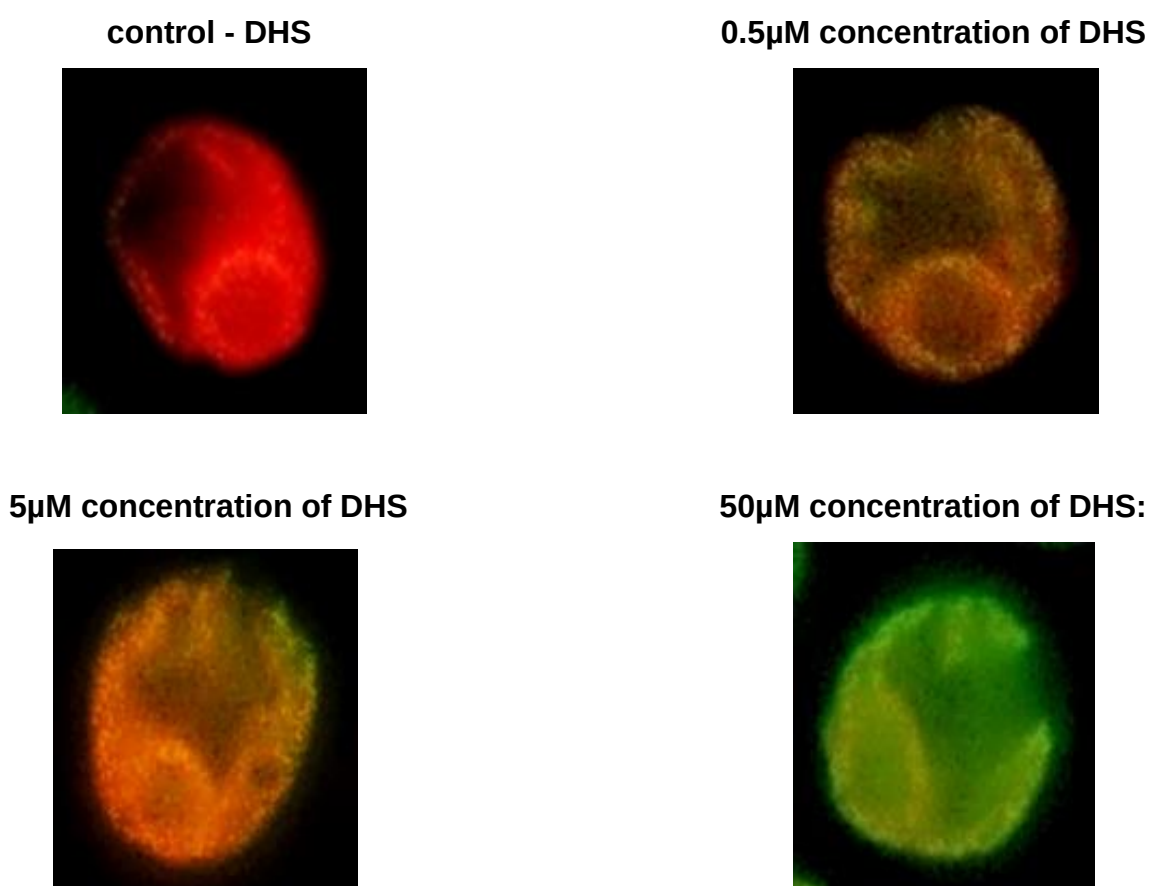


Figure 5.12 – *Chlamydomonas* cells in mitochondrial membrane potential

Graphical representation:

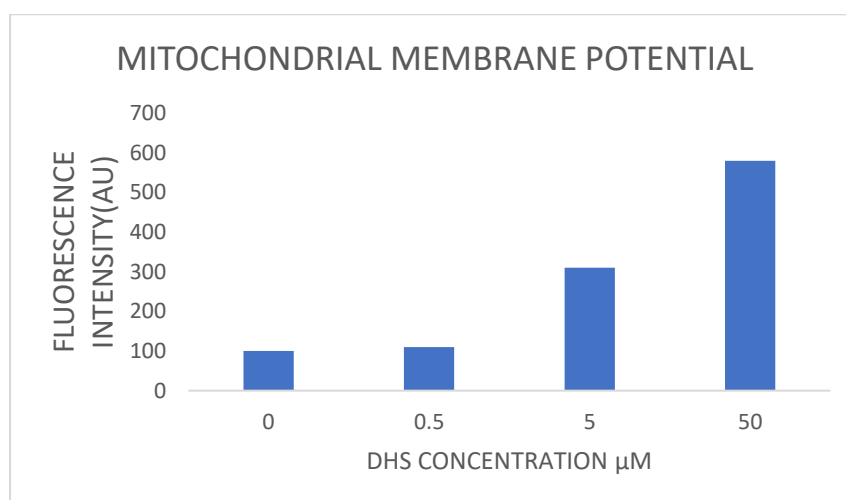


Figure 5.13 – Graphical representation of mitochondrial membrane potential

The graph states that at the 0 concentration of DHS, there is no membrane damage, at 0.5 μM concentration of DHS membrane damage is less, and at “5 μM ” concentration of DHS the membrane damage will be more compared with “0.5 μM ” concentration finally at “50 μM ” of DHS concentration the membrane is damaged fully as the stress increased.

5.6. CALCIUM SIGNALING:

Cytosolic calcium is a key secondary messenger in the stress response and regulated cell death processes. Previous studies reported that ferroptotic cell death was associated with lipid peroxidation and increased calcium fluxes through specific channels [47,48]. In this study, Calcium Green-1, a fluorescent dye, was used to measure the cytosolic calcium levels in *Chlamydomonas* cells subjected to stress. Cells showed a significant increase in cytosolic calcium and were viewed under fluorescence microscopy. This highlights the involvement of cytosolic calcium during stress-induced apoptotic cell death in *Chlamydomonas*.

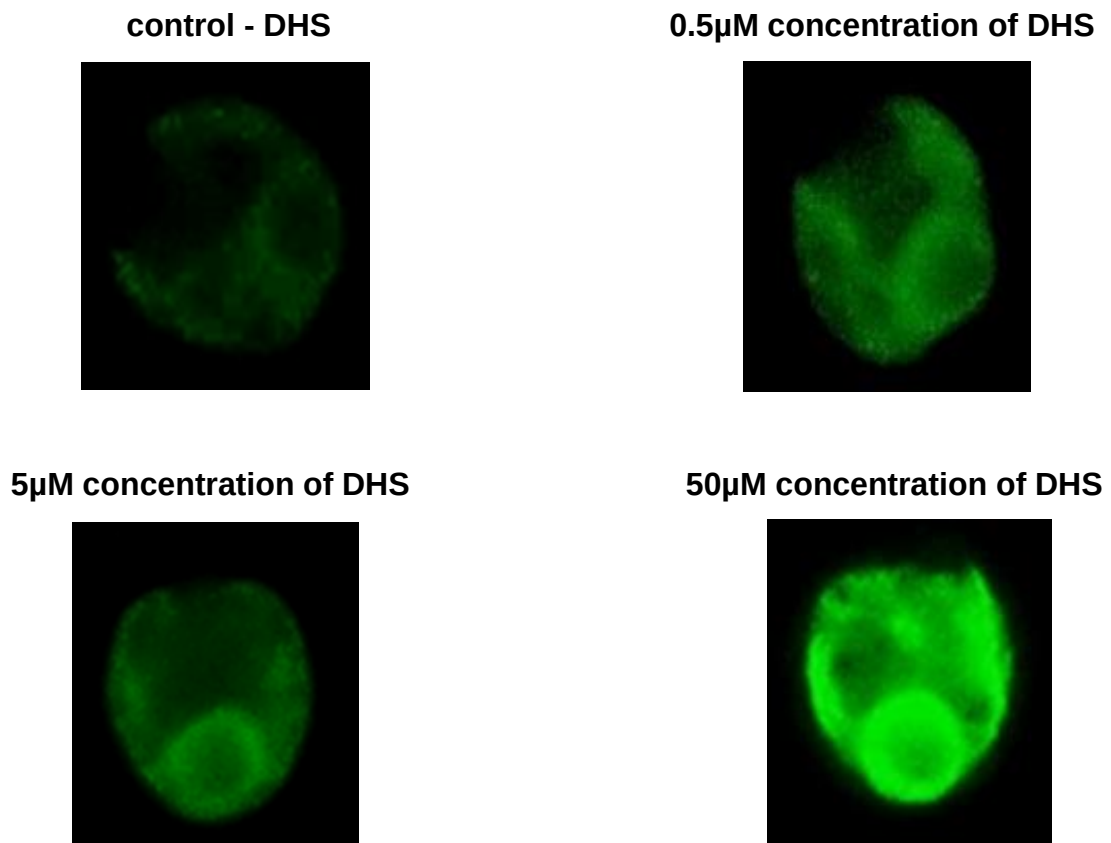


Figure 5.14 – *Chlamydomonas* cells in calcium signaling

Graphical representation:

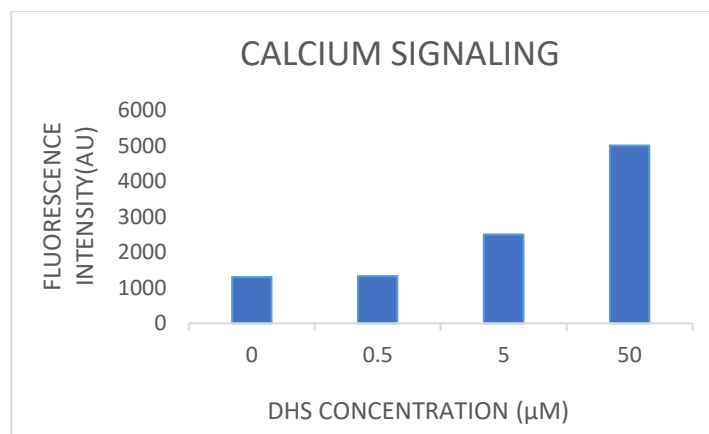


Figure 5.15 – Graphical representation of calcium signaling

The graph demonstrates that at the 0 concentration of DHS, the stress is less and there are no dead cells, at 0.5 μ M concentration of DHS some cells may be stressed or dead, and at “5 μ M” concentration of DHS more cells are stressed and dead

compared with “0.5 μ M” concentration finally at “50 μ M” of DHS concentration the stress is increased and more number dead cells are seen.

5.7. COMET ASSAY:

To gain additional information about the effect of DHS on DNA disintegration, the cells were analyzed by the Comet assay which is a sensitive method for measuring double- and single-strand breaks in DNA. The samples were collected from cell suspensions exposed to 1 mM DHS for 10 min, 30 min, 2 h and 24 h. No comet pattern appeared in control cells, which indicated intact DNA. Slight DNA degradation was observed 10 min after administration of DHS. The comet tail was extended 30 min after the treatment, showing that DNA was undergoing a relaxation of the structural loop with an increasing number of breaks. Two hours after DHS administration, DNA appeared granular both in the tail and in the comet head which is a symptom of progressive breakage. After 24 h of DHS treatment, class 4 comets, lacking a compact comet head and with almost all DNA fragments in the tail, indicated irreversible DNA damage. Fluorescence images reflected the increase in the percentage of comet nuclei found at the consecutive time points of analysis. The results of the Comet assay provided supportive evidence that in DHS-treated *C. reinhardtii* DNA cleavage proceeded concomitantly with the progression of cell death [43].

Graphical representation:

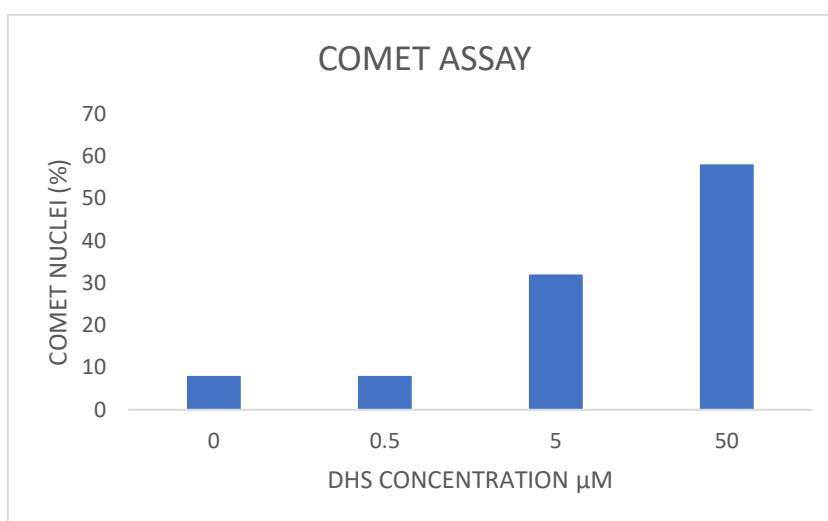


Figure 5.16 – Graphical representation of COMET assay

The graph states that at the 0 concentration of DHS, the stress is less so there is no DHA damage, at 0.5 μ M concentration of DHS the DHA damage is less, and at “5 μ M” concentration of DHS the DNA damage will be more compared with “0.5 μ M” concentration finally at “50 μ M” of DHS concentration the DNA is damaged as the stress increased.

5.9. CASPASE ASSAY:

To determine the caspase-like activity in *Chlamydomonas* during cell death, this study suggested that a caspase-like activity was involved in the pathway triggered by adding DHS. Programmed cell death, such as apoptosis, has been widely reported in *Chlamydomonas* and appears to share certain morphological characteristics with apoptosis-like cell death in multicellular organisms, including DNA fragmentation [44]. In previous studies, DNA fragmentation in *Chlamydomonas* under heat stress (50 °C for 10 min) was observed only after 14–16 h of treatment but not at different time points after heat treatment [35,49].

Graphical representation:

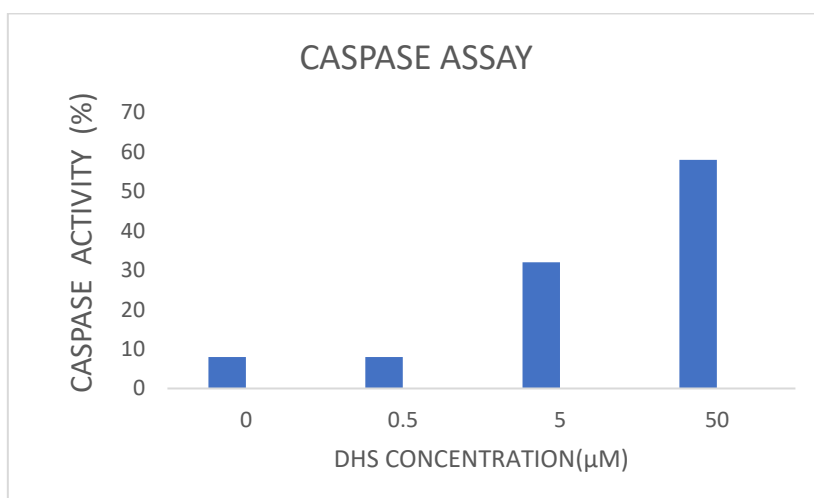


Figure 5.17 – Graphical representation of caspase assay

The graph states that at the 0 concentration of DHS, the stress is less so the caspase activity is less, at 0.5 μ M concentration of DHS the caspase activity may increase or remain the same, and at “5 μ M” concentration of DHS the caspase activity will be more compared with “0.5 μ M” concentration finally at “50 μ M” of DHS concentration the caspase activity has increased the maximum as the stress.

CHAPTER - 6

6. CONCLUSION

According to the current study, oxidative stress causes apoptotic cell death, which results in an increase in ROS and lipid peroxidation. These *Chlamydomonas* biochemical and physiological alterations show that DHS treatment of the microalgae caused apoptotic cell death, which is well-defined in both plant and mammalian cells. *Chlamydomonas* cells were saved from apoptotic cell death during oxidative stress by anti-apoptotic proteins like Bcl-2. *Chlamydomonas*'s occurrence of apoptotic-mediated cell death offers fresh perspectives [36] on the evolutionarily conserved processes of programmed cell death and cell survival in eukaryotic cells under stress. Additionally, knowing the specifics of how apoptotic cell death is regulated in microalgae may help with the efficient management and control of a number of naturally occurring and anthropogenically produced events. For instance, having this knowledge could enable us to manage toxic algal blooms brought on by climate change and the effects they have on ecosystems. Despite the advancements mentioned above, there are currently very few investigations on sphingolipid metabolism and signaling in plants when compared to those in animals. Because plant sphingolipids and their signaling functions remain largely unknown in the sphingolipid signaling pathway's receptors, targets, and mediators, there is a huge opportunity to study these components. Sphingolipids' function in PCD has been extensively researched. Although there has been significant advancement, the particular sphingolipids involved in this process are yet unknown. It is obvious that PCD is strictly controlled, and additional thought should be given to the various pressures that cause PCD.

CHAPTER - 7

7. REFERENCES

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