A STUDY ON PHOTOCHEMICAL ACTIVITY STABILITY OF CHLOROPLAST AND ITS RELATIONSHIP WITH ANTI-OXIDATIVE ENZYMES FROM SELECTED PLANT LEAVES

By

YEOH LOO YEW

A dissertation submitted to the Department of Chemical Science,
Faculty of Science,
Universiti Tunku Abdul Rahman,
in partial fulfillment of the requirements for the degree of
Master of Science

March 2019

ABSTRACT

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Yeoh Loo Yew

Chloroplasts are organelles involved in oxygenic photosynthesis and biosynthesis of important nutrients. The electron transport properties of isolated chloroplasts are being utilized to build biofuel cells or biosensors. To understand the plant chloroplast photochemical activity stability, this study evaluated the functionality parameters of isolated chloroplasts, stability of photochemical activity of the isolated and stored chloroplast, response of indigenous plant leaf towards water and salinity stress and also relationship of the leave's anti-oxidative enzymes and photochemical activity and stability of chloroplast of four plant samples, namely *Amaranthus tricolor* Linn., *Pandanus amaryllifolius*, *Elaeis guineensis* and *Pistia stratiotes*. The chloroplast was isolated followed by the determination of chlorophyll content, microscopic examination and storage. *A. tricolor* Linn. exhibited highest chlorophyll content, *E. guineensis* exhibited highest Hill reaction activity (HRA) and greatest storage stability. Rapid freezing with liquid nitrogen and stored at -20^{o}C was proven to better preserve the isolated chloroplast than only stored at 4^{o}C without pre-freezing. In the study of plant leaf, moisture content and electrolyte leakage were determined and salt tolerance of plant leaf was studied. *E.*

guineensis and *P. amaryllifolius* exhibited higher tolerance towards salinity stress due to high proportion of lignified tissues, thick cuticle and low moisture content. For the extraction of crude enzyme, the protein content was determined followed by the qualitative study of anti-oxidative enzyme activity: ascorbate peroxidase (APX), catalase (CAT), peroxidase (POX), guaiacol peroxidase (GPX), pyrogallol oxidase (PO), catechol oxidase (CO), and superoxide dismutase (SOD). *E. guineensis* exhibited highest APX, POX and CO activity; *P. stratiotes* exhibited highest CAT, GPX and PO activity; while *P. amaryllifolius* exhibited highest SOD activity. Correlation analysis suggested that APX, CAT, PO, and CO can probably be used as a biomarker for chloroplast photochemical activity; while CAT, GPX, and PO may be used as a biomarker for isolated chloroplast photochemical stability at prolonged storage.

ACKNOWLEDGEMENTS

Firstly, I would like to express my gratitude to my supervisor, Dr Chang Ying Ping and my co-supervisors, Dr Chee Swee Yong and Dr Lim Tuck Meng, for giving me a chance to work under their supervision for this project, thereby completing my study in Master of Science. With great pleasure, I also like to express my sincere thanks to all of my supervisors for their constant encouragement, moral support, critical suggestion and friendly guidance along my project.

In addition, I also wish to thank the lab assistants for their kind help and advices in supporting me to complete this project. They had also helped me a lot in providing me glassware and materials that I needed, and taught me a lot in handling different instruments so that this project can go on smoothly. Moreover, I would also like to express my gratitude to my family members who had always been there to support me throughout this period. Their encouragement had helped me a lot in overcoming stress and other problems encountered.

Apart from that, I would also like to thank my colleagues as they had mentally supported me all the time and showing me how important friendship is in this period. They had also helped me by pointing out the mistakes that I made during the project and during the process of completing this dissertation. Lastly, I wish to express my gratitude to everyone who had helped me towards the completion of my project. Without the proper direction and encouragement, this project could not have been completed. On top of that, I would like to express my

apologies toward any inconveniences caused. I welcome comments and critiques in order to help me improve even more. Thank you.

APPROVAL SHEET

This dissertation entitled "**A STUDY ON PHOTOCHEMICAL ACTIVITY STABILITY OF CHLOROPLAST AND ITS RELATIONSHIP WITH ANTI-OXIDATIVE ENZYMES FROM SELECTED PLANT LEAVES**" was prepared by YEOH LOO YEW and submitted as partial fulfilment of the requirements for the degree of Master of Science at Universiti Tunku Abdul Rahman.

Approved by:

_____
(Dr. CHANG YING PING)
Date: ……..
Supervisor
Department of Chemical Science
Faculty of Science
Universiti Tunku Abdul Rahman

_____
(Dr. CHEE SWEE YONG)
Date: ……..
Co-Supervisor
Department of Chemical Science
Faculty of Science
Universiti Tunku Abdul Rahman

_____
(Dr. LIM TUCK MENG)
Date: ……..
Co-Supervisor
Department of Chemical Science
Faculty of Science
Universiti Tunku Abdul Rahman

FACULTY OF SCIENCE

UNIVERSITI TUNKU ABDUL RAHMAN

Date: _____

SUBMISSION OF DISSERTATION

It is hereby certified that **YEOH LOO YEW** (ID No: **14ADM07927**) has completed this dissertation entitled "**A STUDY ON PHOTOCHEMICAL ACTIVITY STABILITY OF CHLOROPLAST AND ITS RELATIONSHIP WITH ANTI-OXIDATIVE ENZYMES FROM SELECTED PLANT LEAVES**" under the supervision of DR CHANG YING PING (Supervisor) from the Department of CHEMICAL SCIENCE, Faculty of Science, DR CHEE SWEE YONG (Co-Supervisor) from the Department of CHEMICAL SCIENCE, Faculty of Science, and DR LIM TUCK MENG (Co-Supervisor) from the Department of CHEMICAL SCIENCE, Faculty of Science.

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_____
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DECLARATION

I hereby declare that the dissertation is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UTAR or other institutions.

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LIST OF ABBREVATIONS

HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
DCPIP	2,6-dichlorophenolindophenol
Δa*	a* value difference
NH_4Cl	Ammonium chloride
Δb*	b* value difference
CO_2	Carbon dioxide
CO	Catechol oxidase
ΔC*	Chroma difference
DHAR	Dehydroascorbate reductase
DNA	Deoxyribonucleic acid
NADPH	Dihydronicotinamide adenine dinucleotide phosphate
dm	Dry matter
g	Earth's gravitational field
EDTA	Ethylenediaminetetraacetate
GR	Glutathione reductase
GPX	Guaiacol peroxidase
ΔH*	Hue metric difference
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
OH·	Hydroxyl radicals
ΔL*	Lightness difference
MDA	Malondialdehyde
ε	Molar extinction coefficient

NADP	Nicotinamide adenine dinucleotide phosphate
O_2	Oxygen
POX	Peroxidase
PET	Photosynthetic electron transport chain
PSI	Photosystem I
PSII	Photosystem II
Pt	Platinum
KCl	Potassium chloride
PO	Pyrogallol oxidase
ROS	Reactive oxygen species
RNA	Ribonucleic acid
$^1O_2$	Singlet oxygen
NaCl	Sodium chloride
$O_2{\cdot}^-$	Superoxide anion
SOD	Superoxide dismutase
ΔE*ab	Total color difference
UV	Ultraviolet
v/v	Volume per volume
H_2O	Water

CHAPTER 1

INTRODUCTION

Plants carry out photosynthesis by harnessing the energy of sunlight to generate glucose, oxygen, and ATP (Holding and Streich, 2013). The overall photosynthesis process can be summarized as the following reaction scheme:

$$6\ CO_2 + 12\ H_2O + \text{light} \longrightarrow C_6H_{12}O_6 + 6\ H_2O + 6\ O_2$$

carbon dioxide water glucose water oxygen

According to the data obtained from International Energy Agency (International Energy Agency, 2012), the energy consumption level in the world raised more than 78% over the past three decades. Alternative renewable energy sources gaining increasing attention due to the serious environmental problems caused by usage and limited amount of fossil fuels as well as the continuing fluctuation of cost due to unstable political disturbances (Schmidt and Dauenhauer, 2007). The world fossil oil is approximated to be exhausted in less than 50 years and the replacement of petroleum oil by carbon neutral biofuels will further cause global warming with the emission of greenhouse gases (Rodolfi et al., 2009). The development of renewable, sustainable and environmental friendly energy sources became the main focus in this current situation.

The global energy consumption was estimated to be around 17 TW per year and the current available renewable energy sources such as wind, tidal, energy of waves, rain energy, geothermal heat, and solar energy only able to generate approximately 16% of the energy used (Sekar and Ramasamy, 2015; Lewis and Nocera, 2006). The need for renewable energy sources foresees to gain increasing attentions if the global energy consumption continues to rise. Solar energy conversion is considered as a reliable solution for the energy crisis. Annually, there is about 5,500 × 1021 J of solar energy from the Sun reaches the Earth's atmosphere (Smil, 2003). There is about 100 PW of sunlight emission near the ground surface, which already 5000 times exceeds our current energy demands (Gratzel, 2007). Even in the presence of clouds, the sun is still an attractive source of energy which make it the most accessible and reliable source over other alternative renewable energy sources.

Chloroplasts are plant-specific organelles that involved directly in photosynthesis. Chloroplasts possess many advantages as an energy source (Greenbaum, 1985) because they are available in abundance and extractable from plant material, (Giebel and Street, n.d.). Besides, they also possessed own mechanism to harvest light energy, store energy and self-repair upon injury. Chloroplasts also allow scalable extraction, in both smaller and larger scale with the use of mortar and pestle or a grinding mill (Kwiatkowski et al., 2006). By adapting electrochemical conversion principle, scientists have made use of electron transport functions of isolated chloroplast in the study of energy conversion (Sekar and Ramasamy, 2015; Voloshin et al., 2015; Voloshin et al., 2016)

According to Carpentier (1989), thylakoid membrane from spinach leaves was first isolated to be used as a photosensitizer. A photosensitizer is able to conduct light-induced molecular transformation with the help of electron or energy transfer processes by utilize its abundant light absorbance and also redox property. (Carpentier et al., 1989). Thylakoids and chloroplasts isolated from spinach were then further used by a few researchers in several studies of photobioelectrochemical cells and dye-sensitized solar cells (Chang et al., 2010; Bedford et al., 2011; Rasmussen et al., 2014; Syafinar et al., 2015). Spinach is an edible vegetable. This approach may raise controversial issue in terms of utilization a food material for non-food application. For example, the United States is the leading exporter of maize, but a third of the production of this crop went to biofuels in 2007. According to a World Bank report, biofuels were found to account for the increase of approximately 70-75% of food prices from the year 2002 to 2008. This is mainly due to the cut down of food supply and the replacement of crops for food with crops for biofuels (Mitchell, 2008; Berthelot, 2008). Thus, various inedible agricultural by-products such as leaves and shoots may be explored for its photosynthetic components properties and functions. This would provide a higher value options in utilizing the phytomass waste.

Photosynthesis is the natural process which offered a low cost pathway for solar energy transformation (Blankenship et al., 2011) with a negative carbon footprint (Hall et al., 1995), however, the instability of the extracellular protein caused by the reactive oxygen species (ROS) limited its bioreactor application (Rooke et al., 2008). Isolated chloroplasts from plant cells are susceptible to irreversible protein damage due to ROS, which constraint their

extracellular photoactive lifetime and further affect their integration into the application of synthetic, light harvesting devices. It is crucial to prolong chloroplast photostability through buffer alteration, encapsulation, light condition and temperature control in order to delay the inevitable and irreversible photodegradation of the organelle for only a few days. Some researchers postulate that the use of regenerative ROS-scavenging compound probably can prolong organelle photoactivity and promote extracellular regeneration (Giebel and Street, n.d.; Rooke et al., 2008). The rational of this approach is considering the nature of photosynthesis process in generating ROS.

Photosynthesis is well known as the major generation source of ROS in plants due to the electron flow in an aerobic condition which is high in oxygen level, reductants, as well as high-energy intermediates (Asada, 2006). The photo-production of ROS is also largely influenced by physiological and environmental factors, for example: drought and salinity stress (Asada, 2006). The presence of high salt concentration from salinity stress can causes several deleterious consequences which start with an ionic imbalance (Niu et al., 1995; Zhu et al., 1997). The salt-induced water stress could inhibit photosynthesis by reducing chloroplast stromal volume and accumulation of ROS in chloroplast (Price and Hendry, 1991). When the levels of ROS generated exceed the ability of plant's antioxidant system to cope with, various damaging effects occur on cellular components (Zimmermann and Zentgraf, 2005). Under normal circumstances, production and concentration of ROS remains low and well-regulated (Polle, 2001). This is due to presence of protective enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), polyphenol oxidase

(PPO) and catalase (CAT) (Asada, 1984) which provide defence mechanisms for aerobic organisms (Beyer et al., 1991) in which SOD, CAT, and various peroxidases for example guaiacol peroxidase (GPX) and APX are the primary anti-oxidative enzymes (Kuk et al., 2003). In order to protect against the cellular damage caused by ROS, anti-oxidative enzymes function as defence mechanisms against deleterious free radicals in plants (Slooten et al., 1995; Ding et al., 2013).

In this study, the functionality parameters studied included the chlorophyll content, the microscopic examination and the initial Hill reaction activity of isolated chloroplast. Besides, the relationship between electron transferring properties of isolated chloroplasts based on Hill reaction, various anti-oxidative enzyme activities of crude enzyme extracts and response to stress study of 4 plant leaf samples were investigated. Four different types of plant samples were studied in order to compare the chloroplast activity, plant leaf properties and anti-oxidative enzymes activities of plants from different living environments. Four plant samples were studied in which *Amaranthus tricolor* Linn. is an edible vegetable, *Pandanus amaryllifolius* is a vascular plant, *Elaeis guineensis* is a woody plant, and *Pistia stratiotes* is an aquatic plant. The specific objectives of this study were:

1) To screen on the functionality parameters of isolated chloroplasts, quantitatively and qualitatively in selected plant leaves.
2) To assess the stability of photochemical activity of the isolated and stored chloroplast from selected plant leaves.
3) To assess the response of indigenous plant leaf towards water and salinity

stress.

4) To evaluate the relationship of the leave's anti-oxidative enzymes and photochemical activity and stability of chloroplast.

CHAPTER 2

LITERATURE REVIEW

2.1 Plant and its Photosynthetic Components

2.1.1 Photosynthesis

Plants are autotrophic in which they are able to convert simple molecules into complex compound such as carbon dioxide from the atmosphere and minerals from the soil into the carbohydrates, proteins, and fats. This forms the basis of living organisms. Plants carry out photosynthesis by harnessing the energy of sunlight to generate sugar, oxygen, and a molecule called ATP (Holding and Streich, 2013).

There are three stages of photosynthesis: (1) harvesting energy from sunlight; (2) storing the energy in the form of ATP and reducing power in the form of NADPH; (3) synthesising organic molecules from carbon dioxide with the use of ATP and NADPH (carbon fixation).

Light reactions take place in the first two stages, in which the presence of light is needed, while dark reaction which is known as Calvin cycle takes place in the third stage, which required a supply of ATP and NADPH.

The overall photosynthesis process can be summarized as the following reaction scheme:

$$6\ CO_2 + 12\ H_2O + light \longrightarrow C_6H_{12}O_6 + 6\ H_2O + 6\ O_2$$

carbon dioxide water glucose water oxygen

2.1.2 Anatomy of Leaf

The main function of the leaf is for absorption of light, their anatomy is highly specialized for them to carry out their photosynthesis (Terashima and Hikosaka, 1995). Epidermis, which is the outermost cell layer of leaf is typically transparent to visible light. Epidermal cells are often convex which help in magnifying the intensity of light and focusing light to the chloroplasts (Taiz and Zeiger, 2002).

Palisade cells are the top layers of photosynthetic cells which are located below the epidermis. They are pillars in shape and stand in parallel columns that might range from one to three layers deep. The sieve effect and light channeling allowed more light to penetrate the first layer of palisade cells while the high surface-to-volume ratios of chloroplast structure helped increasing the efficiency of photosynthesis (Evans et al., 2009).

The unevenly distributed chlorophyll in the chloroplasts contributed to the sieve effect as this created shading and gaps between the chloroplasts which allowed light to penetrate. The arrangement of the central vacuoles of the palisade cells and air spaces that presence in between allowed the occurrence of light channeling in which the incident light is propagated and transmitted into

the leaf interior (Vogelmann, 1993)

Spongy mesophyll cells are located below the palisade layers. Spongy mesophyll cells have irregular shape and surrounded by large air space. The large air space allowed interface light scattering as light are reflected and refracted between interfaces of air and water. Light scattering plays a crucial role in leaves as it allowed multiple reflections between cell and air interfaces which increase the length of path for photon to travel, thus increase the rate of absorption. The properties of palisade cell and spongy mesophyll cell of light channelling and scattering ensure uniform light absorption throughout the leaf (Taiz and Zeiger, 2002).

2.1.3 The Structure of the Chloroplast and Photosynthetic Membranes

Chloroplasts are plant-specific organelles that involved directly in many essential metabolic functions including oxygenic photosynthesis and biosynthesis of crucial nutrients for the development of plant. The internal membranes of chloroplasts are assembled into sacs called thylakoids. The thylakoid is the structural unit of photosynthesis. The thylakoid membrane keeps photosynthetic pigments and forms a photosystem for light energy capturing and the machinery for ATP synthesis. Thylakoids are stacked on one another and form grana. The grana are surrounded by stroma. Stroma is responsible in housing the necessary enzymes for the assembly of carbon molecules (Johnson and Raven, 2002).

Photosystem is comprised of chlorophyll *a* molecules, accessory pigments and associated proteins on the surface of the photosynthetic membrane. A photosystem are comparted into two components: (1) an antenna complex; (2) a reaction center. The antenna complex is a web of chlorophyll molecules on the thylakoid membrane which captures photons from sunlight. The antenna complex functions by funneling the energy from electrons to the reaction center. The reaction center is made up of transmembrane protein-pigment complex which allows the transfer of energy to the primary electron acceptor for the conversion of light to chemical energy (Johnson and Raven, 2002).

2.1.4 Role of Photosystems and the Transferring of Electron

When a photon of light energy strikes on the pigment molecule in photosystem II, an electron was excited. The excited electron is coupled to a proton that stripped from water through photolysis is then transferred through a chain of membrane-bound cytochrome electron carriers to the primary electron-acceptor molecule. Electrons are generated with the breakdown of another water molecule, which then passed to the electron deficient photosystem II. Oxygen is released from the cell whenever water is split and the hydrogen ions remain in the thylakoid space. The photon then supplied the energy to transport a proton across the membrane into the thylakoid through the proton pump (b6-f complex) which leads to a increase of the concentration of hydrogen ions within the thylakoid. The reduced primary electron acceptor now donates electrons to the down-stream components of the electron transport chain and finally passed onto the reaction centre of photosystem I (P700). Energy is released and stored in the form of ATP during photophosphorylation when proton diffuse back through

ATP synthase (Johnson and Raven, 2002).

When another photon of light strikes the photosystem I, second high-energy electron is passes to a reduction complex, which results in the generation of NADPH. Similarly, photosystem I transfers its electrons to another primary electron acceptor molecule when it absorbs light. The P700 is oxidized and draws electrons from photosystem II. The reduced primary electron acceptor is then transfers its electrons to Nicotinamide Adenine Dinucleotide Phosphate (NADP) via other electron carrier to produce $NADPH_2$ which is then utilized in the reduction of CO_2 to carbohydrate in the biosynthetic pathway (Johnson and Raven, 2002).

2.1.5 Chloroplast and its Pigments

Pigments are molecules that are good light absorbers in the visible wavelength range. There are generally two types of pigments in green plant photosynthesis, which are chlorophylls and carotenoids. Chlorophylls contain a complex ring structure, known as porphyrin ring, with alternating single and double bonds along with a magnesium atom at the center of the ring. Chlorophylls can absorb photons within a narrow energy range (Johnson and Raven, 2002). The chlorophylls present in plants are chlorophyll *a* and chlorophyll *b*. They preferentially absorb violet-blue and red light, which mean neither of them are able to absorb photons that fall at 500 to 600 nm (Johnson and Raven, 2002). Chlorophyll a is the main and only photosynthetic pigment that can carry out direct conversion of light energy to chemical energy. The light energy was absorbed and converted into chemical energy in the thylakoid

membrane. An antenna complex contains pigments which function to absorb light energy and also a reaction center that comprised of a complex of proteins together with two chlorophyll a molecules can be found in a photosystem. Each antenna contains one or more light-harvesting complexes that capture energy and funnel to the two chlorophylls in the reaction center.

Other than chlorophyll a, antenna of plants also contains accessory pigments such as chlorophyll b and carotenoids. Accessory pigments allows absorption at a broader range of wavelength, thus allow more energy to be captured from sunlight (Bullerjahn and Post, 1993). Accessory pigments only absorb energy and transfer it to chlorophyll a instead of directly participating in the process of energy conversion. The absorption spectrum of chlorophyll b tend to shift towards green wavelengths, thus enabled plants to harvest higher proportion of photons in sunlight (Johnson and Raven, 2002). In most of plants, the ratio of chlorophyll a to chlorophyll b is 3:1. Carotenoids are pigments that absorb violet and blue-green light, which assists in photosynthesis by capturing energy that are not efficiently absorbed by chlorophyll (Johnson and Raven, 2002). Carotenoids helps in absorbing excess energy and dissipating it as heat when a leaf is exposed to excess photon irradiance to prevent the possible deleterious effect on photosynthetic machinery. In molar terms, carotenoids present at about one third the abundance of chlorophyll (a + b) in mature leaves (Atwell et al., 1999).

2.2 Biotic and Abiotic Stress Affecting Plants' Photosynthesis

2.2.1 Biotic Stresses and its Effects on Crops Production

Biotic plant stress can be defined as the interactions between plants with biological entities which result in negative effects in certain level of metabolic disturbance (Lichtenthaler, 1998). Under natural condition, plants constantly facing threat of infection by biotic agents such as bacteria, fungal pathogens, viruses, parasitic plants and attack by herbivore pests (Pimentel, 1991; Atkinson and Urwin, 2012). Different strategies were employed by pathogens in the attack of plants. Necrotrophic kills plant tissue before the colonization, biotrophic attack the plant while the plant cells remain alive, whereas hemibiotrophic acts by attacking the plant cells and allow the cell to remain alive in a first phase and then followed by necrotrophic phase (Dangl and Jones, 2001). Once plants were invaded by biotic agents, plant metabolism must balance the allocation of resources either to support the defence mechanism or for cellular maintenance, growth and reproduction (Herms and Mattson, 1992; Zangerl and Berenbaum, 1997; Berger et al., 2007).

Biotic threats account for up to 30% loss of crops production both pre- and post-harvest across the world (Oerke, 2006; Flood, 2010; Bebber and Gurr, 2015). Millions of human death and migration to other countries in the past due to the devastation of food production. Nowadays, crop production are threatened by new pathogen races and insect biotypes, which account for about 15% losses in global food production (Sanghera et al., 2014). In the middle of the 19th century, massive migration of Irish farmers to North America after the attack of potato field by *Phytophtora infestans* in which it killed or displaced

about 25% of the Irish potato field (Fraser, 2003). Insect pests contributed more than 10% to the yield gap with field losses (Kerchev et al., 2012), and 50-80% is expected in the absence of control measures (Bruce, 2010). Only a small percentage among 4-6 million of arthropods species are classified as pests. They destroyed approximately 14% of the world annual crop production and contributed to 20% losses of stored grains, which is around US$100 billion of damage each year (Nicholson, 2007). The major threat to food production for human consumption goes to herbivorous insects and mites. The larval forms of lepidopterans of heliothine species account for the use of about 40% of all insecticides (Brooks and Hines, 1999). Insect pests can induce damage on crop plants by direct feeding in the field or by infesting the stored products and indirect damage through the transmission of viral diseases or by secondary microbial infections of crop plants (Ferry and Gatehouse, 2010).

2.2.2 Influence of Abiotic Factors on Leaf Morphology

Abiotic factors influence leaf eco-physiological traits. Plants tend to alter their physiology, morphology and development when they experiencing abiotic stress. The leaves of plant are the crucial organs to carry out photosynthesis and have a significant role in survival and growth of a plant. Previous studies suggested that the variations in leaf traits are the result of adaptation to growing environment (Sisó et al., 2001; Pandey and Nagar, 2002). The leaf size of plant leaf can be restricted by deficient water supply and light considering leaf area and dry mass. The reduction in plant leaf size as exposed to stressful environments can be rationalized on the basis of leaf boundary-layer conductance that function in heat and gaseous transport (Royer et al., 2005).

Besides, plant leaf size decline may be due to limited resources in stressful environments (Niinemets et al., 2007). During the short supply of water, the cell walls of lamina become thicker and lignified and these changes help to enhance the epidermal resistance of water vapor through cuticles in order to reduce water loss of leaf. Besides, the higher dry mass per area of leaves during the drought helps to prolong the lifespan of leaf so it is able to compensate for fewer units of the photosynthetic apparatus and carry out limited activity. Drought also caused the leaves to become narrower and reduces the size of the boundary layer in order to reduce the transpiration (Lusk and Warton, 2007).

Light is one of the factors that monitors the development of the photosynthetic apparatus in plants. There is a positive correlation between the intensity of visible and UV light with the thickness of cuticle on plant leaf and integration of protective flavonoid compounds in the cuticle. Leaves tend to develop thicker, smaller, more developed palisade tissues and higher stomatal density under intense light as compared with shade leaves (Anderson and Osmond, 1987; Murchie and Horton, 1997). This can protect the leaves from intense light damage (Davi et al., 2008). Shade-type chloroplasts tend to have more appressed thylakoid membranes as compared to sun-type chloroplasts. In fact, sun leaves have much higher photosynthetic capacity than shade leaves on a leaf area basis. Besides, sun leaves also have higher amounts of ribulose bisphosphate carboxylase/ oxygenase and more electron transfer carriers. This made the sun leaves have better high light tolerance due to their high carbon assimilation rate and also increased ability to dissipate excess light energy. Shade leaves tend to have increased susceptibility to damage when exposed to

intense light (Demmig-Adams and Adams III, 1992; Osmond and Forster, 2008).

2.2.3 Membrane Permeability as Stress Damage Indicator

The survival and production of plant when facing stresses from environment are managed by complex mechanisms. Studies show that cell membrane is the major target of environmental stresses and first site experiences stress injury (Levitt, 1972). Membranes are dynamic structures which assist the biochemical and biophysical reactions in plant (Leshem, 1992). Initial response of plant towards stress is often shown on membrane (Heckman et al., 2002; Marcum, 1998). The structure and function of membrane tend to damage drastically when plants experience intense environmental condition (Lieberman et al., 1958; Siminovitch et al., 1964; Mckersie and Tomes, 1980; McKersie et al., 1982).

Membranes play its roles in the discrimination and transport processes at the level of tissues and organelles as it provides metabolic interfaces between the plant and its surrounding environment. The damage to plant due to environmental stress can causes cellular membrane modification, leading to membrane dysfunction in which causing increase of the cell permeability and electrolyte leakage from the cell (Ahmadizadeh et al., 2011). Cell death occurs when membrane permeability is drastically altered (Hancock, 1972). Different environmental stresses such as drought, salinity, heavy metals and acute or extreme temperature changes can cause major effects on membranes (Rachmilevitch et al., 2006).

Plants experience wilting, limiting carbon dioxide diffusion to chloroplast by closure of stomata, reduced photosynthesis rate, decrease in cell enlargement and growth and accelerated leaf senescence when drought stress occurrs (Beltrano et al., 1994; Beltrano et al., 1999). The increase of solute concentration in surrounding environment results in osmotic flow of water out of plant cells. The increase of solute concentration inside plant cells then lower the water potential. Lipid peroxidation induced by drought results in the leaking out of solutes from the organelles and cell which further damage the membrane function and inhibit certain metabolic processes (Blokhina et al., 2003). Drought or water stress also induces several alterations on cell membrane properties in term of selective permeability, fluidity and microviscosity (Beltrano et al., 1999, 1994; Navari-Izzo et al., 1993). Water tends to loss from leaf tissues causing disorganization of membranes structure and its function (Somerville et al., 2015). For example thylakoids lose their intactness when water diffuses out. The instability of protein complexes caused a reduction of photosynthetic pigments as chlorophyll degrading enzymes and chlorophyllase tend to increase under stressful situation. Chlorophyll content of cotton (Massacci et al., 2008) and *Catharanthus roseus* (Jaleel et al., 2008) was reduced significantly due to drought stress.

Cell membrane is also a primary site of physiological injury by heat stress. Heat stress causes damage to the cells integrity which lead to several physiological changes (Larcher, 2003). Cell membrane are constituted of protein and larger polyunsaturated fatty acids. High temperature changes fluidity, permeability, cellular metabolic functions and lipid composition in

membrane. Thus cell membranes become more fluid and highly permeable or 'leak' (Behzadipour et al., 1998; Wahid et al., 2007). High temperature stress damages cell membrane through lipid peroxidation as it increases the formation of free radical that can oxidize lipid in membrane and lead to leakage of ions (Xu et al., 2006; Liu and Huang, 2000). Furthermore, heat stress reduces photosynthesis activity of plants as it causes several alteration on photosynthetic components such as PSII complex, thylakoid membrane and ribulose 1'5 bis phosphate carboxylase (Rubisco) enzyme (De Ronde et al., 2004; Allakhverdiev et al., 2008). In addition, the over production of ROS promotes oxidative damage to lipid membrane and inhibits the repairing process of PSII (Møller et al., 2007). Other than heat stress, freezing injury can also induces cellular dehydration on plant cells, which damages the membrane structure, alter the cellular functions and increases the membrane permeability (Uemura et al., 1995; Xin and Browse, 2000). Apart from that, freezing injury can also decreases enzymatic activities and reduces photosynthetic capacity of plant (Dubey, 2005). Thylakoid and plasma membrane are known to be the primary injury site for chilling injury (Leshem, 1992). When temperature get lower, lipid membrane experiences phase transitions and its permeability is affected. Chilling injury not only can inhibit photosynthesis process, carbohydrate translocation, but also protein synthesis. Besides, chilling injury also causes degradation of existing proteins, leads to either partial or complete loss of membrane function (Leshem, 1992; Simon, 1974).

Heavy metals such as cadmium and copper have been reported to alter the cellular redox state, which increase the production of ROS like hydrogen

peroxide (H_2O_2), superoxide anion ($O_2^{\bullet -}$), and hydroxyl radical (•OH) (Benavides et al., 2005; Romero-Puertas et al., 2004), thereby change the permeability and selectively of the movement of nutrients across the membrane, thus causing electrolyte leakage (Ke, 2007). Accumulation of ROS is able to cause damage to components such as DNA, protein, and lipids, results in lipid peroxidation, protein denaturation, DNA mutation, disintegration and reduction of chlorophyll content in plants (Bowler et al., 1994; Tian et al., 2012). There is evidence of correlations between the rate of increase in H_2O_2 content and the extent of membrane lipid peroxidation (Bandeoğlu et al., 2004; Tian et al., 2012). The elevated amount of ROS in plant cells induces a cyclic cascade of reaction causing the distortion of lipid bilayer of membrane proteins by Fenton reaction.

Cell membrane is considered the first targets of plants (Levitt, 1972). Injury inflicted on leaf tissues by stresses such as heat, drought (Blokhina et al., 2003) and freezing due to lipid peroxidation weakens the cell membrane. Injured cells are unable to maintain the chemical compositions of their contents which release electrolytes through damaged membrane. The leakage of electrolyte is generally proportional to the stress damage, thus the ability of plant cell to control ion movement rate across the membrane can be used as a common biochemical marker to test the damage in plants under stress (Bandeoğlu et al., 2004; Tian et al., 2012; Valentovic et al., 2006; Simova-Stoilova et al., 2008). Cell membrane stability can be estimated by measuring the leaked cellular electrolyte from a particular plant tissues based on electrolyte leakage method (Murray et al., 1989).

2.2.4 Abiotic Stresses and its Effects on Plant's Photosynthesis

Photosynthesis is the key phenomenon that promotes plant growth and development other than physiological, biochemical, and molecular processes. Photosynthesis is capable to convert light energy into a usable chemical energy through a series of metabolic processes (Ashraf and Harris, 2013). However, stressful environments such as salinity, temperatures, and drought could affect the photosynthesis in plants by causing the alteration in the ultrastructure of their organelles and make changes on the concentration of various pigments and metabolites involved. (Taiz and Zeiger, 2010; Dulai et al., 2011)

Chloroplast is the key site for photosynthesis and yet highly sensitive to different stresses induced by surrounding environment included drought, flooding, salinity, extremes of temperature, different light intensity, and UV radiation (Biswal et al., 2008; Saravanavel et al., 2011). The photosynthesis rate of plants are largely affected by these stresses either through stress-induced stomatal or nonstomatal limitations (Saibo et al., 2009; Rahnama et al., 2010)

For example, drought stress, plants react to it by rapid closure of stomata to control the transpiration rate in order to avoid further loss of water during mild intensity of drought (Lawlor, 1995). The closure of stomata often shows more inhibitory effect on transpiration of water than carbon dioxide diffusion into the leaf tissues (Chaves et al., 2009; Sikuku et al., 2010). However, during severe drought stress, mesophyll cells tend to dehydrate which further causes inhibition of basic metabolic processes of photosynthesis, reduces the efficiency of to use the available CO_2 and reduces water-use efficiency of plant (Karaba et

al., 2007; Dias and Brüggemann, 2010a; Dias and Brüggemann, 2010b; Damayanthi et al., 2010; Anjum et al., 2011).

When plants are subjected to heat stress, several effects such as growth retardation, water loss, a change in photosynthetic efficiency, and oxidative stress are observable (Hasanuzzaman et al., 2013). The plants are then experience wilting, necrosis, loss of pigment and repression of leaf elongation as they are exposed to longer periods of heat stress (Scafaro et al., 2010). Heat stress reduces the photosynthetic capacity of plants by causing membrane disruption, especially membranes of thylakoid thus influences the membrane-associated electron carriers and inhibits certain enzymes activities (Ristic et al., 2008; Rexroth et al., 2011).

Excess photosynthetically active radiation absorbed by leaves and unable to be fully utilized lead to the excess of excitation energy (Müller et al., 2001). Dissipation of excess energy is an essential process to ensure the survival of photosynthetic organisms. Photooxidative damage can be prevented if excited chlorophyll molecules properly transfer their higher energy state to neighbouring molecules or oxygen (Asada, 1999; Heber et al., 2001; Mullineaux and Karpinski, 2002). If the excess energy absorbed is not channelled into the reduction of carbon dioxide, photooxidative damage could occur (Mittler, 2002). The maintenance of electron flow through the photosynthetic membrane is vital to prevent the damage to plant cells (Heber et al., 2001).

Environment pollution as well can cause stress to plant. Heavy metal pollution produced from human activity especially near urban and industrial areas caused metal stress towards plant. Those heavy metals included cadmium, copper, mercury, nickel, lead and zinc (Sbartai et al., 2011). Metal compounds are one of the most sustained pollutants in both soil and aquatic environments. Unlike organic pollutants, metal compounds are hardly degradable and they tend to accumulate by organisms in a process called bioaccumulation throughout the food chain which results in a negative impacts on food production and human health (Kong et al., 1995). Several studies demonstrated that heavy metal pollution can cause disadvantageous towards both plant growth and development that eventually lead to environmental deterioration (Atici et al., 2005).

The presence of heavy metal toxicity could release ions that able to cause inhibitory effects by repressing or destroying plant resistance and tolerance (Ali et al., 2013). For example, high concentration of heavy metal can inhibit seed germinating and plant growth and interrupt with their biochemical and physiological processes (Clemens, 2001; Farooq et al., 2013). High levels of heavy metal ions can cause several negative impacts on plant tolerance to both abiotic and biotic stresses by inhibiting their photosynthetic functionality, slowing protein synthesis and disturbing the anti-oxidative enzymes activities (Atici et al., 2005; Shao et al., 2008). Although trace amount of some heavy metals are essential for various metabolic processes in organisms, high concentration of heavy metal could cause physiological stress to organisms. For example, copper is essential for the synthesis of chlorophyll and maintenance

of optimum plant metabolism but deficiency of copper in plant can cause reduced synthesis of electron carriers and results in reduced photosynthesis and respiration (Baron et al., 1995).

2.2.5 Salinity Stress and its Effects on Leaf Senescence

Leaf senescence is a process which structurally, physiologically and genetically coordinated and also the last stage of leaf development. The organelles in the leaf and their constituents and contents are broken down while the nutrients are remobilized to actively growing organs of the plant (Noodén, 1988; Smart, 1994; Lim et al., 2007). Leaf senescence is not just governed by the developmental age, it is also regulated by exogenous and endogenous factors, including abiotic and biotic factors (Lim et al., 2007). Early leaf senescence that induced by age and environmental stresses may result in degradation of chlorophyll and other macromolecules and thus the decline of photosynthetic activity (Guo et al., 2004). Senescence of leaf also manifested in ROS accumulation (Rosenvasser et al., 2006; Vanacker et al., 2006).

Salinity is certainly one of the major environmental stress that limiting the crop productivity (Ashraf and Wu, 1994). Salinity causes membrane destabilization (Hasegawa et al., 2000), nutrient imbalance (Munns, 1993), inhibition of photosynthetic machinery (Munns and Termaat, 1986) and irreversible damage to plant cells and tissues (Meyer and Boyer, 1981). Salinity decreases photosynthetic rates through stomatal closure (Heuer, 2005) and non-stomatal events such as impairments in chlorophyll content (Seeman and Critchley, 1985; Neocleous and Vasilakakis, 2007), chlorophyll degradation

(Jiang et al., 2012; Yang and Lu, 2005) chloroplast ultrastructure damage (Shu et al., 2013; Zhao et al., 2015), photosystem II activity inhibition (Everard et al., 1994; Neocleous and Vasilakakis, 2007), and damage to membrane and enzymatic proteins in the photosynthetic apparatus such as key enzymes involved in the photosynthetic carbon reduction cycle (Seeman and Critchley, 1985; Reddy et al., 1992; Chaves et al., 2009; Mittal et al., 2012). Previous studies demonstrated that high concentration of inorganic salts irreversibly affect the photosynthesis of plants by inactivating electron transferring and photophosphorylation of isolated thylakoid membranes (Veiga et al., 2007; Mittal et al., 2012).

The presence of high salt concentration causes several deleterious consequences which start with an ionic imbalance (Niu et al., 1995; Zhu et al., 1997). The most common type of salt stress are due to an excess of sodium chloride (NaCl). The elevated intracellular concentration of Na and Cl ions can be deleterious to cellular systems (Serrano et al., 1999), this affects the homeostasis of K and Ca ions as well (Serrano et al., 1999; Hasegawa et al., 2000; Rodriguez-Navarro, 2000). In order to survive and grow, plant have to adapt and re-establish ionic homeostasis to reduce cellular exposure to ionic imbalance. Besides of ionic imbalance, plant could experience hyperosmotic shock and loss in plant cell turgidity due to high salt concentration. Lastly, salt-induced water stress could inhibit photosynthesis by reducing chloroplast stromal volume and accumulated ROS (Price and Hendry, 1991). The excitation energy from chlorophyll is transferred to produce a singlet oxygen or through reduction of univalent oxygen at PS I in the Mehler reaction which promotes

the generation of ROS in the chloroplast (Foyer and Harbinson, 1994; Allen, 1995).

2.3 Overproduction of ROS and How Plants Cope with It.

2.3.1 ROS as the Indicator for Secondary Stress

Abiotic stresses such as heat and drought stress can cause overproduction and accumulation of ROS in plants. Various subcellular organelles in plant cell such as chloroplast, mitochondrion and peroxisomes are the common sites of ROS production. Accumulation of ROS leads to destructive effects on various cellular mechanisms, not only on enzyme inhibition and protein degradation, but also DNA and RNA damage, as well as membrane lipid per-oxidation, which finally lead to cell death. Photosynthesis and respiration processes of plants can be inhibited as well due to oxidative stress (Ishikawa et al., 2010).

The overproduction of ROS is the best indicator for secondary stress as it can affect the biochemical processes in many plant cells (Ali et al., 2013). In higher plants, ROS including $O_2^{\bullet -}$, H_2O_2, •OH, and singlet oxygen ($^1O_2$) are generated with the presence of excessive heavy metals (Rama Devi and Prasad, 1998). When the levels of ROS generated exceed the ability of plant's antioxidant system to cope with, various damaging effects occur on cellular components. For example plants experience oxidative stress during leaf senescence and produce malondialdehyde (MDA) as a result of membrane lipid peroxidation. Lipid peroxidation is a comprehensive feature of membrane deterioration which eventually lead to cell death (Zimmermann and Zentgraf,

2005). Photosynthesis is the most sensitive biological process influenced by high temperature stress and ROS can be overproduced (Li et al., 2013; Mathur and Jajoo, 2014). Besides, under heavy metals stresses such lead stress, plants experience changes in cellular membrane permeability which lead to the damages of organelles such as chloroplasts, mitochondria and nuclei in plant cells (Clemens, 2001; Ali et al., 2012). The production of ROS due to lead stress was measured by electrolyte leakage, MDA and H_2O_2 contents (MacFarlane, 2003).

In a cell, H_2O_2 and $O_2\bullet^-$ are produced from the generation of •OH radicals, which is known to be the most toxic among all the ROS. Biomolecules in cell such as amino acids, membrane lipids along with organic acids are directly attacked by •OH radical, and lead to cells and organelles leak, which eventually turned to irreparable metabolic dysfunction and cell death (Halliwell, 1991). $^1O_2$ is more reactive than H_2O_2 and $O_2\bullet^-$, which causes chlorophyll breakdown and lipid peroxidation, thus further exerts destruction effect towards membrane integrity and loss of cell function. Finally, more ROS are generated and further damage the cell (Anthony et al., 2005). Studies suggested that $^1O_2$ is a crucial damaging species during photoinhibition induced by stresses such as high light intensities or PSII herbicide treatment (Rutherford and Krieger-liszkay, 2001). ROS scavengers can significantly suppress the damage caused by ROS as ROS generation is an earlier event before mesophyll cells injury. This suggests that the production of ROS cause injury on cellular components through lipid peroxidation and oxidative damage (Chen et al., 2012).

2.3.2 Chloroplast – Major Site of ROS Generation

The major sources of ROS production include photorespiration process in peroxisomes and mitochondrial respiration. Besides, ROS can also be produced by plasma membrane NADPH oxidases, xanthine oxidase, along with cell wall oxidases and peroxidases. Photosynthesis in chloroplasts is certainly one of the major site of ROS production (Brosché et al., 2010; Apel and Hirt, 2004). The reaction centers of PSI and PSII that located in chloroplast thylakoids are the site of photosynthesis in cells. Photosynthesis is well known as the major generation source of ROS in plants due to the electron flow in the transport chain that operates in an aerobic environment which is rich in oxygen, reductants, and high-energy intermediates (Asada, 2006). The photo-production of ROS is also largely affected by physiological and environmental factors (Asada, 2006). The accumulation of $^1O_2$ in chloroplast caused the generation of ROS when chloroplast absorbed light energy which exceeded the capacity for it to carry out CO_2 assimilation or due to inactivation of PSII reaction centers or from the production of $O_2^{\bullet -}$ as the photoreduction of oxygen occurred at PSI when exposed to high light intensities (Laloi et al., 2004). Thus, regulatory systems and efficient antioxidant network are essential to minimize ROS production and to ensure the intracellular ROS pools are always at low levels.

Under normal circumstances, production and concentration of ROS remains low and well-regulated (Polle, 2001). This is due to presence of protective enzymes such as superoxide dismutase (SOD), catalase (CAT), polyphenol oxidase (PPO), and ascorbate peroxidase (APX) (Asada, 1984) which provide a defence mechanism for aerobic organisms (Beyer et al., 1991).

ROS may act as cellular indicators of stresses and secondary messengers in the stress-response signalling pathways. However, ROS tend to accumulate under stress conditions if it is not regulated well (Edreva, 2005). Oxidative stress leads to cellular damage when this critical balance is disrupted when there is an occurrence of ROS burst and antioxidant depletion (Scandalios, 2002).

2.3.3 The Scavenging of ROS by Anti-oxidative Enzymes

Anti-oxidative metabolism is absolutely essential in the defence response of plants. Plants possess both enzymatic and non-enzymatic defence systems to protect cells from oxidative stress. In order to protect against the cellular damage caused by ROS, anti-oxidative enzymes function as a defence system against deleterious free radicals in plant cells (Slooten et al., 1995; Ding et al., 2013). SOD, CAT, and various peroxidases such as guaiacol peroxidase (GPX) and APX are the primary anti-oxidative enzymes (Kuk et al., 2003). SOD are metalloenzymes which serve as the first line of antioxidant defence against ROS by catalyzing the dismutation of $O_2^{\bullet -}$ to produce H_2O_2 and O_2 (Alscher et al., 2002; Raychaudhuri, 2000). APX is believed to affect the fine modulation of ROS in signalling and CAT is mainly responsible in the excess ROS scavenging (Mittler, 2002; Feng et al., 2013). CAT dismutates H_2O_2 and converts it to H_2O and O_2. H_2O_2 as well scavenged by peroxidase such as ascorbate peroxidase and peroxidase indirectly by combining it with antioxidant compounds such as ascorbate (Lu et al., 2010; Yang et al., 2011). Ascorbate-glutathione cycle is well-known as the major hydrogen peroxide detoxifying system in which ascorbate peroxidase utilizes ascorbate as an electron donor to

reduce H_2O_2 to water (Asada, 1992). Its high efficiency protects the chloroplast from oxidative damage under abiotic and biotic stress (Asada, 2006; Kuzniak and Skodowska, 2001). In the ascorbate-glutathione cycle, ascorbate peroxidase is responsible in removing H_2O_2, while dehydroascorbate reductase (DHAR) and reductase (GR) are responsible for providing substrate such as the reduced ascorbate and glutathione for APX (Shi et al., 2005). Different APX isozymes can be found in cytosol, peroxisomes, mitochondria and chloroplasts. One of the two isoforms of chloroplast APX is localized in the stroma while another is associated with the thylakoid membranes (Shigeoka et al., 2002; Jiménez et al., 1998).

2.3.4 Protective Role of Anti-oxidative Enzyme in Photosynthesis

Major source of ROS is from photosynthesis process in plants. The photosynthetic electron transport chain (PET) always operates in an aerobic condition, thus an efficient regulatory systems such as antioxidant network are necessary to minimize and process ROS production effectively in order to maintain the low level of intracellular ROS pools. The oxidation of water on the lumen side of PS II is a vital step of photosynthesis, in which the light-driven PET system further drives electrons from water to NADP and generates the proton gradient for the ATP synthesis. PSII reaction center had to rebuild once every 30 minutes as it is vulnerable to light-induced damage, due to the production of $^1O_2$ in the PSII reaction center (Vass and Cser, 2009), which leads to an irreversible oxidation of the D1 protein (Krieger-Liszkay et al., 2008). Photoinhibition is the consequence due to the rate of damage is greater than that

of repair on PET system (Vass and Aro, 2007). However, the rate of repair is just as fast as the rate of damage under normal circumstances, thus, high level of PSII activity is still able to be maintained (Ohad et al., 1984). PET is widely accepted with its role in minimizing the production of singlet oxygen at PSII and generation of $O_2^{\bullet -}$ and H_2O_2 mainly on the reducing side of PSI (Genty and Harbinson, 1996).

H_2O_2 is a potent inhibitor of photosynthesis as it inhibits CO_2 fixation by 50% even at low concentrations (10 μM). Therefore, it is crucial to maintain the balance between the production of ROS and ROS scavenging in chloroplasts. Several studies have been done on the enhancement of plants tolerance towards abiotic stress in order to protect photosynthesis against inhibition caused by stress. This includes molecular genetics and genetic engineering approach (Mittler and Blumwald, 2010). The overexpression of SOD, GR, and DHAR was proven to improve plants tolerance to abiotic stress through enhancing the water-water cycle capacity (Logan et al., 2006). The overexpression of thylakoid membrane-bound APX in chloroplasts on transgenic tobacco (*Nicotiana tabacum*) and *Arabidopsis* plants were effective to enhance plant tolerance to high light and paraquat treatment (Ishikawa and Shigeoka, 2008). Thus, enhancing the antioxidant defences of chloroplasts apparently was the most effective ways to protect the plant cells from abiotic stress and stabilizing photosynthesis under stressful condition (Ishikawa and Shigeoka, 2008; Chang et al., 2009).

2.4 *In vitro* Study of Isolated Chloroplast's Photochemical Activity and its Stability

2.4.1 *In vitro* Photochemical Analysis: Hill Reaction

During 1937, Robert Hill found that isolated chloroplasts were able to evolve oxygen in the absence of carbon dioxide which indicates that source of the electrons are from water in the light reaction. He added artificial electron acceptor to the chloroplast during the *in vitro* study of electron movement. An artificial electron acceptor is able to draw electrons from the electron transport chain, thus intercepts the electrons between the photosystem II and I. This reaction is named after Robert Hill: The Hill reaction. It involves the photoreduction of an electron acceptor in the presence of hydrogens of water while in the absence of carbon fixation (Funkhouser and Balint, 1994).

In vivo, the final electron acceptor in the light reactions is NADP+. For *in vitro* study on isolated chloroplasts, various dyes such as 2,6-dichlorophenolindophenol (DCPIP) can be used as the artificial electron acceptor to measure the rate of the Hill reaction as an observable difference in color at the reduced and oxidized form. DCPIP is blue in color in oxidized form and then turns colorless in its reduced form. This change in colour allows us to monitor the movement of electrons by measuring the change in absorbance at 600 nm for the determination of the Hill reaction rate (Funkhouser and Balint, 1994). The overall movement of electrons during the Hill reaction can be summarized as follows:

$$2H_2O \rightarrow \text{PSII} \rightarrow \text{electron transport chain} \rightarrow \text{DCPIP}$$

2.4.2 *In vitro* Photochemical Analysis: Oxygen Evolution

There are two major pathway in photosynthesis. One of it is light reaction. Light reactions include the splitting of water and electron transporting in the grana of the chloroplast. The evolution of oxygen can be used to monitor the rate of photosynthesis of chloroplasts. *In vivo*, the final electron acceptor is NADP. Artificial electron acceptors such as potassium ferricyanide are commonly used to transport electrons from the splitting of water at PS II across the electron transport chain in the study of oxygen evolution. The dissolved O_2 produced from the isolated chloroplasts can be measured electrochemically with a Clark O_2 sensor, in which the cathode is made of gold (Au) or platinum (Pt) and the anode is silver (Ag) with saturated potassium chloride (KCl) as the electrolyte.

$$\text{Cathode reaction: } O_2 + 2H_2O + 4e^- \rightarrow 4OH$$

$$\text{Anode reaction: } 4Ag + 4Cl^- \rightarrow 4AgCl + 4e^-$$

At the cathode, molecular O_2 and electrons are consumed while the electrons move from the anode to the cathode then creates a current that can be measured. A proportional current travels through the circuit as each molecule of oxygen contact with the cathode. The change in current indicates a change in O_2 partial pressure in the solution (Clark et al., 1953; Renger and Hanssum, 2009).

2.4.3 Application of Isolated Chloroplast: Biofuel Cell

Biofuel production is one of the applications which take the advantage of the photosynthetic properties from isolated chloroplast. Photosynthetic

production of hydrogen provides a clean, sustainable, and renewable source of hydrogen as compared with other conventional methods (Hankamer et al., 2007). First generation of biofuels was produced from corn starch and sugarcane, but the issues with food and energy arise as the scale of the production increase. Second generation biofuels was produced from lignocellulosic biomass, but one of the drawback is that intensive energy pre-treatment is needed for the degradation of lignocellulosic biomass (Shimizu, 2014). Third generation of biofuel production was from photosynthetic organisms, the productivity of the metabolites is significantly low due to low cell growth rate (Shimizu, 2014). Despite the low productivity of the biofuel and biochemical production, the ability to fix CO_2 with sunlight still make photosynthesis apparatus an attractive source for biofuel production. Previous study reported the production of nanodevice which utilized PSI and a hydrogenase for hydrogen production. Transferring of electron from Au electrode to phenazine methosulfate, followed by passing it to PSI tethered to the surface and lastly to hydrogenase for the use in hydrogen production. This device yielded hydrogen production rate of 120 pmol s^{-1} cm^{-2} (Krassen et al., 2009).

2.4.4 Application of Isolated Chloroplast: Biosensor

Herbicides are widely used in agriculture and domestic gardening, in which further caused contamination towards both surface and groundwater. Contamination occurs through run-off, direct application and aerial spraying, either one can causes negative effects on aquatic ecosystems. Most commercial herbicides function by targeting photosynthesis pathway and inhibit it in the plant, however, most herbicides are also affecting photosynthetic aquatic

microorganisms and aquatic plant as well (Villeneuve et al., 2011). The long term exposure of drinking water towards trace pollutants could cause disruption to endocrine system and possibly cancer in humans even with low concentration of ng L^{-1} (Murray et al., 2010). Various herbicides, such as atrazine, bromacil, and diuron are detectable with concentration at least 5 mg L^{-1} in surface and groundwater. Besides, the concentration of herbicides are vary not only depend on different location and season, but also included climatic events and size of the watershed (Villeneuve et al., 2011). Thus, it is crucial to develop quick, easy yet accurate methods to detect the presence of trace pollutants in the field. The present instrument techniques for the detection of herbicides included gas chromatography-mass spectrometry, liquid chromatography-tandem mass spectrometry (Picó et al., 2004), capillary electrophoresis (Robledo and Smyth, 2009; Menzinger et al., 2000; Dinelli et al., 1996), bioluminescence (Jia et al., 2012), and immunosensing (Tran et al., 2012) in which mostly with the limits of detection of mg L^{-1}. Most commonly used method involving chromatography that operates by separating the sample into its components before detection, however, pre-concentration step (Menzinger et al., 2000; Dinelli et al., 1996) and extended analysis time are needed. Thus, the most ideal detection method in field have to be no sample preparation and large instrumentation required.

In 2001, the first self-powered biosensors were reported with the use of two enzymatic biofuel cells in the detecting of glucose or lactate over the concentration range 1–80 mM (Katz et al., 2001). Processes in electron transport chain of photosynthesis pathway include oxidation of water to O_2, generation of NADPH, and generation of a proton gradient for cyclic and noncyclic

photophosphorylation (Meunier et al., 2010) Most herbicides operate by blocking or inhibiting complexes along the electron transport chain and preventing photosynthesis (Villeneuve et al., 2011). This properties allow the detection of herbicides by simply measuring the changes in current or power output of the biosolar cell. Rasmussen and Minteer (2013) showed the application of thylakoid-based solar cells in the self-powered herbicide biosensor allowed the determination of herbicide in solution when the photobioelectrocatalysis is inhibited with different sensitivities dependent on the sources of thylakoids used (Rasmussen et al., 2014). Besides, the research on self-powered biosensor based on thylakoid membranes at the bioanode also reported detection of several commercial herbicides such as atrazine, bromacil, and diuron that give a linear response as high as concentrations of 15 mg L^{-1} and also limits of detection below 0.5 mg L^{-1}, way below than the Environmental Protection Agency limits (Rasmussen and Minteer, 2013).

2.4.5 Storage Condition and its Impacts on the Functionality and Stability of Isolated Chloroplast

There were plenty of potential application of isolated chloroplast, however, plenty of difficulty and challenges needed to be resolved especially on its physical and chemical instabilities in aqueous suspension for post-extraction storage. Isolated chloroplasts and its Hill activity were found to deteriorate very rapidly when stored at room temperature (Hill and Scarisbrick, 1940; Constantopoulos and Kenyon, 1968), thus, optimum storage condition is crucial in order to maintain the stability and functionality of isolated chloroplast. There are various stabilization and preservation of isolated chloroplast such as cryopreservation (Farkas and Malkin, 1979), immobilization (Campàs et al.,

2008) and freeze drying (Zimmermann et al., 1996) with the purpose for long term stability and functionality of the biological unit and its photochemical activity.

One way to preserve the stability of photochemical activity in isolated chloroplasts is by rapidly freezing with dry ice or with temperature low enough to prevent deterioration and damage to the photochemical mechanisms (Gorham and Clendenning, 1950). Gorham and Clendenning (1950) in their research stored their crude chloroplast at -40^{o}C for one year in the presence of sucrose gained new discovery with the deterioration of the photochemical activity after thawing proceeds as rapidly as in freshly isolated chloroplasts. The use of sucrose also helps in stabilizing the activities of isolated chloroplasts at low temperatures (Neuberg and Roberts, 1946; Tressler, 1933). Meanwhile, storage of chloroplasts in the dark was found to better preserve the intactness of isolated chloroplast. The intactness of chloroplasts ensure the high activity of photosystem II, coupling between the electron transport and phosphorylation as well high efficiency of thylakoid energization (Droppa et al., 1981).

It is essential for the chloroplast to go rapid freezing with dry ice before storage at very low temperature as different cooling rate during the freezing could cause different magnitude of damages (Mazur, 1984). Intracellular ice tend to form as cytoplasmic supercooling is associated with cell destruction at fast cooling rates. Although intracellular ice formation will not cause cell death during the freezing process, however, cell death tends to occur during the thawing process (Mazur, 2004). In contrast, ice tend to form outside the cell

during slow cooling in which hydration would occur due to water moves out of the membrane by osmosis. The elution of water from membrane created solute effects and thus endogenous biomolecules exposed to high concentration of solutes and damaged (Mazur, 1984). The distinct differences between fast and slow cooling is that fast cooling can cause formation of intracellular ice while the membrane still in fluid state while dehydrating freezing effect of slow cooling would create fluid-to-gel phase transitions. Farkas and Malkin (1979) in their research found out that the photosynthetic activities of chloroplast were better preserved by higher rate of cooling and thawing, though different cell type with own characterized membrane permeability towards water have different critical cooling rate for maximum survival.

As compared to past research, there was insufficient study on the relationship between plants' photosynthesis ability of its isolated chloroplast and their anti-oxidative enzymes activities. It is crucial for us to have better understanding on this so that we are able to learn about the role of anti-oxidative enzymes in preserving plant's photosynthetic capability either in their native growing environment or under stressful condition. The photosynthetic ability and anti-oxidative enzymes activities of some plant samples in the study such as *Amaranthus tricolor* Linn., *Pandanus amaryllifolius*, and *Elaeis guineensis* are not well studied before. This study can serves to fill in the research gap in which no previous study has done before.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals and Reagents

Chemicals used in this research (Table 3.1) were of analytical reagent (AR) grade.

Table 3.1: List and details of chemicals and reagents used.

Chemicals	Brands
(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (HEPES)	Nacalai Tesque
2,6-Dichlorophenolindophenol (DCPIP)	Sigma Aldrich
2-Propanol	QRëC
Acetone	Bendosen
Ammonium chloride	Merck
Ascorbic acid	HmbG Chemicals
Bovine serum albumin (BSA)	Merck
Catechol	Fisher Scientific
Coomassie Brilliant Blue (CBB)	Nacalai Tesque
Disodium ethylenediaminetetraacetate dihydrate	Duksan
Dithiothreitol	Acros Organics
Guaiacol	Sigma Aldrich
Hydrochloric acid	Fisher Scientific
Hydrogen peroxide	Merck
Magnesium Chloride Hexahydrate	QRëC
Polyvinylpyrrolidone (PVP)	Acros Organics
Phenylmethylsulfonyl fluoride (PMSF)	Merck
Potassium dihydrogen phosphate	Systerm
Potassium hydrogen phosphate	Systerm
Pyrogallol	Merck
Sodium chloride	RCI Labscan
Sodium dihydrogen phosphate	R&M Chemicals
Sodium hydrogen phosphate	Systerm
Sodium hydroxide	QRëC
Sucrose	QRëC

Table 3.1 continued

Chemicals	Brands
Tricine	Amresco
Tris(hydroxymethyl)aminomethane hydrochloride	Fisher Scientific
Liquid nitrogen	Linde Malaysia

3.1.2 Equipment

Table 3.2: List and details of equipment used.

Equipment	Brands
Centrifuge machine	Beckman Coulter
Conductivity meter	Mettler Toledo
Digital water bath	Memmert
Electronic balance	Sartorius Cubis
Hotplate stirrer	Stuart
Ice collector machine	Scotsman
Light source	Overhead Projector, 3M™, Model 1708
Micropipettor	Sartorius
Microscope	Labo Microsystems GmbH, AXL
Omni-mixer homogenizer	Sorvall
Oven	Memmert
Portable spectrophotometer	Konica Minolta, CM-700d
pH meter	Mettler Toledo
Refrigerator	Toshiba
UV-VIS spectrophotometer	Geneys WS, Thermo Fisher Scientific
Vortex mixer	Scientific Industries Genie 2,

3.2 Overview of Research Study

Figure 3.1 shows the three major parts of this research study, which included study on photochemical activities and storage stability of isolated chloroplast; study on leaves' parameters in response to salinity stress; study on crude anti-oxidative enzymes activities extracted from leaves.

Figure 3.1. Three major parts of research study.

3.3 Plant Samples

The plant samples studied were Chinese spinach *(Amaranthus tricolor* Linn.*)*, pandan *(Pandanus amaryllifolius)*, oil palm *(Elaeis guineensis)* and water lettuce *(Pistia stratiotes)***.** All of the samples were collected from the Kampar region in Perak state, Malaysia. Fresh leaf was harvested in the morning between 9-10 a.m. Only matured leaf was selected in which leaf with approximately 10-15 cm of length and 9-12 cm of width for Chinese spinach leaf, 45-50 cm of length and 3-4 cm of width for pandan, 90-110 cm of length and 5-6 cm of width for oil palm while 3-4 cm of length and 2-3 cm of width for water lettuce. Harvested leaf was immediately processed for further extraction and experiments use. The species of the plant samples were authenticated by Dr. Rahmad Zakaria at the Institute of Biological Sciences, Universiti Sains Malaysia, Malaysia (Appendix A-D).

3.4 Study on Isolated Chloroplast

3.4.1 Isolation of Chloroplasts

Chloroplast was isolated from Chinese spinach, pandan leaves, oil palm fronds and water lettuce based on the method of Cuello and Quiles (2007). Freshly harvested leaves were washed with normal tap water and then wiped dry. The leaves were cut into small pieces and placed in a Sorvall omni-mixer homogenizer. Extraction buffer consisted of 0.35 M sucrose, 25 mM HEPES, 2 mM disodium-EDTA, 2 mM ascorbic acid, 4 mM dithiothreitol, 10 mM magnesium chloride and 1 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.6 was added in at a ratio of 1:6 (weight to volume). The leave and buffer were homogenized. The homogenate was filtered and centrifuged with an Avanti JE Centrifuge at 200 × g for 5 min. The supernatant was further centrifuged at 2500 × *g* for 10 min. The pellet obtained was mixed with 40 mL of the extraction buffer and centrifuged at 2500 × *g* for 10 min. The pellet which contained the chloroplasts was then resuspended in a hypotonic buffer solution with 10 mM tricine, 10 mM sodium chloride and 10 mM magnesium chloride at pH 7.8.

3.4.2 Microscopic Examination of Isolated Chloroplasts

The prepared chloroplast suspension was dispensed on a microscope slide and covered with a slide cover. The image of the slide was captured using a light microscope (Labo Microsystems GmbH, AXL, Germany) with the magnification of 100 times.

3.4.3 Determination of Chlorophyll Content

The prepared chloroplast suspension was mixed with distilled water to form a 5% (v/v) suspension. This diluted chloroplast suspension was added with 100% acetone at a ratio of 1:4. The mixture was vortexed and centrifuged at 2500 × *g* for 5 min. The absorbance of the supernatant was recorded at 646 and 663 nm with a Genesys WS UV-VIS spectrophotometer with 80% (v/v) acetone as the blank. The chlorophyll content of isolated chloroplasts was calculated according to the Lichtenthaler and Wellburn formula with some modifications:

$$\text{Total Chlorophyll (mg/mL)} = (17.32A_{646} + 7.18A_{663})/10$$

$$\text{Total Chlorophyll (mg/ g FW)} = \text{Total chlorophyll (mg/mL)}*V/ (W*DM/100)$$

A_{646} = Absorbance at 646 nm

A_{663} = Absorbance at 663 nm

V = Volume of chloroplast isolated

W = Weight of plant sample

DM = Dry matter (100% - moisture content)

3.4.4 Storage of Isolated Chloroplasts

The isolated chloroplasts were dispensed into small vials for storage. The chloroplast-containing vials were divided into two groups for each type of leaves. One group was stored at 4ºC for 30, 60 and 90 days while the other was immersed into liquid nitrogen before storage at -20ºC. The photoreduction rate of the isolated chloroplast was determined based on the Hill's reaction.

3.4.5 Determination of Photoreduction Rate Based on Hill Reaction

Photoreduction rate of isolated chloroplasts was assessed based on the method of Trebst (Trebst, 2007). We prepared a reaction mixture containing 3 mL of Hill Reaction buffer (50 mM Tricine, 1 mM NH_4Cl, 100 mM Sucrose and 80 μM DCPIP) and 15 μL chloroplasts. The absorbance of the reaction mixture was recorded at 620 nm. The blank was a mixture containing only the chloroplasts sample and the Hill Reaction buffer without the DCPIP. The reaction mixture was then exposed to a light source. The absorbance at 620 nm was recorded at a time interval of 30 s of light exposure for 5 min. Frozen samples were allowed to thaw in a water bath at ~25°C and kept in an ice bath before further analysis. The Hill reaction activity was expressed in term of μmol (DCPIP)/ mg (chl)/ hr.

3.5 Study on Plant Leaves' Parameters

3.5.1 Determination of Moisture Content

The moisture content of plant leaf was determined gravimetrically after drying at 105°C in an oven (Memmert, Universal Oven U, Germany) until a constant weight.

3.5.2 Determination of Electrical Conductivity

Electrical conductivity was measured based on the method of Bajji et al. Ten 6 mm-diameter leaf disks from randomly collected leaves were cut with a puncher and weighed. The leaf disks were washed twice with 100 mL deionized water with constant stirring at room temperature. Then, they were soaked in 40 mL deionized water and the electrical conductivity was measured with a bench

top conductivity meter at a 15 min-interval. The electrical conductivity was expressed in μs cm^{-1} g^{-1} of leaf fresh weight.

3.5.3 Salt Tolerance Test

Salt tolerance test was carried out based on the method of Liu et al with some modifications (Liu et al., 2014).Three leaf disks of 9 mm were floated on 5 mL of 400 mM NaCl solution before incubated for 96 h at 24 °C using photoperiods of 16 h light/8 h dark. Leaf disks floated in sterile distilled water were served as the experimental control. The morphology of the leaf disks was examined under microscope and the color changes was measure with a portable spectrophotometer (Konica Minolta, CM-700d, US). The total color difference (ΔE*ab), difference in lightness/ darkness value (ΔL*), difference in red/ green axis (Δa*), difference in yellow/ blue axis (Δb*), difference in chroma (ΔC*) and difference in hue (ΔH*) were calculated:

$\Delta E^*ab = [(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2]^{1/2}$

$\Delta L^* = L^* - L^*t$

$\Delta a^* = a^* - a^*t$

$\Delta b^* = b^* - b^*t$

$\Delta C^* = [(a^*)^2 + (\Delta b^*)^2]^{1/2} - [(a^*t)^2 + (\Delta b^*t)^2]^{1/2}$

$\Delta H^* = [(\Delta E^*ab)^2 - (\Delta L^*)^2 - (\Delta C^*)^2]^{1/2}$

3.6 Study on Extracted Crude Anti-oxidative Enzymes

3.6.1 Extraction of Crude Enzyme

Crude enzyme extract was isolated from spinach, pandan leaves, oil palm fronds and water lettuce according to the procedure as described by

Mohammadian et al. (2012) with some modification. Freshly harvested leaves were washed with normal tap water and then wiped dry. The leaves were cut into small pieces and placed in a Sorvall omni-mixer homogenizer. Extraction buffer solution consisted of 0.1M sodium phosphate buffer, 0.5mM EDTA and 2% polyvinylpyrrolidone at pH 7.5 was added at a ratio of 1:6 (weight to volume) to the leaf samples and homogenized. The homogenate was centrifuged at 15000 rpm for 15 min at 4ºC. The supernatant was collected as the crude enzyme extract.

3.6.2 Determination of Protein Content

The prepared crude enzyme extract was diluted 40 times with distilled water. Then, 1mL of Coomassie Brilliant Blue reagent was added to 100 µL of crude enzyme extract. The mixture was inverted and mixed and allowed to stand at ~25ºC for 2 min. The absorbance of the mixture was determined at 595 nm, using a spectrophotometer with a mixture of Coomassie Brilliant Blue reagent and extraction buffer as the blank. A standard curve was constructed using 0 – 100 µg/mL of final BSA concentration (Appendix E). Protein content was expressed as mg/ mL (AOAC, 2012).

3.6.3 Quantitative Analysis of Anti-oxidative Enzyme Activities

The anti-oxidative enzymes studied included ascorbate peroxidase, catalase, peroxidase, guaiacol peroxidase, pyrogallol oxidase, catechol oxidase and superoxide dismutase.

3.6.4 Ascorbate Peroxidase (APX)

The spectrophotometric determination of ascorbate peroxidase activity was carried out based on the method of Nakano and Asada (Nakano and Asada, 1987). The final reaction volume of 3 mL contained: 100 µL crude enzyme extract, 50 µL H_2O_2 (0.3%, v/v) and 2850 µL sodium phosphate buffer-ascorbate (50 mM sodium phosphate buffer, 0.5 mM ascorbate, pH 7.2). A blank was prepared in the absence of the enzyme extract. The absorbance of the reaction mixture was determined at 290 nm for 1 min at an interval of 15 s (molar extinction coefficient,ε = 2800 $M^{-1}.cm^{-1}$). The unit of APX activity was calculated as µmole of ascorbic acid oxidized per min under specified conditions. APX specific activity was expressed in U mg^{-1} protein.

3.6.5 Catalase (CAT)

The spectrophotometric determination of catalase activity was carried out based on the method of Cakmak and Horst (1991). The final reaction volume of 3 mL contained of 100 µL of crude enzyme extract, 50 µL H_2O_2 (0.3% v/v) and 2850 µL sodium phosphate buffer (50 mM, pH 7.2). A blank was prepared in the absence of the enzyme extract. The decrease in absorbance of the reaction mixture was determined at 240 nm for 3 min at an interval of 15s (molar extinction coefficient, ε = 39400 $M^{-1}.cm^{-1}$). The reaction was initiated after the addition of hydrogen peroxide. CAT specific activity was expressed in U mg^{-1} protein.

3.6.6 Peroxidase (POX)

Peroxidase was assayed as described by Vestena et al. (2011). The assay mixture contained 1.95 mL potassium phosphate (0.1 M, pH 6.8), 0.8 mL pyrogallol (20 mM), 0.15 mL H_2O_2 (20 mM) and 0.1 mL of crude enzyme extract. The absorbance of the reaction mixture was determined at 420 nm for 2 min at an interval of 15 s (molar extinction coefficient, $\varepsilon = 2470\ M^{-1}.cm^{-1}$). POX specific activity was expressed in U mg^{-1} protein

3.6.7 Guaiacol Peroxidase (GPX)

Guaiacol peroxidase was assayed as described by Chance and Maehly (1955). The assay mixture contained 2 mL potassium phosphate (0.1 M, pH 7), 1 mL of guaiacol (20 mM), 0.05 mL of H_2O_2 (20 mM) and 0.5 mL of crude enzyme extract. The absorbance of the reaction mixture was determined at 470 nm (molar extinction coefficient, $\varepsilon = 26600\ M^{-1}.cm^{-1}$) for 2 min at an interval of 15 s. One unit is defined as the amount of enzyme that required to catalyze the conversion of one μmole of hydrogen peroxide, in the presence of guaiacol as hydrogen donor, per min under specified conditions. GPX specific activity was expressed in U mg^{-1} protein.

3.6.8 Pyrogallol Oxidase (PO)

Pyrogallol oxidase activity was assayed as described by Murao et al. (1992) with slight modifications. Crude enzyme extract of 0.05 mL was added to 2 mL of assay buffer (5mM pyrogallol, 50 mM potassium phosphate buffer, pH 6.0) at 37°C. After 5 to 25 min of incubation, the absorbance was measured at 440 nm against a blank without the substrate. One unit of PO activity was

defined as the amount of the enzyme which increases the absorbance by 0.1 per min. PO specific activity was expressed in U mg^{-1} protein.

3.6.9 Catechol Oxidase (CO)

The spectrophotometric determination of catechol oxidase activity was carried out based on the method of Ögel et al. (2006). The assay mixture contained 2 mL potassium phosphate buffer (0.1 M, pH 6) and 1 mL catechol (0.1 M). The assay mixture was allowed to incubate for 3 min at room temperature. Then, 0.5 mL of crude enzyme extract was added to the cuvette containing the assay mixture. The reaction was monitored at 410 nm for 2 min at room temperature, absorbance was recorded every 15 s (molar extinction coefficient, ε = 3450 $M^{-1}.cm^{-1}$). CO specific activity was expressed in U mg^{-1} protein.

3.6.10 Superoxide Dismutase (SOD)

Superoxide dismutase activity was assayed as described by Marklund and Marklund (1974) with slight modifications. The reaction mixture consisted of 2.85 mL Tris-HCl buffer (50 mM, pH 8.2) and 0.1 mL of crude enzyme extract and 50 µL of pyrogallol (20 mM, in 10 mM hydrochloric acid) was added as the initiator the assay. The absorbance reading was recorded at 320 nm for 3 min. The auto-oxidation of pyrogallol was the reaction mixture contained only 2.85 mL of 50 mM Tris-HCl buffer in pH 8.2 and 50 µL of 20 mM pyrogallol. The SOD specific activity was expressed in U mg^{-1} protein.

3.7 Data Analysis

All experimental data are expressed as mean ± standard deviation. All experiments were carried out in triplicate. Microsoft Excel t-Test Paired Two Sample was applied for Section 4.4.1 to 4.4.4 to identify the significant between means (P <0.05). Statistical Software SPSS 20.0 was used for statistically analysis and comparison of means. One way descriptive analysis and one way ANOVA were applied for Section 4.3 and 4.10.1 to 4.10.7. Turkey *post hoc* test was applied for mean comparing in all analysis. Difference was considered as significant at the level of $P < 0.05$. Pearson (Bivariate) Correlation analysis was carried out using Microsoft Excel 2007. Correlation relationship was presented as Pearson Correlation Coefficient. In statistics, the correlation coefficient r is used to determine the strength and direction of a linear relationship of two variables on a scatterplot. The obtained value of r must between +1 and –1.The r values of exactly –1 and +1 show a prefect negative and perfect positive linear relationship. The range of r value in the range of 0.0 to 0.19 (0.0 to -0.19) indicates very weak positive (negative) linear relationship, 0.20 to 0.39 (-0.20 to -0.39) indicates weak positive (negative) linear relationship, 0.4 to 0.59 (-0.40 to -0.59) indicates moderate positive (negative) linear relationship, 0.60 to 0.79 (-0.60 to -0.79) indicates strong positive (negative) linear relationship while 0.80 to 1.0 (-0.8 to -1.0) indicates very strong positive (negative) linear relationship, 0 means no relationship at all between the two variables (Evans, 1996).

CHAPTER 4

RESULTS

4.1 Chlorophyll Content of Isolated Chloroplast

Table 4.1 shows the chlorophyll content of isolated chloroplasts of 4 leaf samples. Among the 4 leaf samples, Chinese spinach had the highest chlorophyll content of 2.40 mg g^{-1} dry basis (db), followed by pandan of 1.07 mg g^{-1} (db), water lettuce of 0.55 mg g^{-1} (db), while oil palm had the lowest chlorophyll content of 0.12 mg g^{-1} (db).

Table 4.1. Chlorophyll content of isolated chloroplasts of 4 leaf samples.

Leaf source of chloroplasts	Chlorophyll content (mg g^{-1} db)
Chinese spinach	2.40±0.03
Pandan	1.07±0.01
Oil palm	0.12±0.00
Water lettuce	0.55±0.01

Values represent mean ± SD (n=3).

4.2 Microscopic Examination of Isolated Chloroplast

Figure 4.1 shows the microscopy image of chloroplast isolated from the leaves of Chinese spinach at 100 × magnification. These chloroplasts were tiny in size and abundance in number, which were arranged in a compact manner.

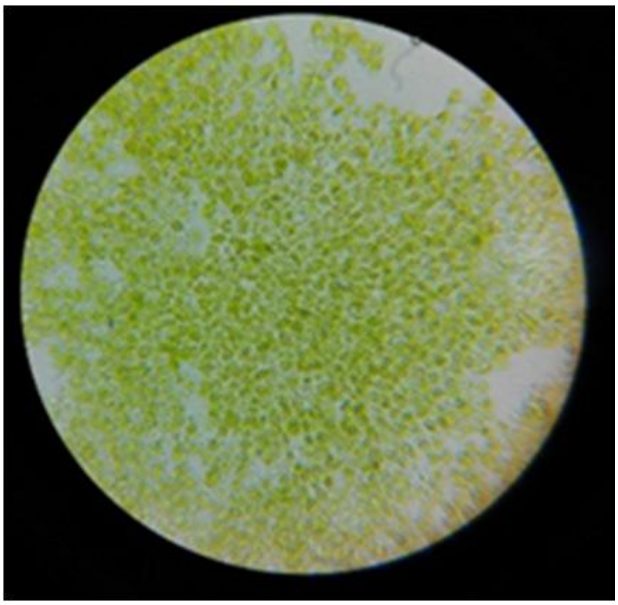

Figure 4.1. Microscopy image of chloroplasts isolated from the leaves of Chinese spinach at 100 × magnification.

Figure 4.2 shows the microscopy image of chloroplasts isolated from the leaves of pandan at 100 × magnification. These chloroplasts were tiny in size and slightly less abundance in number as compared with Chinese spinach chloroplasts, they were arranged in a slightly less compact manner than the Chinese spinach chloroplasts.

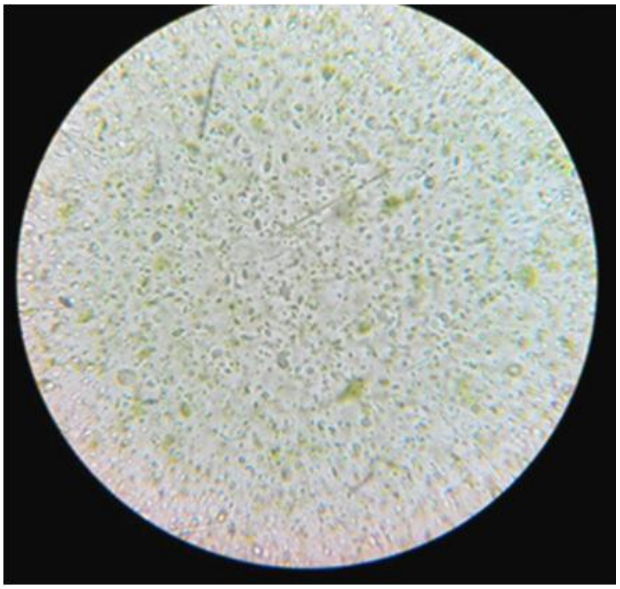

Figure 4.2. Microscopy image of chloroplasts isolated from the leaves of pandan at 100 × magnification.

Figure 4.3 shows the microscopy image of chloroplast isolated from the leaves of oil palm at 100 × magnification. These chloroplasts were bigger in size and moderate in number, which were arranged in a slightly less compact manner than Chinese spinach chloroplast.

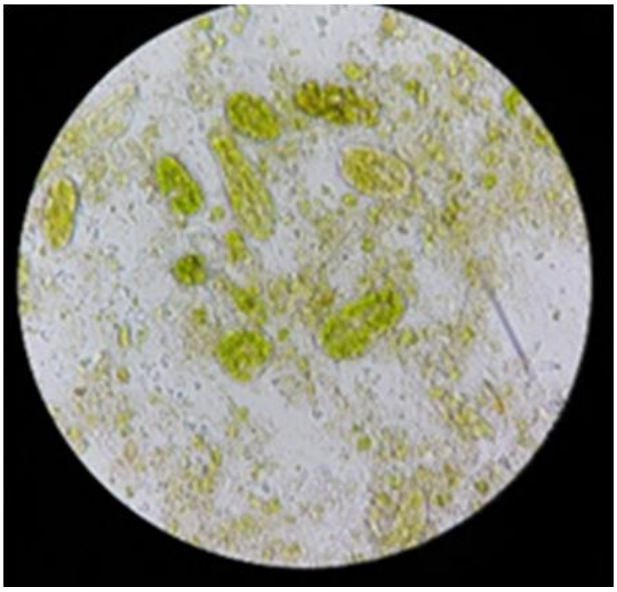

Figure 4.3. Microscopy image of chloroplasts isolate from the leaves of oil palm at 100 × magnification.

Figure 4.4 shows the microscopy image of chloroplasts isolated from the leaves of water lettuce at 100 × magnification. These chloroplasts were moderate in size and fewer in number, which were arranged in a ‘loose’ manner.

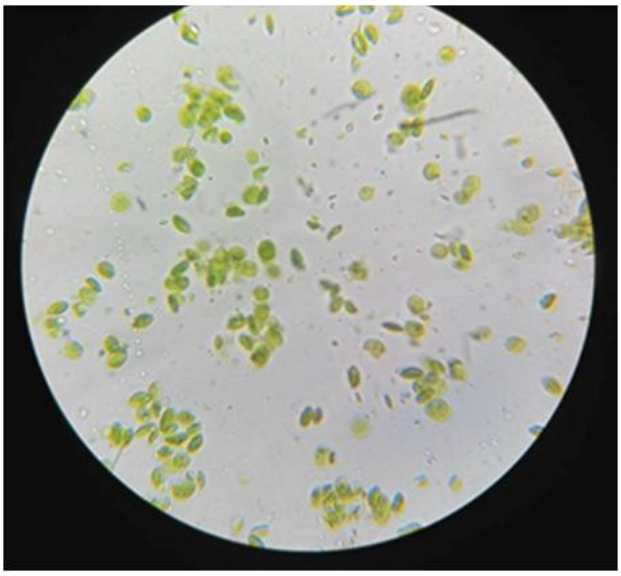

Figure 4.4. Microscopy image of chloroplasts isolate from the leaves of water lettuce at 100 × magnification.

4.3 Initial Hill Reaction Activity

Figure 4.5 shows the initial Hill Reaction Activity (HRA) of isolated chloroplasts of 4 leaf samples. Among the chloroplasts-rich extract, oil palm showed the highest initial HRA of 95.6 µmole DCPIP mg^{-1} chl hr^{-1}, followed by Chinese spinach of 81.2 µmole DCPIP mg^{-1} chl hr^{-1} and pandan of 80.8 µmole DCPIP mg^{-1} chl hr^{-1}. Water lettuce showed the lowest HRA of 64.0 µmole DCPIP mg^{-1} chl hr^{-1}.

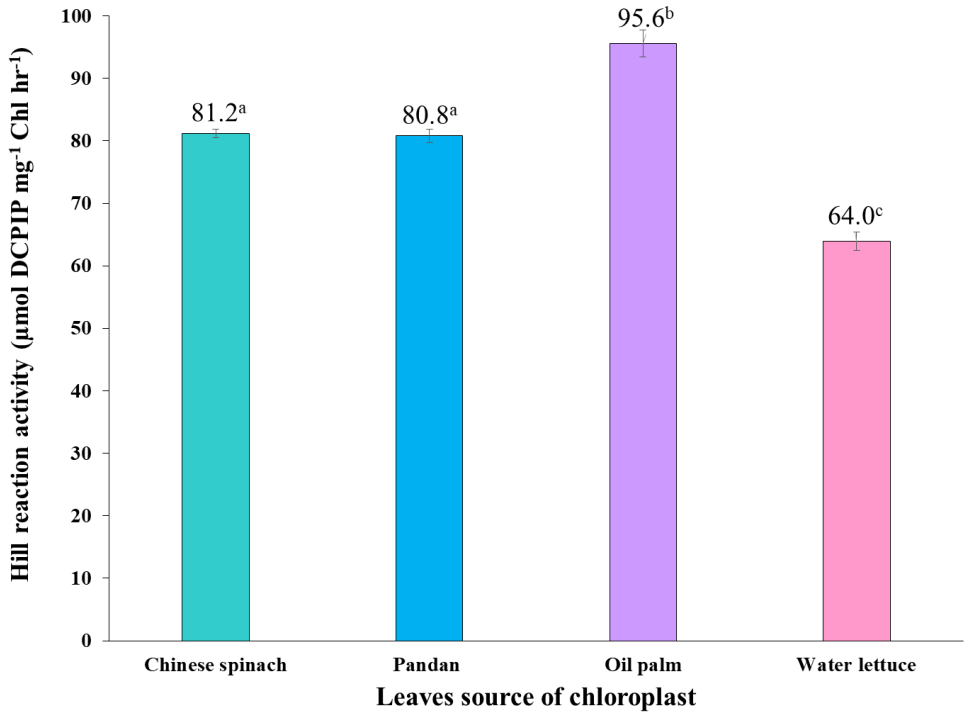

Figure 4.5. Initial Hill reaction activity of the 4 chloroplast suspension of leaf samples. Values represent mean ± SD (n=3).

4.4 Hill Reaction Activity (HRA) on Storage

4.4.1 Chinese Spinach Chloroplasts

Figure 4.6 shows the HRA of chloroplast isolate of Chinese spinach over 90 days of storage with 10 day-interval under both chilled and frozen storage conditions. The initial HRA of Chinese spinach chloroplast was 81.2 µmole DCPIP mg^{-1} chl hr^{-1}. The HRA of chill-stored chloroplast dropped to 33.7 µmole µmole DCPIP mg^{-1} chl hr^{-1} by day 30, and further dropped to 14.2 µmole DCPIP mg^{-1} chl hr^{-1} by day 60 and became relatively constant to 11.1 µmole DCPIP mg^{-1} chl hr^{-1} after 90-day storage. For frozen chloroplast, the HRA dropped to 59.4 µmole µmole DCPIP mg^{-1} chl hr^{-1} by day 30 and decreased slowly to 45.0 µmole DCPIP mg^{-1} chl hr^{-1} by day 60 and dropped to 36. µmole DCPIP mg^{-1} chl hr^{-1} of HRA by after 90-day storage.

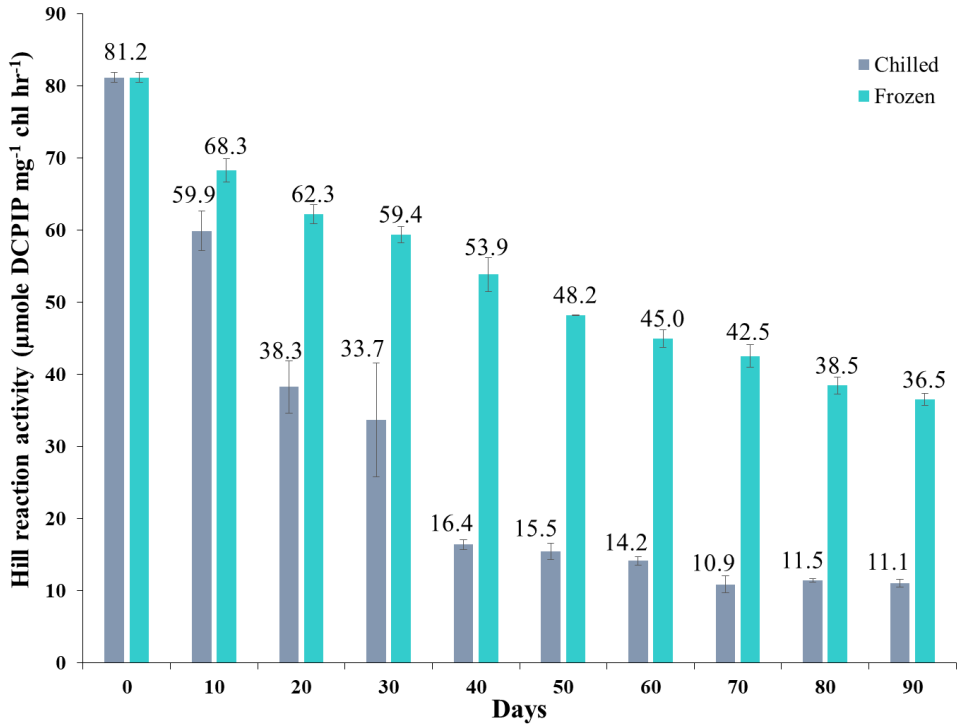

Figure 4.6. Hill reaction activity of chloroplast isolate of Chinese spinach over 90 days of storage with 10 day-interval. Values represent mean ± SD (n=3). There is no significant difference in the Hill reaction activity between chilled and frozen conditions ($p>0.05$).

4.4.2 Pandan Leaves Chloroplasts

Figure 4.7 shows the HRA of chloroplast isolate of pandan leaves over 90 days of storage with 10 day-interval under both chilled and frozen storage conditions. The initial HRA of pandan chloroplast was 80.8 µmole DCPIP mg^{-1} chl hr^{-1}. The HRA of chill-stored chloroplast dropped to 36.6 µmole DCPIP mg^{-1} chl hr^{-1} by day 30, further dropped to 20.6 µmole DCPIP mg^{-1} chl hr^{-1} by day 60 and further reduced to 9.4 µmole DCPIP mg^{-1} chl hr^{-1} by day 90. The HRA of frozen chloroplast dropped to 46.5 µmole DCPIP mg^{-1} chl hr^{-1} by day 30 and maintained rather steadily with storage to reach 38.3 µmole DCPIP mg^{-1} chl hr^{-1} of HRA on day 60 and 37.7 µmole DCPIP mg^{-1} chl hr^{-1} of HRA on day 90.

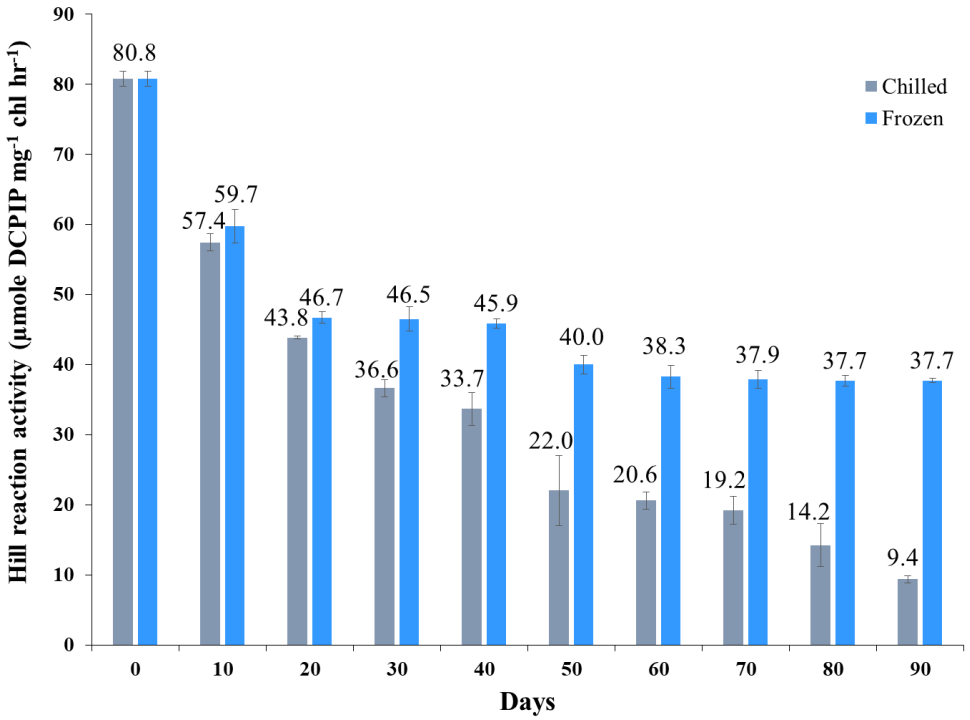

Figure 4.7. Hill reaction activity of chloroplast isolate of pandan leaves over 90 days of storage with 10 day-interval. Values represent mean ± SD (n=3). There is no significant difference in the Hill reaction activity between chilled and frozen conditions ($p>0.05$).

4.4.3 Oil Palm Frond Chloroplasts

Figure 4.8 shows the HRA of chloroplast isolate of oil palm frond over 90 days of storage with 10 day-interval under both chilled and frozen storage conditions. The oil palm chloroplast showed HRA as high as 95.6 µmole DCPIP mg^{-1} chl hr^{-1} initially. The HRA of chill-stored chloroplast on day 30 was 78.7 µmole DCPIP mg^{-1} chl hr^{-1}, further dropped to 56.7 µmole DCPIP mg^{-1} chl hr^{-1} on day 60 and 37.7 µmole DCPIP mg^{-1} chl hr^{-1} on day 90. The frozen chloroplast's HRA dropped in a slower manner in which HRA on day 30 was 89.1 µmole DCPIP mg^{-1} chl hr^{-1}, 67.2 µmole DCPIP mg^{-1} chl hr^{-1} on day 60 and finally 53.0 µmole DCPIP mg^{-1} chl hr^{-1} on day 90.

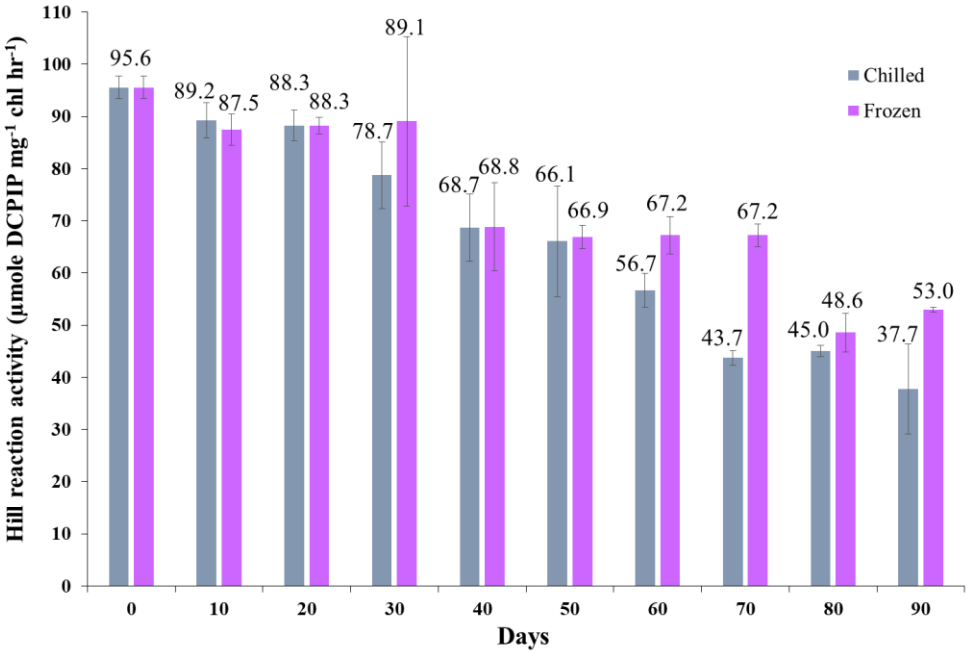

Figure 4.8. Hill reaction activity of chloroplast isolate of oil palm frond over 90 days of storage with 10 day-interval. Values represent mean ± SD (n=3). There is no significant difference in the Hill reaction activity between chilled and frozen conditions ($p>0.05$).

4.4.4 Water Lettuce Chloroplasts

Figure 4.9 shows the HRA of chloroplast isolate of water lettuce over 90 days of storage with 10 day-interval under both chilled and frozen storage conditions. Water lettuce showed the lowest initial HRA, which was 64.0 µmole DCPIP mg^{-1} chl hr^{-1}. The HRA of chill-stored chloroplast on day 30 was 58.3 µmole DCPIP mg^{-1} chl hr^{-1}, and slightly dropped to 57.9 µmole DCPIP mg^{-1} chl hr^{-1} by day 60 and further dropped to 41.1 µmole DCPIP mg^{-1} chl hr^{-1} by day 90. The frozen chloroplast showed quite similar magnitude as chilled chloroplast over the period of storage, in which the HRA on day 30 was 61.0 µmole DCPIP mg^{-1} chl hr^{-1}, 55.0 µmole DCPIP mg^{-1} chl hr^{-1} on day 60 and 46.0 µmole DCPIP mg^{-1} chl hr^{-1}.

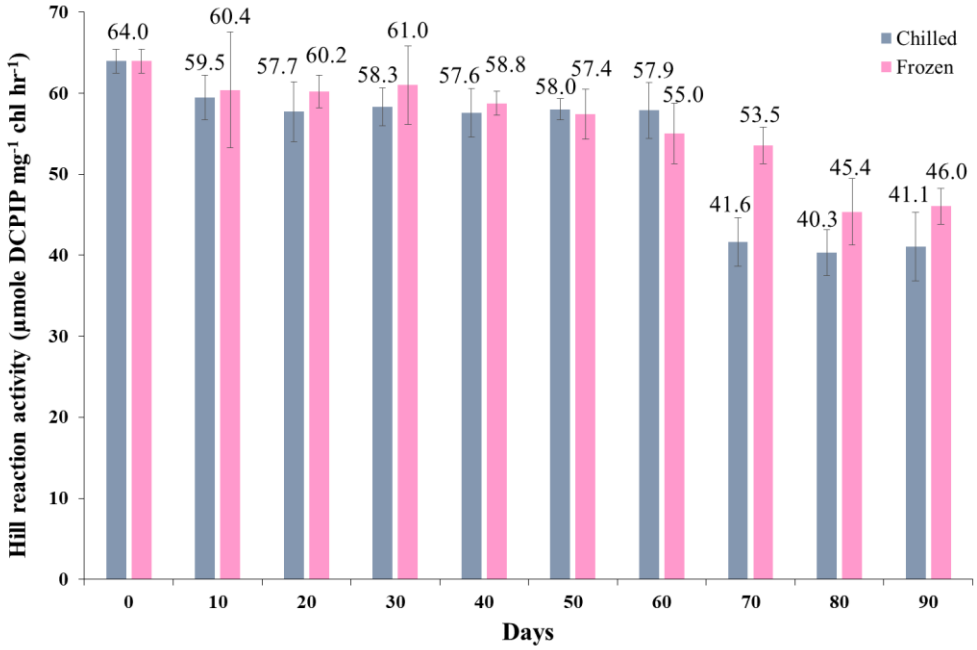

Figure 4.9. Hill reaction activity of chloroplast isolate of water lettuce over 90 days of storage with 10 day-interval. Values represent mean ± SD (n=3). There is no significant difference in the Hill reaction activity between chilled and frozen conditions ($p>0.05$).

In order to compare the storage stability of different chloroplasts objectively, we calculated the degradation rate based on the initial HRA for each type of chloroplast.

4.5 Percent Degradation Based on Hill Reaction Activity

4.5.1 Chinese Spinach Chloroplasts

Figure 4.10 shows the degradation of HRA of Chinese spinach chloroplast isolate over 90 days of storage with 10 day-interval in both chilled and frozen storage conditions. Both chilled and frozen chloroplasts showed a low degree of degradation initially: 26.2% for chilled and 15.9% for frozen storage on day-10. However, the degradation of chilled chloroplast exceeded 50% by day-20 and attained 58.3% by day 30, further degraded to 82.6% by day 60. The percent of degradation attained up to 86.4% by the end of 90 day-storage.

The degradation for frozen chloroplast increased slowly from 26.8% on day 30 to 44.6% on day 60 and finally 55.0% on day 90.

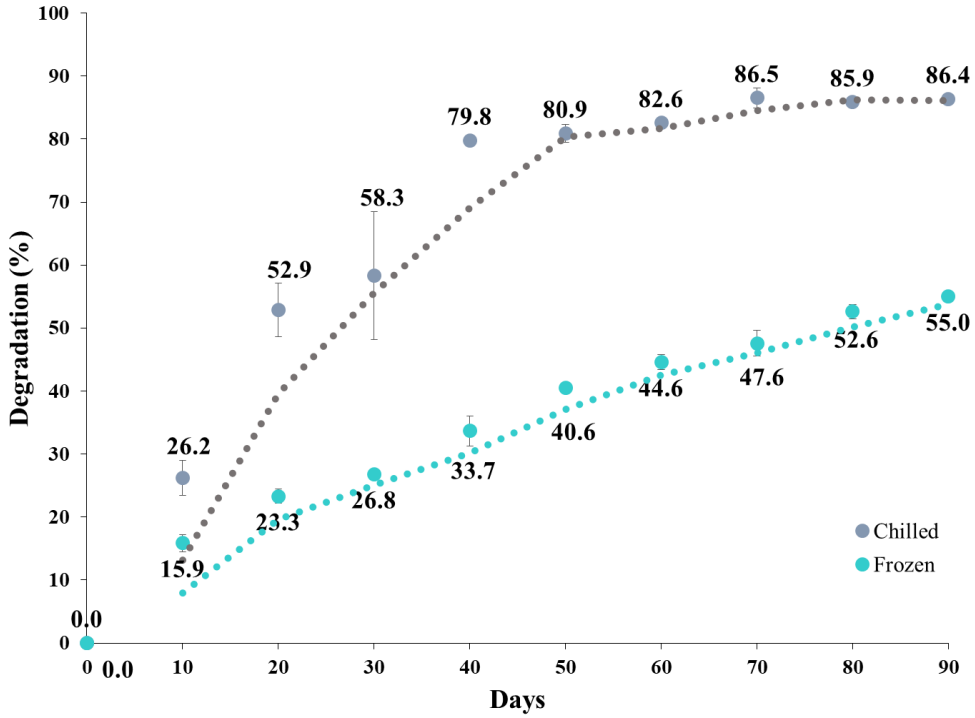

Figure 4.10. Degradation (%) of Hill reaction activity of Chinese spinach over 90 days of storage with 10 day-interval. Values represent mean ± SD (n=3).

4.5.2 Pandan Leaves Chloroplasts

Figure 4.11 shows the degradation of HRA of pandan leaves chloroplast over 90 days of storage with 10 day-interval. Both chilled and frozen chloroplasts attained similar percent of degradation: 28.9% for chilled and 26.0% for frozen condition by day-10. The degradation of chilled chloroplast further increased to 54.6% by day 30, 74.5% by day 60 and 88.4% by day 90. The degradation of frozen chloroplast increased to 42.5% by day 30, 52.6% by day 60 and up to 53.3% by day 90.

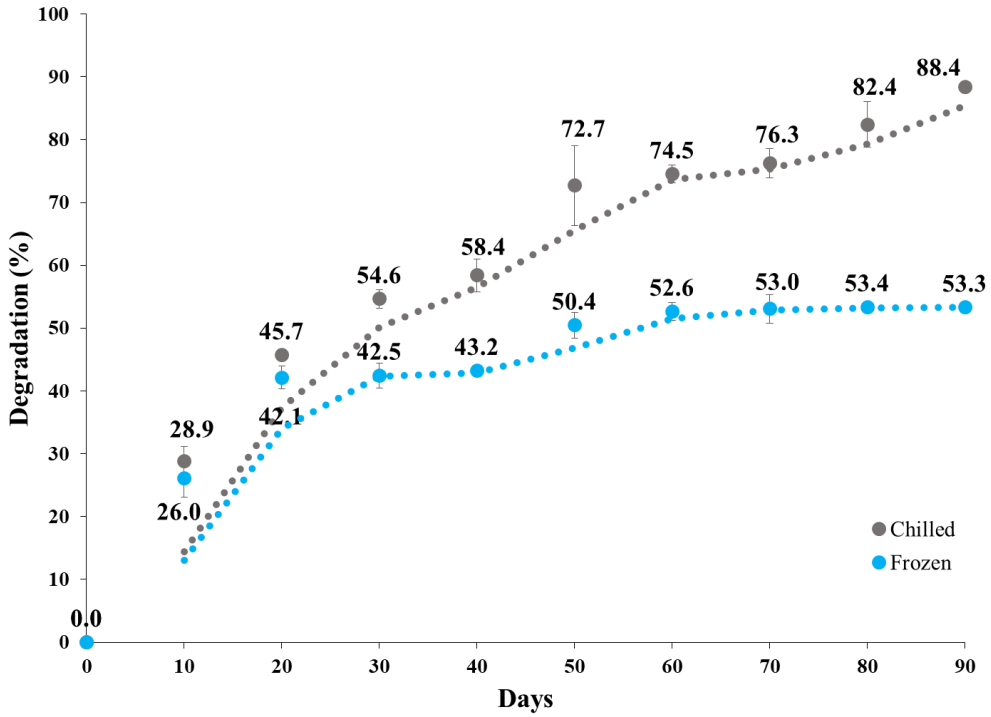

Figure 4.11. Degradation (%) of Hill reaction activity of chloroplast isolate of pandan leaves over 90 days of storage with 10 day-interval. Values represent mean ± SD (n=3).

4.5.3 Oil Palm Frond Chloroplasts

Figure 4.12 shows the degradation of HRA of oil palm frond chloroplast isolate over 90 days of storage with 10 day-interval. Both chilled and frozen chloroplast showed similar degradation until day 60 in which 17.6% and 40.8% on day 30 and day 60 for chilled chloroplast while 6.9% and 29.7% on day 30 and day 60 for frozen chloroplast. By the end of 90-day storage, chilled chloroplast degraded by 60.2% whereas frozen chloroplast degraded by 44.5%.

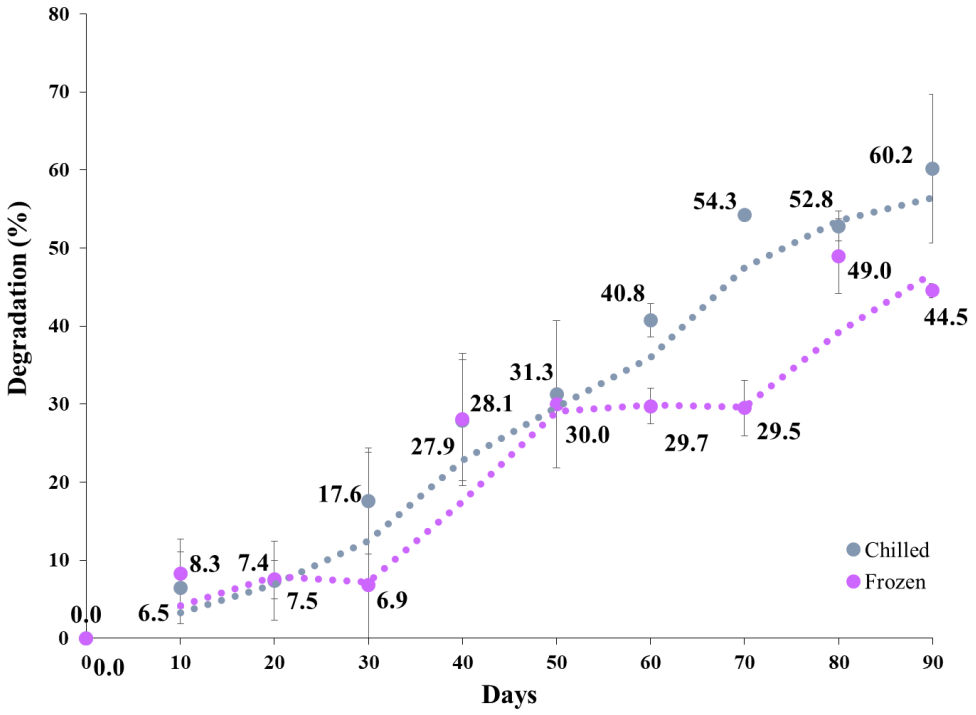

Figure 4.12. Degradation (%) of Hill reaction activity of chloroplast isolate of oil palm frond over 90 days of storage with 10 day-interval. Values represent mean ± SD (n=3).

4.5.4 Water Lettuce Chloroplasts

Figure 4.13 shows the degradation of HRA of chloroplast isolate of water lettuce over 90-days of storage with 10 day–interval. Water lettuce showed similar extent of degradation for both chilled and frozen chloroplast until day 60, in which 8.6% and 9.3% on day 30 and day 60 for chilled chloroplast, while 4.8% and 13.7% on day 30 and day 60 for frozen chloroplast. Both chilled and frozen chloroplast experienced 35.8% and 28.1% of degradation by day 90.

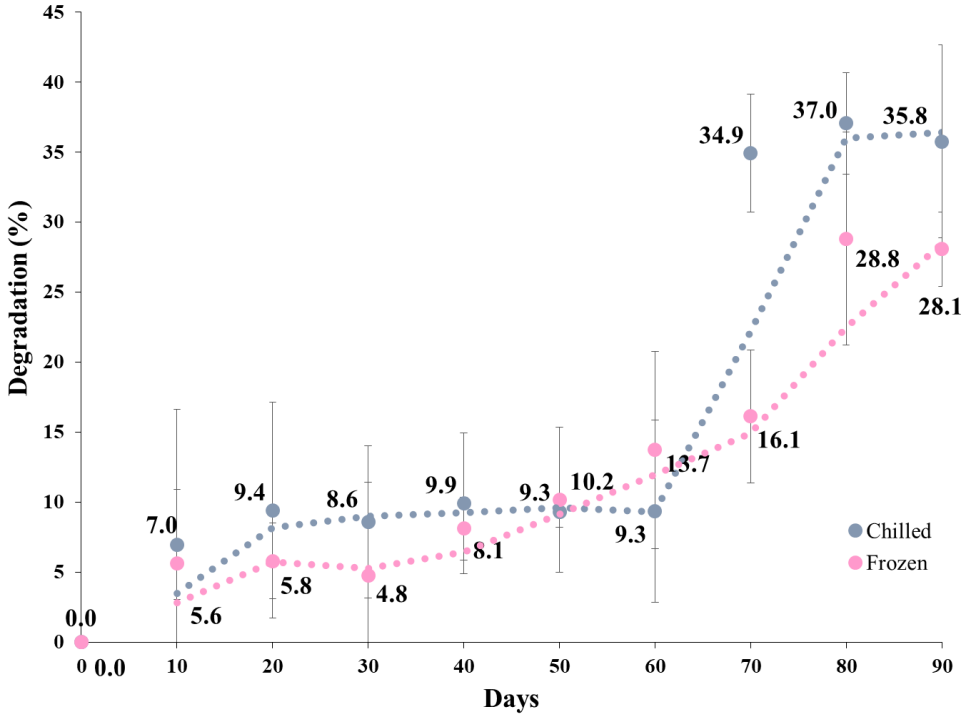

Figure 4.13. Degradation (%) of Hill reaction activity of chloroplast isolate of water lettuce over 90 days of storage with 10 day-interval. Values represent mean ± SD (n=3).

4.6 Moisture Content of Plant Leaf

Table 4.2 shows the moisture content of the leaf of 4 plant samples. Water lettuce showed the highest moisture content followed by Chinese spinach and pandan. Oil palm frond showed the lowest moisture content among all plant samples.

Table 4.2. Moisture content of 4 plant leaf.

Leaf sample	Moisture content (%)
Chinese spinach	90.1±0.1
Pandan	77.1±0.1
Oil palm	59.5±0.3
Water lettuce	90.3±0.2

Values represent mean ± SD (n=3).

4.7 Electrolyte Leakage

Figure 4.14 shows the electrolyte leakage activity of four leaf samples over 90 min with 15-min interval. Chinese spinach showed the highest initial electrical conductivity reading of 18.5 µs cm^{-1} g^{-1} followed by pandan and oil palm of 15.9 µs cm^{-1} g^{-1}. Water lettuce showed the lowest initial electrical conductivity reading of 11.5 µs cm^{-1} g^{-1}. Four plant samples showed a drastic increase of activity after 15 min and increased steadily over 90 min. After 90 min of incubation, oil palm showed the highest electrical conductivity reading of 63.0 µs/ cm/ g, followed by pandan and Chinese spinach. Water lettuce remained the lowest throughout the incubation time.

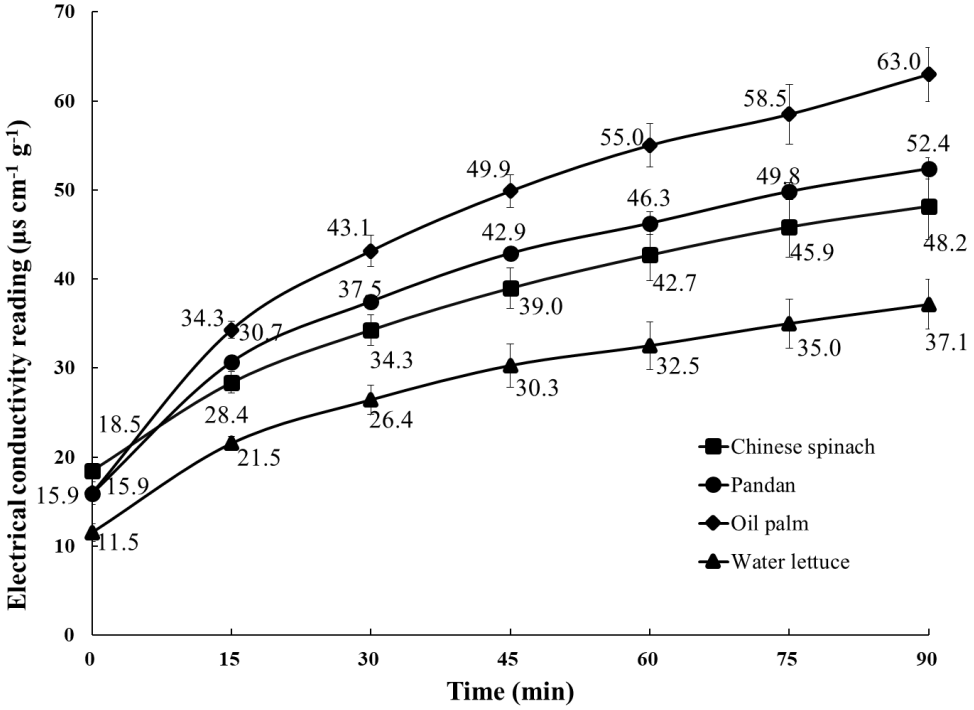

Figure 4.14. Electrical conductivity reading of 4 plant samples over 90 min with 15-min interval. Values represent mean ± SD (n=3).

The leakage of electrolyte is generally related to the vulnerability of a plant towards stress damage (Valentovic et al., 2006; Simova-Stoilova et al.,

2008). Thus, the study of salt tolerance on leaf samples provides further insight on how the leaf samples respond to the stress experienced.

4.8 Salt Tolerance Leaf Senescence Test

These leaf disks were treated with sterile distilled water and 400 mM NaCl solution for 96 h separately. The water treatment acted as the control experiment. Different responses were shown by leaf samples on both treatments, as compared with its initial condition.

4.8.1 Leaf Disk Morphology

Figure 4.15 to Figure 4.18 show the morphology of leaf disks of 4 leaf samples after 96 h of water and salinity treatment, as compared with their initial condition. After 96 h of treatment, oil palm showed no obvious changes on the leaf disks of both water and saline treatments. Both pandan leaf disks on both water and saline treatments showed yellow coloration and brighter after 96 h, in which saline-treated leaf disks showed more intense in turning yellow as compared to water-treated leaf disks. Water-treated water lettuce leaf disks showed only yellow coloration after 96 hours, whereas saline-treated leaf disks were more severe in which they turned yellow along with the appearance of injury ring on the edge of leaf disks. Water-treated leaf disks of Chinese spinach showed obvious sign of injury and thinning of leaf disks, along with discoloration from green to yellowish light green, whereas saline-treated leaf disks not only showed severe thinning of leaf disks, but also discoloration from green to partial transparent.

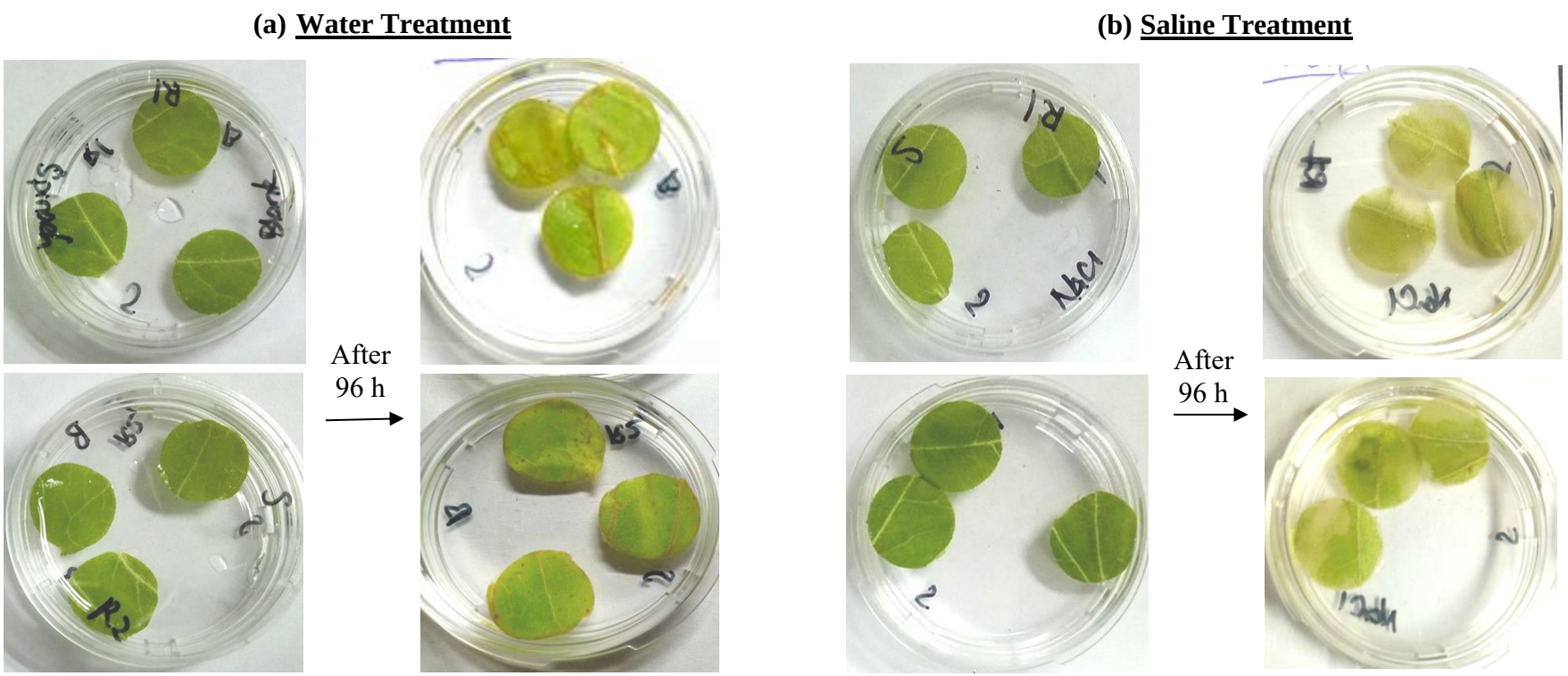

Figure 4.15. Morphology of leaf disks of Chinese spinach after 96 h of (a) water treatment and (b) saline treatment.

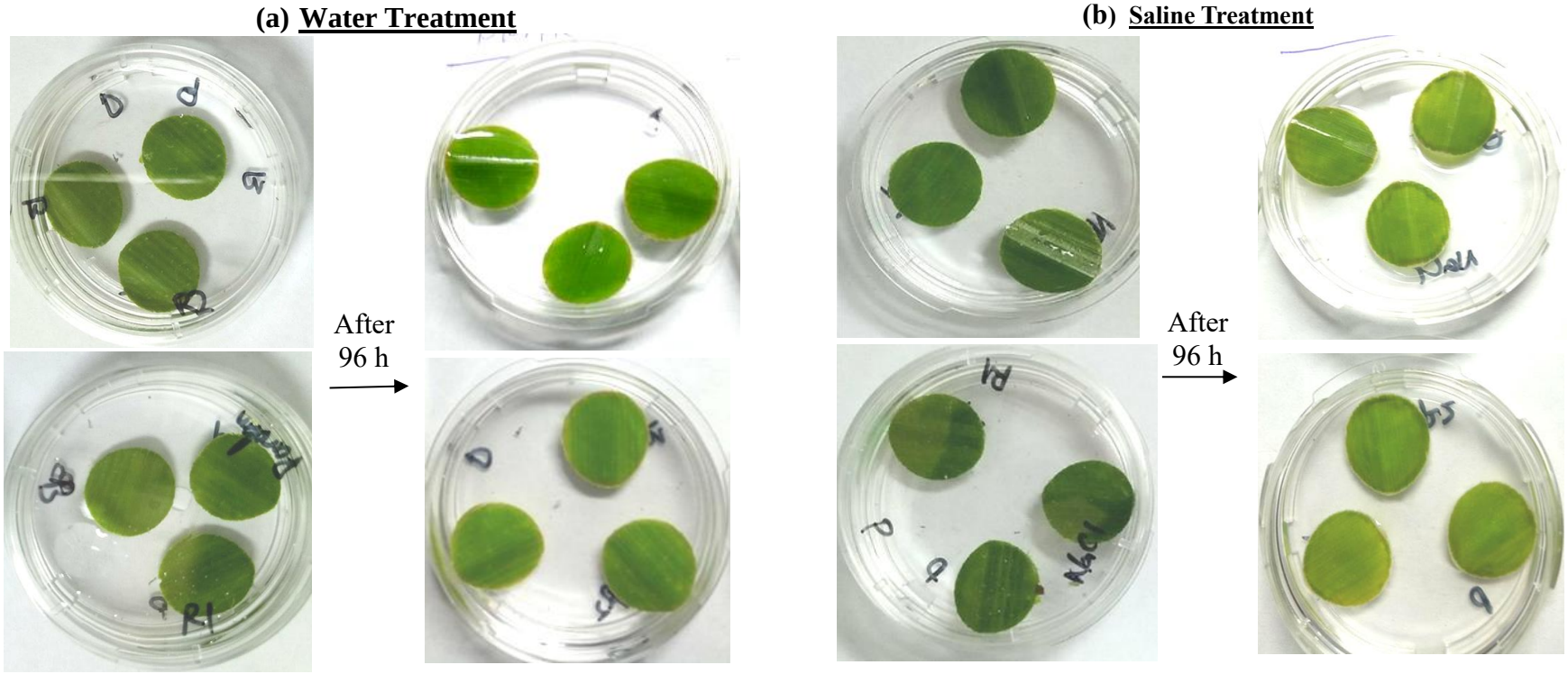

Figure 4.16. Morphology of leaf disks of pandan after 96 h of (a) water treatment and (b) saline treatment.

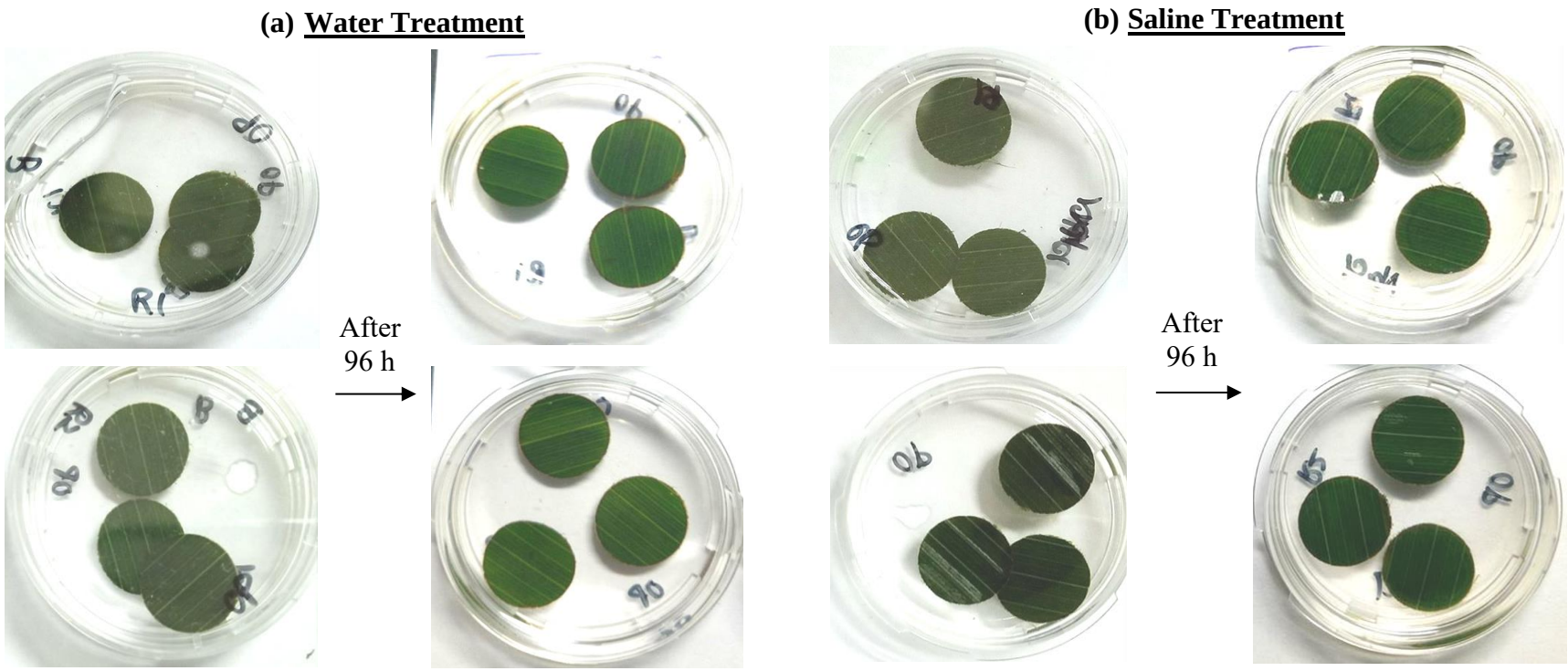

Figure 4.17. Morphology of leaf disks of oil palm after 96 h of (a) water treatment and (b) saline treatment.

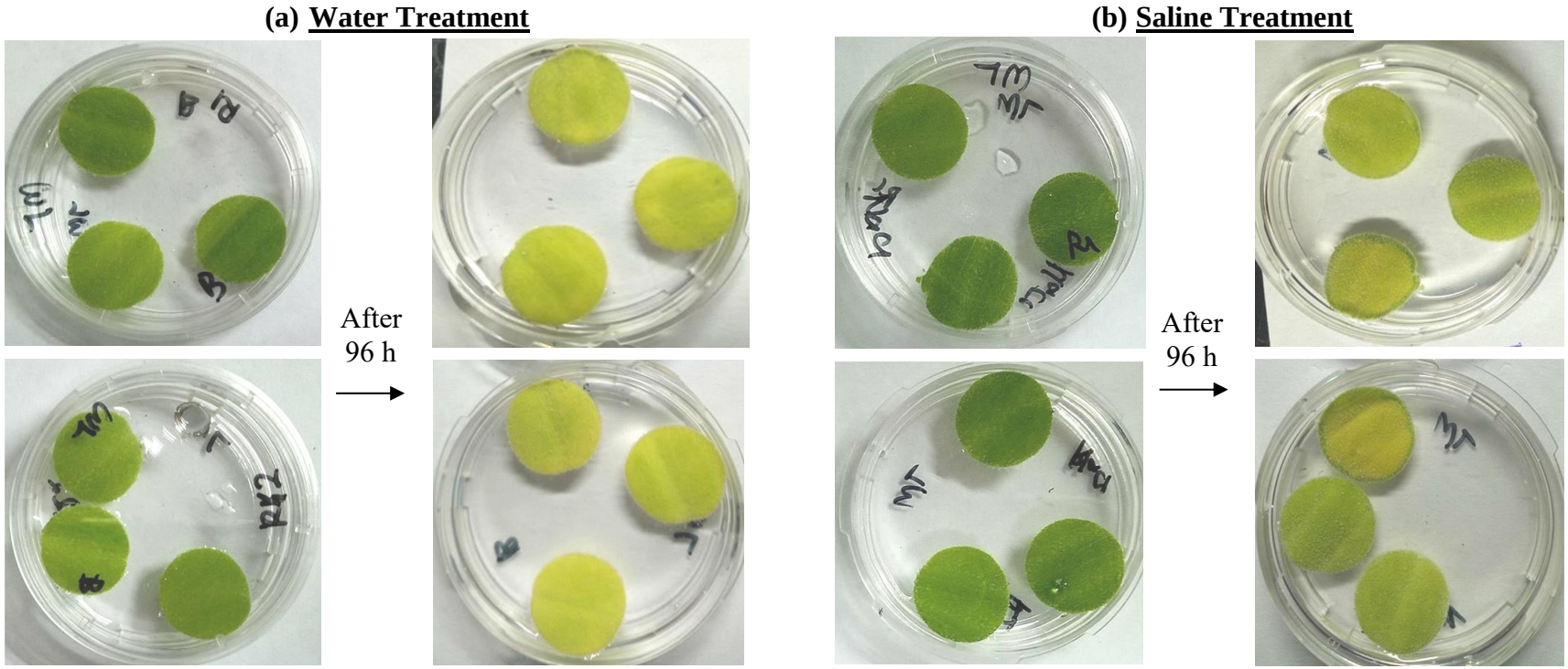

Figure 4.18. Morphology of leaf disks of water lettuce after 96 h of (a) water treatment and (b) saline treatment.

4.8.2 Comparison of Leaf Disk Microscopic Image

Figure 4.19 to Figure 4.22 show the microscopic image of 4 types of leaf disks at 10 × magnification at initial condition, after water treatment and saline treatment. Generally all four plant samples showed green in color on their leaf surface under the microscope for 10× magnification at their initial condition. There was no sign of injury can be observed and features on leaf surface such as trichomes of water lettuce were obviously observed in good condition at their initial state.

For Chinese spinach, leaf disks decreased in green intensity and brighter in color and only minor shrinkage occurred after 96 h of water treatment. Chinese spinach leaf disks in saline treatment were yellower in color and shrinkage of cells were observed due to occurrence of plasmolysis. Nucleus of plant cells was observed after the saline treatment. The pandan leaf disks remained green in color and no sign of injury was observed in both treatments. Obvious plasmolysis can be observed on leaf disks in saline treatment. Similar intensity of greenness was observed and no distinct differences can be observed in oil palm leaf disks in both water and saline treatments. Numerous multicellular projections called trichomes were observed on both the surface of water lettuce leaf disks in the water treatment experienced shrinkage as compared with those of initial condition, and moisture was retained as compared with those in saline treatment. Besides, obvious change of color was observed in the microscopic image in both treatments. Severe plasmolysis and pores formed due to the shrinkage of cells after the saline treatment.

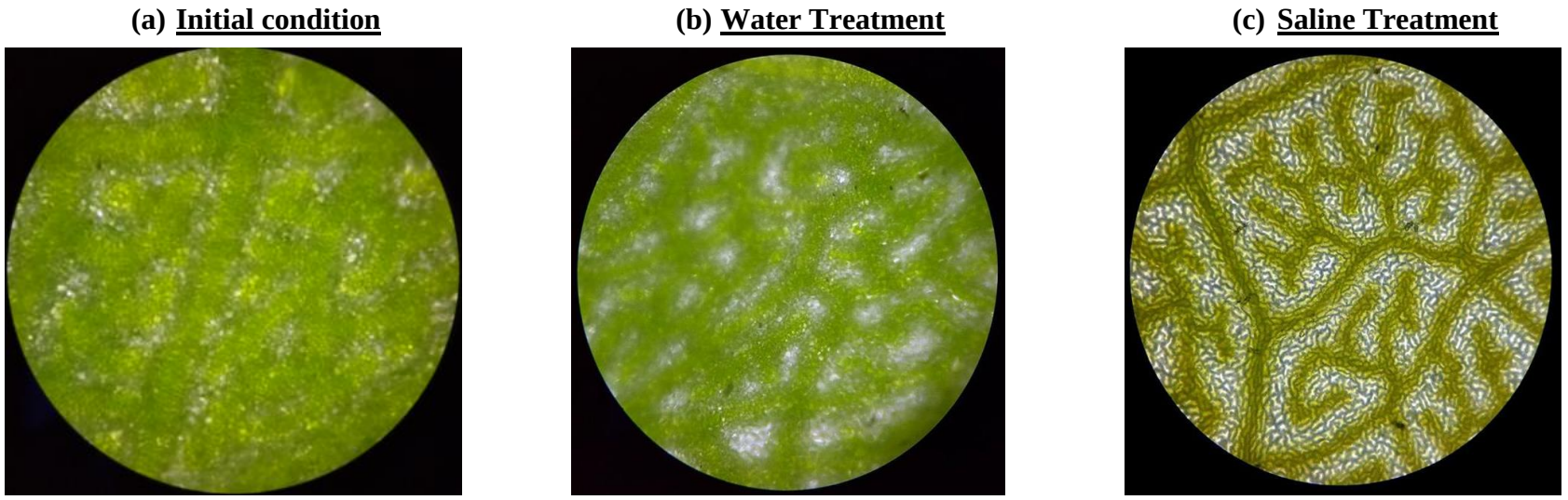

Figure 4.19. Microscopic image of leaf disks of Chinese spinach at 10× magnification at (a) initial condition, (b) water and (c) saline treatment after 96 hr.

(a) Initial condition **(b) Water Treatment** **(c) Saline Treatment**

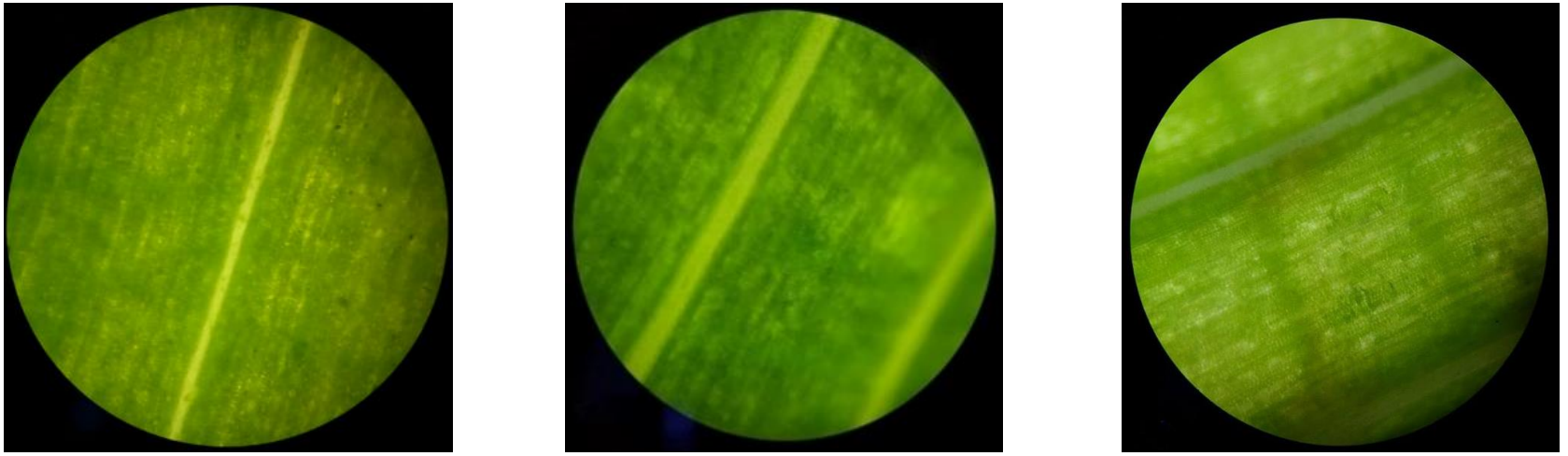

Figure 4.20. Microscopic image of leaf disks of pandan at 10× magnification at (a) initial condition, (b) water and (c) saline treatment after 96 hr.

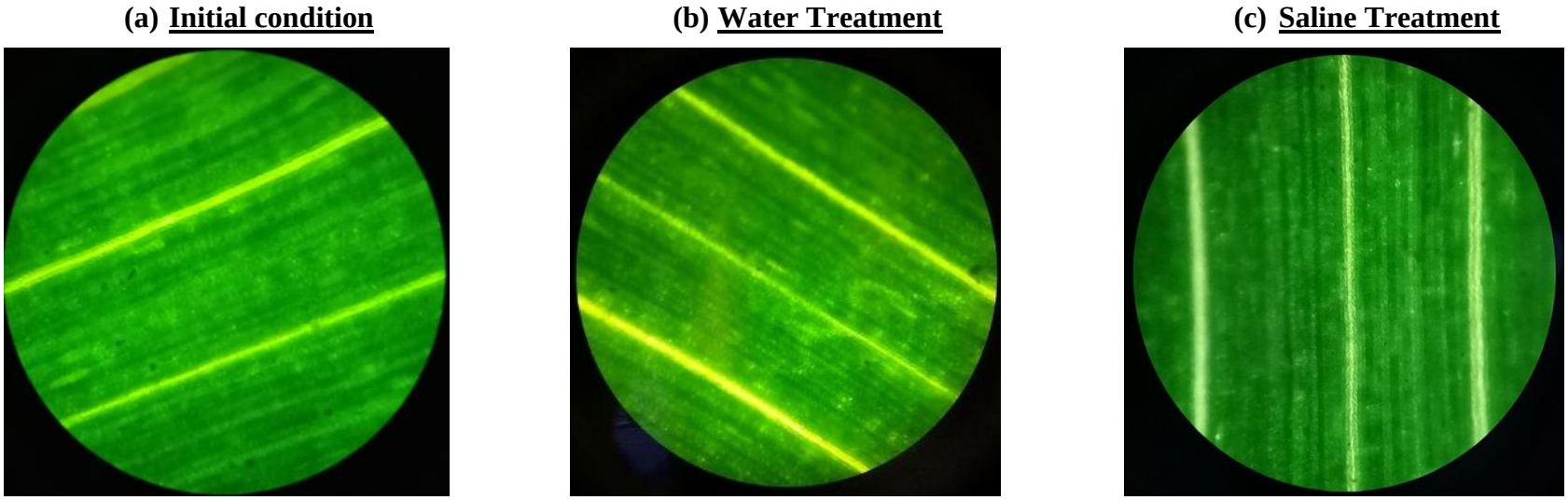

Figure 4.21. Microscopic image of leaf disks of oil palm at 10× magnification at (a) initial condition, (b) water and (c) saline treatment after 96 hr.

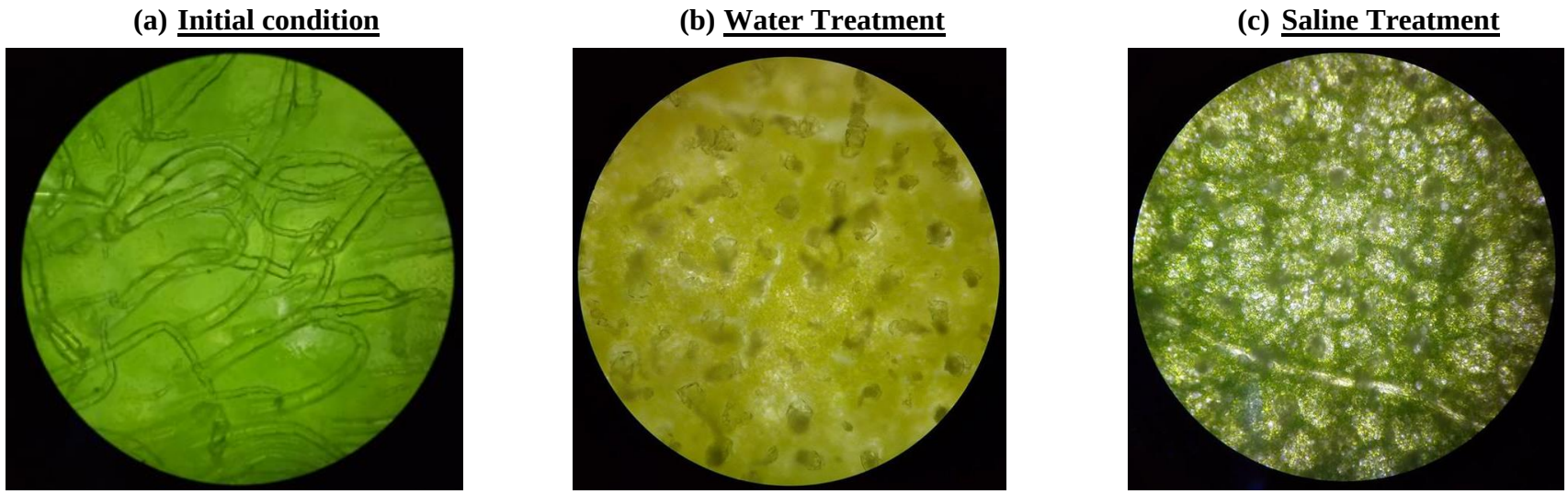

Figure 4.22. Microscopic image of leaf disks of water lettuce at 10× magnification at (a) initial condition, (b) water and (c) saline treatment after 96 hr

In order for us to quantify the color changes of leaves in response to water and salinity test, various color parameters were studied using a portable spectrophotometer to detect the color changes on the surface of leaves.

4.8.3 Color Parameters of Salinity Test

Table 4.3 shows various color parameters of 4 leaf samples that have been determined in salinity test, which included lightness difference (ΔL*), a* value difference (Δa*), b* value difference (Δb*), total color difference (ΔE*ab), chromas angle difference (ΔC*), and hue metric difference (ΔH*).

Lightness (Δ L*):

For L*, it represents darkness and lightness, in which the scale is given as 0 which is black and scale of 100 as white (Konica Minolta, 2003). After water treatment, leaf disks of water lettuce showed the highest positive lightness changes (ΔL*= +18.9), followed by Chinese spinach (ΔL*= +12.1) and pandan (ΔL*= +1.0) while oil palm showed negative lightness changes (ΔL*= -1.1)

After saline treatment, water lettuce leaf disks showed the highest positive lightness changes (ΔL*= +13.0), followed by Chinese spinach (ΔL*= +8.6). Oil palm leaf disks showed negative on lightness changes (ΔL*= -6.2). It is interesting to note that pandan showed the least positive lightness changes (ΔL*= +0.9).

Table 4.3. Color parameters of leaf disks of 4 leaf samples in experience of water and salinity treatment.

Leaf sample	Treatment	Δ L*	Δ a*	Δ b*	Δ E*ab	Δ C*	Δ H*
Chinese spinach	Water	12.1±0.7	1.0±1.0	12.4±0.8	17.6±2.0	11.7±0.8	4.4±0.9
Pandan		1.0±0.2	-0.2±0.2	-0.2±0.7	1.8±0.7	-0.1±0.7	0.3±0.1
Oil palm		-1.1±0.4	0.4±0.1	-0.1±0.2	1.4±0.8	-0.3±0.2	0.3±0.1
Water lettuce		18.9±0.8	5.4±0.5	25.2±1.0	32±3.1	23.3±0.9	11.1±1.5
Chinese spinach	Saline	8.6±1.0	0.0±0.8	12.1±1.6	11.2±2.7	11.7±1.5	3.2±0.9
Pandan		0.9±0.2	-0.1±0.1	1.4±0.5	9.2±3.4	1.3±0.5	0.5±0.2
Oil palm		-6.2±0.1	2.2±0.1	-4.7±0.1	2.8±1.4	-5.2±0.2	0.2±0.1
Water lettuce		13.0±0.6	1.8±0.4	16.1±0.3	23±3.8	15.0±0.3	6.0±0.8

Δ L*= lightness difference; Δ a*= a* value difference; Δ b*= b* value difference; Δ E*ab = total color difference; Δ C*= chroma angle difference; and Δ H*= hue metric difference. Values represent mean ± SD (n=3).

a* value difference (Δ a*):

For a*, negative value indicates greenness whereas positive value indicates reddish (Konica Minolta, 2003). Water lettuce leaf disk showed the highest positive a* value difference (Δ a*= +5.4) after water treatment, followed by Chinese spinach (Δa*= +1.0) and oil palm (Δa*= +0.4). Pandan showed a negative a* value changes (Δa*= -0.2) in this case.

After saline treatment, a negative difference of Δ a* obtained for pandan (Δa*= -0.1), whereas oil palm showed the highest positive difference (Δa*= +2.2) followed by water lettuce (Δa*= +1.8). Chinese spinach showed no difference on this parameter (Δa*= 0).

b* value difference (Δ b*)

For b*, negative value means blue whereas positive indicates yellowish hue. Both water lettuce and Chinese spinach showed positive difference on b* value (Δb*= +25.2 and Δb*= +12.4) whereas pandan and oil palm showed negative difference on b* value (Δb*= -0.2 and Δb*= -0.1) after water treatment.

After saline treatment, water lettuce showed the highest difference on b* value (Δb*= +16.1), followed by Chinese spinach (Δb*= +12.1) and pandan (Δb*= +1.4), whereas negative difference obtained for oil palm (Δb*= -4.7) leaf disks.

Total color difference (Δ E*ab):

ΔE*ab, which indicates the size of the color difference but does not indicates in what way the colors are different (Konica Minolta, 2003). All leaf disks showed positive total color difference (ΔE*ab) on water treatment. Water lettuce showed the highest total color difference (ΔE*ab= +32), followed by Chinese spinach (ΔE*ab= +17.6), pandan (ΔE*ab= +1.8) and oil palm (ΔE*ab= +1.4).

Upon saline treatment, water lettuce showed the highest positive total color difference (ΔE*ab= +23), followed by Chinese spinach (ΔE*ab= +11.2), pandan (ΔE*ab= +9.2) and oil palm (ΔE*ab= +2.8) on their leaf disks.

Chroma angle difference (Δ C*):

Chroma (C) measures color saturation or intensity (Konica Minolta, 2003). After water treatment, water lettuce showed the highest positive chroma angle difference (ΔC*= +23.3), followed by Chinese spinach (ΔC*= +11.7) while oil palm and pandan showed negative chroma angle difference (ΔC*= -0.3 and ΔC*= -0.1).

For saline treatment, water lettuce showed a great difference (ΔC*= +15.0), followed by Chinese spinach (ΔC*= +11.7) and pandan (ΔC*= +1.3). Oil palm in this case showed negative chorma angle difference (ΔC*= -5.2).

Hue metric difference (Δ H*):

The hue metric difference is positive only if the metric hue angle (h) of the specimen is found greater than the target while negative if the metric hue angle of the specimen is found less than that of the target (Konica Minolta, 2003). Same trend of metric hue difference (ΔH*) shown on leaf disk after water treatment in which water lettuce showed the highest positive difference (ΔH*= +11.1), followed by Chinese spinach (ΔH*= +4.4), pandan (ΔH*= +0.3) and oil palm (ΔH*= +0.3).

Similar trend was observed on saline treatment as well, in which water lettuce showed the highest positive difference (ΔH*= +6), followed by Chinese spinach (ΔH*= +3.2), pandan (ΔH*= +0.5) and oil palm (ΔH*= +0.2).

Difference in leaf morphology changes and its response to salt stress were observed among 4 leaf samples. Thus, we extracted crude enzymes of 4 leaf samples in order to study their anti-oxidative enzyme activities at their natural state.

4.9 Protein Content of Extracted Crude Enzyme

Table 4.4 shows the protein content of extract crude enzyme of 4 leaf samples. Among the four plant samples, Chinese spinach showed the highest protein content followed by oil palm and pandan in the range of 10.7 – 12.6 mg g^{-1} fresh weight basis. Water lettuce contained the lowest protein content among all plant samples, which is 3.9 mg g^{-1} fresh weight basis.

Table 4.4. Protein content of crude enzyme extract of 4 leaf samples.

Leaf source of crude enzyme	***Protein content**
Chinese spinach	12.6±0.06
Pandan	10.7±0.12
Oil palm	12.1±0.12
Water lettuce	3.9±0.12

* (mg g^{-1} fresh weight basis). Values represent mean±SD (n=3)

4.10 Quantitative Anti-oxidative Enzyme Analysis

From the crude enzymes we extracted, 7 anti-oxidative enzyme activities were studied, which included ascorbate peroxidase, catalase, peroxidase, guaiacol peroxidase, pyrogallol oxidase, catechol oxidase, and superoxide dismutase.

4.10.1 Ascorbate Peroxidase Activity

Figure 4.23 shows the ascorbate peroxidase (APX) activity of 4 leaf crude enzymes. Oil palm had the highest APX activity, whereas pandan had the lowest APX activity, in which the APX activity obtained was in a range of 0.16 – 1.80 U mg^{-1} protein. APX activity of oil palm was 11-fold higher than that of pandan.

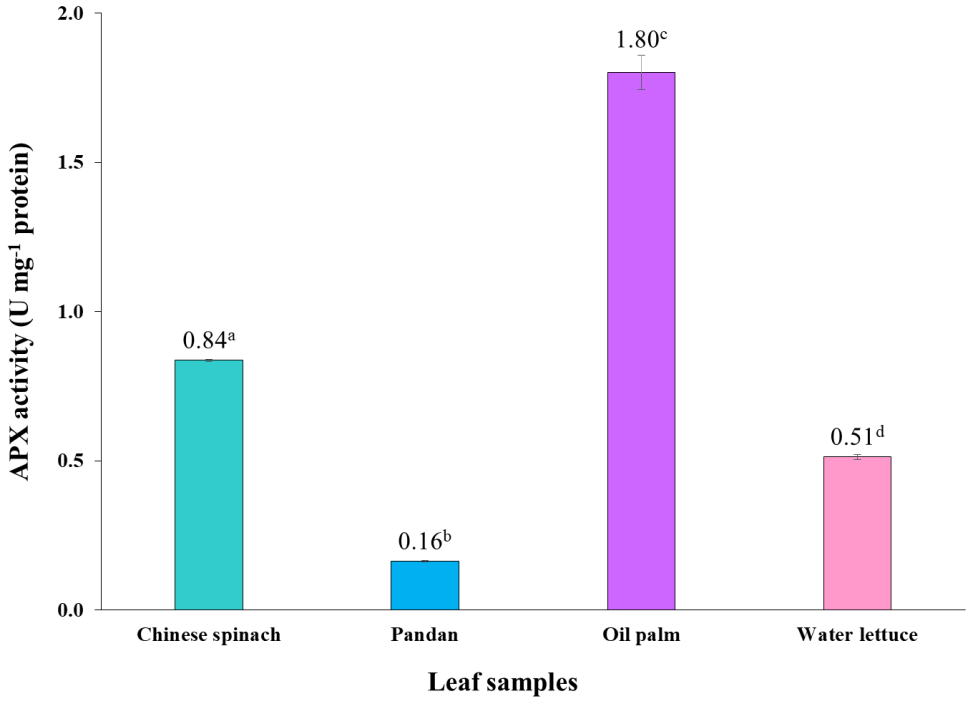

Figure 4.23. Ascorbate peroxidase (APX) activity of 4 crude extract of leaf samples. Values represent mean ± SD (n=3).

4.10.2 Catalase Activity

Figure 4.24 shows the catalase (CAT) activity of 4 leaf crude enzymes. All four plant samples showed similar magnitude of CAT activity and water lettuce had the highest CAT activity. The CAT activity obtained was ranged from 0.1 – 0.2 U mg^{-1} protein.

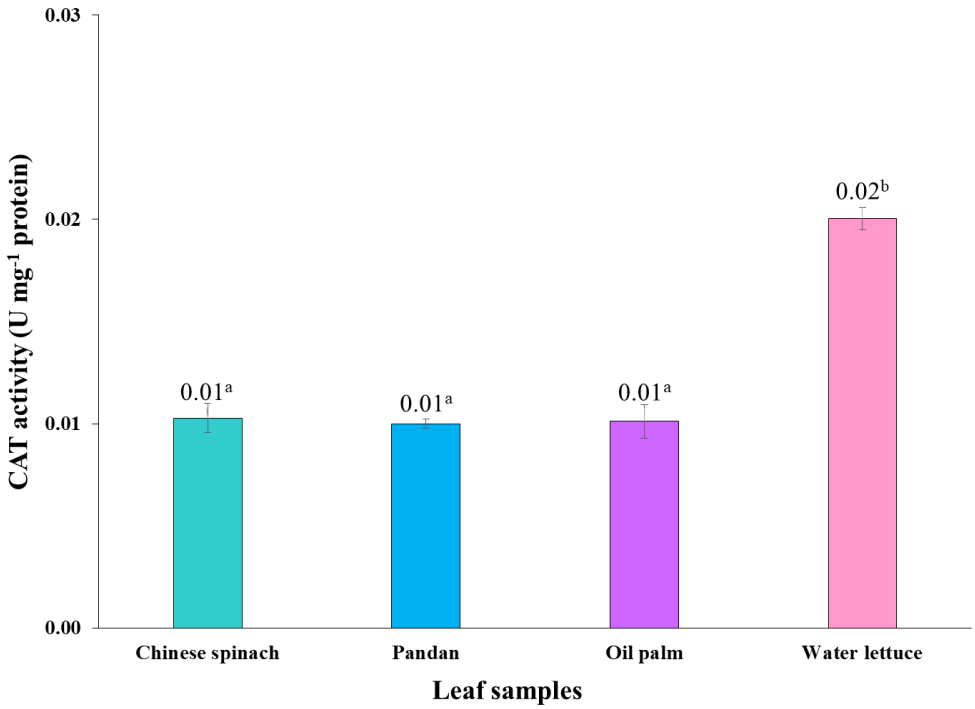

Figure 4.24. Catalase (CAT) activity of 4 leaf samples. Values represent mean ± SD (n=3).

4.10.3 Peroxidase Activity

Figure 4.25 shows the peroxidase (POX) activity of 4 leaf crude enzymes. Oil palm had the highest POX activity followed by water lettuce and Chinese spinach. Pandan had the lowest POX activity, which is in the range of 0.68 – 4.87 U mg^{-1} protein.The POX activity of oil palm was 7-fold higher compared with that of pandan.

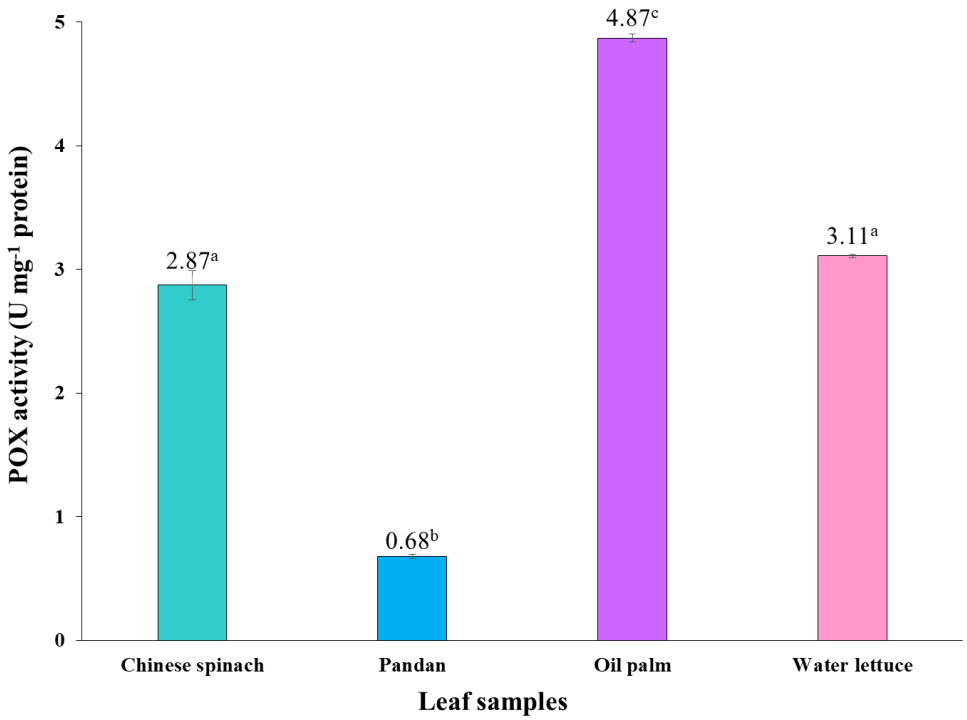

Figure 4.25. Peroxidase (POX) activity of 4 crude extract of leaf samples. Values represent mean ± SD (n=3).

4.10.4 Guaiacol Peroxidase Activity

Figure 4.26 shows the guaiacol peroxidase (GPX) activity of 4 leaf crude enzymes. Water lettuce had the highest GPX activity, whereas pandan had the lowest GPX activity. The GPX activity obtained was in the range of 0.06 – 0.25 U mg^{-1} protein. GPX activity of water lettuce was 4-fold higher than that of pandan.

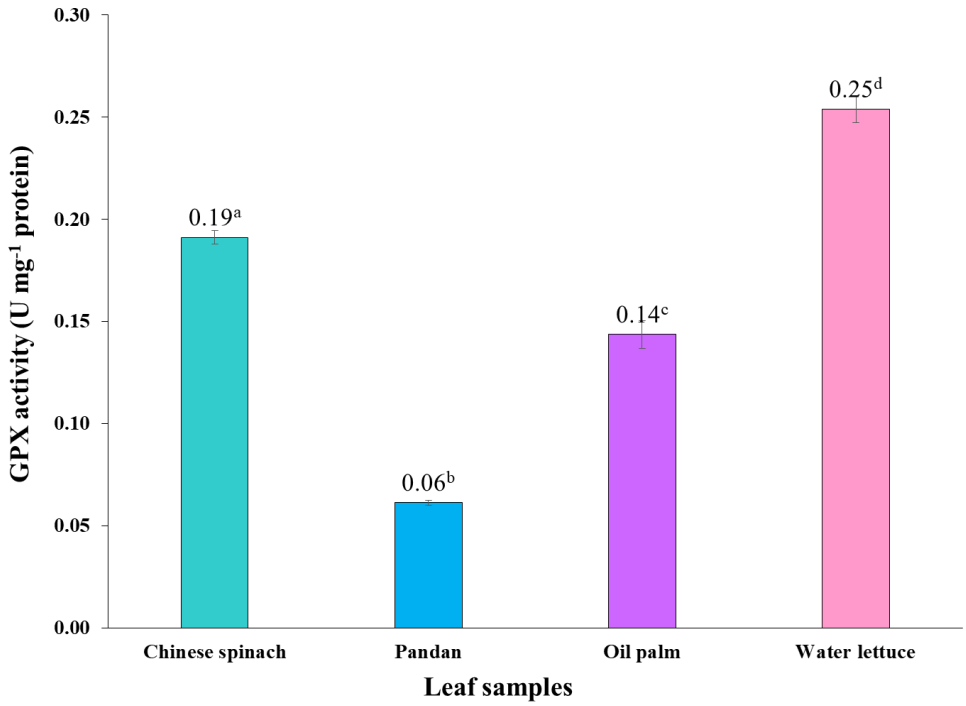

Figure 4.26. Guaiacol peroxidase (GPX) activity of 4 crude extract of leaf samples. Values represent mean ± SD (n=3).

4.10.5 Pyrogallol Oxidase Activity

Figure 4.27 shows the pyrogallol oxidase (PO) activity of 4 leaf crude enzymes. Water lettuce had the highest PO activity whereas pandan had the lowest PO activity. The PO activity obtained was in the range of 0.93 – 3.74 U mg^{-1} protein. The PO activity of water lettuce was around 4-fold higher compared with that of pandan.

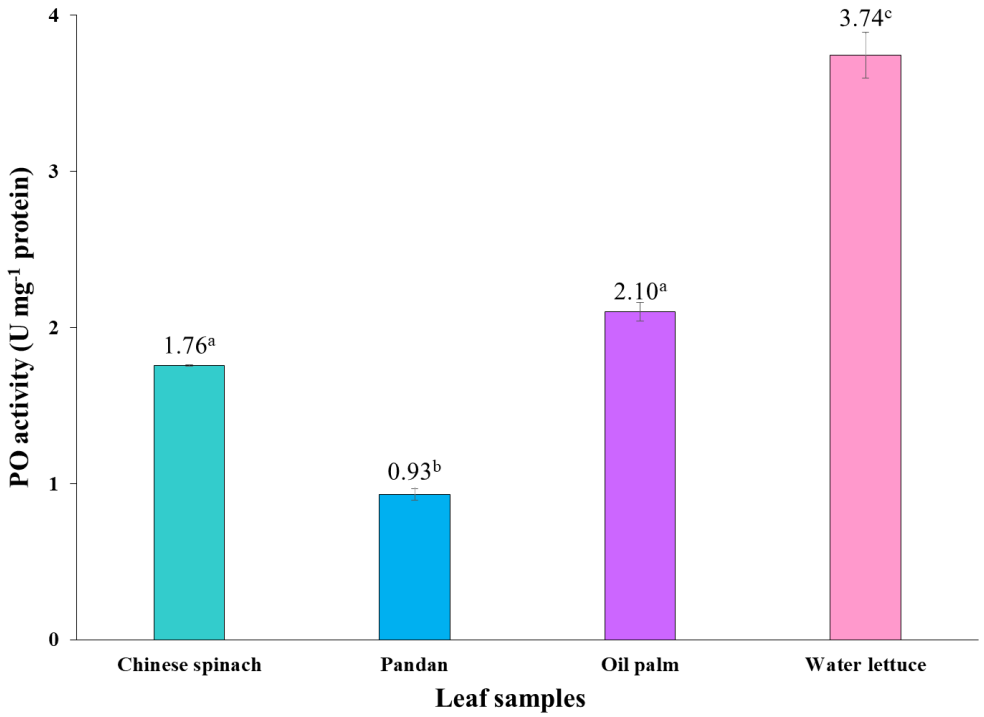

Figure 4.27. Pyrogallol oxidase (PO) activity of 4 crude extract of leaf samples. Values represent mean ± SD (n=3).

4.10.6 Catechol Oxidase Activity

Figure 4.28 showed the catechol oxidase (CO) activity of 4 leaf crude enzymes. Based on our results, CO activity was only detected in oil palm and Chinese spinach. Both oil palm and Chinese spinach showed similar level of CO activity, which is in the range of 0.037 – 0.040 U mg^{-1} protein.

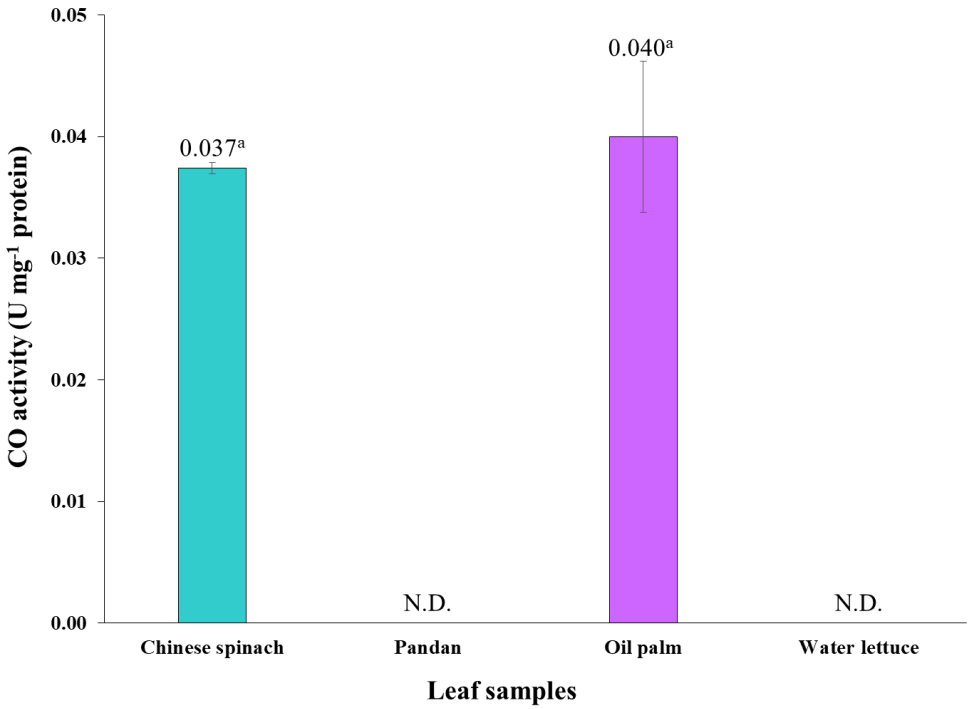

Figure 4.28. Catechol oxidase (CO) activity of 4 crude extract of leaf samples. Values represent mean ± SD (n=3).

4.10.7 Superoxide Dismutase Activity

Figure 4.29 showed the superoxide dismutase (SOD) activity of 4 leaf crude enzymes. Based on our results, SOD activity was only detected in pandan and oil palm. Pandan showed the highest SOD activity followed by oil palm, in the range of 2.79 – 6.19 U mg^{-1} protein.

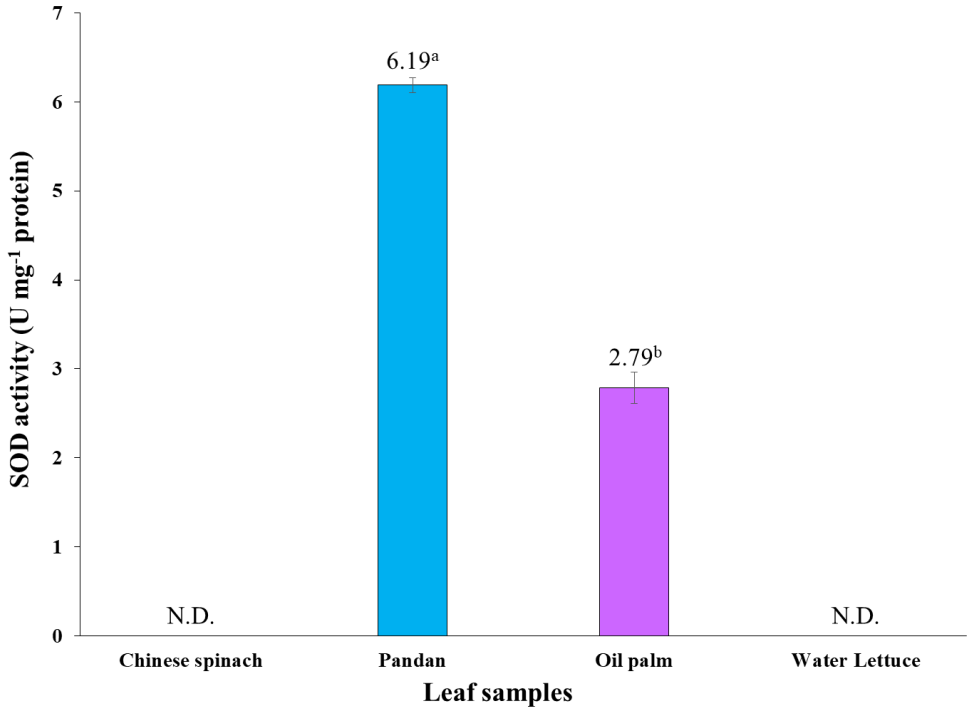

Figure 4.29. Superoxide dismutase (SOD) activity of 4 crude extract of leaf samples. Values represent mean ± SD (n=3).

4.11 Correlation Analysis

The correlation analysis was carried out to study the relationship between leaf parameters, leaf crude enzyme, isolated chloroplast quality and its storage stability. Figure 4.23 shows the possible factors that could influence the photosynthesis of isolated chloroplasts as well its functionality and stability over the storage periods in our research.

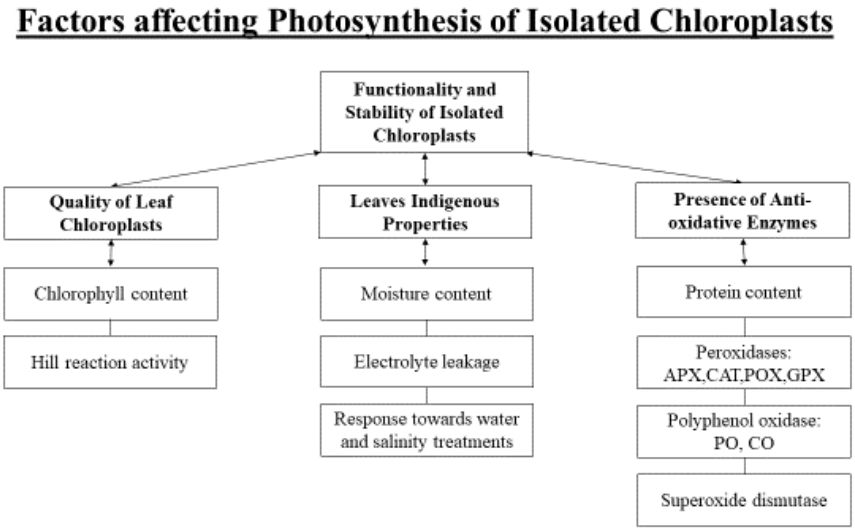

Figure 4.30. Schematic diagram of the factors affecting photosynthesis of isolated chloroplasts in term of functionality and stability.

From the sign of the correlation coefficient, we can determine whether the correlation is positive or negative, while the magnitude of the correlation coefficient allowed us to determine the strength of the correlation. In statistics, the correlation coefficient r is used to determine the strength and direction of a linear relationship of two variables on a scatterplot. The obtained value of r must between +1 and –1.The r values of exactly –1 and +1 show a prefect negative and perfect positive linear relationship. The range of r value in the range of 0.0 to 0.19 (0.0 to -0.19) indicates very weak positive (negative) linear relationship, 0.20 to 0.39 (-0.20 to -0.39) indicates weak positive (negative) linear relationship, 0.4 to 0.59 (-0.40 to -0.59) indicates moderate positive (negative) linear relationship, 0.60 to 0.79 (-0.60 to -0.79) indicates strong positive (negative) linear relationship while 0.80 to 1.0 (-0.8 to -1.0) indicates very strong positive (negative) linear relationship, 0 means no relationship at all between the two variables (Evans, 1996).

4.11.1 Correlation of Electrolyte Leakage and Moisture Content

Table 4.5 shows the correlation between electrolyte leakage after 90 min and moisture content of the leaf samples. Strong negative correlation was obtained between leaf moisture content and electrolyte leakage at the 90th min (r = -0.743, $P < 0.05$).

Table 4.5. Correlation between electrolyte leakage after 90 min (Fig. 4.14) and moisture content (Table 4.2) of 4 leaf samples.

Variable		Pearson Coefficient	Correlation Strength
Electrolyte leakage after 90 min	Moisture content	-0.7427	Strong

4.11.2 Correlation of Electrolyte Leakage and Chlorophyll Content

The increase of relative electrolyte leakage percentage in the leaf tissues induced by stress could lead to the degradation of total chlorophyll as it directly injured the photosynthetic pigments (Cha-Um et al., 2010). Table 4.6 shows the correlation between electrolyte leakage of leaf sample after 90 min and the chlorophyll content (mg g^{-1}) of the chloroplast isolate. A strong negative correlation was obtained between chlorophyll content and electrolyte leakage after 90 min (r = -0.791, $P < 0.05$).

Table 4.6. Correlation between electrolyte leakage of 4 leaf samples after 90 min (Fig. 4.14) and the chlorophyll content of the chloroplast isolate (Table 4.1).

Variable		Pearson Coefficient	Correlation Strength
Electrolyte leakage after 90 min	Chlorophyll content	-0.7911	Strong

4.11.3 Correlation of Chlorophyll Content and Moisture Content

Table 4.7 shows the correlation between moisture content of 4 leaf samples and the chlorophyll content (mg g^{-1}) of the chloroplast isolate. A strong positive correlation was obtained between moisture content and chlorophyll content ($r = 0.627$, $P < 0.05$).

Table 4.7. Correlation between moisture content of 4 leaf sample (Table 4.2) and the chlorophyll content of the chloroplast isolate (Table 4.1).

Variable		Pearson Coefficient	Correlation Strength
Moisture content	Chlorophyll content	0.6267	Strong

4.11.4 Correlation of Initial DCPIP Photoreduction Rate and Chlorophyll Content

Table 4.8 shows the correlation between initial DCPIP photoreduction rate and chlorophyll content of the isolated chloroplast. Initial DCPIP photoreduction rate (µmole DCPIP hr^{-1}) was used instead of initial Hill reaction activity (µmole DCPIP mg^{-1} chl hr^{-1}) in which chlorophyll content is not included in initial DCPIP photoreduction rate in order to obtain the correlation with chlorophyll content. A very strong positive correlation was obtained between initial DCPIP photoreduction rate and chlorophyll content (mg mL^{-1}) ($r = 0.995$, $P < 0.05$).

Table 4.8. Correlation between initial DCPIP photoreduction rate (Appendix E) and chlorophyll content of the chloroplast isolates (Table 4.1).

Variable		Pearson Coefficient	Correlation Strength
Initial DCPIP photoreduction rate	Chlorophyll content	0.9952	Very strong

4.11.5 Correlation of Initial Hill Reaction Activity and Moisture Content

Table 4.9 shows the correlation between initial Hill reaction activity and moisture content of 4 leaf samples. A very strong negative correlation was obtained between initial Hill reaction activity of isolated chloroplast (µmole DCPIP mg^{-1} chl hr^{-1}) on day 0 and moisture content of 4 leaf samples (r = -0.833, $P < 0.05$).

Table 4.9. Correlation between initial Hill reaction activity of the chloroplast isolate (Fig. 4.5) and moisture content of 4 leaf samples (Table 4.2).

Variable		Pearson Coefficient	Correlation Strength
Initial HRA	Moisture content	-0.8332	Very strong

4.11.6 Correlation of Initial Hill Reaction Activity and Protein Content

Table 4.10 shows the correlation between initial Hill reaction activity of the chloroplast isolate and protein content of the crude enzyme extract. A very strong positive correlation was obtained between initial Hill Reaction activity of isolated chloroplast (µmole DCPIP mg^{-1} chl hr^{-1}) on day 0 and protein content of extracted crude enzyme (mg g^{-1}) (r = 0.840, $P < 0.05$).

Table 4.10. Correlation between initial Hill reaction activity of the chloroplast isolate (Fig. 4.5) and protein content of the crude enzyme extract (Table 4.4).

Variable		Pearson Coefficient	Correlation Strength
Initial HRA	Protein content	0.8397	Very strong

4.11.7 Correlation of Color Parameters of Leaves and Moisture Content

Table 4.11 shows the correlations between various color parameters and moisture content of 4 leaf samples. These correlations allowed us to evaluate how the initial moisture content of leaf samples could affect the response of plant leaf disk towards water and salinity treatment.

All leaf disks show very strong positive correlation with both water (r = 0.837, $P < 0.05$) and salinity treatment (r = 0.823, $P < 0.05$) for the correlation between lightness and moisture content. For the correlation between a* value difference and moisture, strong positive correlations were observed in both water treatment (r = 0.733, $P < 0.05$) and salinity treatment (r = 0.717, $P < 0.05$). A strong positive correlation were shown in the water treatment (r = 0.752, $P <$ 0.05) as well as salinity treatment (r = 0.785, $P < 0.05$) in the correlation study between b* value difference and moisture content.

Similarly, strong and positive correlations were observed in both water (r = 0.787, $P < 0.05$) and saline treatment (r = 0.707, $P < 0.05$) between total color changes and moisture content. For the correlations between chroma difference and moisture content, a strong yet positive correlation was observed in water treatment (r = 0.7907, $P < 0.05$), whereas, a very strong positive correlation in saline treatment (r = 0.930, $P < 0.05$). Lastly, there is also a strong positive correlation was obtained in water treatment (r = 0.774, $P < 0.05$) whereas a very strong positive correlation in saline treatment (r = 0.881, $P <$ 0.05) in the correlation study between metric hue difference and moisture content.

Table 4.11. Correlations between color parameters of leaf disks (Table 4.3) and moisture content (Table 4.2) of 4 leaf samples.

Variable		Pearson Coefficient	Correlation Strength
Lightness difference in water treatment	Moisture content	0.8366	Very strong

Lightness difference in saline treatment	Moisture content	0.8232	Very strong
a* value difference in water treatment	Moisture content	0.7329	Strong
a* value difference in saline treatment	Moisture content	0.7166	Strong
b* value difference in water treatment	Moisture content	0.7515	Strong
b* value difference in saline treatment	Moisture content	0.7851	Strong
Total color difference in water treatment	Moisture content	0.7871	Strong
Total color difference in saline treatment	Moisture content	0.7071	Strong
Chroma angle difference in water treatment	Moisture content	0.7907	Strong
Chroma angle difference in saline treatment	Moisture content	0.9295	Very strong
Metric hue difference in water treatment	Moisture content	0.7736	Strong
Metric hue difference in saline treatment	Moisture content	0.8810	Very strong

4.11.8 Correlation of Color Parameters of Leaves and Initial Hill Reaction Activity of Isolated Chloroplast

Table 4.12 shows the correlations between color parameters and initial Hill reaction activity of isolated chloroplast (μmole DCPIP mg^{-1} chl hr^{-1}). This correlation enabled us to figure out how the isolated chloroplast quality could influence the response of plant leaf disk when subjected to water and salinity stress treatment. Strong and very strong negative correlations were obtained between all the color parameters and moisture content.

Very strong and negative correlations were obtained in water (r = -0.852, $P < 0.05$) and salinity treatment (r = -0.949, $P < 0.05$) for the correlation between lightness difference and HRA day 0. For the correlation between a* value difference and HRA day 0, a very strong negative correlation observed on in water treatment (r = -0.803, $P < 0.05$), whereas strong negative correlation observed in saline treatment (r = -0.718, $P < 0.05$). A very strong negative correlation were shown in water treatment (r = -0.825, $P < 0.05$), and saline treatment (r = -0.903, $P < 0.05$) in the correlation study between b* value difference and HRA day 0.

Very strong and negative correlations were observed in both water (r = -0.855, $P < 0.05$) and saline treatment (r = -0.906, $P < 0.05$) between HRA on day 0 and total color changes. For the correlation between chroma difference and HRA day 0, a very significant negative correlation was for both water treatment (r = -0.845, $P < 0.05$) and saline treatment (r = -0.810, $P < 0.05$). A very strong negative correlation was obtained in both water (r = -0.845, $P < 0.05$) and saline treatment (r = -0.843, $P < 0.05$) in the correlation study between metric hue difference and initial HRA on day.

Table 4.12. Correlation between color parameters of leaf disks (Table 4.3) and initial Hill reaction activity of isolated chloroplast (Fig. 4.5).

Variable		Pearson Coefficient	Correlation Strength
Lightness difference in water treatment	Initial HRA	-0.8522	Very strong
Lightness difference in saline treatment	Initial HRA	-0.9485	Very strong

a* value difference in water treatment	Initial HRA	-0.8028	Very strong
a* value difference in saline treatment	Initial HRA	-0.7179	Strong
b* value difference in water treatment	Initial HRA	-0.8249	Very strong
b* value difference in saline treatment	Initial HRA	-0.9034	Very strong
Total color difference in water treatment	Initial HRA	-0.8554	Very strong
Total color difference in saline treatment	Initial HRA	-0.9059	Very strong
Chroma angle difference in water treatment	Initial HRA	-0.8446	Very strong
Chroma angle difference in saline treatment	Initial HRA	-0.8096	Very strong
Metric hue difference in water treatment	Initial HRA	-0.8450	Very strong
Metric hue difference in saline treatment	Initial HRA	-0.8429	Very strong

4.11.9 Correlation of Color Parameters of Leaves and Protein Content of Crude Enzyme Extract

Table 4.13 shows the correlations between color parameters and protein content of crude enzyme extract. Negative correlation was obtained in all the correlation between color parameters and moisture content. Based on correlation analysis between lightness difference and protein content, a strong and negative correlation were obtained for water treatment (r = -0.750, P < 0.05). Whereas a very significant negative correlation was shown in saline treatment (r = -0.871, P < 0.05). For the correlation between a* value difference and protein content, a strong negative correlation observed in water treatment (r = -

0.764, $P < 0.05$), as well as in saline treatment ($r = -0.624$, $P < 0.05$). A strong and negative correlation were shown in both water ($r = -0.788$, $P < 0.05$) and saline treatment ($r = -0.771$, $P < 0.05$) for the correlation study between b* value difference and protein content.

A strong and negative correlation was obtained between total color changes of leaf disks in the water treatment in salinity test and protein content of extracted crude enzyme ($r = -0.793$, $P < 0.05$), also, a very significant and negative correlation was obtained between the total color change of leaf disks in saline treatment and protein content($r = -0.905$, $P < 0.05$). For the correlation between chroma difference and protein content, a strong negative correlation was observed in water treatment ($r = -0.780$, $P < 0.05$) while only a moderate negative correlation in saline treatment ($r = -0.446$, $P < 0.05$). Strong negative correlations were obtained in both water ($r = -0.789$, $P < 0.05$) and saline treatment ($r = -0.694$, $P < 0.05$) in the correlation study between metric hue difference and protein content.

Table 4.13. Correlation between color parameters of leaf disks (Table 4.3) and protein content of crude enzyme extract (Table 4.4).

Variable		Pearson Coefficient	Correlation Strength
Lightness difference in water treatment	Protein content	-0.7500	Strong
Lightness difference in saline treatment	Protein content	-0.8709	Very strong
a* value difference in water treatment	Protein content	-0.7642	Strong
a* value difference in saline treatment	Protein content	-0.6236	Strong

b* value difference in water treatment	Protein content	-0.7876	Strong
b* value difference in saline treatment	Protein content	-0.7709	Strong
Total color difference in water treatment	Protein content	-0.7934	Strong
Total color difference in saline treatment	Protein content	-0.9051	Very strong
Chroma angle difference in water treatment	Protein content	-0.7795	Strong
Chroma angle difference in saline treatment	Protein content	-0.4459	Moderate
Metric hue difference in water treatment	Protein content	-0.7888	Strong
Metric hue difference in saline treatment	Protein content	-0.6938	Strong

4.11.10 Correlation among Leaves Crude Enzyme Activity

Table 4.14 shows the correlations among anti-oxidative enzymes activity of crude enzyme extract. Negative correlation was obtained in all the correlation between color parameters and moisture content. Very strong positive correlation were obtained between APX and POX activity ($r = 0.913$, $P < 0.05$), APX and CO activity ($r = 0.801$, $P < 0.05$), CAT and PO activity ($r = 0.886$, $P < 0.05$) and between GPX and PO activity ($r = 0.888$, $P < 0.05$). There are strong positive correlations were obtained between CAT and GPX ($r = 0.762$, $P < 0.05$) and between POX and CO activity ($r = 0.671$, $P < 0.05$).

There is a very strong negative correlation obtained between GPX and SOD activity (r = 0.945, P < 0.05), whereas strong negative correlation was obtained between PO and SOD activity (r = 0.717, P < 0.05),

Table 4.14. Correlation among leaves crude enzyme activity (Figs. 4.23 - Fig 4.29).

Variable		Pearson Coefficient	Correlation Strength
APX activity	CAT activity	-0.2868	Weak
APX activity	POX activity	0.9128	Very strong
APX activity	GPX activity	0.1156	Very weak
APX activity	PO activity	0.1065	Very weak
APX activity	CO activity	0.8011	Very strong
APX activity	SOD activity	-0.2243	Weak
CAT activity	POX activity	0.0928	Very weak
CAT activity	GPX activity	0.7621	Strong
CAT activity	PO activity	0.8855	Very strong
CAT activity	CO activity	-0.5636	Moderate
CAT activity	SOD activity	-0.5225	Moderate
POX activity	GPX activity	0.4867	Moderate
POX activity	PO activity	0.4817	Moderate
POX activity	CO activity	0.6705	Strong
POX activity	SOD activity	-0.5320	Moderate
GPX activity	PO activity	0.8876	Very strong
GPX activity	CO activity	0.0467	Very weak
GPX activity	SOD activity	-0.9451	Very strong
PO activity	CO activity	-0.1872	Very weak
PO activity	SOD activity	-0.7166	Strong
CO activity	SOD activity	-0.2992	Weak

4.11.11 Correlation of Hill Reaction Activity Degradation and Chlorophyll Content of the Isolated Chloroplast

Table 4.15 shows the correlations between Hill reaction activity degradation rate on day 90 of chilled and frozen chloroplast and chlorophyll content of the chloroplast isolate. Strong positive correlations obtained between chlorophyll content (mg g^{-1}) and degradation of chilled and frozen-stored chloroplast on 90 days (r = 0.614 and r = 0.607, P < 0.05).

Table 4.15. Correlation between Hill reaction activity degradation on day 90 of chilled and frozen chloroplast (Figs. 4.10- 4.13) and chlorophyll content of the chloroplast isolate (Table 4.1).

Variable		Pearson Coefficient	Correlation Strength
HRA degradation of chilled chloroplast	Chlorophyll content	0.6138	Strong
HRA degradation of frozen chloroplast	Chlorophyll content	0.6070	Strong

4.11.12 Correlation of Initial Hill Reaction Activity of Isolated Chloroplast and Leaves Crude Enzyme Activity

Table 4.16 shows the correlations between HRA of the chloroplasts isolate (µmole (DCPIP) mg^{-1} (chl) hr^{-1}) and specific enzyme activities of the crude enzyme extracts. Strong positive correlations were observed on initial HRA with APX activity (r = 0.706, $P < 0.05$) and CO activity (r = 0.680, $P < 0.05$). A very strong negative correlation was obtained on initial HRA with CAT activity (r = -0.804, $P < 0.05$), while a strong negative correlation with PO activity (r= -0.603, $P < 0.05$).

Table 4.16. Correlation between initial Hill reaction activity of the chloroplasts isolate (Fig. 4.5) and specific enzyme activities of the crude enzyme extracts (Figs. 4.23 – Fig 4.29).

Variable		Pearson Coefficient	Correlation Strength
Initial HRA	APX activity	0.7062	Strong
Initial HRA	CAT activity	-0.8036	Very strong
Initial HRA	POX activity	0.3791	Weak
Initial HRA	GPX activity	-0.5567	Moderate
Initial HRA	PO activity	-0.6027	Strong
Initial HRA	CO activity	0.6802	Strong
Initial HRA	SOD activity	0.3797	Weak

4.11.13 Correlation of Hill Reaction Activity Degradation of Isolated Chloroplasts and Leaves Crude Enzyme Activity

Table 4.17 shows the correlations between Hill reaction activity degradation on day 90 of chilled and frozen chloroplast and specific enzyme activities of 4 plant samples. This correlation was carried out in order to determine whether the degradation rate of HRA of isolated chloroplast could relate to leaves crude enzyme activity. Strong negative correlation was obtained between catalase activity and degradation percentage of chilled-stored chloroplast in term of Hill Reaction activity on 90-day storage (r = -0.729, P < 0.05), whereas very strong negative correlation obtained on 90-day of frozen-stored chloroplast (r = -0.899, P < 0.05).

GPX activity showed a strong negative correlation with degradation percentage of chilled-stored chloroplast on day 90 (r = -0.641, P < 0.05) and as well as frozen-stored chloroplast on day 90 (r = -0.686, P < 0.05). PO activity showed a very strong negative correlation with chilled-stored chloroplast on day 90 (r = -0.881, P < 0.05) as well as frozen-stored chloroplast on both day 60 and day 90 (r = -0.906, P < 0.05).

Table 4.19. Correlation between Hill reaction activity degradation on day 90 of chilled and frozen chloroplast (Fig 4.10 – Fig 4.13) and specific enzyme activities of 4 plant samples (Figs. 4.23 – Fig 4.29).

Variable		Pearson Coefficient	Correlation Strength
HRA degradation of chilled chloroplast	APX activity	-0.1854	Very weak
HRA degradation of frozen chloroplast	APX activity	-0.0174	Very weak

HRA degradation of chilled chloroplast	CAT activity	-0.7286	Strong
HRA degradation of frozen chloroplast	CAT activity	-0.8989	Very strong
HRA degradation of chilled chloroplast	POX activity	-0.4945	Moderate
HRA degradation of frozen chloroplast	POX activity	-0.3597	Weak
HRA degradation of chilled chloroplast	GPX activity	-0.6414	Strong
HRA degradation of frozen chloroplast	GPX activity	-0.6857	Strong
HRA degradation of chilled chloroplast	PO activity	-0.8813	Very strong
HRA degradation of frozen chloroplast	PO activity	-0.9061	Very strong
HRA degradation of chilled chloroplast	CO activity	0.1537	Very weak
HRA degradation of frozen chloroplast	CO activity	0.3777	Weak
HRA degradation of chilled chloroplast	SOD activity	0.4458	Moderate
HRA degradation of frozen chloroplast	SOD activity	0.4289	Moderate

CHAPTER 5

DISCUSSION

5.1 Screening on Isolated Chloroplast in Selected Plant Leaves

5.1.1 Chlorophyll Content of Isolated Chloroplast

Among the 4 plant samples, Chinese spinach had the highest chlorophyll content while oil palm had the lowest chlorophyll content (Table 4.1). Previous study reported on chlorophyll content of pandan was around 1-1.2 mg/ g FW (Han et al., 2014), which is comparable to our result (1.07 mg/ g dry basis). Shu et al. (2009) reported the chlorophyll content of Chinese spinach of 2.07 mg g-1 FW while Wang and Nii (2000) found that Chinese spinach contained approximately 2.2 mg g^{-1} dry mass of chlorophyll. We found a higher chlorophyll content of chloroplast isolated in this study (2.4 mg g^{-1}dry basis). The chlorophyll content of oil palm frond obtained was 0.12 mg g^{-1}dry basis, and it agreed with those reported by other studies, which are 0.289 mg g^{-1} FW (Mahmood et al., 2013) and 0.391 mg g^{-1} FW (Chaum et al., 2012). Chlorophyll content of 0.55 mg g^{-1}dry basis from water lettuce was found in this study, which was comparable to the 0.28 mg g^{-1} FW and 1.09 mg g^{-1} FW as documented by other researchers (Mahesh et al., 2014; Singh and Pandey, 2011).

5.1.2 Microscopic Examination of Isolated Chloroplast

The microscopic image of isolated chloroplast of four plant samples showed the variations in terms of number and size of the isolated chloroplast

(Figs. 4.1 - 4.4). Isolated chloroplast of Chinese spinach and pandan were tiny in size and present in great number, in which isolated chloroplast of Chinese spinach distributed in a compact manner, while loosely arranged for pandan. The isolated chloroplast of water lettuce was slightly bigger than both Chinese spinach and pandan, however, lower in density. Oil palm possessed the biggest size of chloroplast among all and fewer in number. In a study conducted by Jones et al. (1993) found that low level of chlorophyll, reduced chloroplast numbers and thylakoids per granum contributed to reduced rate of photosynthesis. According to Kariya and Tsunoda, chlorophyll content per unit leaf-area was strongly affected by change in chloroplast number, and the correlation between the rate and the chloroplast number was more distinct than that between the rate and the chlorophyll content. Thus, this suggested an increase in the chlorophyll content does not guarantee an increase in photosynthetic rate, unless the high content is associated with an increase in chloroplast number (Kariya and Tsunoda, 1972). In this case, both Chinese spinach and pandan displayed greater number of chloroplast, which explained the higher chlorophyll content isolated, thus, the high photosynthetic rate of Chinese spinach and pandan (Figure 4.5) were contributed by their high chloroplast number as well as high chlorophyll content, which will be discussed in the following section (Section 5.2).

5.1.3 Correlation of Moisture Content and Chlorophyll Content

There was only a strong positive correlation ($r = 0.627$, $P < 0.05$) obtained between moisture content of leaf samples and chlorophyll content of isolated chloroplast (Table 4.7). Surendar et al. (2013) found that relative water

content, soluble protein and total chlorophyll content of ratoon banana decreased under water deficit condition, thus, it can be used as a drought tolerance index to the selection of tolerant genotype under water deficit conditions. Aref et al. (2014) found that water stress caused reduction not only on the relative water content but also the chlorophyll content of *Juniperus procera Hochst. ex Endlicher*. This finding also supported by Neto et al. (2017) in which water index and chlorophyll content index of sunflower plant were reduced when the plant encountered water stress in their study. Therefore, chlorophyll content and moisture content of plants are strongly linked and reduction in moisture content is accompanied by the reduction of chlorophyll content when plants encountered stress. The presence of positive correlation between two parameters also indicates the decrease of chlorophyll content followed with the decrease of moisture content in plant and vice versa.

5.1.4 Correlation of Initial DCPIP Photoreduction Rate and Chlorophyll Content

Correlation relationship was observed between the initial DCPIP photoreduction rate and chlorophyll content (Table 4.8). The high chlorophyll content was observed on pandan and Chinese spinach samples, which agreed with previous study that the high chlorophyll content contributed to the high photosynthetic rate. A very strong positive correlation was obtained between initial photoreduction rate of DCPIP (μmol DCPIP/ hr) and chlorophyll content ($r = 0.995$, $P < 0.05$), which indicated the higher the chlorophyll content isolated from plant sample, the higher the photosynthetic rate will be observed. Significant and positive correlation between total chlorophyll content and photosynthesis were observed in other studies (Lobato et al., 2010), in which

slightly decrease in chlorophyll content caused a drastic reduction in photosynthetic rate (Nagaraj et al., 2002). The loss of photosynthesis is mainly due to the loss of a functional unit of photosynthesis or as a result of decrease of the number of whole chloroplasts (Kura-Hotta et al., 1987).

5.1.5 Correlation of Initial Hill Reaction Activity and Moisture Content

A very strong negative correlation (r = -0.833, $P < 0.05$) was obtained between moisture content of leaf samples and initial HRA of isolated chloroplast on day 0 (Table 4.9). Dastur (1935) noticed three important facts of the relations between water content and photosynthesis. He noticed that the decrease in assimilation of leaves is always followed by a decrease in water content, however, the extent of decrease varies with different plants. Besides, leaves with low water content tend to fall greater in assimilation is, with a small decrease of the water content. Lastly, the fact that leaves with a low water content were found to assimilate more rapidly if compared to those of leaves with a high water content compliance with the negative correlation obtained in our study. This might indicates that plant leaves with lower water content tend to have higher photosynthetic ability as compared to those with higher moisture content. On the other hand, Stålfelt (1937) also found that the photosynthesis curve for *Hylocomium splendens* was declining at high water content. This may be due to the excess of water lead to much slower diffusion of gases through water than diffusion through air, thus, restricting the exchange of gas at the low concentration gradients available for CO_2 uptake (Dilks and Proctor, 1979). The slower rate of CO_2 uptake lead to reduced CO_2 assimilation, which contributed to lower electron transfer during the Hill reaction.

5.1.6 Influence of Chloroplasts' Size and Number on Photosynthetic Ability

Chloroplast size and the chloroplast number per cell are regulated by both genetic and environmental factors, in which high CO_2 concentration (Bockers et al., 1997; Kutík et al., 1995; Teng et al., 2006) increases while high temperature (Jin et al., 2011; Xu et al., 2012) decreases the size of chloroplast and number. Mesophyll cells in higher plants are constituted with numerous small chloroplasts instead of one or fewer larger chloroplasts due the ability of small chloroplasts to rapidly change their positions or directions dependent on the changes in irradiance, which fully utilize the limiting irradiance or to minimize the photodamage when experiencing excess of light (Jeong, 2002). Besides, large population of small chloroplasts in mesophyll cells is able to enhance the CO_2 diffusion conductance and increase the photosynthetic nitrogen use efficiency (PNUE). The conclusion obtained was the increase of chloroplast number and decrease of chloroplast size can improve nitrogen use efficiency in plants (Xiong et al., 2017). The increase of leaf nitrogen content per leaf area were reported to cause a decrease of PNUE, lower Rubisco activation status and insufficient CO_2 supplementation, in which chloroplast size is significantly increased by high nitrogen supplementation. Large chloroplasts with smaller surface area to volume ratio eventually reduce the exposure to intercellular airspace and thus lead to lower rate of CO_2 diffusion into the chloroplast (Xiong et al., 2015). PNUE and activity of Rubisco were found negatively related to chloroplast size (Li et al., 2013). Both Chinese spinach and pandan possessed greater chloroplast number and smaller chloroplast size might contributed to better nitrogen use efficiency and thus high photosynthetic rate (Figs. 4.1, 4.2 and 4.5) . The large chloroplast size along

with great chloroplast number of oil palm also contributed to the high photosynthetic rate whereas the slightly bigger chloroplast size of water lettuce with low chloroplast number lead to lower photosynthetic rate (Figs. 4.3, 4.4 and 4.5).

5.2 Assessment on The Stability of Photochemical Activity of The Isolated and Stored Chloroplast from Selected Plant Leaves

5.2.1 Hill Reaction Activity and its Storage Stability

Oil palm showed the highest HRA followed by Chinese spinach, pandan and water lettuce (Fig. 4.5). Chinese spinach chloroplast showed the highest degradation rate in frozen storage while pandan chloroplast showed the highest degradation rate in chilled storage, whereas, water lettuce chloroplast showed the lowest degradation rate in both storage conditions (Figs. 4.10 – 4.13). Pandan showed the highest differences between chilled and frozen chloroplast, which is 35.1%, followed by Chinese spinach of 31.4%, oil palm of 15.7% and water lettuce of 7.7% at the end of 90-day storage (Figs. 4.10 – 4.13). Water lettuce was considered as the most stable among all plant samples as only small differences existed between two conditions in term of stability (Fig. 4.13). The high differences between two storage conditions of pandan indicated specific condition is needed for the storage of its chloroplast (Fig. 4.11). This also indicating storage of pandan chloroplast under frozen condition greatly preserved its electron transferring capacity and sustained its stability over time as compared with chilling condition.

5.2.2 Correlation of Hill Reaction Activity Degradation Rate and Chlorophyll Content of Isolated Chloroplasts

Fleischer (1935) made a conclusion in his study that the rate of photosynthesis of plants is proportional to the chlorophyll content by varying the iron supply (Fleischer, 1935). In a study conducted by Buttery and Buzzell (1977) stated that there is a direct relationship between quantity of chlorophyll of plant leaves and its rate of CO_2 assimilation, in which photosynthesis rate of soybean is positive correlated with chlorophyll content (Buttery and Buzzell, 1977). Kura-Hotta et al (1987) had this interpretation in their research study in which loss of photosynthesis during senescence mainly due to a decrease in the number of functional unit of the entire photosynthetic process, or in the number of whole chloroplasts (Kura-Hotta et al., 1987). These observations suggested that chlorophyll content of leaf is closely related to the degradation degree of its photosynthesis rate (Hill Reaction activity) during senescence or damage experienced. Injury occurred to the leaf chloroplast leads to the reduction of photosynthesis rate. We postulate that, plants with originally higher chlorophyll content with high photosynthesis rate probably experience larger extent of photosynthesis degradation when subjected to damage or prolonged storage. This explained the positive correlation between degradation of Hill Reaction activity in both chilled ($r = 0.614$, $P < 0.05$) and frozen condition ($r = 0.607$, $P < 0.05$) with chlorophyll content (Table 4.15).

5.3 Response of Indigenous Plant Leaf towards Water and Salinity Stress

5.3.1 Electrolyte Leakage

During the study of electrolyte leakage study, leaf samples were subjected to deionized water for 90 min in order to study the leaking rate of electrolyte from the leaf disks. The deionized water is considered as a hypotonic solution and osmosis occurred where water moved from a solution of lower solute concentration to one of higher solute concentration. The rigid cell wall that surrounded the plant cells supposes to prevent the plant cell from swelling and maintain the turgor pressure in response to the osmotic influx of water (Lodish et al., 2000). However, the disruption of cell wall due to the extraction of leaf disk from the whole leaf might contribute to the leakage of electrolyte when the water flows in from the salvation layer. The presence of Na+/K+ pump on the plasma membrane as well as vacuolar Na+/H+ antiporter help regulate osmotic level through ion homeostasis (Zepeda-Jazo et al., 2008; Lodish et al., 2000; Blumwald et al., 2000). Under hypotonic condition, movement of ions out of cells is essential to draw the water out the cell through osmotic potential gradient. The detected electrolyte leakage might be due to the ions eluted of from leaf disks in order to regulate the osmotic pressure caused by hypotonic solution. Although subjected to similar treatment, but different intensity of electrolyte leakage was observed among four samples (Fig. 4.14). Oil palm showed the highest electrolyte leakage after 90 min, this might due to the low moisture content of oil palm caused larger osmotic potential gradient that drawn larger amount of water flowing into the membranes, thus higher electrolyte leaked from leaf disks due to the disruption of cell wall as well as the ion homeostatic regulation during the hypotonic condition.

5.3.2 Correlation of Electrolyte Leakage and Moisture Content

Bolat *et al.* (2014) concluded that both relative water content of leaf and chlorophyll index were found decreased as the electrolyte leakage increased when there is an increase of water stress in both rootstocks in their research study. This suggests that higher moisture content of leaf contributed to lower electrolyte leakage and vice versa. Research conducted by Masoumi *et al.* (2010) indicates that relative water content of leaves decreased while the electrolyte leakage increased upon exposure to stress treatment as compared with a control study. This suggests that the initial leaf water status is highly related to the stress level and environmental conditions of their habitat, thus, contributed to the leakage of electrolyte. This explained the strong and negative correlation (r = -0.743, $P < 0.05$) between electrolyte leakage after 90 min and moisture content of 4 leaf samples (Fig. 4.5).

5.3.3 Correlation of Electrolyte Leakage and Chlorophyll Content

Besides, the leakage of electrolyte is generally proportional to the stress damage (Valentovic et al., 2006; Simova-Stoilova et al., 2008), thus the ability of plant cell to control ion movement rate across the membrane can be used as a common biochemical marker to test the damage in plants under stress (Bandeoğlu et al., 2004; Tian et al., 2012). The lower electrolyte leakage indicated higher cell membrane stability. We found only strong negative correlation (r = -0.791, $P < 0.05$) between chlorophyll content and electrolyte leakage after 90 min of 4 leaf samples (Table 4.6). Therefore higher chlorophyll content contributed to lower electrolyte leakage, or, the increase of electrolyte leakage upon stress exposure may lead to the decrease of chlorophyll contents

(Tatari et al., 2012). Cha-Um et al. (2010) proved that the increase of relative electrolyte leakage percentage in leaf tissues induced by stress could lead to the degradation of total chlorophyll as it directly injured the photosynthetic pigments (Cha-Um et al., 2010). This reflects that electrolyte leaked from injured photosynthetic pigment contributed to the increases of electrolyte leakage and at the same time caused the decrease of chlorophyll content. The ability of plant cell to control ion movement rate across the membrane can be used as biochemical marker to test the damage in plants when encounters stress (Bandeoğlu et al., 2004; Tian et al., 2012). The negative correlation of electrolyte leakage of plant leaves and chlorophyll content of chloroplast isolate in this case can be used as a biomarker to indicate the chlorophyll content of plant leaves by determining the cellular electrolyte leakage from affected plant tissues.

5.3.4 Leaf Disks Morphology of Salt Tolerance Leaf Senescence Test

After 96 h of treatment, oil palm turned slightly greener with no other obvious changes on the leaf disks of both water and saline treatments (Fig. 4.17). The leaf structure is highly resistant to possible destruction due to osmosis or plasmolysis. The oil palm frond composed of high proportion of lignified tissue, along with the cells of the epidermis that have a thick cuticle and lie on top of the hypodermis was more highly developed on the adaxial surface. They are semi-xermorphic, in which its structure is important to prevent severe dehydration over long periods of drought (Corley and Tinker, 2003).

Pandan leaf disks on both water and saline treatments showed yellow coloration, brighter and appearance of light injury ring after 96 h, in which saline-treated leaf disks showed more intense in turning yellow and injury ring as compared to the water-treated leaf disks (Fig. 4.16). The leaf of pandan is dorsiventral and hairs are absent. The leaf is formed by well-developed surface layers, in which each of them is made up of a shallow, strongly cutinized but not markedly thick-walled epidermis and often slightly thick-walled hypodermal layers, usually three or four cells deep. Their adaxial hypodermal layers are usually thicker than abaxial (Tomlinson, 1965). It is possible that unique leaf anatomy of pandan provided certain degree of protection towards water and salinity treatments during the salt tolerance stress study.

Water-treated water lettuce leaf disks showed only yellow coloration after 96 h, whereas saline-treated leaf disks experience severe plasmolysis in which they turned yellow along with the appearance of injury ring on the edge of leaf disks (Fig. 4.18). The epidermis of leaf of water lettuce is generally uniseriate with thin walled cells and cuticularised. Leaves of water lettuce have a complex three-level hierarchical surface architecture. The epidermis consists of numerous multicellular projections called trichomes, which contain microscale convex cells superimposed with nanoscale wax crystals (Koch and Barthlott, 2009). In addition to water repellency, the trichome-covered leaves retain a stable air layer on their surface, which ensures buoyancy and survival of the plant when it is dragged underwater (Mayser et al., 2014). Both adaxial and abaxial foliar epidermis are perforated by numerous paracytic stomata (Omprakash et al., 2011). The unique feature of trichomes of water lettuce acted

only to maintain the buoyancy of plant, though with single layer of cuticularised cell wall, however, it is unable to protect the leaf disks against salinity treatment.

Water-treated leaf disks of Chinese spinach showed obvious sign of injury and thinning of leaf disks, along with discoloration from green to yellow, whereas saline-treated leaf disks not only showed severe thinning of leaf disks, but also discoloration from green to transparent, which indicated the bleaching of chlorophyll (Fig 4.15). The leaf morphology of Chinese spinach was not widely studied. According to the limited information on Chinese spinach leaf morphology, three layers of chlorenchyma are located on the vascular bundle periphery, in which one layer of mesophyll parenchyma cells along with two layers of bundle sheath cells with chloroplasts (Hong et al., 2005). Reasonable observation on water and salinity treatments on Chinese spinach leaf disks were obtained given that the absence of protective layer such as lignified tissue and cuticle, which made it more vulnerable not only to salinity but also water treatment.

The presence of high proportion of lignified tissue and thick cuticle of epidermis protected the oil palm leaf disks against the salinity treatment, while pandan with its strongly cutinized leaf protected the leaf disk against salinity treatment where the leaf disks turned yellow after 96 h. The leaf morphology of water lettuce such as cuticularised epidermis, presence of trichome and wax crystal helped protect the leaf disk to the extent of turning yellow and slightly injured. The thin layer of Chinese spinach leaf disks, however, injured and turned transparent as chlorophyll was bleaching due to the salinity treatment.

In order to probe further the leaf morphology in response to water and salinity stress, those subjected leaf disks were observed under microscope.

5.3.5 Comparison of Leaf Disk Microscopic Image on Salt Tolerance

The surface of four plants leaf disks in water treatment still able to retain the greenness and moisture after 96 h (Figs. 4.19 – 4.22). Chinese spinach leaf disks showed plasmolysis, in which cell shrinkage occurred, obvious veins and nucleus were observed in saline treatment. There was only minor injury observed on pandan leaf disks in saline treatment. Oil palm leaf disks do not show obvious difference for both water treatment and saline. Formation of pores was observed due to the occurrence of plasmolysis in saline treatment. Plasmolysis occurs as the protoplast shrink and the plasma membrane separates from the wall when the osmotic pressure of solution is higher than the cells (Munns, 2002). The high moisture content in water lettuce and Chinese spinach contributed to higher amount water drained out of cell to the saline due to high osmotic gradient, thus, cell experienced plasmolysis (Table 4.2, Figs. 4.19 and 4.22). Moderate moisture content of pandan contributed to moderate osmotic gradient, in which only minor injury occurred to the leaf disks (Table 4.2, Fig 4.20). Leaf disk of oil palm in saline treatment does not show significant injury or difference as compared with those in water treatment (Fig. 4.21). The 5 mL of 400 mM NaCl in saline treatment does not create large osmotic gradient to oil palm, in which it might considered as isotonic condition to the oil palm leaf disks, thus, no injury or plasmolysis occur after 96 h. Besides, the presence of highly lignified tissue on leaf and thick cuticle on top of the hypodermis possibly played a role in protecting the cell wall from destruction.

5.3.6 Color Parameters Studied in Salt Tolerance Test

The parameters studied in the salt tolerance test on plant leaves included lightness difference (ΔL*), a* value difference (Δa*), b* value difference (Δb*), total color difference (ΔE*ab), chroma difference (ΔC*) and metric hue difference (ΔH*). L*, it represents darkness and lightness, in which the scale 0 as black whereas 100 as white (Konica Minolta, 2003); for a*, negative value indicates greenness whereas positive value indicates reddish (Konica Minolta, 2003); b*, negative value means blue whereas positive indicates yellowish (Konica Minolta, 2003). An increase in the b* values indicates an increase in leaf yellowing (Kasim and Kasim, 2012) ; ΔE*ab, which indicates the size of the color difference but does not indicates in what way the colors are different (Konica Minolta, 2003); chroma (C) measures color saturation or intensity (Konica Minolta, 2003); while hue metric difference is positive only if the metric hue angle (h) of the specimen is found greater than the target while negative if the metric hue angle of the specimen is found less than that of the target (Konica Minolta, 2003).

Chinese spinach showed positive ΔL* in both treatments, in which the increase in the magnitude of ΔL* was much greater in water treatment instead of saline treatment (Table 4.3). For Δa* value, similar trend was observed as with ΔL*. In term of Δb* value, Chinese spinach showed almost similar degree of positive Δb* in both treatment. The positive ΔL* value indicated increased in lightness; positive Δa* value indicated reddish; while positive Δb* value indicated yellowish. The positive difference of these three parameters of Chinese spinach can be interpreted as decreased in greenness as well as

chlorophyll bleaching (Meir *et al*., 1992). Chinese spinach showed moderate positive ΔE*ab as compared with pandan, oil palm and water lettuce. The leaf disks surface of spinach in water and saline treatment showed a same positive ΔC* value. This showed that both water and saline treatment exerted same degree of effect in term of ΔC*. Water treatment showed higher magnitude than that of saline treatment in term of ΔH* value. Chinese spinach with a positive ΔE*ab which reflects it has experienced certain extent of color changes after 96 hr of water and salinity treatment, as well as increased in color saturation (positive ΔC* value) and closer to +b* (yellow) axis (positive ΔH* value).

Pandan showed relatively low and similar extent of lightness increment in both water and saline treatment (Table 4.3). This suggests that both water and saline treatment have no significant effect for pandan. Pandan showed a negative Δa* in both water and saline treatment, which suggested the increase of greenness instead of losing it. Pandan showed a negative Δb* in water treatment and positive Δb* in saline treatment. From the results obtained, water treatment does not causes much effect on pandan as no yellowing in term of coloration occurred, whereas the saline treatment caused some leaf yellowing as we can see from the positive Δb* value. Pandan showed considerably low ΔE*ab value in overall in water and saline treatment. In term of ΔC* value, negative ΔC* value was obtained in water treatment while positive ΔC* value for saline treatment. Relatively low positive ΔH* value was obtained in both treatment. These indicated pandan experienced very little color changes and relatively low changes of color saturation. Positive ΔH* value indicated hue angle shifted closer to +b* (yellow) axis, however, very low extent of yellowing

occurred to pandan leaves as relatively low positive ΔH* value was obtained.

Oil palm frond showed negative ΔL* values, indicated on both treatments, but with greater magnitude on saline treatment as compared to water treatment (Table 4.3). This suggests that oil palm leaf disks decreased in lightness and turned darker to dark green in both treatments, however, much darker in saline treatment. Oil palm showed greater positive Δa* in saline treatment than in water treatment. This indicates the reduction of chlorophyll concentration is higher in saline treatment for oil palm than in water treatment. Oil palm showed negative Δb* in both treatments, however, greater degree of negative Δb* in saline treatment. This indicates that water treatment exerted only minimal effect on oil palm and suggested that the ability of oil palm leaf to maintain its chlorophyll content from losing during the saline treatment. In term of ΔE*ab value, oil palm showed the lowest ΔE*ab as compared with other leaf samples. Negative ΔC* value was observed on oil palm in both treatments, however, there was greater magnitude of negative ΔC* observed on saline treatment. This suggested the greenness of oil palm were decreased in saturation or duller, which turned the leaf disk into darker green. Oil palm showed the lowest positive ΔH* among all samples, which indicated oil palm was able to retain its greenness than pandan, Chinese spinach and water lettuce in this case.

Water lettuce showed positive ΔL* value in both treatments, however, the increase in the magnitude of ΔL* was much greater in water treatment instead of saline treatment and similar trend was observed in Δa* as well (Table 4.3). This suggests that water treatment caused greater loss of greenness or chlorophyll content in water lettuce than saline treatment. Water lettuce showed

a greater positive Δb* value in water treatment than in saline treatment, which hinted that certain degree of leaf yellowing and chlorophyll loss in both treatments, in which greater degree of yellowing in water treatment. Water lettuce showed the highest ΔE*ab among all leaf samples, this suggests that the treatment with water and saline could cause major changes to water lettuce in term of total color difference after 96 hr. There was greater ΔE*ab value observed in water treatment. Higher positive ΔC* was observed in water treatment than in saline treatment. This indicated the increase of saturation or brightness of leaf disks after both water and saline treatment. Lastly, water lettuce showed the highest positive ΔH* value among all samples. Both showed positive ΔH* value indicated closer to +b* axis, in which leaf disks became yellower, however, greater extent of yellowing was shown in water treatment.

The loss in greenness in plant is often used as an indicator of maturity, senescence or both (Kingston, 2010). Color change is usually the first visible symptom of senescence shown (Ferrante et al., 2008). Water lettuce showed the highest positive lightness difference, a* value difference, b* value difference, total color difference, chroma angle difference and metric hue difference. Meir et al. (1992) observed an increase in lightness from the color changes of green young leaves (L*= +38) to yellow aged leaves (L*= +75). The increase of lightness indicated chlorophyll bleaching. A positive a* value difference reflected a greenness loss in the leaf disks and the positive increase of b* value difference indicated the yellowing of leaf disks. Total color difference ΔE, included the combination of parameters L*, a* and b* values. This colorimetric parameter extensively used to characterize and determine the variation of colors

depending on processing conditions (Maskan, 2001). High total color difference of water lettuce indicated the higher variation of color after subjection of salinity. Besides, the positive chroma angle difference indicated the leaf disk increase in saturation and brighter reflected a greenness loss in the leaf disks while positive metric hue difference indicated the shifting from greenness to yellow in leaf disks.

The differences in color parameters allowed us to compare the vulnerability of 4 leaf samples towards salinity stress. Among 4 leaf samples, water lettuce constituted the highest in all color parameters followed by Chinese spinach. Water treatment on water lettuce and Chinese spinach showed greater differences than saline treatment. This suggests Chinese spinach and water lettuce are more susceptible to water treatment instead of saline treatment. As for pandan and oil palm, greater differences was observed in saline treatment. As compared with water lettuce and Chinese spinach, much lower differences can be observed, which indicates that oil palm and pandan experienced the least damage in both water and saline treatment and somehow showed better resistance and tolerance towards salinity stress subjected based on the color parameters studied (Table 4.3).

Several correlations were carried out in order to study the possible relationship of color parameters of leaf samples with other leaves' properties.

5.3.7 Correlation of Color Parameters of Leaf Disks and Moisture Content

All 4 leaf samples showed strong and very strong positive correlations between color parameters (ΔL*, Δa*, Δb*, ΔE*ab, ΔC* and ΔH*) and moisture content (Table 4.11). Keskin et al. (2013) found a positive and high correlation between moisture content and brightness and between moisture content and yellowness (b*) in which they utilized the color parameter in the estimation of moisture content and nutrient levels of peanut leaves. Therefore, moisture content of peanut leaves can be possibly predicted from color data. However, the strong and positive correlation of color parameters and moisture content suggests that the higher the moisture content, the higher the difference of color parameters' value as compared to its initial color parameters' value regardless the increase or decrease of it. Boudhrioua et al. (2009) in their study to compare the color of infrared dried olive leaves showed an increase of L* value, a* value and b* value along with the decrease of moisture content of leaves. In this case, the increase of these color parameters as the moisture content decrease were observed. Based on the positive correlation obtained in our study, it might hints that as the moisture content decreases, the magnitude of the changes of color parameters tend to decrease as well regardless the initial value of individual color parameter.

5.3.8 Correlation of Color Parameters of Leaf Disks and Initial Hill Reaction Activity of Isolated Chloroplast

Colorimetry parameters (ΔL*, Δa*, Δb*, ΔE*ab, ΔC* and ΔH*) studied in salt tolerance of 4 leaf samples showed correlative relationship with initial HRA. Negative correlation was shown between all colorimetry parameters and

HRA day-0 (Table 4.12). The changes in the colorimetry parameters represent the susceptibility of plant leaf disks towards subjected stress. The higher the HRA, the lower the changes in the colorimetry parameters. Photosynthetic carbon fixation and photosynthetic electron transport rate can be affected by various environmental stress factors. Under stress circumstances or encountered stress, plant would generate excess electron which cannot be consumed. These excessive production of electron can react with O_2, producing reactive oxygen species (Cavalcanti et al., 2004). The good electron transferring properties that contributed to high HRA enabled plants to have higher turnover rate on excess production of electron under stress condition. Due to their better ability in coping with excess of electron, the plant itself will less likely to experience oxidative damage in the salinity study. The negative correlation suggesting plant sample with high HRA tend to be less susceptible towards stress encountered. Through the analysis of colorimetry parameters, the photosynthetic ability of plants can be estimated. The high photosynthetic rate of plant might provide certain degree of protective role against stress.

5.4 Evaluation of the Role of the Leaves' Anti-oxidative Enzymes in Photochemical Activity and Stability of Chloroplast

5.4.1 Protein Content of Extracted Crude Enzyme

Crude enzyme extract of Chinese spinach consisted the highest protein content of 12.6 mg g^{-1} FW which is equivalent to 2.3 mg mL^{-1} of protein content. Information on the protein content of Chinese spinach was so far not reported in any study. Oil palm crude enzyme extract consisted slightly lower protein content than Chinese spinach, which is 12.1 mg g^{-1} FW, which is equivalent to 2.4 mg mL^{-1} of protein content (Table 4.4). Deepa and Arumughan (2002)

obtained 34.74 mg of total protein in the total volume of 500 mL of crude extract of peroxidase while Mahmood et al (2013) obtained 5.5 to 46.1 mg g^{-1} FW of soluble protein in crude soluble fraction in oil palm leaves. Crude enzyme extract of pandan showed 10.7 mg g^{-1} FW or 2.2 mg mL^{-1} of protein content (Table 4.4), while Ningrum and Schreiner (2014) showed 16.46 mg mL^{-1} of total soluble protein in their crude enzymes extraction., which is approximately 7-fold higher from what we obtained. The protein content of the crude enzyme extract of water lettuce was 3.9 mg g^{-1} FW or 0.65 mg mL^{-1}, while Tewari et al. (2008) obtained approximately 7 mg g^{-1} FW of protein content, which is approximately 2-fold higher from our obtained result. The variation of protein content extracted most possibly due to different extraction method used plus the uncontrolled growing condition of plant samples in this study as compared to other studies.

5.4.2 Anti-oxidative Enzyme Activities of Crude Leaves Extract

Chinese spinach had 0.84 U mg^{-1} protein of ascorbate peroxidase (APX) activity in the study (Fig. 4.23). There is no previous study reported on the APX activity of Chinese spinach. The catalase (CAT) activity obtained was 0.01 U mg^{-1} protein in this study (Fig. 4.24). Previous studies reported CAT activity of Chinese spinach of approximately 2 U mg^{-1} protein, which is much higher to what we obtained (Qin et al., 2013). The peroxidase (POX) activity of Chinese spinach was 2.87 U mg^{-1} protein in our study (Fig. 4.25) and approximately 5 U mg^{-1} protein reported in previous study (Qin et al., 2013). Chinese spinach obtained 0.19 U mg^{-1} protein of guaiacol peroxidase (GPX) activity (Fig. 4.26), 1.76 U mg^{-1} protein of pyrogallol oxidase (PO) activity (Fig. 4.27) and 0.037 U mg^{-1} protein of catechol oxidase (CO) activity in the study (Fig. 4.28). In term

of superoxide dismutase (SOD) activity, Chinese spinach showed no activity in this study (Fig. 4.29).

Pandan showed the lowest APX activity, which is 0.16 U mg^{-1} protein; CAT activity of 0.01 U mg^{-1} protein; lowest POX activity of 0.68 U mg^{-1} protein; lowest GPX activity of 0.06 U mg^{-1} protein; and lowest PO activity of 0.93 U mg^{-1} protein in the study (Figs. 4.23 – 4.27). There was no CO activity detected (Fig. 4.28), however, highest SOD activity of 6.19 U mg^{-1} protein was obtained on pandan (Fig. 4.29). No study of APX, CAT, POX, GPX, PO, CO and SOD activity on pandan have been reported before.

Oil palm exhibited the highest APX activity in the study, which is 1.80 U mg^{-1} protein; CAT activity of 0.01 U mg^{-1} protein; highest POX activity of 4.87 0.01 U mg^{-1} protein; GPX activity of 0.14 U mg^{-1} protein; PO activity of 2.10 U mg^{-1} protein; highest CO activity of 0.04 U mg^{-1} protein and SOD activity of 2.79 U mg^{-1} protein (Figs. 4.23 – 4.29). In the study conducted by Deepa and Arumughan (2002), 585.06 U mg^{-1} protein of POX activity was obtained with 34.74 mg of total protein in 500 mL of total volume. Apart from this, there is no study of APX, CAT, GPX, PO, CO and SOD activity on oil palm has been reported before.

Water lettuce showed 0.51 U mg^{-1} protein of APX activity in the study (Fig. 4.23). Previous studies reported water lettuce with 13.10 U g^{-1} fresh weigh (3.36 U mg^{-1} protein) (Upadhyay et al., 2011) and approximately 100 µmol min^{-1} g^{-1} fresh weigh (25.64 U mg^{-1} protein) (Sinha et al., 2005) of APX activity,

which is higher than what we obtained in this study. Water lettuce contained 0.02 U mg^{-1} protein of CAT activity (Fig. 4.24). CAT activity reported from previous studies on water lettuce was approximately 0.45 μmol mg^{-1} protein (0.45 U mg^{-1} protein) (Odjegba and Fasidi, 2007) and 1.89 U g^{-1} fresh weigh (0.48 U mg^{-1} protein) (Upadhyay et al., 2011) which was higher as compared to our study. Water lettuce obtained 3.11 U mg^{-1} protein of POX activity (Fig. 4.25) while POX activity reported from previous studies on water lettuce was 0.015 Δ470 min^{-1} g^{-1} fresh weight (0.0467 U mg^{-1} protein) (Odjegba and Fasidi, 2007) and 1.44 U g^{-1} fresh weigh (0.369 U mg^{-1} protein) (Upadhyay et al., 2011). Water lettuce exhibited the highest GPX activity among all leaf samples, which was 0.25 U mg^{-1} protein (Fig. 4.26) while GPX activity reported from previous studies on water lettuce was 60 μmol min^{-1} g^{-1} fresh weigh (15.38 U mg^{-1} protein) (Sinha et al., 2005). Water lettuce also showed the highest PO activity as well, which was 3.74 U mg^{-1} protein (Fig. 4.27). There was no CO activity detected on water lettuce (Fig. 4.28) and no study of CO activity on water lettuce has been reported before. SOD activity reported from previous studies on water lettuce was 4 units mg^{-1} protein (Odjegba and Fasidi, 2007), 1.72 U g^{-1} fresh weigh (Upadhyay et al., 2011) and approximately 28 U g^{-1} fresh weigh (Sinha et al., 2005), however, SOD activity was not detected in this study (Fig. 4.29). The differences of the anti-oxidative enzymes activity between this study as compared to other studies reported before might be due to the initial different growing condition of plant samples that contributed to the different magnitude of enzyme activities as anti-oxidative enzymes are regulated by its growing environment condition.

The different magnitude of various anti-oxidative enzymes among these four samples might indicate the importance of their roles in coping with existing stresses of the plant growing environment under natural condition and normal circumstances. It varies with different plants. For example, the growing environment of water lettuce (aquatic plant) and oil palm are totally distinct from each other, thus the different magnitude of stresses encountered lead to the different magnitude of anti-oxidative enzyme activities. Plants possess both enzymatic and non-enzymatic defense systems to ensure cells are protected from oxidative stress. In order to protect against the cellular damage caused by reactive oxygen species (ROS), anti-oxidative enzymes play a role in defense system against deleterious free radicals in plant cells (Slooten et al., 1995; Ding et al., 2013).

Members of the enzymatic antioxidant defense system included superoxide dismutase, catalase, ascorbate peroxidase, and phenolic peroxidases, such as guaiacol peroxidase. The superoxide anion (O_2•−) is dismutated to H_2O_2 by SOD, followed by the conversion of hydrogen peroxide (H_2O_2) to water (H_2O) by CAT, APX and GPX. APX and GPX. APX requires reduced ascorbate as specific electron donor while GPX requires a phenolic compound like guaiacol as substrate to function. Figure 5.1 shows the oxidation of ascorbic acid in the presence of hydrogen peroxide by ascorbate peroxidase (Martinello and Luiz da Silva, 2006). Figure 5.2 shows the enzymatic conversion of guaiacol to tetraguaiacol in the presence of hydrogen peroxide by guaiacol peroxidase (Wilkesman et al., 2014).

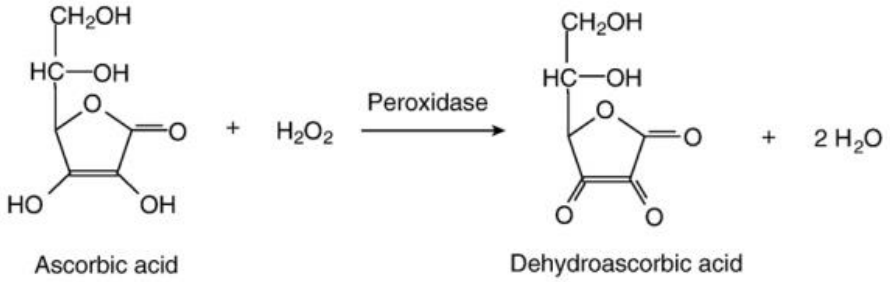

Figure 5.1. Oxidation of ascorbic in the presence of hydrogen peroxide by ascorbate peroxidase (Martinello and Luiz da Silva, 2006).

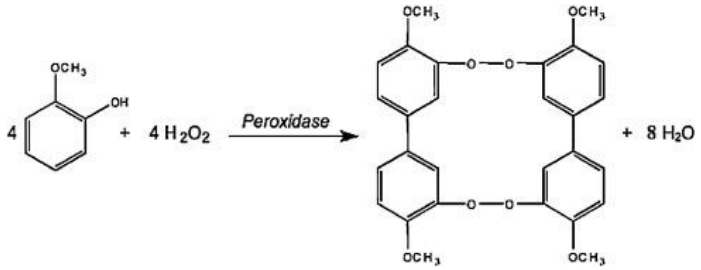

Figure 5.2. Enzymatic conversion of guaiacol to tetraguaiacol in the presence of hydrogen peroxide by guaiacol peroxidase (Wilkesman et al., 2014).

POX and SOD act as the first line of defence against ROS (Moran et al., 1994). SOD is a major scavenger of ROS and it dismutates O_2•− with great efficiency and produces H_2O_2 and oxygen (O_2) (Smirnoff and Wheeler, 2000). Inhibition of the autoxidation of pyrogallol indicated the presence of SOD activity. Figure 5.3 shows the autoxidation of pyrogallol. In the autoxidation, the oxidation substrate pyrogallol will firstly become the high oxidative activity chemicals as superoxide anion radical and semiquinone radical. This reaction will finally be ended as producing the quinone and hydrogen peroxide (Liu et al., 2013; Ramasarma et al., 2014). SOD activity was only shown on pandan and oil palm in the study under normal circumstances (F. The absence of SOD activity in both Chinese spinach and water lettuce does not indicate the inability of a particular plant to dismutate superoxide anion radical, this might indicates the low stress level of particular plant in its natural growing condition.

Superoxide anion radical can also be scavenged by antioxidant compound such as quercetin (Nimse and Pal, 2015) and flavonoid (Robak and Gryglewski, 1988).

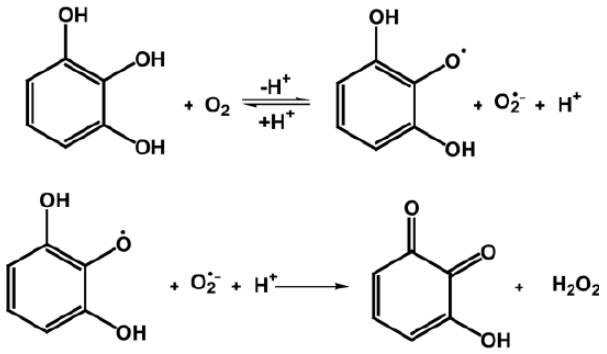

Figure 5.3. Autoxidation process of pyrogallol (Liu et al., 2013).

H_2O_2 scavenger studied included APX, CAT, GPX and POX. APX (class I peroxidase) utilizes ascorbate as the preferential electron donor in scavenging H_2O_2 molecules and maintaining the steady-state level of H_2O_2 in various plant subcellular compartments (Najami et al., 2008). CAT can be found in peroxisomes, glyoxysomes and mitochondria but absent in the chloroplast helps dismutating mostly photorespiratory or respiratory H_2O_2 into H_2O and O_2 (Apel and Hirt, 2004). Class III peroxidases are known with the use of phenolics as the preferential electron donors. Besides, class III peroxidases are also participate in polymerization reactions in the cell wall and the metabolism of indolacetic acid or ethylene (De Gara, 2004). Glycosylated unspecific POX belonging to class III. POX decomposes H_2O_2 through oxidation of co-substrates for example phenolic compounds and/or antioxidants. Figure 5.4 shows the oxidation of pyrogallol into purpurogallin by POX in the presence of H_2O_2. Significant roles of POX have been mentioned in plant development processes (Gaspar et al., 1985), particularly in the scavenging of H_2O_2 produced

in chloroplasts (Parida and Das, 2005). Among all the peroxidase enzymes studied, POX exhibited the highest magnitude over APX, CAT and GPX (Figs. 4.23 – 4.26). Sreenivasulu et al. (1999) found that higher POX activity was discovered to be higher in protecting plants against the oxidative stresses in tolerant plants.

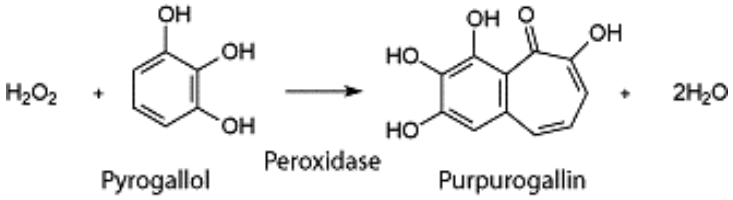

Figure 5.4. Oxidation of pyrogallol into purpurogallin by peroxidase in the presence of hydrogen peroxide (Gaspar et al., 1985).

Figure 5.5 shows the oxidation reaction from catechol to quinone by catechol oxidase which is a polyphenol oxidase (Mishra and Gautam, 2016). PPO can oxidize some phenols to chinone. This enzyme was found to be related to wounding, enzymatic browning (Demir and Kocaçalişkan, 2001) as well as biosynthesis of alkaloids under stressful conditions (Bilková et al., 2005). An increase of PPO activity under stress suggests the ability of plant to oxidize and degrade the toxic substance especially phenolic compounds that tend to accumulate during salt stress. The presence of PPO activity causes browning reaction by oxidation of phenolic substrates, depending on the availability of active PPO enzyme and phenolic compounds that presence in the plant tissue (Mcevily et al., 1992). PO is also a type of PPO where the substrate is pyrogallol with catechol ring structure along with functional group at other position of the ring (-OH at *ortho* position) instead of catechol as in CO (Mishra and Gautam, 2016). PO activity showed in all leaf samples, whereas CO activity showed only

in Chinese spinach and oil palm under normal circumstances (Fig. 4.28), which might hint the level of stress encountered and certain level of toxic substances within the plant which varies with their habitat. The absence of common anti-oxidative enzymes in certain plants might be due to the presence of small amount of substrate and also due to the role of that enzyme being taking by enzyme with similar role. For example, small amount of H_2O_2 play a role in signaling under stress. Under such circumstances, the role of anti-oxidative enzymes is to control the cellular concentrations of H_2O_2 instead of completely remove it (Ivan et al., 2012). According to Ivan et al. (2012), CAT activity was undetectable from some of the collected halophytes like *Plantago maritima, Plantago coronopus* and *Limonium gmelini* because of the presence of only small amounts of H_2O_2 which causes the role of CAT being taken by peroxidase.

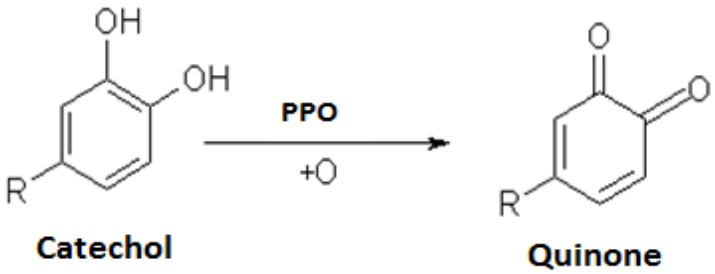

Figure 5.5. Oxidation reaction from catechol to quinone by polyphenol oxidase (Mishra and Gautam, 2016).

Sairam et al. (2000) observed differences in various anti-oxidative enzyme activities among tolerant genotypes of wheat. The study showed very high levels of ascorbic acid and APX in one tolerant genotype while exhibited higher SOD and CAT and intermediate level of ascorbic acid in another tolerant genotype. It is not possible for all anti-oxidative enzymes to increase uniformly and different anti-oxidative enzymes may be more prominent for imparting

tolerance in some genotypes over the others. This explained for both strong negative and positive correlations between selected anti-oxidative enzymes and the other anti-oxidative enzymes (Sairam et al., 2000). Previous study of Queiroz et al. (1998) on cold stress in *Coffea arabica* L. roots showed that both APX and CAT did not change significantly, however POX increased in greater extent under chilling stress over the control plants which summarized that anti-oxidative enzyme activity is not related to stress tolerance in some case. Cavalcanti et al. (2004) documented that SOD, POX, and CAT did not ensure the survival of cowpea leaves under high salinity levels. Although anti-oxidative enzymes always play an important role in the antioxidant capabilities of plants, but there is still some observable variability in term of enzyme activities among species and genotypes.

5.4.3 Correlation of Initial Hill Reaction Activity and Protein Content

From the correlation analysis that we studied, relationship between initial HRA of 4 leaves' chloroplast isolate and protein content of crude enzyme extract were observed. A very strong positive correlation was obtained between HRA of isolated chloroplast on Day-0 and protein content of leaves' extracted crude enzyme ($r = 0.840$, $P < 0.05$) (Table 4.10). Other study has shown a great loss of proteins in leaves whenever there was a decline in rate of light-saturated photosynthesis (P_{max}), though not all proteins are photosynthesis related, however, synchronously degraded during senescence (Jiang et al., 1999). Thus, correlative relationships were obtained between breakdown of proteins and P_{max}. Jiang et al. (1999) found a positive correlation between P_{max} and total soluble proteins level and they suggest that the high photosynthetic capacity of

Akenohoshi leaves is supported by its high content of proteins (Jiang et al., 1999). According to Hardwick and Woolhouse (1967), the variation of the change in photosynthetic activity in different parts of the leaf was highly related to the changes of RNA and protein content. Besides, positive correlation was obtained between photosynthetic oxygen evolution with soluble protein and chlorophyll as well in the study conducted by Shieh and Liao (1988). The presence of significant positive correlation indicated the level of protein might determine the rate of photosynthesis, and at the same time, the decline of photosynthesis would be accompanied with the decrease of protein content. A strong and positive correlation is usually observed between photosynthetic rate and leaf nitrogen content (Yamori et al., 2011). The existence of large amount of total leaf nitrogen in chloroplasts was mainly used to produce photosynthetic proteins in the stroma in some plants. Rubisco which is the key enzyme in photosynthesis accounted for approximately half of the total leaf nitrogen, as well as electron transport proteins (approximately 7%) (Yamori et al., 2011). Besides, similar correlation was also obtained in other study, suggesting this relationship reflected that the proteins of both the Calvin–Benson cycle and thylakoids account for the majority of the leaf nitrogen (Evans, 1989). This suggests that the higher level of protein content, indicating higher amount of precursor for photosynthetic proteins and enzymes, thus contributed to higher photosynthetic capacity.

5.4.4 Correlation of Color Parameters of Leaves and Protein Content of Crude Enzyme Extract

There was a correlation relationship observed between the colorimetry parameters measured and protein content of crude enzyme extract as shown in

Table 4.15. The high protein content suggests higher amount of precursor for the production of photosynthetic proteins and enzymes (Yamori et al., 2011). To interpret in another way, it could be the salinity stress imposed to the plant that contributed to the increase of total color changes lead to the decline of protein content. As leaf senescing, the leaf protein breakdown as well (Jiang et al., 1999).

Proteins are building materials for enzymes and photosynthetic components. Enzymes facilitate chemical reactions without being changed in the process, are known as protein catalyst (Rolfes et al., 2012). The stroma area in chloroplast contains the enzymes that responsible for photosynthetic carbon fixation and also a great many other enzymes and 70S ribosomes. Photosynthetic light reaction take places in thylakoid membranes in the presence of other unique proteins *in vivo* such as complexes of proteins and photosynthetic pigments (Vaughn, 2013). The negative correlation observed between all colorimetry parameter (ΔL*, Δa*, Δb*, ΔE*ab, ΔC* and ΔH*) and protein content of plant leaf (Table 4.13). The higher the protein content in plant leaf, the lesser the changes in term of colorimetry parameters when plant undergo salinity study. This might indicates the high level of protein content acts as crucial building materials necessary for the photosynthetic components as well as the defence components such as anti-oxidative enzyme, thus, protected the plant when stress encountered.

5.4.5 Correlation among Leaves' Crude Enzyme Activity

There are 7 anti-oxidative enzymes being analysed in our research, which included; class I plant peroxidase: ascorbate peroxidase and catalase;

class III plant peroxidase: peroxidase and guaiacol peroxidase; polyphenol oxidase: pyrogallol oxidase and catechol oxidase; and lastly superoxide dismutase. SOD functions by catalysing the dismutation of O_2•− to the production of H_2O_2 and O_2 (Smirnoff and Wheeler, 2000). The H_2O_2 is then dismutates by CAT to H_2O and O_2 directly or by ascorbate peroxidase and peroxidase indirectly by combining it with antioxidant compounds such as ascorbate (Lu et al., 2010; Yang et al., 2011). Polyphenol oxidase also acts in defence mechanism as well as free radical scavenging (Lee et al., 1990; Altunkaya and Gökmen, 2008; Ünal et al., 2011).

From the correlation between specific enzymes' activity, we observed that CAT showed no correlation with APX, in the meantime, class III peroxidases (POX and GPX) does not correlate with each other, neither for CO and PO which belong to PPO group (Table 4.14).

Based on Table 4.14, both APX and CAT individually showed positive correlation with one and different enzyme from class III peroxidase and polyphenol oxidase group. APX showed very strong positive correlation with POX ($r = 0.913$, $P < 0.05$) and CO ($r = 0.801$, $P < 0.05$), whereas CAT showed a strong positive correlation with GPX ($r = 0.762$, $P < 0.05$) and a very strong positive correlation with PO ($r = 0.886$, $P < 0.05$). For the correlation between class III peroxidase and polyphenol oxidase group, each class III peroxidase also showed positive correlations with only one and different polyphenol oxidase group's enzyme. In this case, POX showed a strong positive correlation with CO ($r = 0.671$, $P < 0.05$), while GPX showed a very strong positive

correlation with PO (r = 0.888, $P < 0.05$). For SOD, there was a very strong negative correlation with GPX (r = -0.945, $P < 0.05$), while only a strong negative correlation with PO (r = -0.717, $P < 0.05$).

From the anti-oxidative enzymes correlation obtained from the plant leaves in their natural state, we noticed that the level of APX positively corresponded with POX and CO while level of CAT positively corresponded with GPX and PO (each from class III plant peroxidase and polyphenol oxidase). The negative correlation between SOD with GPX and PO (class III plant peroxidase and polyphenol oxidase) showed that the level of SOD of plant leaves in their natural state is negatively corresponded with GPX and PO. The high level of SOD leaded to low level of GPX and PO, and vice versa.

In general, APX shows similar role as CAT in ROS scavenging, in which their differences are APX requires ascorbate which acts as an electron donor to reduce H_2O_2 to H_2O while CAT can directly dismutates H_2O_2 into H_2O and O_2. APX was found to be superior to CAT in discarding excess electron in plant, however, CAT was found to be more superior in term of enzyme stability as a stress-resistant enzyme where plants are under oxidative stress condition (Shikanai et al., 1998). This explains why there is no correlation between APX and CAT. Class III plant peroxidase reduces H_2O_2 to H_2O using various substrates (Wang et al., 2009). In our study, POX utilized pyrogallol as its substrate while GPX utilized guaiacol as its substrate. This also explained why there is no correlation between POX and GPX. Peroxidase is an enzyme that catalyses the oxidation of a particular substrate by hydrogen peroxide while

oxidase is an enzyme which is known with the function of transferring a hydrogen atom from a particular substrate and accepted by an oxygen molecule in order to form water or hydrogen peroxide (Nicolas et al., 2003; Meister, 2009). The oxidase we study was polyphenol oxidase which included CO and PO, in which the difference between these two enzymes is their substrate. The substrate for CO was catechol while, pyrogallol was the substrate for PO. This explains the absence of correlations between them.

Figure 5.6 shows the schematic diagram of correlation overview among anti-oxidative enzymes' activity of 4 leaf samples. The correlation obtained here showed that each anti-oxidative enzyme acts preferably with selected anti-oxidative enzymes, this allowed us to figure out the relationship among anti-oxidative enzyme of plant leaves in their natural state.

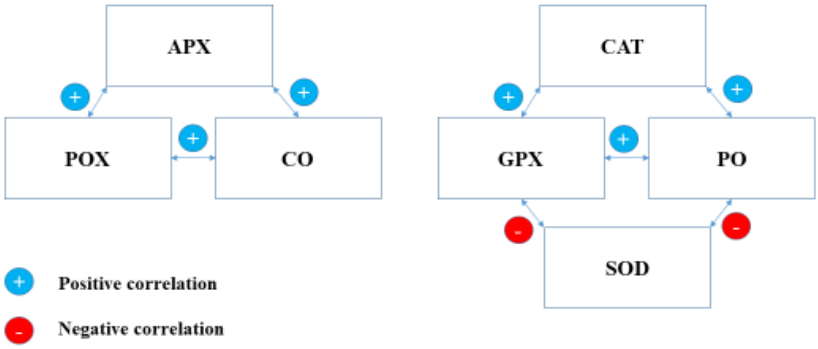

Figure 5.6. Schematic diagram on correlation overview among anti-oxidative enzymes' activity of 4 leaf samples.

5.4.6 Correlation of Initial Hill Reaction Activity of Isolated Chloroplast and Leaves' Crude Enzyme Activity

SOD, CAT, along with various peroxidases such as GPX and APX are the primary anti-oxidative enzymes (Kuk et al., 2003). SOD are metalloenzymes serve as the first line of antioxidant defence against ROS by catalyzing the dismutation of O_2•− to the production of H_2O_2 and O_2(Alscher et al., 2002; Raychaudhuri, 2000). APX is responsible to affect the fine modulation of ROS in signaling and CAT is mainly responsible in the excess ROS scavenging (Mittler, 2002; Feng et al., 2013). CAT dismutates H_2O_2 and converts it to H_2O and O_2. H_2O_2 as well scavenged by peroxidase such as ascorbate peroxidase and peroxidase indirectly by combining it with antioxidant compounds such as ascorbate (Lu et al., 2010; Yang et al., 2011).

A strong positive correlation was observed between Hill Reaction Activity (HRA) on day 0 and APX activity ($r = 0.706$, $P < 0.05$), whereas a very strong negative correlation with CAT activity ($r = -0.804$, $P < 0.05$) (Table 4.16). This suggests that APX play the main role in scavenging H_2O_2 to H_2O and O_2 in the presence of ascorbate as substrate for all four plant samples under their nature state instead of peroxidase (POX), CAT, and GPX. APX plays an important role in H_2O_2 detoxification in green leaves and the key antioxidant enzyme in plants (Foyer and Harbinson, 1994). APX may be superior to CAT in discarding excess electrons, however, under oxidative stress conditions, CAT is more superior in term of enzyme stability as a stress-resistant enzyme (Shikanai et al., 1998), which explain the strong positive correlation of APX and strong negative correlation of CAT with HRA on day-0 in which no stress was induced.

Catechol oxidase (CO) activity was only observed in Chinese spinach and oil palm (Fig. 4.28). A strong positive correlation was observed on Hill Reaction Activity (HRA) on day-0 with CO activity ($r = 0.680$, $P < 0.05$); while strong negative correlation with PO activity ($r = -0.603$, $P < 0.05$) (Table 4.27). There are several possible functions of the involvement of PPO in photosynthesis. PPO activity was found correlated with chloroplast evolving high levels of O_2 (Vaughn and Duke, 1984). Besides, PPO protein also found associated with the photosystems (Sheptovitsky and Brudvig, 1996; Lax and Vaughn, 1991). PPO might possibly plays a role in inhibiting cyclic and/or non-cyclic photophosphorylation by oxidation of potential substrates (Neumann and Drechsler, 1967). Apart from this, PPO might involves in photosynthesis during growth and development (Winters et al., 2008; Webb et al., 2014; Ben-shalom et al., 1977). Lastly, PPO activity can be modulated by environmental effects (Rivero et al., 2001). The differences of correlation between HRA with PO and CO might be due to anti-oxidative enzymes from the same class play different roles and thus contributed to the differences in term of availability in plant leaves.

Figure 5.7 shows the correlation obtained between initial HRA on day-0 of 4 leaves' chloroplast isolate and specific enzyme activity of crude enzyme extract.

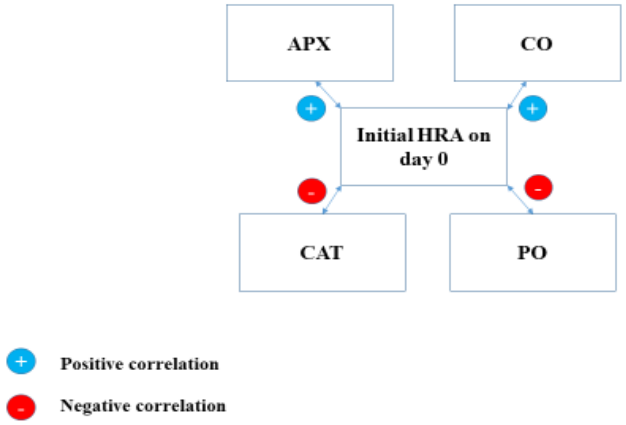

Figure 5.7. Schematic diagram on correlation obtained between initial HRA on day-0 and anti-oxidative enzymes' activity of 4 leaf samples.

5.4.7 Correlation of Degradation Rate of Hill Reaction Activity of Isolated Chloroplasts and Leaves' Crude Enzyme Activity

Correlation analysis between degradation of HRA of 4 leaves' chloroplast isolate and initial specific enzyme activity of crude enzyme extract was done. A strong negative correlation was obtained between initial CAT activity and total degradation of chilled-stored chloroplast in term of Hill Reaction activity on 90-days storage (r = -0.729, P < 0.05) whereas a very strong negative correlation obtained on 90-days of frozen-stored chloroplast (r = -0.899, P < 0.05) (Table 4.17). As the degradation continues to increase over storage period, the level of catalase activity was postulated to decrease as well. CAT functions by scavenging and detoxification of H_2O_2 (Asada, 1992). The negative correlation between degradation of Hill Reaction activity and CAT activity might hinted that CAT activity decreased as the electron transferring capacity of the stored chloroplast decrease. This correlation also suggests that the chloroplast might encounters stress when it was in storage, and this long period of exposing to stress most possibly will lead to the decrease of CAT activity. The decrease in CAT activity would lead to the accumulation of H_2O_2,

which can further react with superoxide anion radical ($O_2^{\bullet -}$) to form hydroxyl radical (•OH) via the Herbert-Weiss reaction. Prolonged exposure to stress eventually caused CAT activity to decline (Bowler et al., 1992). GPX activity showed a strong negative correlation with degradation of chilled-stored chloroplast on day 90 (r = -0.641, $P < 0.05$) and degradation of frozen-stored chloroplast (r = -0.686, $P < 0.05$) on day 90.

Peroxidases share a common catalytic mechanism in degrading H_2O_2 (Nissum et al., 1998). All peroxidases showed negative correlation for the degradation rate of both chilled and frozen-stored chloroplast on day-90. As the storage of chloroplast continue, accumulation of ROS is inevitable. According to Keshavkant and Naithani (2001), an increase in ROS and decrease in anti-oxidative enzyme, such as APX, CAT, SOD and peroxidase were observed in chilling sensitive young sal (*Shorearobusta*) seedling in response to constant chilling exposure. The negative correlation obtained might hints the chilling sensitivity of plant samples, thus CAT activity was postulated to decrease with an increases of the degradation of stored chloroplast.

PO activity also showed a very strong negative correlation with both degradation of chilled (r = -0.881, $P < 0.05$) and frozen-stored chloroplast (r = -0.906, $P < 0.05$) on day-90. The negative correlation might suggests an active antioxidant role of this enzyme in response to the degradation of HRA. Polyphenol oxidase is well known with certain roles in plant photosynthesis (Neumann and Drechsler, 1967; Vaughn and Duke, 1984; Lax and Vaughn, 1991; Sheptovitsky and Brudvig, 1996). Polyphenol oxidase is also found to function

in immunity reactions and defense mechanism against insects and plant pathogens (Constabel et al., 2000), plant components biosynthesis, enzymatic browning reactions as well free radical scavenging (Lee et al., 1990; Altunkaya and Gökmen, 2008; Ünal et al., 2011). The protective role of polyphenol oxidase agreed with the negative correlation of PO activity with degradation of HRA.

Figure 5.8 shows the correlation obtained between total degradation of HRA on day-90 and initial anti-oxidative enzymes' activity of 4 leaf samples on day 0.

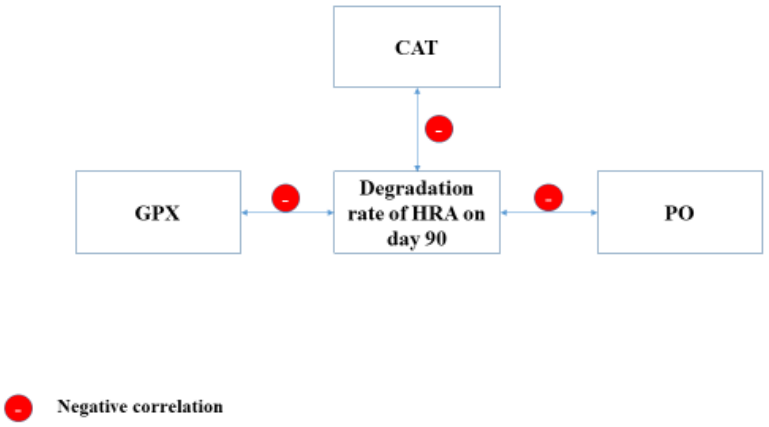

Figure 5.8. Schematic diagram of correlation obtained between degradation rate of HRA on day-90 and anti-oxidative enzymes' activity of 4 leaf samples.

5.4.8 The Relationship between Anti-oxidative Enzyme and Functionality and Stability of Isolated Chloroplast

There is a strong linkage between plants' photosynthetic ability and enzymatic anti-oxidant activity. Both photosynthetic carbon fixation and electron transport rate are greatly influenced by environmental stress factors. Plant tend to generate excess electron that unable to be consumed when subjected to stress circumstances which lead to the formation of ROS

(Cavalcanti et al., 2004). The overproduction of ROS can act as cellular indicators of stresses and secondary messengers in the stress-response signalling pathways which allowed the regulation of ROS scavenging enzymes among different compartment in plant (B. Ali et al., 2013). Chloroplasts are responsible for establishing the connection between ROS signalling and sensing of the environmental conditions and thus when there are any changes of environmental variables, the photosynthetic electron transport in chloroplasts will be affected, which causes the redox state of chloroplast to modify. This in turn regulates the activity of ROS scavenging enzymes (Laloi et al., 2004) even for non-chloroplastic anti-oxidant enzyme such as CAT. CAT is a predominant peroxisome enzyme, but also exists in mitochondria and cytoplasm of cells. Among all ROS, H_2O_2 is the only ROS which is able to pass through the cellular membrane by the membrane water channels aquaporins, which also play roles in H_2O_2 signalling and regulation. Therefore, H_2O_2 can move along with water to sites distant from where it is produced (Bienert et al., 2007; Dynowski et al., 2008; Bienert and Chaumont, 2013; Sofo et al., 2015). This properties of plant aquaporins and role of ROS as stress indicator and secondary messengers in the stress-response signalling pathways enabled non-chloroplastic enzymes such as catalase to be regulated to protect plant cell and its organelles against oxidative damage.

From the correlation study between initial HRA on day-0 and anti-oxidative enzymes' activity, a strong negative correlation was observed on CAT and PO activity, while strong positive correlations were found between initial HRA and both APX and CO activity (Table 4.16). This suggests that initial HRA

on day-0 establish certain co-relationship with certain anti-oxidative enzymes' activity, which means determination on anti-oxidative enzymes' activity in a particular leaf sample enabled us to have a good guess on how good their photosynthetic ability. In this case, we suggests APX, CAT, CO and PO activity in crude leave extract allow us for better prediction of plant leaves initial photosynthetic ability.

From the correlation study between degradation of HRA on day-90 and anti-oxidative enzymes' activity, we noticed a strong negative correlation on CAT (belong to class I plant peroxidase), GPX (belong to class III plant peroxidase) and PO (belong to polyphenol oxidase's group) (Table 4.17). This indicates the higher the initial anti-oxidative enzyme in leave, the lower the HRA degradation rate of the isolated chloroplasts. The negative correlation might indicates an active anti-oxidant role of these anti-oxidative enzymes in protecting the photochemical function of chloroplast. Anti-oxidative enzyme level such as CAT, GPX and PO most probably act to protect photosynthetic apparatus even after isolation and prolonged storage. This correlation enabled us to predict the stability of isolated chloroplast of certain plant leaf samples by first determine the activity of CAT, GPX and PO.

CHAPTER 6

CONCLUSION

Among 4 plant samples, Chinese spinach had the highest chlorophyll content. Oil palm was found with highest HRA and great stability over the storage period. Storage condition of isolated chloroplast that rapid frozen with liquid nitrogen and stored at -20°C was proven to be able to preserve isolated chloroplast better than only stored at 4°C without pre-freezing. The strong positive correlation between degradation rate of HRA in both chilled and frozen condition with chlorophyll content of isolated chloroplasts suggest that plants that originally higher in chlorophyll content probably experience larger extent of photosynthesis degradation when subjected to damage or prolonged storage.

In the study of salt tolerance of plant leaf, oil palm and pandan were found to have higher tolerance salinity stress. This may be due to the initial low moisture content of plant leaf and high proportion of lignified tissues and thick cuticle of leaf morphology. The strong positive correlation between all color parameters studied and moisture content of plant leaf suggests that the higher the moisture content, the higher the magnitude of difference of color parameters. The very strong negative correlation between all color parameters and initial HRA suggests that plant with high photosynthetic rate tend to be less susceptible towards salinity stress encountered.

Both Chinese spinach and oil palm consist of similarly high protein content. Pandan shows highest superoxide dismutase activity (SOD); oil palm shows highest ascorbate peroxide activity (APX), peroxidase activity (POX) and catechol activity (CO); water lettuce shows highest catalase activity (CAT), guaiacol peroxidase activity (GPX) and pyrogallol oxidase activity (PO).

For the correlation among all anti-oxidative enzymes studied, class I peroxidase was positively correlated to class III and polyphenol oxidase, but not interrelated to each other within the same class. Positive correlations was observed between APX, POX, CO, and also between CAT, GPX and PO. SOD was negatively correlated to class III peroxidase (GPX) and polyphenol oxidase (PO). This may be due to anti-oxidative enzymes from the same class carrying similar reaction that leads to such correlations obtained. These correlations provided us the insight on how plants' anti-oxidative enzyme response and interact.

For the correlation analysis between initial HRA and all anti-oxidative enzymes studied, the initial HRA of 4 plant chloroplast isolates was found positively correlated to each of class I peroxidase (APX) and polyphenol oxidase (CO), while negatively correlated to another class I peroxidase (CAT) and polyphenol oxidase (PO). These correlations show the relationship between the enzymatic defense and photosynthetic ability of plant samples in their natural state in which high APX and CO might hint high HRA, while low CAT and PO might suggest high HRA.

In the correlation studies between the degradation of HRA of 4 plant chloroplast isolates and anti-oxidative enzymes activity, negative correlations was obtained. The degradation of HRA after 90 days of storage was found to negatively correlate to each class I peroxidase (CAT), class III peroxidase (GPX) and polyphenol oxidase (PO) initial activities. The negative correlation might indicates an active antioxidant role in protecting the photochemical function of chloroplast, in which the higher the initial anti-oxidative enzyme in leaf, the lower the HRA degradation rate of the isolated chloroplasts. Anti-oxidative enzyme level such as CAT, GPX and PO most probably act to protect photosynthetic apparatus even after isolation and prolonged storage. This correlation allow us to study on how the enzymatic defense react in response to the degradation of plant photosynthetic ability.

Generally, the correlation results allow us to postulate the relationships between the photosynthetic ability (initial HRA), chloroplast stability (degradation rate of HRA) and stress status (anti-oxidative enzyme activities) of a particular plant leaf. Anti-oxidative enzymes such as APX, CAT, PO, and CO can probably be used as a biomarker for chloroplast photochemical activity while anti-oxidative enzymes such as CAT, GPX, PO may be used as a biomarker for predicting isolated chloroplast photochemical stability at prolonged storage.

The main research limitation in this study was that due to time constraint and limited space, it is not possible to grow all the selected plant samples in this study in order to minimize the variation of initial growing environment and

stress encountered of plant samples in the study of anti-oxidative enzyme activities.

In the future research, the isolated chloroplast from selected plant leaf can be further studied in the biosensor which can be applied in the herbicide sensing through the inhibition of electron transferring in the Hill reaction. Apart from that, plant leaf with high anti-oxidative enzyme activities, for example SOD from pandan can be extracted and further studied in different application study.

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APPENDIX A

Herbarium No.:11763

Picture of herbarium

Picture of Label

USM Herbarium
Pusat Pengajian Sains Kajihayat
Universiti Sains Malaysia

Family: **Amaranthaceae**
Genus/Species: *Amaranthus tricolor L.Sinn*
Date: 12/02/2018 Altitude:
Locality: Gunung Pondan Herbal Valley

Habitat: Widely cultivated plant occurs on cultivated land, roadside and wasteland.

Notes: A much branched, vigorous, erect annual plant with a stout stem growing up to 125cm, with long stalked leaves. It is generally the leaves are up to 12cm-20cm long ovate and are notched or rounded or tips and decurrent at the base into petioles. Flowers are small clusters whitish green or red. Meanwhile the seeds are very small, black or red brown.

Collector: Yeoh Loo Yew Identified by: Dr. Rahmad Zakaria

Picture of plant

APPENDIX B

Herbarium No.:11760

Picture of herbarium

Picture of Label

USM Herbarium
Pusat Penggajian Sains Kajihayat
Universiti Sains Malaysia

Family: **Pandanaceae**

Genus Species : *Pandanus amaryllifolius Roxb.*
Date : 11/12/2018
Locality : Ulu Kampar Agro Perak.
Habitat :

Notes : Evergreen herb shrub grown (1-1.6 m tall) or small tree (2 - 4.5 m tall) The tree form of this species has an erect trunk with little no branching (15 cm wide) produces thick aerial roots. leaves are linear with an entire leaf margin and acute apex (2 - 5 cm wide; 25 - 75 cm long). Leaves of the tree form are the same shape, but about twice the size (7-9 cm wide;150-220 cm long).Leaves have a slightly pleated surface; a cross-section of the leaf is shaped like the letter "W" turned upside down. Leaves are spirally arranged. The female inflorescence has not been observed, while the male inflorescence is very rare and only found on the tree form of this species. The male inflorescence is a spike of flowers with a white spathe.

Collector : Yeoh Loo Yee — Identified By : Dr. Rahmad Zakaria

Picture of plant

APPENDIX C

Herbarium No.:11762

Picture of herbarium

Picture of Label

USM Herbarium
Pusat Pengajian Sains Kajihayat
Universiti Sains Malaysia

Family: **Arecaceae**
Genus/Species: *Elaeis guineensis Jacq.*
Date: 26/02/2018 Altitude:
Locality:

Habitat: Grown in large plantation below 50m elevation occur wild in riverine forest or in freshwater swamps.

Notes: Evergreen shrubs grow up to 20m or more at maturity. The trunk is characterized by persistent, spirally arranged leaf, numerous, erect spreading to drooping, reaching 3-6m in adulteness, leaf stalk short with broad base. Spiny fibrous projection exist along the leaf margin from the leaf sheath. Leaf blades have numerous (100-160 pairs) It has many flowers crowded on short branches that develop into large cluster of oval fruit, some 4cm when ripe. The fruit are black with a red base and featured a single oily seed.

Collector: Yeoh Low Yeow Identified by: Dr Rahmad Zakaria

Picture of plant

APPENDIX D

Herbarium No.:11761

Picture of herbarium

Picture of Label

USM Herbarium
Pusat Pengajian Sains Kajihayat
Universiti Sains Malaysia

Family: **Araceae**
Genus/Species: *Pistia stratiotes*
Date: 12/02/2018 Altitude:
Locality:

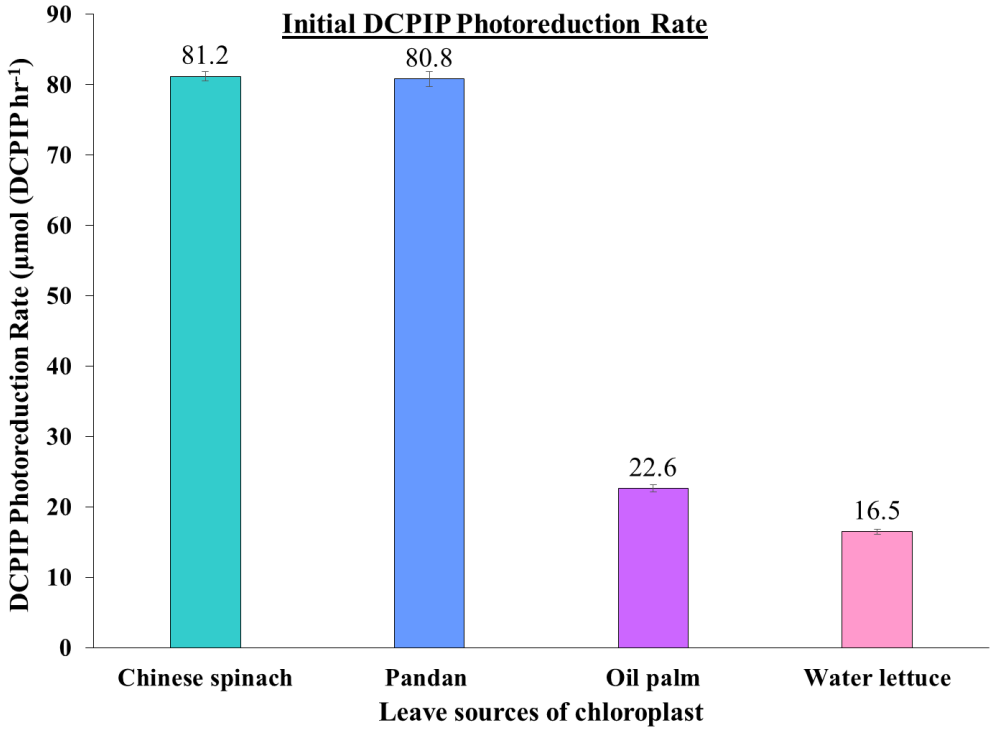
Habitat: A floating aquatic plant. Easily grow on still water occurs on surface of pond, lake and drain.

Notes: A perennial monocotyledon. Free floating fast-Hender aquatic plant with soft thick, rosettes (6-8" across) of wedge-shaped, or enlarging, fluted, velvety, soft green leaves. Measured 2-15cm with repellant. Parallel veins, wavy margins. Tubular, axillary, arum-like, yellowish-green to creamy white flowers. Flowers are dioecious and are hidden in the middle of the plant.

Collector: Yeoh Loo Yew Identified by: Dr. Fahmiel Zakaria

Picture of plant

APPENDIX E


Initial DCPIP Photoreduction Rate
DCPIP Photoreduction Rate (µmol (DCPIP hr-1)
90
80
70
60
50
40
30
20
10
0
81.2
80.8
22.6
16.5
Chinese spinach
Pandan
Oil palm
Water lettuce
Leave sources of chloroplast

APPENDIX F

Final BSA concentration (µg/mL)	Mean of Absorbance
0	0.000
25	0.147
50	0.284
75	0.403
100	0.498