MORPHO-PHYSIOLOGICAL AND PROTEOME CHANGES AND INFLUENCE OF EXOGENOUS APPLICATION OF OIL PALM WOOD VINEGAR (OPWV) ON THE GROWTH OF DROUGHT-STRESSED *PANDANUS AMARYLLIFOLIUS*

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THESIS SUBMITTED IN FULFILLMENT OF THE REQUIREMENT FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

INSTITUTE FOR ADVANCED STUDIES

UNIVERSITI MALAYA

KUALA LUMPUR

2023

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

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Name of Degree : **DOCTOR OF PHILOSOPHY**

Title of Thesis ("This Work"): **MORPHO-PHYSIOLOGICAL AND PROTEOME CHANGES AND INFLUENCE OF EXOGENOUS APPLICATION OF OIL PALM WOOD VINEGAR (OPWV) ON THE GROWTH OF DROUGHT-STRESSED *PANDANUS AMARYLLIFOLIUS***

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MORPHO-PHYSIOLOGICAL AND PROTEOME CHANGES AND INFLUENCE OF EXOGENOUS APPLICATION OF OIL PALM WOOD VINEGAR (OPWV) ON THE GROWTH OF DROUGHT-STRESSED *PANDANUS AMARYLLIFOLIUS*

ABSTRACT

Drought is a significant threat to the agricultural sector, as it can negatively impact plant growth, yield, and survival. However, our understanding of how plants respond to drought stress and recover from it is limited. To address this knowledge gap, further research on the molecular regulation of drought stress signalling in plants is indispensable. Oil palm wood vinegar (OPWV), a waste product from the palm oil industry, potentially mitigates the adverse effects of drought stress on plants. This study hypothesised that drought stress would impact the morphology, physiology, biochemistry, and molecular responses of *P. amaryllifolius*, and exogenously applying OPWV would alleviate the adverse effects of drought stress. To test this hypothesis, *P. amaryllifolius* plants were exposed to drought stress for 0, 4, 7, 10 and 14 days before being sampled for the morphological, biochemical and protein response analyses. The results showed that drought stress caused a decreased leaf relative water content (LRWC) and chlorophyll content in *P. amaryllifolius.* In contrast, the relative electrolyte leakage (REL), proline and malondialdehyde (MDA) contents, and the activities of antioxidant enzymes in the drought-treated and recovered samples were relatively higher than in the well-watered sample. Several abundantly altered proteins of drought-stressed, well-watered, and recovered samples were identified using tandem mass tags (TMT)-based quantitative proteomics. Of the 1415 differentially abundant proteins, 74 were significantly altered. Most proteins differing between them were related to carbon metabolism, photosynthesis, stress response, and antioxidant activity. On the other hand, drought stress enhanced the expression of several drought-responsive genes, such as *heat shock protein 70* (*HSP70*), *thaumatin* (*Thau*), *enolase*

(*ENO*), *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) and *β-fructofuranosidase* (*β-Fruc*). Applying OPWV to drought-stressed *P. amaryllifolius* improved plant morphology, such as increased LWRC, stem circumference, root-to-shoot ratio, chlorophyll pigments, and reduced relative electrolyte leakage, leaf yellowing, and leaf folding percentage. OPWV treatment also decreased stress-related indicators, such as hydrogen peroxide, proline, and malondialdehyde, and increased the antioxidants activity, such as superoxide dismutase, catalase, peroxidase, ascorbate peroxidase and glutathione reductase. In addition, the relative expression of drought-responsive genes, *HSP70* and *Thau*, was significantly enhanced, while *ENO* and *β-Fruc* gene expressions were reduced under drought conditions with OPWV treatment. These findings support the idea that the exogenous application of OPWV enhances drought tolerance in *P. amaryllifolius* by effecting changes at both morphological and molecular levels. This study provides insights into morpho-biochemical and proteome changes in *P. amaryllifolius* under drought stress conditions and how OPWV impacts its drought tolerance. These data may provide a potential strategy for minimising growth inhibition due to abiotic stress in agricultural fields.

Keywords: *Pandanus amaryllifolius*, Crop Improvement, Drought Stress, Oil Palm Wood Vinegar, Proteomics.

PERUBAHAN MORFO-FISIOLOGI DAN PROTEOME SERTA KESAN APLIKASI CUKA KAYU KELAPA SAWIT (OPWV) TERHADAP PERTUMBUHAN *PANDANUS AMARYLLIFOLIUS* DALAM PERSEKITARAN KEMARAU.

ABSTRAK

Kemarau adalah ancaman besar kepada sektor pertanian kerana ia boleh memberi kesan negatif kepada pertumbuhan, hasil dan kemandirian tumbuhan. Walau bagaimanapun, pemahaman mengenai cara tumbuhan bertindak balas terhadap kemarau dan pulih daripadanya adalah terhad. Untuk menangani jurang pengetahuan ini, penyelidikan lanjut mengenai peraturan molekul bagi isyarat kemarau dalam tumbuhan adalah amat diperlukan. Cuka kayu kelapa sawit (OPWV), produk buangan daripada industri minyak sawit, berpotensi mengurangkan kesan buruk kemarau pada tumbuhan. Kajian ini membuat hipotesis bahawa kemarau akan memberi kesan kepada morfologi, fisiologi, biokimia, dan tindak balas molekul *P. amaryllifolius*, dan penggunaan OPWV secara eksogen akan mengurangkan kesan buruk tekanan kemarau. Hipotesis dalam kajian ini adalah kemarau akan memberi kesan kepada morfologi, fisiologi, biokimia, dan tindak balas molekul *P. amaryllifolius*, dan penggunaan cuka kayu kelapa sawit secara luaran akan dapat mengurangkan kesan buruk kemarau. Untuk menguji hipotesis ini, tumbuhan *P. amaryllifolius* telah didedahkan kepada kemarau selama 0, 4, 7, 10 dan 14 hari sebelum sampel diambil untuk analisis tindak balas morfologi, biokimia dan protein. Keputusan menunjukkan bahawa kemarau menyebabkan kandungan air relatif daun (LRWC) dan kandungan klorofil berkurangan dalam tumbuhan *P. amaryllifolius*. Sebaliknya, kandungan kebocoran elektrolit relatif (REL), prolin dan malondialdehid (MDA), dan aktiviti enzim antioksidan dalam sampel yang dirawat dan pulih kemarau adalah lebih tinggi daripada sampel yang disiram dengan baik. Beberapa protein yang bertindakbalas secara berbeza bagi sampel yang didedah kepada kemarau, disiram dengan baik dan pulih

daripada kemarau telah dikenal pasti dengan menggunakan kaedah proteomik kuantitatif berasaskan *tandem mass tag* (TMT). Daripada 1415 protein yang dikenalpasti, 74 daripadanya telah bertindakbalas dengan ketara. Kebanyakan fungsi protein-protein ini adalah berkaitan dengan metabolisma karbon, fotosintesis, tindak balas tekanan, dan aktiviti antioksidan. Sebaliknya, kemarau meningkatkan pengekspresan beberapa gen yang bertindak balas terhadap kemarau, seperti protein kejutan haba 70 (*HSP70*), thaumatin (*Thau*), enolase (*ENO*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) dan β-fructofuranosidase (*β-Fruc*). Penggunaan OPWV pada *P. amaryllifolius* yang didedahkan kepada kemarau telah memberi kesan kepada morfologi tumbuhan, seperti peningkatan LWRC, lilitan batang, nisbah akar-ke-pucuk, pigmen klorofil, dan mengurangkan kebocoran elektrolit relatif, kekuningan daun dan peratusan lipatan daun. Rawatan OPWV juga mengurangkan petanda bio berkaitan tekanan, seperti hidrogen peroksida, prolin, dan malondialdehid dan meningkatkan aktiviti antioksidan seperti 'superoxide dismutase', 'catalase', 'peroxidase', 'ascorbate peroxidase' dan 'glutathione reductase'. Di samping itu, pengekspresan relatif gen responsif kemarau, *HSP70* dan *Thau*, telah meningkat secara ketara manakala pengekspresan gen *ENO* dan *β-Fruc* berkurangan di bawah keadaan kemarau dengan rawatan OPWV. Penemuan ini menyokong idea bahawa aplikasi secara luaran OPWV meningkatkan toleransi kemarau *P. amaryllifolius* dengan mengubah kawalatur morfologi dan molekul tumbuhan tersebut. Kajian ini memberikan pemahaman baru tentang perubahan morfo-biokimia dan proteome dalam *P. amaryllifolius* dan bagaimana OPWV memberi kesan kepada toleransi kemarau tumbuhan tersebut. Data ini mungkin menyediakan strategi yang berpotensi untuk meminimumkan perencatan pertumbuhan dalam bidang pertanian akibat kemarau.

Kata kunci: *Pandanus amaryllifolius*, Kemarau, Cuka Kayu Kelapa Sawit (OPWV), Ketahanan Kemarau, Proteomik.

ACKNOWLEDGEMENT

In the name of Allah, The Most Gracious and The Most Merciful. I am incredibly grateful to have completed my PhD. Without His grace, this accomplishment would not have been possible. Alhamdulillah for the strength and perseverance to overcome the challenges that came with this academic pursuit. I am truly blessed to have had the opportunity to pursue higher education and I am thankful for the knowledge and skills that I have gained throughout the process. I am looking forward to utilizing this knowledge to make a positive impact in my field and in the world.

I would like to express my most sincere gratitude and appreciation to all those who have helped me in any way throughout the years. First and foremost, I am deeply grateful to my main supervisor, Dr. Tan Boon Chin, for his outstanding guidance and support throughout my research journey, from the selection of the title to the presentation of the results. His vast knowledge, motivation, and patience have given me the strength and determination to excel in my research writing. Without his guidance, conducting an academic study on such a challenging topic would not have been as easy. He has been a mentor and an exceptional advisor for my doctoral studies, going above and beyond. Additionally, I would like to express my appreciation to my co-supervisors, Dr. Fiqri Dizar Khaidizar and Assoc. Prof. Wan Mohd Aizat Wan Kamaruddin, for their encouragement and valuable suggestions. They played a crucial role in refining my research writing skills, and their guidance will remain with me throughout my life.

I am also pleased to extend my gratitude to Dr. Aaron Teo Wei Fei and Ms Lau Su Ee for teaching me crucial research techniques and being always helpful in brainstorming and resolving any difficulties encountered in conducting experiments. They have also been incredibly supportive in the labour-intensive and time-consuming task of harvesting the plants, without which this research would not have been possible.

I will always remember their valuable contributions. I would also like to acknowledge and thank my fellow labmates, Ms Teoh Ee Yang, Ms Norhafizah Sidek, Mr Karlson Chou Khai Soong, Ms Nur Zawani Nordin, Ms Leh Lin San, Ms Imène Tatar, Ms Sheueni Ong, Ms Alicia Ng Jia Huey, and Ms Dharane Ravi for the enjoyable times we shared, support to each other and for stimulating discussions about life and research.

I would like to express my deepest appreciation and gratitude to my wife, Nurul Syuhada Jamhari, my parents, Kasmiyati Katiman and Mohd Amnan Sukiman and my family for their unwavering love and support throughout my PhD journey. My wife has been my rock and my constant source of motivation, providing emotional and practical support with every step of the way. I could not have accomplished this without her. My parents and my family have also been my support system, always encouraging me and believing in me, even during the most challenging times. Their love and support have been invaluable, and I am forever grateful to have them in my life. I would not have been able to complete this journey without their love and support. Thank you from the bottom of my heart.

Lastly, I am extremely grateful to have received the Graduate Excellence Programme (GrEP) for my PhD studies. The financial aid provided by the program has been crucial in allowing me to concentrate on my research and complete my degree. Without this support, I may not have had the opportunity to pursue my interest in higher education. I would like to extend my heartfelt thanks to MARA for making this scholarship possible. Thank you for investing in my education and for supporting me in achieving my academic goals. The GrEP financial assistance will always be an invaluable asset in my journey, and I am deeply appreciative of the opportunity.

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LIST OF ABBREVIATIONS

Abbreviation	Description
$	dollar
%	percentage
°C	degree Celcius
µg	microgram
µL	microlitre
µM	micromolar
µS	microsiemens
1D	1-dimension
$^1O_2$	singlet oxygen
2-AP	2-acetyl-1-pyrroline
2D	2-dimension
2PGA	D-2-phosphoglycerate
A-	anion
AA	anthranilic acid
ABA	abscisic acid
ABF	ABRE binding factors
Act	actin
ADP	adenosine diphosphate
ANJ1	46.6-kD polypeptide
ANOVA	analysis of variance
APS	ammonium persulfate
APX	ascorbate peroxidase
AREB	ABA-responsive element binding protein

AsA	ascorbate
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
Ca^{2+}	calcium ions
CAT	catalase
cDNA	complimentary deoxyribose nucleic acid
CE-MS	capillary electrophoresis-mass spectrometry
CHAPS	3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate
Ci	initial electrical conductivity
Cl^-	chloride ion
Cmax	conductivity of lysed cells
CO_2	carbon dioxide
CRISPR-Cas	clustered regularly interspaced short palindromic repeats-Cas
Ct	cycle treshold
CTAB	Cetyl trimethylammonium bromide
Cyt	cytosol
D	drought
DCM	dichloromethane
DHA	dehydroascorbate
DIGE	Differential gel electrophoresis
DNA	deoxyribose nucleic acid
DSS	decision support system
DT	drought + OPWV
DTT	dithiothreitol
DW	dry weight

EDTA	disodium ethylenediaminetetraacetic acid
EF1	elongation factor 1
EFB	empty fruit bunch
ENO	enolase
ESI-MS	electrospray ionization mass spectrometry
F1	6-day intervals
F2	3-day intervals
F3	1-day interval
FAO	Food and Agriculture Organization
FDR	false discovery rate
FT-ICR	Fourier-transform ion cyclotron resonance
FTICR-MS	Fourier-transform ion cyclotron resonance mass spectrometry
FW	fresh weight
g	gram
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GB	glycine betaine
GC-MS	gas chromatography-mass spectrometry
GDP	gross domestic product
GIS	geographic information system
GM	genetically modified
GMO	genetically modified organism
GO	gene ontology
GPS	global positioning system
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione

GUI	graphical user interface
GWAS	genome wide analysis study
h	hour
H^+	hydrogen ion
H_2O	water
H_2O_2	hydrogen peroxide
HCl	hydrocloric acid
HPO_4^{2-}	hydrogen phosphate
HSP70	heat shock protein 70
IAA	indole-3-acetic acid
IEF-MS	isoelectric focusing-mass spectrometry
IPCC	Intergovernmental Panel on Climate Change
iTRAQ	isobaric tags for relative and absolute quantification
IWM	irrigation water management
K^+	potassium ion
KAT1	potassium channel protein 1
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC	liquid chromatography
LC-MS/MS	liquid chromatography with tandem mass spectrometry
LiCl	lithium chloride
LPR1	low density lipoprotein receptor-related protein 1
LRWC	leaf relative water content
M	molar
MALDI	matrix-assisted laser desorption/ionization
MAPS-MS	Multi-Affinity Purification-Mass Spectrometry
MDA	malondialdehyde

MDHA	monodehydroascorbate
MDHAR	monodehydroascorbate reductase
MF	mesocarp fibre
mg	milligram
min	minute
mL	millilitre
mm	millimeter
MS	mass spectrometry
NADH	nicotinamide adenine dinucleotide
$NADP^+$	nicotinamide adenine dinucleotide phosphate
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NBT	nitro blue tetrazolium
NCBI	National Center for Biotechnology Information
ng	nanogram
NI	no information
nM	nanomolar
NO^{3-}	nitrate
NOAA	National Oceanic and Atmospheric Administration
NPK	nitrogen phosphate potassium
O_2	oxygen
$O_2^{\bullet-}$	superoxide
OAT	ornithine-delta-aminotransferase
$^{\bullet}OH$	hydroxyl radical
OPWV	oil palm wood vinegar
P5C	pyrroline-5-carboxylate
P5CR	pyrroline-5-carboxylate reductase

P5CS	pyrroline-5-carboxylate synthetase
Pa	*Pandanus amaryllifolius*
PAGE	polyacrylamide gel electrophoresis
PAHs	polycyclic aromatic hydrocarbons
PCD	programmed cell death
PCR	polymerase chain reaction
PEP	phosphoenolpyruvate
PGPR	plant growth-promoting rhizobacteria
PIN	PIN-FORMED
PKO-p	palm kernel oil polyol
PKS	palm kernel shell
POD	peroxidase
POFA	palm oil fuel ash
POME	palm oil mill effluent
PP2C	protein phosphatase 2C
PR	pathogenicity-related
PR5	pathogenicity-related 5
PSM	peptide-spectrum match
PTM	post translational modification
PUFA	polyunsaturated fatty acid
PVP	polyvinyl pyrrolidone
PYL	pyrabactin resistance-like
PYR	pyrabactin resistance
qPCR	quantitative real-time polymerase chain reaction
RCAR	regulation component of ABA receptors
REL	relative electrolyte leakage

RM	Ringgit Malaysia
RNA	ribonucleic acid
ROS	reactive oxygen species
RP	reverse phase
rpm	revolution per minute
RSPO	Roundtable on Sustainable Palm Oil
RT	reverse transcriptase
RWC	relative water content
SDS	sodium dodecyl sulfate
SLAC1	slow anion channel 1
SnRK2	SNF1-related protein kinases
SOD	superoxide dismutase
SP-MS	synthetic peptide-based mass spectrometry
SPSS	Statistical Package for the Social Sciences
SW	saturated weight
SWAMP	Soil Water Management for Paddies
TBA	thiobarbituric acid
TCA	tricarboxylic acid cycle
TCEP	tris (2-carboxyethyl) phosphine
TEMED	tetramethyl ethylenediamine
Thau	thaumatin
TLP	thaumatin-like-protein
TMT	tandem mass tag
TOF	time of flight
USD	United states dollar
v/v	volume/volume

w/v	weight/volume
WV	wood vinegar
WW	well-watered
WWT	well-watered + OPWV
β-Fruc	β-Fructofuranosidase

CHAPTER 1

INTRODUCTION

Climate change often leads to increased frequency and severity of drought in various agricultural regions. Drought is defined as a condition in which there is a persistent water shortage in the soil due to low levels of precipitation and high evaporation rates (Haile *et al*., 2020). It can threaten plant growth and adversely impact global crop production (Surendran *et al*., 2017). For example, the drought in the United States from 2011 to 2013 resulted in losses of approximately USD 7.6 billion in the agricultural sector (Ray *et al*., 2018). China also reported approximately USD 30 billion in losses due to drought in 2010 (Zhang & Zhou, 2015). If this trend continues, the rising demand for food crops could lead to food insecurity, given the forecasted global population of 10 billion by 2050. Therefore, it is crucial to understand how plants respond and adapt to drought to enhance crop performance under drought stress. This aligns with the United Nation's Sustainable Development Goals, such as the eradication of poverty and hunger.

Drought stress negatively affects plant growth and development, including disruptions to cell membranes, reduced photosynthesis, decreased biomass, and reduced yield. In order to adapt to drought stress, plants may alter their physiology by reducing cell division and development and modifying leaf size and root distribution (Alqurashi *et al*., 2018). Other morpho-physiological changes include reduced relative water content and relative chlorophyll content, wilting, and yellowing of leaves (Sattar *et al.*, 2020; Zhu *et al.*, 2020). In addition to these physiological changes, plants have developed various strategies to cope with drought stress. For example, plants can sense external stimuli through sensors and transmit signals via complex signal transduction pathways, leading to changes in gene expression. Several signal molecules and genes, such as abscisic acid (ABA), reactive oxygen species (ROS), nitric oxide, and soluble sugars, have been shown

to play critical roles in plant responses to drought stress. Therefore, understanding the mechanisms that allow plants to respond and adapt to the adverse effects of drought stress is crucial for improving our understanding of plant responses to this environmental stressor.

Proteomics technologies have revolutionised our understanding of plants' responses and adaptations to drought stress. These techniques allow the study of proteins and their modifications, which play crucial roles in cellular processes. For instance, Cao *et al.* (2017) utilised proteomics approaches to identify 95 drought-responsive proteins in wild peaches. These proteins were categorised into stress and defence, nutrient and energy metabolism, signalling, translation, and transport proteins. This comprehensive analysis revealed novel insights into the underlying mechanisms of drought tolerance in plants (Cao *et al*., 2017). Another study by Azri *et al.* (2020) identified 18 proteins crucial for grapevine drought tolerance using proteomics approaches. Their findings suggest that these proteins could be targeted to enhance the drought tolerance of grapevines. Similarly, proteomics approaches have also been applied to other plant species, such as chickpeas (Gupta *et al*., 2020), tomatoes (Çelik *et al*., 2021), and soybeans (Yahoueian *et al*., 2021). Overall, proteomics technologies have significantly advanced our knowledge of plant proteins and their roles in drought responses and adaptations. Furthermore, these techniques have demonstrated their potential in identifying novel targets for crop improvement programmes to enhance drought tolerance.

Wood vinegar is a reddish-brown translucent liquid produced as a by-product of pyrolyzing wood at temperatures ranging from 200 to 450°C in low oxygen conditions. The smoke generated during this process is channelled into a condensation tube, forming a condensed, smoked liquid (Wei *et al*., 2010). Wood vinegar contains several beneficial compounds. For example, Yang *et al.* (2016) found that the primary compounds in wood vinegar derived from lychee are 2,6-dimethoxyphenol (syringol), 2-methoxyphenol

(guaiacol), and 3,5-dimethoxy-4-hydroxytoluene (pyrogallol). In contrast, acetic acid, hydroxyacetone, and hydroxyacetaldehyde have been identified as the main components in wood vinegar derived from poplar, pine, forest pine wood, and pruning litter (Aguirre *et al*., 2020). Oramahi *et al.*, (2018) found that phenol, acetic acid, and 1-hydroxy-2-propanone are the main components in wood vinegar derived from oil palm trunks.

Wood vinegar has been used in agriculture as a plant growth stimulant, fungicide, and herbicide (Gao *et al.*, 2020; Liu *et al.*, 2021; Zhang *et al.*, 2020). It is an organic and environmentally friendly product that has gained popularity recently. It has been shown to positively affect plant growth and development, making it a promising alternative to synthetic chemical fertilisers and pesticides. Several studies have demonstrated its potential to improve plant performance and growth (Feng *et al.*, 2020; Vannini *et al.*, 2021; Zhu *et al.*, 2021). For example, Wang *et al.* (2019) found that wood vinegar enhanced wheat tolerance to drought by increasing ABA accumulation, boosting antioxidant activity, and reducing ROS. However, research on the potential of wood vinegar to alleviate plant tolerance to abiotic stresses is limited. As the world's population continues to grow, coupled with worsening climate, there is an urgent need to improve crop yields and ensure food security. Wood vinegar's potential to enhance plant performance and growth could have significant implications for sustainable agriculture and, thus, serve as one of the efforts to improve plant performance under stress.

Oil palm wood vinegar (OPWV) is a waste product of the palm oil industry that is abundant in Malaysia. Using this waste product to produce wood vinegar can reduce waste and create a sustainable value chain for the palm oil industry. Furthermore, the underutilisation of OPWV represents a missed opportunity to create a valuable resource from a waste product. Therefore, this study aimed to evaluate the potential of wood vinegar produced from oil palm kernel shells in reducing the adverse effects of drought stress on plants.

Pandanus amaryllifolius is a monocot from the Pandanaceae family that is well-known for its scented leaves. It is widely cultivated in tropical and subtropical regions. *P. amaryllifolius* has several local names, such as pandan (Malaysia), pandan mabango (Philippines), and toei hom (Thailand). In these regions, *Pandanus* is widely used in culinary as flavouring, colouring, and aroma. In addition to its aromatic value, the leaves of *P. amaryllifolius* are also packed with beneficial phenolic compounds. Hence, *Pandanus* was used as herbal medicine to treat diabetes, fever, jaundice, and hypoglycaemia (Wakte *et al*., 2012). A recent study on *P. amaryllifolius* demonstrated the antioxidant and antimicrobial properties of its leaf extract against mouth infection and squamous carcinoma cells (Suwannakul *et al*., 2018). However, despite its economic value, little is known about its ability to tolerate drought stress. This study aimed to determine the drought tolerance mechanisms of *P. amaryllifolius* through proteomics analysis and to fill in the literature gap on its drought stress response. As *P. amaryllifolius* comes from the same genus as other *Pandanus* species known to tolerate drought stress, it is likely that this species may possess similar traits that allow it to survive in low-precipitation areas. This study not only helps to understand plant drought tolerance mechanisms, the findings from this study, particularly the OPWV, might have practical implications for sustainable agriculture, which is vital for addressing the impacts of climate change on agriculture.

1.1 Objectives

Understanding the drought-responsive mechanism of plants is vital for developing climate-resilient crops. However, as of the current knowledge, there are gaps in the literature on *P. amaryllifolius* drought stress and the effects of OPWV application in alleviating drought stress. Hence, the current study aimed to:

1. analyse the impacts of drought stress on the morphological, physiological, biochemical, and proteome levels of *P. amaryllifolius,*
2. analyse the expression of drought-responsive genes in drought-treated *P. amaryllifolius* and
3. determine if OPWV could alleviate the adverse effects of drought stress on plants.

CHAPTER 2

LITERATURE REVIEW

2.1 Agriculture in a changing climate

2.1.1 **Status of climate change**

Climate change refers to the long-term changes in the Earth's climate, including temperature, precipitation, and extreme weather events. The scientific consensus is that climate change is primarily caused by human activities, such as burning fossil fuels, which releases greenhouse gases into the atmosphere, leading to an increase in global temperature. As of 2021, the Earth's average temperature has risen by about 1.1 °C since the pre-industrial era, and the warming trend is projected to continue (Raven, 2022). This warming leads to various impacts, including more frequent and severe heat waves, droughts, heavy precipitation events, rising sea levels, melting glaciers and ice sheets, and changes in precipitation patterns. Consequently, these changes affect ecosystems, agriculture, water resources, human health, and infrastructure worldwide, leading to the displacement of people, food, and water scarcity, increasing in diseases, and destructing habitats and extinction of species. Hence, climate change is a global problem that requires collective action.

The Intergovernmental Panel on Climate Change (IPCC) has stated that without significant global action to reduce emissions, the Earth's average temperature is projected to rise by another 2 °C or more by the end of the century, which would have severe consequences for human and natural systems (Kemp *et al*., 2022). It's worth noting that 2021 had the sixth-highest global surface temperature since 1880, which was 0.84 ºC above the global average temperature (NOAA, 2022). The changes brought about by climate change have made its effects more prominent, and their impact is already being

felt worldwide. Over the past few decades, it has become increasingly apparent that the climate has grown increasingly volatile and unpredictable. This has had far-reaching consequences for the United States, with one of the most notable being the rise in both the frequency and severity of storms and hurricanes. These devastating natural disasters can wreak havoc on everything from homes and businesses to entire communities, leaving in their wake a trail of destruction and economic loss. According to a report released by the United Nations in 2021, the economic cost of the storms and hurricanes that battered the United States between the years 1992 and 2017 was nothing short of staggering, totalling a massive USD 500 billion (UN, 2021).

Climate change is projected to increase the frequency of droughts in many regions of the world due to changes in precipitation patterns and higher temperatures. It is expected to cause more intense and longer droughts in many regions of the world, particularly in the Mediterranean, the Sahel, the western United States, and Australia (Hoegh-Guldberg *et al*., 2019). These regions are projected to experience more frequent and severe droughts because of rising temperatures and changes in precipitation patterns. Drought frequency can also be affected by human activities, such as land-use change, which can lead to the degradation of soil and vegetation and reduce the ability of landscapes to retain water. According to a report by the World Wildlife Fund (WWF), the risk of drought is worsening and affecting about 55 million people annually, which suggests that over 1.5 billion people around the world are currently living in areas experiencing water scarcity, with droughts causing severe economic losses and damaging ecosystems (WWF, 2019). This sobering statistic serves as a stark reminder of the real-world impact that climate change is having on the country and underscores the need for swift action to address this pressing issue.

2.1.2 **Impact of climate change on agriculture**

Drought stress due to climate change is one of the major environmental stresses. The occurrence of flash drought or rapid intensification of drought from 1980 to 2015 has had significant impacts on agriculture, water sources, and ecosystems in global hotspots, such as India, China, Brazil, and the United States (Christian *et al*., 2021). The percentage of agricultural drought occurrence was anticipated to be increased by 24% if the global average temperature increased to 1.5 ºC (Arnell *et al*., 2019). These drought events have added pressure on agricultural sectors to increase food production to ensure global food security in the coming decades. However, the unpredictable nature of the climate has had adverse effects on many agricultural sectors, resulting in global agricultural losses due to drought of approximately $37 billion, with Africa alone losing around $14 billion from 2008 to 2018 (FAO, 2021). In addition, China experienced an average annual loss of $4.2 billion due to drought from 1986-2005, which is projected to increase to $47 billion if the global temperature rises by 1.5 ºC (Su *et al*., 2018).

Due to the inadequacy of current farming practices to meet the global food demand and address the vulnerability of agriculture to climate change, it is crucial to implement significant innovations to ensure sustainable crop production and food security. Furthermore, with the rapidly growing human population, there is a pressing need to address the question of whether it is possible to produce sufficient quality food in a sustainable manner using limited resources. This challenge requires significant research and development efforts in the field of agriculture to ensure food security for future generations.

2.1.3 **Climate change in Malaysia**

Malaysia is a tropical country located in Southeast Asia, one of the most vulnerable countries in the region to the impacts of climate change. The country is already experiencing the effects of climate change, including more frequent and severe heat waves, droughts, heavy precipitation events, and sea level rise. For instance, in the last two decades, Malaysia has experienced irregularities in warming and precipitation (Tang, 2019), affecting ecosystems, agriculture, water resources, human health, and infrastructure.

According to the Climate Risk Country Profile report of Malaysia, the regions of Peninsular Malaysia, Sabah, and Sarawak saw an increase in mean surface temperature of 0.14 °C to 0.25 °C per decade from 1970 to 2013 (The World Bank Group & Asian Bank Development, 2021). During the same period, the maximum surface temperatures in these regions increased by 0.17°C to 0.22°C per decade, while the minimum surface temperatures increased by 0.20°C to 0.32°C per decade (The World Bank Group & Asian Bank Development, 2021). This means that the overall temperatures are getting warmer, and the hottest temperatures and lowest temperatures are also increasing. These temperature changes have significant implications for the region, including impacts on agriculture, water resources, and public health. For instance, Malaysia experiences high maximum temperatures regularly, with an annual maximum of daily maximums averaging around 33°C. However, the current median probability of a heat wave (defined as a period of three or more days where the daily temperature is above the long-term 95th percentile of daily mean temperature) is very low, around 2% (Climate Change Knowledge Portal, 2021).

These circumstances have allowed meteorological and hydrological droughts to occur and severely impact Malaysia's agricultural and industrial sectors. Meteorological

drought, associated with a precipitation deficit, and hydrological drought, associated with a deficit in surface and subsurface water flow, can result in agricultural drought, affecting crop productivity. Malaysia experiences a median probability of severe meteorological drought of around 4% annually, as defined by a standardised precipitation evaporation index (SPEI) of less than -2 (Climate Change Knowledge Portal, 2021). For example, Tan *et al*. (2017) reported that the Kelantan River Basin is particularly vulnerable to severe droughts, often during El Niño events. These severe drought events have caused crop losses and negative impacts on freshwater supply, as seen in the case of the 2014 drought that affected 8,000 paddy farmers and caused USD 22 million in crop losses (Tan *et al*., 2017).

Climate change has also impacted the region's weather patterns in Malaysia. According to Mayowa *et al*. (2015), the trends of rainfall on the east coast of Peninsular Malaysia between 1970 to 2010 have shown a significant increase in annual rainfall and during the monsoon period, which is a seasonal wind system that brings more heavy rainfall more significant than 20 mm to the region between November and March. Additionally, the increase in heavy rainfall days can lead to flooding, which has implications for infrastructure, transportation, and public safety. In 2010, it was estimated that the number of people affected by flooding annually in Malaysia, taking into account protection for events that occur once every 25 years, was around 130,000, whereby the anticipated yearly costs associated with damages from flooding were estimated to be approximately $1.8 billion (Climate Change Knowledge Portal, 2021).

2.1.3.1 **Malaysia agriculture**

To better understand how the Malaysian agriculture industry fared, it's important to examine the current agricultural landscape in Malaysia. Despite being heavily reliant on the agricultural sector for economic development since the 1950s, Malaysia's agricultural industry has experienced a decline, as evident from the decrease in agricultural product exports by 9.5% in 2018 compared to RM126,492 million in 2017, according to the Department of Statistic Malaysia, (2019). Although there was an increase in total agricultural exports from 2019 (RM115.5 billion) to 2020 (RM118.6 billion), the sector's total imports also rose, as reported by the Department of Statistic Malaysia, (2020).

The agricultural sector plays a significant role in the Malaysian economy, accounting for 7.4% of the overall Gross Domestic Product (GDP) in 2020, as reported by the Department of Statistics Malaysia, (2021). However, despite its significance, the sector's growth rate has decreased by 2% compared to the previous year. Several factors have impacted the agricultural sector and food production systems, including climate change, land degradation, water scarcity, the COVID-19 pandemic, and, more recently, armed conflicts or war.

In the agricultural sector, Malaysia produces a wide range of crops, including fruits, vegetables, spices, and various cash crops. According to the contribution to the country’s agricultural output, oil palm is the most significant crop produced in Malaysia, covering approximately 5.9 million hectares of land. In 2020, Malaysia produced 19.5 million metric tonnes of crude palm oil (CPO) and was the second-largest producer of palm oil in the world after Indonesia (Department of Statistics Malaysia, 2021). The oil palm sector contributed about RM 34.8 billion to Malaysia’s gross domestic product in 2021 (Department of Statistics Malaysia, 2022), indicating its importance to the country.

Malaysia is also the third-largest producer of natural rubber in the world, with rubber plantations covering approximately 1.04 million hectares of land. Of which the Department of Statistics Malaysia (2022) reported 469,669 tonnes of natural rubber was produced in 2021. In addition, paddy is also an essential crop in Malaysia, and the country produces various types of rice, including fragrant rice, glutinous rice, and ordinary rice. According to Zakaria & Nik Abdul Ghani (2022), the average rice production by state in Peninsula Malaysia, Sabah, and Sarawak was 3,770 million metric tonnes in 2018, while the average production from 2019 to 2020 was 7,136 million metric tonnes.

Malaysia's agricultural sector is also affected by the ever-worsening climate change. The unpredictable nature of the recent climate has impacted the important crops in Malaysia, such as rice, oil palm and rubber. Based on the analysis of the minimum and maximum yield over the last 28 years and macro cases of national data from 1980 to 2008, the study predicts that the yield of paddy in Malaysia would decrease between 43% and 61% if there was a 1 °C temperature increase and one millimetre (mm) rainfall increase (Alam *et al*., 2017). In addition, an increase in precipitation would cause the northern region of Peninsular Malaysia to be prone to flooding during the wet seasons, which lead to reduced paddy yields as previously recorded from 2015-2017, whereby rice yield was reduced up to 14% due to flooding (Firdaus *et al*., 2020). Moreover, a recent report by the Department of Statistics Malaysia (2023) in Malaysia rubber statistics December 2022 showed a decline in the production of natural rubber from 469,669 tonnes (2021) to 377,047 tonnes (2022). Increasing rainfall and flooding in recent years might contribute to this trend as rainy days lead to loss of tapping days.

The increase in global average temperature has also impacted the oil palm industry in Malaysia. Based on the report by Sarkar *et al*. (2020), oil palm production is negatively affected by climate change. Through multiple regression analyses of oil palm production and climate change data collected from 1980 to 2010, a 1°C increase in temperature could

lead to a 7.5% decrease in oil palm yield. For example, Khor *et al*. (2021) reported that El Niño events, which are associated with warmer and drier conditions in Southeast Asia, has significantly decreased oil palm yield in Malaysia. During the 2015-2016 El Niño event, oil palm yield in Malaysia decreased by an average of 16.6% compared to the previous year. The greatest declines in yield occurred in August, September, and October, which are typically the driest months of the year in Malaysia.

Malaysia has taken steps to address climate change, such as increasing the use of renewable energy, improving energy efficiency, reducing deforestation and land-use change, increasing carbon sequestration, and investing in climate-resilient infrastructure and communities. Between 2001 to 2017, there were multiple policies as mitigation strategies against climate change deployed by the Malaysian government related to environmental conservation, green technology, greenhouse gasses emission, oil palm sustainability and food security (Tang, 2019). The National Agrofood Policy 2021-2030, which was recently introduced, has identified four crucial subsectors for food security. The policy aims to promote the development of the agro-food sector over the next ten years. Among the key subsectors identified are fruits and vegetables. However, the country needs to continue working towards reducing its greenhouse gas emissions, sustainable agriculture and developing plans for climate change adaptation to mitigate the impacts of climate change on its economy, society and environment.

2.1.4 **Current agricultural strategy to cope with the adverse effects of climate change**

Many efforts have been made to transform the current agricultural practices to be more resilient to the impacts of a changing climate. These include plant breeding, advanced agricultural practices, modern biotechnology, and genome editing approaches.

2.1.4.1 **Plant breeding**

Plant breeding is one of the strategies to improve plant adaptation and tolerance against climate change. With changes in temperature, precipitation patterns and other environmental conditions, crops must be selectively bred to withstand these changes to maintain stable food production. One approach is to identify, select, and incorporate genetic traits from wild relatives of crops that can adapt to extreme environmental conditions or disease resistance via breeding. By repeatedly selecting plants with these desirable traits, breeders can create new varieties that are well-adapted to changing conditions. For instance, a cross-breed dwarfed rice with a large panicle and increased spikelet number produced 15-20% more yield than the semi-dwarfed variety (Panigrahi *et al*., 2019). However, despite its success in improving crop yields, plant breeding has several limitations. These include the availability of desirable genes in the breeding pool, the time and resources required for breeding programmes, and the challenges in predicting the performance of new plant varieties under varying environmental conditions (Hamdan *et al*., 2023). Additionally, the slow pace of traditional plant breeding methods has highlighted the urgent need for alternative approaches to plant breeding.

Marker-assisted breeding (MAB) has been widely used to speed up the plant breeding process. It is a plant breeding technique that combines traditional breeding with molecular biology tools to identify and select plants with desirable traits more efficiently and accurately. MAB involves three main steps: marker discovery, marker screening, and

marker-assisted selection. This technique is particularly useful for traits that are difficult or time-consuming to select using traditional methods, such as disease resistance, drought tolerance, or yield potential, and can reduce the time and cost required to develop new plant varieties. For example, genome sequencing and genetic markers can be used to identify specific genes responsible for stress tolerance, which can then be incorporated into new crop varieties through gene editing (Zhu *et al.*, 2020).

Recent evidence has shown the effectiveness of the MAB technique in developing multiple stress-tolerance plants in a stress-prone environment. Sandhu *et al*. (2019) reported a successful release of drought and submergence tolerance rice with pyramided multiple quantitative trait locus (QTLs) through MAB, which could produce a high yield despite being exposed to the respective abiotic stresses. Similarly, a salinity stress tolerance rice germplasm has been developed via single nucleotide polymorphism (SNP) marker-assisted selection. The *hst1* gene from "Kaijin" (salinity tolerant) variety was introgressed into a high-yielding "Yukinko-mai" variety resulting in a better performance of the salinity-tolerant germplasm with a significantly higher survival rate and increased shoot and root biomasses compared to wild-type under high salinity conditions (Rana *et al*., 2019).

Overall, plant breeding programmes have been used to increase crop performance under climate change scenarios. However, this method can be laborious and time-consuming, even aided by marker-assisted selection. Given the critical need for sustainable crop yield, the challenge faced by the scientific community is to enhance current technologies or develop novel approaches to increase crop productivity.

2.1.4.2 **Advanced agricultural practices (Integration with technology)**

Advanced agricultural practices integrated with technology can help farmers adapt to the impacts of climate change. These practices include using precise and climate-smart agricultural technology that can improve the efficiency of farming operations. This includes using a global positioning system (GPS), geographic information system (GIS) and remote sensing technologies to collect data on crop growth and soil conditions for optimising fertiliser and water applications. For instance, a web-based Geospatial Decision Support System (DSS) called Soil Water Management for Paddies (SWAMP), integrated with simple access graphical user interface (GUI), allows a user to easily monitor different views for the rice irrigation water management scheme (Mohd *et al*., 2014). This innovation also provides real-time information by visualising the presented results together with data on irrigation water demand and supply. The generated information can then be used to optimise water and fertiliser management, making informed decisions about planting, and harvesting times.

The utilisation of Unmanned Aerial Vehicles (UAVs) or drones revolutionised the agriculture industry to be more efficient, precise, and cost-effective. According to Ahirwar *et al*. (2019), UAVs or drones provide a less stressful working environment, better decision-making capabilities, and increased safety as they can fly for longer durations without human fatigue. Unlike traditional aircraft, the UAV does not require a qualified pilot, and it can carry out repetitive tasks and perform precise raster scans of regions, day, or night, even in unfavourable conditions, such as darkness or fog, under computer control. In addition to geological surveys, the UAV can capture visual or thermal imaging and measure cell phone, radio, or TV coverage over any terrain. The handoff of control from one operator to another is seamless, with no operational downtime. Furthermore, drones are capable of achieving pinpoint accuracy from much greater distances.

A recent report by Bhoi *et al*. (2021) has demonstrated the capability of UAVs equipped with a camera and Internet of Things (IoT) sensors to detect pests in rice fields. The authors collected the data using UAVs, including images of the rice crops and environmental data, such as temperature and humidity, and analysed them using an artificial intelligence (AI) algorithm. The AI algorithm was trained using deep learning techniques to identify the presence of pests in the rice fields. This allows the UAV system to achieve high accuracy in detecting rice pests, with an average precision of 95.2% and an average recall of 96.7%. Bhoi *et al*. (2021) highlighted that the UAV system effectively detects pests in real-time, allowing for timely intervention and pest control measures. Furthermore, the authors suggest that the system is cost-effective and efficient, providing a way to improve crop yield and reduce pesticide use in rice fields.

Apart from a monitoring system, UAVs could also be utilised as a pest control application. For instance, using a quadrotor UAV, Chen *et al*. (2020) investigated the effectiveness of different nozzle types for controlling planthoppers in two-stage rice. The study revealed that the type of nozzle used significantly affected the droplet deposition and control of planthoppers, with the rotary atomiser nozzle proving more effective than the standard hydraulic nozzle. The authors suggest that equipping a UAV with a rotary atomiser nozzle could offer an efficient and cost-effective way to control planthoppers in two-stage rice. However, further research is necessary to optimise the use of UAVs for crop protection in the development of precision agriculture, which utilises technology to improve crop production while minimising environmental impact. Nevertheless, the authors emphasise the potential of UAVs and advanced spraying technologies to enhance pest control in agriculture.

2.1.4.3 **Modern Biotechnology**

Another strategy to increase crop yield and food production is through modern biotechnology. This strategy enables the creation of crops that are better equipped to withstand the impacts of a changing climate. One such method of modern biotechnology is genetically modified organisms (GMOs). GMOs are plants whose genetic material has been altered through methods not naturally possible through mating or recombination. These technologies have the potential to overcome the limitations of conventional plant breeding. For example, Nguyen *et al.* (2019) successfully developed GM soybeans that can withstand soil salinity by introducing the dehydration-responsive element binding proteins (DREBs) genes into the plants. The transgenic plants exhibited higher levels of proline accumulation in response to salt stress, which was associated with reduced levels of oxidative stress and improved plant growth and survival under high salinity conditions.

Recently, Tang *et al*. (2019) investigated the potential of a MYB family gene, *OsMYB6*, to enhance the tolerance of rice to drought and salinity stress. The overexpression of the *OsMYB6* gene in the rice plants significantly increased their tolerance to drought and salinity stress (Tang *et al*., 2019). The transgenic rice plants showed higher survival rates and biomass accumulation under drought and salinity stress conditions compared to the control plants. Further analysis of the transgenic rice showed that they had increased levels of stress-related hormones such as abscisic acid (ABA) and proline, as well as higher activities of antioxidant enzymes like catalase, peroxidase, and superoxide dismutase. These changes are believed to have contributed to the enhanced stress tolerance of transgenic plants.

Another example of a GM plant with the MYB family gene was reported by Wu *et al*. (2019), which explores the potential of *ZmMYB3R*, a MYB transcription factor gene from maize, to enhance stress tolerance in *Arabidopsis thaliana*. The overexpression of

ZmMYB3R in *A. thaliana* improved the transgenic plants' tolerance towards drought and salt stress compared to wild-type plants. The transgenic plants showed higher survival rates, increased root length, and reduced water loss under drought stress conditions, as well as lower Na^+ accumulation and higher K^+/Na^+ ratios under salt stress conditions. These morphological changes were accompanied by higher levels of stress-related hormones, such as ABA and proline, as well as increased activities of antioxidant enzymes. Moreover, further transcriptome analysis has identified several stress-responsive genes that were upregulated in transgenic plants, including genes involved in ABA signalling and stress response.

As recombinant DNA technology advances, integrating multiple traits within a single GM plant becomes possible. This process is known as 'gene stacking'. This approach has become increasingly popular in GM crop production, particularly in the United States. In 2020, the cultivation of gene-stacked GM corn and GM cotton accounted for 86% and 87% of their respective total cultivation areas, compared to 4% and 33% in the year 2000 (Hamdan *et al*., 2022).

Despite its application benefits, developing GMO crops is controversial and highly debated. In addition, low public acceptance of GM crops and strict safety regulatory procedures have limited its development or entered the market. For example, a few countries, such as Switzerland, strictly restricted or legally prohibited GMO cultivation (Turnbull *et al*., 2021). In China, GM foods received a positive view from only 11.9% of the population. On the other hand, 41.4% and 46.7% held neutral and negative views, respectively (Cui & Shoemaker, 2018). Hence, transparency in the development, risk assessment, and regulatory processes for modern biotechnology tools and their products are critical for improving their applications in agriculture and fostering public trust.

2.1.4.4 **Genome editing**

Advances in genome editing have facilitated the development of crops with desirable traits. Unlike GM technology, genome editing tools employ sequence-specific nucleases to manipulate predetermined DNA sequences in the host plant genome, leveraging the inherent DNA repair mechanisms. Due to their specificity, genome editing has been utilised to enhance crop yield, tolerance to environmental stressors, and nutritional quality. Currently, there are four main genome editing systems, namely meganucleases, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced palindromic repeat (CRISPR)/CRISPR-associated protein (Cas) system.

ZFNs and TALENs consist of a FokI nuclease domain and a DNA-binding domain. For proper function, the FokI nuclease domain must be dimerised, requiring a specific design with appropriate spacing between the two domains. This specificity contributes to the effectiveness of ZFNs and TALENs, but their synthesis is often challenging and costly. Both technologies have been tested in several crops, such as soybean, sugarcane, maize, and wheat (Zhang *et al.*, 2018). However, despite their potential for crop improvement, several limitations of TALENs have hindered their applications. A key challenge is the inefficient delivery of the TALEN system into the target cells, owing to the large size of the cDNA encoding TALEN, which is approximately 3 kilobases in length. Additionally, the construction of TALE repeats remains a bottleneck, and the efficiency of TALENs in targeting specific genes is highly variable (Zhang *et al.*, 2018). In contrast, the implementation of ZFNs as a genome editing tool in crops is restricted due to the intricate and costly process of constructing the protein for each targeted site, as well as the possibility of inducing cytotoxicity from cleavage at unintended sites (Rasheed *et al*., 2021).

The advent of the CRISPR/Cas9 genome editing system has dramatically impacted the disciplines of functional genomics in both animal and plant biology. Derived from bacteria and archaea as a means of adaptive immunity, the CRISPR/Cas9 system has emerged as a powerful tool for targeted genome editing in both prokaryotes and eukaryotes. The CRISPR/Cas system utilises the complementary binding between a guide RNA (gRNA) and target DNA sequence to direct the cleavage of the target by the Cas nuclease (Hamdan *et al*., 2022). The gRNA-target complementarity and a protospacer-adjacent motif (PAM) are crucial for successful targeting. Compared to ZFNs and TALENs, which require protein-DNA interaction and reconstruction of large DNA sequences (500-1500 bp) for each new target site, CRISPR-Cas9 is more flexible (Cong *et al*., 2013). It can be applied to a wider range of target sites by simply modifying the 20-bp protospacer of the guide RNA in the gRNA plasmid while retaining the unaltered Cas9 protein (Cong *et al*., 2013). The simplicity and efficiency of the CRISPR system have made it the most widely used genome editing technology in various plant species, including model plants, food crops, industrial crops, and ornamental plants.

The CRISPR/Cas technology allows precision and efficient editing of the plant genome, enabling the introduction of desirable traits, such as increased drought tolerance, improved heat resistance, and enhanced nutritional value. For example, Zhang *et al.* (2019) successfully developed salinity-tolerant rice via CRISPR/Cas9 systems. The authors found that the CRISPR-edited rice showed higher plant height and biomass than the wild-type at the seedling stage when exposed to saline soil (Zhang *et al.*, 2019). A recent investigation revealed the significance of *OsNAC041* as a key transcription factor in the response of rice to salt stress. The utilisation of the CRISPR/Cas9 technique resulted in the creation of a targeted *osnac041* mutant, which displayed an increase in plant height compared to the wild-type (Bo *et al*., 2019).

Overall, CRISPR/Cas technology has the potential to play a critical role in the adaptation of crops to climate change since it can accelerate the breeding process, allowing for the rapid introduction of desirable traits into crops (Ahmar *et al*., 2020). However, there is still a need for further technological advancements, particularly concerning the accuracy of editing and the direct delivery of gene engineering agents. To enhance CRISPR delivery into the host genome, reducing the payload size of the delivery vehicle to allow for the transfer of CRISPR proteins through the cell is one strategy that can be employed. An alternative approach is to utilise a hypercompact CRISPR CasΦ system. The CasΦ protein, which has a molecular weight of approximately 70 kDa, is approximately half the size of the Cas9 and Cas12a enzymes (Pausch *et al*., 2020).

2.2 Plant performance under drought stress

2.2.1 **Drought stress**

Drought stress is a significant challenge that crops face and is becoming increasingly prevalent due to climate change. Climate change is causing prolonged and severe droughts in many parts of the world, affecting agricultural productivity and food security. Drought stress occurs when plants experience a water deficit due to insufficient rainfall or water supply, leading to a reduction in plant growth, development, and overall productivity (Lisar *et al*., 2012). The impact of drought stress on crops can be severe. It can result in reduced crop yields or complete crop failure, which can have significant economic implications for farmers and food production systems. Furthermore, drought stress also has negative effects on soil health. Soil moisture is depleted, leading to soil degradation, erosion, and reduced soil fertility. As a result, it becomes difficult for vegetation to hold soil in place, and new plants struggle to establish and grow (Masroor *et al*., 2022).

In addition to its impact on crop yields and soil health, drought stress also has broader ecological consequences. The adverse conditions associated with drought stress can lead to biodiversity loss. Many plant and animal species are unable to adapt to dry conditions, leading to the collapse of ecosystems and the loss of valuable habitats. This can further exacerbate the impact of drought stress on agriculture and food security. Therefore, there is an urgent need to develop crops that are resilient to drought stress to mitigate the negative impact of climate change on agriculture and the environment. Research in breeding drought-tolerant crops using various techniques, such as marker-assisted breeding and genetic engineering, is ongoing. Developing crops that can withstand drought stress is crucial for ensuring food security and the sustainability of agricultural systems in the face of changing climatic conditions.

2.2.2 **Impact of drought on plants**

Plants have a range of responses to drought stress, including changes in morphology-physiology and biochemistry, as well as short- and long-term developmental adaptations (Abobatta, 2019). For example, in response to drought stress, plants undergo physiological changes to conserve water, such as closing their stomata (pores on the leaves) to reduce water loss through transpiration and slowing down their growth rate, including reducing their leaf number and size. Moreover, some plants could also change their leaf colour, such as turning yellow or brown, as a response to water stress (Lamaoui *et al*., 2018). However, in extreme cases, the plant may die.

Plant root systems are compromised under drought stress, which affects the ability of plants to take up water and nutrients from the soil. As soil moisture is reduced, it causes the roots of plants to shrink and changes the root architecture, such as a reduction in the number of root hairs (Duddek *et al*., 2022). These changes might alter the plant root

exudate composition, leading to disruption of the interaction with soil microorganisms in the rhizosphere (Williams & de Vries, 2020). Nonetheless, certain plant species have developed a coping mechanism to sustain the microbial community in the rhizosphere despite unfavourable conditions. For example, de Vries *et al.* (2019) reported that drought stress would trigger plants to modify the composition of their root exudates to increase microbial activity during stress. However, this activity might decrease plant root diameter and length. Some drought-tolerant plants survive severe drought stress by enhancing their root systems to source water in the deeper soil layers. For instance, when both drought-tolerant and sensitive potatoes were exposed to drought stress, the tolerant cultivars showed a significantly higher root elongation than the sensitive cultivars (Boguszewska-Mańkowska *et al*., 2020). Taken together, these findings suggest that plant roots are critical for drought adaptation and drought adaptation and water and nutrient uptakes to ensure their survival.

At the cellular level, drought significantly affects the structure and composition of plant cell walls, affecting the ability of plants to withstand water stress. To reduce water loss, drought-stressed plants would increase the cell wall thickness. This modification depends on the distribution of cellulose, hemicellulose, pectin, and lignin, as it may affect the mechanical strength and flexibility to respond against biotic and abiotic stresses (Bacete & Hamann, 2020). Besides, several components of the cell wall, such as expansin, cellulase, and pectinase, might undergo changes in response to drought stress. For example, the expansin genes help to maintain cell surface expansion and enlargement in drought-stressed wheat plants (Yang *et al.*, 2020). The authors found that those plants with elevated expansin expression showed better survivability, photosynthesis rate and water-retaining ability than wild-type (Yang *et al.*, 2020). These findings demonstrated that changes in cell wall composition and structure are crucial for plants to withstand water stress.

2.2.3 **Plant responses against drought stress**

Understanding how plants respond to drought stress is crucial for improving crop productivity in the face of global climate change. As previously discussed, plants have evolved various mechanisms to conserve water, increase water uptake efficiency, and maintain their water balance to cope with the adverse effect of drought stress. For example, plants alter their water relations by adjusting the osmotic potential in cells by accumulating compatible solutes, such as sugars, proline, and amino acids (Seleiman *et al*., 2021). This allows plants to maintain turgor pressure under low external water potential, which is essential for metabolic function. Other mechanisms include expressing drought-responsive genes, producing signalling molecules, such as ABA, calcium ions (Ca^{2+}), hormones, and osmolytes, and activating reactive oxygen species (ROS) scavenging mechanisms.

ABA is a lipophilic hormone known to function in plant growth, seed germination, and senescence. It is also crucial in regulating plant responses to various external stresses, such as drought. ABA is synthesised from carotenoids (C40) derivatives of isopentenyl diphosphate through the methylerythritol phosphate (MEP) pathway in plastids (Muhammad Aslam *et al*., 2022). ABA biosynthesis involves a multistep process. The initial step involves the conversion of zeaxanthin to all-trans-violaxanthin through the cyclic hydroxylation of epoxycarotenoids, which is catalysed by the enzyme zeaxanthin epoxidase via an intermediate molecule, antheraxanthin. Subsequently, cis-isomerisation of all-trans-violaxanthin to either violaxanthin or cis-neoxanthin occurs through an unknown enzymatic reaction. The cis-isomers of violaxanthin and neoxanthin are then split by 9-cis-epoxycarotenoid dioxygenase enzymes, yielding a C15 intermediate product known as xanthoxin, which is subsequently exported to the cytosol. In the cytosol, xanthoxin is converted into ABA through two enzymatic reactions. Firstly,

xanthoxin is transformed into abscisic aldehyde by short-chain alcohol dehydrogenase/reductase, followed by the oxidation of the abscisic aldehyde to ABA by aldehyde oxidase.

Plants perceive external drought stimuli through sensors located on the membrane and initiate a series of signalling cascades through multiple signal transduction pathways. This led to an alteration in drought-responsive gene expression. There are two categories of drought-responsive genes, namely ABA-dependent and ABA-independent. Several drought-responsive genes are activated by ABA, including late embryogenesis abundant (LEA) proteins, which are widely reported as ABA-dependent, as well as enzymes involved in the production of osmolytes, detoxification, and metabolism, transporters, such as ion transporters and channel proteins, transcription factors, and protein kinases (Lau *et al*., 2021). However, some genes do not respond to ABA. The ABA-independent pathway in response to drought stress is characterised by proteins, such as DREB2, members of the AP2/ERF transcription factor family, which play a significant role.

Under drought stress, plants exhibit a significant increase in ABA levels. ABA is then bound to the pyrabactin-resistance 1/pyrabactin resistance like/regulatory component of aba receptor (PYR/PYL/RCAR) receptor, sequestering the negative regulator of PP2C from hindering the positive regulator, sucrose nonfermenting related kinases 2 (SnRK2) (**Figure 2.1**). This action activates the SnRK2 via autophosphorylation of the activation loop, thus phosphorylating the downstream transcription factors, such as AREB/ABF, SLAC1, and KAT1. Those transcription factors are crucial to the expression of ion transport, drought-responsive genes, and proteins (Ali *et al*., 2020).

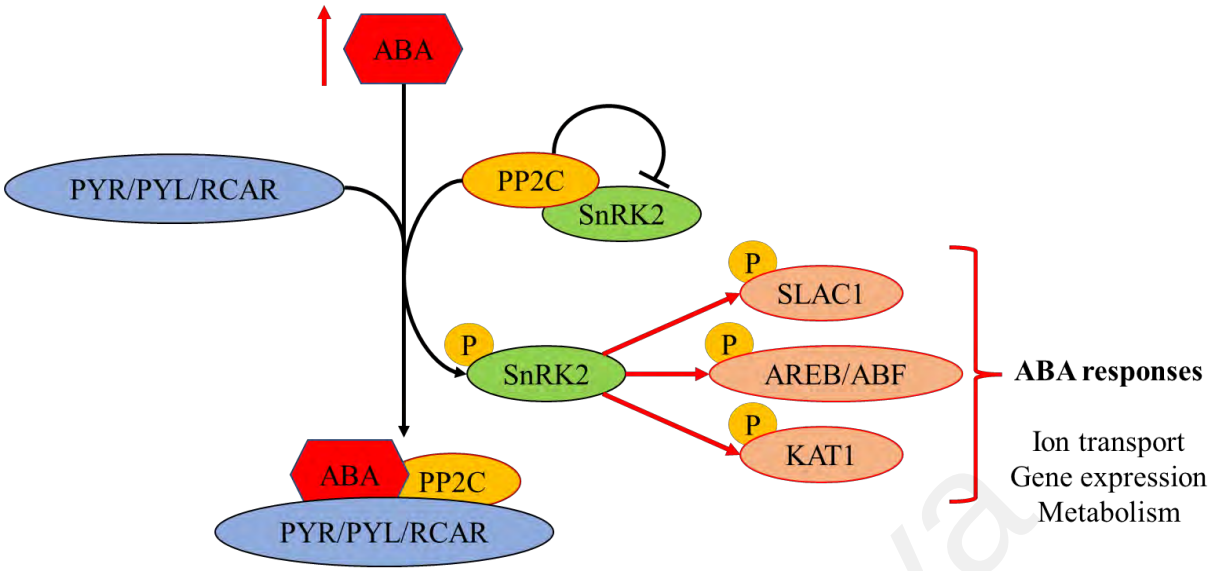

Figure 2.1. Increase in concentration of ABA initiates the binding with PYR/PYL/RCAR thus promotes the binding interaction with PP2C that negatively regulates SnRK2 activation. This interaction releases the SnRK2 and activates its kinase phosphorylation targeting ABA-responsive genes.

Ca^{2+}, the first messenger in many plant signalling systems, plays a crucial role in maintaining cell membrane stability, structure, intracellular homeostasis, growth, and development. The intracellular increase of Ca^{2+} in of *Arabidopsis thaliana* showed that exogenous application of Ca^{2+} could diffuse into the cell through a calcium-sensing receptor on the plasma membrane of guard cells (Wang *et al.*, 2012). Under drought conditions, plants produce ABA to reduce water loss through stomatal closure and remain closed due to the build-up of Ca^{2+} concentration in the cytoplasm. The closure of stomata helps the plant to conserve its water content and improve water use efficiency, allowing it to adapt to a water-scarce environment. In addition, plasma membrane calcium channels are activated by ABA and stimulate intracellular calcium reservoirs to release Ca^{2+}, inhibiting potassium ion influx (Kudla *et al*., 2010).

During stomatal closure, the increase of ABA in the plants triggers the accumulation of cytosolic calcium ([Ca^{2+}]cyt) via the Ca^{2+} channel protein (**Figure 2.2**). The [Ca^{2+}]cyt build-up allows the activation of anion (A^-) channels, such as slow-activating sustained (S-type) and rapid transient (R-type), which trigger depolarisation via the release of A^- from the guard cells (Schroeder *et al*., 2001). This action causes the potential difference in the membrane, thus inhibiting the potassium ions (K^+) influx into the cell by deactivating the K^+_{in} channel and promoting the K^+ efflux out of the cell via the K^+_{out} channel activation. The out K^+ efflux movement is also enhanced by the guard cell alkalisation due to ABA build-up.

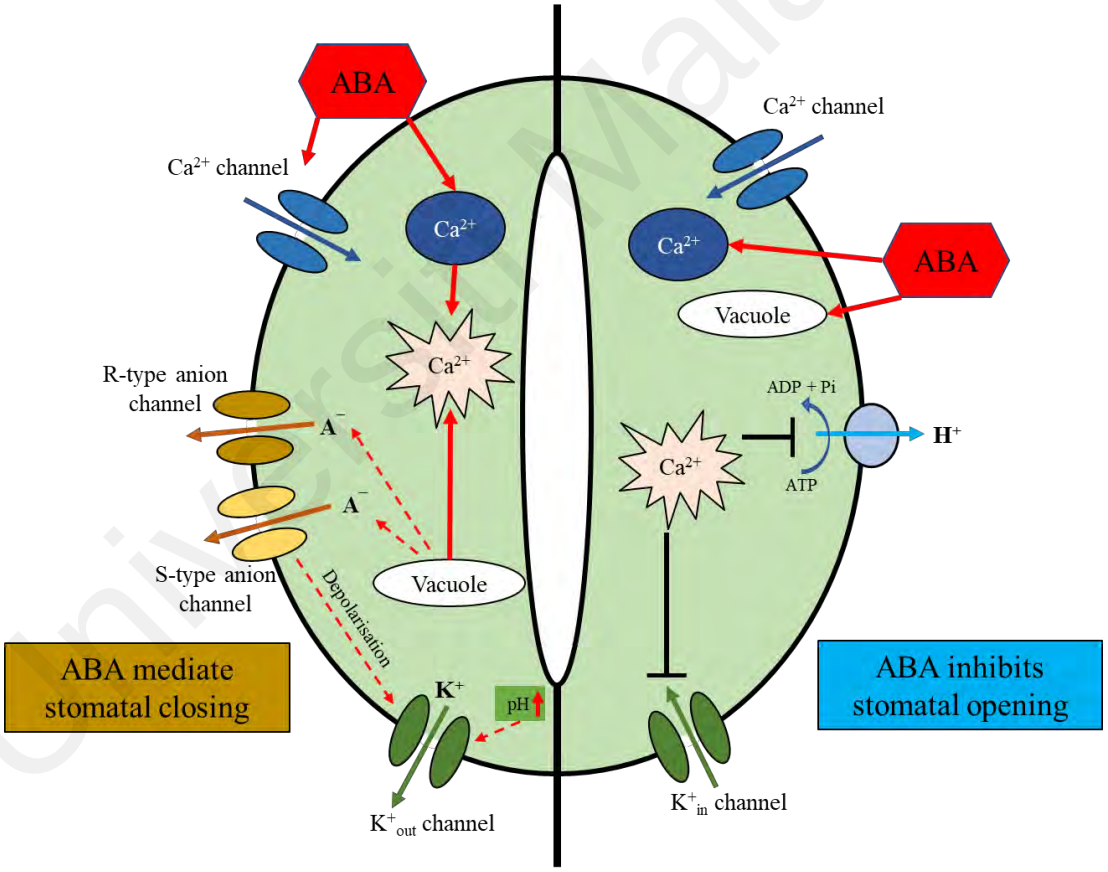

Figure 2.2. The overall ABA-mediated stomatal closure via the movement of calcium ions (Ca^{2+}). The increased accumulation of Ca^{2+}in the guard cells triggers the anion (A^-) releases and inhibits the potassium ion (K^+) influx into the cells.

ROS are free radicals of oxygen produced under stress conditions. Plants inherently produce ROS for cell signalling, but excess production leads to oxidative stress. Antioxidant defence mechanisms balance ROS production under normal conditions, but various stresses disrupt this equilibrium, resulting in a sudden increase in intracellular ROS levels. Approximately 1-3% of oxygen consumed by plants generates ROS in plant tissues (Meitha *et al*., 2020). One important ROS is hydrogen peroxide (H_2O_2). H_2O_2 is known for its stability, long half-life, and high diffusivity. It is mainly produced in peroxisomes, chloroplasts, and mitochondria (Das & Roychoudhury, 2014), although mitochondria are the most vulnerable to oxidative damage. When H_2O_2 concentration becomes high due to intensified oxidative stress, it becomes an important alarm signal to up-regulate antioxidant defence mechanisms or trigger programmed cell death. In recent years, the regulation of calcium mobilisation, protein phosphorylation, and gene expression by H_2O_2 has been extensively studied. H_2O_2 can regulate Ca^{2+} influx in protoplasts and increase $[Ca^{2+}]$cyt in guard cells in *Arabidopsis thaliana* by activating plasma membrane calcium channels in guard cells (Pei *et al*., 2000). H_2O_2 can also be induced by ABA, which is essential in ABA-induced stomatal closure. ABA can promote ROS production, and the accumulation of ROS, such as H_2O_2, becomes a signalling cue in regulating stomatal closure (Mori & Schroeder, 2004; Yan *et al*., 2007).

Upon the increased ROS production, such as singlet oxygen ($^1O_2$), hydroxyl radical ($^{\bullet}OH$), superoxide ($O_2^{\bullet-}$), and H_2O_2, plants induce their innate non-enzymatic and enzymatic ROS scavenging actions to protect the cells from oxidative damages. The enzymatic ROS scavenger includes superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), monodehydroascorbate (MDHAR), dehydroascorbate reductase (DHAR), and glutathione reductase (GR) (**Figure 2.3**). The frontline of plant defence started with SOD, a metalloenzyme that catalyses the harmful

$O_2^{\bullet-}$ into H_2O_2 and suppresses the generation of $^{\bullet}OH$ (Gill *et al*., 2015). The abundance of H_2O_2 in the cells is then converted into H_2O and O_2 by CAT, POD, and APX. CAT is known to be the most efficient enzyme in catalysing H_2O_2 into H_2O and O_2 due to its tetrameric heme structure (Garg & Manchanda, 2009), while POD is a glycoprotein containing proximal and distal heme-binding domains specialise in oxidising phenolics and utilise H_2O_2 as an electron acceptor in the conversion of oxidised phenolics into $2H_2O$ (Martínez-Rubio *et al*., 2018). Nonetheless, APX is also involved in detoxifying the H_2O_2 as part of the AsA-GSH cycle by utilising the ascorbate (AsA) as an electron donor to produce MDHA. In the AsA-GSH cycle, MDHAR contained a thiol group that is crucial in reducing phenoxyl radical and AsA regeneration from MDHA (Hasanuzzaman *et al*., 2019). In addition, DHA formed from MDHA through spontaneous reaction is recycled into AsA while converting glutathione (GSH) into oxidised glutathione (GSSG) (García-Caparrós *et al*., 2019). The redox homeostasis is further regulated by GR, which reduces the GSSG into GSH.

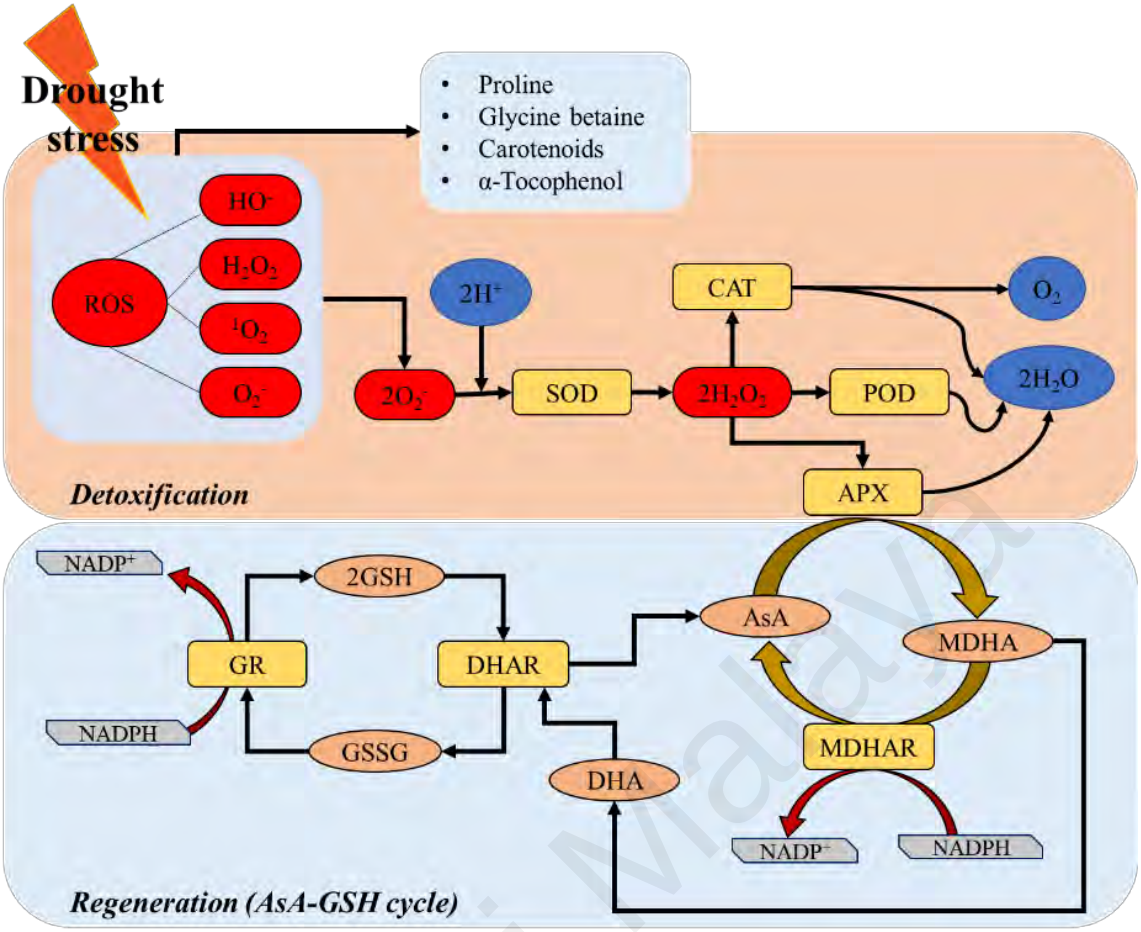

Figure 2.3. The overall antioxidant activities triggered during drought stress which started with the build-up of ROS in the cells. ROS, reactive oxygen species; $^1O_2$, singlet oxygen; $^{\bullet}OH$, hydroxyl radical; $O_2^{\bullet-}$, superoxide; H_2O_2, hydrogen peroxide; H^+, hydrogen ion; SOD, superoxide dismutase; CAT, catalase; POD, peroxidase; APX, ascorbate peroxidase; O_2, oxygen; H_2O, water; AsA, ascorbate; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; $NADP^+$, nicotinamideadenine dinucleotide phosphate; NADPH, reduced form of nicotinamide adenine dinucleotide phosphate; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GR, glutathione reductase.

On the other hand, the non-enzymatic ROS scavenger consisting of AsA and GSH, which are also crucial in the AsA-GSH (regeneration), aids the protection against ROS oxidative damages. AsA is also involved in the ROS scavenging activity as a co-enzyme to donate electrons (Hasanuzzaman *et al*., 2019). Besides being involved in the AsA-GSH cycle to maintain the redox balance, GSH is also important in recycling AsA and α-Tocopherol (Singh *et al*., 2016). Moreover, proline, glycine betaine (GB), carotenoids, and α-Tocopherol were also involved in the non-enzymatic ROS scavenging activity. The osmolytes proline and GB are widely known as small solutes utilised by cells and tissue to maintain cell volume and integrity. Proline is known to be a ROS scavenger aside from its roles as a protein stabiliser and redox balancer (Heuer, 2010), while GB is known to be enhancing the plant tolerance against abiotic stresses by regulating osmotic potential, enzymatic activities and integrity of cell membrane (Ashraf & Foolad, 2007). Furthermore, carotenoids could play a role as ROS scavengers during photosynthesis apart from being part of the photosynthetic machinery whereby they are involved in the light spectra absorption, protecting other light absorbing proteins and ensuring the thylakoid membrane stabilisation (Hussain *et al.*, 2019; Young & Lowe, 2018). Despite their effective scavenging of ROS, carotenoids have received comparatively less attention, possibly due to their vulnerability to oxidative degradation (Impa *et al*., 2012). On the other hand, α-tocopherol is one of the important ROS scavengers, particularly singlet oxygen ($^1O_2$) and HO^- formation in the photosynthetic membrane (Hasanuzzaman *et al*., 2014).

A more comprehensive and cautious comprehension of the various ROS signalling mechanisms in cellular stress responses is necessary to differentiate between a particular compound's suggested involvement as a consequence of general stress or genuinely stress-specific response. Additionally, the swift progress in "omics" technologies, such as proteomics and metabolomics analyses, can aid in identifying

obscure connections and cross-talks among diverse stress signalling pathways. These discoveries could augment plants' resilience to one of the most destructive abiotic stresses.

2.3 Omics technology in abiotic stress research

Omics is a field of study encompassing a wide range of biological disciplines, including genomics, transcriptomics, proteomics, and metabolomics. The prefix "omics" refers to a large set of molecules, such as genes, proteins, or metabolites, and the study of these molecules. Omics technologies, such as high-throughput sequencing and mass spectrometry, have revolutionised biological research by allowing scientists to simultaneously study large sets of biological molecules (Micheel *et al*., 2012). Omics approaches have been applied in a wide range of research areas, including basic biology, drug discovery, and personalised medicine (Pulido *et al*., 2016; Ramos *et al*., 2018).

Genomics studies the complete genetic material, such as the DNA of an organism. In plants, genomics analysis aims to understand the genetics, growth, development, and adaptation of the subject. One of the important genomics studies in plant research is the sequencing of the *Arabidopsis thaliana* genome, which has provided insights into plant genetics and important processes (The Arabidopsis Genome Initiative, 2000). In addition, genomic research on crops, such as rice, maize, and soybeans, has led to the discovery and development of new, disease and pest-resistant, high-yielding, and nutritious crop varieties. For instance, a recent pan-genomic (the entire set of genes from all strains within a clade) analysis of sunflower has revealed the over-presented biotic resistance genes among the introgressed pan-genome of cultivated species introgressions from wild species, which confers the plant disease resistance (Hübner *et al*., 2019). Genomics is also useful for studying the plants' response and adaptation toward stress at the molecular

level. As plants activate specific genes to cope with certain stresses, the genomics tool could identify expressed genes and genetic variation that confers tolerance against abiotic stress. For example, a genome-wide analysis study (GWAS) of 300 rice accession exposed to different intensities of salt stress uncovered 1200 candidate genes comprised of both known and unknown salinity-tolerance cation transporters and transcription factors (Patishtan *et al*., 2018). Thus, the genomics analysis tool can contribute to the development of more resilient crop varieties by identifying important genes responsible for the advantageous traits in plants.

Transcriptomics studies the complete set of RNA molecules and their role in gene regulation. This technology could analyse the transcripts of an organism in response to different environmental conditions, such as abiotic stress, pathogens, and nutrient availability. Recently, next-generation sequencing technologies have revolutionised molecular research, enabling deep investigation into the molecular mechanisms and pathways involved in developmental processes, adaptation, and responses to environmental cues at the whole-genome level (Baldoni, 2022). With the ability to analyse the entire transcriptomic landscape in real-time, researchers can obtain an accurate snapshot of all transcripts present in a cell or tissue, making it possible to assess gene expression rewiring in different tissues and/or conditions and capture dynamic genotype/environment interactions. For example, by analysing plant transcriptomes under different conditions, researchers can determine which genes are active/inactive and how they change in response to stress. For instance, a comparative transcriptomic analysis between cold-sensitive and resistant varieties of tea plants showed the importance of cold-resistant genes related to the plants' photoinhibition, ABA signal conduction, and plant immunity to allow greater tolerance against cold conditions (Li *et al.*, 2019). In addition, a combination of shot-read sequence and full-length transcriptome data of pearl millet exposed to 48 hours of heat and drought stress showed significant improvement in the

number of annotated genes related to the stresses by 20%, allowing more transcription factors and transcription regulator to be identified (Sun *et al*., 2020). The utilisation of transcriptomics analysis, such as RNA-sequencing (RNA-seq), would help to develop more resilient crop varieties and study gene expression during plant development and hormone response. As a result, transcriptomic studies have become a valuable tool for identifying genes and regulatory networks that control plant responses to environmental fluctuations (Zandalinas *et al*., 2020). Currently, the comprehension of transcriptional regulation of gene expression in plant research is primarily based on technologies, such as microarray and bulk RNA-seq. These methods deliver information on targeted and bulk gene expression, which constrains our understanding of the intricate molecular mechanisms underlying diverse regulatory processes.

Metabolomics is a powerful tool to analyse the response of organisms to external factors like abiotic stress and nutrient availability at the metabolite level (Ghatak *et al*., 2018). For example, metabolomics is often used to study stress biology in plants, with the potential to elucidate the mechanisms contributing to plant tolerance to abiotic stress (Carrera *et al*., 2021). Qualitative and quantitative analyses of plant metabolites subjected to biotic and abiotic stresses not only describe the responses of stressed plants but can also reveal underlying genetic and biochemical mechanisms, as well as distinguish the plant's ability to resist and tolerate stress. Most metabolomics studies have compared the response of stress-susceptible and stress-tolerant cultivars. For instance, Guo *et al.* (2020) evaluated the metabolome profiles of drought-tolerant and sensitive wheat varieties. They found that thymine, the aminoacids L-cysteinylglycine and fructoselysine, and several phenolic compounds were more highly accumulated in leaves in the drought-tolerant variety than in the sensitive one after drought treatment (Guo *et al*., 2020). When comparing drought-tolerant and susceptible peanuts, agmatine and cadaverine were found to be exclusively present in drought-tolerant peanuts during drought. Moreover,

polyphenols, including syringic acid and vanillic acid, were more highly accumulated in drought-tolerant than in drought-susceptible peanuts, while catechin production was higher in drought susceptible than in drought-tolerant during drought. Besides studying plant stress biology, metabolomics can also track changes in plant development and hormone responses and identify natural variations in wild crop relatives for improved crop productivity. A novel technique called metabolomic quantitative trait locus (mQTL) mapping is a new approach that helps identify genetic components associated with variations in metabolic profiles. For example, an mQTL analysis between wild maize and inbred maize uncovers about 350 QTLs that are responsible to the 65 primary metabolites which important for the plant growth and development (Li *et al*., 2019). This technique is useful for developing stress-tolerant crops because it can help discover new genes that regulate metabolic pathways, identify metabolites that can be used as stress-resistant biomarkers, and bridge the gap between phenotype and genotype for complex traits (Abdelrahman *et al*., 2018).

Proteomics studies an organism's complete set of proteins, their interactions, and how they are expressed. Techniques used in plant proteomics include mass spectrometry, two-dimensional gel electrophoresis (2-DE), and protein microarray. Mass spectrometry (MS) identifies proteins based on their mass-to-charge (*m/z*) ratio and quantified based on peptides' MS signal intensity or counts. 2-D gel electrophoresis separates proteins based on charge and size. Protein microarrays analyse multiple proteins by immobilising proteins or lysates on a solid support and probing with specific protein-recognising antibodies. Proteomics in plants is a field of study that analyse the complete set of proteins expressed by a plant and their interactions. It helps researcher to understand plant responses to environmental changes and stress regulation. By comparing normal and stress samples, researchers can identify changes in protein expression during plant development, hormone response and in wild relatives to improve crop breeding.

2.4 Proteomics

2.4.1 **Principle of proteomics analysis**

Proteins are composed of building blocks of amino acids, which are essential biological molecules that play important roles in structural, transport, metabolic, signalling, and regulatory functions. The term “proteome” was first described as all proteins an organism can express, which are unique to each species (Wilkins, 2009). In contrast to the genome, the proteome composition of an organism is in constant flux, which may differ among cell types, tissues, or organs at any given time. To some extent, the proteome could reflect transcriptomics changes, although protein activity is also regulated by many factors, such as degradation and modifications.

Proteome studies, also known as proteomics, include identifying different protein interactions and their functions within an organism (McArdle & Menikou, 2021). Although other approaches, such as mRNA expression analysis, could be utilised to study protein expression, this method would be inaccurate as the protein expression level does not always correlate with the mRNA expression level. Moreover, the key to functional proteins, such as protein post-translational modifications (PTMs), cleavage, folding, and localisation, are not considered in the study of mRNA. Therefore, proteomics is a useful technique to investigate the timing and location of protein expression, protein abundance that includes production and degradation, and protein modifications through phosphorylation. Using these data, protein activities, such as subcellular protein movements, protein alterations in metabolic pathways, and protein-protein interactions, could be discovered (**Figure 2.4**).

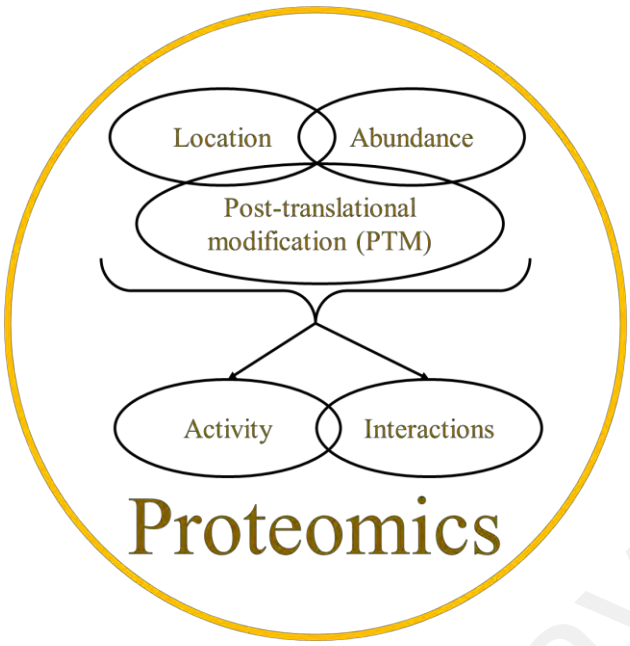

Figure 2.4. Proteomic study focussed on the collection of data on protein location, abundance, and post-translational modifications (PTMs). Combination of these collected data could be utilised to uncover the protein interactions, co-localisations, and protein activity after PTMs.

2.4.2 **Types of proteomics analysis**

Proteomics analysis in the general workflow involves protein extraction and preparation, high-resolution separation, protein identification, and bioinformatics. In general, proteomic analysis can be classified into two types: top-down and bottom-up. Top-down proteomics analyses intact proteins rather than breaking them down into smaller peptides (Catherman *et al*., 2014). This approach allows the identification and characterisation of PTMs and alternative splicing events, which can be missed by bottom-up methods. Common techniques used in top-down proteomics include electrospray ionisation mass spectrometry (ESI-MS), matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF-MS), Fourier-transform ion cyclotron resonance mass spectrometry (FTICR-MS), and high-resolution mass spectrometry. This method uses an electrical charge to create a spray of ions from a protein sample, which

can then be analysed by mass spectrometry. MALDI-TOF-MS uses a laser to create ions from a protein sample, which are then analysed by time-of-flight mass spectrometry. In contrast, FTICR-MS uses a magnetic field to trap and analyse ions, allowing the high-resolution analysis of intact proteins. On the other hand, high-resolution mass spectrometry utilises high-resolution mass analysers such as Orbitrap and FT-ICR, which provide high mass accuracy and resolution for identifying intact proteins. A recent large-scale, top-down proteomics in *Arabidopsis thaliana* leaf and chloroplast revealed over 4700 unique proteome forms, of which 1300 have post-translational modifications (Wang *et al.*, 2022). Top-down proteomics has the advantage of providing more information about the protein of interest. However, it is also more challenging and technically demanding than bottom-up methods.

In contrast, bottom-up proteomics refers to the method of analysing proteins by breaking them down into smaller peptides, which are then identified and characterised by mass spectrometry (Zhang *et al.*, 2013). In this method, proteins are digested by specific enzymes, such as trypsin and chymotrypsin, which cleave the protein at specific locations, generating a mixture of peptides. The cleaved peptide mixture was then subjected to liquid chromatography-mass spectrometry (LC-MS), whereby the peptides were separated by liquid chromatography and analysed by mass spectrometry, allowing for the identification and characterisation of the peptides. Further analysis with tandem mass spectrometry (MS/MS) allowed the peptides to be fragmented by collision-induced dissociation in the mass spectrometer, and the resulting fragment ions were analysed to identify the peptide sequence (Medzihradszky *et al*., 2000). This approach is often used to study the overall composition of a sample, such as a cell lysate or tissue extract, and can provide information regarding the relative abundance of different proteins in the sample. In plant study, bottom-up proteomic analysis is less technically demanding than

top-down proteomics and allows the analysis of complex protein mixtures. The major disadvantage is that they may miss PTMs and alternative splicing events.

2.4.2.1 **Gel-based proteomics**

Gel-based proteomic techniques are a group of methods that use gel electrophoresis to separate proteins based on their size and charge. These techniques can be used to analyse the overall composition of a sample, such as a cell lysate or tissue extract. They can provide information about the relative abundance of different proteins in the sample. Gel-based proteomics techniques are useful for analysing complex protein mixtures and can provide information regarding the relative abundance and presence of specific proteins in a sample (Wu *et al*., 2006). These techniques involve a separation step (usually 2-DE) and an identification step (MS/MS). At the separation step, 2-DE resolves proteins according to the isoelectric point and molecular mass. Isoelectric focusing (IEF) was usually performed to separate proteins based on their isoelectric point until the pH of each protein reached a net charge of zero. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), also known as 1D-gel analysis, is commonly used to separate proteins according to their molecular mass. This method uses a detergent, sodium dodecyl sulfate (SDS), to denature and uniformly charge proteins, allowing for separation based on size. The separated protein spots may be dyed using Coomassie brilliant blue, silver nitrate, or SYPRO Ruby. This analysis can also be paired with Western blotting, which uses specific antibodies to detect and identify specific proteins in a gel, followed by separation by SDS-PAGE. After separation and gel staining, the selected protein spots were analysed using an MS, which allows hundreds of proteins to be characterised in a single polyacrylamide gel.

Differential gel electrophoresis (DIGE) was developed to enhance the consistency of 2-DE and to address the variations between gels. This technique uses fluorescent dyes, such as CyDye2, CyDye3, and CyDye5, which are specific to certain pH ranges to label the proteins in each sample, allowing for the simultaneous comparison of multiple samples in a single gel (Lilley & Friedman, 2004). This method can identify a single protein with as little as 150 pg while also decreasing the number of gels necessary for a single experiment (Tan *et al*., 2017). DIGE has several advantages over traditional 2D-gel electrophoresis, such as increased sensitivity and improved reproducibility, making it a powerful tool for the detection of small changes in protein expression. In addition, DIGE can be combined with mass spectrometry for protein identification and quantification, making it a powerful tool for discovering new biomarkers and therapeutic targets. Despite its efficacy, the utilisation of DIGE has been constrained due to the relatively expensive DIGE equipment, software, and consumables (Tan *et al*., 2017).

Gel-based proteomics techniques, however, have some limitations, such as resolution and sensitivity, which limit their ability to resolve closely related proteins, similar-sized proteins and charges, and difficulty in detecting low-abundance proteins (Chevalier, 2010). It is also not well suited for proteins larger than ~200 kDa, and many proteins larger than 100 kDa cannot be resolved using traditional gel-based methods. Furthermore, post-translational modifications (PTMs) and alternate splicing events may not always be detected, making them difficult to separate and detect by gel electrophoresis. Additionally, protein quantification via gel-based analysis is often unreliable and is mostly used for qualitative analysis, which could be time-consuming and labour-intensive, requiring multiple steps and manual interventions, and the results can be affected by the operator's experience (Zuckerman *et al*., 2009). Owing to these limitations, gel-based techniques are often used in combination with other methods, such as mass spectrometry, to provide a complete picture of the proteome.

2.4.2.2 **Gel-free proteomics techniques**

Gel-based methods alone cannot sufficiently tackle the challenges posed by proteomics research. Consequently, the disadvantages of gel-based approaches have inspired the creation of alternative gel-free proteomics techniques, which aim to either surmount these constraints or completely supplant gel-based methods. Gel-free proteomic techniques are a group of methods that do not use gel electrophoresis to separate proteins. Instead, these techniques rely on liquid chromatography (LC) or other methods to separate proteins based on their size, charge, or other properties. Common gel-free proteomic techniques include LC-MS, multi-affinity purification-mass spectrometry (MAP-MS), and synthetic peptide-based mass spectrometry (SP-MS). Gel-free proteomics techniques have several advantages over gel-based techniques, including improved sensitivity and resolution (Tan *et al*., 2017). It can be more sensitive and provide better resolution than gel-based techniques, making it easier to detect low-abundance proteins and allowing for the identification of closely related proteins or proteins that are similar in size and charge (Vuckovic *et al*., 2013). In addition, gel-free techniques can have a wider dynamic range, flexibility, and greater throughput than gel-based techniques, allowing for the accurate representation of proteins that are present in widely varying amounts and the separation of proteins based on different properties, such as size, charge, and hydrophobicity. They can be more automated and, therefore, have higher throughput than gel-based techniques, speeding up the process and reducing the need for manual intervention.

2.4.2.3 **Free-labelled LC-MS**

Free-labelled LC-MS is a proteomic technique to identify and quantify proteins in a sample without prior labelling. This contrasts with labelled protein LC-MS, where the proteins in a sample are labelled with a specific tag, such as an isotope or small molecule, before analysis. In free-labelled LC-MS, the proteins in a sample are first digested with a protease to produce peptides. The digested peptides were then separated by liquid chromatography and analysed by mass spectrometry. MS data were then used to identify the peptides and their corresponding proteins using database searching techniques and computational tools. One of the early applications of label-free proteomics in plant research was in a study reported by Dani *et al*. (2005). In this study, the authors used 2-DE combined with MS to analyse changes in protein expression in tobacco (*Nicotiana tabacum*) leaves in response to salt stress. The authors performed two-dimensional electrophoretic analyses and identified about 150 polypeptide spots in the pH range of 3.0 to 10.0 and conducted quantitative evaluations and statistical analyses of the resolved spots in treated and untreated samples. They managed to identify 20 differentially expressed proteins in response to salt stress of which an enhanced accumulation of protein species known to be induced by biotic and abiotic stresses was observed. In particular, two chitinases and a germin-like protein increased significantly, and two lipid transfer proteins. This study demonstrated the utility of label-free proteomics in identifying changes in protein expression and modification in response to environmental stress in plants.

Label-free proteomics has been used in many other plant research studies, including studies on the response of plants to biotic and abiotic stress, changes in growth conditions, and the identification of protein-protein interactions. For instance, Yu *et al*. (2018) reported an investigation on the changes in protein expression of Southern rice black-streaked dwarf virus (SRBSDV)-infected rice and the potential use of

Cytosinpeptidemycin (Cpm) as a treatment for the virus. The authors implemented a label-free quantitative proteomics analysis to compare the protein expression profiles of healthy rice plants and SRBSDV-infected rice plants, with and without Cpm treatment. The results showed that SRBSDV infection caused significant changes in the protein expression profiles of rice plants, with 103 differentially expressed proteins identified, mostly downregulated. These proteins were involved in processes such as photosynthesis and energy metabolism. Moreover, the Cpm treatment altered the protein expression profiles of infected plants, with 61 differentially expressed proteins identified, mainly involved in stress response, energy metabolism, and cell wall synthesis. Upregulation of proteins involved in stress response and energy metabolism suggested that Cpm treatment could enhance the defence response and improve the energy metabolism of infected plants.

Furthermore, Salvato *et al*. (2019) reported the changes in protein expression profiles in the nuclei of sugarcane stems in response to drought stress by utilising a label-free quantitative proteomic technique. In total, 5742 proteins were identified, out of which 282 proteins were found to be differentially expressed in response to drought stress. The differentially expressed proteins were found to be involved in various biological processes, including signal transduction, transcriptional regulation, and stress response, which are related to the response to oxidative stress and the regulation of gene expression. Proteins involved in oxidative stress response, such as peroxidases, catalases, and superoxide dismutase, were found to be upregulated under drought stress, indicating the importance of antioxidant defence mechanisms in sugarcane plants under drought stress. The differentially expressed proteins involved in the regulation of gene expression, such as histones, transcription factors, and chromatin, suggesting the involvement of epigenetic regulation in response to drought stress. These findings provide insights into the molecular mechanisms underlying the response of sugarcane plants to drought stress

at the nuclear level, such as the activation of multiple defence mechanisms to cope with drought stress, including the upregulation of antioxidant defence mechanisms and the epigenetic regulation of gene expression.

Overall, label-free proteomics has proven to be a powerful tool for investigating the complex changes in protein expression and modification that occur in response to environmental stress in plants without the need for chemical labelling. Free-labelled LC-MS has several advantages over labelled protein LC-MS, such as not requiring a labelling step, which can be time-consuming and expensive. Additionally, free-labelled LC-MS is less prone to bias and can identify PTMs, such as phosphorylation, ubiquitination, and acetylation, which can affect the activity and stability of proteins and play a critical role in the regulation of various cellular processes (Levin & Bahn, 2010).

2.4.2.4 **Labelled LCMS**

Labelled LC-MS analysis is a technique used to identify and quantify proteins in a sample by labelling each peptide, which acts as a tracer for identifying and quantifying the sample in the mass spectrometer. Prior to analysis, in labelled protein LC-MS, the proteins in a sample are labelled with a specific tag, such as an isotope or a small molecule, to distinguish them from the proteins in other samples. The labelled proteins were then separated by liquid chromatography, which separated them based on their physical and chemical properties. The separated proteins were then analysed using mass spectrometry, which measures the mass-to-charge ratio of the proteins, allowing for the identification and quantification of the labelled proteins (Xie *et al*., 2011). One of the earliest labelled LC-MS proteomic analyses in plants was published by Agrawal *et al*. (2002). In this study, the authors used isotope-coded affinity tag (ICAT) labelling coupled with LC-MS to quantitatively analyse changes in protein expression in *Arabidopsis*

thaliana in response to ozone stress. The ICAT method involves the covalent labelling of cysteine residues in proteins with different isotopes of the same reagent, enabling relative quantification of protein abundance between samples. Nowadays, the most common labelling type used in LC-MS is isobaric tagging, such as isobaric tags for relative and absolute quantification (iTRAQ) or tandem mass tags (TMT), which allows for the simultaneous quantification of multiple samples in a single run.

iTRAQ and TMT are labelling techniques used for the quantitative proteomic analysis of multiple samples. They are a type of isobaric tagging in which different samples are labelled with different isobaric tags, which are small molecules that are chemically similar but have different mass spectrometric properties. The labelling process typically involves the following steps: protein preparation by a protease to digest the protein into peptides prior to labelling with different isobaric tags, which are small molecules that are chemically similar but have different mass spectrometric properties (Rauniyar & Yates, 2014). The labelled peptides from each sample were mixed and analysed by mass spectrometry. The data were processed using data analysis software to identify and quantify the peptides and their corresponding proteins. For example, Parrine *et al*. (2018) reported the utilisation of iTRAQ LC-MS/MS analysis to investigate the proteome modifications in tomato plants under extreme high light stress. A total of 323 differentially expressed proteins were identified, with roles in photosynthesis, energy metabolism, stress response, and protein folding. These findings provide insights into the complex proteome-level changes that occur in tomato plants under high light stress and highlight the importance of proteomic approaches for understanding plant stress responses.

In addition, Zhan *et al*. (2019) demonstrated the TMT-labelled LC-MS/MS analysis in investigating the proteomic changes between okra seedlings grown under 0 and 300 mmol L^{-1} NaCl (salt stress). The results showed that salt stress led to significant

changes in the okra seedling proteome, with a total of 43 differentially expressed proteins identified, of which the differentially expressed proteins were involved in a wide range of cellular processes, including photosynthesis, carbohydrate metabolism, protein synthesis and degradation, stress response, and signal transduction. From this analysis, a number of stress-responsive proteins were identified, including heat shock proteins, peroxidases, and antioxidant defence proteins, which were upregulated in response to salt stress, indicating their crucial role in protecting okra seedlings from salt stress. Overall, the study highlights the significance of proteomic approaches for understanding plant stress responses and provides insights into the proteomic changes that occur in okra seedlings under salt stress.

2.4.3 **Applications of proteomics in plants**

Plants are constantly exposed to various environmental stresses, such as drought, heat, cold, salinity, and heavy metal toxicity, among others. These stresses can significantly affect the metabolism of plants, particularly in relation to the uptake, assimilation, and utilisation of nutrients, as well as the production and storage of energy (Khan *et al*., 2017). As a response to these stresses, plants often activate complex regulatory mechanisms and metabolic pathways that involve a variety of proteins. Proteins that are involved in carbohydrate, nitrogen, and energy metabolism are particularly important in this regard, as they play critical roles in the maintenance of plant growth, development, and survival under adverse conditions (Tan *et al*., 2017). By analysing the proteome under different abiotic stress conditions, researchers better understand the molecular mechanisms underlying the plant response to stress and identify potential targets for improving stress tolerance.

Recent proteomic investigations on abiotic stress tolerance plants under unfavourable conditions have helped pave the way for devising a more efficient agriculture strategy and developing plant variety with greater resilience towards climate change. For instance, Jiang *et al*. (2021) performed a TMT proteomic analysis on citrus plants that had been exposed to short-term cold stress. The analysis revealed approximately 400 differentially altered proteins that were found to play a significant role in the regulation of various metabolic processes, including starch and sucrose metabolism, biosynthesis of secondary metabolites, and phenylpropanoid. These metabolic pathways are crucial for the growth and development of plants, and their alteration is a common response to environmental stresses. In another study reported by Jamshidi Goharrizi *et al.* (2020), a MALDI-TOF/TOF proteomic analysis was conducted on pistachio rootstock that had been subjected to salinity stress. The analysis identified the involvement of several proteins that had not been previously reported to be involved in the plant response to salinity stress, including ribonucleoside-diphosphate reductase small chain, polcalcin Phl p 7-like, golgin subfamily A member 5, and 5 unknown proteins. The authors thought these proteins to be important for the regulation of various cellular processes under stress conditions, such as DNA synthesis and repair, calcium signalling, and vesicular transport.

Zhu *et al*. (2021) reported the physiological and proteomic responses of wheat seedling leaves to salt and osmotic stresses. Both salt and osmotic stresses significantly reduced the growth of wheat seedling leaves. The accumulation of ions, such as Na^+ and Cl^-, were observed to increase under salt stress, while proline accumulation was increased under osmotic stress. Interestingly, proteomic analysis of the chloroplast proteins of wheat seedling leaves under both stresses exhibit a total of 152 differentially expressed proteins, with 80 proteins upregulated and 72 proteins downregulated. The differentially expressed proteins were involved in various biological processes, such as photosynthesis,

carbon metabolism, stress response, and protein synthesis. From these findings, salt stress reduces photosynthetic efficiency, disrupts carbon metabolism, and significantly reduces protein synthesis, while osmotic stress affects mainly the wheat stress response and protein synthesis.

Several research on drought-responsive proteins in plants via proteomics technique has been widely reported. For instance, a recent investigation by Gu *et al*. (2021) utilised a TMT LC-MS/MS analysis to examine the response of two-year-old tea plants to drought stress by withholding watering for 96 hours under a controlled environment. Of the 4789 proteins identified, 11 proteins were upregulated, while 100 proteins showed a significant downregulation, which is involved in the biosynthesis of lignin, flavonoids, and long-chain fatty acids. This study highlights the potential of proteomics techniques in identifying critical proteins involved in plant stress responses. The proteomics analysis enables the identification of numerous proteins and their alterations in response to stress, offering a comprehensive understanding of the molecular mechanisms responsible for stress tolerance in plants.

A comparative study on the leaf proteome of two tomato varieties, one drought-tolerant and one drought-sensitive, by Rai *et al*. (2021), demonstrated the importance of proteomics analysis in identifying key proteins involved in drought tolerance. The drought-tolerant variety expressed 77 differential proteins, while the drought-sensitive variety only expressed four proteins. These differentially expressed proteins were related to important cellular processes, such as stress response, photosynthesis, electron transport, and protein synthesis. This result highlights the potential of proteomics analysis in the development of drought-tolerant crops.

The identification of drought-responsive proteins in plants via proteomics analysis is crucial for understanding the mechanisms underlying plant responses to drought stress

and developing crops with improved drought tolerance. This knowledge can be used in breeding programs to produce crops that can withstand drought stress and maintain productivity even in adverse environmental conditions. Therefore, proteomics analysis is a powerful tool in plant abiotic stress research and can aid in the development of higher abiotic tolerance crops, particularly drought stress. The integration of high-throughput technologies has become essential to crop improvement strategies, allowing researchers to more efficiently address current and future demands in food and agriculture production, which have been exacerbated by global climate change.

2.5 *Pandanus amaryllifolius*

2.5.1 **Pandanus family**

Pandanus species, commonly referred to as screw pines, are a collection of plants from the genus *Pandanus*. They are commonly found in tropical and subtropical areas and are recognizable by their unique aerial roots and leaves that are arranged in a spiral pattern. There are over 700 different species of *Pandanus*, each possessing distinct features and characteristics. These plants range in size from small shrubs to towering trees, and their shapes and colours vary greatly from deep green to yellow and red. Some *Pandanus* species produce fragrant flowers, whereas others bear edible fruits consumed locally, such as *Pandanus odaratissimus* and *Pandanus tectorius* (**Figures 2.5A & B**).

Pandanus species have a wide range of practical and cultural applications. For example, leaves of some species can be used to thatch roofs, weave mats, baskets, and bags. The fruit of some species is used as a food source, whereas wood is used for construction and carving. In traditional medicine, various parts of the plant are used to treat ailments, such as wounds, headaches, and digestive issues. Overall, *Pandanus*

species are a diverse and versatile group of plants that play crucial roles in the lives and cultures of many tropical communities.

Figure 2.5. Other species of *Pandanus* spp. A) *Pandanus odaratissumus* (Adkar & Bhaskar, 2014). B) *Pandanus tectorius* (Gallaher, 2014).

2.5.2 **Characteristics**

Pandanus amaryllifolius Roxb. or Pandan is a tropical herbaceous plant belonging to the Pandanaceae family, also known as "pandan-wangi" or "fragrant screw pine." The plant has a spiral arrangement of long, narrow green leaves that resemble pineapple leaves and are fragrant. Approximately 600 species of *Pandanus* in the family are found in tropical and subtropical regions. *P. amaryllifolius* is the only known member of the *Pandanus* species capable of producing scented leaves by accumulating the compound 2-acetyl-1-pyrroline (2-AP). It is native to Southeast Asia and is widely cultivated for various purposes, including cooking and traditional medicine.

Taxonomical classification:

Kingdom: Plantae
Phylum: Tracheophyta
Class: Liliopsida
Order: Pandanales
Family: Pandanaceae
Genus: Pandanus
Species: *Pandanus amaryllifolius* Roxb.

P. amaryllifolius is a dioecious, woody, palm-like tree or shrub without secondary growth, with bark covered in leaf scars arranged in a spiral pattern sheathing the rounded to articulate base. The plant prop roots emerged at the base of the trunk (**Figure 2.6A**). The leaves were arranged alternately on a central stem (**Figure 2.6B)** and did not have thorny characteristics, although they had small thorns at the tip of the leaf. The size of *P. amaryllifolius* plants can vary from shrub-like to less than two feet tall, with a moderate growth rate (Routray & Rayaguru, 2010). The mass propagation method is suitable for planting in a monocrop or intercrop system, yielding approximately 6 tonnes per hectare per year with two harvests (Useful Tropical Plants, 2022). Based on the current market

prices in Malaysia, a kilogram of pandan leaves is worth around RM 8-10, giving the plant a potential economic value of up to RM 50,000/ha/year.

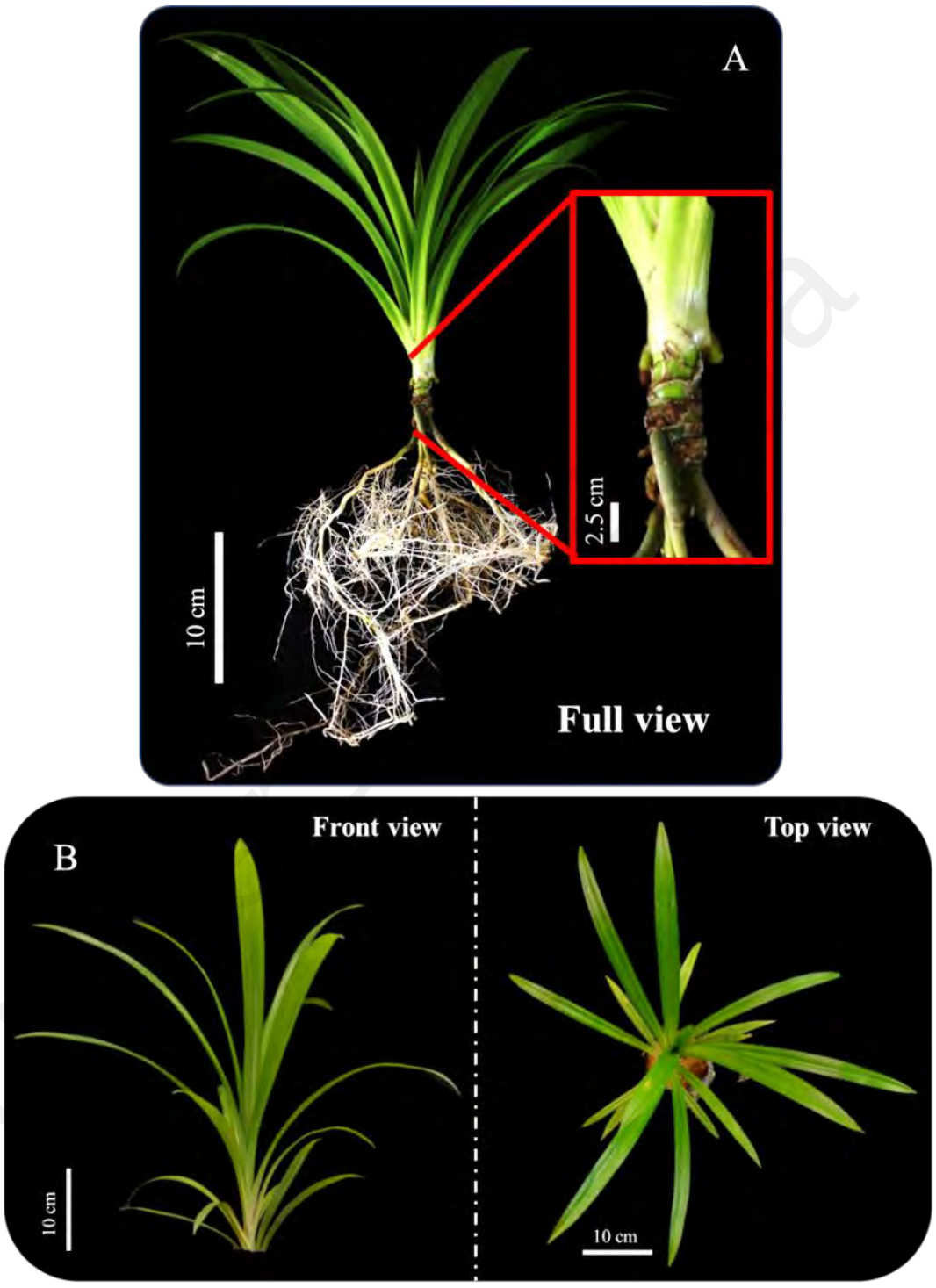

Figure 2.6. The morphology of 3-month-old *Pandanus amaryllifolius* from the front and top view. The line bar indicates 10 cm.

2.5.3 **General uses**

P. amaryllifolius is commonly used as a cooking ingredient due to its distinctive natural flavour and fragrance. The pandan leaves have a sweet, nutty, fresh hay flavour similar to the fragrance found in expensive aromatic rice grown in Southeast Asia, such as Jasmine-Thai rice, Kaorimai-Japan rice, and Basmati-India rice (Bhuyan & Sonowal, 2021). In addition, the unique fragrance *P. amaryllifolius* has been incorporated into one of the national dishes of Malaysia, 'Nasi lemak', to enhance the aroma and flavour of the rice (**Figure 2.7A)**. Similarly, the pandan-layered cake made from the *P. amaryllifolius* extract gives the dessert a distinct colour, flavour, and fragrance of pandan (**Figure 2.7B**).

Figure 2.7. The food products utilising the aroma and extract from *P. amaryllifolius* as flavouring and colour. **A**) Nasi lemak (Khoo, 2019) **B**) Pandan-layered cake (Khang Yi, 2021).

P. amaryllifolius is also widely used in traditional medicine to control diabetes as a diuretic and to treat skin diseases (Wakte *et al*., 2009). For instance, a recent study demonstrated the effectiveness of *P. amaryllifolius* leaf extract in controlling blood sugar levels and found that it contains high levels of antioxidants and anticancer compounds (Chiabchalard & Nooron, 2015; Ghasemzadeh & Jaafar, 2013). In addition, *Pandanus*

spp. leaf extracts, including *P. amaryllifolius*, have the potential to be used in the synthesis of nanoparticles through a green synthesis method (Alajmi *et al.*, 2018; Hussain *et al.*, 2019; Jabbar *et al.*, 2020; Patil *et al.*, 2021).

P. amaryllifolius was chosen for this study because of its ability to grow under low soil moisture conditions and its hardy, low-maintenance nature (Han *et al*., 2014). A study of two related *Pandanus* spp., *P. dubius* and *P. tectorius*, showed that these species could tolerate a humid, lowland tropical climate by maintaining leaf osmotic potential after eight weeks of water withholding (Marler *et al*., 1996). It has been reported that 2-AP, which is responsible for the fragrance of *P. amaryllifolius*, is enhanced under osmotic stress in fragrant rice (Fitzgerald *et al*., 2008). However, *Pandanus* species that produce high levels of 2-AP are more tolerant to osmotic stress than fragrant rice (Bhatt *et al*., 2021). Luo *et al.* (2020) also found that 2-AP accumulation is increased with the exogenous application of proline, indicating a relationship between drought stress and fragrance release. Proline is a molecular marker of osmolytes that is used as an indicator of water stress. However, more research is needed to fully understand *P. amaryllifolius*'s tolerance to drought stress. A comprehensive study of the ability of plants to tolerate drought stress would be useful for further understanding its mechanisms, thus helping to develop an effective strategy to overcome agricultural issues in arid environments.

2.6 Plant biostimulants

Plant biostimulants have gained recognition as a potential eco-friendly agronomic tool, as evidenced by the rise in scientific publications and the consistent expansion of their market (Bulgari *et al*., 2019). Consequently, they have emerged as one of the prominent areas of research in agriculture and have been extensively reviewed (Baltazar *et al*., 2021; Sible *et al*., 2021).

Plant biostimulants refer to any substances or microorganisms applied to plants to promote nutrient uptake, improve environmental stress tolerance, and enhance crop quality attributes, ultimately resulting in a higher yield (Del Buono, 2021). They were first defined by Zhang and Schmidt (1997) as "materials that, in minute quantities, promote plant growth". Here, the term "in minute quantities" differentiates the plant biostimulants from nutrients, soil improvers, and pesticides applied in larger quantities. However, plant biostimulants were redefined in the Fertilising Products Regulation (FPR) as "*EU fertilising products stimulating plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere: nutrient use efficiency, tolerance to abiotic stress, quality traits, availability of confined nutrients in soil or rhizosphere*" (European Parliament and European, 2019). The new definition clarifies the categorisation of plant biostimulant products.

2.6.1 **Source of plant biostimulants**

As stated earlier, plant biostimulants can be formulated using various microorganisms or naturally occurring bioactive compounds. Unlike conventional fertilisers, plant biostimulants do not directly supply nutrients to plants or target pests and pathogens but instead promote nutrient uptake and facilitate plant growth and

development. There have been various methods of categorising plant biostimulants based on their mode of action, contents, or a combination of both (Lau *et al*., 2022). However, under the Fertiliser Products Regulation (FPR), plant biostimulants are classified into two categories: microbial plant biostimulants and non-microbial plant biostimulants.

In recent decades, the availability of high-quality agricultural land has declined, and this trend is expected to continue as climate change progresses. This underscores the need to maintain crop yields production under increasingly stressful conditions despite on a smaller land area. Microbial-based plant biostimulants have emerged as a promising tool to enhance crop yields under low-input conditions and alleviate the impacts of climate change on crops (Lau *et al*., 2022). These formulations contain free-living beneficial bacteria and/or fungi under Component Material Categories number 7 (Castiglione *et al*., 2021). Various bacteria and fungi have been studied for their potential use as biostimulants, with Rhizobium and rhizospheric plant growth-promoting rhizobacteria (PGPR) being the most widely reported group (Philippot *et al*., 2013). Over 20 commercially available microbial-based plant biostimulants are currently derived from PGPR. Apart from PGPR, arbuscular mycorrhizal fungi (AMF) have also enhanced plant performance. These fungi form early symbiotic partnerships with many plant species, with more than 80% of land plant species capable of forming symbiotic associations with AMF (Spatafora *et al*., 2016). Like PGPR, some AMF inoculants have been commercialized by companies, utilising either single or mixtures of AMF species, with *Funneliformis mosseae* and *Rhizoglomus irregulare* being the most commonly used (Giovannini *et al*., 2020).

Non-microbial plant biostimulants comprise bioactive substances, including humic substances, protein hydrolysates, nitrogen-containing compounds, seaweed extracts, chitosan, biopolymers, and inorganic compounds. These plant biostimulants account for over 33% of the biostimulant market globally (Eef *et al*., 2018), with seaweed

extracts being the most commonly utilised (El Boukhari *et al*., 2020). Seaweeds are highly nutritious, containing proteins, enzymes, and polysaccharides. Applying seaweed extracts in agriculture has been documented since the first century (Newton, 1951). Currently, seaweed extracts are applied to plants to alleviate abiotic stress, enhance nutrient efficiency, and promote root growth and microbial activity in the root zone. Seaweeds are classified into three groups based on their pigmentation: Phaeophyta (brown), Rhodophyta (red), and Chlorophyta (green). Commercially available seaweed extracts are predominantly derived from brown seaweeds, such as *Ascophyllum*, *Fucus*, and *Laminaria*, possibly due to their rich organic and mineral components, such as laminarin, fucoidan and alginates, mannitol, plant hormones, and minerals (Fe, I, K, Mg, and S) (Kapoore *et al*., 2021).

Humic and fulvic acids are organic molecules originating from the biological or chemical transformation of plant and animal waste and chemical reactions. These substances have been shown to elicit physiological changes in roots and shoots that enhance crop nutrient assimilation and distribution, ultimately improving growth and stress tolerance. Humic substances are typically administered through soil drenching, although the foliar application is also used in some cases. The beneficial effects of humic substances include improved soil structure, enhanced phosphorus availability, soil pH neutralisation, induction of lateral root growth, and stimulation of nitrate assimilation in crops. Despite their demonstrated efficacy, the precise mechanisms by which humic substances affect plant physiology remain elusive due to the complex nature of these substances and their varied effects on plant responses.

Protein hydrolysates are a popular type of plant biostimulants used in agriculture to improve crop performance under stress. This category of plant biostimulants is composed of a mixture of free amino acids, oligo- and polypeptides derived from plant and animal sources. They are mainly applied as foliar spray but can also be applied as a

substrate drench or seed treatment. Protein hydrolysates have demonstrated biostimulatory effects by inducing critical enzymes involved in nitrogen assimilation and carbon metabolism and increasing antioxidant enzyme activity and secondary metabolite production (Rouphael & Colla, 2018).

2.6.2 **Wood vinegar**

Wood vinegar (WV) is a type of plant biostimulant. It is a concentrated reddish-brown liquid produced by the pyrolysis of organic biomass during the production of biochar or charcoal. WV is a liquid containing a complex mixture of compounds, including acetic acid (the main component of vinegar), methanol, acetone, and various types of organic acids and aldehydes (Theapparat *et al*., 2018). WV has also been referred to as pyroligneous acid and concentrated smoke water in several publications. WV has been utilised in many sectors, such as agriculture, farming, industry, and the environment, because of its versatility. For instance, WV is used as a natural pesticide, fungicide, soil conditioner, and plant growth promoter to control diseases in plants and increase crop yields.

The production of WV is an environmentally friendly process because it does not generate greenhouse gases; instead, it can be used as an energy source. This pyrolysis process also helps reduce soil degradation and greenhouse gas emissions (Fidel *et al*., 2019). This is because most biomass waste from industry is discarded in landfills or burned to ash in open burning, which damages soil biota and releases harmful particulates, volatile compounds, ash, and trace gases into the atmosphere. Between 2017 and 2018, it was reported that the global biomass waste from the agricultural sector, including maize, rice, sugarcane, oil palm, and wheat, was 1.33 billion metric tonnes (Millati *et al*., 2019). This can lead to environmental pollution, global climate change, biodiversity loss, and

socioeconomic and health problems. However, the long-term effects of WV are not yet clear. Further research is needed to understand the safety and efficacy of WV in various applications.

2.6.3 **Wood vinegar production**

The pyrolysis process occurs at temperatures ranging from 200 to 450 °C under anaerobic conditions, producing biochar, smoke, and WV (Grewal *et al*., 2018; Lu *et al*., 2019). In a closed system, biomass is heated to a specific temperature, which allows it to release moisture. Because the oxygen concentration is low, the heated biomass also releases smoke, which is channelled into a cooling tube where smoke and moisture are precipitated and collected. Producing WV typically involves several steps starting from the collection of starting materials, such as forestry residues, industrial wood residues, sawdust, or wood chips. These materials are subjected to pyrolysis in a reactor or kiln, where they are heated in the absence of oxygen at temperatures between 200 and 600 °C (Xin *et al*., 2021) (**Figure 2.8**).

The intense combustion causes wood to break into gases and liquids, known as pyrolysis products. Next, the pyrolysis gases are cooled and condensed to form liquid WV containing acetic acid, methanol, and other organic compounds. At this stage, concentrated WV is mixed with impurities, such as smoke particles. To purify the product, filtration and distillation processes need to be deployed, whereby liquid WV is then distilled to separate and purify the different components. This process increases the concentration of acetic acid in the final product, making it an effective pesticide and fungicide.

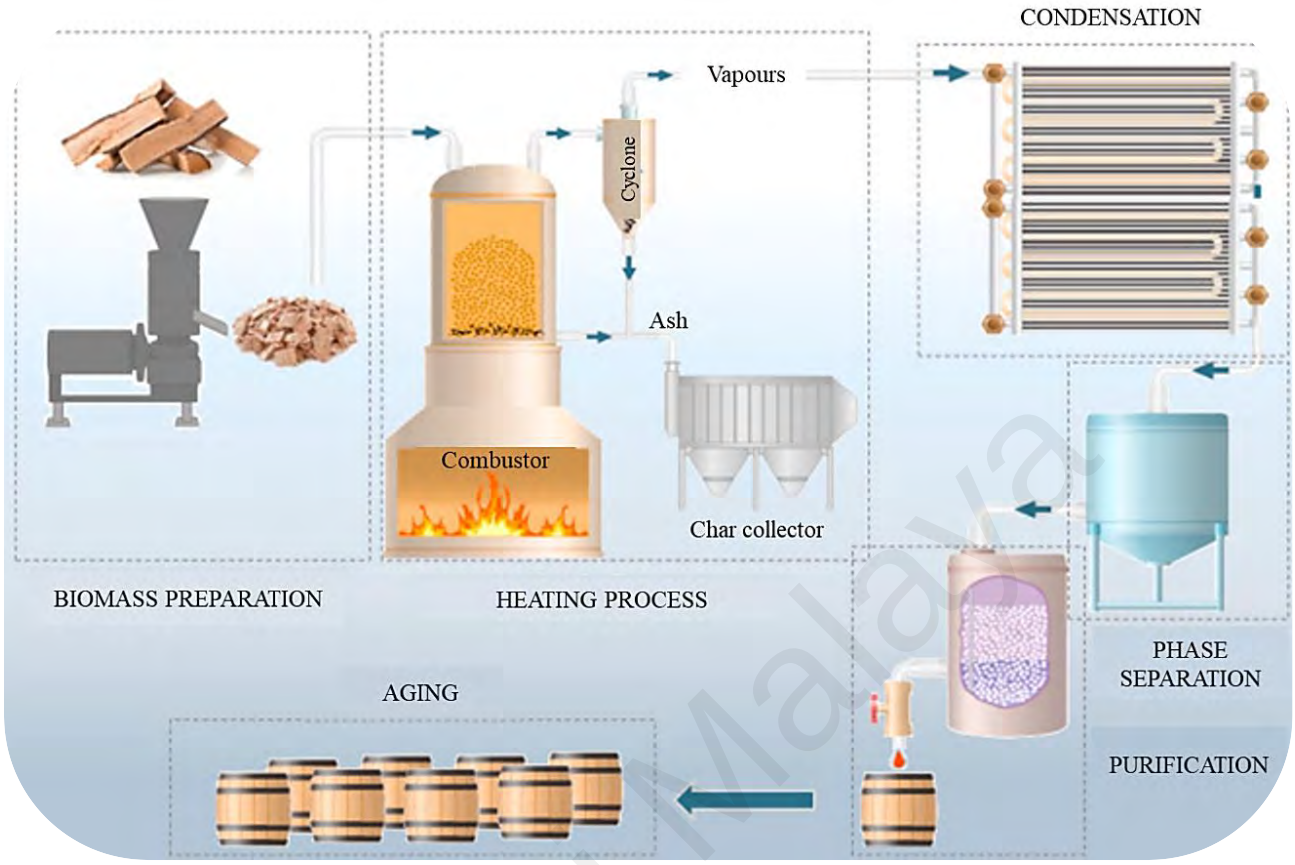

Figure 2.8. Overview process of WV production (Xin *et al*., 2021).

The pyrolysis process can be carried out in different ways, and the yield, composition, and properties of the final product depend on the type of wood, the conditions of the pyrolysis process, and the methods used for distillation and purification (Xin *et al*., 2021) (**Table 2.1**). It is important to note that WV should be performed under controlled conditions to avoid the production of harmful compounds, such as polycyclic aromatic hydrocarbons (PAHs) and to avoid the release of harmful emissions to the environment (Bucheli *et al*., 2015).

Table 2.1. Pyrolysis condition based on the biomass and type of reactor of WV production (Xin *et al*., 2021).

Biomass waste	Reactor	Pyrolysis/smoking conditions
Durian peel	Batch slow pyrolysis reactor	Pyrolysis at 300, 340 and 380 °C
Corncob	Batch slow pyrolysis reactor	Pyrolysis at 300, 350, 400, 450 and 500 °C
Cocoa bean skin	Batch slow pyrolysis reactor	Heating rates of 5, 10 and 15 °C/min to achieve pyrolysis temperature of 450–550 °C
Palm kernel shells	Batch slow pyrolysis reactor	Temperatures ranging from 280 to 400 °C
Pineapple stem and leave	Kiln	Pyrolysis temperatures ranging between 200 and 500 °C, Holding time of 48 h
Coconut shell	Batch slow pyrolysis reactor	Heating rate of 30 °C/min, Pyrolysis temperatures ranging between 150 and 450 °C, Holding time of 2 hours
Rice hulls	Carbonisation furnace	Processing time of 10 days

2.6.4 **Wood vinegar compositions**

It is important to note that not all types of WV are created equally. WV is a complex mixture that contains a large number of organic compounds, such as acids, phenols, aldehydes, ketones, esters, and sugars. The composition of WV can vary depending on different factors, such as the type of wood used, the temperature, and duration of the distillation process. The main constituents of WV include acetic acid, which is responsible for its sour taste and pungent odour, phenols that contribute to its smoky flavour and aroma, furfural that has a nutty smell and is used in the production of resins and solvents, and pyrones that have a fruity or floral odour and are used in the production of flavours, fragrances, and pharmaceuticals. Additionally, the quality and the composition of the WV is dependent on various factors, with the type of wood and the conditions of the pyrolysis process being the key considerations. Different origin of starting materials with varying chemical compositions can impact the properties of the resulting WV. For example, different sources of biomass and pyrolysis condition such as temperature have been reported to produce various combinations of compounds, as reviewed by Mattos *et al.* (2019) (**Table 2.2**).

Table 2.2. Chemical composition of WV derived from biomass pyrolysis (Mattos *et al*., 2019).

Biomass origin	Temperature(°C)	Major compounds
Coffee grounds and its fractions	400, 450, 500, 550 and 600	**Phenolic compounds**: phenol; 2-methylphenol; 3-methylphenol; 4-methylphenol; 2,3-dimethylphenol; 2,5-dimethylphenol; 2,6-dimethylphenol; 2,4-dimethylphenol; 3-ethylphenol; 3,5-dimethylphenol; 2-ethyl-6-

		methylphenol; **Acids and esters and Alcohols:** trifluoroacetic acid; tetradecyl ester; 9,12-octadecandien-1-ol; **Ketone:** 2-cyclopenten-1-one; **Hydrocarbons:** pentadec-1-ene; 2-(1-methyl-1-pyrrolinidyl);4-tetradecene; octadec-9-ene; dodec-1-ene; dec-1-ene; tridec-1-ene; bicyclo[4.3.0]octa-1,3,5-triene; eicos-5-ene; pentadecane; eicos-9-ene; eicos-3-ene; docos-1-ene; **Nitrogenated compound**: pyridine; 2-(1-methyl-1-pyrrolinidyl)
Dry tomato residue and its fractions	300 and 500	**Phenolic compounds**: diene; neophytadiene; 2-methylphenol, 3-methylphenol; 2,4-dimethylphenol; 4-ethylphenol **Nitrogenated compounds:** benzonitrile; indole linear alpha-olefins (LAOs), C15-C19
Lignin, cellulose, hemicellulose isolated and 2 batches of ESP lignin	450 and 550	**Polycyclic aromatic hydrocarbons**: fluoranthene; phenanthrene; 4,5-methylenephenanthrene; pyrene; 1-phenylnaphthalene; 2-phenylnaphthalene; anthracene; benzoanthracene; 1-methylphenanthrene; **Ester and Alcohols:** dodecyl acrylate; dodecan-1-ol; phytol

Macadamia nutshell	NI	**Polycyclic aromatic hydrocarbons**: acenaphthene; acenaphthylene; anthracene; pyrene; benzo[a]anthracene; benzo[a] pyrene; benzo[ghi]perylene; benzo[k + b]fluoranthene; crysene; dibenz(ah)anthracene; ethylbenzene; fluoranthene; fluorene; indeno[1,2,3-cd] pyrene; octadecane; nonadecane; **Phenolic compounds**: naphthalene; phenanthrene; **Aromatic hydrocarbons:** benzene; 1,2,4 trimethylbenzene; 1,3,5 trimethylbenzene; toluene; xylene; **Hydrocarbons:** decane; undecane; dodecane; tridecane; tetradecane; pentadecane; hexadecane; heptadecane; phenol; 2-methylphenol; 2,4 dimethylphenol
Balsam fir and white spruce mixed bark residues	450	**Phenolic compounds:** phenol; 3-methylphenol; 3-methoxyphenol; 2-methoxy-4-methylphenol; 4-ethyl-2-methoxyphenol; 4-hydroxy-3-methoxybenzenacetic acid 1,2-benzendiol; 3-methyl-1,2-benzendiol; 4-methyl-1,2-benzendiol **Furan derivatives:** 2-furancarboxaldehyde **Ketone:** 3-methyl-1,2-cyclopentanedione
Yellow poplar (*L. tulipifera*)	500	**Phenolic compounds**: phenol; 2,6-dimethoxyphenol; 4-hydroxy-3-methoxybenzoic acid; 1-(4-hydroxy-3,5-dimethoxyphenyl) ethenone; 1,6-anhydro-β-D-glucopyranose

Bamboo (*D. asper*)	400	**Phenolic compounds**: 2-methylphenol; 4-propyl-2-methoxyphenol; 4-methyl-2-methoxyphenol; 4-ethyl-2-methoxyphenol; 2-methoxyphenol; eugenol;
Eucalyptus wood residues	500	**Phenolic compounds**: phenol; 2,6-dimethoxyphenol; 4-hydroxy-3,5-dimethoxybenzaldehyde; 1-(4-hydroxy-3,5-dimethoxyphenyl) ethanone; 4-hydroxy-3-methoxybenzaldehyde; 1,2-benzendiol; 4-allyl-syringol; pyranone; 2-methoxyphenol; hydroquinone; hydroxy-guaiacol; eugenol; acetoguiacone; guacylcetone; 4-propenyl-syringol (trans); homosyringaldehyde; conyferyl aldehyde (trans); synapyl alcohol (allyl); Furan; furanone; 1,6-anhydro-β-D-glucopyranose; 4-hydroxy-4-methyl-2-pentanone; 1,4:3,6-dianhydro-α-D-glucopyranose
Palm tree bark	350 and 600	**Phenolic compounds**: 2,6-dimethoxyphenol; 2-methoxyphenol; 4-methyl-2-methoxyphenol; 4-methylphenol; 3-methoxy-1,2-benzenediol; 4-ethyl-2-methoxyphenol; 1,2-benzenediol; 2-methylphenol; 4-hydroxy-3-methoxybenzoic acid;
Solid pineapple biomass	350 and 600	**Phenolic compounds**: phenol; 2,6-dimethoxyphenol, 1,2-benzenediol; 3-methoxy-1,2-benzenediol; 4-methylphenol; 2-methoxy-4-methylphenol; 4-methyl-1,2-benzenediol; 2-

		methoxyphenol; ρ-carbomethoxy phenol; 1-(4-hydroxy-3,5-dimethoxyphenyl)-ethanone; 1-(4-hydroxy-3-methoxyphenyl)-ethanone; 3,5-dimethoxy-4-hydroxyphenyl; acetic acid;1,4:3,6-dianhydro-α-D-glucopyranose; 3-methyl-1,2-cyclopentanedione; 1,2,4-trimethoxybenzene, maltol
Walnut branches	90-230, 230-370 and 370-450	**Acids**: acetic; 2-methyl-propanoic anhydride; propanoic acid; **Phenolic compounds**: 2,6-dimethoxyphenol; 1,2-benzenediol; 2-methoxyphenol; 2-methoxy-4-methyphenol; 3-methoxy-1,2-benzenediol; trimethoxybenzene; 1,2,3-trimethoxy-5-methylbenzene;furfural; maltol; 4-ethyl-2-methoxyphenol; hydroquinone; **Ketones:** 3-methyl-1,2-cyclopentanedione; 3-ethyl-2-hydroxy-2-cyclopenten-1-ona; 2-cyclopenten-1-ona; **Aromatic hydrocarbons**: 1,2,4-trimethoxybenzene; 1,2,3-trimethoxy-5-methylbenzene; furfural; maltol
Japanese red pine (*P. densiflora*)	500	**Furans derivates:** 2-methylfuran; 2,5-dimethoxytetrahydrofuran; 2(5 H)-furanone; 2,3,4-trimethylfuran; 5-(hydroxymethyl)-2-(dimethoxymethyl)furan; **Phenols derivates:** Phenol; 2-methylphenol; 4-methylphenol; 2,5-xylenol; phenol (2-methoxy-6-(1-propenol); **Benzenes** 4-ethylresorcinol; 1,3-bis

	(trimethyl siloxy) benzene; benzenemethanol 4-(phenylmethyl); benzene-methanamine **Acids**: butanoic; benzoic; **Ketones:**propan-2-one; 2-methyl-2-cyclopentanone; **Others**: trimethyl orthoacetate; 6-methyl-4-indanol; 1,4-methanoazulene; 1,1,N,N-(tetramethylbuta)-1,3-diene-4-amine; erythrite-tetramethyl ether;
	naphthalene; 4-oxo-5-methoxy-2-penten-5-olide; p-ethylanisole; 5,4-dimethyl-2-methylbibenzyl hexanalldimethyl acetal; benzaldehyde dimethyl acetal

The acetic acid found in WV has potential agricultural uses. Organic acids in WV, including acetic acid, can act as natural herbicides in organic farming. For instance, Webber *et al*. (2018) reported increasing the concentration of acetic acid from 5% to 20% increased the efficacy of weed control, with the higher concentration resulting in a greater percentage of weed desiccation and reduced plant biomass. However, it also resulted in more severe damage to non-target plants. In addition, phenolic compounds are naturally occurring organic compounds that are found in various plants, including wood. Phenolic compounds are responsible for the smoky flavour and aroma of WV and are known to have antimicrobial, antifungal, and antioxidant properties (Winarni *et al*., 2021). WV-containing phenolic compounds can be used for a variety of purposes, including as a natural insecticide, herbicide, and fungicide. For example, a study by Setiawati *et al*. (2019) found that the neutralised WV from durian wood had a pH of 7.0, containing various organic acids, phenols, and other compounds that were toxic to the larvae of diamondback moths, which is a common pest in cabbage. It can also be used as a soil conditioner, as phenolic compounds have been shown to promote plant growth and enhance soil fertility. A recent investigation by Zhang *et al*. (2020) has shown the multiple purposes of WV containing high phenolic content in improving plant growth as well as soil fertility. The authors found that the co-application of biochar and WV significantly increased the yield of blueberry fruits by 38.5% and improved the nutritional quality of the fruits, including total phenolic content and antioxidant activity. The study also found that the co-application of biochar and WV increased the soil pH, nutrient availability, and microbial biomass compared to the individual application of either biochar or wood vinegar.

Pyrones are cyclic compounds found in WV that have a pleasant fruity or floral scent. These compounds are used in the production of fragrances, flavours, and medicines, as they have anti-inflammatory and analgesic properties. Researchers are

exploring their potential in treating serious illnesses like Alzheimer's and cancer (Bhat *et al*., 2017; McGlacken & Fairlamb, 2005). Pyrones can also be used as natural insecticides and plant growth promoters in agriculture. They have been shown to suppress the growth of some insect pests while enhancing the growth of crops. For example, a biocontrol agent, *Trichoderma* spp. was reported to consist of antibiotic properties contributed by the 6-pentyl-*α*-pyrone (6PP) (Vinale *et al*., 2008). Moreover, Garnica-Vergara *et al*. (2016) demonstrated the effectiveness of 6PP in inducing the formation of lateral roots via modulation of auxin transporters gene expression by interfering with the pathway of auxin and cytokinin in plants. The presence of pyrones in WV makes it a valuable and eco-friendly product that can be used in various industries.

Apart from the useful components in the WV, there is also toxic compound produced during the pyrolysis process, such as PAHs. PAHs are not intentionally used in the production of WV. However, they can be present in small amounts as impurities due to the heating of wood during the distillation process. PAHs are a group of organic compounds that are formed during incomplete combustion or pyrolysis of organic materials, including wood (De la Rosa *et al*., 2019). They are known to be toxic and carcinogenic and can pose a health risk if present in high concentrations. Therefore, the levels of PAHs in WV are closely monitored and regulated to ensure that they are within safe limits for human and environmental health.

2.6.5 **Wood vinegar applications**

WV has a long history, dating back to ancient civilisations, whereby the earliest discovery, estimated about 80,000 years old, was found in Germany and Italy (Sauter *et al*., 2001). The condensate with tar viscosity produced from birch wood was first produced by burning wood and collecting the condensate and used as an adhesive to craft tools and weapons while other usage as a pesticide and fertiliser has also been recorded

(Tiilikkala *et al*., 2010). However, the production and use of WV have declined with the advent of synthetic chemicals and modern agriculture. Nonetheless, there has been a resurgence in popularity as a natural and sustainable alternative to chemical products in recent years.

Controlled thermal degradation of organic biomass and by-products can produce a concentrated liquid known as crude WV. WV contains over 200 compounds, including organic acids, esters, furans, phenols, alcohols, and pyran derivatives (Liu *et al*., 2020). These concentrated compounds can potentially be used as biostimulants in agriculture because of their history of use in alternative medicines, pesticides, and fungicides. Biostimulants are substances or microorganisms that improve crop quality, nutrient efficiency, and tolerance to biotic or abiotic stress, regardless of their nutrient content (du Jardin, 2015). A recent review of biostimulant effectiveness based on various field experiments found that biostimulants can significantly improve crop yields, particularly in arid climates, low soil organic matter, and low nutrient levels (Li *et al.*, 2022). Several studies have demonstrated the impact of biostimulants on plant growth and resilience. For example, natural biostimulants derived from quillay extract, sesame oil, seaweed, and neem seed cake improve tomato growth and reduce the number of nematode eggs on the root (D'Addabbo *et al*., 2019). In addition, a study on cyanobacteria-based biostimulants (hydrolysates) showed promising effects on basil growth in a hydroponic system (Santini *et al*., 2022). Biostimulant products have been shown to improve common bean plant quality under regular irrigation and drought conditions (Petropoulos *et al*., 2020).

WV has been shown to have biostimulatory effects on plants. For example, eucalyptus wood-derived WV improved the biomass, yield, and total soluble solutes in tomato plants when applied via foliar or soil drenching (Mungkunkamchao *et al*., 2013). Similarly, various sources of WV significantly improve the growth of eggplant plants, including the number of leaves, plant height, stem circumference, and the number of

branches (Siriwardena *et al*., 2020). WV has also been shown to improve the growth and yield of other crops, such as tomatoes (Ofoe *et al*., 2022). However, there are concerns about the environmental impact of WV application in agriculture, as heavy metals or trace elements can accumulate and cause toxicity in soil or water sources. However, a study of the aquatic fern *Azolla filiculoides* showed that the trace element concentrations of WV in a water source were low and did not pose any toxicological concerns (Fačkovcová *et al*., 2020). Based on this evidence, WV has promising potential as a biostimulant, and its availability is abundant, as it can be produced from various waste biomasses. One potential source worth exploring is WV derived from oil palm waste biomass.

Besides improving plant growth and development, research has been conducted to investigate the potential of WV application in conferring environmental stress tolerance in several plant species. For instance, Zhu *et al.* (2022) reported the effect of poplar WV on the growth and low-temperature tolerance of rapeseed seedlings. Applying WV increased the growth of rapeseed seedlings and improved their tolerance to low temperatures. In addition, the treated seedlings exhibited higher shoot and root growth, as well as increased chlorophyll content and antioxidant enzyme activity, compared to untreated seedlings. Furthermore, the treated seedlings were better able to maintain their membrane integrity and avoid oxidative damage under low-temperature stress.

The recent investigation by Ma *et al*. (2022) showed that the application of WV produced from peach shells significantly improved the salinity tolerance of rapeseed plants. The treated plants exhibited higher biomass accumulation, lower electrolyte leakage, and lower levels of oxidative stress compared to untreated plants under salinity stress conditions (Ma *et al*., 2022). Additionally, the treated plants had higher chlorophyll content and photosynthetic efficiency, indicating that WV protected the photosystem II complex from damage caused by salinity stress. The beneficial effects of WV on salinity tolerance in rapeseed plants may be due to its ability to enhance antioxidant activity,

reduce membrane damage, and improve nutrient uptake. WV was also found to promote the growth of beneficial soil microorganisms, which may contribute to the observed improvements in plant growth and stress tolerance.

Apart from enhancing plant growth and alleviating stresses in plants, WV could also be applied as a herbicide and pesticide when used at high and optimum concentrations. For example, Adfa *et al*. (2017) reported the potential use of Chinese mahogany WV as a termite control agent against *Coptotermes curvignathus* Holmgren, a common and destructive termite species in Southeast Asia to cause damage to wooden structures and crops. Different concentrations (0%, 2%, 4%, 6%, and 8%) of Chinese mahogany WV against *C. curvignathus* in a no-choice test experiment. All concentrations of the WV exhibited significant termiticidal activity against the termite, whereby the highest concentration (8%) achieved complete mortality of termites within 3 days (Adfa *et al*., 2017). Moreover, the author observed a significant correlation between termite mortality with the WV concentration.

Similarly, Chu *et al*. (2022) demonstrated the potential of apple tree branch WV as a natural biocontrol agent herbicide for several weed species. Several concentrations of WV (0.5% to 1.1%) applied were able to reduce 50% of seeds germination of several weed species, such as motherwort (*Leonurus cardiaca* L.), redroot pigweed (*Amaranthus retroflexus* L.), Spanish needles (*Bidens pilosa* L.), and tall fescue (*Festuca arundinacea* L.). The author discussed the efficacy and effectiveness of WV in affecting weeds depending on the application rate (spray volume), weed species, and condition of the environment, such as light. As demonstrated, the WV application rates required to provide 50% control of motherwort and Spanish needles were relatively high, at 1911 L/ha and 653 L/ha, respectively (Chu *et al*., 2022). However, the highest evaluated rate of 4000 L/ha was able to control 35% of tall fescue by 10 days after treatment (DAT). Additionally, WV was found to be more effective in dark conditions than in light

conditions for controlling common purslane. By 5 DAT, the WV treatment provided 95% and 87% control of common purslane in dark and light conditions, respectively (Chu *et al*., 2022). This evidence demonstrates WV's potential as an alternative biocontrol agent to synthetic pesticides and herbicides. However, further research is needed to optimize the concentration and application method of the WV for maximum efficacy and minimal environmental impact.

These studies provide evidence for the potential of WV as a natural and sustainable solution for improving plant growth and resilience in challenging environments. Overall, the study contributes to the growing body of research on using natural products and sustainable agricultural practices, highlighting the potential of WV as a tool for enhancing crop performance and environmental sustainability.

2.6.6 **Oil palm wood vinegar**

Elaeis guineensis, or oil palm, is the most efficient oil-producing plant, producing more oil per hectare than other plants, such as rapeseed, coconut, soy, and sunflower (Qaim *et al*., 2020; WWF, 2022). It is a tropical tree grown for its fruit and produces high-yield oil, known as palm oil. Oil is extracted from the mesocarp of the fruit (the fleshy part surrounding the seed) and is used in a wide range of products, such as food, cosmetics, and biofuels. Oil palm is native to West Africa but is now grown in tropical regions around the world, including Southeast Asia, Africa, and South America. Oil palm supplies almost one-third of the world's demand for vegetable oil, with Malaysia and Indonesia being major producers. The market value of oil palms in Indonesia was reported to be USD 21 billion in 2018, while Malaysia was valued at USD 23 billion by 2021 (Parveez *et al*., 2022; Purnomo *et al*., 2020). However, the cultivation of palm oil has been associated with several environmental and social issues that suppress the full utilisation of palm oil globally as a challenge, particularly in the European market.

The expansion of oil palm cultivation has led to deforestation, biodiversity loss, and greenhouse gas emissions. Additionally, converting natural habitats to oil palm plantations has led to the displacement of local communities and the loss of traditional livelihoods. To address these issues, the Roundtable on Sustainable Palm Oil (RSPO) was established to promote sustainable palm oil production and certify palm oil that has been produced in accordance with a set of environmental and social criteria. Many companies and organizations have also committed to sourcing sustainable palm oil and reducing or eliminating its use of palm oil in their products.

The waste generated from oil palm, including empty fruit bunches (EFB), palm kernel shells (PKS), mesocarp fibres (MF), and palm oil mill effluent (POME), possess significant potential for the production of value-added products. These resources can be

utilized for the creation of biodegradable items such as composite, construction materials, and feedstock. For instance, palm kernel oil polyol (PKO-p) produced from oil palm waste was found to be a promising bio-asphalt for pavement applications as a binder that can potentially be used to replace bituminous binder, which is carcinogenic and hazardous material (Alamawi *et al*., 2019). In addition, palm oil fuel ash (POFA) has high amounts of silica and alumina that make it a potential supplementary cementitious materials which when added into the concrete production can improve the strength, durability of concrete and reduce the amount of cement needed that subsequently lowers greenhouse gas emissions (Hamada *et al*., 2021). In addition, POME has a variety of potential uses as a source of organic fertilisers in agriculture. For example, a recent study has shown a promising utilisation of POME as fertiliser, which is a good source of nitrogen, phosphorous and potassium (NPK) when anaerobically treated prior to usage (Loh *et al*., 2019). This can improve soil fertility and crop yields while reducing the need for synthetic fertilisers.

Although these efforts have greatly improved the oil palm market, there is still underutilised oil palm waste that could potentially add more value to the oil palm industry, one of which is WV. Exploring the potential of WV from palm oil waste could add value to the market. Several studies have been conducted on the production of WV from oil palm biomass. For example, WV produced from oil palm trunks contains high concentrations of 1-hydroxy-propanone, 3-hydroxy-2-butanone, acetic acid, propanoic acid, and phenol, which have shown fungicidal effects against white-rot and brown-rot fungi as well as antitermitic properties (Oramahi *et al*., 2018). Abas *et al.* (2018) also reported the antimicrobial properties of WV from oil palm fibres against *Bacillus cereus*, *Lactobacillus plantarum*, *Escherichia coli*, and *Staphylococcus aureus*. In addition, WV produced from oil palm kernel shells, containing phenols, esters, and

ketones, has shown strong antioxidant and anti-inflammatory activities, effectively inhibiting nitric oxide activity (Mahmud *et al*., 2020).

In summary, oil palm waste can positively impact the environment and economy by providing alternative sources of energy and valuable products. Proper management and utilisation of oil palm waste can reduce the environmental impacts of the oil palm industry and promote sustainable development. The application of OPWV as biostimulants has been shown to have a beneficial effect on crop growth and yield under stress conditions and is considered an important factor for maintaining soil health and sustainable food production. However, these practices are not widely adopted in commercial agriculture, possibly due to the lack of empirical data on the risks and benefits across multiple crop systems, as well as farmers' limited knowledge of practical applications. Additionally, some farmers may be hesitant to adopt WV biostimulants due to perceived increases in cultivation costs.

CHAPTER 3

METHODOLOGY

3.1 Plant material

Three-month-old disease-free *Pandanus amaryllifolius* were acquired from the I Green Nursery Sdn. Bhd., Muar, Johor, Malaysia. Total of 88 plants were transferred to polybags (20 × 11 cm^2) containing 600 g clay silt loam soil. The plants were acclimatised for two weeks in a growth room at Universiti Malaya, Malaysia, which were maintained at 28 ± 2 °C and 80 ± 5% relative humidity with 1500 lux light intensity under a 12:12 h photoperiod cycle. All plants were watered 20 mL once a day and foliar fertilised (BABA Mr Ganick Foliar Fertilizer 20×) once per week in the morning (8 am-10 am) prior to treatment.

3.2 Drought stress treatment

After two weeks of acclimatisation, the plants were exposed to drought stress by withholding water for 4, 7, 10, and 14 days and re-watered for additional 7 days (**Figure 3.1A**). The control plants of each time point received 20 mL water daily throughout the 21-day experimental period. The soil moisture content in all pots was monitored daily using a soil moisture meter (HH2 moisture meter, Delta-T, UK) (**Appendix 1A**). The experiments were carried out in a complete randomised block design with 8 biological replicates for each treatment. After drought treatment, leaf samples at positions 2 to 6 from the centre leaf (**Figure 3.1B**) were harvested, wiped with 70% ethanol (EMSURE, Merck, Germany) and snap-frozen in liquid nitrogen. These samples were stored at -80°C until use.

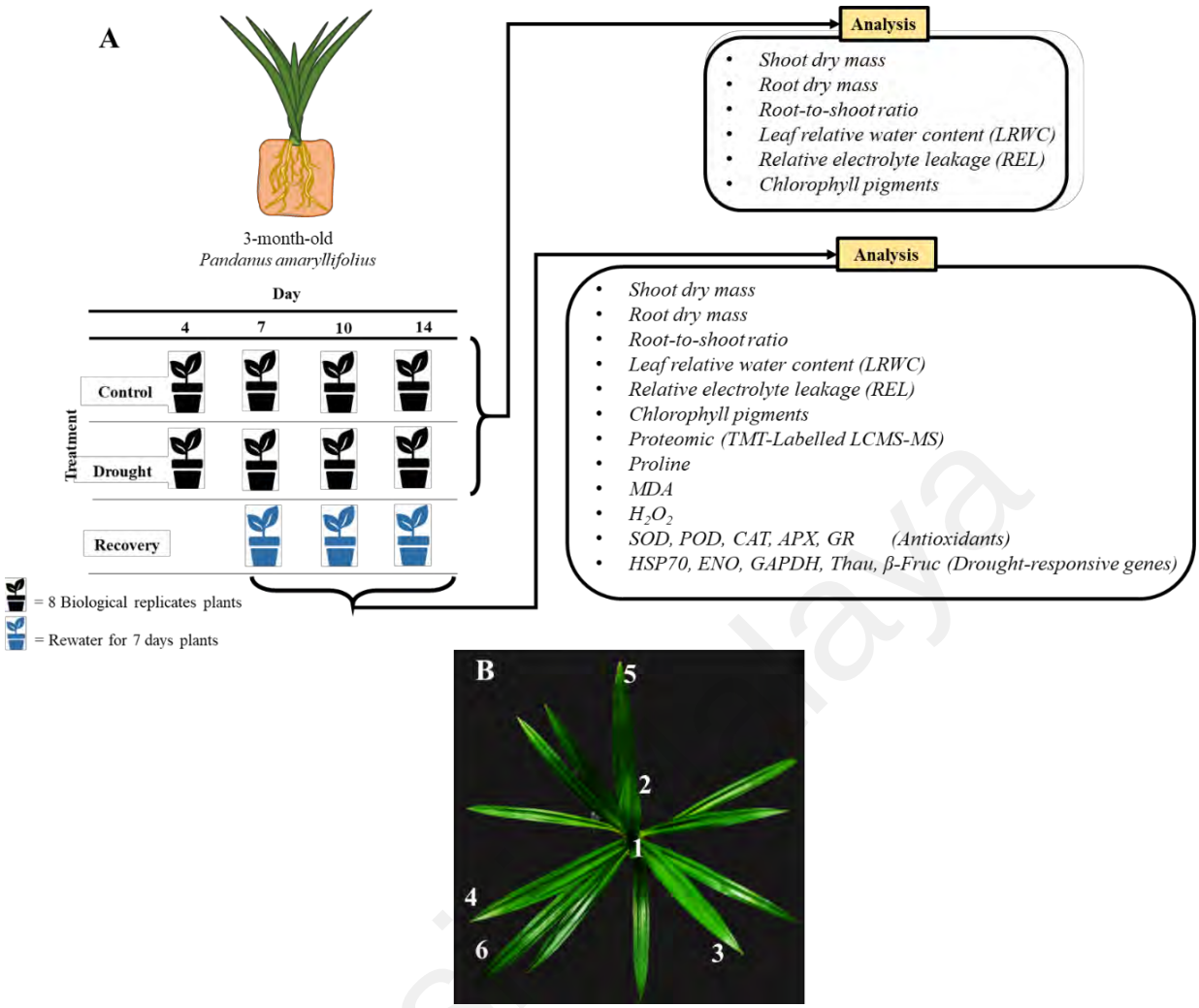

Figure 3.1. (**A**) Experimental design for the application of drought stress and recovery treatment on *P. amaryllifolius*. (**B**) Leaf number position guide for harvesting from the top view of *P. amaryllifolius* starting at leaf number 1 from the centre.

3.3 Morphological analysis

3.3.1 **Measurement of plant biomass**

The plant biomass was analysed by taking the fresh and dry weights of the samples. Immediately after the harvest, the leaves and roots were measured separately and stored in a paper bag. The stored samples were dried in an oven at 65 °C for 7 days or until a constant weight was achieved to measure their dry weight (DW). The root and shoot dry

weights were combined to get the total plant biomass and calculated for the root-to-shoot ratio.

3.3.2 **Determination of leaf relative water content**

The leaf relative water content (LRWC) of *P. amaryllifolius* was analysed according to Turner (1981). The harvested leaf samples were wiped with 70% ethanol, cut into small pieces (2 cm^2), and weighed for their fresh weight (FW) before being transferred to a Petri dish. The leaf pieces were taped to the bottom part of the dish to allow full submergence of the sample. Then, the samples were submerged in 20 mL of distilled water for 6 h at room temperature. The saturated weight (SW) of the samples was measured before oven-dried (65 °C) for 2 days. The dried samples were then measured when achieving the constant weight. Leaf RWC was calculated using the following formula:

$$\text{RWC}\ (\%) = \left(\frac{\text{FW} - \text{DW}}{\text{SW} - \text{DW}}\right) \times\ 100$$

3.3.3 **Measurement of relative electrolyte leakage**

The relative electrolyte leakage (REL) was measured according to Quan *et al.* (2018). The harvested leaf samples were cut into half with a 5 cm in length. The leaf pieces were then immersed in 10 mL deionised water in a 50 mL falcon tube and shaken on an orbital shaker (Cole-Palmer, United States) at 150 rpm. After incubating 6 h at room temperature, the initial electrical conductivity (C_i) was measured using a conductivity meter (Cyberscan CON 11, Eutech Instrument, Thermofisher, Singapore) in µS/ cm conductivity range. Once the C_i was measured, the leaf samples were boiled for 20 minutes and cooled on ice for 10 minutes to ensure all cells were lysed and released all of the electrolyte content. After cooling, the conductivity of lysed cells (C_{max}) was measured. REL was calculated as:

$$\text{REL}\ (\%) = \frac{Ci}{Cmax} \times 100$$

3.3.4 **Determination of photosynthetic pigmentation content**

The chlorophyll content was analysed according to Lichtenthaler, (1987). The processed leaf powder was subjected to a freeze dryer (Labconco Freezone 4.5) overnight. Once freeze-dried, 0.01 g of the lyophilised leaf powder was mixed well with 2 mL 80% (v/v) acetone (EMSURE, Merck, Germany) in a 2 mL microcentrifuge tube. The mixture was incubated in the dark for 20 minutes with occasional vortexing every 10 minutes before being centrifuged at 10,000 × *g* for 15 minutes. The supernatant (100 µL) was mixed with 900 µL 80% (v/v) acetone for dilution of pigment. The absorbance of the mixture was loaded in 1 mL quartz cuvette and measured by a spectrophotometer at 470, 647 and 663 nm. The chlorophyll and carotenoid contents were calculated according to the formula below:

$$\text{Chlorophyll } a\ (\mu\text{gmg}^{-1}) = 12.25\text{A}_{663} - 2.79\text{A}_{647}$$

$$\text{Chlorophyll } b\ (\mu\text{gmg}^{-1}) = 21.5\text{A}_{647} - 5.1\text{A}_{663}$$

$$\text{Total chlorophyll}\ (\mu\text{gmg}^{-1}) = 7.15\text{A}_{663} - 18.71\text{A}_{647}$$

$$\text{Carotenoids}\ (\mu\text{gmg}^{-1}) = (1000\text{A}_{470} - 1.82\text{C}_a - 85.02\text{C}_b) \div 198$$

$C_a = Chlorophyll\ a$

$C_b = Chlorophyll\ b$

3.3.5 **Determination of malondialdehyde content**

Malondialdehyde (MDA) content was measured as described by Heath and Packer (1968). Fresh leaf samples (100 mg) were homogenised in liquid nitrogen, and then extracted with freshly prepared 1.5 mL 0.1% (w/v) trichloroacetic acid (Chemiz (M) Sdn Bhd, Malaysia). The ground pulp was collected in 2 mL centrifuge tube. The mixture was centrifuged at 13,000 × *g* for 10 minutes at 4 °C. About 300 µL supernatant was mixed with 1 mL reaction mixture containing 0.5% (v/v) thiobarbituric acid (TBA) (Merck, Germany) in 20% (w/v) trichloroacetic acid. The mixture was heated at 95 °C for 30 minutes in fume hood, cooled on ice, and centrifuged at 10,000 × *g* for 10 minutes. The supernatant was transferred to a 1 mL quartz cuvette. The absorbance of the mixture was measured using a nanophotometer (IMPLEN P300, Germany) at 532 and 600 nm and calculated using the following formula:

$$\text{MDA} = \frac{(A_{532} - A_{600}) \times \text{VTr} \times 1000}{\text{Extinction coefficient MDA} \times 1\ \text{cm} \times \text{Ve}} \div \text{g FW}$$

A_{532} - A_{600} = Absorbance of MDA - TBA

VTr = Volume of reaction (mL)

Ve= Volume of enzyme extract (mL)

Extinction coefficient of this MDA-TBA adduct at 532 nm is 155 mM^{-1} cm^{-1}.

FW= Fresh weight of the sample

3.3.6 **Measurement of proline content**

Proline content was measured according to Bates *et al.* (1973). About 200 mg leaf samples were ground with 2 mL 70% (v/v) ethanol. The mixture was centrifuged at 13,000 × *g* for 20 minutes. The supernatant was added to a reaction mixture containing

500 µL glacial acetic acid (EMSURE, Merck, Germany):500 µL freshly prepared acid-ninhydrin reagent (Loba Chemie, India) :500 µL sample extract or proline standards (Merck, Germany). The mixture was mixed in a 2 mL centrifuge tube with a hole on its cap to release pressure when boiled at 100 °C in a heat block for 1 hour. After placing on ice for 30 minutes, the mixture was mixed with 1 mL toluene (EMSURE, Merck, Germany). The toluene phase was carefully collected into a test tube. The absorbance of the fraction was measured in a 1 mL quartz cuvette at 520 nm with toluene as blank. The proline standard curve was constructed at concentrations of 1, 5, 10, 25, 50, 100, and 200 µM (**Appendix 2A**). The proline content was calculated based on the following formula:

$$\text{Proline}(\mu\text{Mg}^{-1}\text{FW}) = \frac{\left[\left(\frac{\mu\text{g Proline}}{\text{mL}} \times \text{mL Toluene}\right) \div \frac{\mu\text{g } 115.5}{\mu\text{mole}}\right]}{\frac{\text{g FW}}{5}}$$

3.3.7 **Quantification of hydrogen peroxide**

The H_2O_2 level in the leaf samples was determined according to Velikova *et al.* (2000). About 100 mg leaf powder was homogenised with freshly prepared 1.5 mL 0.1% (w/v) TCA in an ice bath. The homogenised samples were collected in a 2 mL centrifuge tube before being centrifuged at 10,000 × *g* for 15 minutes. About 250 µL supernatant was mixed with 1 mL reaction mixture containing 2.5 mM potassium phosphate (Merck, Germany) buffer (pH 7.0), and 0.5 M potassium iodide (Merck, Germany). The H_2O_2 concentration was measured at 390 nm with a 1 mL quartz cuvette and calculated using a H_2O_2 (Merck, Germany) standard curve with concentrations of 2.5 to 100 µM (**Appendix 2B**). The blank was prepared by replacing the sample extract or H_2O_2 standards with 0.1% (w/v) TCA.

3.4 Antioxidant enzymatic assays

3.4.1 **Enzyme extraction**

About 200 mg leaf samples were mixed with 2 mL cold extraction buffer (100 mM phosphate buffer, pH 7.0, containing 0.1 mM disodium ethylenediaminetetraacetic acid (EDTA) (Merck, Germany), and 0.1 g polyvinylpyrrolidone (PVP) (Merck, Germany). The mixture was ground in a 2 mL centrifuge tube. The pulp mixture was centrifuged at 13,000 × g for 10 minutes at 4 °C. The supernatant was used for the subsequent antioxidant enzymatic assays. The enzyme activity for CAT, APX, POD, and GR assay was calculated using the following formula:

$$\text{Enzyme activity (Mmin}^{-1}\text{ g}^{-1}\text{ FW)} = \frac{\Delta A \times VTr}{\varepsilon \times \Delta t \times 1\text{cm} \times Ve \times \text{g FW}} \times 1000$$

ΔA = Difference in absorbance

VTr = Volume of reaction (mL)

Ve= Volume of enzyme extract (mL)

Δt = Difference in time of absorbance (min)

$For\ CAT, \varepsilon(\text{Hydrogen peroxide}) = 36.0\ mol^{-1}\ cm^{-1}$

$For\ APX, \varepsilon(\text{Ascorbic acid}) = 2.8\ mmol^{-1}\ cm^{-1}$

$For\ POD, \varepsilon(\text{Guaiacol}) = 26.6\ mol^{-1}\ cm^{-1}$

$For\ GR, \varepsilon(\text{NADPH}) = 6220\ mol^{-1}\ cm^{-1}$

3.4.2 **Superoxide dismutase**

SOD activity was determined according to Dhindsa *et al.*, (1981). The 3 mL of reaction mixture containing 50 mM phosphate buffer (pH 7.0), 9.9 mM L-methionine (Merck, Germany), 55 µM nitro blue tetrazolium (NBT) (Goldbio, USA), 0.025% (v/v) Triton X-100 (Sharlau, Turkey), 100 µL enzyme extract, and 4.8 µM riboflavin (Merck, Germany)

were prepared in a test tube and covered by aluminium foil. The riboflavin was added last to initiate the reaction. The mixture was shaken before incubating at 30 °C for 10 minutes under a white light source (35 W) placed at 20 cm height above the test tubes. The mixture was measured at 560 nm in a 3 mL quartz cuvette. The blank was prepared by replacing the sample with the enzyme extraction buffer. The blank was prepared in 2 sets, whereby one was exposed to the light while the other was kept in the dark. The SOD activity was calculated based on the formula below:

$$\text{SOD (Unit g}^{-1}\text{FW)} = \frac{[(\text{Blank} - \text{Sample})\ A_{560\text{nm}}]}{(\text{Blank}\ A_{560\text{nm}})} \times \frac{\text{Volume reaction}}{\text{Volume enzyme}} \times 100 \times \frac{1}{50} \div 0.1\ \text{g FW}$$

3.4.3 **Catalase**

CAT assay was performed as described by Aebi (1974). The 3 mL reaction mixture containing 50 mM phosphate buffer (pH 7.0), freshly prepared 8.33 mM H_2O_2, 100 µL enzyme extract, and distilled water was prepared. The enzyme extract was added last to initiate the reaction. This reaction was measured at 240 nm in 2 minutes with a 15-s interval of each reading. The blank was prepared by replacing the enzyme extract with an extraction buffer. The CAT activity was measured and calculated with the decrease of absorbance due to the utilisation of H_2O_2 by CAT to form water and oxygen.

3.4.4 **Peroxidase**

POD assay was carried out according to Chance and Maehly (1955). About 100 µL extracted enzymes were added into the reaction mixture containing 100 mM phosphate buffer (pH 7.0), 0.5 mM guaiacol (Merck, Germany), and 0.0833 mM H_2O_2. The mixture was topped up with distilled water to a final volume of 1 mL reaction. The guaiacol and H_2O_2 solution were prepared fresh prior to the reaction. The H_2O_2 was added last, and the

mixture was incubated at room temperature for 10-s to initiate the reaction. The POD activity was measured at 470 nm wavelength for 2 minutes with a 15-s interval reading.

3.4.5 **Glutathione reductase**

GR activity was determined as described by Mannervik (1999). The 1 mL reaction mixture contained 500 µL assay buffer (0.2 M potassium phosphate buffer, pH 7.0, 0.2 mM EDTA, 50 µL 20 mM freshly prepared glutathione disulfide (GSSG) or oxidised glutathione (Nacalai Tesque, Japan), 50 µL 2 mM NADPH (Merck, Germany) solution, and 300 µL enzyme extract) and distilled water. The extracted enzyme was added last into the reaction mixture in a 1 mL quartz cuvette to initiate the reaction. In the presence of GR and NADPH, GSSG is reduced into reduced glutathione (GSH). Thus, the decrease in absorbance due to the reduction of GSSG at 340 nm was monitored for 1 minute with a 15-s interval reading. The blank was prepared by replacing the enzyme extract with the enzyme extraction buffer.

3.4.6 **Ascorbate peroxidase**

APX activity was measured according to Chen and Asada (1992). The 1 mL reaction mixture contained 50 mM phosphate buffer (pH 7.0), 200 µL enzyme extract, and freshly prepared 0.5 mM ascorbic acid (Merck, Germany), and 0.42 mM H_2O_2. The blank was prepared by replacing the enzyme extract with an extraction buffer. The H_2O_2 was added last to initiate the reaction of ascorbic acid oxidation by the APX enzyme, which caused the reduction of absorbance value. The reduced absorbance value was measured at 290 nm wavelength for 2 minutes with a 15-s interval reading, and the APX activity was calculated.

3.5 Proteomics analysis

3.5.1 **Protein extraction**

The total protein of the leaf sample was extracted using trichloroacetic acid/acetone precipitation with the phenol method as described by Wu *et al*. (2014). Leaf powder (200 mg) was suspended in 4 mL cold 10% (w/v) trichloroacetic acid/acetone containing 10 mM dithiothreitol (DTT) (Goldbio, USA) and 0.001% (v/v) protease inhibitor (Nacalai Tesque, Japan) and centrifuged at 20,000 × *g* for 10 minutes at 4°C. The pellet was resuspended with 1.5 mL 10% (w/v) trichloroacetic acid/acetone before centrifuging 20,000 × *g* for 5 minutes at 4°C. Ice-cold acetone (80%) containing 10 mM DTT was added to the pellet, mixed by using a vortex machine and centrifuged at 20,000 × *g* for 5 minutes at 4°C. The pellet was resuspended 1.5 mL sodium dodecyl sulfate (SDS) (Merck, Germany) extraction buffer (1% (w/v) SDS, 0.15 M Tris-HCl (pH 8.8), 0.1 M DTT, 1 mM EDTA, and 0.001% (v/v) protease inhibitor) and the mixture was incubated at 65°C for 1 hour. After incubation, the mixture was centrifuged at 20,000 × *g* for 5 minutes at 4°C before adding an equal volume of Tris-buffered phenol (Merck, Germany) (pH 8.0). The mixture was shaken and vortexed for about 3-5 minutes before centrifuging at 20,000 × *g* for 5 minutes at room temperature. The phenol phase at the bottom layer was extracted carefully by using a small tip pipette or a syringe and mixed with washing buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 0.7 M sucrose (Merck, Germany)]. The mixture was shaken and vortexed for about 3-5 minutes before being centrifuged at 20,000 × *g* for 5 minutes at 4 °C. The upper layer of phenol was collected and mixed with 1.5 mL 0.1 M ammonium acetate (Merck, Germany) in methanol. After incubating at -20 °C overnight, the mixture was centrifuged at 20,000 × *g* for 10 minutes at 4°C. The pellet was washed with 1.5 mL 0.1 M ammonium acetate in methanol, centrifuged at 20,000 × *g* for 5 minutes at 4°C, and mixed with 1 mL 80% (v/v) ice-cold acetone containing 20

mM DTT. Next, the pellet was vacuum dried in a desiccator for 5 minutes and resuspended in either ToPI-DIGE lysis buffer (ITSI Biosciences, USA) for LCMS/MS analysis or a resuspension buffer (7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) (Nacalai Tesque, Japan), 30 mM Tris-HCl (pH 8.0), and 1% DTT) for protein content measurement.

3.5.2 **Protein quantification and 1D-sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE)**

The protein content was determined using the Bradford reagent (Bradford, 1976), where bovine serum albumin (BSA) (Merck, Germany) was used as a protein standard. About 10 µL of protein sample or BSA standard were added with 1 mL of Bradford reagent according to the manufacturer's protocol (Bio-Rad Protein Assay Dye, USA). After incubation in the dark for 5 minutes, the absorption was measured at 595 nm wavelength using a spectrophotometer (NanoPhotometer P300, IMPLEN GmbH, Germany). A series of BSA solutions ranging in concentration from 0.2, 0.3, 0.5, 0.7 and 0.9 mg/mL were prepared in the resuspension buffer and used to generate a standard curve (**Appendix 1C**).

The quality of extracted protein was analysed by using SDS-PAGE (Brunelle & Green, 2014). First, the gel casting glass was assembled in a sandwich position and placed into a casting stand ensuring the bottom part is tightly sealed with the stopper foam. In order to have a consistent gel, the glass was marked with a line for the resolving gel height, which is about 1 cm below the bottom of the comb. SDS-PAGE gels were prepared by mixing the resolving gel, which consists of acrylamide/bis (19:1) (Nacalai Tesque, Japan) solution (12%), 0.1% SDS, 375 mM Tris-HCl (pH 8.8), and distilled water. The mixture was then mixed with 50 µL of 10% ammonium persulfate (APS) (Merck, Germany) and 5 µL of tetramethylethylenediamine (TEMED) (Merck, Germany) to initiate the

polymerisation. The resolving gel was then pipetted into the gel casting up to the line marked and overlaid with 500 mL of isopropanol (EMSURE, Merck, Germany) to obtain a smooth top gel surface. After 40 minutes of polymerisation, the isopropanol on top of the resolving gel was carefully removed by using tissue paper. The stacking gel was prepared which contained 5% acrylamide/bis (19:1) solution, 0.1% (w/v) SDS, 125 mM Tris-HCl (pH 6.8), and distilled water. The mixture was mixed gently with 50 µL of 10% (w/v) APS and 5 µL of TEMED to initiate the polymerisation. The stacking gel was then pipetted into the gel casting on top of the resolving gel and a well comb was quickly placed on top of the stacking gel before it polymerised in about 30 minutes. The prepared SDS-PAGE gel was then fixed onto the electrophoresis tank and filled with a running buffer containing 25 mM Tris-base (Merck, Germany), 192 mM glycine (Merck, Germany), and 0.1% SDS. The pouring of running buffer was done carefully to ensure minimum bubble formation in the upper chamber of the tank. Based on the protein quantification done earlier, about 20 µL to 30 µL of protein sample was added in a 5 µL loading buffer containing 120 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 750 mM β-mercaptoethanol (Merck, Germany), and 0.05% (w/v) bromophenol blue (Merck, Germany) prior incubation at 95°C for 5 minutes. After incubation, the protein samples and the protein ladder were loaded into the wells and fractionated at 150 V for 45 minutes. Once the electrophoresis was completed, the gel was washed with distilled water and soaked with a staining solution containing 0.1% (w/v) Coomassie-Blue R250 (Merck, Germany), 40% (v/v) methanol, and 10% (v/v) acetic acid for 30 minutes with gentle shaking on an orbital shaker (60 rpm). Another washing with distilled water was performed to remove the excess staining solution before soaking with a de-staining solution consisting of 40% (v/v) methanol and 10% (v/v) acetic acid for 2 hours. After de-staining, the gel was washed with distilled water to remove the excess methanol and acetic acid before viewing with a gel viewer (GELSCAN-2, iMeasure Inc.).

3.5.3 **Liquid chromatography-mass spectrometry/mass spectrometry (LCMS/MS) analysis**

The protein resuspended in the ToPI-DIGE lysis buffer was reduced with Tris (2-carboxyethyl) phosphine (TCEP) and alkylated with iodoacetamide. About 25 µg of each sample was then precipitated to remove contaminant before being resuspended in 100 mM triethylammonium bicarbonate buffer and digested overnight with trypsin. The digested protein samples were then labelled with TMT reagent according to the manufacturer's protocols. The samples were pooled prior to SCX fractionation via cation exchange column (SCIEX, Framingham, MA USA) with one drop/second flow rate. The column was then washed before 3 different concentrations of ammonium acetate (75 mM, 150 mM, and 450 mM) were loaded separately with a rate of one drop/second. Each fractionation was collected and desalted with C18 cartridges before being dried in a vacuum centrifuge. The peptides were subjected to in-line reverse phase (RP) chromatography and tandem mass spectrometry (MS/MS). The sample was loaded onto a Picofrit C18 nanospray column with the aid of a Thermo Scientific Surveyor Autosampler operated in the no-waste injection mode at a constant flow rate of 600 nL/min. The peptides were eluted along the column using a linear acetonitrile gradient from 5 to 45 % over 120 minutes, followed by high and low organic washes for another 30 minutes into the LTQ XL mass spectrometer (Thermo Scientific, USA) via a nanoelectrospray source in positive mode with a spray voltage of 1.8 kV. The temperature for the ion tube transport was set at 180 °C. A data-dependent of Top five method was used to perform a full MS scan range (300-1,600 *m/z*), followed by MS/MS scans on the five most abundant ions.

3.5.4 **Proteomics data analysis**

The raw data files generated were searched against *Viridiplantae* clade (NCBI protein; 33090) (Accessed on 20th February 2020) using the MudPIT option in Proteome Discoverer 2.2 (Thermo Scientific) and the Sequest HT search algorithm, allowing up to two missed cleavages per peptide. Carbonylation of cysteine, N-Terminal TMT 6-plex, and lysine TMT 6-plex was used as a fixed modification, whereas oxidation of methionine and acetylation of the protein N-terminal was used as a variable modification. Only high-confidence peptides were selected using a percolator algorithm for the peptide-spectrum match (PSM) in database searches with a false discovery rate (FDR) threshold set at 0.01 for protein identification. A *p*-value of less than 0.05 (Student's *t*-test) and a 1.5-fold change of abundance was used to define significantly changed proteins between the control and drought-treated samples. The identified proteins were filtered based on their abundance ratio of drought-treated to the well-watered samples in a range of lower than 0.5 and higher than 1.2. The significantly changed proteins were visualised by hierarchical clustering using Perseus software (version 1.6.0.7) (Tyanova *et al.* 2016).

3.6 Gene expression analysis

3.6.1 **RNA extraction, DNase treatment and cDNA conversion**

Total RNA was extracted by using the cetyltrimethylammonium bromide (CTAB) conventional method (Asif *et al*., 2000). About 100 mg of finely ground leaf powder was extracted with 1 mL of CTAB extraction buffer containing 1 M Tris-HCl (pH 8.0), 0.5 M EDTA (pH 8.0), 5 M NaCl (Merck, Germany), 10% (w/v) CTAB (Fisher scientific, USA), 2% (w/v) PVP, and 2% (v/v) β-mercaptoethanol in a chilled pestle and mortar. Both PVP and β-mercaptoethanol were added fresh in the extraction buffer before being used. The ground mixture was then aliquoted into 2 mL centrifuge tube, vortexed for 2 minutes, and incubated at 65 °C for 30 minutes. The mixture was cooled down to room temperature before adding with 1 mL of chilled chloroform (Merck, Germany): isoamyl alcohol (49:1) (Emsure, Merck, Germany) and vortex for 2 minutes prior to centrifugation at 13000 × *g* for 20 minutes (4 °C). The upper layer of the supernatant was transferred into a new 2 mL tube and added with 1 mL of chilled chloroform: isoamyl alcohol (49:1) and vortexed for 2 minutes prior to centrifugation at 13000 × *g* for 20 minutes at 4 °C. The upper layer of the supernatant was transferred into a new 2 mL tube and added with 1 mL of chilled pure chloroform at 1:1 ratio. About 800 µL of upper layer supernatant was transferred into 2 new 1.5 mL centrifuge tubes containing 400 µL supernatant each tube. The mixture was mixed with 181.8 µL of 8 M ice-cold lithium chloride (Fisher scientific, USA) (LiCl) to make the final concentration of LiCl to be 2.5 M. After overnight incubation at -80 °C, the mixture was centrifuged at 13,000 × *g* for 30 minutes at 4 °C. The pellet was then washed with 400 µL of 70% ethanol by flicking the tube several times before centrifuging at 13,000 × *g* for 20 minutes at 4 °C. The pellet was washed with 400 µl of absolute ethanol (molecular grade) by flicking the tube several times before centrifuging at 13,000 × *g* for 20 minutes (4 °C). The pellet was air dried for 10 minutes in a fume hood before eluted with 20 µL of nuclease-free water. The

concentration (ng/µL) and purity of the samples were measured at A260/280 and A260/230 wavelength by using a spectrophotometer. The extracted RNA was added with 10 µL of RDD buffer (Qiagen, Germany) and 2.5 µL of DNase (Fisher scientific, USA), making up the final concentration of 10 µg RNA per tube. The mixture was incubated for 10 minutes at room temperature. After being precipitated with 45.5 µL of 8M ice-cold LiCl for 30 minutes at -20 °C, the treated RNA was centrifuged at 16,000 × *g* for 15 minutes at 4 °C. The pellet was washed with ice-cold 70% ethanol. After centrifuging at 16,000 × *g* for 15 minutes (4 °C), the pellet was briefly air-dried before being eluted with 20 µL of RNase-free water. The concentration was measured using a spectrophotometer. The RNA was then subjected to gel electrophoresis at 1% agarose gel to analyse the nucleic acid quality.

DNase-free RNA was converted to cDNA in a PCR tube. The 20 µL PCR reaction contained 4 µL of 5× RT buffer, 2 µL dNTPs mixture, 1 µL RNase inhibitor, 1 µL random primer, 1 µL ReverTra Ace (Toyobo), 1 µg of RNA, and nucleases-free distilled water. The prepared mixture was amplified at the following conditions: 42 °C for 30 minutes and 99 °C for 5 minutes. The cDNA was stored at -80 °C until further use.

3.6.2 **Polymerase chain reaction (PCR), gradient PCR, primer efficiency test and quantitative real-time PCR (qPCR)**

The most differentially altered proteins from the proteome data were selected for relative gene expression analysis. The primers for the selected proteins were designed (**Table 3.1**). The 20 µL PCR reaction contained 30 ng cDNA, 2 µL of 10× EasyTaq buffer (TransGen Biotech, Beijing, China), 1.6 µL dNTPs mixture (Toyobo, Japan), 10 µM of forward and reverse primers, and nuclease-free distilled water. The mixture was amplified using a PCR machine (T100™ Thermal Cycler, BioRad, USA) under the following

conditions: Step 1: 94 °C for 5 minutes, Step 2: 94 °C for 30 seconds, followed by 55-65 °C for 30 seconds. This step was repeated for 30 cycles, and Step 3: 72 °C for 10 minutes (1-2 kb/min) and 4 °C for 5 minutes. The PCR product was kept at -20 °C until use or mixed with sample loading buffer (6x loading buffer) (NEB, USA) for quality check. The PCR product was loaded into the well with a 1.5 µL 1 kb ladder (Promega, USA) next to it. The electrophoresis was run at 110 V for 12 minutes. The gel was viewed under a gel viewer (proBLUEVIEW, Cleaver Scientific).

Table 3.1. List of genes and their primer sequences used for relative expression analysis.

Gene	Sequences 5'-3'
HSP70_Pandan (*PaHSP70*)	F- ACCTACAAGGGTGAGGAGAAG R- GAAATAGGCAGGGACAGTGATG
GAPDH_Pandan (*PaGAPDH*) (Arora *et al*., 2017)	F: AGGGTGGTGCCAAGAAGGT R: CCACCTCTCCAGTCCTT
Enolase_Pandan (*PaENO*)	F- TGAGTGATGGCACTTACGCC R- ACGTTCTCCACAGCCTTGAG
Thaumatin_Pandan (*PaThau*)	F- TCGCTGTCCTTCTCCTTTGG R- CACCTTGTGAGGAATGCAGC
β-fructofuranosidase_Pandan (*Paβ-Fruc*)	F- GAACCCTGGATGGTATCGGG R- CCGGCAAATGCTCCTAAGTG
*Actin_Pandan (*PaActin)*	F- GAGGCTATTCCTTCACCACTAC R- GTCTCAAGCTCCTCCTCATAATC
*Elongation factor 1_Pandan (*PaEF1)*	F- TCTTCACAAAGCCAGCATCTC R- GACTGCCACACCTCTCATATTG

The asterisk (*) indicated genes used as housekeeping gene during the qPCR analysis.

The designed primers were subjected to a primer efficiency test. The 10 µL PCR reaction contained 10 µM of forward and reverse primers, 5 µL 1×SG Fast qPCR Master Mix (Sangon Biotech Co., Ltd., China) and 4 µL cDNA template (2.5 to 40.0 ng/µL). The mixture was amplified in a qPCR machine (Applied Biosystems ViiA7) under the following conditions: 42 °C for 30 minutes and 99 °C for 5 minutes. The Ct value obtained from the qPCR analysis with QuantStudio Real-Time PCR software v 1.3 (Thermofisher, USA) was used to calculate the primer efficiency. The primer efficiency of each designed primer was calculated by using the formula below. The accepted primer efficiency percentage is between 90% and 110% (**Appendix 3**).

$$\text{Primer efficiency} = (10^{\frac{-1}{\text{slope}}} - 1) \times 100$$

The quantitative real-time PCR (qPCR) was performed to analyse the expression of drought-stressed responsive genes (**Table 3.1**). The qPCR consisted of a reaction volume of 10 µL containing 40 ng of cDNA, 0.2 µM primers, and 1×SG Fast qPCR Master Mix (Sangon Biotech Co., Ltd., China), with the elongation factor-1 and actin as reference genes. The qPCR assay was conducted according to the manufacturer's protocol. The relative expression levels were calculated according to Pfaffl (2001). The qPCR analysis was conducted with three biological replicates and three technical replicates for each gene.

3.7 Oil palm wood vinegar

The OPWV was acquired from Palm Leaf Trader Sdn. Bhd., Banting, Selangor, Malaysia. Prior to usage, the OPWV was mixed well and aliquoted into a smaller vessel. The OPWV was freshly prepared.

3.8 Optimisation of oil palm wood vinegar applications

The OPWV dilution factor and frequency of application were determined. A series of dilution factors (1:100, 1:250, 1:500, and 1:1000) were selected **(Figure 3.2A)** to be applied onto 5 plants for each dilution including the control treatment. Distilled water was used as the control treatment. Once the optimum concentration of OPWV was determined, different frequencies of application were optimised, whereby 5 plant samples were foliar sprayed each at 6-day intervals (F1), 3-day intervals (F2), and 1-day intervals (F3) (**Figure 3.2B**). OPWV was foliar applied together with 0.1% Tween 20 at 20 mL per plant using a small handheld sprayer (**Figure 3.3**). The plants were sprayed adaxially and abaxially until run-off. The treated plant was briefly air-dried before randomly placing it in the growing plot. All samples received 20 mL of distilled water every day. After 7 days of treatment, the samples were harvested. The mass, root-to-shoot ratio, and chlorophyll content were analysed according to Section 3.3.

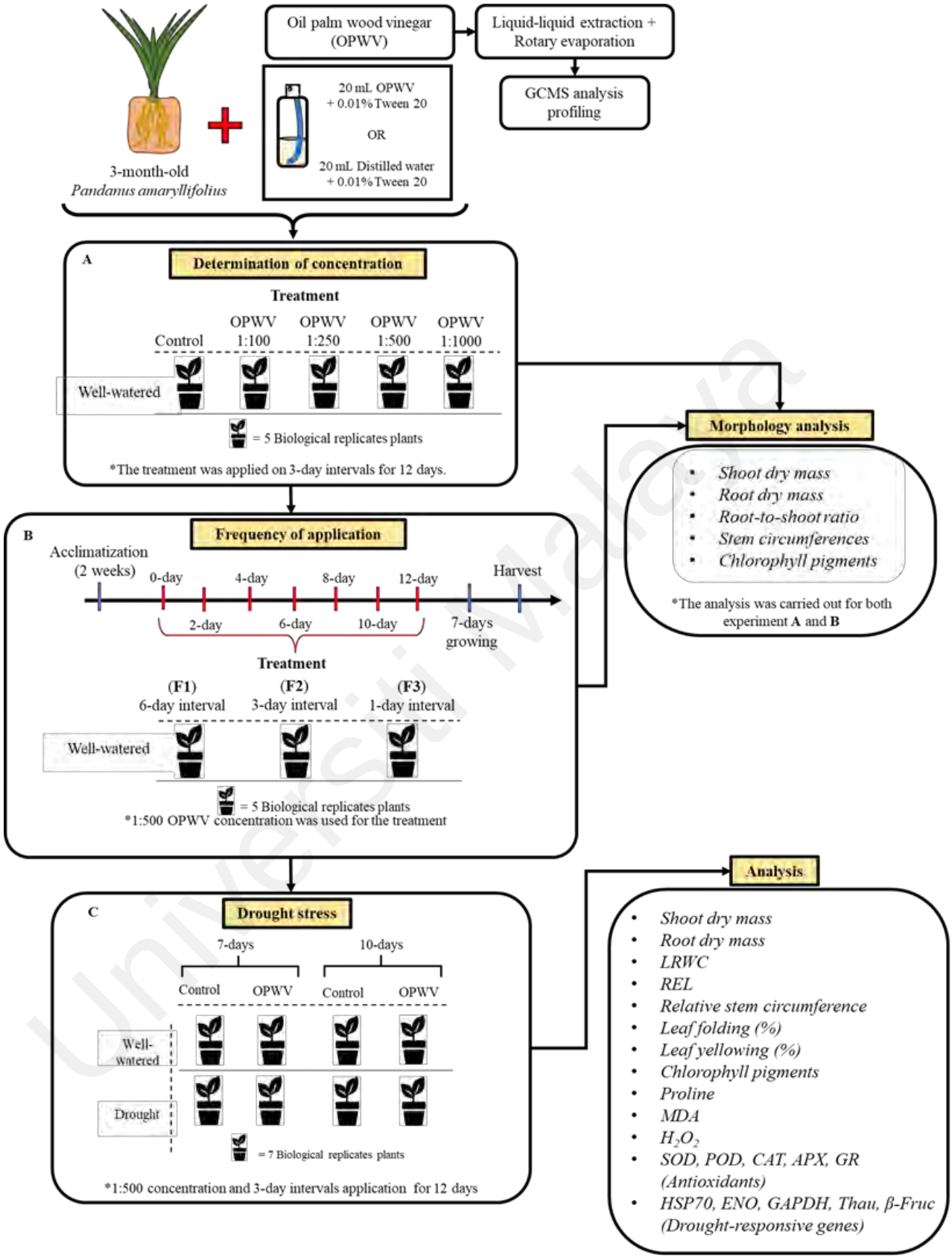

Figure 3.2. The schematic diagram of the experimental setup. (**A**) *Pandanus amaryllifolius* plants were sprayed with oil palm wood vinegar (OPWV) at 1:100, 1:250, 1:500, and 1:1000 dilutions in 3-day intervals until 12 days. The plants were allowed to grow for additional 7 days before harvesting. (**B**) *P. amaryllifolius* plants were sprayed with OPWV at 1:500 dilution at 6-day intervals (F1), 3-day intervals (F2), and 1-day intervals (F3). Well-watered plants served as control. (**C**) The optimised concentration and application frequency of OPWV was used to determine its potential to mitigate the drought stress effects. *P. amaryllifolius* plants were well-watered or treated with 7- or 10-day drought stress with or without OPWV.

Figure 3.3. Commercial handheld sprayer with 120 mL capacity used to deploy foliar spray application of 20 mL of freshly prepared OPWV diluent containing 0.1% Tween 20. Each concentration of OPWV treatment were separated into different sprayers.

3.9 Relative stem circumference

The stem circumference of each plant was recorded on 0, 4, 8, 12 and 19 days to analyse and compare the relative stem circumference between treatments. It was measured by wrapping a 1-metre flexible tape around the meeting point between the node where the oldest leaf is attached to the stem of *P. amaryllifolius*, as shown in **Figure 3.4**. The relative stem circumference was calculated as follows:

$$\text{Relative stem circumference (cm)} = \text{(Harvest day)} - \text{(Post OPWV treatment)}$$

Figure 3.4. The region between the node where the oldest leaf is attached to the stem of *P. amaryllifolius* used for the stem circumference measurement. A flexible 1 metre tape was utilised measure the circumference by wrapping the tape surrounding the region.

3.10 Drought and oil palm wood vinegar (OPWV) treatments

A new batch of *P. amaryllifolius* plants was sprayed with 20 mL 1:500 OPWV solution containing 0.1% (v/v) Tween 20 until run-off. The plants received OPWV solution at 3-day intervals for 12 days before being exposed to drought treatment (**Figure 3.2C**). Drought treatment was conducted by withholding water for 7 and 10 days (Section 3.2).

Each treatment of each timepoint consisted of 7 biological replicates. The soil moisture meter (HH2 moisture meter, Delta-T, UK) was used to monitor the soil moisture of each treatment throughout the experiment (**Appendix 1B**). The harvested samples were analysed as described in Section 3.3.

3.11 Leaf analysis of drought-stressed *P. amaryllifolius* with or without OPWV application

Throughout the experiment, the leaf shape and colour of *P. amaryllifolius* were observed and recorded. The leaf shape was categorised into 3 groups: No folding (Normal), less than 50% folding (Stage 1), and more than 50% folding (Stage 2) (**Figure 3.5A**). The number of folded leaves per plant was calculated accordingly to its stage as follows:

$$\text{Percentage of folding leaves (\%)} = \frac{\text{Number of folded leaves per plant}}{\text{Total number of leaves per plant}} \times 100$$

In addition, the leaf colour was recorded based on the yellowing percentage scale shown in **Figure 3.5B**. The number of leaves with a yellowing percentage of 25% and above was considered yellowing and recorded. The number of yellowing leaves per plant was calculated as follows:

$$\text{Percentage of yellowing leaves (\%)} = \frac{\text{Number of yellowing leaves per plant}}{\text{Total number of leaves per plant}} \times 100$$

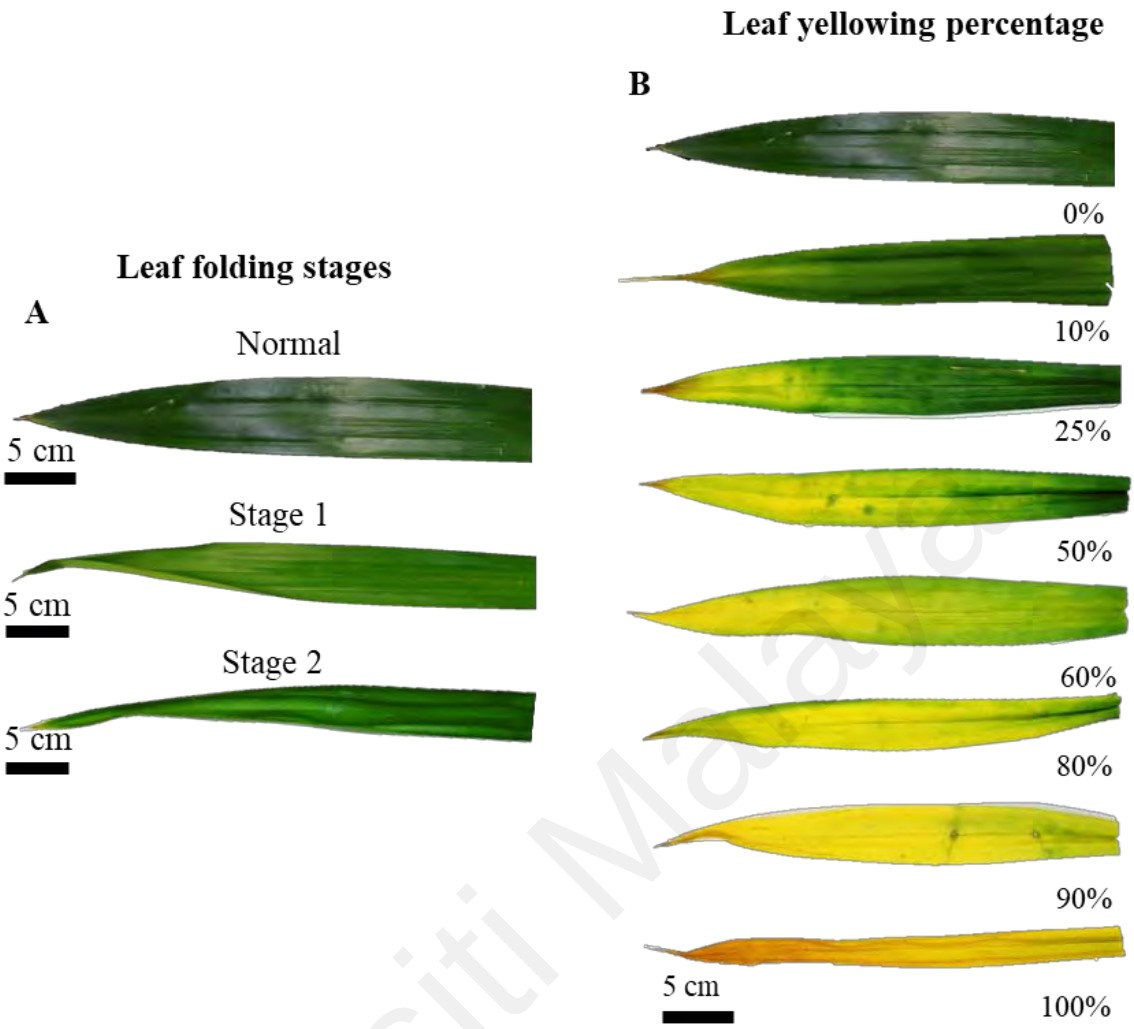

Figure 3.5. The yellowing percentage scale was created based on the leaf observation in this experiment. (**A**) Normal leaf and different stages of leaf folding. (**B**) The percentage of yellowing leaves from 0% to 100% yellowing.

3.12 Drought stress indicators and antioxidant activities analyses

The accumulation of proline, MDA and H_2O_2 was quantified according to the protocol described in Section 3.4. On the other hand, the antioxidant activities analysis, such as SOD, CAT, POD, APX, and GR, were carried out according to Section 3.5.

3.13 Relative expression of drought-responsive genes

Total RNA from leaf samples was extracted and converted to cDNA as described in Section 3.6.1. The amplification was performed as described in Section 3.6.2 using the same primers.

3.14 Metabolite extraction and gas chromatography mass spectrometry (GC-MS) analysis

About 50 mL of the crude samples were extracted with 50 mL dichloromethane (DCM) in a separating funnel. The DCM partition was collected. This step was repeated twice. The DCM partition extract (about 150 mL) was before being subjected to rotary evaporation at 40 °C for 1 hour. The dried extract powder was weighed and reconstituted in 10 mL methanol prior to GC-MS profiling. About 100 ppm of collidine was added to the methanol extract with a final volume of 1 mL. The mixture was then diluted at 1:30 with methanol before the GC-MS injection.

The diluted OPWV extract mixture was characterised using GC-MS (Shimadzu Manufacturing Co. Ltd, Kyoto, Japan, QP-2010). The GC-MS analysis parameters were set as follows: capillary columns (SH-5MS); 30 m × 0.25 mm diameter × 0.25 µm thickness; the temperature of injection: 250 °C; column temperature program: 50-220 °C and helium flow rate: 4.7 mL/min. The GC-MS was arranged in the electron ionisation mode at 70 eV with an Ion source and interface temperature of 250°C and 300 °C, respectively. 1 µL of the sample was injected into a column and held at 50 °C for 2 minutes with an increasing rate of 8 °C/min until 250 °C at which the temperature was held for another 2 minutes. The compounds were identified by comparison with the

standard library data calculated by the integrated peak areas relative to the internal standard peak area.

3.15 Root analysis

The roots of each plant were carefully harvested from the soil, washed with distilled water, and blotted dry with a paper towel. The dried primary roots were cut into small parts and placed on a transparent film. All root parts were scanned using a scanner (Canon Pixma, MG3570). The scanned root images were analysed for their diameter using RhizoVision Explorer (Seethepalli *et al*., 2021). The root diameter ranges were: Range 1 root diameter: 0-0.53 mm, Range 2 root diameter: 0.53 mm-1.32 mm and Range 3 root diameter: more than 1.32 mm.

3.16 Statistical analyses

The data were analysed by Student's t-test followed by a Post hoc Tukey range using SPSS Statistics software (version 23.0; IBM). The analysed data are considered to be statistically significant when its p-value < 0.05.

CHAPTER 4

RESULTS

4.1 Drought stress altered the morpho-physiology of *Pandanus amaryllifolius*

The morpho-physiological changes of *P. amaryllifolius* at different time points of drought stress were examined (**Figure 4.1**). In this study, *P. amaryllifolius* started to show an early sign of clamping on the leaf tips after 7 days of drought stress (**Figure 4.1A**). The plants exhibited severe wilting with yellowing leaves after 10 and 14 days of drought stress. The percentage of LRWC was reduced significantly after 4 days of drought, whereas the percentage of REL for drought-stressed samples was significantly increased after 10 and 14 days of drought treatment (**Figures 4.1B** and **C**). The shoot and root biomass of the *P. amaryllifolius* were analysed based on its FW and DW. The well-watered plants exhibited higher FW and DW in the shoots and roots compared to the drought-stressed samples (**Figures 4.1D-G**). Intriguingly, the 7-day drought-stressed plants showed significantly higher root DW than well-watered plants (**Figure 4.1F**). This pattern is reflected in the higher root-to-shoot ratio of the 7-day drought-stress samples compared to the well-watered plants (**Figure 4.1H**). Nonetheless, chlorophyll constituents, such as chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoid contents, were generally reduced in the drought-stress samples compared to well-watered plants throughout the experiments (**Figures 4.2A-D**).

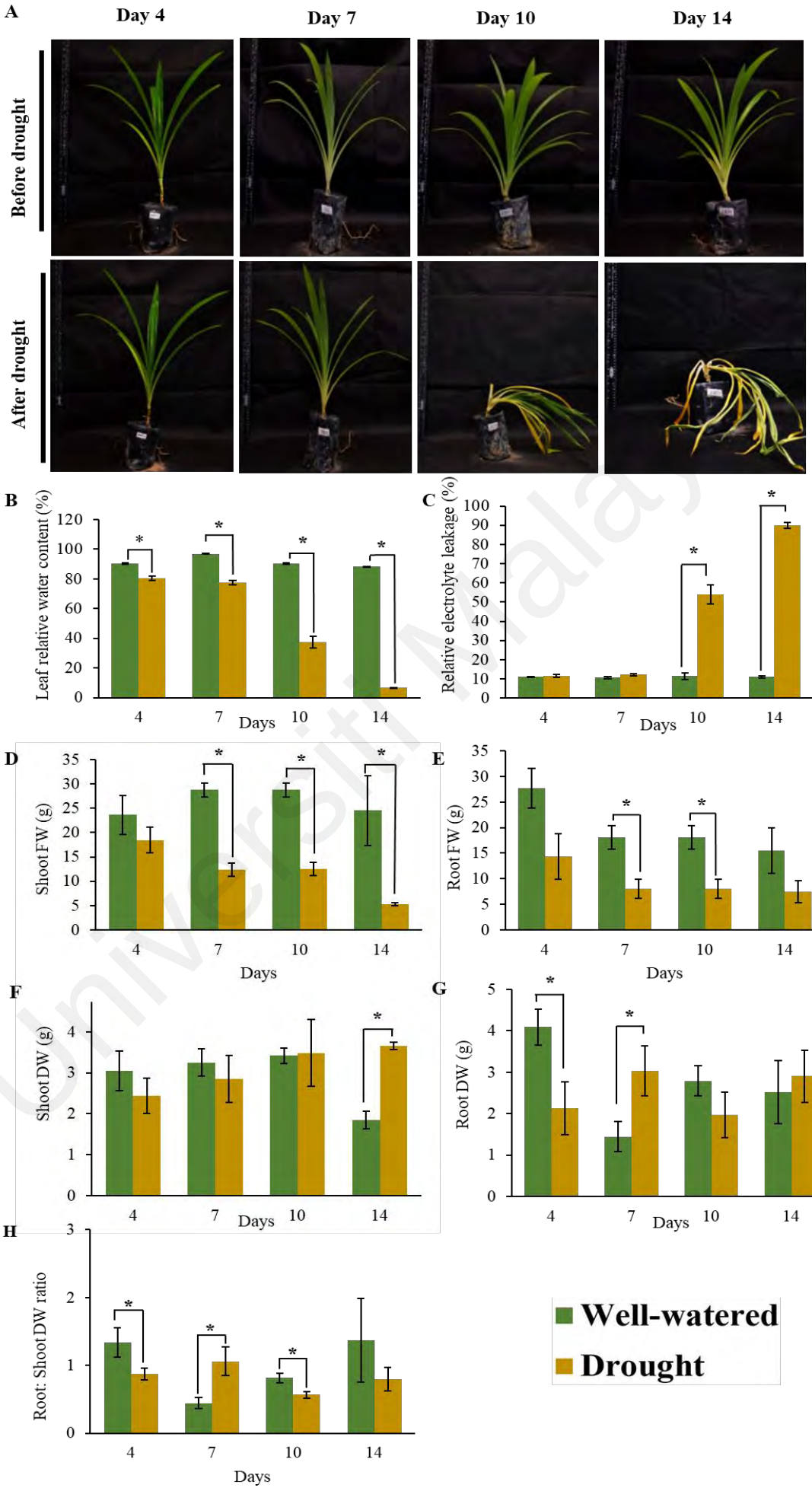

Figure 4.1. Morphological changes of drought-stressed and well-watered *Pandanus amaryllifolius. P. amaryllifolius* were subjected to drought stress conditions for 4, 7, 10 and 14 days by water withholding. The well-watered *P. amaryllifolius* constitute as control. **(A)** A comparison of *P. amaryllifolius* plants was taken before and after drought stress treatment for 4, 7, 10 and 14 days. **(B)** The LRWC percentage of *P. amaryllifolius* leaves at different drought stress time points. **(C)** The REL percentage for each sample at different harvest points. **(D)** The fresh weight of *P. amaryllifolius* shoots. **(E)** Root fresh weight of *P. amaryllifolius*; **(F)** Shoot dry weight of *P. amaryllifolius*. **(G)** Root fresh weight of *P. amaryllifolius*. **(H)** The root-to-shoot ratio of *P. amaryllifolius* was based on the dry weight. The means labelled with an asterisk were significantly different based on the Student's *t*-test when its *p*-value < 0.05.

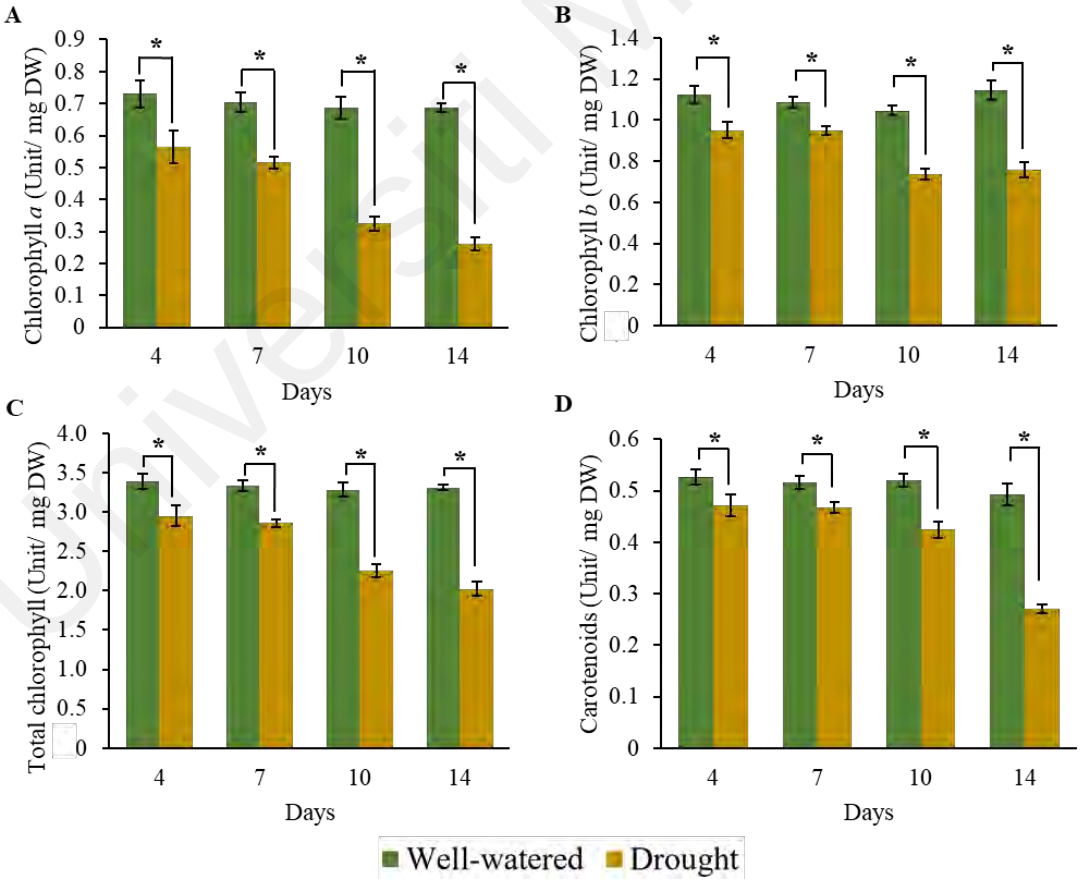

Figure 4.2. Leaf pigment constituent of the well-watered and drought-stressed *Pandanus amaryllifolius* across drought stress time points. (**A**) Chlorophyll *a* concentration (µg mg-

^{1} DW). (**B**) Chlorophyll *b* concentration (µg mg^{-1} DW). (**C**) Total chlorophyll content (µg mg^{-1} DW). (**D**) Carotenoid concentration (µg mg^{-1} DW). The means labelled with an asterisk (*****) were significantly different based on the Student's *t*-test when its *p*-value < 0.05.

All drought-stressed *Pandanus* were rewatered for 7 days. However, only 7-day drought-stressed *Pandanus* plants were able to recover, with visible necrosis damage on the leaf tips and several outer or matured leaves (**Figure 4.3A**). Both 10- and 14-day drought-stressed plants displayed severe symptoms, such as losing turgid structure and total collapse during the stress. Furthermore, they were unable to recover after rewatering. Therefore, a 7-day drought treatment was selected for the subsequent experiments.

A new set of experiments comprising 7-day well-watered, drought-stressed, and rewatered plants were carried out. The drought-stressed samples showed leaf wilting and folding (**Figure 4.3A**). Nonetheless, the rewatered drought-stressed *Pandanus* demonstrated its ability to recover from the stress by regaining its leaf greenness and structural turgidity (**Figure 4.3A**).

The *Pandanus* plants recorded a 20% LRWC reduction when exposed to 7-day drought stress (**Figure 4.3B**). In contrast, the recovered samples showed higher LRWC than the drought-stressed plants (**Figure 4.3B**). In addition, the percentage of REL of the drought-stressed *Pandanus* plant was significantly higher than in the water-recovered sample (**Figure 4.3C**). On the other hand, the shoot and root DW of the plants were not affected by the imposed drought stress **(Figures 4.3D** and **E**). However, the root-to-shoot ratio in drought-stressed *Pandanus* was significantly higher than in other treatments (**Figure 4.3F**). In addition, the leaf pigmentation constituents of the drought-stressed and recovered plant were lower than the well-watered sample, which could be related to insufficient time for pigment recovery (**Figures 4.4A-D**).

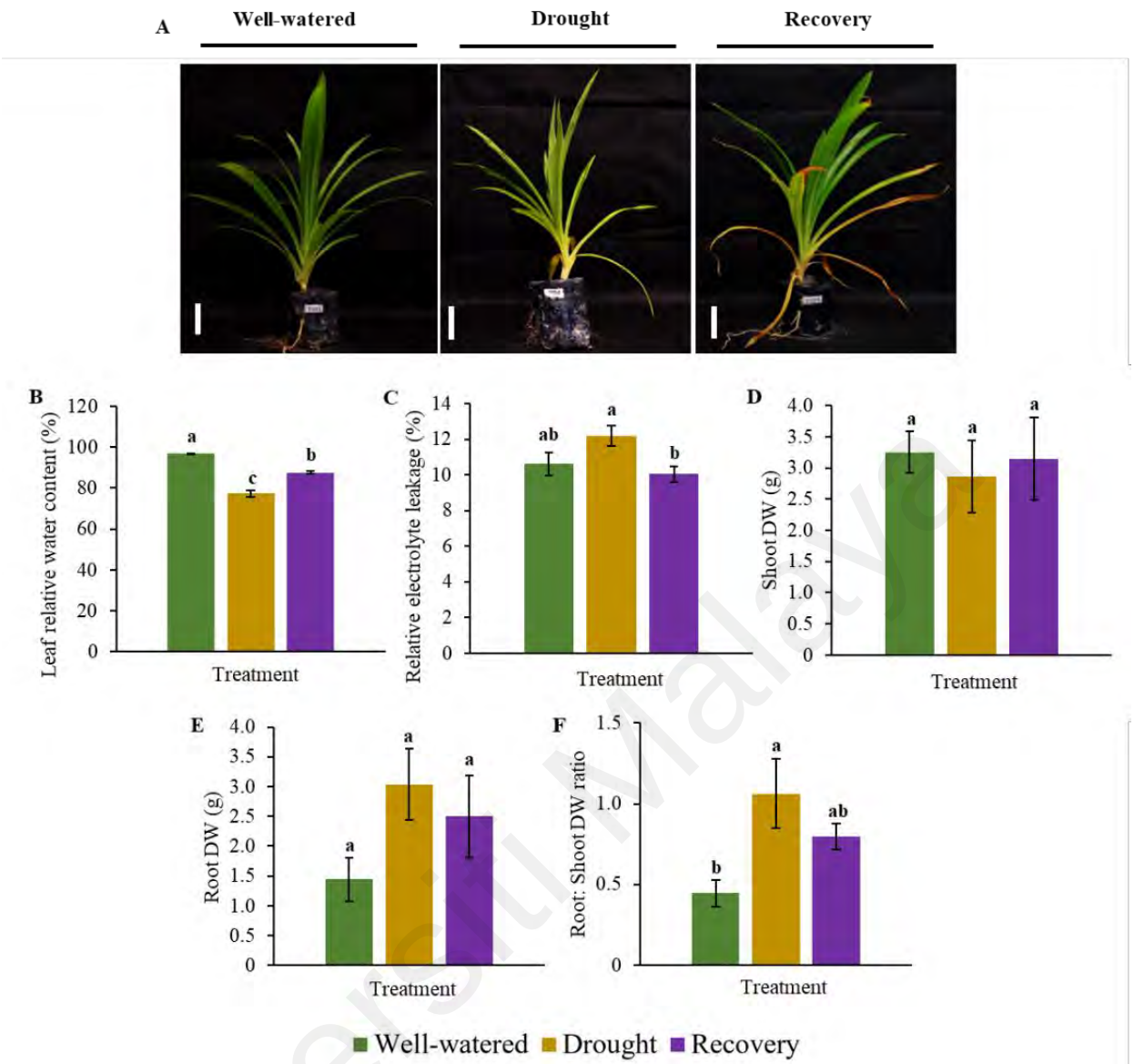

Figure 4.3. The morphology responses of *Pandanus amaryllifolius* plants of 7-day well-watered, drought-stressed, and water recovery. (**A**) *P. amaryllifolius* plants were exposed to drought stress by withholding water for 7 days, whereas the post-drought recovery plants were rewatered for 7 days after the 7-day drought conditions. The well-watered *P. amaryllifolius* served as control. The line bar indicates the scale of the plant = 10 cm. (**B**) The LRWC percentage of *P. amaryllifolius* leaves. (**C**) The REL percentage of the leaves of each sample. (**D**) Shoot dry weight (DW) of *Pandanus*; (**E**) Root dry weight (DW) of *Pandanus*. (**F**) The root-to-shoot ratio based on the biomass of *P. amaryllifolius*. The means labelled with the letters were significantly different based on the ANOVA, followed by the post hoc Tukey test when its p-value < 0.05.

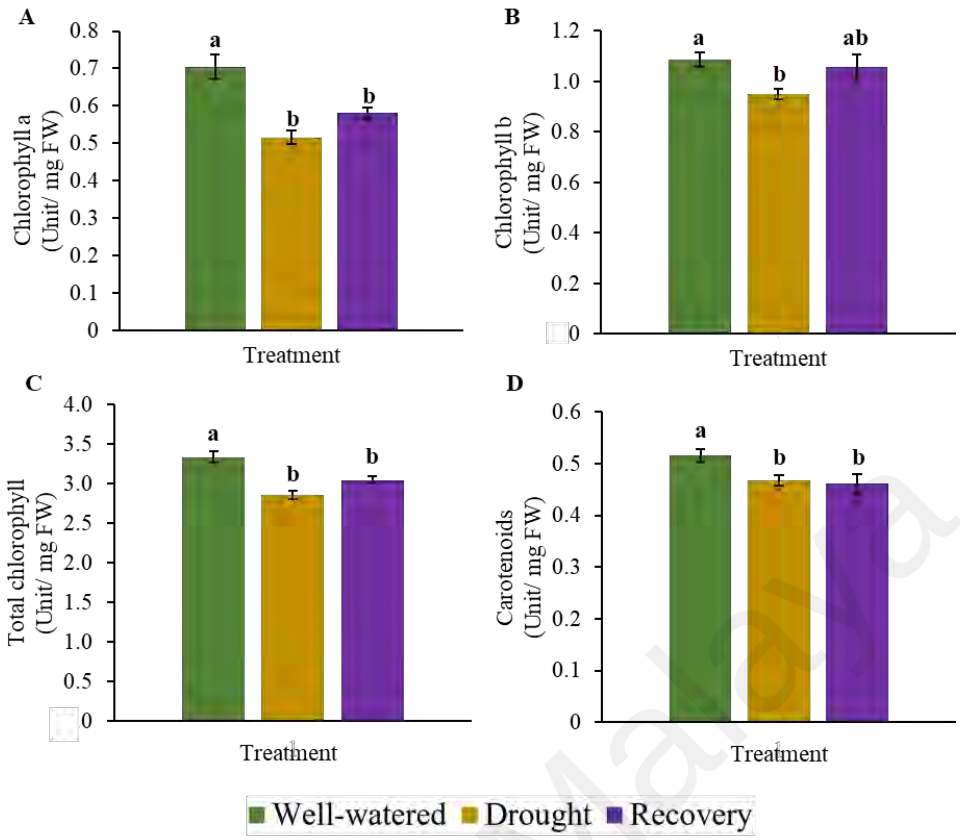

Figure 4.4. The leaf sample pigmentation content for well-watered, 7-day drought-stressed, and recovered *Pandanus amaryllifolius*. (**A**) Chlorophyll *a* concentration (µg mg^{-1} DW). (**B**) Chlorophyll *b* concentration (µg mg^{-1} DW). (**C**) The total chlorophyll content (µg mg^{-1} DW). (**D**) Carotenoids concentration (µg mg^{-1} DW). Means labelled with the letter were significantly different based on the ANOVA, followed by the Tukey test when its *p*-value < 0.05.

4.2 Hydrogen peroxide, malondialdehyde and proline contents

ROS is generally accumulated in drought-stressed samples. However, in this study, H_2O_2 concentration in drought-stressed and well-watered samples did not show significant changes (**Figure 4.5A**). In contrast, the recovered plants exhibited the highest accumulation of H_2O_2 (30.0 µM mL^{-1} g^{-1} FW).

The drought-stressed *P. amaryllifolius* plants recorded higher proline content (1.6 µM g^{-1} FW) than well-watered (1.3 µM g^{-1} FW) (**Figure 4.5B**). However, the recovered plants exhibited much lower proline accumulation (0.8 µM g^{-1} FW) than other treatments. On the other hand, the accumulation of MDA was highest (9.2 nM g^{-1} FW) in drought-stressed plants (**Figure 4.5C**). The well-watered and recovered samples recorded similar MDA levels.

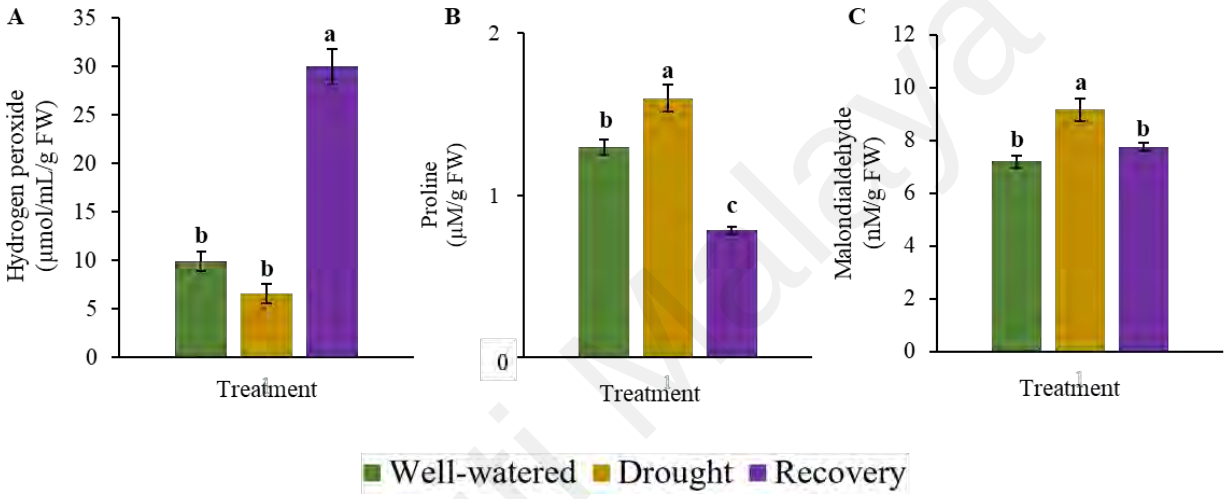

Figure 4.5. The reactive oxygen species accumulation, osmolyte and lipid peroxidation changes of the well-watered, drought-stressed, and water-recovered *Pandanus amaryllifolius*. (**A**) Hydrogen peroxide (H_2O_2) activity (µM mL^{-1} g^{-1} fresh weight (FW)). (**B**) Proline accumulation of *P. amaryllifolius* leaves (µM g^{-1} fresh weight (FW)). (**C**) Malondialdehyde (MDA) accumulation in *P. amaryllifolius* leaves as lipid peroxidation by-product quantified in nM g^{-1} fresh weight (FW). The means labelled with the letters were significantly different based on the ANOVA, followed by the post hoc Tukey test when its *p*-value < 0.05.

4.3 Antioxidant enzyme activities in *Pandanus* plants in response to drought stress

The antioxidant enzyme activities of the 7-day well-watered, drought-stressed, and recovered samples were analysed. In general, the drought-stressed plants showed higher activity of POD, CAT, APX and GR than other treatments, except SOD (**Figures 4.6B-E**). In the SOD activity, the drought-recovered samples recorded the highest value (462.1 Ug^{-1} FW) (**Figure 4.6A**). The POD and CAT for drought-stressed and recovered plants showed significantly higher activity than in well-watered plants (**Figures 4.6B** and **C**). For APX and GR, the highest activity was recorded in the drought-stressed samples, followed by recovery and well-watered treatments (**Figures 4.6D** and **E**).

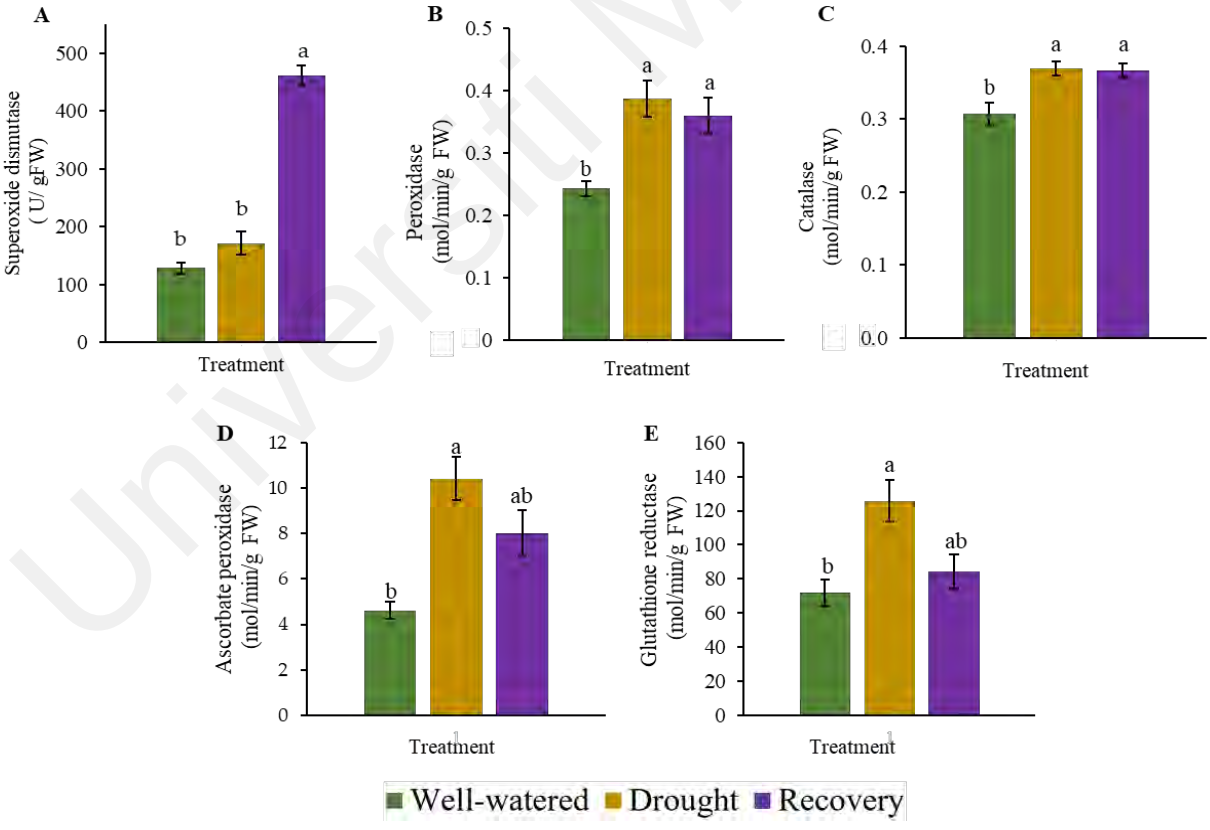

Figure 4.6. The antioxidant enzyme activities of the *Pandanus amaryllifolius* leaves in response to drought stress and post-drought recovery. (**A**) The superoxide dismutase

(SOD) activity was shown as U g^{-1} FW based on NBT colouration and inhibition. (**B**) Peroxidase (POD) (**C**) Catalase (CAT). (**D**) Ascorbate peroxidase (APX). (**E**) Glutathione reductase (GR). CAT, POD, APX and GR were measured on the absorbance changes according to their reaction and the activity presented in M min^{-1} g^{-1} FW. The means labelled with the letters were significantly different based on the ANOVA, followed by the post hoc Tukey test when its *p*-value < 0.05.

4.4 Protein extraction, quantification, and visualisation in response to drought stress

Total proteins from *P. amaryllifolius* leaf samples were extracted and the protein concentration of each sample was measured and calculated based on the generated BSA standard curve (**Table 4.1; Appendix 2C**). After separating through SDS-PAGE, multiple protein bands with different sizes were detected (**Figure 4.7**).

Table 4.1. Protein content of the day 7 sample for well-watered (WW), drought (D), and recovery (R) calculated based on the BSA standard curve.

Sample		Mean Abs.	Mean conc. (µg/mL)
Well-watered	7W1	0.967	777
	7W2	0.443	356
	7W3	0.456	367
	7W4	0.877	704
	7W5	0.945	759
Drought	7D1	1.020	820
	7D2	0.957	769
	7D3	0.900	723
	7D4	1.029	826
	7D5	1.041	836
Recovery	7R1	0.884	710
	7R2	0.997	801
	7R3	0.948	761
	7R4	0.754	606
	7R5	0.893	717

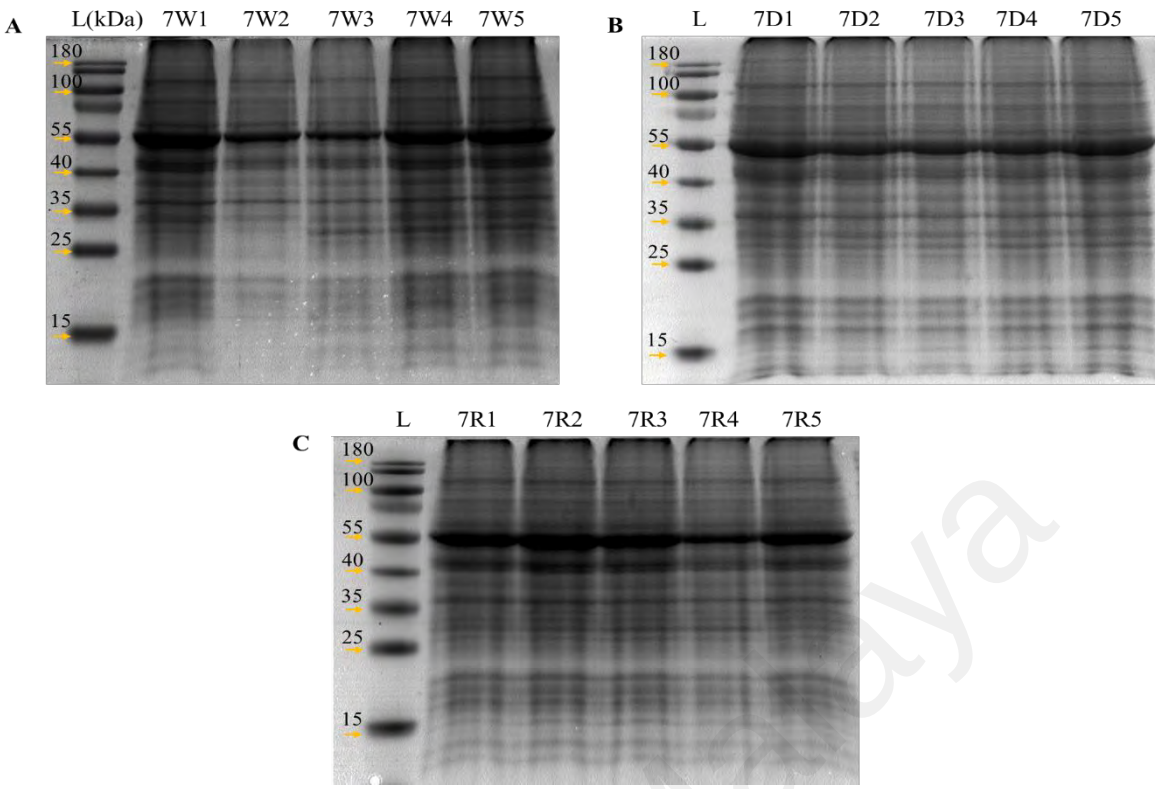

Figure 4.7. One-dimensional SDS-PAGE gel profile of protein samples extracted from day 7 samples. (**A**) Well-watered samples protein profile. (**B**) Drought-stressed samples protein profile. (**C**) Recovery samples protein profile. The biological samples were labelled as well-watered (W), drought-stressed (D), and recovered (R).

A total of 1,415 proteins were detected, and the mass spectrometry data have been deposited to ProteomeXchange with the dataset identifier PXD028784. Of these, 74 proteins were significantly changed (**Table 4.2**). These proteins were visualised with clustergram via hierarchical clustering and the log ratio expression (**Figures 4.8A** and **4.8B**). The clustering analysis exhibited four groups of protein abundance profiles, whereby Cluster 1 showed decreasing trend when comparing well-watered, drought-stressed and recovered samples. Clusters 2 and 3 showed increased or decreased in drought-stressed samples. However, Cluster 3 showed an increasing trend among the three samples.

Table 4.2. List of abundantly altered proteins between well-watered, drought-stressed, and recovered *P. amaryllifolius*.

Function	Accession	Protein	Biological process	Cluster
4Fe-4S cluster binding	Q6STH5	Fe-S cluster assembly factor HCF101, chloroplastic	Iron-sulphur cluster assembly	3
Actin binding protein	Q9LUI2	Protein NETWORKED 1A	Cytoskeleton	2
Alloesteric enzyme	P24846	4-hydroxy-tetrahydrodipicolinate synthase 1, chloroplastic	Amino acid biosynthesis	3
Aminotransferase	Q940M2	Alanine-glyoxylate aminotransferase 2 homolog 1, mitochondrial	Photorespiration	2
	Q84P54	Gamma aminobutyrate transaminase 1, mitochondrial	Biotin biosynthesis	3
Calcium ion binding	O22925	Vacuolar-sorting receptor 2	Protein transport	2
	Q41932	Oxygen-evolving enhancer protein 3-2, chloroplastic	Photosynthesis (ET)	4
Catalase	O65660	PLAT domain-containing protein 1	Stress response	4
Chaperone	P43644	DnaJ protein homolog ANJ1	Stress response	2
	P11143	Heat shock 70 kDa protein	Stress response	2
	Q04960	DnaJ protein homolog	Stress response	2
	Q0E3C8	Chaperone protein ClpB3, mitochondrial	Stress response	3
	Q39613	Peptidyl-prolyl cis-trans isomerase	Protein folding	3
	Q75GT3	Chaperone protein ClpB2, chloroplastic	Stress response	3
DNA binding	A4S6Y4	Lon protease homolog, mitochondrial	Oxidative stress	3
	Q94LW3	Homeobox protein knotted-1-like 3	Mucilage biosynthesis	3
Eleongation factor	Q9SI75	Elongation factor G, chloroplastic	Protein biosynthesis	3
Fatty acid biosynthesis	Q9LN49	3-ketoacyl-CoA synthase 4	Acyltransferase	3
	Q9C992	3-ketoacyl-CoA synthase 7	Acyltransferase	3

	Q570C8	3-ketoacyl-CoA thiolase 5, peroxisomal	Acyltransferase	4
Glycosidase	P48980	Beta-galactosidase	Carbohydrate metabolism	3
	P26792	Beta-fructofuranosidase, insoluble isoenzyme 1	Carbohydrate metabolism	3
	Q8W0A1	Beta-galactosidase 2	Carbohydrate metabolism	3
	A5JTQ2	Beta-xylosidase/alpha-L-arabinofuranosidase 1 (Fragment)	Carbohydrate metabolism	3
	O04931	Alpha-glucosidase	Carbohydrate metabolism	3
	Q56UD0	Beta-fructofuranosidase, insoluble isoenzyme 6	Carbohydrate metabolism	3
	Q8L7S6	Beta-hexosaminidase 3	Carbohydrate metabolism	3
Glycosyltransferase	O82627	Granule-bound starch synthase 1, chloroplastic/amyloplastic	Starch biosynthesis	3
	Q9ZQ94	UDP-glycosyltransferase 73C5	Brassinosteroid metabolism	3
GTPase activity	O24461	Ras-related protein Rab7	Protein transport	1
	Q05737	GTP-binding protein YPTM2	Protein transport	2
Histidine kinase binding	Q6ZIV7	Hypersensitive-induced response protein 1	Potassium ion channel regulation	3
Hydrolase	P49043	Vacuolar-processing enzyme	Cysteine-type endopeptidase	1
	Q6DBP4	Pectin acetylesterase 8	Cell wall biogenesis/degradation	3
	Q0DM51	DEAD-box ATP-dependent RNA helicase 3, chloroplastic	Ribosome biogenesis	3
	O80731	Pectin acetylesterase 3	Cell wall biogenesis/degradation	3
	P50246	Adenosylhomocysteinase	One-carbon metabolism	3
	Q9SG80	Alpha-L-arabinofuranosidase 1	L-arabinose metabolic	4
Isomerase	Q40147	Glutamate-1-semialdehyde 2,1-aminomutase, chloroplastic	Chlorophyll biosynthesis	3
Kinase	Q9LIK0	Plastidial pyruvate kinase 1, chloroplastic	Glycolysis	3
Ligase	O22436	Magnesium-chelatase subunit ChlI, chloroplastic	Chlorophyll biosynthesis	3

Magnesium ion binding	P48711	Ribulose bisphosphate carboxylase large chain	Photorespiration	1
Mediator complex binding	Q9XIM0	CCG-binding protein 1	Cellular response to hypoxia	3
Methyltransferase	O23760	Caffeic acid 3-O-methyltransferase	Lignin biosynthesis	1
Monooxygenase	Q42600	Cytochrome P450 84A1	Phenylpropanoid biosynthesis	3
mRNA binding	Q9FLN4	50S ribosomal protein L27, chloroplastic	Ribonucleoprotein	1
Non-photochemical quenching	A2WXD9	Photosystem II 22 kDa protein 1, chloroplastic	Photosynthesis	1
	Q9XF91	Photosystem II 22 kDa protein, chloroplastic	Photosynthesis	1
Oxidoreductase	F1SWA0	Zerumbone synthase	Protein synthesis	1
	A4QLY6	Photosystem I iron-sulfur center	Photosynthesis (ET)	2
	Q9FLC0	Peroxidase 52	Hydrogen peroxide	3
	Q96520	Peroxidase 12	Hydrogen peroxide	3
	Q01289	Protochlorophyllide reducwtase, chloroplastic	Chlorophyll biosynthesis	3
	Q42850	Protochlorophyllide reductase B, chloroplastic	Chlorophyll biosynthesis	3
	Q9SD46	Peroxidase 36	Hydrogen peroxide	3
	Q5ZE07	Multicopper oxidase LPR1 homolog 2	Phosphate homeostasis	3
	Q9SJ20	Ribonucleoside-diphosphate reductase large subunit	DNA replication	3
	Q9M591	Magnesium-protoporphyrin IX monomethyl ester [oxidative] cyclase, chloroplastic	Chlorophyll biosynthesis	3
	Q9CA67	Geranylgeranyl diphosphate reductase, chloroplastic	Chlorophyll biosynthesis	3
	P25795	Aldehyde dehydrogenase family 7 member A1	Stress response	4
	Q9AXH0	Catalase	Hydrogen peroxide	4
	A2YH64	Catalase isozyme B	Hydrogen peroxide	4
	Q0E4K1	Catalase isozyme A	Hydrogen peroxide	4
Pathogenesis	P81370	Thaumatin-like protein	Plant defence	3
Ribonucleoprotein	Q08937	29 kDa ribonucleoprotein B, chloroplastic	mRNA processing	3

	Q9ZUU4	RNA-binding protein CP29B, chloroplastic	mRNA processing	3
rRNA binding	A0A357	30S ribosomal protein S18, chloroplastic	Ribonucleoprotein	1
	A1E9N5	30S ribosomal protein S7, chloroplastic	Ribonucleoprotein	1
	Q32RY4	30S ribosomal protein S4, chloroplastic	Ribonucleoprotein	1
Structural constituent of ribosome	B2LMP1	30S ribosomal protein S15, chloroplastic	Ribonucleoprotein	1
Sucrose biosynthesis	O04932	Probable sucrose-phosphate synthase 1	Glycosyltransferase	4
Transferase	F6H7K5	Thiamine thiazole synthase 2, chloroplastic	Thiamine biosynthesis	3
	Q75LR2	Phospho-2-dehydro-3-deoxyheptonate aldolase 1, chloroplastic	Amino acid biosynthesis	3
	O23787	Thiamine thiazole synthase, chloroplastic	Thiamine biosynthesis	3

Cluster 3 recorded the highest number of proteins altered (45 proteins) between treatments, followed by Cluster 1 (12 proteins), Cluster 4 (9 proteins), and Cluster 2 (8 proteins) (**Figure 4.8B**). The abundance of the 74 proteins altered was further compared with heatmap visualisation according to its respective pairing comparison (**Figure 4.9**).

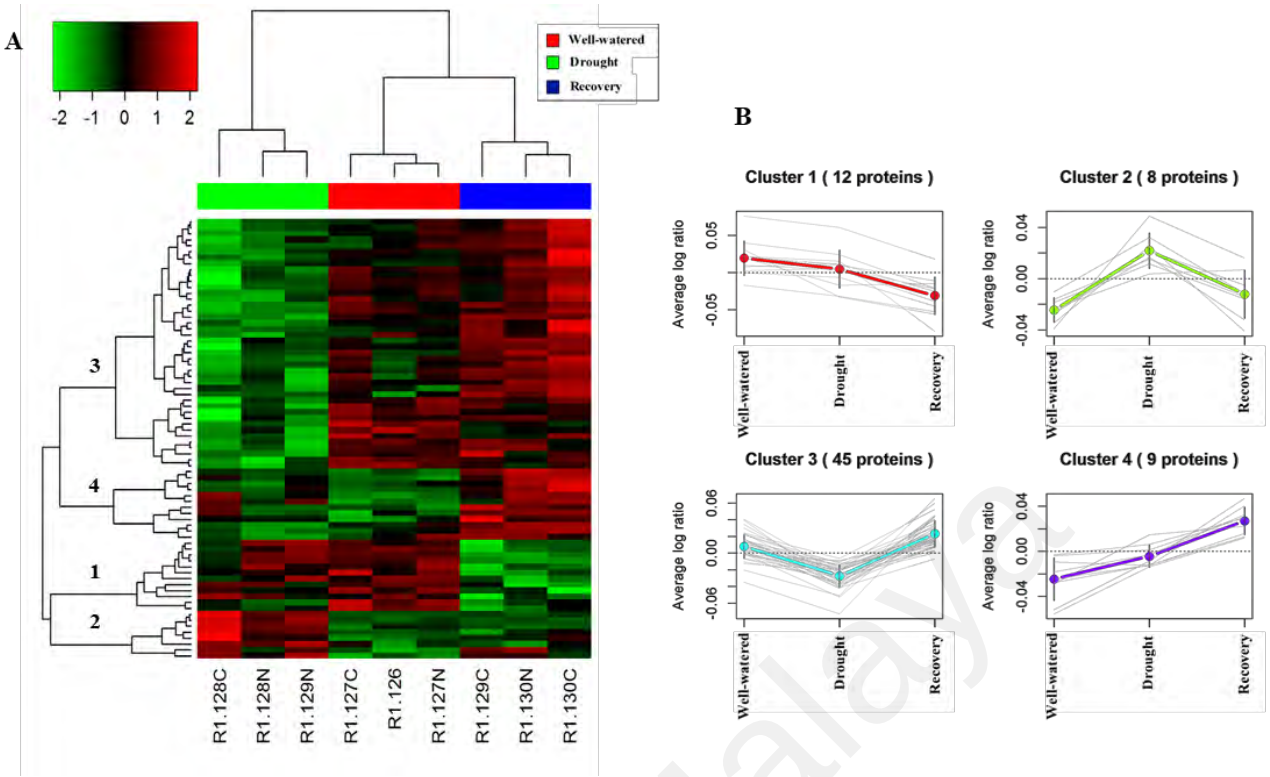

Figure 4.8. The hierarchal clustering of mean protein abundance and clustering between treatment comparisons for well-watered, drought-stressed, and recovered *Pandanus amaryllifolius*. (**A**) Clustergram visualised based on hierarchical clustering and heatmap of log ratio expression of the significant protein abundance comparison between treatments according to its cluster group. The intensity scale indicates the range of upregulation (red) or downregulation (green) of proteins between treatments. (**B**) Log ratio expression of differentially abundant proteins between treatments clustered into 4 groups.

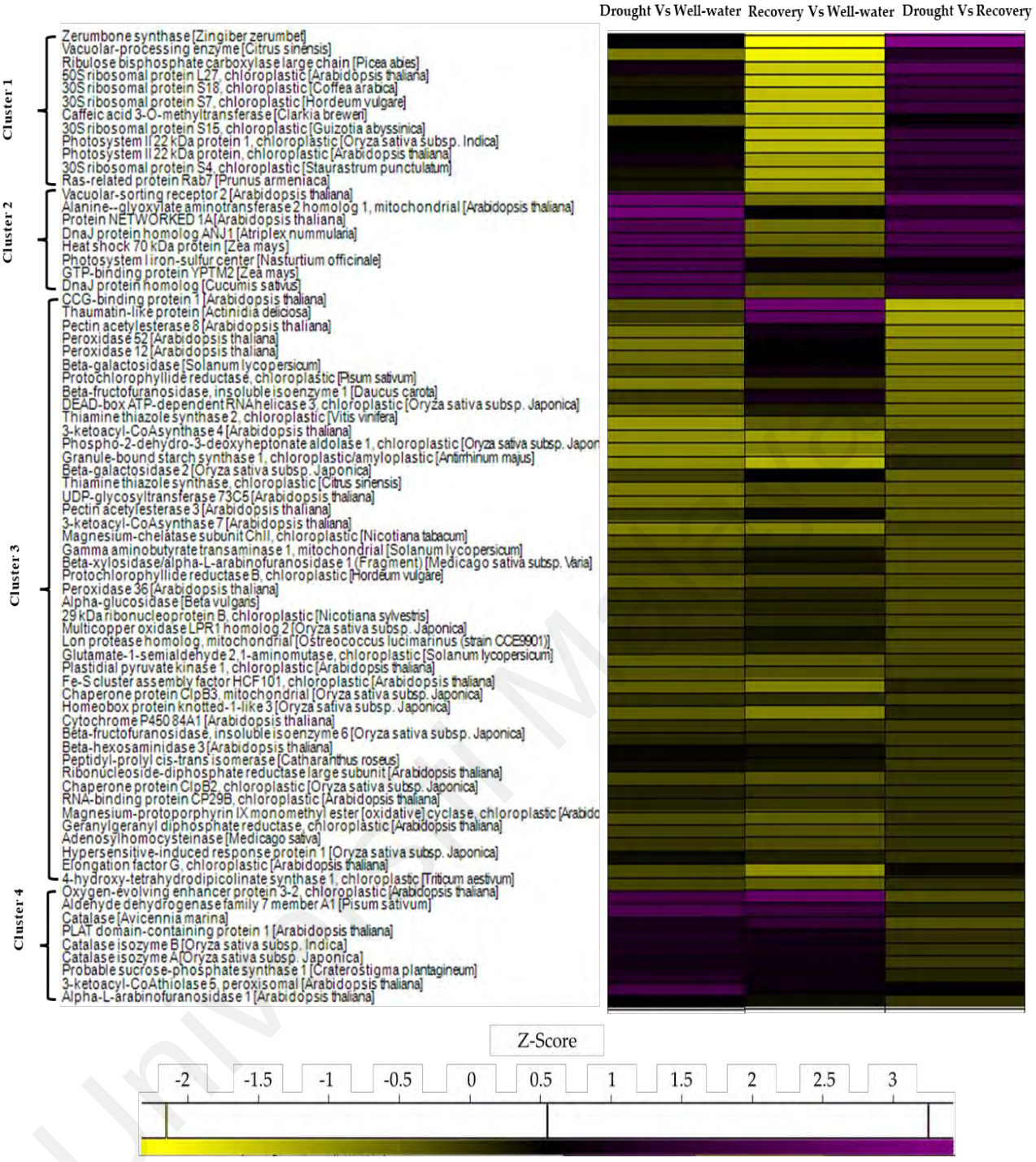

Figure 4.9. Differentially altered proteins between the comparison combination of well-watered, drought-stressed, and post-drought recovery *Pandanus amaryllifolius* according to their clusters. The Z-Score scale indicates the range of upregulation (purple) or downregulation (yellow) of proteins between treatments.

Of the 74 abundantly altered proteins, carbohydrate- and stress-related proteins were the largest differentially changed protein group (**Figures 4.10** and **4.11**).

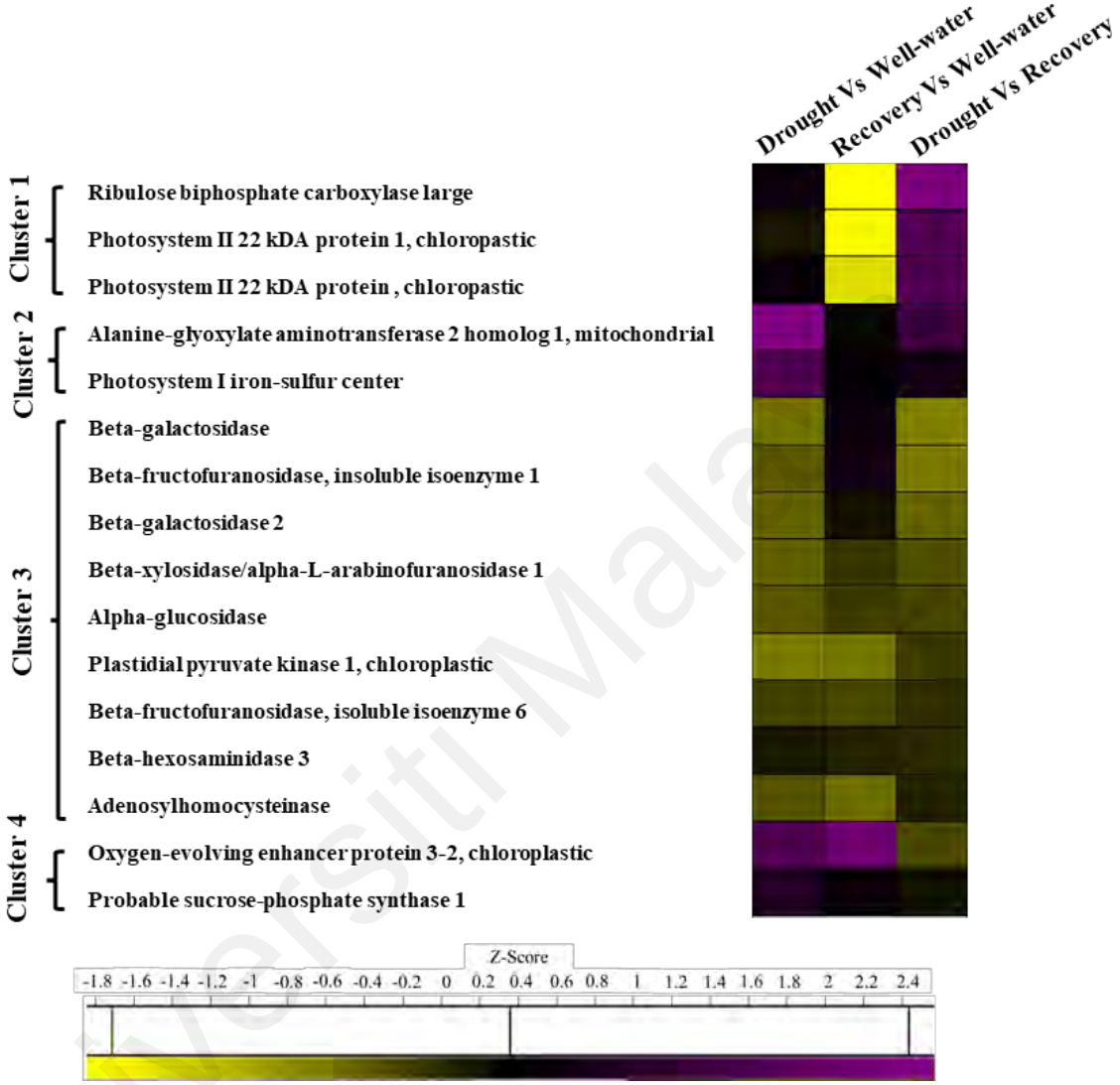

Figure 4.10. The differentially altered protein classes linked to the carbon metabolism processes between well-watered, drought-stressed, and watered-recovered *Pandanus amaryllifolius*. The Z-Score scale indicates the range of upregulation (purple) or downregulation (yellow) of proteins between treatments.

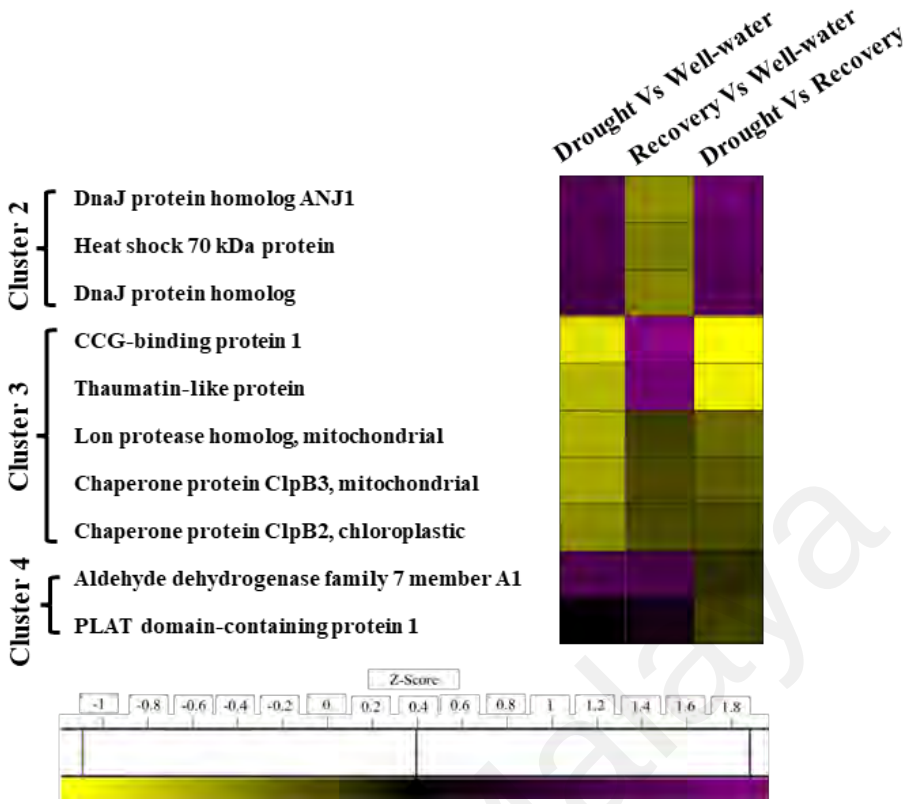

Figure 4.11. The differentially altered protein classes related to the stress-related processes between well-watered, drought-stressed, and watered-recovered *Pandanus amaryllifolius*. The Z-Score scale indicates the range of upregulation (purple) or downregulation (yellow) of proteins between treatments.

Venn diagram revealed 39 unique proteins were found in Comparison 1 (Drought vs Well-watered), 40 proteins in Comparison 2 (Recovery vs Well-watered), and 57 proteins in Comparison 3 (Recovery vs Drought) (**Figure 4.12A**). KEGG pathway enrichment analysis was performed to classify the function of the 74 differentially changed proteins (**Figure 4.12B**). The analysis indicated that 24% of differentially changed proteins were involved in carbohydrate metabolism, another 13% in genetic information processing and 8% in cofactors and vitamin metabolism (**Figure 4.12B**). In addition, the functional characterisation of these proteins via gene ontology (GO) showed that most differentially changed proteins were involved in photosynthesis processes and stress responses (**Figure 4.12C**).

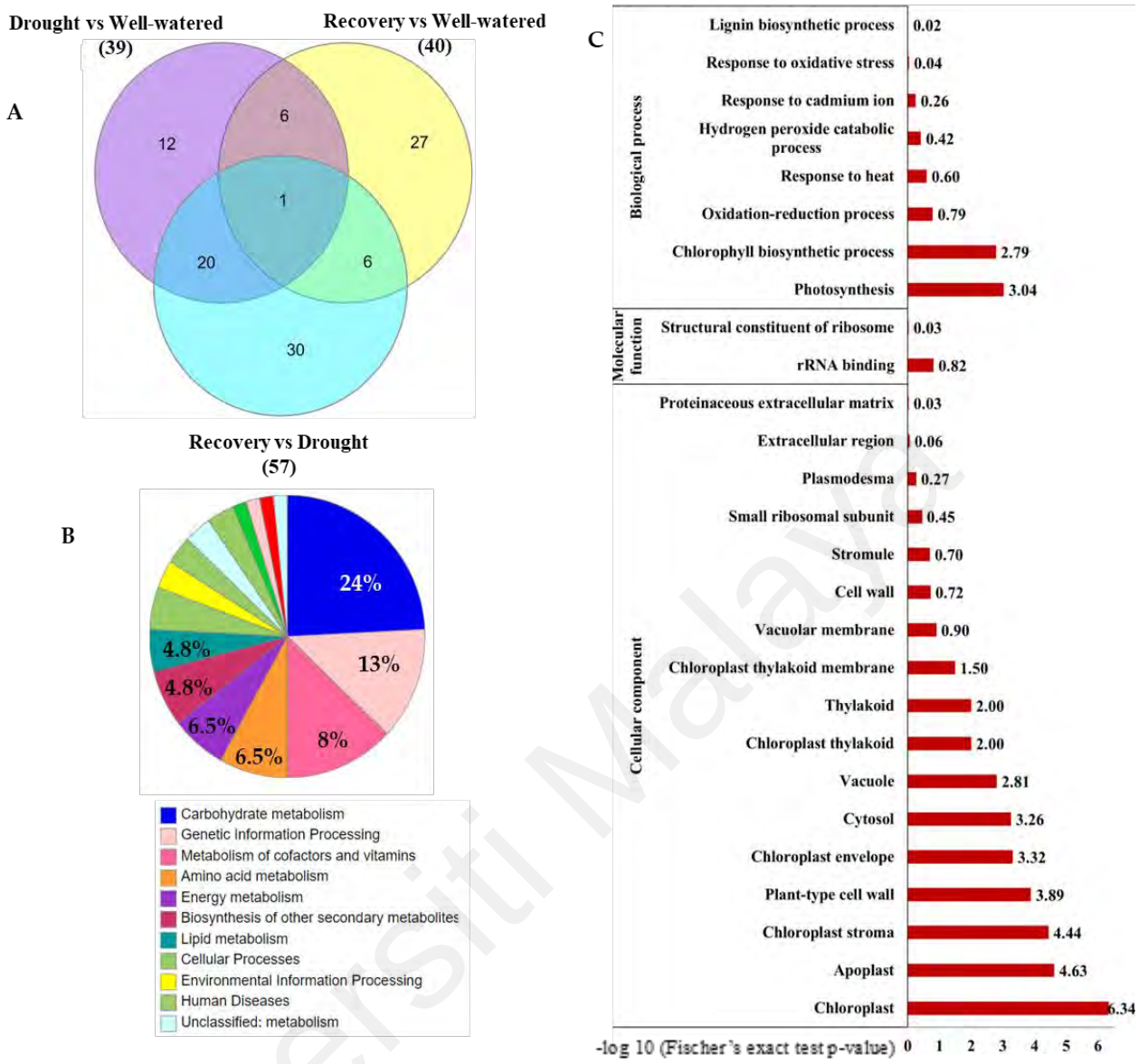

Figure 4.12. Abundantly altered protein comparison, functional categorisation, and enrichment between well-watered, drought, and post-drought recovery samples. (**A**) The Venn diagram represents the comparison of differentially abundant proteins in the leaves of *P. amaryllifolius* plants treated with or without drought stress and recovery. (**B**) KEGG enrichment of differentially altered proteins based on functional category. (**C**) Gene ontology enrichment is based on the KEGG pathway according to biological processes, molecular functions, and cellular components.

4.5 Drought-responsive genes triggered under drought stress and post-drought recovery

The total RNA of all samples was extracted, quantified using a spectrophotometer, and separated using gel electrophoresis. The RNA extracted was in good quantity and quality (**Table 4.3**) before proceeding with gel electrophoresis. The agarose gel showed two distinct bands on 1500 bp and 1000 bp, indicating 25S and 18S plant RNA were successfully extracted. Several candidate drought-responsive genes/proteins were selected based on previous studies (Davoudi *et al.*, 2022; Chen *et al.*, 2021; He *et al.*, 2021; Liu *et al.*, 2020; Kappachery *et al.*, 2015). The primer pairs for the gene sequence of the selected proteins were designed (**Table 3.1**) and subjected to gradient PCR. The PCR products were visualised in agarose gel and showed a single band at a higher annealing temperature (60 ºC) (**Figure 4.13A-C**). These primers showed the desired range of PCR products as predicted, such as *HSP70* (135 bp), *GAPDH* (250 bp), *ENO* (130 bp), *Thau* (110 bp), *B-Fruc* (167 bp), *EF1* (127 bp), and *Actin* (100 bp) (**Figures 4.14A-G**).

Table 4.3. RNA concentration and quality of the 7-day well-watered, drought, and recovery sample.

Sample	A260/280	A260/230	ng/µL
7C1	2.031	2.081	433
7C2	1.997	2.027	266
7C3	2.02	1.83	159
7C4	2.023	2.04	279
7C5	2.031	2.056	332
7D1	2.007	1.968	118
7D2	2.033	2.028	285
7D3	2.025	2.11	353
7D4	2.016	2.125	384
7D5	2.067	2.191	340
7R1	2.05	2.225	274
7R2	2.028	2.221	364
7R3	2.02	2.165	389
7R4	2.041	2.207	412
7R5	2.027	2.156	448

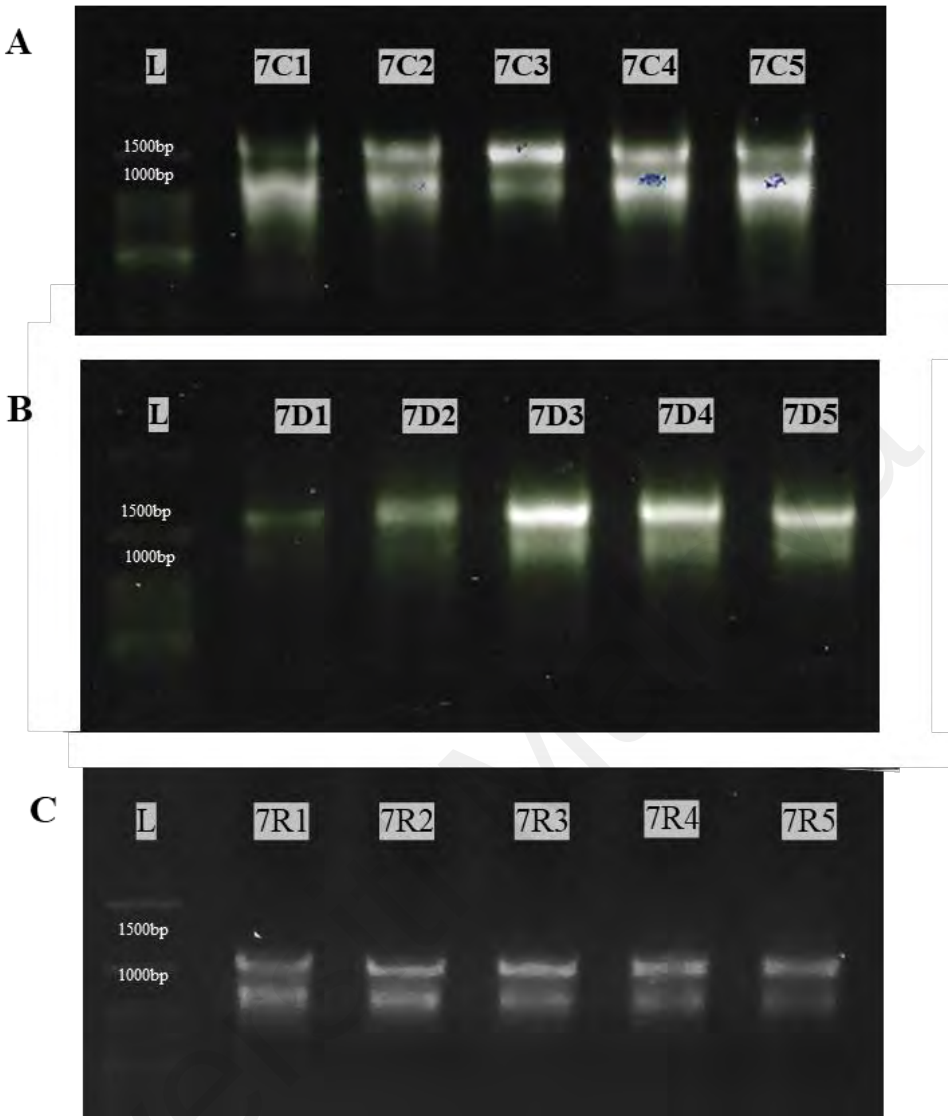

Figure 4.13. The quality of DNase-treated RNA extracted from the leaf sample of *Pandanus amaryllifolius*. (**A**) RNA quality of the 7-day well-watered sample. **(B**) RNA quality of the 7-day drought-stressed sample. **(C**) RNA quality of the 7-day post-drought recovery sample.

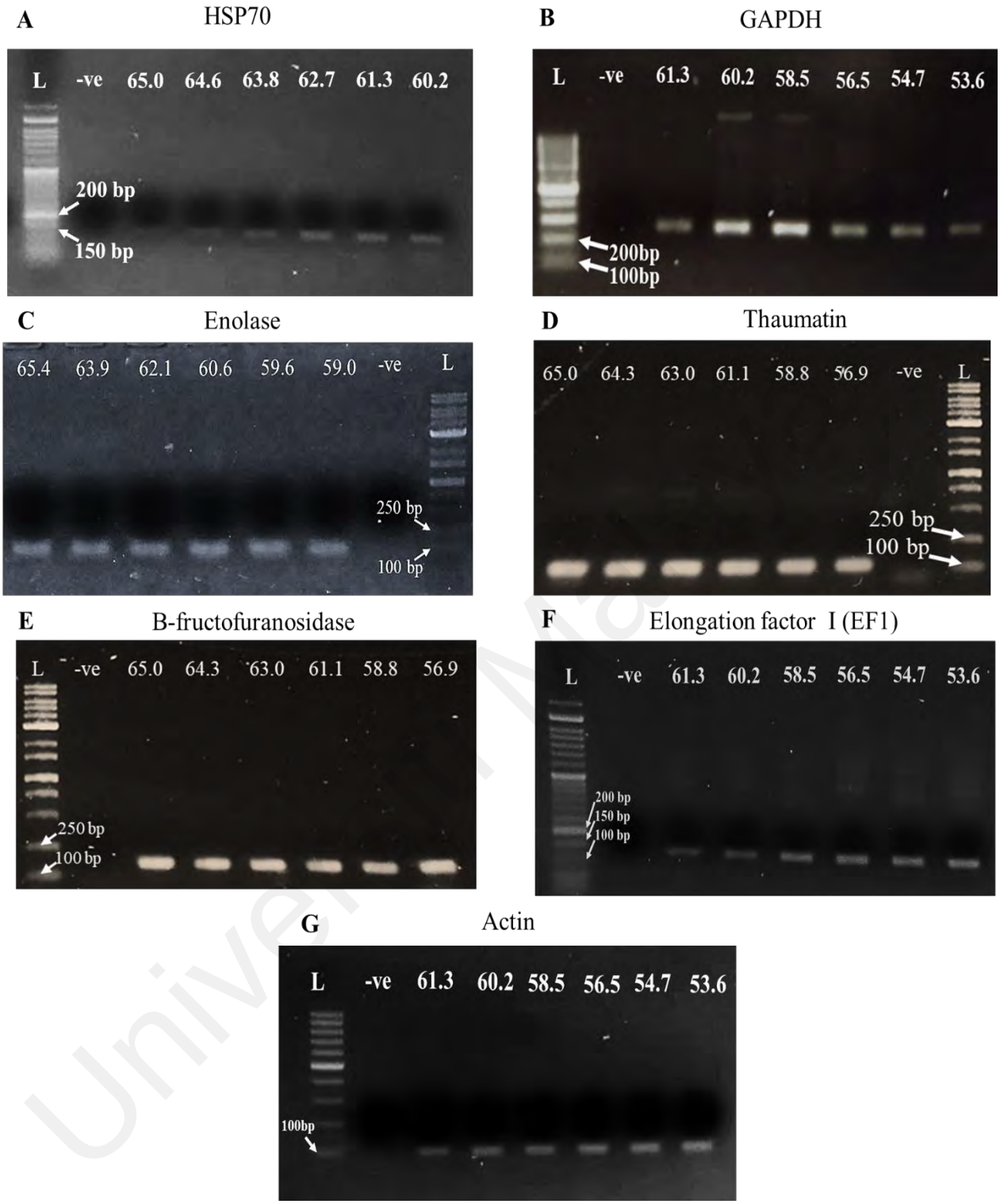

Figure 4.14. The gel electrophoresis of PCR products amplified at different annealing temperatures (50-65 ºC). (**A**) Heat shock protein 70 (HSP70). **(B**) (GAPDH). **(C**) Enolase (ENO). (**D**) Thaumatin (Thau). **(E**) β-Fructofuranosidase (β-Fruc). **(F**) Elongation factor 1 (EF1). **(G**) Actin.

The efficiency of the designed primer pairs was evaluated based on the single peak melt curve produced from a serial dilution of cDNA (**Figures 4.15A-G**). The Ct value of each cDNA dilution was used to construct the standard curves of each primer, and the efficiency of the primers was calculated. Each of the primers passed the efficiency range of 90-110%, such as *Actin* (107%), *EF1* (106%), *HSP70* (93%), *Thau* (95%), *GAPDH* (92%), *ENO* (98%), and *B-Fruc* (102%) (**Appendices 3A-G**).

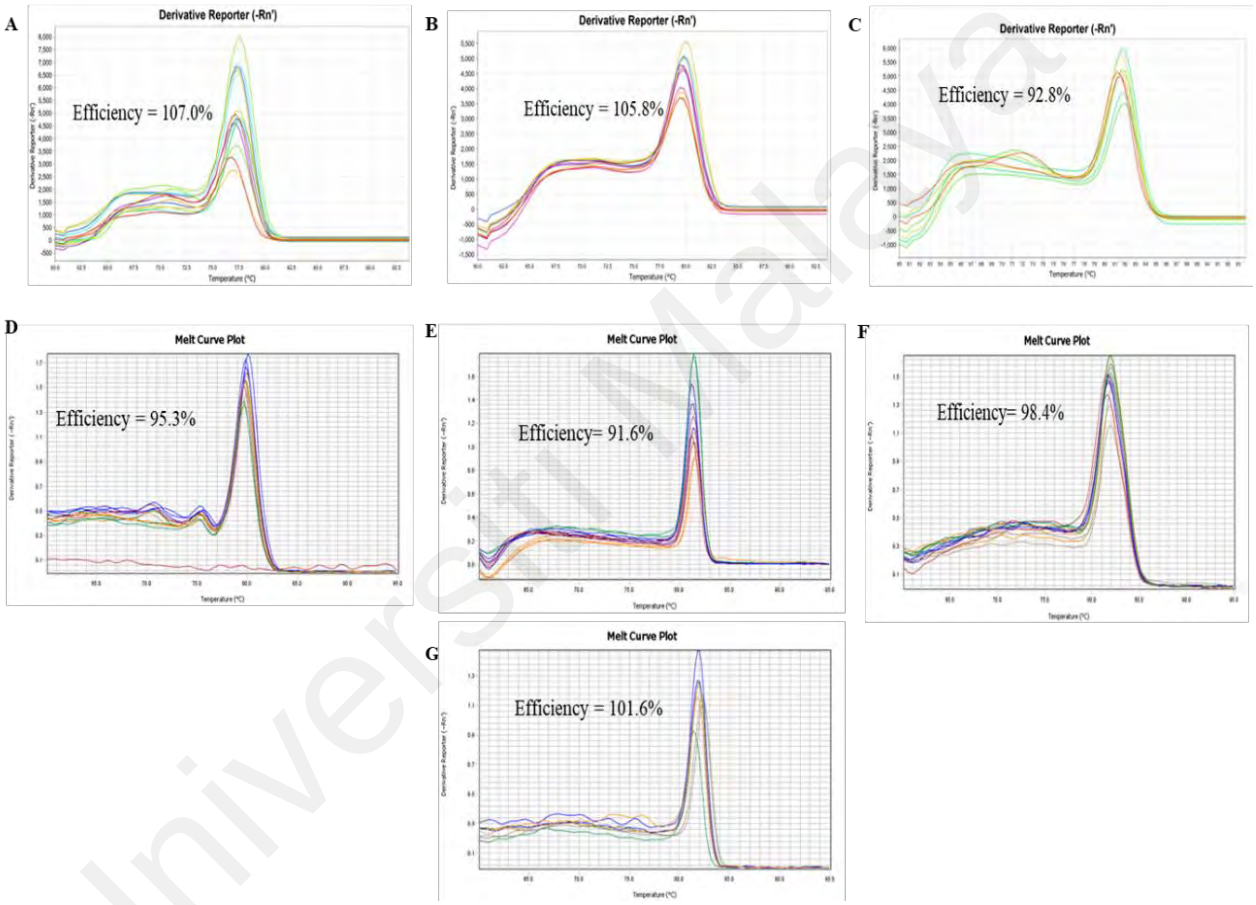

Figure 4.15. Melt curves and primer efficiency percentages for reference gene and gene of interests. The Ct value of the primer product with starting template of a series of cDNA dilution (**A**) *Actin*. (**B**) *Elongation factor 1* (*EF1*). (**C**) *Heat shock protein 70* (*HSP70*). (**D**) *Thaumatin* (*Thau*). (**E**) *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). (**F**) *Enolase* (*ENO*). (**G**) *β-fructofuranosidase* (*β-Fruc*).

The expression of the selected drought-responsive genes, such as *HSP70, β-Fruc, ENO,* and *Thau,* were analysed in all samples (**Figures 4.16A-E**). All tested genes exhibited the highest expression in drought-stressed samples, except *GAPDH*. It is noteworthy that *HSP70, β-Fruc, ENO,* and *Thau* gene expressions in recovered plants were significantly lower than in drought-stress samples (**Figures 4.16A, C, D** and **E**).

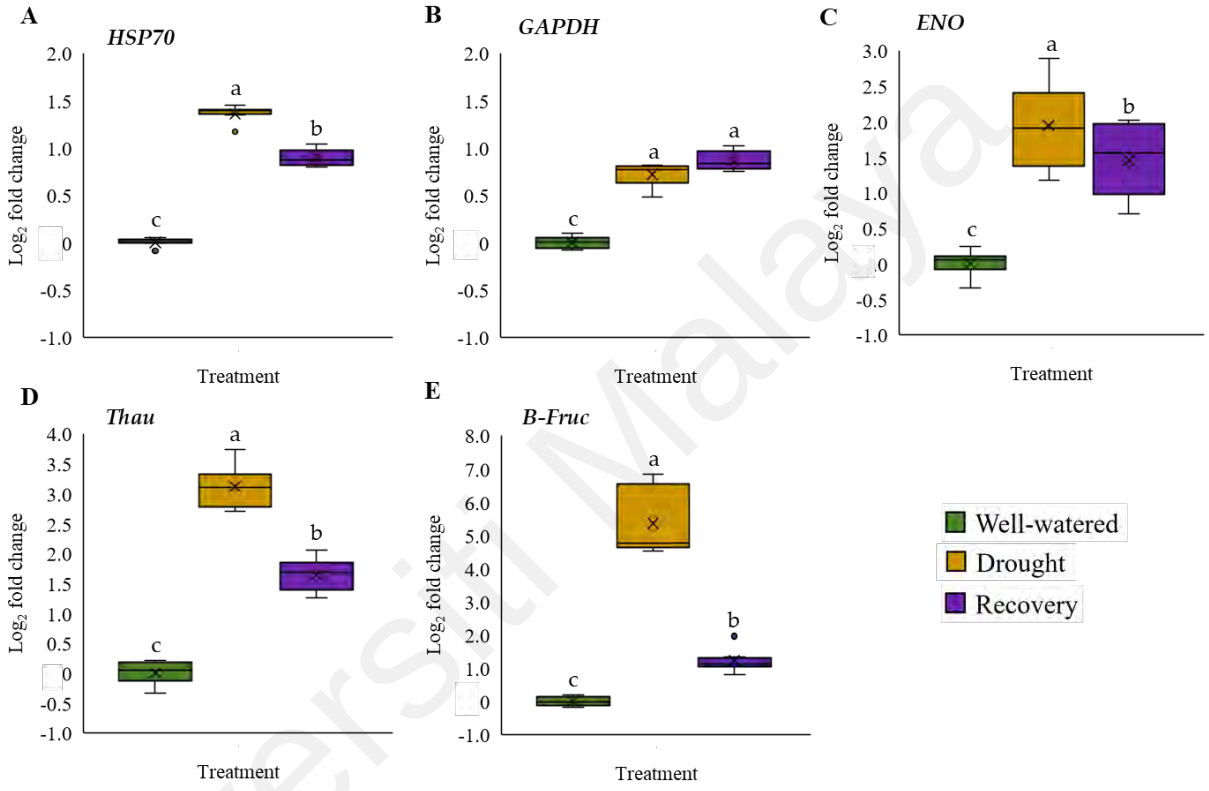

Figure 4.16. The relative expression of the selected drought-responsive genes in well-watered, 7-day drought-stressed and post-drought recovery leaf samples. (**A**) *Heat shock protein 70* (*HSP70*). (**B**) *Glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*). (**C**) *Enolase* (*ENO*) (**D**) *Thaumatin* (*Thau*). (**E**) *β-fructofuranosidase* (*β-Fruc*). Actin and Elongation factor 1 of *P. amaryllifolius* were used as internal standard genes. The letter labelled on the mean value indicates the significance level between treatments based on the ANOVA, followed by the post hoc Tukey test when its *p*-value < 0.05.

4.6 Comparison of different oil palm wood vinegar (OPWV) dilution factors and application frequencies

OPWV was diluted to 1:100, 1:250, 1:500, and 1:1000 with distilled water before applying it to plants. The untreated and treated plants showed no visible phenotypic differences after 19 days of growing (**Figure 4.17**). However, applying 1:500 diluted OPWV increased stem circumference (**Figure 4.18A**) and shoot and root DW (**Figures 4.18B** and **C**). In contrast, samples treated with 1:250 dilution accumulated the highest chlorophyll *a*, *b*, total chlorophyll, and carotenoid content (**Figures 4.18E-H**). Nonetheless, since the 1:500 dilution OPWV demonstrated a more significant impact on the *Pandanus* morphological improvement, such as stem growth and biomass, it was selected for the subsequent experiment.

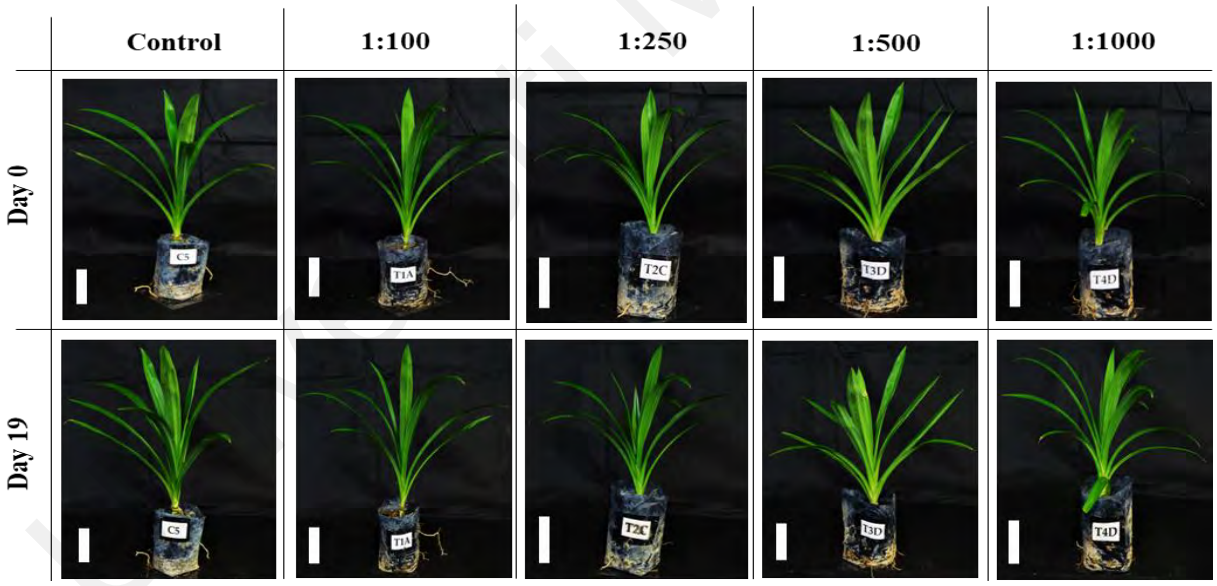

Figure 4.17. The morphological and pigment analyses of the *Pandanus amaryllifolius* treated with different dilution factors of OPWV at 3-day intervals for 12 days. The plants were grown for another 7 days in well-watered conditions. Photographs of *P. amaryllifolius* treated with 1:100, 1:250, 1:500, 1:1000 OPWV and distilled water (control) on days 0 and 19. The line bar indicates 10 cm.

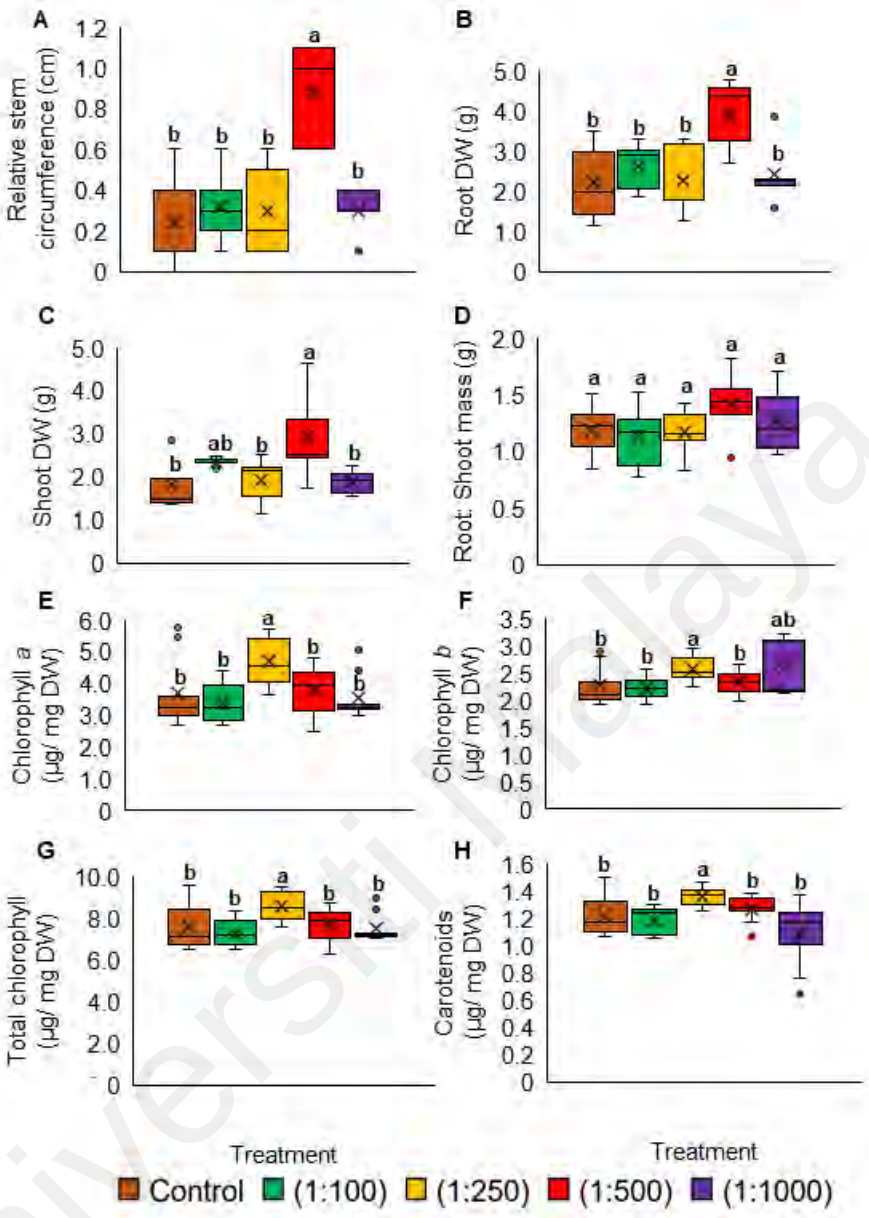

Figure 4.18. (**A**) Relative stem circumference comparison between dilution treatments. (**B**) Shoot dry weight (DW). (C) Root dry weight (DW). (D) The root-to-shoot DW ratio. (**E**) Chlorophyll *a* (U mg^{-1} DW). (**F**) Chlorophyll *b* (U mg^{-1} DW). (**G**) Total chlorophyll (U mg^{-1} DW). (**H**) Carotenoid (U mg^{-1} DW). The letter labelled on the mean value indicates the significant level between treatments based on the ANOVA, followed by the post hoc Tukey test when its p-value < 0.05.

The selected 1:500 OPWV dilution was applied to plants at 6-day intervals (F1), 3-day intervals (F2), and 1-day intervals (F3) for 12 days. After OPWV application, the plants were allowed to grow for another 7 days. The results showed no visible morphological changes for each treatment (**Figure 4.19**). Nevertheless, the plants treated with F2 exhibited significantly improved stem circumference and shoot and root DW compared to the F1 (**Figures 4.20A-D**). However, differences in the root-to-shoot ratio were insignificant (**Figure 4.20D**). On the other hand, F1 and F2 significantly increased pigment constituents compared to F3 (**Figures 4.20E-H**). Altogether, a 1:500 dilution of OPWV applied at F2 frequency was selected for the subsequent experiments.

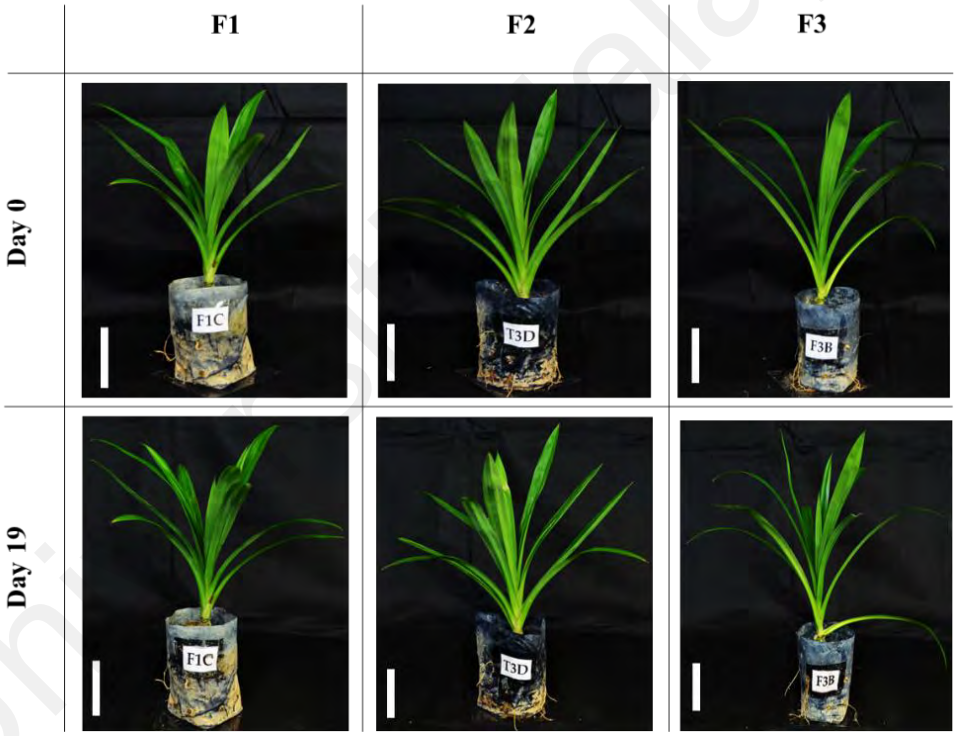

Figure 4.19. The morphological and pigment analyses of the 1:500 OPWV-treated *Pandanus amaryllifolius* at different application frequencies for the first 12 days. F1: OPWV was applied at 6-day intervals; F2: at 3-day intervals; and F3: every 1-day intervals. (**A**) Photographs of *P. amaryllifolius* treated with F1, F2, and F3 application frequency on days 0 and 19. The line bar indicates 10 cm.

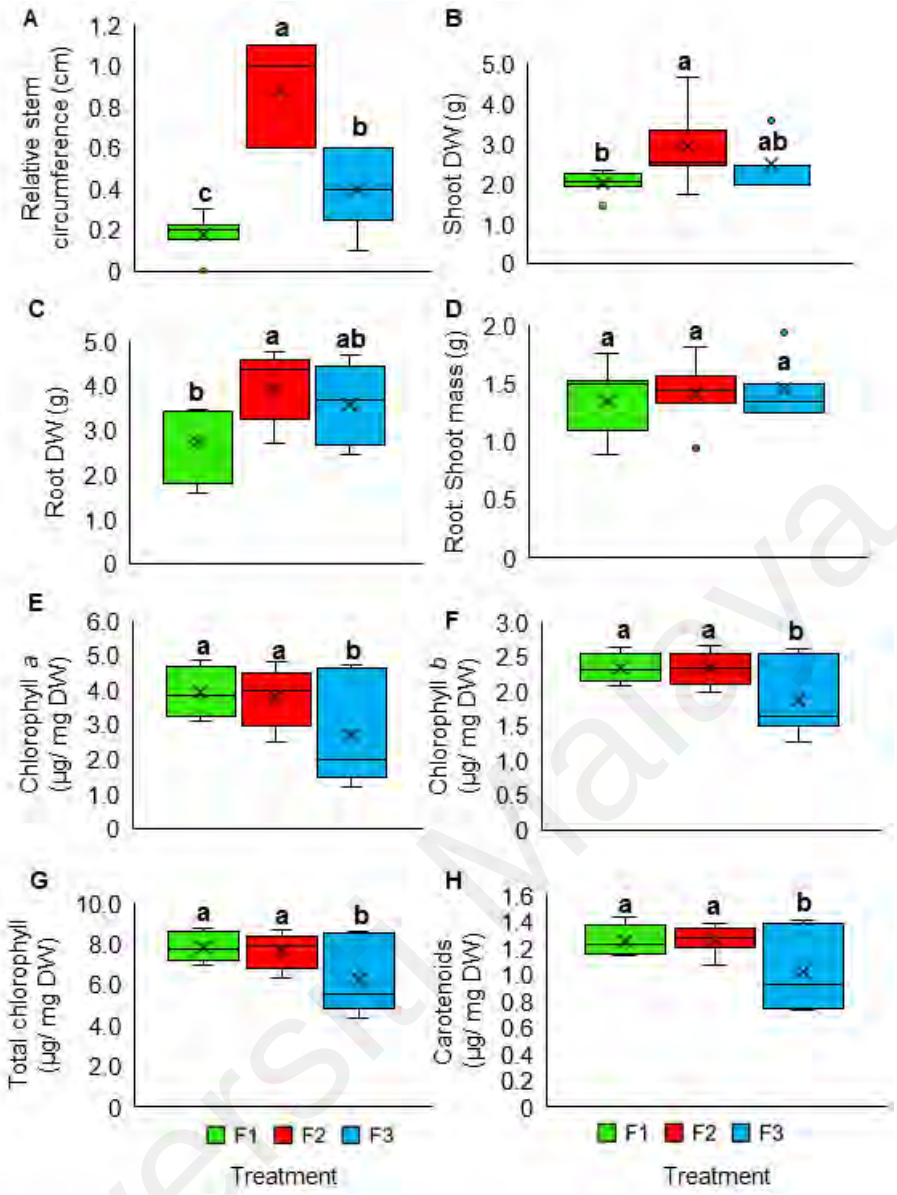

Figure 4.20. The morphological and pigment analyses of the 1:500 OPWV-treated *Pandanus amaryllifolius* at different application frequencies for the first 12 days. F1: OPWV was applied at 6-day intervals; F2: at 3-day intervals; and F3: every 1-day intervals. (**A**) The relative stem circumference of each treatment. (**B**) Dry weight (DW) of the shoots. (**C**) DW of the root samples. (**D**) The root-to-shoot ratio of sample biomass. (**E**) Chlorophyll *a* (U mg^{-1} DW). (**F**) Chlorophyll *b* (U mg^{-1} DW). (**G**) Total chlorophyll (U mg^{-1} DW). (**I**) Carotenoid (U mg^{-1} DW). The letter on the mean value indicates the significant level between treatments based on the ANOVA, followed by the post hoc Tukey test when its *p*-value < 0.05.

4.7 Oil palm wood vinegar (OPWV) improved drought-stressed *Pandanus amaryllifolius*

The experiment evaluated the protective role of OPWV in alleviating the adverse effects of drought stress. The morphological changes of all plants were recorded on days 7 and 10 (**Figure 4.21**). Similar to the previous results, drought stress increased leaf wilting and folding after 7 days of drought stress (**Figure 4.21**). However, applying OPWV reduced the leaf wilting and folding of the drought-stressed plants (**Figure 4.21**). In addition, the OPWV-treated drought-stressed plants maintained their structural turgidity compared to the collapsed structure of the non-treated *Pandanus*.

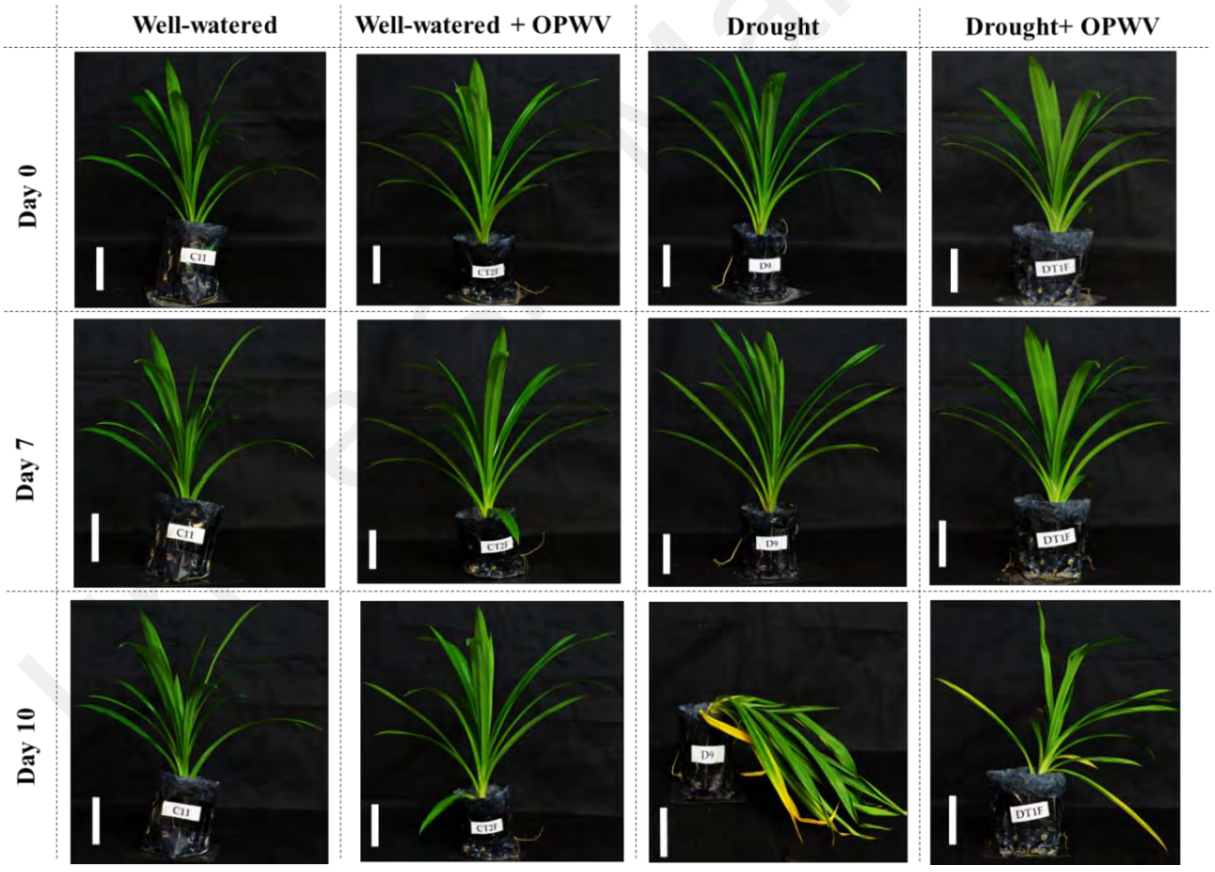

Figure 4.21. The morphology analysis of the *P. amaryllifolius* treated with 1:500 OPWV dilution and F2 application frequency under well-watered and drought stress conditions for 7 and 10 days. The line indicates 10 cm. Photographs of *P. amaryllifolius* treated with well-watered, well-watered + OPWV, drought, and drought + OPWV.

The LRWC percentage of drought-stressed plants was reduced to 50% on day 7 and recorded the lowest percentage on day 10 (18%) (**Figure 4.22A**). In contrast, the LRWC percentage of the well-watered and OPWV-treated plants was generally higher than 80% (**Figure 4.22A**). When *Pandanus* was primed with OPWV, the plants increased the LRWC to 65% and 30% on days 7 and 10 of drought stress, respectively. In addition, the REL percentage in the drought-stressed *Pandanus* increased from 12 to 27% on days 7 and 10, respectively (**Figure 4.22B**), whereas the well-watered plants have the lowest REL (4%). Notably, OPWV treatment reduced the REL percentage in drought-stressed plants (**Figure 4.22B**).

On day 7, the well-watered plants with or without OPWV showed higher shoot DW than the drought-stressed samples (**Figure 4.22C**). The OPWV-treated plant showed lower shoot DW after 10 days of drought stress than the well-watered sample. However, differences between OPWV-non-treated, well-watered and drought-stressed plants were not significant (**Figure 4.22C**). On the other hand, the root DW of 7-day OPWV-treated well-watered and drought-stressed samples exhibited higher biomass than those without OPWV treatment (**Figure 4.22D**). Similarly, the OPWV-treated samples showed the highest root-to-shoot ratio on day 7 (**Figure 4.22E**).

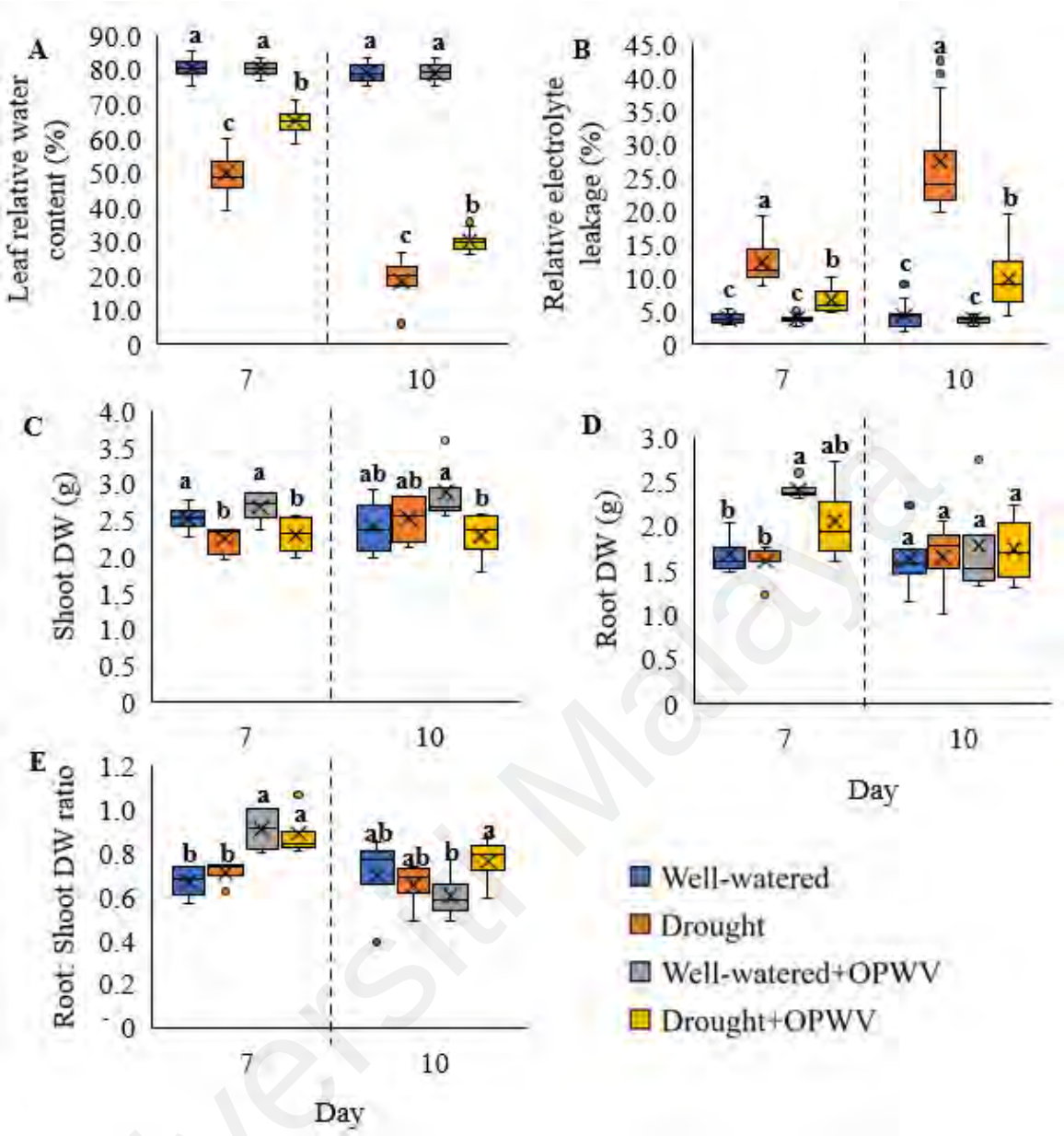

Figure 4.22. The morphology analysis of the *Pandanus amaryllifolius* treated with 1:500 OPWV dilution and F2 application frequency under well-watered and drought stress conditions for 7 and 10 days. (**A**) Leaf relative water content (LRWC). (**B**) Leaf relative electrolyte leakage (REL). (**C**) Dry weight (DW) of the shoot samples. (**D**) Dry weight (DW) of the root samples. (**E**) Root-to-shoot DW ratio. The letter labelled on the mean value indicates a significant level between treatments based on the ANOVA, followed by the post hoc Tukey test when its p-value < 0.05.

The well-watered *P. amaryllifolius* treated with OPWV recorded the highest stem growth for days 7 (0.3 cm) and 10 (0.4 cm), followed by well-watered (0.1 cm for both 7 and 10 days), OPWV-treated drought-stressed (0.2 and 0.7 cm for 7 and 10 days, respectively), and drought-stressed samples (0.5 and 1.4 cm for 7 and 10 days, respectively) (**Figure 4.23A**).

The percentage of leaf yellowing was calculated based on the number of yellowing leaves per plant following the colour reference (**Figure 3.5B**). No visible yellowing symptoms were observed in well-watered plants treated with or without OPWV (**Figure 4.21** and **Figure 4.23B**). Thus, both treatments were calculated as null. On the contrary, drought-stressed plants exhibited visible leaf yellowing, 16% and 37% yellowing leaves on days 7 and 10, respectively (**Figure 4.23B**). In contrast, applying OPWV to drought-stressed samples reduced their yellowing leaves to 8% and 23% on days 7 and 10, respectively.

The percentage of leaf folding was recorded by determining the number of folded leaves according to the leaf folding references (**Figure 3.5A**). The drought-stressed plants showed the highest percentage of Stage 1 folding (73%), followed by OPWV-treated drought-stressed samples (68%) (**Figure 4.23C**). In contrast, 10-day drought-stressed plants showed a lower percentage of Stage 1 folding (51%) than the OPWV-treated samples (69%) (**Figure 4.23C**). This reduction was due to the shifting of leaf folding severity from Stage 1 to Stage 2. Nonetheless, applying OPWV to drought-stressed plants significantly reduced the leaf folding in Stage 2 (**Figure 4.23D**).

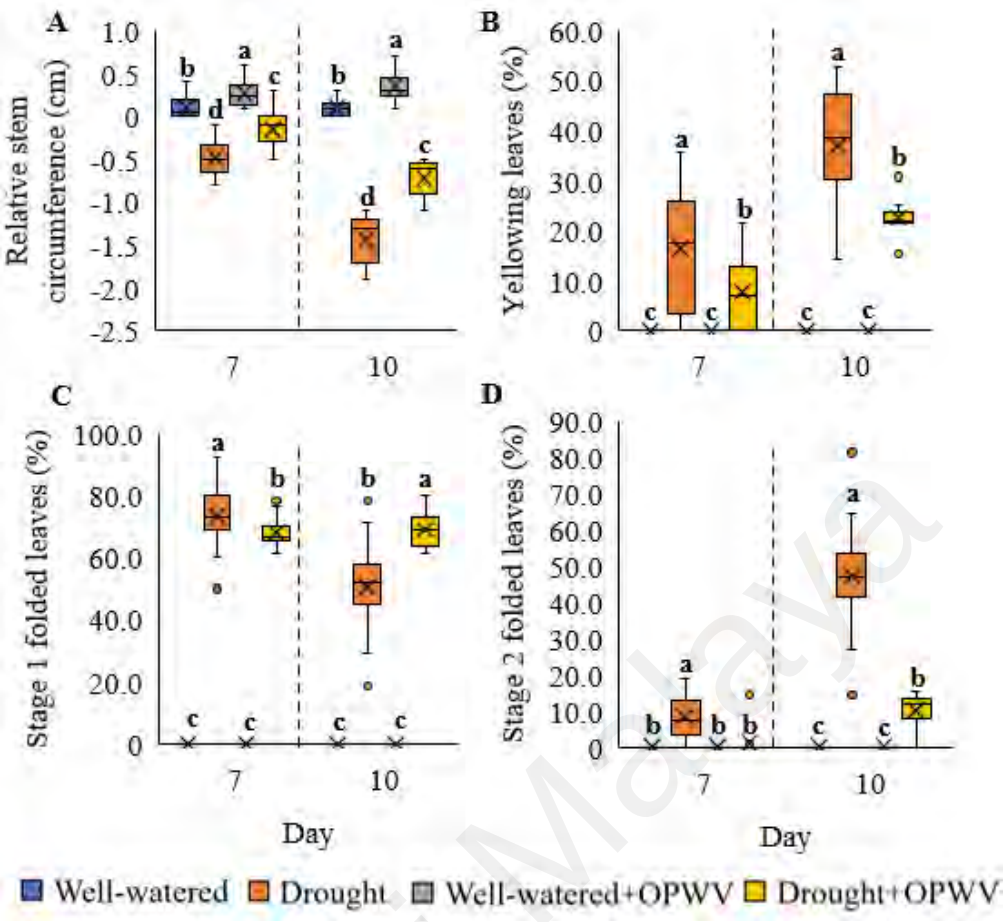

Figure 4.23. The stem growth and leaf morphology analyses of the *Pandanus amaryllifolius* under well-watered and drought stress conditions for 7 and 10 days with and without the treatment of 1:500 OPWV dilution and F2 application frequency (**A**) Relative stem circumference. (**B**) The percentage of yellowing leaves (%). (**C**) Stage 1 leaf folding percentage (%). (**D**) Stage 2 leaf folding percentage (%). The letter labelled on the mean value indicates the significant level between treatments based on the ANOVA, followed by the post hoc Tukey test when its *p*-value < 0.05.

4.8 Leaf pigment constituents in *Pandanus amaryllifolius* in response to OPWV

In this experiment, the drought-stressed *P. amaryllifolius* exhibited a significant reduction of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids on days 7 and 10 compared to well-watered samples (**Figures 4.24A-D**). Interestingly, when the plant was primed with OPWV, the pigmentation degradation was reduced in the drought-stressed samples (**Figures 4.24A-D**).

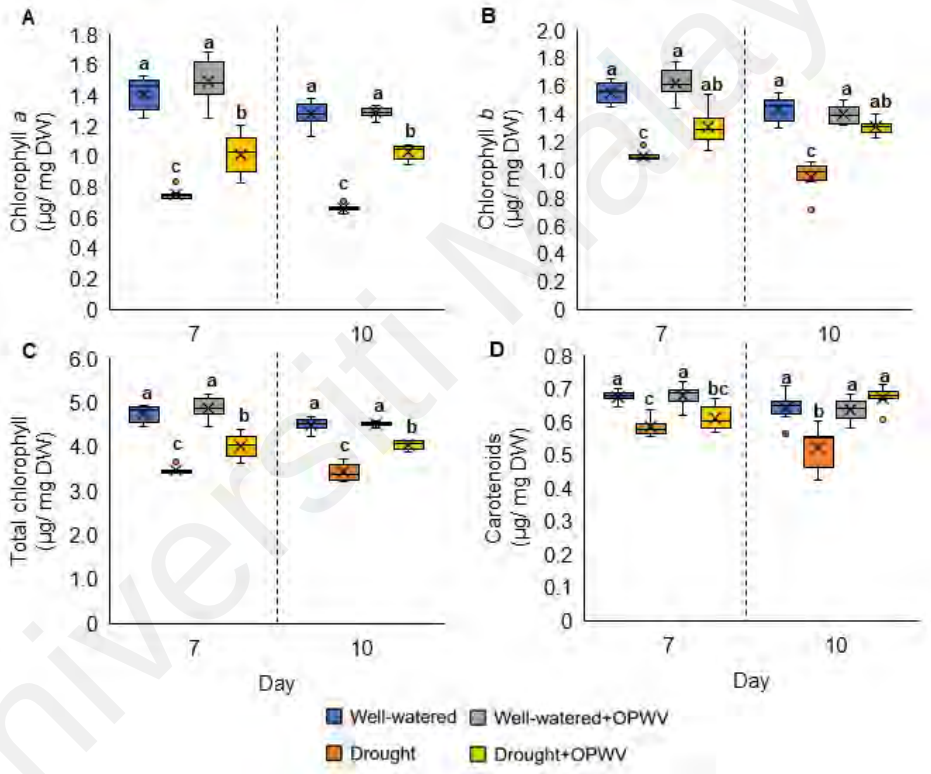

Figure 4.24. The leaf pigment content of the OPWV-treated samples under well-watered and drought stress for 7 and 10 days. (**A**) Chlorophyll *a* (U mg^{-1} DW). (**B**) Chlorophyll *b* (U mg^{-1} DW). (**C**) Total chlorophyll (U mg^{-1} DW). (**D**) Carotenoid (U mg^{-1} DW). The letter labelled on the mean value indicates the significant level between treatments based on the ANOVA followed by the post hoc Tukey test when its p-value < 0.05.

4.9 Root structure analysis

The root structure of all treatments was determined (**Figure 4.25**). Under drought stress of 7-day and 10-day, drought-stressed *Pandanus* showed more root formation compared to well-watered plants (**Figures 4.25A** and **B**). However, the OPWV-treated well-watered and OPWV-treated drought-stressed *Pandanus* showed much denser root formation compared to the non-treated *Pandanus* of both soil conditions (**Figures 4.25A** and **B**).

Figure 4.25. The root structure morphology *Pandanus amaryllifolius* according to the treatment. (**A**) Root structure comparison of well-watered, drought, well-watered + OPWV, and drought + OPWV samples on day 7. (**B**) Root structure comparison of well-watered, drought, well-watered + OPWV, and drought + OPWV samples on day 10.

The root length of all range sizes was significantly increased in drought-stressed plants after 7 and 10 days of drought stress compared to the well-watered sample (**Figures 4.26A, C** and **E**). Only the small root (Range 1) of OPWV-treated drought-stressed *Pandanus* showed an increment in length during early drought stress (7-day) but decreased on day 10. However, the root length of medium and large size roots (Ranges 2 and 3) of OPWV-treated drought-stressed plants significantly increased on day 10 of drought (**Figures 4.26A, C** and **E**). Interestingly, the OPWV-treated well-watered plant consistently exhibited enhanced root length of all root sizes on 10-day drought and both range 1 and 2 for 7-day drought-stress (**Figures 4.26A, C** and **E**).

In contrast, drought-stressed *Pandanus* only showed improvement in the root surface area of range 2 root size, while the surface area of range 3 root size was reduced significantly on day 7 compared to the well-watered plant (**Figures 4.26D** and **F)**. Furthermore, the OPWV-treated drought-stressed *Pandanus* showed a surface area reduction of the range 2 root size on day 7 but enhanced on day 10 (**Figure 4.26D**). Intriguingly, the large root size (range 3) increased significantly in the OPWV-treated drought-stressed *Pandanus* on both 7-day and 10-day drought (**Figure 4.26F**). Nevertheless, OPWV-treated well-watered *Pandanus* demonstrates enhanced root surface area on both time points (**Figures 4.26B, D,** and **F**).

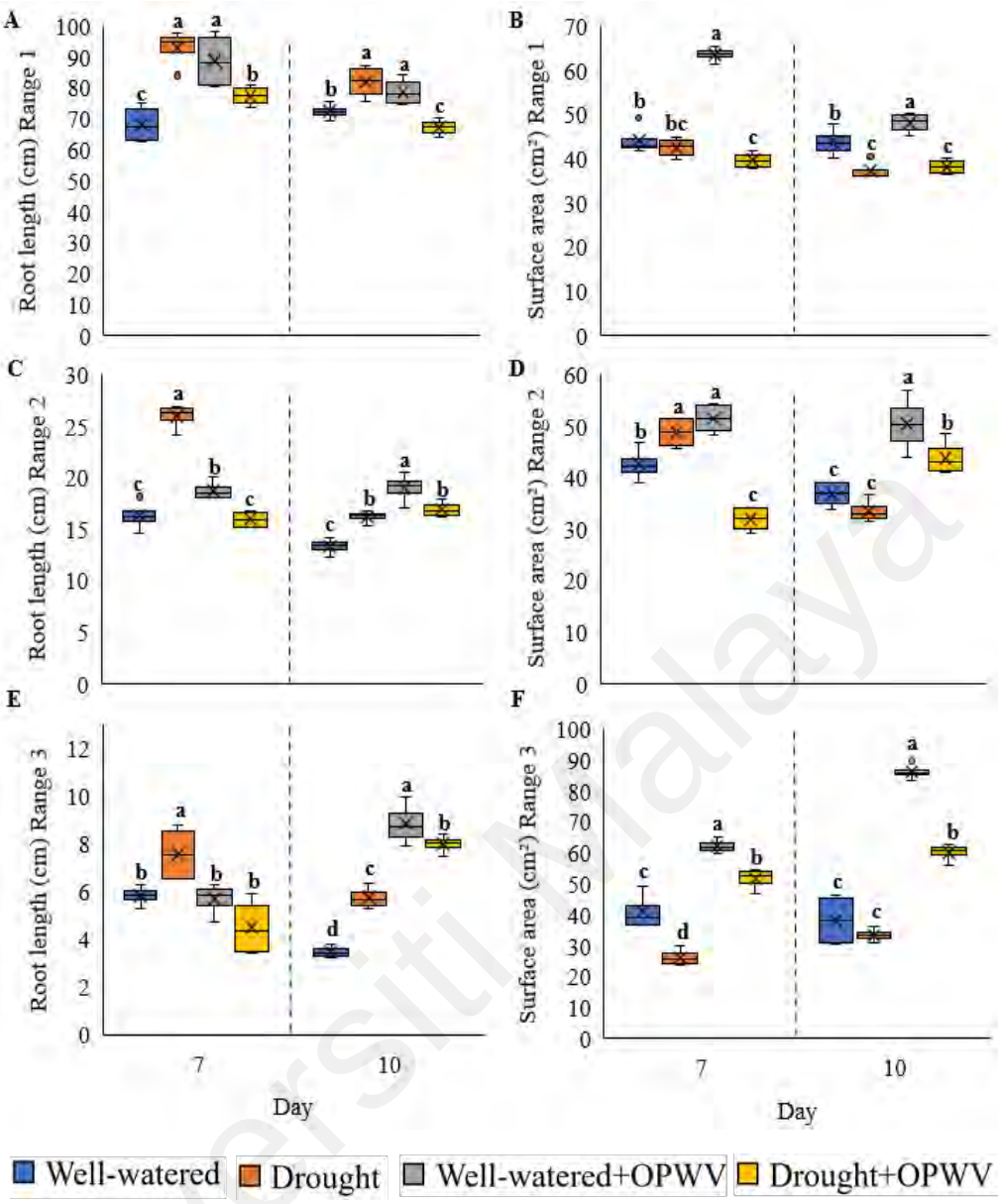

Figure 4.26. The root structure of *Pandanus amaryllifolius* was analysed according to the root diameter sizes which categorised into Range 1: 0-0.53 mm, Range 2: 0.53-1.53 mm and Range 3: more than 1.53 mm. (**A**) Root length range 1. (**B**) Root length range 2. (**C**) Root length range 3. (**D**) Surface area range 1. (**E**) Surface area range 2. (**F**) Surface area range 3. The letter labelled on the mean value indicates the significant level between treatments based on the ANOVA, followed by the post hoc Tukey test when its *p*-value < 0.05.

4.10 Hydrogen peroxide, osmolyte, and malondialdehyde of OPWV-treated *P. amaryllifolius*

The drought-stressed plants showed increased H_2O_2, proline and MDA contents (**Figures 4.27A-C**). However, OPWV application reduced the H_2O_2, proline and MDA contents on days 7 and 10 (**Figures 4.27A-C**). Surprisingly, well-watered plants treated with OPWV showed significantly increased H_2O_2, proline and MDA contents after day 7 of application.

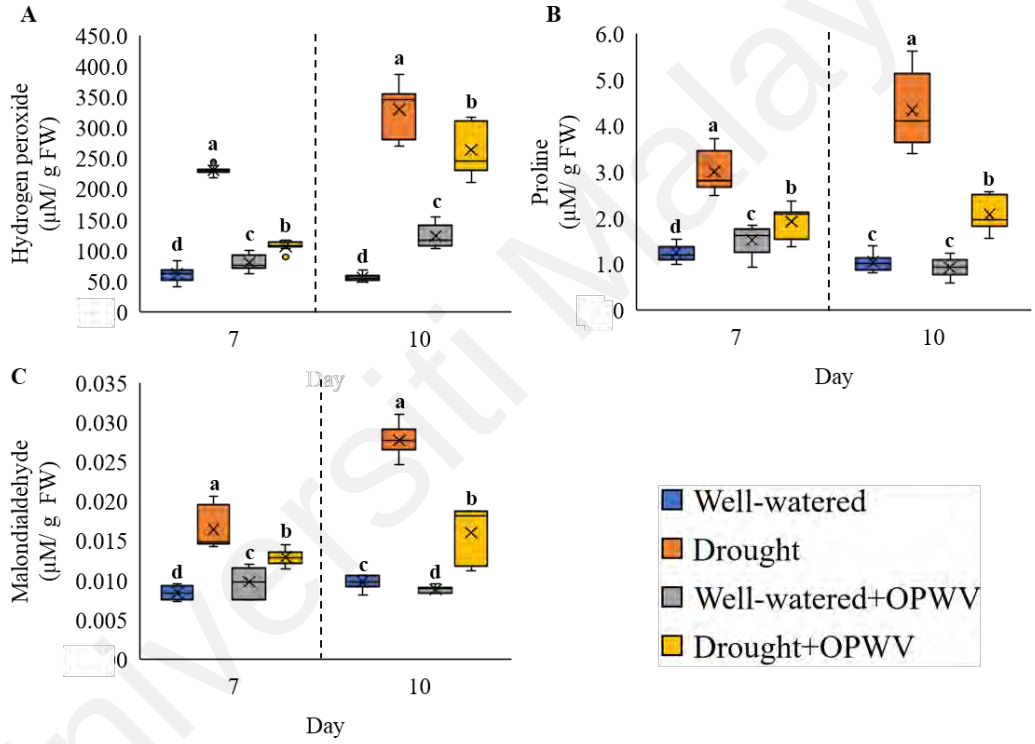

Figure 4.27. The accumulation of stress-related marker compounds in the OPWV-treated samples under well-watered and drought stress for 7- and 10-days. (**A**) Hydrogen peroxide (H_2O_2). (**B**) Proline. (**C**) Malondialdehyde (MDA). The letter labelled on the mean value indicates the significant level between treatments based on the ANOVA, followed by the post hoc Tukey test when its p-value < 0.05.

4.11 Antioxidant enzyme altered in *Pandanus amaryllifolius* in response to OPWV and drought stress

The OPWV application generally reduced the activity of the SOD, CAT, POD, APX and GR in drought-stressed plants (**Figures 4.28A-E**), except for SOD on day 10. Besides, applying OPWV to well-watered *Pandanus* plants increased the SOD, APX and GR activities for both days 7 and 10 (**Figures 4.28A**, **D**, &**E**).

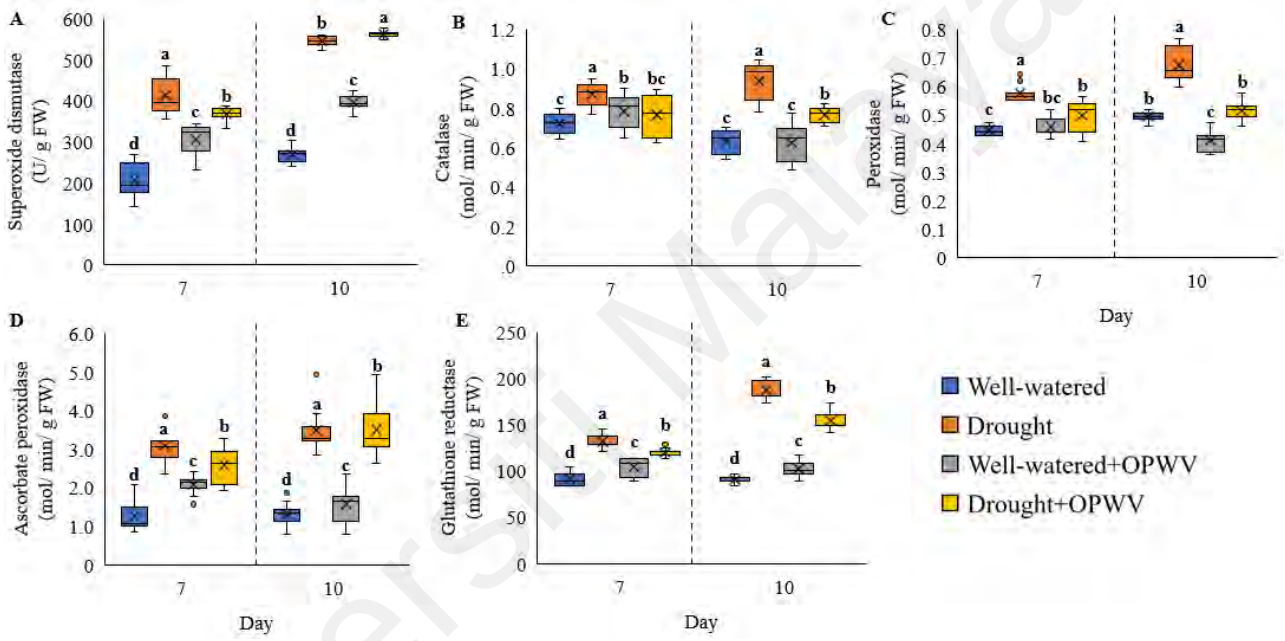

Figure 4.28. The antioxidant enzyme activities of the *Pandanus amaryllifolius* treated with or without 1:500 OPWV dilution at F2 frequency under well-watered and drought stress conditions for 7 and 10 days. (**A**) Superoxide dismutase (SOD). (**B**) Catalase (CAT). (**C**) Peroxidase (POD). (**D**) Ascorbate peroxidase (APX) and (**E**) Glutathione reductase (GR). The letter labelled on the mean value indicates the significant level between treatments based on the ANOVA, followed by the post hoc Tukey test when its p-value < 0.05.

4.12 Drought-responsive genes were enhanced with OPWV application

The total RNA of the leaf samples was extracted. The DNase-treated RNAs were quantified using a spectrophotometer and separated using gel electrophoresis. All RNA samples showed A260/280 and A260/230 values of more than 2, indicating the RNA is of good quality and integrity (**Table 4.4**; **Figure 4.29**).

Table 4.4. The RNA quantity and quality of Well-watered (WW), Drought (D), Well-watered + OPWV (WWT), and Drought + OPWV (DT).

	A260/280	**A260/230**	**ng/µL**
7WW1	2.103	2.404	848
7WW2	2.038	2.415	566
7WW3	2.053	2.411	1009
7D1	2.060	2.489	770
7D2	2.042	2.306	681
7D3	2.085	2.416	978
7WWT1	2.087	2.441	750
7WWT2	2.051	2.351	698
7WWT3	2.047	1.878	988
7D1	2.047	2.280	1074
7D2	2.071	2.422	867
7D3	2.080	2.353	585

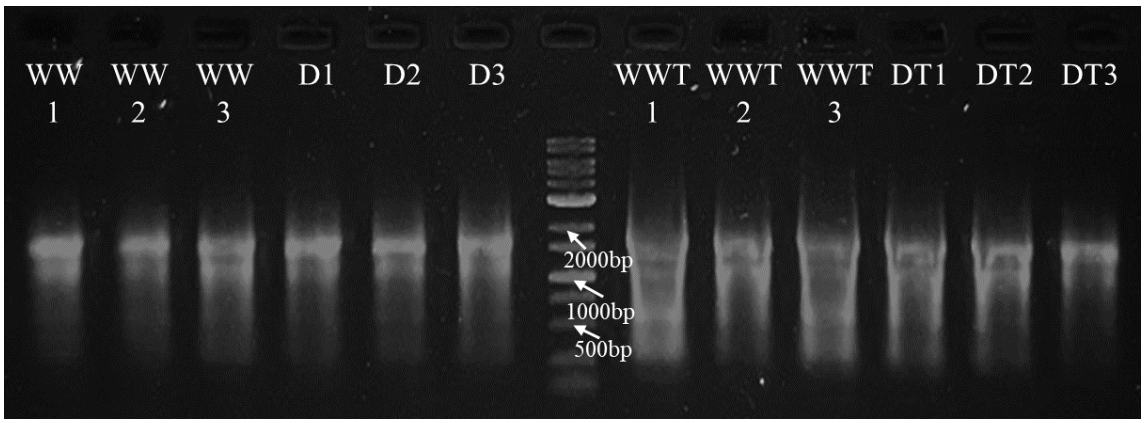

Figure 4.29. The quantity and quality of DNase-treated RNA extracted from the 7-day *Pandanus amaryllifolius* leaf samples. Well-watered (WW), Drought (D), Well-watered + OPWV (WWT), and Drought + OPWV (DT).

The expression of drought-responsive genes in well-watered and drought-stressed *Pandanus* plants treated with or without OPWV was analysed. In drought-stressed plants, the expression of *PaHSP70*, *PaGAPDH, PaENO*, *PaThau*, and *Paβ-Fruc* genes was significantly enhanced (**Figure 4.30**). Applying OPWV to drought-stressed plants elevated *PaHSP70*, *PaGAPDH,* and *PaThau* expressions (**Figures 4.30A, B,** and **D**). In contrast, the *PaENO* and *Paβ-Fruc* gene expressions of the drought-stressed *Pandanus* were significantly reduced after OPWV treatment (**Figures 4.30C** and **D**). The *GAPDH* and *Thau* gene expressions were upregulated, while *ENO* and *β-Fruc* were downregulated after 7 days of OPWV application (**Figures 4.30B, C, D** and **E**).

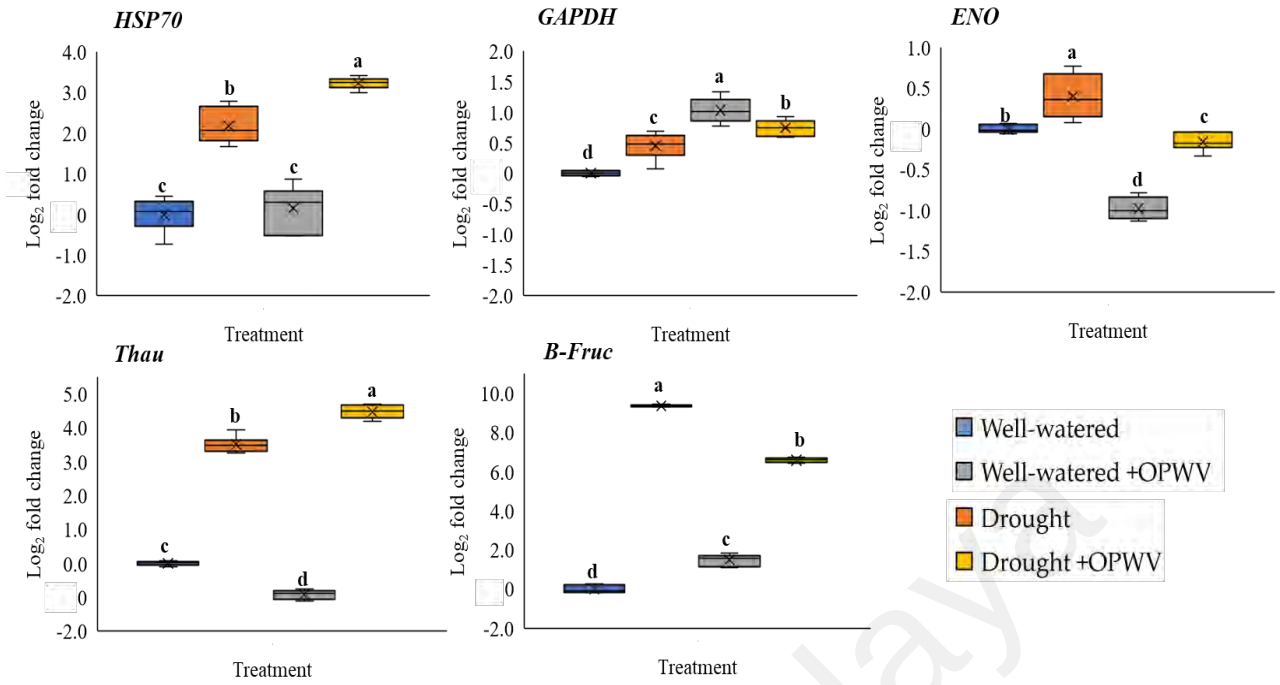

Figure 4.30. The effects of OPWV treatment on the drought-responsive gene expression of 7-day *Pandanus amaryllifolius* samples under well-watered and drought-stress conditions. (**A**) *Heat shock 70* (*PaHSP70*). (**B**) *Glyceraldehyde-3-phosphate dehydrogenase* (*PaGAPDH*). (**C**) *Enolase* (*PaENO*). (**D**) *Thaumatin* (*PaThau*). (**E**) *β-fructofuranosidase* (*Paβ-Fruc*). *Actin* and *elongation factor 1* of *P. amaryllifolius* were used as housekeeping genes for expression normalisation. The letter labelled on the mean value indicates the significant level between treatments based on the ANOVA, followed by the post hoc Tukey test when its p-value < 0.05.

4.13 OPWV GC-MS profile

The OPWV profile was analysed using GC-MS analysis. Overall, 26 peaks were identified from the chromatogram, including the internal standard (pyridine) (**Appendix 4**). These peaks were analysed and calculated in ppm/mL. As shown in **Table 4.5**, the major components of OPWV were phenyl carbamate or anthranilic acid (64%), followed by phenol (16%), guaiacol (4%), syringol (3%), nonamethylcyclopentasiloxane (2%), and tetrasiloxane (2%).

Table 4.5. The summarised identified compound of oil palm wood vinegar (OPWV). The asterisk (*) indicates the internal standard spiked prior to injection.

No.	Identified Compound	Molecular Formula	Retention Time (RT)	m/z	Concentration ppm/mL
1	Phenyl carbamate	$C_7H_7NO_2$	7.111	94.15	6.81
2	Phenol	C_6H_6O	6.868	94.05	1.73
3*	Pyridine, 2,4,6-trimethyl-	$C_8H_{11}N$	7.191	121.05	1.00
4	Guaiacol	$C_7H_8O_2$	9.079	109.05	0.47
5	Syringol	$C_8H_{10}O_3$	13.856	154.05	0.31
6	2-(2',4',4',6',6',8',8'-Heptamethyltetrasiloxan-2'-yloxy)-2,4,4,6,6,8,8,10,10-nonamethylcyclopentasiloxane	$C_{16}H_{48}O_{10}Si_9$	19.103	73.1	0.26
7	3-Isopropoxy-1,1,1,7,7,7-hexamethyl-3,5,5-tris (trimethylsiloxy) tetrasiloxane	$C_{18}H_{52}O_7Si_7$	23.366	73.1	0.24
8	Catechol	$C_6H_6O_2$	11.458	110.05	0.15
9	3-Oxabicyclo [3.3.0] oct-7-en-2-one, 4-methoxy-	$C_9H_{12}O_3$	8.946	109.05	0.13
10	o-Creosol	C_7H_8O	8.271	108.05	0.11
11	1,2-Cyclopentanedione, 3-methyl-	$C_6H_8O_2$	8.011	112.05	0.11
12	1,2,4-Trimethoxybenzene	$C_9H_{12}O_3$	15.875	168.1	0.08
13	4-Ethylguaiacol	$C_9H_{12}O_2$	12.918	137.05	0.07
14	3-Methoxyycatechol	$C_7H_8O_3$	12.644	140.05	0.07
15	Methoxyacetylene	$C_{10}H_{14}O_3$	17.18	167.1	0.03
16	Creosol	$C_8H_{10}O_2$	10.938	123	0.03
17	Methyl palmitate	$C_{17}H_{34}O_2$	22.761	74.1	0.02

CHAPTER 5

DISCUSSION

5.1 Drought stress implication to the morpho-physiology of *Pandanus amaryllifolius*

5.1.1 **Biomass and root structure**

It has been well-documented that drought affects plant growth in terms of biomass and root structure. In the present study, the leaf biomass of *P. amaryllifolius* was reduced by drought stress. Recent studies have reported a reduction in leaf biomass in several plant species, such as red sage, kiwi fruit, and tobacco (García-Caparrós *et al*., 2019; Liang *et al*., 2019; Liu *et al*., 2021). During drought stress, plants experience water loss via photosynthesis and transpiration. However, limited water availability in the soil causes the plant to experience a water deficit and is unable to maintain optimum turgidity in the cell. This unfavourable condition ultimately slows down plant metabolic processes, thus hindering plant growth. Water availability is correlated with plant metabolic processes, development, and growth, as plant cells would require optimum hydration or turgidity to perform efficient cell activity (Filipović, 2020; Giménez *et al*., 2013).

The root biomass, including all root lengths of *P. amaryllifolius*, was enhanced under drought stress. However, the root surface area was unchanged. This is reflected in the improved root DW and the root-to-shoot ratio of the drought-stressed *P. amaryllifolius*, suggesting that the plants adapt to drought stress by inducing root elongation instead of increasing surface area via root branching. Similar findings were also reported. For example, root biomass in several plant species, such as tobacco, soybean, and rice, was increased when exposed to drought stress (Du *et al*., 2020; Hossen *et al*., 2022; Liu *et al*., 2021). One of the plant mechanisms used to encounter drought

stress is the extensive growth of roots when searching for water sources. It is generally accepted that during drought stress, drought-tolerant plants would exhibit root modifications, including changes in size, length, density, growth rate, and proliferation, as the main strategy for plants to tolerate water deficits. According to Poorter *et al*. (2012), drought-tolerant plants usually showed increased biomass in their root systems in response to drought stress, thus enhancing the root-to-shoot ratio and root length. In addition, a recent report has shown the ability of plants to pause root branching in the absence of water, allowing the root extension until it reaches a water source and continues branching (Mehra *et al*., 2022).

5.1.2 **Leaf relative water content, leaf folding, and stem circumference**

In a water-scarce environment, plants would experience water loss at a higher rate through photosynthesis and transpiration than the root systems, which could scavenge moisture in the soil to supply water for the plant's metabolic processes. In the present study, drought-stressed *P. amaryllifolius* plants showed a significant reduction in LRWC, relative stem circumference, and a high percentage of severe leaf folding compared to well-watered plants. These findings are consistent with other studies on different plant species, such as maize and soybean (Sacita *et al*., 2018; Xu *et al*., 2018; Zhou *et al*., 2020). Besides observing reduced stem circumference, stem shrinkage in chestnut plants has also been reported when exposed to drought conditions (Knipfer *et al*., 2019). Other observations, such as reduced branch diameter, has been reported in drought-stressed lavender plants (Lamacque *et al*., 2020).

Water loss affects the LRWC of cells and their cell wall extensibility by either relaxation or tightening (Moore *et al*., 2008), causing plant cells to lose turgidity and become flaccid. Furthermore, a reduction in cell turgor pressure compromises the

mechanical structure of plants (Kumar *et al*., 2018). This condition ultimately led to visible drought stress symptoms in leaf morphology, such as folding/rolling and wilting. Leaf folding/rolling occurs when the leaf epidermal and bulliform cells experience changes in water potential (Ali *et al*., 2022). The purpose of this modification is to reduce the rate of transpiration and allow the photosynthetic process to be more efficient under water deficit conditions (Kadioglu & Terzi, 2007). Moreover, extensive damage to plants can be observed through stem circumference measurements. Water is predominantly stored in the stem or, specifically, in the xylem. Water release from the xylem depends on the water potential created by transpiration, which induces shrinkage of the stem (Skelton, 2019).

5.1.3 **Leaf yellowing and pigmentation**

Generally, plant metabolic processes and growth require energy and is the building block for photosynthesis. However, its machinery components, such as chloroplasts, are highly susceptible to environmental stresses, such as drought. Because chlorophyll pigments are damaged by drought stress, the plant photosynthesis rate is significantly depleted and lead to leaf senescence (Adiba *et al*., 2021; Zahedi *et al*., 2022). The earliest visible sign of leaf senescence is commonly acknowledged to be leaf yellowing, which occurs due to the degradation of pigmentation, predominantly chlorophyll. Leaf yellowing is a drought stress symptom (Seleiman *et al*., 2021), the severity of which is influenced by the stress intensity. In this study, leaf yellowing and pigmentation degradation, such as chlorophylls and carotenoids, were recorded in drought-stressed *P. amaryllifolius*. Similar results have been reported for multiple plant species when exposed to a water-deficient environment. For instance, Ramkumar *et al*. (2019) demonstrated an increase in leaf yellowing incidence and total chlorophyll

degradation in rice after 10 d of drought stress. Moreover, drought stress imposed on the Japanese spindle and wheat plants has led to the development of leaf yellowing, which reduces chlorophyll *a*, *b*, and carotenoids in the leaves (Lin *et al*., 2021; Todorova *et al*., 2022).

Naturally, leaf senescence occurs due to plant growth, ageing, and the transition from the vegetative to the reproductive stage. However, the onset and development of this process could also be influenced by other factors, such as mechanical wounding and biotic and abiotic stresses. These factors regulate endogenous signalling molecules, such as phytohormones and ROS. These signalling molecules mediate metabolic reprogramming and physiological changes, such as reduced photosynthesis, remobilisation of nutrients, degradation of pigments, and expression of senescence-associated genes (SAGs) (Buet *et al*., 2019; Zentgraf, 2019). ROS, such as H_2O_2, $^1O_2$, and O_2-, are known to be involved in the induction of plant senescence (Garapati *et al*., 2015), whereas chloroplast-produced ROS are key regulators of programmed cell death (PCD) and chlorophyll degradation (Ambastha *et al*., 2015). In general, ROS production in plant cells is attributed to energy production from mitochondrial metabolism, chloroplast electron transport chains, and peroxisomal photorespiration (Noctor & Foyer, 2016; Rogers & Munné-Bosch, 2016). Nevertheless, leaf senescence is required for nutrient remobilisation from the accumulated nutrients in the mature leaf to other parts of the plant, such as the young shoots, flowers, and roots, as an adaptive mechanism to survive stress (Munné-Bosch & Alegre, 2004). This suggests that leaf yellowing via pigmentation degradation is one of the mechanisms by which *P. amaryllifolius* adapts to a water-deficient environment. The degraded chlorophyll protects the plant from phototoxic chlorophyll intermediates and allows nitrogen recycling and other nutrients for remobilisation to other parts of *P. amaryllifolius*, such as the root, for elongation.

5.2 Stress indicator of *Pandanus amaryllifolius* changes under drought

5.2.1 **Hydrogen peroxide, relative electrolyte leakage and malondialdehyde**

Under water-deficient conditions, plants are susceptible to oxidative damage. The overaccumulation of ROS, such as H_2O_2, has detrimental effects on cells, including lipid peroxidation and electrolyte leakage. In this study, drought stress treatment of *P. amaryllifolius* resulted in elevated production of H_2O_2 and MDA and a higher percentage of REL compared to the well-watered sample. These findings are consistent with those reported for other drought-stressed plants. For example, the leaves of fang feng plants showed a significant increase in H_2O_2, MDA, and REL when subjected to drought stress for 20 days (Men *et al*., 2018). In addition, a similar trend of H_2O_2, MDA, and REL upsurge was demonstrated in pansy and blanket grass plants under drought but reduced when rewatered and allowed for recovery (Nawaz & Wang, 2020; Oraee & Tehranifar, 2020).

Under a water-deficient environment, stomatal closure reduces the CO_2-to-O_2 ratio in mesophyll cells, which elevates photorespiration and glycolate production in the chloroplast (Miller *et al*., 2008). Glycolate accumulated in cells is then oxidized in peroxisomes, which accounts for the majority of H_2O_2 production during photorespiration (Karpinski *et al*., 2003). As H_2O_2 works in a concentration-dependent manner, it acts as a signalling molecule at low concentrations but initiates cellular death at higher concentrations (Gechev & Hille, 2005). Furthermore, electrolyte leakage is related to the efflux of potassium ions (K^+) in cells mediated by plasma membrane cation conductance and counterions (Cl^-, HPO_4^{2-}, and NO_3^-) (Bajji *et al*., 2002). A high concentration of ROS activates K^+ efflux through the GORK channel, resulting in plant cells losing K^+, which, in turn stimulates endonucleases and proteases, thus promoting PCD (Demidchik *et al*., 2014). Upon ROS overaccumulation, elevated electrolyte leakage and cell damage could

be induced via lipid peroxidation of arachidonic acid and large polyunsaturated fatty acid (PUFA), producing MDA (Esterbauer *et al*., 1991). Hence, MDA is generally used as a biomarker to measure extensive damage to cells due to stress (Morales & Munné-Bosch, 2019).

It was notable that in the first experiment of drought stress on *P. amaryllifolius*, the H_2O_2 level in the plant was at the same level as the well-watered sample, whereas the H_2O_2 level of the drought-stressed *P. amaryllifolius* in the second experiment was significantly enhanced compared to the well-watered sample. This variation in the results could be one of the limitations of this study. Although the *P. amaryllifolius* plants obtained from the same source and their sizes, conditions, and ages were standardised for both experiments, the exact physiology of the plants could vary. This is because the propagation of *P. amaryllifolius* occurred through offshoot replanting. Therefore, in future investigations, the actual offshoot age before propagation should also be considered to obtain more consistent data.

5.2.2 **Proline**

In non-stress environments, proline production occurs predominantly in the cytosol. Proline biosynthesis in plants is known to occur through either the glutamate or ornithine pathways. In the glutamate pathway, proline biosynthesis from glutamate can occur in the cytoplasm or chloroplast, which involves two steps of enzymatic reactions involving pyrroline-5-carboxylate synthetase (P5CS) and pyrroline-5-carboxylate reductase (P5CR) (Sekhar *et al*., 2007). P5CS converts glutamate into pyrroline-5-carboxylate (P5C), which is further converted into the final product proline by P5CR. In the alternate pathway, proline biosynthesis occurs with ornithine deamination before the transamination process is regulated by ornithine-delta-aminotransferase (OAT) to

become P5C (Verbruggen & Hermans, 2008). However, under drought stress, the imbalance of osmotic pressure in the cell triggers proline biosynthesis to favour the glutamate proline metabolism pathway in chloroplasts instead of the ornithine pathway in the cytosol (Per *et al*., 2017). Proline is also considered as a stress indicator in cells that functions as an osmolyte and serves as a metal chelator, antioxidant, and signalling molecule (Hayat *et al*., 2012).

In this study, proline accumulation was demonstrated in the drought-stressed *P. amaryllifolius*. It was downregulated to a normal level as compared to the well-watered plants during the post-drought recovery stage. This finding is consistent with that of other plant species exposed to drought stress. For instance, sugar beet and barley plants showed increased proline build-up under drought conditions, whereby the sugar beet was treated with 75% (mild drought) and 50% (severe drought) water, whereas the barley was treated with one irrigation per growing season instead of five irrigations (Abdelaal *et al*., 2020; Ghaffari *et al*., 2019). This finding indicates the importance of proline accumulation in *P. amaryllifolius* to maintain the cell's osmotic balance, allowing cellular and metabolic processes to occur optimally under drought stress. In addition, rice and maize plants exhibit high proline content under drought stress and are significantly reduced when allowed to recover by re-watering (Dien *et al*., 2019; Voronin *et al*., 2019). During proline biosynthesis, ROS production may occur as P5C is converted, thereby inducing apoptosis and PCD (Székely *et al*., 2008). Therefore, when stress was relieved, the undue amount of proline accumulated during the stress period needed to be degraded quickly. This mechanism has been demonstrated in the post-drought recovery of *P. amaryllifolius* and other plant species.

5.3 *P. amaryllifolius* antioxidant activities under drought stress

Antioxidants play a crucial role in protecting cells from oxidative stress caused by the accumulation of ROS. Drought stress can disrupt the normal metabolism regulation in cells, leading to the overproduction of ROS, which can cause oxidative damage and, ultimately, cell death. To prevent this damage, plants have developed a complex antioxidant system that can scavenge excess ROS and neutralise them. According to Laxa *et al*. (2019), all plants could induce antioxidant systems. However, drought-tolerant plants have developed a higher capacity for antioxidant defence compared to drought-sensitive plants. This is due to their ability to accumulate higher levels of osmolytes, such as proline and glycine betaine, which can act as ROS scavengers and protect cellular structures from oxidative damage. In addition to osmolytes, drought-tolerant plants also have a higher affinity for non-protein antioxidants, such as ascorbic acid, tocopherols, and carotenoids. These compounds can act as scavengers of ROS and protect the plant from oxidative damage. Furthermore, drought-tolerant plants undergo metabolic restructuring to improve their antioxidant capacity. This involves the upregulation of genes involved in antioxidant biosynthesis and the downregulation of genes involved in ROS production. This restructuring helps to suppress ROS accumulation and protects the plant from oxidative stress.

In this investigation, drought-stressed *P. amaryllifolius* plants exhibit increased antioxidant activities, such as SOD, POD, CAT, APX and GR, indicating the plants were coping with the stress by neutralising deleterious ROS. These results correspond with other studies that showed similar enhanced antioxidant activities under a water-deficit environment. For example, milk thistle and tomato plants showed elevated activities of SOD, POD, CAT, APX and GR when exposed to drought stress (Altaf *et al*., 2022; ElSayed *et al*., 2019). This evidence suggests the importance of antioxidant enzyme

regulation in the adaptive responses of *P. amaryllifolius* in tolerating and surviving drought stress.

There is an inconsistency in the result of SOD activity, similar to the H_2O_2, as described in Section 5.2.1. The SOD activity level in the drought-stressed *P. amaryllifolius* in the first experiment was similar to the well-watered sample, while in the second experiment, drought-stressed *P. amaryllifolius* exhibited significant enhancement of SOD activity. It is hypothesised that the plant in the first experiment might be slightly matured and was able to detoxify O_2^- into H_2O_2 faster, which then efficiently converted most of the H_2O_2 into water by other antioxidant enzymes, such as APX and CAT. It was known that APX has a higher affinity to reduce H_2O_2 predominantly by utilising ascorbate as electron in donor apoplastic spaces, chloroplast, cytosol, peroxisomes, and mitochondria (Sofo *et al*., 2015; Tiwari *et al*., 2017). However, the antioxidant affinity in the *P. amaryllifolius* is still unclear and would require a thorough investigation.

5.4 Protein altered in *Pandanus amaryllifolius* during drought and recovery

Plants respond to drought stress conditions by changing regulatory circuits in transcription and protein expression, reorganising metabolic pathways and physiological processes (Amnan *et al*., 2021). This is the first study to determine protein changes in response to drought stress in *P. amaryllifolius* using a TMT-labelled proteomics approach. Proteins involved in stress-responsive and carbohydrate metabolism were identified as the largest differentially changed protein group in this study, suggesting their essential roles in response to drought stress conditions.

5.4.1 **Carbohydrate-related proteins**

In this study, several carbohydrate-related proteins such as enolase (ENO), GAPDH, β-fructofuranosidase (β-Fruc), and sucrose phosphate synthase (SPS) were found to be highly altered in drought-stressed and post-drought recovery *P. amaryllifolius*. Upregulation of ENO and GAPDH proteins was previously reported in several plant species, such as wheat, spider flower, foxtail millet, and maize (Cheng *et al*., 2015; Pan *et al*., 2018; Yildiz *et al*., 2021; Zeng *et al*., 2019). ENO is an enzyme involved in the glycolysis process to convert 2-phosphoglycerate to phosphoenolpyruvate (Yang *et al*., 2013), While GAPDH is involved in the oxidative modification of glyceraldehyde-3-phosphate to 1,3-bisphosphoglycerate during glycolysis, the conversion of glycerate-3-phosphate to glyceraldehyde-3-phosphate (G3P) is performed by the enzyme triose phosphate isomerase (TPI) (Hildebrandt *et al*., 2015). Yu *et al*. (2017) have shown the ENO involvement in part of the upregulation of glycolysis proteins in enhancing drought tolerance in the soybean plants. On the other hand, GAPDH in *Arabidopsis* was demonstrated to improve the drought tolerance of the plant via root length enhancement and ROS suppression (Zhang *et al*., 2019).

GAPDH is a key enzyme in the glycolytic pathway that converts glyceraldehyde-3-phosphate into 1,3-bisphosphoglycerate which further processed by TPI to become G3P. It is a highly conserved enzyme in all living organisms and is crucial in energy metabolism. A recent report by Li *et al*. (2020) demonstrated the key role of the GAPDH enzyme in the response of wheat plants towards drought stress. The authors found that the TaWRKY transcription factors are induced by drought stress in wheat and found that TaWRKY20 and TaWRKY93 bind specifically to the promoter of the *GAPC5* gene, which encodes a GAPDH enzyme involved in energy metabolism. The expression level of *TaGAPC5* varied significantly among the drought-tolerant wheat cultivars, and the promoter activities were significantly improved. This finding suggests the interaction

between WRKY transcription factor with energy metabolism via GAPDH regulation. The functionality of GAPDH in conferring tolerance in the plant could also be observed in other abiotic stress, such as salinity stress. For instance, Lim *et al*. (2021) demonstrated an enhanced salinity tolerance of transgenic rice plants overexpressing *PsGAPDH* cloned from a salt-tolerant halophyte, *Puccinellia tenuiflora*. The transgenic rice seedling growth and germination rates were improved significantly compared to the wild-type plants, indicating their enhanced tolerance towards saline conditions. Further transcriptome profiling analysis revealed 1124 differentially expressed transcripts in the leaves of the *PsGAPDH*-overexpressing rice under salt stress conditions are mainly involved in starch and sucrose metabolism. This evidence evinces the importance of GAPDH protein in providing *P. amaryllifolius* with more energy to tolerate and recover from drought stress.

The protein β-Fruc, also known as invertase, has been identified as playing a crucial role in the response of plants to drought stress. In fact, it has been reported that the β-Fruc protein is highly upregulated in alfalfa when exposed to severe drought stress (Ma *et al*., 2021). This highlights the significance of invertase in enabling plants to adapt to harsh environmental conditions. In recent years, researchers have also turned their attention to the role of β-Fruc in the potato response to drought stress. In a genome-wide study, Abbas *et al*. (2022) investigated the expression of *β-Fruc* genes in potato plants under drought stress conditions. The study revealed that under drought stress, the abundance of β-Fruc was significantly upregulated, particularly in the vacuole and cell wall which suggests that β-Fruc plays a critical role in the potato's response to drought stress by facilitating the storage and mobilization of sugars. The findings of this study are particularly significant, as they shed light on the importance of β-Fruc in potato plants' ability to tolerate drought stress. By increasing our understanding of the role of invertase in plant adaptation to drought stress, we may be able to develop strategies to improve crop resilience to environmental stresses. This suggests the importance of the β-Fruc in *P.*

amaryllifolius to tolerate drought stress. By uncovering the significance of β-Fruc in plant response to drought stress, we can develop strategies to improve crop resilience to environmental stresses and pave the way for more sustainable agriculture.

The protein SPS is a vital component in the category of proteins that help plants cope with drought stress. SPS is responsible for catalysing the conversion of uridine diphosphate-glucose and fructose 6-phosphate into sucrose, which can provide additional energy to cells during periods of water scarcity. Research studies have shown that the abundance of SPS changes in response to drought stress in various plant species. One such study conducted by Nemati *et al*. (2019) found that SPS abundance in wheat seedlings increased after exposure to drought stress. Additionally, other studies have reported that drought treatment markedly increases SPS activity in different plant species, including apple and rice (La *et al*., 2019; Yang *et al*., 2019). These findings suggest that SPS plays a crucial role in plants' ability to cope with drought stress by providing extra energy to cells. In *P. amaryllifolius*, alterations in carbohydrate-related proteins, including SPS, are crucial for the plant's survival under drought stress. The plant requires more energy to adapt, survive, and recover from drought stress, and SPS is one of the proteins that can provide this energy. Therefore, the alteration of these carbohydrate-related proteins in *P. amaryllifolius* is significant to ensure the plant can acquire the required energy to withstand, adapt, survive, and recover from drought stress.

5.4.2 **Drought-responsive proteins**

In the present study, the abundance of heat shock proteins 70 (HSP70), thaumatin (Thau), and polycystin, lipoxygenase, alpha-toxin, and triacylglycerol lipase (PLAT) proteins were highly altered in drought-stressed samples compared to well-watered treatments.

Drought stress can impact both the amount and quality of plant proteins. When faced with environmental stressors, plants may generate increased amounts of ribosomal and chaperone proteins, which are crucial in maintaining proper protein synthesis and structure. Additionally, proteins such as HSPs can aid in breaking down misfolded proteins and assisting in stress adaptation. HSPs are well-established as molecular chaperones that help in the maintenance of proper protein folding, which prevents the aggregation of proteins (Nagaraju *et al*., 2020). Cell survivability under a stressful environment depends on the efficiency of protein folding and disaggregation. Several HSP70 gene families have been reported to be involved in the drought stress response in various plant species. For example, a recent proteomic study on liquorice root revealed the HSP70 protein role in protein refolding, stabilisation, and signal transduction (Zhang *et al*., 2022). In addition, HSP70 proteins in peanuts increased in abundance in response to drought (Carmo *et al*., 2019). The ability of plants to express a high level of HSPs confers to their tolerance against abiotic stress, particularly drought and heat stress. A recent study by Ni *et al*. (2021) has shown the importance of the HSP70 protein coded by the *GhHSP70-26* gene was significantly induced in cotton leaves and roots under drought stress. Overexpressing the *GhHSP70-26* gene in tobacco plants showed improved tolerance to drought stress compared to wild-type plants. The transgenic plants had lower leaf wilting, higher survival rates, lower leaf water loss, as well as increased root length and chlorophyll content. They also had higher proline contents, SOD and POD enzyme activities, and lower MDA and ROS contents than wild-type plants. This evidence shows the importance of the HSP70 in alleviating the deleterious effects of drought stress and aiding the plants to adapt to such conditions.

A pathogenicity-related (PR) protein of the PR5 family, thaumatin-like protein (TLP), was first described to be involved in biotic stress response of plants (Jung *et al*., 2005). Nonetheless, several reports have shown another function of TLP particularly in

enhancing plant tolerance against different abiotic stresses, such as salt and drought stress, via the maintenance of lower Na^+ and higher K^+ levels in plant cells (Munis *et al*., 2010). Misra *et al*. (2016) also reported the elevated expression of *TLP* in basil plants that exhibited tolerance against fungal, salinity, and drought stress. Furthermore, specific induction of *TLP* expression was reported in the transcriptomic analysis of drought-stressed Aleppo pine (Fox *et al*., 2018). The induction of thaumatin proteins during drought stress has also been documented in several plant species. For instance, the constitutive expression of *TLP* improves the yield and drought tolerance in *Arabidopsis*, while *BoTLP1* was found to be regulating the salt and drought tolerance of broccoli (He *et al*., 2021; Muoki *et al*., 2021). In this study, TLP was downregulated in drought-stressed *P. amaryllifolius* but significantly upregulated in the recovered samples. Although this finding contrasts with other studies, it suggests that TLP might be involved in the recovery process. Nonetheless, the exact mechanism of TLP that confers drought tolerance in *P. amaryllifolius* is still unclear and requires further investigation.

Plants have the ability to respond to various abiotic stresses, such as drought, salt, and cold, by inducing specific proteins that play a crucial role in stress signalling and tolerance. Among these proteins, PLAT domain proteins have been shown to be induced by the stress hormone ABA, which mediates stress responses in plants. These proteins bind to basic leucine zipper (bZIP) transcription factors ABA-Responsive Element Binding Protein/ABA-Binding Factors (AREB/ABFs), which in turn activate stress-responsive genes. In a recent study, PLAT was found to be abundantly accumulated in response to abiotic stress in plants. Interestingly, this protein was also observed to increase in salinity-stressed quinoa, indicating its potential role in multiple stress responses. Additionally, a study by Hyun *et al*. (2014) investigated the function of *Arabidopsis* PLAT, *AtPLAT1*, in tobacco. The authors found that its expression promoted growth and increased stress tolerance towards cold, drought, and salt (Hyun *et al*., 2014).

Taken together, these findings suggest that PLAT proteins are key players in the drought stress-responsive mechanisms of *P. amaryllifolius* and may serve as potential targets for improving stress tolerance in crops. However, despite these findings, the precise role of PLAT proteins in the *P. amaryllifolius* drought stress response remains unclear and requires further exploration.

5.4.3 **Cell-membrane related proteins**

During the investigation, it was observed that *P. amaryllifolius* plants exposed to drought stress showed an increase in the quantity of networked (NET) actin-binding proteins. These proteins are thought to have a vital function in preserving the structural stability of plant cells. NET proteins have been implicated in various other cellular processes. For instance, they are involved in cytokinesis, cell expansion, endocytosis, and vesicle trafficking (Hawkins *et al*., 2014). The precise mechanism of how NET proteins are induced in response to drought stress is still not completely understood. However, it is thought that the activation of specific signalling pathways in response to drought stress may be responsible for the increased expression of NET genes (Li *et al*., 2019). Furthermore, it has been suggested that the accumulation of NET proteins under stress conditions may help to reinforce the actin cytoskeleton, thereby enhancing the mechanical strength of the cell wall (Hawkins *et al*., 2014). Interestingly, a recent study showed that NET proteins might play a role in the regulation of ion channels and transporters. For example, NET proteins were found to interact with the KAT1 potassium channel in *Arabidopsis*, suggesting that they may be involved in the regulation of ion transport in response to environmental stresses (Li *et al*., 2015). Overall, the high abundance of NET proteins in drought-stressed *P. amaryllifolius* indicates their critical role in maintaining

cell integrity and function during drought stress. Nevertheless, further research is required to fully understand the underlying molecular mechanisms.

Alpha-L-arabinofuranosidase (ASD) is an important cell wall protein crucial in catalysing the hydrolysis of L-arabinofuranosidic bonds present in L-arabinose-containing hemicelluloses (Saha, 2000). These hemicelluloses are important components of the plant cell wall and play a vital role in maintaining its structural integrity. Recent studies have shown that ASD1 protein levels increase in response to drought stress. This finding suggests that ASD may play a critical role in the response of plants to drought stress. In particular, studies in maize (Zhu *et al*., 2007) and maritime pine (López-Hinojosa *et al*., 2021) have demonstrated the role of ASD in drought stress. These studies indicate that the increased expression of ASD1 in response to drought stress may be a key mechanism by which plants cope with water deficit conditions. By breaking down the hemicellulose components of the cell wall, ASD1 may help plants to maintain their cellular integrity and survive under drought-stress conditions. Based on this evidence, the high accumulation of ASD protein in *P. amaryllifolius* in response to drought stress suggests its crucial role in stress-responsive mechanisms. The findings from this study might be useful for crop improvement programmes to develop climate-resilient crops.

The carbohydrate-related (Enolase, GAPDH, B-fruc, and SPS), drought-responsive (HSP70, thaumatin, and PLAT), and cell-membrane related (NET and ASD) proteins could be used as protein markers to identify drought tolerance in plants and may further assist plant biologists in selecting desired genes to develop new drought-tolerant varieties.

5.5 *Pandanus amaryllifolius* carbohydrate-related and drought-responsive gene expression under drought and recovery

Based on the proteomic analysis, several abundantly altered proteins related to carbohydrate metabolism and drought response were selected for gene expression analysis. In order to survive under stress, plants would respond to stress by activating their stress response and carbohydrate-related genes. The expression of *PaGAPDH*, *PaENO* and *Paβ-Fruc* (carbohydrate-related) and *PaHSP70* and *PaThau* (drought-responsive) were determined. Under drought stress, the expressions of *PaHSP70*, *PaENO*, *PaThau, PaGAPDH,* and *Paβ-Fruc* in *P. amaryllifolius* were significantly enhanced compared to well-watered plants. These findings were in consensus with previous studies.

Carbohydrates have been identified to have multiple crucial roles in the response of plants to drought stress. It plays several roles in drought stress response such as energy production, osmoregulation, and osmoprotectant (Figueroa & Lunn, 2016). Moreover, it is involved in the production of secondary metabolites, such as flavonoids, stilbenes, and lignin (Jeandet *et al*., 2021). These metabolites have been shown to have antioxidant and antimicrobial properties and play a crucial role in plant defence against abiotic stresses. Carbohydrates are essential as an energy source for metabolic reactions and pathways within cells. They are crucial for maintaining cellular homeostasis and respiration, even under drought stress conditions, and also serve as signalling molecules. Additionally, carbohydrates play a significant role in abiotic stress responses and in the regulation of other stress-responsive genes (Price *et al.*, 2014).

The overexpression of the *GAPDH* gene in wheat and *Arabidopsis* has significantly improved the plant's tolerance against drought stress (Zhang *et al*., 2019). The study aimed to investigate the role of the *TaWRKY40* transcription factor in

regulating the expression of the *TaGAPC1* gene, which encodes the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) enzyme, and its contribution to drought tolerance in wheat. They found that *TaWRKY40* was upregulated by drought stress, and its overexpression in wheat enhanced drought tolerance, as indicated by improved growth performance and reduced water loss. The study also demonstrated that *TaWRKY40* directly binds to the promoter region of the *TaGAPC1* gene and activates its expression. Of which, the overexpression of *TaGAPC1* in wheat increased GAPDH enzyme activity and enhanced drought tolerance.

ENO2 gene-encoded MBP-1-like protein plays an important role in regulating the response of *Arabidopsis thaliana* to drought and salt stresses (Liu *et al*., 2020). When exogenously applying AtMBP-1 (a protein coded by *AtENO2*) to *A. thaliana*, the *eno2*-mutant plants (*ENO2* knockdown) showed increased drought and salt tolerance, although it was not able to completely restore the phenotype of *eno2*- to that of the wild-type (Liu *et al*., 2020). These findings suggest that *AtMBP-1* plays a key role in the tolerance of plants to abiotic stresses, providing additional evidence of the critical function of *AtENO2* in the growth and development of plants. Similarly, the increased *ENO* gene expression in *P. amaryllifolius* in response to drought stress suggests its importance in regulating carbohydrate metabolism to provide more energy to plants during stress.

Invertase enzymes or β-Fructofuranosidase play a vital role in regulating sugar metabolism, especially when plants are exposed to stresses. For example, Dahro *et al*. (2016) reported that 14 *β-Fructofuranosidase* or *Invertase* genes in the trifoliate orange were highly upregulated when exposed to different abiotic stress conditions, such as drought, salt, and cold stress. These include seven alkaline/neutral INV genes (*PtrA/NINV1–7*), two vacuolar INV genes (*PtrVINV1–2*), and five cell wall INV isoforms (*PtrCWINV1–5*) (Dahro *et al*., 2016). Besides that, several key genes, including *PtCWINV5* and *PtLIN6,* were highly upregulated in response to the imposed abiotic

stresses (Dahro *et al*., 2016). Additionally, the authors found that the expression of these genes was positively correlated with the activity of invertase enzymes, suggesting that they play a role in regulating sugar metabolism under abiotic stress conditions.

The expression of drought-responsive genes is essential for the synthesis of functional proteins that aid in stress tolerance, including osmolytes, water channels, and ROS detoxification enzymes. Additionally, drought-responsive genes regulate signal transduction pathways through the production of proteins such as transcription factors, protein kinases, and ABA biosynthesis enzymes (Shinozaki & Yamaguchi-Shinozaki, 2007). For instance, the expression of the *HSP* gene in drought-tolerant sugarcane was elevated when treated with drought stress (Selvi *et al*., 2020). Apart from its known function as a chaperone to maintain protein functional configuration, *HSP* was reported to be the key modulator for the formation of interlocking marginal lobes (IMLs) that are associated with plant growth which is regulated by auxin-binding protein, Rho GTPases and actin (Augustine *et al*., 2015). Davoudi *et al*. (2022) showed that the *HSP70* gene family in pumpkin (*Cucurbita moschata*) rootstock is essential in response to drought stress. About 21 HSP70 in pumpkins were identified, mostly located in different cellular compartments, including the cytoplasm, chloroplast, mitochondria, nucleus, and endoplasmic reticulum (ER). The authors found that those *HSP70* genes contain different cis-regulatory elements, including stress-responsive and hormone elements, suggesting that the *HSP70* gene family might have a potential role in stress tolerance.

TLP has also been reported to be involved in plant abiotic responses, such as salinity, osmotic, drought and cold (Ahmed *et al.*, 2013). A recent study by Hasan *et al*. (2019) reported that the *thau* gene was highly expressed in drought-tolerant cotton, while the drought-sensitive cotton exhibited downregulation of the *thau* gene under drought stress. Another study by He *et al*. (2021) showed that the *BolTLP1* gene is essential in conferring tolerance to salt and drought stresses in broccoli plants, as its expression was

upregulated under such conditions. This finding was further confirmed when overexpressing *BolTLP1* in broccoli plants. He *et al*. (2021) found increased tolerance to salt and drought stresses in *BolTLP1*-expressing broccoli plants. In addition, the *BolTLP1*-overexpressing plants showed higher survival rates, increased biomass, and improved physiological characteristics compared to control plants under salt and drought stress conditions. On the other hand, when analysing *TLP* gene expression in wheat, Sharma *et al*. (2022) found that several *TLP* genes were upregulated in response to drought, salt and heat stress conditions, indicating their potential roles in stress response. These TLPs may be involved in signalling pathways, such as MAPK and CDPK pathways, and in the regulation of gene expression. Sharma *et al*. (2022) speculated that TLPs might interact with other proteins, such as chitinases and peroxidases, to enhance stress tolerance in bread wheat. Nonetheless, the mechanism of TLP in modulating drought tolerance response in the plant remains unclear.

Taken together, this evidence demonstrates the importance of *GAPDH*, *ENO*, *β-Fruc*, *HSP70*, and *Thau* genes in *P. amaryllifolius* to trigger adaptive responses during drought stress.

5.6 Performance of optimum OPWV concentration and application frequency

In this study, the 1:500 dilution of OPWV and application frequency of 3-day intervals in 12 days period were determined to be the optimum factors that gave the best morphological effects on the *P. amaryllifolius* under well-watered conditions. Based on the current literature, this is the first reported optimisation of OPWV for foliar application on the plant. Of the 1:100, 1:250, 1:500, and 1:1000 dilution factors applied, 1:500 dilution enhanced the stem growth and biomass DW of *P. amaryllifolius*. Moreover, the

3-day intervals (F2) application frequency exhibits similar enhancement of the stem growth and biomass DW compared with the 6-day intervals (F1) and 1-day intervals (F3) application. It was notable that the F3 application showed lower accumulation of the chlorophyll content despite showing improved stem growth and biomass DW.

Several research studying on a variety of WV have demonstrated optimisation of concentration and application frequency. For instance, 1:50 and 1:500 dilution applied for seed priming was shown to have the optimum effects on pepper and tomato plants, such as root and shoot length and biomass, when compared to the other concentrations (Luo *et al*., 2019). In addition, wheat seed priming with a 1:900 dilution of WV demonstrated the highest enhancement of seedling root and shoot biomass compared to other dilutions ranging from 1:300 to 1:1500 (Wang *et al*., 2019). In comparison, the application of a higher concentration of WV was reported to inflict negative effects on plant performance. For example, Liu *et al*. (2021) reported severe damage to the annual bluegrass weed when 25% to 100% concentration of apple branches WV was applied, while the less concentrated (less than 12.5%) apple branches WV showed less harmful effects on the plant. However, these effects could be varied depending on the volume applied to the subjects. Similarly, high concentrations of pine WV were utilised as a herbicide against nitrophilous plant communities, whereby 25% concentration was sufficient to reduce about 70% of the plant biomass (Aguirre *et al*., 2020).

This evidence suggests the optimum concentration of WV to be applied to plants depends on the subject or objective of application, whereby the diluted or less concentrated WV would give the optimal effects when utilised for seed priming. In addition, the optimal concentration of WV would improve the plant's morpho-physiology more effectively. In contrast, due to its potent phenolic content, a high concentration of WV is best used as a pesticide, fungicide, and herbicide. Furthermore, the higher the application frequency, the higher the WV dosage would accumulate in the plant system,

which would be harmful or toxic to the plant. Therefore, based on this evidence, the synergistic relation between WV concentration and application frequency must be considered when treating the plants.

5.7 OPWV alleviate drought stress on *P. amaryllifolius* morpho-physiology

5.7.1 **Leaf and root biomass**

Plant growth and development are susceptible to drought stress and would adapt to this unfavourable condition by altering their morphology, biochemical and molecular mechanism. In this study, drought stress has been shown to negatively affect the morpho-physiology of *P. amaryllifolius*, such as biomass, leaf structure, and pigmentation. Intriguingly, OPWV-treated drought-stressed *P. amaryllifolius* exhibit significant improvement in the root length and surface area of the large root size. Low soil moisture increased the hardness of the soil texture, which could be one of the factors that caused the improvement in size, specifically in the large root of *P. amaryllifolius*. In addition, root elongation and branching have been established as plant adaptations to tolerate drought stress (Fenta *et al*., 2014; Jochum *et al*., 2019). This phenomenon is known as hydropatterning, or a condition allowing the plant's lateral roots to branch towards the high-water availability region and preventing the root emergence in the low-water availability region (Robbins & Dinneny, 2018).

Interestingly, the supplementation of OPWV on the *P. amaryllifolius* increase the shoot and root DW and overall root length and surface area of all root sizes under well-watered condition. This finding corresponds with the application of different sources of WV reported to improve the root growth of several plant species, such as Chinese fir, wheat, and rapeseed (Lu *et al*., 2019; Wang *et al*., 2019; Zhu *et al*., 2021). Hence, this

suggests the potential of OPWV as a biostimulant or priming agent to induce plant root proliferation via elongation and branching. Moreover, it can also be hypothesized that OPWV might contain an active compound that could stimulate root growth. One of the compounds that could be responsible for this phenomenon is anthranilic acid (AA). AA was known to be one of the early precursors of indole-3-acetic acid and was recently reported to regulate the auxin flow via auxin transporter, such as PIN-FORMED (PIN) efflux carrier, which is important in root gravitropism (Doyle *et al*., 2019).

5.7.2 **Leaf relative water content, leaf folding, and stem circumference**

As the drought stress exposed the plant to a water deficit condition, a general symptom developed by the stressed plant includes reduced water content, leaf wilting and folding, and reduction of stem circumference. In this investigation, the drought-stressed *P. amaryllifolius* exhibit lower relative water content, higher severe leaf folding percentage and reduced stem circumference. These results are consistence with other studies on drought stress effects on the plant. For example, in the screening of drought-tolerant variety cowpea, the seedlings' leaf area and stem sizes were significantly reduced under drought stress (Ajayi *et al*., 2018). Moreover, Misra *et al*. (2020) reported a reduction in the leaf area and width of sugarcane plants exposed to drought stress for 60 days which indicates the plant's adaptive mechanism in response to the stress. Additionally, morphological and physiological analysis of the zinnia plants in response to drought stress exhibit a 25% reduction of RWC (Toscano & Romano, 2021). This evidence indicates the general response of plants in tolerating drought stress which includes reducing water content in the cells and lowering surface area and girth sizes in leaves and stems, respectively.

Remarkably, drought-stressed *P. amaryllifolius* primed with OPWV showed improved LRWC, relative stem circumference and significant reduction of severe leaf folding percentage. These results are in line with other studies in drought-stressed wheat and *Arabidopsis* primed with WV and seaweed extract, respectively (Rasul *et al*., 2021; Wang *et al*., 2019). Having a lower leaf folding/rolling is important for the plant to sustain growth and tolerate drought (Ye *et al*., 2020). In addition to that, the higher LRWC and stem circumference suggest the potential of OPWV application in enhancing the water-holding capacity of plant cells and maintaining their structural integrity, hence, enhancing their tolerance against drought stress. These findings highlight the potential of OPWV as a promising tool for improving drought tolerance in plants, which could have significant implications for agriculture in regions prone to drought. Nonetheless, further research is needed to elucidate the underlying mechanisms of how OPWV primes plants for improved drought tolerance.

5.7.3 **Leaf yellowing and pigmentation**

The upsurge of ROS build-up in plant cells during drought stress might induce oxidative damage to the pigment constituents and could lead to cell apoptosis under extreme conditions. In this experiment, drought stress induced the leaf yellowing percentage of *P. amaryllifolius*, specifically in the old and mature leaves. Furthermore, the pigment constituents such as chlorophyll *a*, *b*, and carotenoids were significantly reduced under drought stress. Nevertheless, the OPWV-priming of *P. amaryllifolius* prior to the drought treatment allows the plant to exhibit lower leaf yellowing and sustained lower damage on the pigment's constituents. The alleviation of these detrimental stress effects could also be observed in other studies. For example, sweet potato primed with garlic WV formulation prior to the *Fusarium oxysporum* f.sp. batatas inoculation

exhibited lower disease incidence, such as leaf yellowing (Masangcay & Galian, 2021). From a different perspective, the priming application of melatonin on the kiwi plant was able to suppress the degradation of chlorophyll when exposed to drought stress (Liang *et al*., 2019).

The OPWV-treated well-watered *P. amaryllifolius* recorded a higher chlorophyll accumulation than the non-treated samples. This finding is aligned with the report by Vannini *et al*. (2021), whereby lettuce plants primed with sweet chestnut wood distillate demonstrate higher chlorophyll content than the non-treated plants. Furthermore, another study by Ma *et al*. (2022b) showed a higher accumulation of chlorophyll content in peach WV primed-rapeseed plants before exposure to salt stress. In addition, chlorophyll pigments of soybean seedlings were enhanced when foliar was sprayed with acetic acid while reducing the yellowing leaf incidence when exposed to drought stress (Rahman *et al*., 2021). Chlorophyll is an essential pigment in plants that captures light energy during photosynthesis. The higher accumulation of chlorophyll content in OPWV-treated *P. amaryllifolius* suggests that the treatment may have positively impacted the plant's photosynthetic capacity, possibly due to various bioactive compounds, such as catechol and phenyl carbamate, in OPWV that stimulate the plant's physiological processes. This enhanced chlorophyll content could contribute to the overall growth and development of the plant. For example, a recent report by Khan *et al*. (2023) showed that steam-mediated foliar application of catechol and plant growth regulators significantly enhanced the growth attributes, photosynthesis, and essential oil production of lemongrass. Specifically, the treated plants showed a significant increase in plant height, leaf area, leaf number, and chlorophyll content, as well as an increase in net photosynthetic rate, stomatal conductance, and transpiration rate, indicating that the treatment improved the photosynthetic activity of the plants. In contrast, pesticide compounds, such as phenyl carbamate or carbamic acid, have been found to reduce plant growth and photosynthetic

pigment rates, according to Chahid *et al*. (2013). For example, Mishra *et al*. (2008) showed the exposure of cowpea seedlings to dimethoate resulted in a decrease in plant growth, as well as a decrease in photosynthetic activity, but did not significantly affect chlorophyll content. This suggests that the insecticide may be more harmful to the photosynthetic system of the cowpea seedlings than to the production of chlorophyll. However, further studies are necessary to understand the underlying mechanisms of how OPWV treatment affects chlorophyll content and photosynthetic capacity in plants.

5.8 OPWV impact on drought stress indicator of *Pandanus amaryllifolius*

5.8.1 **Hydrogen peroxide, relative electrolyte leakage and malondialdehydes**

In this study, drought-stressed *P. amaryllifolius* exhibited elevated accumulation of H_2O_2, MDA, and REL percentage. These findings are aligned with multiple reports on different plant species (Alvarez-Morezuelas *et al*., 2022; Jamshidi Goharrizi *et al*., 2020; Jothimani & Arulbalachandran, 2020; Men *et al*., 2018; Oraee & Tehranifar, 2020; Pamuta *et al*., 2022). Interestingly, the OPWV foliar application on *P. amaryllifolius* managed to mitigate these detrimental effects under drought stress, whereby H_2O_2, MDA, and REL levels were reduced significantly. Similar effects of priming application of various biostimulants on several plant species, which enhanced the plant drought tolerance have been reported. For example, H_2O_2, MDA, and REL levels in rice, pea, and lentil plants were all enhanced under drought stress when primed with chitosan, *Bacillus thuringiensis* with carrot extract, and melatonin, respectively (Arafa *et al*., 2021; Moolphuerk & Pattanagul, 2020; Yasmeen *et al*., 2022). Although reports on WV application as a priming agent under stress were scarce, a recent study by Ofoe *et al*.

(2022) on aluminium-stressed tomato has shown a significant reduction of H_2O_2 and MDA under drought stress when primed with poplar WV.

OPWV-treated well-watered *P. amaryllifolius* showed a complex interaction whereby the H_2O_2 and MDA accumulation was higher than the non-treated samples, while the REL percentage remained unchanged throughout the time points. The elevated levels of H_2O_2 observed in the treated plants may have resulted from increased ROS production by the plant cells, which was induced by the OPWV. This finding contrasts with the reported effects of biostimulants on plants (Arafa *et al*., 2021; Yasmeen *et al*., 2022). Nonetheless, a recent report on chitosan-primed rice has shown increased H_2O_2 and MDA accumulation under well-watered conditions (Moolphuerk & Pattanagul, 2020). Overall, the OPWV application on the plants could trigger the plant H_2O_2 accumulation, which acts as a signalling molecule in cells to stimulate downstream responses, such as antioxidant activation, proline build-up and stress-responsive gene expression at the expense of slight elevation of lipid peroxidation.

5.8.2 **Proline**

In this study, drought-stressed *P. amaryllifolius* showed significant proline accumulation compared to the well-watered plant. A similar trend has been reported in multiple plant species, such as Guinea grass, soybean, and watermelon (Moreno-Galván *et al*., 2020; Sheteiwy *et al*., 2021; Wang *et al*., 2022). On the contrary, OPWV-treated drought-stress *P. amaryllifolius* experienced lower proline accumulation. This finding is aligned with other studies (Dien *et al*., 2019; Furlan *et al*., 2020). For example, Abdelaal *et al*. (2020) reported a reduction of proline in salt-stressed-sweet pepper after foliar sprayed the plant with silicon. The authors found that exogenous silicon could mitigate the adverse effects of salt stress by enhancing water status, photosynthesis, and

antioxidant enzyme activity, resulting in increased fruit yield. Similar finding has also been reported in sugar beet. AlKahtani *et al*. (2021) found that sugar beet treated with silicone and proline exhibited lower proline content when exposed to drought stress. However, in this investigation, OPWV-treated *P. amaryllifolius* showed an elevated proline content after 7 days of growth under well-watered conditions but was on the same concentration as the OPWV-non-treated sample after 10 days of growth. This suggests that the effects of OPWV in enhancing the proline content would only last for a certain period, in this case, 7 days after application.

Proline is an important amino acid that acts as an osmoprotectant and helps maintain cell turgor pressure during stress conditions, such as drought. Hence, when drought-stress was imposed, the OPWV-treated *P. amaryllifolius* plant would possess a high accumulation of proline which are ready to be utilised in combating the drought stress response in cells, such as ROS production and loss of turgor pressure. Nonetheless, this finding implies that OPWV could alleviate the detrimental effects of drought stress. However, the detailed mechanisms of OPWV in regulating proline remain unclear, and further research is needed to determine the underlying mechanisms involved in the OPWV-induced stimulation of proline biosynthesis in plants. Understanding these mechanisms could provide valuable insights into developing more effective strategies for enhancing the drought tolerance of plants and improving crop productivity under adverse environmental conditions.

5.9 Influence of OPWV on antioxidant activities of *Pandanus amaryllifolius* during drought stress

Under stress, the overaccumulation of ROS in the plant would enhance the activity of antioxidant enzymes to suppress the deleterious effects of the oxygen-containing radicals. This study has shown the ability of drought-stressed *P. amaryllifolius* to cope with stress by elevating antioxidant enzyme activities to scavenge damaging ROS. On the contrary, the priming application of OPWV on *P. amaryllifolius* suppressed the antioxidant activities under drought stress. This finding is in contrast with other priming application reports. For example, Rady *et al*. (2021) demonstrated that diluted honey application to fava beans enhanced their antioxidant activities in both well-watered and drought-stressed plants. Moreover, exogenous melatonin priming on tomato plants improved the SOD, CAT, POD, APX, and GR activities significantly under drought stress (Altaf *et al*., 2022). The difference in response in *P. amaryllifolius* may suggest the OPWV application triggers the antioxidant enzymes activities, which became readily available to scavenge ROS produced when drought stress was imposed.

Intriguingly, the OPWV-treated well-watered plant showed remarkable effects of the priming towards *P. amaryllifolius* antioxidant activities, whereby the SOD, POD, CAT, APX and GR activities were significantly higher than the non-treated plants. This finding is in agreement with other biostimulant studies, such as melatonin application on lentil plants. After the treatment, SOD, POD, CAT, and APX activities were improved under well-watered conditions (Yasmeen *et al*., 2022). In addition, Zhu *et al*. (2021) reported the antioxidant activities enhancement of rapeseed plants primed with poplar-derived WV. On the other hand, it could be speculated that the antioxidant enhancement was also contributed by the active compounds in OPWV. In this study, the GC-MS profile of OPWV has identified high concentrations of phenyl carbamate, syringol, guaiacol,

catechol, and tetrasiloxane. These compounds have been reported to demonstrate antioxidant properties (Azadfar *et al*., 2015; Liu *et al*., 2017; Loo *et al*., 2008; Singh *et al*., 2021). Due to the antioxidant nature of these compounds, we could speculate priming plants prior to the stress might help them cope with the drought, as demonstrated in the reduction of antioxidant enzyme activities in OPWV-treated plants. Nonetheless, the exact mechanism of these compounds interacting with plants' intrinsic antioxidant systems remains unknown.

Altogether, drought tolerance of *P. amaryllifolius* could be attributed to the enhancement of the overall antioxidant system when primed with OPWV, which consists of several antioxidant compounds. Later, when drought stress was imposed, the primed antioxidant system was immediately utilised to suppress the ROS accumulation, which could be detrimental to the *P. amaryllifolius* plant. Nonetheless, drought tolerance in plants is the perception capability of the antioxidant capacity in realising drought stress (Laxa *et al*., 2019). This means that the plant's ability to sense and respond to drought stress depends on its antioxidant capacity, whereby a plant with a higher antioxidant capacity would be more likely to tolerate drought stress than a plant with a lower antioxidant capacity. This perception of the antioxidant capacity is complex and involves several molecular and biochemical pathways. Nevertheless, more research is needed to understand the specific molecular mechanisms involved in the perception of the antioxidant capacity in plants and how OPWV priming affects these mechanisms.

5.10 OPWV effects on the carbohydrate-related and drought-responsive gene expression in *Pandanus amaryllifolius* during drought stress

Activation of the stress-responsive gene in cells is crucial in regulating the molecular signalling and metabolic process to respond and adapt to stress. In this investigation, the application of OPWV on *P. amaryllifolius* was found to influence the expression of the carbohydrate-related and drought-responsive genes. As mentioned previously, drought stress upregulates the expression of *PaHSP70*, *PaENO*, *PaThau, PaGAPDH,* and *Paβ-Fruc* genes in *P. amaryllifolius.* These molecular responses are crucial in improving the *P. amaryllifolius* tolerance against drought stress via morpho-physiological and biochemical changes.

Interestingly, the priming of OPWV on *P. amaryllifolius* demonstrates remarkable effects on the gene expression of the plant. For instance, the expression of *PaGAPDH*, *PaHSP70* and *PaThau* genes were upregulated in the OPWV-treated drought-stressed samples. These genes are important in plant abiotic tolerance, as shown by previous studies (Davoudi *et al*., 2022; Kappachery *et al*., 2015; Misra *et al*., 2016; Ni *et al*., 2021; Sharma *et al*., 2022). For example, Kappachery *et al*. (2015) demonstrated that overexpression of the *GAPDH* gene increased plant tolerance to water-deficit stress by inducing antioxidant enzymes and reducing ROS. The enhanced stress tolerance of the *GAPDH*-overexpressing transgenic potatoes could be due to several reasons. One possible reason is that expressing *GAPDH* might help increase the energy needed for growth and development. Another reason is that it helps to limit the formation of a harmful substance called methyl-glyoxal, which is produced during glycolysis under stress (Kappachery *et al*., 2015). GAPDH could reduce the accumulation of triose-phosphates and, in turn, decrease methyl-glyoxal production.

HSP was found to be upregulated in this study. These genes have been reported to be highly expressed in various drought-stressed plants. For instance, Ni *et al*. (2021) reported that a 70-kDa HSP called *GhHSP70-26* in cotton plants positively responded to drought stress. Besides, the *GhHSP70-26*-overexpressing transgenic cottons showed less water loss and wilting, as well as higher photosynthetic rates and antioxidant enzyme activities compared to wild-type plants under drought conditions (Ni *et al*., 2021).

Thaumatin-like protein has been shown to play an important role in conferring tolerance to environmental stresses. For example, He *et al*. (2021) found that *BolTLP*1 in broccoli plants was upregulated in response to drought stress. The authors also found that expressing *BolTLP*1 increased proline content and antioxidant enzyme activities (He *et al*., 2021). Similar to these studies, applying OPWV-priming on *P. amaryllifolius* prior to drought stress enhanced the expression of *GAPDH*, *Thaumatin*, and *HSP70*, suggesting that OPWV-priming has the potential to increase plant stress tolerance by modulating drought-responsive genes, such as *GAPDH*, *Thaumatin*, and *HSP70*.

PaENO and *Paβ-Fruc* expressions were downregulated in the OPWV-treated drought-stressed samples. This finding is in contrast with studies whereby PGPR application rice demonstrated upregulation of *ENO* under normal and stressful environments (Bisht *et al*., 2020). Nevertheless, the downregulation of these genes did not affect the overall plant tolerance against drought stress, as the exact mechanism is unclear.

OPWV-treated well-watered *P. amaryllifolius* showed upregulation of *PaGAPDH* and *Paβ-Fruc,* while *PaENO* and *PaThau* expressions were downregulated. This evidence suggests the OPWV potential to influence carbohydrate-related and drought-responsive genes to improve plant drought tolerance. Overall, the regulation of *PaGAPDH* might serve as an important key regulator in *P. amaryllifolius* responses.

Drought stress upregulates the *PaGAPDH* expression, while the supplementation of OPWV further improves the *PaGAPDH* fold expression in *P. amaryllifolius* in both well-watered and drought-stress conditions. Thus, it can be speculated that *PaGAPDH* elevated expression contributes to the improved tolerance of *P. amaryllifolius*. Similarly, the expression of *PaHSP70* and *PaThau* in the drought-stressed *P. amaryllifolius* was further enhanced with the OPWV treatment. As both *HSP70* and *thaumatin* are important in the plant response toward abiotic stress, OPWV supplementation managed to amplify the expression of the gene, which will become important functional proteins in improving the drought tolerance of *P. amaryllifolius.*

β-frucfuranosidase, also known as invertase, is important in regulating sucrose, storage, and utilisation in plants by converting sucrose into glucose and fructose via hydrolysis. Overexpression of *β-Fruc* or *Invertase* gene from trifoliate orange in tobacco plants exhibits higher accumulation of the protein encoded in chloroplast and mitochondrion. This accumulation was responsible for enhancing sucrose degradation activity into reducing sugar, which improved the cold, salinity and drought tolerance of tobacco (Dahro *et al*., 2016). In this study, drought-stressed *P. amaryllifolius* showed upregulation of *Paβ-Fruc*, indicating the importance of this protein to provide sufficient reducing sugar in regulating drought response in the plant. Nonetheless, OPWV priming induced this response in *P. amaryllifolius* under a well-watered environment. This suggests the abundance of readily processed reduced sugar in the OPWV-treated plants to be utilised as energy sources for shoot and root growth, thus increasing the total plant biomass.

Enolase is an enzyme that helps reverse the conversion of D-2-phosphoglycerate (2PGA) into phosphoenolpyruvate (PEP) in glycolysis and gluconeogenesis pathway, which later be further processed in the tricarboxylic acid cycle. In this study, the OPWV application on *P. amaryllifolius* suppressed the expression of *PaENO* in the well-watered

and drought-stressed samples. This indicates that the OPWV treatment suppressed or switched off the tricarboxylic acid cycle pathway in *P. amaryllifolius* by lowering the expression of *PaENO*. Generally, the tricarboxylic acid cycle is often inhibited or suppressed due to the ample amount of energy available in the system, which includes high concentrations of ATP, acetyl-CoA, and NADH. Therefore, it could be speculated that this scenario is related to the enhanced expression of *Paβ-Fruc*.

CHAPTER 6

CONCLUSION

In summary, this study investigated the responses of *Pandanus amaryllifolius* to drought stress by examining the changes in its morphological, biochemical, and molecular responses. Drought stress reduces LRWC. It causes cellular damage, as indicated by the accumulation of MDA and the degradation of leaf pigments, such as chlorophyll. These adverse effects can be observed through wilting or folding of leaves and yellowing of the leaves. Stress intensity can also be measured through the accumulation of proline and hydrogen peroxide in plants exposed to drought. Proteomic analysis has identified several proteins responsible for the tolerance of plants to water-deprived conditions, including stress and defence proteins, and carbohydrate metabolism-related proteins. When the gene expression of these proteins, such as *HSP70*, *thaumatin*, *GAPDH*, *enolase*, and *β-fructofuranosidase*, were analysed, higher expression of these genes in drought-stressed plants indicated their importance in the plant response mechanism to drought (**Figures 6.1A** and **B**). The higher expression of HSP70 and thaumatin suggested *P. amaryllifolius* tolerating to the drought stress by improving ROS scavenging activity, maintaining cell osmotic balance and protein structure (**Figure. 6.1A**). Moreover, highly expressed genes involved in the glycolysis pathway such as *GAPDH*, *enolase*, and *β-fructofuranosidase* proven to be important in supplying extra energy to the plant under drought stress (**Figure. 6.1B)**. To enhance plant tolerance to drought stress, the application of WV, produced from palm oil waste, was explored. The results showed that exogenous foliar spraying of OPWV at 1:500 dilution at 3-day intervals before drought exposure significantly improved plant growth, including stem circumference, leaf structure, pigmentation, and root growth. In addition, proline content, ROS, cell damage, and antioxidant activity were reduced. Expression of drought-

responsive genes, such as *HSP70*, *Thau*, and *GAPDH*, was also enhanced by OPWV priming during drought. Interestingly, OPWV application to well-watered plants showed priming effects, triggering the upregulation of drought-related markers and antioxidant activity.

Nonetheless, further research is needed to understand the relationship of these compounds in OPWV to plant cell biological processes. For instance, the proteomic analysis of the OPWV-treated sample would be an interesting area for further investigation. It will help to further understand the proteins altered with OPWV treatment under well-watered and drought-stressed plants. Moreover, it will be interesting to observe the cell wall and stomatal changes with microscopy analysis of the leaf cross-section morphology of the OPWV-treated sample. This analysis would uncover the effect of the treatment and whether it enhanced the overall structural integrity of the plant cells, as discussed previously. In addition, the OPWV application in this study was only limited to the foliar spray method. Although several studies of other OPWV source origins have reported the effectiveness of foliar spray over the soil-drenching method in promoting plant growth, the literature on soil-drenching of OPWV is still limited. Hence, further analysis of different application methods of OPWV with varied concentrations might have different effects on the plant. In addition, follow-up studies based on the identified compounds in OPWV are crucial to understanding the interaction of the compound with plants. This will answer several questions in this research with regard to which compound is responsible for the OPWV effects on plants or whether it is a complex interaction of several compounds that contributes to the effectiveness of OPWV.

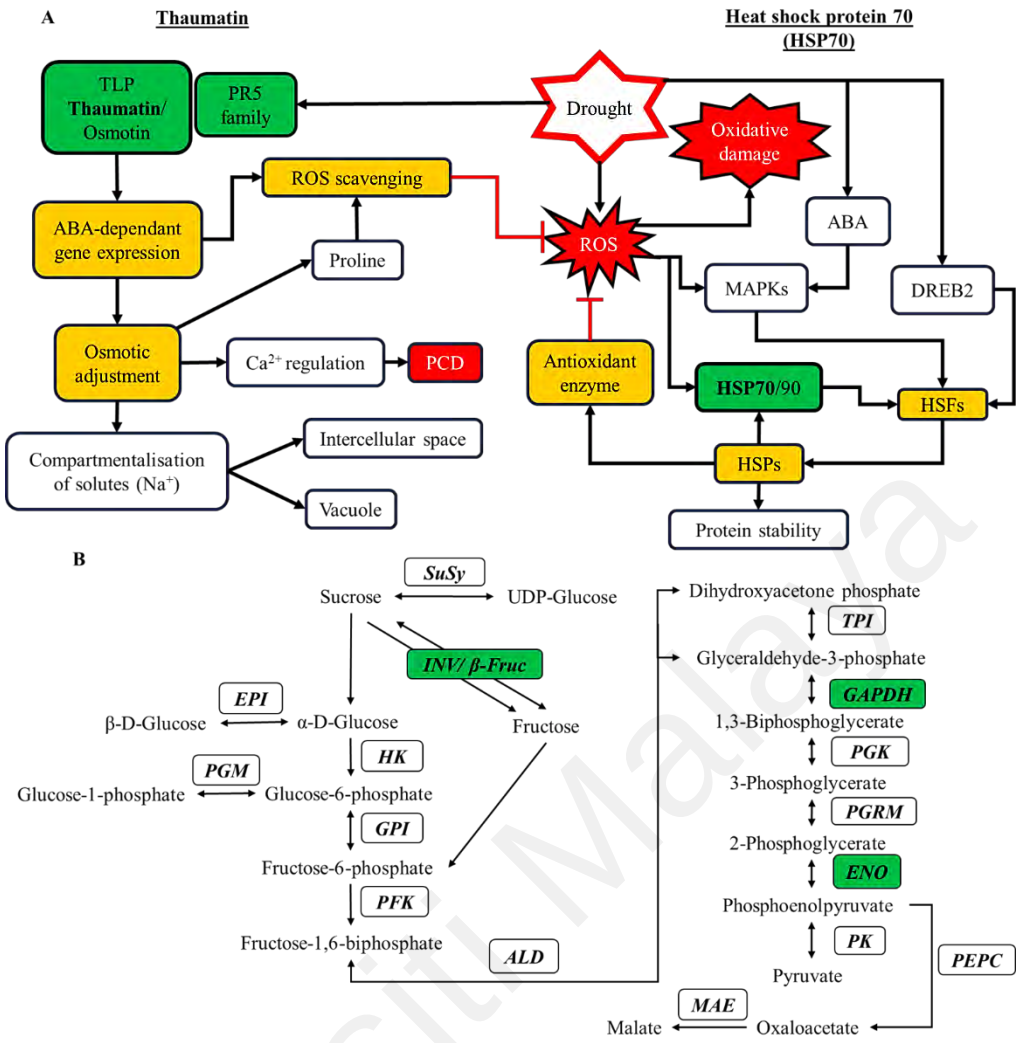

Figure 6.1. Schematic diagram of pathway related to drought-responsive genes investigated in this study. (**A**) Drought stress-responsive gene pathway involved between thaumatin (Thau) and heat shock protein 70 (HSP70). (**B**) Drought stress-responsive gene induced in glycolysis cycle. Fructose phosphate aldolase (***ALD***), enolase (***ENO***), aldose-1-epimerase (***EPI***), phosphorylating glyceraldehyde-3-phosphate dehydrogenase (***GAPDH***), glucose-6-phosphate isomerase (***GPI***), hexokinase (***HK***), malate dehydrogenase (***MAE***), invertase/β-Fructofuranosidase (***INV/β-Fruc***), phosphoenolpyruvate carboxylase (***PEPC***), phosphofructokinase (***PFK***), phosphoglycerate kinase (***PGK***), phosphoglucomutase (***PGM***), phosphoglycerate mutase (***PGRM***), pyruvate kinase (***PK***), sucrose synthase (***SuSy***), triose-phosphate isomerse (***TPI***).

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