

ROOT ACCLIMATION OF RICE EXPOSED TO
ALTERNATE AND CONTINUOUS DROUGHT STRESS

BY

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the degree of Master of Science

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ABSTRACT

Drought conditions as a result of climate change have detrimentally reduced rice production with roots playing a pivotal role in water uptake and providing valuable insights into adaptation to water scarcity through their anatomical phenotype. Thus, this study aimed to elucidate how drought stress affects the agronomic traits, root anatomical phenotype and identify the differentially expressed genes (DEGs) correspond to drought tolerance. Six rice varieties, Moroberekan, MR297, Apami, Merah Wangi, Biris, and Nona Manis, were grown and subjected to normal watering, alternating two cycles of drought and continuous drought. A preliminary study was conducted to evaluate agronomic traits. Anatomical study was done with the root samples harvested at panicle initiation. Following the findings from morphological and histological study, the RNA from the roots of MR297 and Merah Wangi were then selected for comprehensive transcriptomic analysis using next generation sequencing (NGS). Based on the agronomic analysis, Merah Wangi produced the highest grain yield under both drought stress, followed by Nona Manis and MR297. The results for root phenotype showed great plasticity among the varieties. Under alternating drought stress, Moroberekan had the highest values for anatomical root area, root diameter, stele area, cortex area and metaxylem traits, followed by Merah Wangi. Despite severe drought stress, Merah Wangi exhibited the highest grain yield with better root anatomical structure. Meanwhile, transcriptome analysis of Merah Wangi against MR297 unveiled 35257 and 77115 DEGs responsive to alternate drought and continuous drought stress, respectively. The study revealed an abundance of DEGs in Merah Wangi, with more genes downregulated than upregulated, especially under both drought stress. Significantly, the expression of *DEEPER ROOTING 1* and enrichment of DEGs in pathways associated with 'plant hormone signal transduction' and 'metabolite synthesis' underscores their potential relevance to the mechanism underlying drought tolerance in Merah Wangi. The continuous drought stress has triggered the upregulation of hormone related genes, particularly auxin, and cytokinin. In conclusion, Merah Wangi exhibited the highest drought resilience among the varieties studied, a trait attributed to its superior agronomic characteristics, root anatomical phenotypes, and the activation of genes associated with drought response. The findings provide key insights into root acclimation, paving the way for developing more resilient rice varieties under drought stress.

خلاصة البحث

أدت ظروف الجفاف نتيجة لتغير المناخ إلى انخفاض إنتاج الأرز بشكل ضار مع لعب الجذور دورًا محوريًا. في امتصاص المياه وتوفير رؤية قيمة في التكيف مع ندرة المياه من خلال النمط الظاهري التشريحي لها. وبالتالي، تهدف هذه الدراسة إلى توضيح كيفية تأثير الإجهاد الناجم عن الجفاف على السمات الزراعية التي تتوافق (DEGs) والنمط الظاهري التشريحي للجذور وتحديد الجينات المعبر عنها بشكل تفاضلي Apami وMR297 وMoroberekan، مع تحمل الجفاف. تمت زراعة ستة أصناف من الأرز وتعرضت للري العادي، بالتناوب بين دورتين، Nona Manis وBiris وMerah Wangi من الجفاف والجفاف المستمر. أجريت دراسة أولية لتقييم السمات الزراعية. أجريت دراسة تشريحية على عينات الجذر التي تم حصادها عند بدء النورات. بناءً على النتائج المستخلصة من الدراسة المورفولوجية للتحليل Merah Wangi وMR297 والنسجية، تم اختيار الحمض النووي الريبي من جذور وبناءً على التحليل الزراعي، أنتجت (NGS) النسخي الشامل باستخدام تسلسل الجيل التالي Nona Manis أعلى محصول حبوب تحت كل من إجهاد الجفاف، تليها Merah Wangi وأظهرت نتائج النمط الظاهري للجذر مرونة كبيرة بين الأصناف. وسجل MR297 أعلى القيم لجميع الملامح التشريحية للجذر تحت إجهاد الجفاف المتناوب، تليها Moroberekan أعلى Merah Wangi وعلى الرغم من إجهاد الجفاف الشديد، أظهرت Merah Wangi محصول حبوب مع بنية تشريحية أفضل للجذر. وفي الوقت نفسه، كشف تحليل النسخ الجيني لـ Merah Wangi للجفاف المتناوب DEGs عن 35257 و77115 MR297 مقابل Wangi مع، Merah Wangi في DEGs والجفاف المستمر، على التوالي. وكشفت الدراسة عن وفرة من انخفاض تنظيم الجينات أكثر من تنظيمها لأعلى، وخاصة تحت كل من إجهاد الجفاف. ومن الجدير في المسارات المرتبطة بـ DEGs وإثراء 1 DEEPER ROOTING بالذكر أن التعبير عن نقل إشارة الهرمونات النباتية "و"تخليق المستقلبات" يؤكد على أهميتها المحتملة للآلية الكامنة وراء تحمل" وقد أدى الإجهاد المستمر للجفاف إلى تحفيز زيادة تنظيم الجينات. Merah Wangi الجفاف في المرتبطة بالهرمونات، وخاصة الأوكسين والسيبتوكينين. وفي الختام، أظهر صنف ميراه وانجي أعلى قدرة على تحمل الجفاف بين الأصناف المدروسة، وهي سمة تُعزى إلى خصائصه الزراعية المتفوقة، والأنماط الظاهرية التشريحية للجذور، وتنشيط الجينات المرتبطة باستجابة الجفاف. وتوفر النتائج رؤية رئيسية حول تأقلم الجذور، مما يمهد الطريق لتطوير أصناف أرز أكثر مرونة تحت ضغط الجفاف.

APPROVAL PAGE

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

Nur Nazifah binti Saimi

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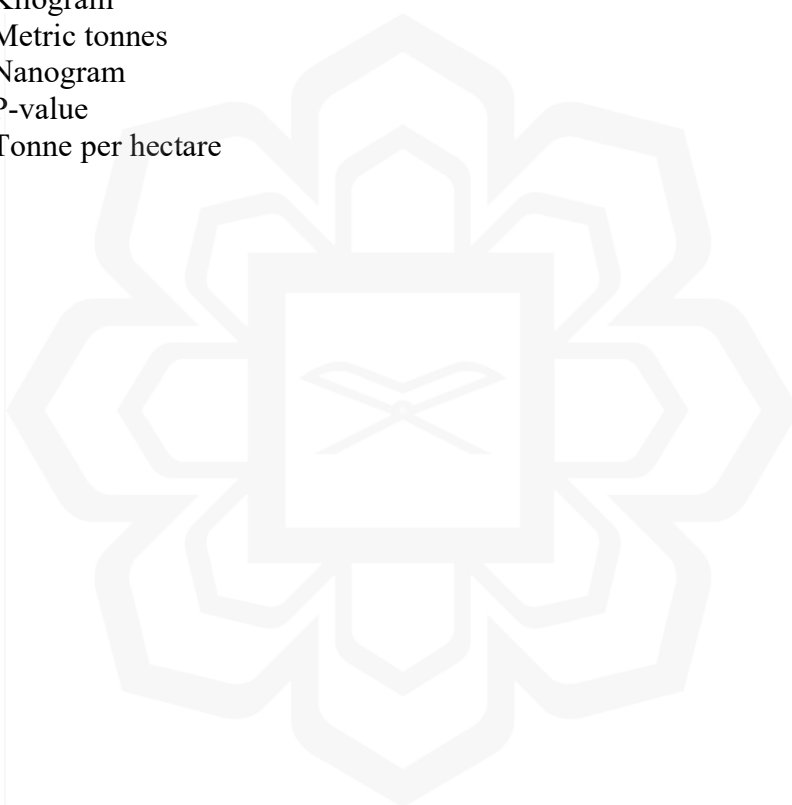
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LIST OF SYMBOLS

%	Percentage
$\leq$	Lower than
°C	Degree Celsius
μl	Microlitre
μM	Micromolar
μm^2	Square micrometres
g	Gram
g/m^3	Metre cubic
kg	Kilogram
mt	Metric tonnes
ng	Nanogram
p	P-value
t/ha	Tonne per hectare



LIST OF ABBREVIATIONS

AWD	Alternate Wetting and Drying
COG	Cluster of Orthologous Group
DAS	Days After Sowing
DEG	Differentially Expressed Genes
FAO	Food And Agriculture Organization
GO	Gene Ontology
IAA	Indole Acetic Acid
KEGG	Kyoto Encyclopedia of Genes and Genomes
NGS	Next Generation Sequencing
PCA	Principal Component Analysis
RCA	Root Cortical Aerenchyma
RCP	Root Cortical Parenchyma
RNA	Ribosomal Nucleic Acid
ROS	Reactive Oxygen Species
RSA	Root System Architecture
SSL	Self Sufficiency Level
USDA	United States Department of Agriculture
WUE	Water Use Efficiency

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND OF THE STUDY

Rice (*Oryza sativa* L.) has been cultivated as the crucial food source for global production. The cultivation of rice is primarily concentrated in the Asia region, which accounts for 90% of total rice production in the world (Khush, 2005). This production simultaneously makes Asia the main producer and consumer of rice, with China being the leading exporter (USDA, 2023). In Malaysia, the paddy industry is one of the precious sectors highlighted in the recent 12th year Malaysia Plan (2021-2025). In 2022, Malaysia's rice self-sufficiency level (SSL) accounted for 62% (Carvalho et al., 2024). The increasing demand in the population linked to the outbreak of food security has urged governments to implement multiple strategies to achieve 80% SSL by 2030 (Carvalho et al., 2024).

Despite the numerous intervention strategies, the availability of water in several regions is impacted by climate change. Food security is at risk due to water scarcity caused by global warming (Rockstrom et al., 2009). The drought condition, which refers to a period of water deficiency in the soil, has a severe impact on rice physiological processes, particularly grain production (Yang et al., 2019). Several agronomic traits including plant height, grain length, grain yield, and tillers quantity are highly influenced by sufficient water availability (Zhang et al., 2018). Notably, the impact of drought stress is dependent on the intensity of drought stress (Kottmann et al., 2016). Therefore, this study covers two conditions of drought stress. Alternating drought stress involves interspersing two drought phases with normal watering. This method of irrigation, widely known as intermittent irrigation, has been utilized 20 years ago (Kwanyuen et al., 2021). The integration of alternating drought cycles is important, as it imitates the common irregularity of rainfall distribution in Malaysia, where certain granary regions often experience prolonged water shortages. This treatment discovers plants' acclimation, which involves rapid responses to subsequent dehydration (Kleine et al., 2020). Meanwhile, the second treatment, continuous drought stress, reflects the

prolonged water scarcity, which enables the plant to develop a long-term adaptation strategy for better resilience to drought stress.

Interestingly, drought stress is first detected by the roots, underground part of the plants (Basu et al., 2016). Kristensen et al. (2016) reported that the rooting environment influences the agronomic traits under water deficit conditions as it has a functional role in water uptake. Furthermore, the well-developed root system architecture (RSA) is the main contributor to a stable yield under drought (Dodd et al., 2011). The RSA and its alteration in a dynamic environment could be viewed through anatomical analysis. Various studies have integrated the plant anatomy to visualize and characterize the phenotypic variation in plants through anatomical analysis (Chinmungu et al., 2015; Hazman & Brown, 2018; Lynch et al., 2021). In some studies, drought stress has been found to alter the anatomical features of root rice, and this could be seen through the development of the vascular structure and changes in cell division (Fonta et al., 2022; Siangliw et al., 2022). Therefore, understanding the RSA is utmost necessary to evaluate a plant's resilience to drought stress, yet little research has incorporated the root analysis.

Meanwhile, understanding root acclimation and adaptation remains incomplete without evaluating the genetic basis underlying drought tolerance. In addition, Basu et al. (2016) reported that the adaptation of root cells requires validation from gene expression analysis. Through transcriptomic analysis, previous studies have exploited the potential genes that stimulate the signalling cascades of drought tolerance mechanism (Siangliw et al., 2022; Wei et al., 2016). Several studies have also validated the crucial role of phytohormones as signal molecules engaged in drought stress (Iqbal et al., 2022; Shaffique et al., 2023). Therefore, identification of the beneficial genes in correlation to adaptational roots could serve as a resource in the breeding of tolerant rice varieties.

1.2 PROBLEM STATEMENT

Drought stress becomes a major constraint in agriculture and causes great crop losses. As climate change is predicted to get worse, developing rice drought-tolerant varieties have gained interest in plant breeding programs. Basu et al. (2016) addressed the

importance of biochemical, anatomical, and morpho-physiological analysis in developing drought tolerant rice. Roots have a fundamental role in absorbing water from the soil. As water scarcity constrains cell division (Setter & Flannigan, 2001), Mostajeran and Rahimi (2008) reported that drought stress results a detrimental effect on the anatomical root features of rice. The variation in anatomical plasticity is regulated by a specific gene (Fu et al., 2021). Expression of these genes controls the modification and alteration of the root cell. Thus, studies delving into root anatomical phenotypes and its genetic basis contributes to valuable donors in the development of tolerant varieties.

Although anatomical study offers visible perspectives on root alteration, it becomes a bottleneck in research due to its methodology's challenges (Lynch et al., 2021; Strock et al., 2022). Previous research has predominantly concentrated on the aerial parts of the rice, leaving the root system underexplored. Unlike shoots and leaves, roots are underground part and can be easily damaged during sampling. The intricate branching patterns of roots require extra precautions to prevent disruption on their natural growth pattern (Lynch, 1995; Mairhofer et al., 2011). Recently, Okamura et al. (2020) reported that the delicate structure of rice root needs specialized techniques to observe its anatomical structure as well as to ensure the integrity of ribonucleic acid (RNA). Hence, the complexity of the root system makes it time-consuming and challenging to standardize experiments (Brunner et al., 2015). There remains a significant gap in the literature regarding the molecular mechanism associated with root development under drought stress. Therefore, this study aims to address this gap by evaluating the plasticity of roots and its adaptation to drought stress.

1.3 RESEARCH OBJECTIVES

This study complies three different fields of study. Chapter 3 is the preliminary analysis of the agronomic traits for six rice varieties under different drought conditions. Meanwhile, Chapter 4 examines the variation in root anatomical phenotypes such as area of cross-sectional root, stele, cortex, and metaxylem. In Chapter 5, focus is given on the genetic basis underlying the outstanding drought-tolerant variety. Ultimately, this analysis aims to discover the root acclimation to alternating drought stress and its adaptation to prolonged drought exposure. The specific research objectives were:

1. To evaluate the effects of drought stress on the agronomic traits of six rice varieties: MR297, Apami, Merah Wangi, Moroberekan, Biris, and Nona Manis.
2. To investigate the variation in root anatomical characteristics of these six paddy varieties under normal condition, alternate drought and continuous drought condition.
3. To analyze the genetic expression and identify the differentially expressed genes in the selected tolerant rice varieties that are associated with root acclimation and adaptation to drought stress.

1.4 HYPOTHESIS

The detrimental impact of drought stress on agronomic traits and root anatomical phenotype will be less pronounced under alternating two cycles, compared to continuous drought stress. The tolerant paddy varieties will develop better root anatomical structures and agronomic traits in response to drought stress. Through gene analysis, these tolerant varieties will express some tolerance trait genes associated with drought condition.

CHAPTER TWO

LITERATURE REVIEW

2.1 RICE CULTIVATION

According to the United States Department of Agriculture (USDA), as of 2022, China is the leading rice producer, followed by India and Indonesia, with the annual average rice production accounting for about 148.28 million tons (Mt), 120.54 Mt, and 35.96 Mt, respectively. The cultivation of paddy not only provides food for consumption but serves as income for the target groups. In Malaysia, rice cultivation is considered small-scale farming, concentrated in several granary areas in peninsular Malaysia (Firdaus et al., 2020). Based on the Malaysian Department of Agriculture (DOA), Malaysia allocated 0.64 million hectares for paddy cultivation in 2020, and this total area is far too low than its neighbour, Indonesia, with allocation of 11.50 million hectares (DOA, 2021; USDA, 2020). Moreover, the production of paddy in Malaysia only aids 70% of domestic demand, while the remaining is supplied by Asian countries such as Thailand, Cambodia, and Vietnam (Rahim et al., 2017).

Globally, rice production in 2022/2023 was forecasted to decline to 507.99 Mt compared to the 2021/2022 which recorded 515.08 Mt (USDA, 2022). In addition, the average paddy production in granary areas of Malaysia in 2020 only accounted for about 4,414 kg/ha, which shows a declining pattern from 4,941kg/ha in 2016 (DOA, 2021). Taking this into consideration, some constraints in paddy cultivation, such as biotic and abiotic stress, high production rate, lack of arable land, post-harvest loss, and lack of mechanization, must be mitigated to achieve the targeted SSL of 100% (Firdaus et al., 2020). Therefore, in the enacted National Agriculture Policy 2.0 (2021-2030), the policy that emphasizes the importance of using natural resources efficiently, improving livelihoods and farmers' income has been implemented to boost rice production, simultaneously upgrading the SSL (DOA, 2021).

2.2 FACTORS CONTRIBUTING TO ENVIRONMENTAL STRESS

Previous studies revealed that plants face various environmental stresses, as a consequence of uncontrollable nature conditions such as changes in precipitation, temperature, and humidity (Alam et al., 2014; Firdaus et al., 2020; Vaghefi et al., 2016). External factors, which include heat, waterlogging, extreme cold temperatures, drought, mineral deficiency or availability, salinity, are collectively referred to as “abiotic stress” (Ashraf et al., 2018; Sharma et al., 2019; & Mareri et al., 2022). Among various abiotic stresses, drought stress is recognized as the most severe and has a prominent impact on plants (Alam et al., 2014). Drought occurs not only because of fewer precipitation, but various contributing factors such as low soil moisture, low humidity, hot and dry wind, high evaporation from the soil, and osmotic stress (Alam et al., 2014).

Climate change due to global warming is also one of the main factors that causes drought. Drought conditions have received great attention in the global forum of the 21st century. The term ‘climate change’ is frequently interchangeable with global warming. However, global warming is different as it refers to the rising of the earth’s temperature, thus causing climate change over a long period (Ninawe et al., 2018). Global warming has been outpaced by the rise of greenhouse gases trapped in the atmosphere, the burning of fossil fuels and deforestation (Rossati, 2017). Based on the World Meteorological Organization (2022), the past seven years were the warmest years on record. Malaysia also experiences the effects of climate change. The warmest year was recorded in 2019, after the El-Nino phenomenon in 2016 (Nullis, 2020). This scenario worsens as it is anticipated that El-Nino phenomenon will occur in either 2023 or 2024 (NOAA, 2023). Hence, cooperation among various agencies, including policymakers, scientists, and stakeholders, is essential to tackle climate change.

2.3 IMPACT OF DROUGHT STRESS ON AGRICULTURE INDUSTRY

The Food and Agriculture Organization (FAO) reported that agriculture became the highest-impacted sector due to drought stress, accounting for 82% of all drought impacts compared to other industries (FAO, 2023). In low-developed countries, 34% of agriculture losses from 2008 to 2018 have been attributed to drought stress (FAO, 2023). The prolonged drought as a result of global warming has a deleterious effect on

crop productivity, as water availability is highly associated with healthy plant growth. These rising temperatures have caused groundwater depletion, the extermination of biodiversity, and an imbalance in ecosystems (Rockstrom et al., 2009). Under ongoing drought conditions, the reduction in annual crop yield leads to the loss of major food sources, which concurrently exposes people to food insecurity.

A previous study by Zhao et al. (2017) concluded that maize had been identified as the crop most adversely affected by the temperature elevation, followed by wheat and rice, which had yield reductions of 7.4%, 6%, and 3.2%, respectively. In Malaysia, paddy, the secured commodity, is also negatively impacted by drought stress. According to Khalijah et al. (2015), a one percent incline drought pattern would impede 0.03% of paddy production.

The prolonged impact of drought stress could be the reason for hunger and malnutrition (Mansoor et al., 2022). The global hunger index (GHI) for Malaysia scored 10.9 in 2014 and increased to 12.5 in 2022 (GHI, 2023). Although Malaysia's score is considered moderate, this upward trend is the worst situation, which is linked to food insecurity. Therefore, it is necessary to understand the underlying responses of paddy to drought stress in order to sustain grain yield in water-limiting areas.

2.4 IMPACT OF DROUGHT STRESS ON PADDY AGRONOMY TRAIT

The intensity of the drought condition causes a serious impact on paddy agronomic traits, which could be observed through a series of visible symptoms. Zhang et al. (2018) reported that the symptoms of drought stress depend on the severity, period of drought stress, and growth phases of paddy plant. The impact of drought stress becomes more intense during the reproductive phase than the vegetative phase (Patnaik et al., 2023; Zhang et al., 2018). The reproductive and booting stages represent the most critically affected phases (Landli et al., 2017; Ru et al., 2023). This finding aligns with Yang et al. (2019), who reported that rice yield shows a greater reduction when drought occurs during the reproductive phase, as this stage involves panicle formation.

Drought conditions interfere with morphological and agronomic traits by reducing plant height, panicle length, productive tiller number, and harvestable yield (Servestani et al., 2018). The ubiquitous symptoms of drought stress could be observed

through the leaf as it could suffer damage, senescence, scrolling, and brittleness (Darmadi et al., 2021; Herawati et al., 2017). As the leaf is the vital medium for gas exchange, Mohamad et al. (2021) found that the photosynthesis rate and chlorophyll content significantly dropped under drought due to low carbon dioxide (CO₂). Moreover, drought stress induces turgor loss and prevents cell elongation and expansion (Fen et al., 2015). This finding was similar to Sarvestani et al. (2018), who mentioned that prolonged drought during the vegetative state has retarded paddy height and crop growth.

Moreover, water scarcity limits the cell division of panicles and spikelet, which are important agronomy traits for rice. Furthermore, Kadam et al. (2014) revealed that water deficit during anthesis reduces the seed set, and when drought prolongs to post anthesis, it will reduce the individual grain weight. Besides that, a recent study by Wang et al. (2022) found that the amylose content in grain is totally low due to the slow grain filling rate. Notably, high-quality grain yield requires sufficient water during the booting stage (Wang et al., 2022). As the drought condition shows a negative association with grain yield, drought stress in paddy has been extensively studied with integration of plant physiology, molecular biology, and biochemistry aspects.

2.5 THE SYNERGISTIC EFFECT OF DROUGHT AND HEAT STRESS ON RICE CULTIVATION

Heat stress occurs when there is an elevated environmental temperature above the specific limit of the threshold level as a consequence of continuous global warming (Ahmad et al., 2022). Drought stress, as a result of inadequate rainfall and lack of soil moisture can occur simultaneously with heat stress (Lal, 2016). Temperature is the pivotal environmental factor that governs the growth, development, and grain yield (Tesfaye et al., 2017). Temperatures exceeding 33°C have resulted in plant dehydration and negatively impacted the worldwide rice production (Mohamed Arshad et al., 2017; Zhao et al., 2020).

Recent studies highlighted the synergistic effect of these heat and drought stresses, which causes great challenges in agriculture production as compared to their individual effects (Dreesen et al., 2012). Basu et al. (2022) reported the physiological mechanism underlying the drought and heat tolerance differ among crop traits. Drought

stress limits the gas exchange by causing stomatal closure, whereas heat stress impacts the photosynthesis by inhibiting the electron transport and PSII activity (Basu et al., 2022). Based on Figure 2.1, both stresses negatively impact the plant-water relations, leading to a low leaf relative water content (RWC), water potential, osmotic potential, and turgor pressure (Basu et al., 2022; Bhardwaj et al., 2021).

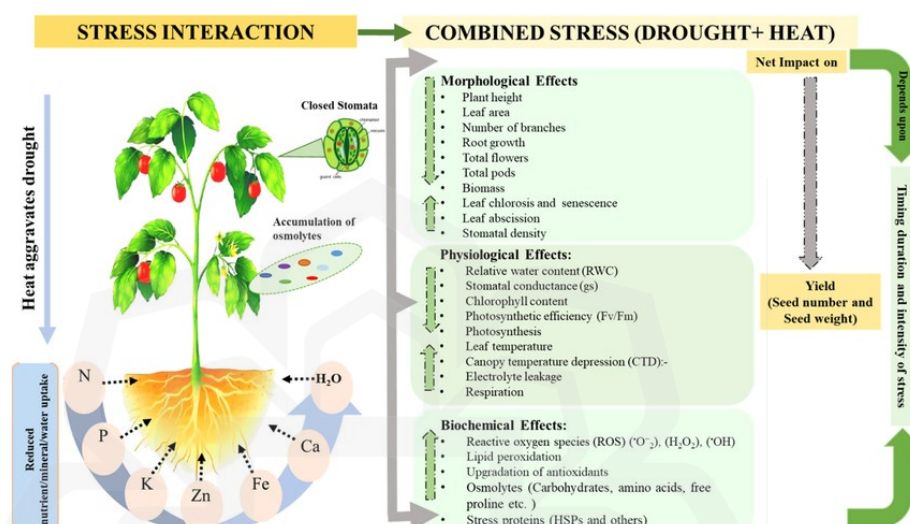


Figure 2.1: The synergistic effect of drought and heat stress on various traits of the plants. (Bhardwaj et al., 2021)

Plants adapt these challenges by developing several adaptations such as increased antioxidant production, osmolytes and activation as stress proteins. Georgi et al. (2017) revealed that transcriptome and metabolic level under drought and heat stress shared a similar response, but drought responses dominated more differential regulation than heat response. In combined stress, Ru et al. (2023) and Alhaithloul (2019) reported leaf water potential and nitrogen metabolism enzyme activity experience significant decreases, consequently diminishing photosynthetic productivity. Moreover, Garcia et al. (2023) highlighted that the acute temperature in the root region causes meristematic cell death, which later terminates root growth and proliferation. Thus, it can be noted that drought and heat are pivotal abiotic stressors that influence plant-water relations.

2.6 IMPACT OF DROUGHT STRESS ON ROOT ANATOMICAL PHENOTYPE

The anatomical characteristics of rice roots have been affected by drought stress. Darmadi et al. (2021) identified the root shape as the most noticeable change in the cross section of rice roots. The root shape in the optimum condition is circular, whereas the roots of rice in water scarcity are flat (Figure 2.2). Previous studies conducted by Hazman and Brown (2018) found that the lateral rice roots under normal condition have more lignified epidermis, exodermis, and sclerenchyma compared to drought stressed rice roots (Figure 2.3). However, heavy lignification was found on stele of drought stressed root (Hazman & Brown, 2018).

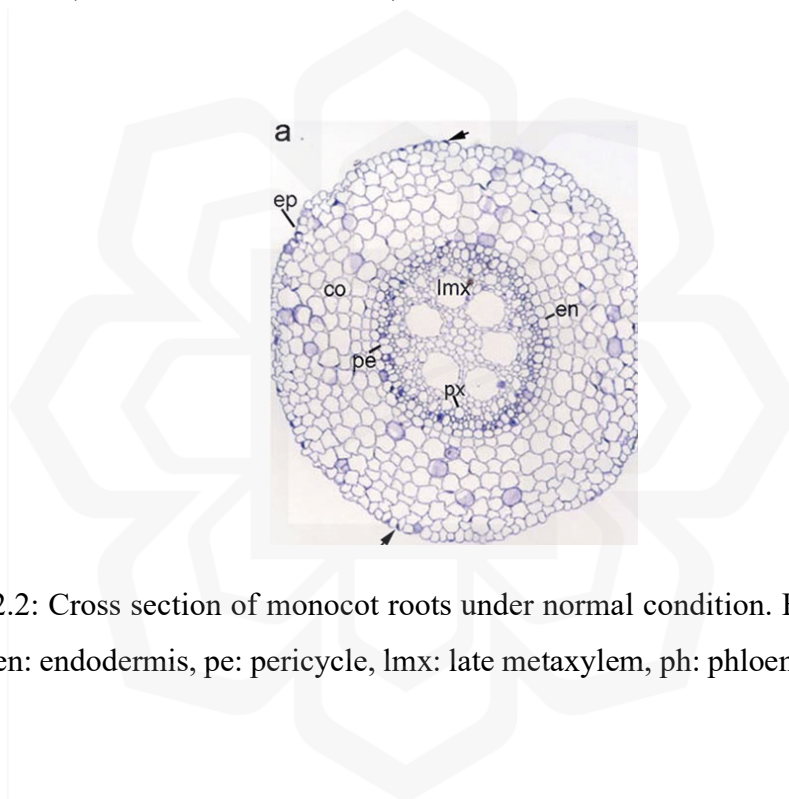


Figure 2.2: Cross section of monocot roots under normal condition. Ep epidermis, co: cortex, en: endodermis, pe: pericycle, lmx: late metaxylem, ph: phloem. (Alarcon et al., 2014)



Figure 2.3: Cross section of rice roots under well-watered (a) and drought stress (b). Water deficit condition caused a shrinkage structure on root with a high lignification on stele. Horizontal bar represents 100 μ m. (Hazman & Brown, 2018)

Moreover, suberin, which functions as a physical barrier, is fewer in sclerenchyma cells but high in the endodermis under drought conditions (Henry et al., 2012). Moreover, aerenchyma cells that facilitate gaseous exchange are not well developed under drought conditions (Hazman & Brown, 2018). Although the formation of aerenchyma is beneficial in the development of drought-tolerant maize (Zhu et al., 2010), Yang et al. (2012) stated that aerenchyma cells in stressed root rice constrain the radial water movement and reduce the water uptake. In terms of water conductive tissue, drought affects metaxylem vessels by constricting them to smaller sizes and fewer in number (Kadam et al., 2015). Hence, further analysis on root anatomy certainly merits valuable understanding of the root acclimation against water stress.

2.7 IMPACT OF DROUGHT STRESS ON ROOT TRANSCRIPTOMIC ANALYSIS

Under water-scarcity conditions, the changes in plants' growth have affected the transcriptomic responses of rice roots. Several research has successfully revealed the transcriptomic analysis of root rice and roots emerged as the focal point of research of drought stress (Huang et al., 2014; Kawakatsu et al., 2021; Lou et al., 2017; Zhou et al., 2020b). Further research on the genome drought-stressed rice has been reported by Wang et al. (2011), who exploited 5284 differentially expressed genes (DEGs) under drought stress. Similarly, Lou et al. (2017) revealed the candidate genes that functioned in root energy metabolism by exploiting 1052 DEGs in deep and shallow roots. Meanwhile, transcriptome profiling of rice root tips by Liang et al. (2021) revealed the functional role reactive oxygen species in mitigating drought stress. Therefore, the variation in transcriptomic analysis could be attributed to gene expression, which later allows the exploitation of DEGs in drought-tolerant varieties.

2.8 ROOT ADAPTATION TO DROUGHT STRESS

Recently, research has increasingly focused on root adaptative traits, particularly root system architecture as it has a pivotal role in absorbing water. Bandurska et al. (2022) mentioned that stress resistance and stress tolerance are often used interchangeably. Generally, plants use three adaptative strategies to endure water deficit conditions: drought tolerance, avoidance, and escape. Drought tolerance refers to the capability of plants to endure water scarcity conditions, even at low tissue water content (Levitt, 1980). Plants adapt to this situation by preserving the cell turgor via osmotic adjustments, cellular elasticity, and high protoplasmic resistance (Abobatta, 2019; Meshra et al., 2022). Meanwhile, drought avoidance aids plants to preserve high turgor by reducing water loss and optimizing water uptake through stomatal regulation, hydraulic conductance, and radiation absorption (Basu et al., 2016; Khan et al., 2018). Lastly, drought escape occurs when plants control their life cycle based on water availability (Basu et al., 2016). Plants will escape the water deficit condition by developing rapidly, shortening the vegetative phase, and causing early flowering. These three strategies, known as drought resistance mechanisms, are associated with physiological, biochemical, morphological, and molecular processes (Abobatta, 2019).

This shows that stress tolerance is a broader term that encompasses the plant's overall ability to withstand stress. Some of the well-known processes that take place in adaptive mechanisms are described in the following sections.

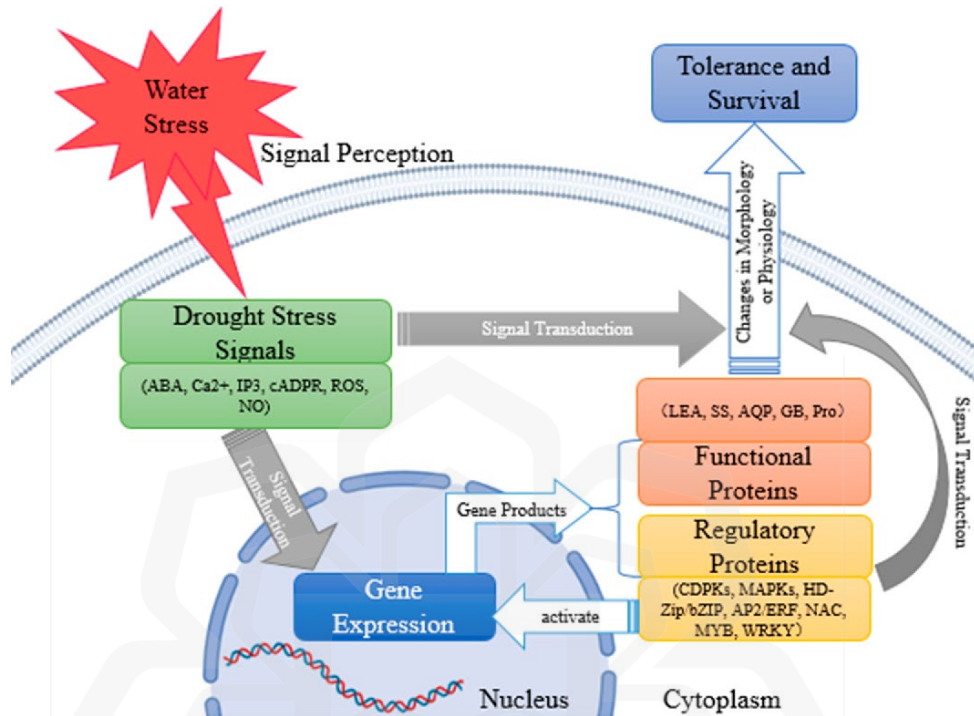


Figure 2.4: The mechanism of plants in response to drought stress (Yang et al., 2021)

2.8.1 Physiological Adaptation to Drought Stress

Under severe drought, plants will close the stomata to minimize transpiration and maximize water use efficiency (WUE) (Abobatta, 2019). The WUE refers to the amount of carbon assimilated in grain produced per unit of water released either through transpiration or used by the crop (Fatichi et al., 2022). WUE in plants plays a significant role in plant survival due to its ability to withstand water-deficient soil. In addition, Hussain et al. (2022) reported that varieties with high WUE exhibited lower water consumption and enhanced grain productivity under drought stress conditions. Thus, increasing WUE in crops becomes the next target for breeding tolerant species (Schulz et al., 2021).

2.8.2 Anatomical Adaptation to Drought Stress

According to Nicotra et al. (2010), varieties with better root anatomical traits in response to drought stress are believed to be promising targets for breeding drought-tolerant plants. Various studies have reported that the anatomical plasticity of roots offers several benefits to plants, such as maintaining water status and enhancing grain yield (Henry et al., 2012; Phoura et al., 2020). Previous studies by Lynch et al. (2021) and Ryser (2004) have shown that roots with a larger diameter are more effective at penetrating compact, fine, and densely packed soils. Furthermore, an increased root area is necessary to provide hydraulic resistance in dry soils, as reported by Kim et al. (2020) and Ryser (2004). A wider root stele has been found to improve water transport under drought conditions (Phoura et al., 2020). Meanwhile, a larger cortical cell in maize, promoted deeper rooting which enhanced the plants adaptation to water deficiency (Chinmungu et al., 2014). In terms of vascular development, Siangliw et al. (2022) reported that the larger xylem area enhances the rice yield under drought stress. Besides that, Comas et al. (2012) also addressed the fact that larger xylem diameters contribute positively to grain yields amidst water deficit conditions. Therefore, root anatomical studies provide valuable insights into root adaptative traits in response to drought stress.

2.8.3 Biochemical Adaptation to Drought Stress

Although stomata closure exhibits drought tolerance traits, it also reduces photosynthesis, eventually restricting carbon dioxide (CO₂) fixation (Wei et al., 2019). The limitation of CO₂ concentration accelerates photorespiration and reduces the role of nicotinamide adenine dinucleotide phosphate (NADP) as a photosynthetic electron transporter, resulting in a high generation of reactive oxygen species (ROS) (Carvalho et al., 2009).

This ROS comprises free radicals and non-free radicals, which can attack and damage proteins, deoxyribonucleic acid (DNA), and lipid molecules (Wei et al., 2019). However, ROS at a low or moderate level could act as signal molecules that induce pathways for plant acclimation under adverse conditions (Choudhury et al., 2017). This beneficial effect, primarily displayed by superoxide and hydrogen peroxide have been reported to promote plant growth and protection against environmental stress (Das &

Roychoudhury, 2014; Nimse & Pal, 2015). In a study of trifoliate citrus, Wei et al. (2019) suggested that increasing the ROS scavenging capacity and sugar accumulation promotes drought tolerance in a stressed plant. Moreover, the level of superoxide and hydrogen peroxide were elevated in the rice leaves under drought stress, suggesting that the changes of free radicals are correlated to drought resistance in rice hybrids (Wang et al., 2010). Recently, Jia et al. (2023) reported the rice OsANN9 promotes drought tolerance mechanism by regulating the ROS scavenging activity. A root tips study by Duan et al. (2010) revealed that stimulating ROS production could stimulate programmed cell death and regulate root system, thus enhancing drought tolerance.

In response to low plant water levels, osmotic adjustments (OA) occur by accumulating solutes such as organic acids, carbohydrates, and free amino acids in the cytoplasm (Blum, 2016). Urmi et al. (2023) also addressed that the accumulation of proline in rice varieties positively affects drought tolerance. As the solute potential is one of the elements in water potential, low water potential results in high turgor maintenance (Turner, 2018). Previous studies on wheat and barley genotypes under drought stress have proved that the OA plays a positive role in increasing grain yield despite water scarcity because it can maintain higher stomatal conductance and delay dehydration (Serraj & Sinclair, 2002; Gonzalez et al., 2008; Moinuddin et al., 2005). In addition, Kang et al. (2022) reported that OA aids plants to keep the water balance and ensure the turgidity of root cells. Thus, the OA adjustments in roots have been identified as an adaptive mechanism of rice under drought stress (Kim et al., 2020).

The role of phytohormones in inducing drought tolerance has been extensively studied by Iqbal et al. (2022). Moreover, Kim et al. (2022a) mentioned that hormonal regulation and its association with root suberization increase drought stress tolerance. Upon exposure to drought, plants incite a signalling cascade, leading to the accumulation of endogenous hormones and the activation of defense mechanisms (Osakabe et al., 2014). The balance of the auxin/cytokinin ratio is vital for plants' adaptation to water deficiency (Kurepa et al., 2022). Auxin mediates drought stress by increasing relative water content (Kim et al., 2022b; Zhang et al., 2020). Besides that, gibberellin-related genes at low levels also trigger drought tolerance and other abiotic stresses. Ito et al. (2018) reported that inhibition of the gibberellin activity pathway displays a tolerance effect against drought stress. Studies by Allagulova et al. (2020)

have proved that the low application of methyl jasmonate (0.1 μ M), shows a protective effect on the growth and membrane integrity of wheat, upon exposure to drought treatment.

2.8.4 Molecular Adaptation to Drought Stress

Root molecular analysis by Lou et al. (2017) reported the crucial role of metabolic pathways in contributing to deeper rooting. Interestingly, few studies addressed the protein DEEPER ROOTING1 (DRO1), which promotes deeper rooting under drought stress (Arai-Sanoh et al., 2014; Vinarao et al., 2023). The mechanism of deeper rooting has been identified as drought avoidance, which enables the root to access water from deep soil layers (Singh et al., 2021; Uga et al., 2013). Thus, DRO1 has been identified as a beneficial gene in the root under drought stress. Besides that, previous studies reported hormonal-related genes mediating drought resistance (Iqbal et al., 2022). The modulation of RSA is influenced by the phytohormones, which affect cell division, elongation, and differentiation (Yetgin, 2024). Notably, Zhang et al. (2020) discovered that the indole acetic acid (IAA) gene was involved in mitigating plant dehydration by stimulating the relative water content. Specifically, the GRETCHEN HAGEN gene, which is an auxin response gene, influences cell division, expansion, and root elongation (Feng et al., 2011; Guo et al., 2022; Hu et al., 2006). In addition, higher expression of deactivator cytokinin results in a larger root system, which promotes drought tolerance (Ramireddy et al., 2018; Werner et al., 2010). In another root study, Barker et al. (2020) stated that the gibberellin oxidase gene is a necessary gene in both the meristematic and root elongation zones. Therefore, the elucidation of hormonal-related genes is beneficial to enhance understanding of drought adaptation mechanisms.

2.9 SUBSEQUENT DROUGHT STRESS AND RICE ACCLIMATION

According to Kwanyuen et al. (2021), alternate wetting and drying (AWD) has been introduced by International Rice Research (IRRI) about 20 years ago. Previous literatures reported AWD as ‘intermittent irrigation’, ‘pulsed irrigation’, and ‘cyclic wetting and drying’ (Dahlgree & Parr, 2024). Recently, Jing et al. (2023), AWD has become a widespread practice in rice production in several Asian countries. The

recurring drying phases have opened up the concept of acclimation to water deficits. Charng et al. (2023) reported that acclimation aids the plant's stress memory by responding more effectively to subsequent stresses. In addition, Godwin et al. (2020) provided a molecular perspective on stress memory under abiotic stresses. Generally, acclimation refers to the ability of plants to adapt rapidly at the physiological level in response to environmental stress (Kleine et al., 2020). In contrast to adaptation, which involves evolutionary changes, acclimation is a temporary and reversible adjustment of non-genetic changes in phenotype (Bennett & Lenski, 1997). Bandurska et al. (2022) reported that acclimation is associated with plant plasticity and requires activating various complex cellular and molecular responses. Therefore, integration of alternating drought stress could provide insights on the root acclimation to drought stress.

2.10 CHALLENGES IN ROOT TRANSCRIPTOMIC ANALYSIS

Although the root plays a pivotal role in water uptake, it is noteworthy to mention that RNA extraction using belowground parts is troublesome and seldomly used for transcriptomic studies (Matsunami et al., 2018; Wang et al., 2011). RNA extraction using the root becomes challenging as the quality and quantity of RNA are highly dependent on the type of genotype, planting condition, and growth stage (Matsunami et al., 2018). Earlier studies have demonstrated alternative strategies to extract adequate and pure RNA from root plants without soil contamination. These steps include reducing the root-washing time during sample collection, which should be within one minute to avoid affecting gene expression (Okamura et al., 2020).

Besides that, the observation of the total RNA amounts in the rice roots declined as the paddy growth progressed, especially after the panicle emergence. Matsunami et al. (2018) extracted a high yield of RNA from paddy root, which accounts for about 404 ng/ μ l at the early stage, instead of 34 ng/ μ l at the booting stage. In addition, the RNA yield extracted from fluminol paddy was 918 ± 143 ng/ μ l at 58th days after sowing (DAS), compared to 684 ± 92 ng/ μ l at 83rd DAS. Therefore, root collection during the early stages of paddy growth is recommended to improve RNA quality.

2.11 APPROACHES ON BREEDING OF DROUGHT-TOLERANT RICE

In developing countries where rice is a main source of livelihoods, ensuring its production becomes critical focus especially in regions with prone to water scarcity (Wassmann et al., 2009). The increasing temperatures and robust rainfall patterns, due to climate change, cause several traditional rice varieties are less viable. Hence, the implementation of drought-tolerant rice offers significant potential in addressing key global challenges. Considering the importance of food security, the adaptability of drought tolerant rice aids farmers to sustain yields even under adverse environmental conditions, thus making grain production more resilient. The Malaysian Agricultural Research and Development Institute (MARDI) has evaluated the performance of several rice drought tolerant such as MR219, MRQ76, MR253 and MRQ74 (Razak et al., 2021). Aside from food security, cultivation of drought tolerant rice can reduce the usage of irrigation, leading to more sustainable water needs in agriculture. This trait benefits the smallholder farmers by reducing their reliance on freshwater supplies, simultaneously promoting the overall sustainability of agricultural systems (Xu et al., 2014). Therefore, it becomes vital for breeders to implement the most effective strategies in breeding drought-tolerant rice.

The conventional breeding approaches in rice involve two major components: the genetic variation in germplasm and the effective screening protocol under several field conditions (Anilkumar et al., 2023). Conventional breeding involves selective methodology based on superior performance. Usually, grain yield becomes the key selector for the superior cultivars (Madhu et al., 2022; Upadhaya et al., 2013). Several traditional breeding strategies have been developed, such as pedigree selection, recurrent selection, backcrossing breeding, and induced mutation (Madhu et al., 2022). Poli et al. (2013) reported that pure-selection from traditional rice landraces has successfully developed heat- and drought tolerant rice varieties such as N22. In spite of these approaches, conventional breeding is time-consuming, as it would take more than 4 to 5 years to disseminate a new cultivar (Bharti & Chimata, 2018; Breseghello & Coelho, 2013). In addition, the success of these methods relies on the skill and subjective analysis of breeders (Lamichane & Thapa, 2022). Therefore, conventional breeding is less effective and less reliable.

With the advances of science and technology, the modern breeding strategies have paved the way for developing drought tolerant rice. Quantitative analysis through mapping of quantitative trait loci (QTL) aids in screening of targeted phenotypes and unravelling the diverse genetic backgrounds (Andrade et al., 2020). Molecular markers are widely utilized to evaluate the genetic control of the traits interest (Marsh et al., 2021). This field of study known as molecular biology is integrated into a plant breeding programme, which enables the exploitation of beneficial genes that corresponds to the tolerant traits (Breseghello & Coelho, 2013). Recently, the advanced technology of marker assisted selection (MAS) such as amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), and random amplified polymorphic sequences (RAPD) has become popular and widely utilized in breeding programmes (Amiteye et al., 2021).

In addition, the new biotechnologies such as clustered regularly interspaced short palindromic repeats (CRISPR), and next-generation sequencing (NGS) offers effective solution in breeding of stress tolerance crop (Zhou et al., 2018; Zhou et al., 2022). The accuracy of CRISPR-associated protein 9 (Cas 9) allows the specific editing of targeted genome sequences, minimizing the risk of unexpected and off-target changes in the genome (Gan & Ling, 2022; Park et al., 2022; Schiml et al., 2016). For example, Park et al. (2022) reported the edited of the drought-induced gene *Oryza sativa* Senescence-associated protein (OsSAP) using the CRISPR/Cas9 positively regulates drought responses in rice. Meanwhile, NGS, coupled with RNA-sequencing, has significantly refined the rice annotation while providing a wide array of genetic expressions and elucidation of the transcription component at the whole-genome scale (Gu et al., 2022). Furthermore, Zhou et al. (2018) reported the efficient application of NGS in the identification of the functional genes. This high-throughput sequencing system offers several benefits over microarrays such as enabling the precise and reproducible measurement of transcript abundance (Gan & Ling, 2022). It also provides a wider dynamic range by using a few RNA samples (Fullwood et al., 2009). Therefore, these advanced genomic and novel sequencing technologies reshaped the breeding revolution into a great rice improvement programmes.

CHAPTER THREE

EFFECT OF ALTERNATE AND CONTINUOUS DROUGHT STRESS ON AGRONOMIC TRAITS OF RICE

3.1 INTRODUCTION

A number of studies have integrated assessments of the agronomic performance of rice to evaluate its susceptibility to abiotic stress. Agronomic traits such as panicle length, grain length, number of effective panicles, number of filled grains, seed length, growth period, and grain weight have been major criteria in the selection of breeding initiatives. (Chen et al., 2021a; Huang et al., 2013; Tshikunde et al., 2019). However, amongst them, yield potential becomes the paramount parameter, profoundly affected by water availability (Araus, 2002; Li et al., 2019). Notably, rice varieties that produce higher grain yields despite drought stress have become a targeted selection for rice breeders. Therefore, understanding the effect of drought stress on the agronomic traits of rice is essential in crop breeding programmes (Tshikunde et al., 2019).

According to Jing et al. (2023), alternating wetting and drying (AWD) irrigation has become a common practice in rice production in several Asian countries, such as China, Vietnam, Bangladesh, and India. Instead of continuously flooding the field, AWD allows the soil to dry out for several days before re-flooding, which improves water savings as well as enhances grain yields (Jing et al., 2023). It was assumed that the integration of alternate drought cycles broadened the concept of root acclimation to water deficits. Nevertheless, Zhang et al. (2018) reported that the high intensity of drought stress caused severe and irreversible damage to agronomic traits, depending on the intensity, period of drought, and growth phases of the paddy plant. As water deficit condition is a critical factor in paddy plantations (Zeng et al., 2023), it was hypothesized that grain yield under alternate drought stress was higher than that under continuous drought stress. This preliminary study aimed to evaluate the relationship between drought stress and agronomic traits of rice. Taken together, the findings of this study provided insights on agronomic traits, particularly yield stability under drought stress.

3.2 METHODOLOGY

3.2.1 Experimental Design

The experiment was conducted in the Glasshouse and Nursery Complex (GNC) at International Islamic University Malaysia, Kuantan Campus, Kuantan, Pahang. The six rice varieties were selected, which were Nona Manis, Merah Wangi, Moroberekan, MR297, Biris, and Apami (Table 3.1). The selection of these varieties was based on its superior performance in root mitotic activity, as observed in the previous study (Rosely, 2023). MR297 was selected as the control plant. This domestic grown rice variety has a high harvesting yield (Esa et al., 2020). Three treatments; control, alternate drought, and continuous drought were performed with a minimum of three replicates. For each replication, three seedlings were planted in the polybag. Temperature and ventilation were maintained throughout the experiment. The seeds were pre-germinated for five days before being transferred into 30 cm x 12 cm polybags. All polybags were filled with 100% topsoil and kept under water-saturated conditions before the transplanting of the seedlings was done.

Table 3.1 The Six Rice Varieties and its Accession Number

Rice Variety	Accession Number
MR297	MRGB12120
Merah Wangi	MRGB03693
Moroberekan	IRGC117621
Apami	MRGB00142
Biris	MRGB06891
Nona Manis	IIUM-R00213

Referring to Figure 3.1a, under the control treatment, the standing water was maintained 2 to 3 cm above the soil surface throughout the experiment. Meanwhile, the

alternate drought treatment was initiated at the 83rd day after sowing (DAS) by allowing the soil to dry gradually for seven days and then restored to normal water levels for another seven days (Figure 3.1b). This treatment was repeated twice, and it simulates the intermittent irrigation that was implemented two decades ago (Kwanyuen et al., 2021). Similarly, the continuous drought treatment was conducted on the 83rd DAS. The standing water was removed from the soil surface and maintained dry during the study period (Figure 3.1c). Both drought stress was applied during milking stage in the reproductive phase. The fertilizers Nitrogen: Phosphate: Potassium (NPK 15:15:15) was applied once every two weeks. During the reproductive phase, fertilizer NPK 12:12:17 was applied. Meanwhile, weed and pesticide management were conducted weekly.

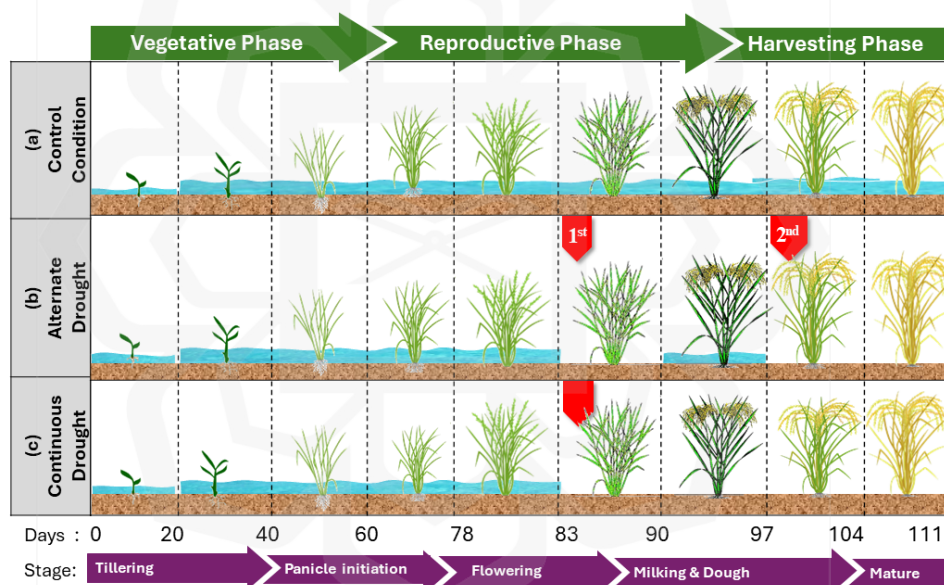


Figure 3.1: Infographic on the experimental design of agronomic analysis. The paddies were planted in the polybags with three replicates for each variety. Control treatment involves normal watering throughout the experiment (a). Alternate drought stress involves two recurring drought phases. The first drought cycle started on 83rd DAS. The water was removed from the polybags. After seven days, the water was restored on 90th DAS. The second drought cycle started on 97th DAS. The water was removed and the soil remained dry till the sample collection (b). Continuous drought stress involves removing the water on 83rd DAS and the soil was maintained dry till the sample

collection (c). The red arrow refers to the day when water was removed from the polybag, signaling the onset of the drought phase.

3.2.2 Data Collection

The determination of plant growth parameters and harvest index was based on the methods described by Amanullah & Inamullah (2016) and Elixon et al. (2019). The six rice samples (three plants per variety) within each treatment were harvested on the 104th DAS. The tillers were counted during the flowering stage. The leaves, culms, and panicles were segregated and dried in paper bags in an oven dryer at 60°C until a stable weight was attained. The dried plant components were then weighed, and the data were recorded as aboveground biomass (g/plant). The filled grains were detached from panicles to calculate the total number of seeds. Then, the grains were weighed to record the grain weight (g). The grain yield was measured by converting the gram per area of polybag (g/m³) to ton per hectare (t/ha). Based on Paiman and Effendey (2020), the harvest index was calculated accordingly:

$$\text{Harvest index (\%)} = \frac{\text{Grain weight}}{\text{Aboveground biomass}} \times 100$$

3.2.3 Statistical Analysis

The data's means between varieties were subjected to one-way analysis of variance (ANOVA) with Fisher's LSD (least significant difference) test ($p \leq 0.05$) using the GraphPad Prism version 9.0. The results were tabulated using the bar graph. The letter groups were designated for each variety means within the treatment, and means with the same letters were referred to as not statistically different. The stars indicate the level of significance of the means in comparison with the control MR297.

3.3 RESULTS

3.3.1 Effect of Drought Stress on Grain Yield

Li et al. (2021) demonstrated that grain weight depends on the dimensions of the grain, including length, width, and thickness. The analysis of a two-way ANOVA shows that the interaction between variety and treatment did not yield statistical significance concerning grain yield among all treatments. However, within the control treatments, there was a significant variation between the varieties. Merah Wangi produced the highest grain yield, significantly followed by MR297, whilst Biris recorded the lowest yield (Figure 3.2). Remarkably, Merah Wangi was the only variety that displayed a significant deviation from MR297. In addition, there was no significant difference in the yields of Merah Wangi relative to Moroberekan, Apami, and Nona Manis.

Under the condition of alternate drought, Merah Wangi demonstrated the highest grain yield of all other varieties, notably surpassing that of MR297 - grown under normal watering. However, it was observed that Nona Manis, Apami and Biris had significant differences while being lower than control MR297. Moreover, these three varieties ranked among the lowest yield producers. Under continuous water shortage, all the varieties had significant differences from the control MR297, while the least significance was seen in Merah Wangi. In addition, the drought-stressed Merah Wangi and Moroberekan were ranked among the highest yields. Meanwhile, Biris and Apami were among the lowest yields and extremely different from control MR297.

However, significant differences were noted in Nona Manis, Apami, and Biris, all of which yielded lower than the control, MR297. Furthermore, these three varieties ranked among the lowest grain producers. Amid continuous water scarcity, all varieties exhibited significant deviations from the control, MR297, with Merah Wangi demonstrating the least divergence. Additionally, drought-stressed Merah Wangi and Moroberekan ranked among the highest yielders, while Biris and Apami were among the lowest, displaying substantial differences from the control, MR297 (Figure 3.2).

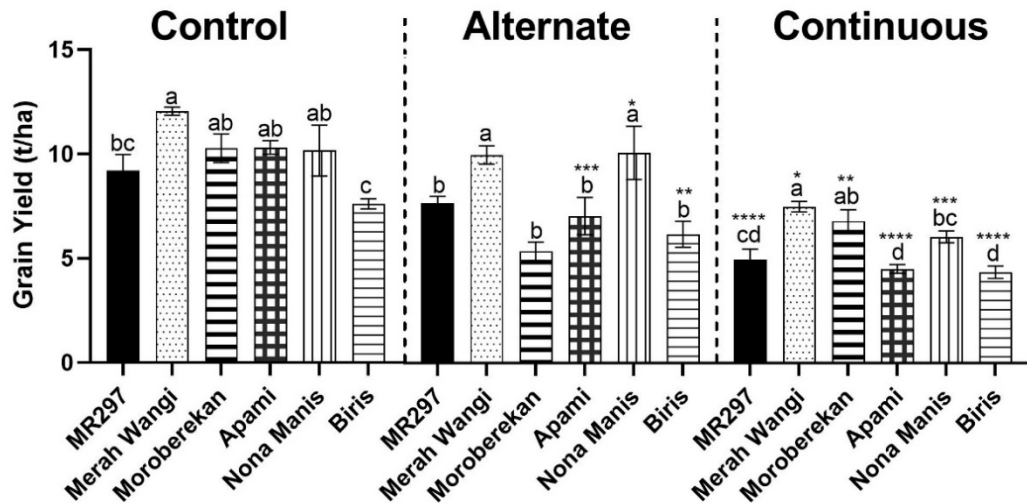


Figure 3.2: Grain yield (t/ha) of six rice varieties in response to control, alternate drought and continuous drought treatment. Data were analysed using one-way ANOVA to test significant differences ($p \leq 0.05$) among the treatments means. Means with same letters are not significantly different. The data exhibited was the comparisons within the same water treatments. The stars (****) exhibited a significant level of variety as compared to the control MR297.

3.3.2 Effect of Drought Stress on the Number of Grains/Plant

Filled grains per panicle is a direct trait that influences the rice's yield potential (Zeng et al., 2017), thus becoming a main focus in rice breeding programmes. In this experiment, the number of filled grains was calculated for each panicle. A two-way ANOVA analysis revealed that the interaction between varieties and treatments was extremely significant. Under control treatment, the highest number of filled grains was produced in Merah Wangi, surpassing the MR297 while being significantly different from the other varieties (Figure 3.3). Conversely, Apami and Moroberekan displayed the lowest filled grain counts. In the condition of alternate drought stress, it was observed that Merah Wangi and Apami produced the highest and lowest number of grains, respectively, showing significant differences from other varieties. Meanwhile, there was no significant difference observed between Merah Wangi and MR297 as top seed producers, indicating their resilience to water scarcity relative to Apami, Moroberekan, Biris and Nona Manis under the same treatment (Figure 3.3).

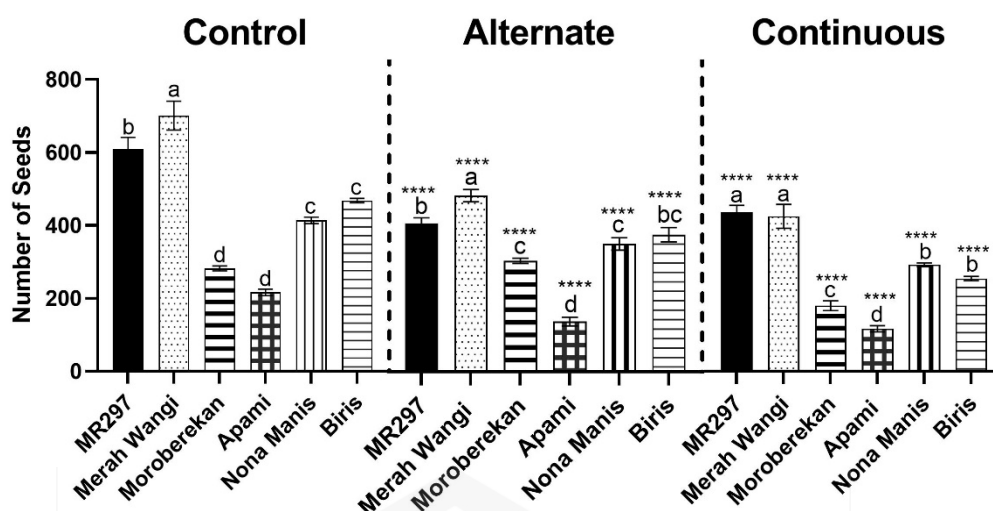


Figure 3.3: Number of seeds in response to control, alternate drought and continuous drought treatment. Data were analysed using one-way ANOVA to test significant differences ($p \leq 0.05$) among the treatments means. Means with same letters are not significantly different. The data exhibited was the comparisons within the same water treatments. The stars (****) exhibited a significant level of variety as compared to the Control MR297.

3.3.3 Effect of Drought Stress on Harvest Index (HI)

The harvest index played a crucial role in the enhancement of grain yield (Zhang et al., 2013). The analysis of a two-way ANOVA revealed a significant interaction effect between variety and treatment on the HI. Under control treatments, there was a significant variation between the varieties studied (Figure 3.4). Merah Wangi and MR297 have shown significantly HI than that of other varieties, whilst Biris recorded the lowest. After alternating two cycles of drought treatment, no significant difference was detected amongst the varieties. However, relative to MR297 grown under normal conditions, all varieties had significantly lower HI. Meanwhile, MR297, Merah Wangi, and Biris were among the varieties with high HI. In the continuous drought stress treatment, Merah Wangi had the highest HI but scored no significant differences with other varieties except for Nona Manis (Figure 3.4). Although one-way ANOVA analysis at $p \leq 0.05$ did not indicate significant treatment effects on HI, all varieties tested were markedly different from the MR297 grown in the control condition.

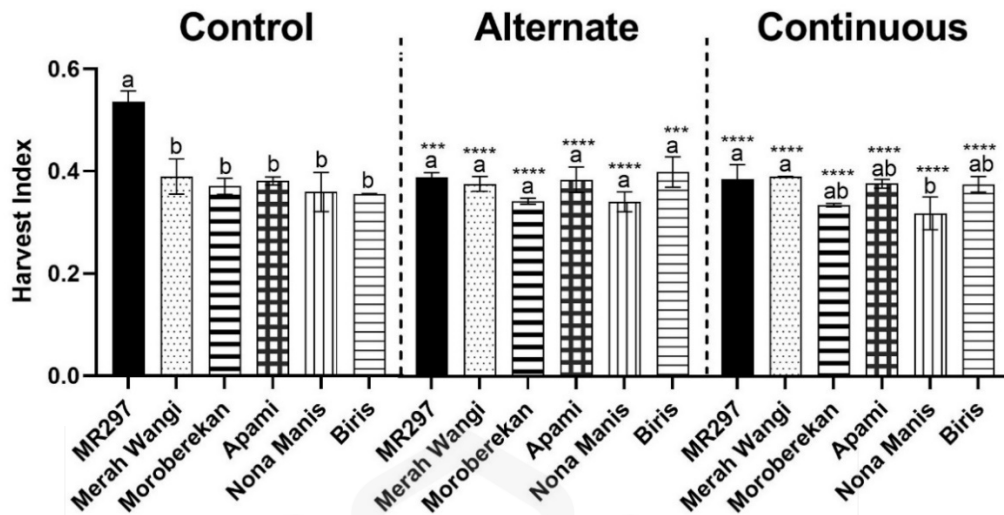


Figure 3.4: Harvest index of six rice varieties in response to control, alternate drought and continuous drought treatment. Data were analysed using one-way ANOVA to test significant differences ($p \leq 0.05$) among the treatments means. Means with same letters are not significantly different. The data exhibited was the comparisons within the same water treatments. The stars (****) exhibited a significant level of variety as compared to the Control MR297

3.3.4 Effect of Drought Stress on Number of Effective Tillers

According to Zhou et al. (2022), effective tillers refer to the tillers that are able to produce panicles. Panicle architecture is one of the most important agricultural traits that will determine branch and grain numbers in rice. Based on a two-way ANOVA, it was discovered that the interaction between treatments and rice varieties was significant. Under normal conditions, the results showed that Merah Wangi had the highest effective tiller numbers, followed by MR297, Apami and Moroberekan, although no significant difference was found amongst them (Figure 3.5). In contrast, significantly lower effective tiller numbers were recorded for Nona Manis and Biris. When plants were treated in an alternating drought, it was observed that MR297 produced the highest effective tillers with no significant difference compared to Merah Wangi (Figure 3.5). Meanwhile, Moroberakan had the lowest tiller number, which was not statistically different when compared to Nona Manis and Biris. Moreover, these varieties exhibited a significant difference in tiller counts compared to MR297 under

well-watered conditions. Under continuous drought, MR297 and Merah Wangi were identified as the top tiller producers, while Apami exhibited the lowest numbers (Figure 3.5). Notably, the tiller numbers across all cultivars were significantly lower compared to the control, MR297, indicating a substantial impact of prolonged drought stress on tiller production.

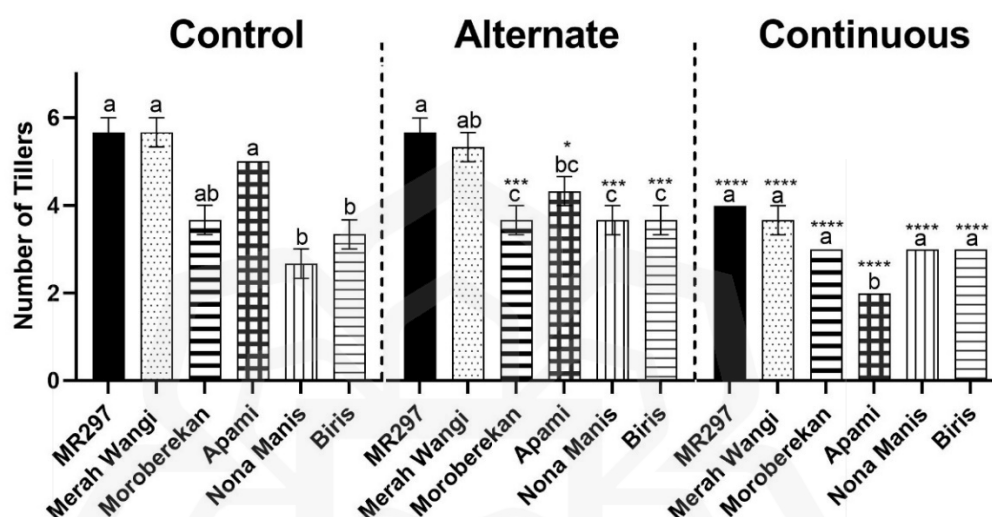


Figure 3.5: Number of effective tillers in response to control, alternate drought and continuous drought treatment. Data were analysed using one-way ANOVA to test significant differences ($p \leq 0.05$) among the treatments means. Means with same letters are not significantly different. The data exhibited was the comparisons within the same water treatments. The stars (****) exhibited a significant level of variety as compared to the Control MR297.

3.4 DISCUSSION

3.4.1 Effect of Drought Stress on Grain Yield

Based on Figure 3.3, Merah Wangi produced the highest number of grains under normal watering, outyielding MR297. A similar pattern was also observed in grain yield in the control treatment, with Merah Wangi recording 12.01 t/ha (Figure 3.2). According to Grisp (2013), irrigated lowland rice systems, including Malaysia's granary areas have an average production of 5.4 t/ha. Meanwhile, the national average rice yield accounts for 4.2 t/ha. It was assumed that the great production of Merah Wangi was attributed to

the growth conditions because this plant was grown in pots, in contrast to the field conditions that have higher plant density (Saju et al., 2019). Moreover, Sales et al. (2022) also discovered the phenotypic variation of wheat under glasshouse relative to field condition. It is noteworthy to mention that the growth environment influences crop productivity.

In the alternate drought treatments, upon reaching the milking phase, the water supply interruption during the second cycle reduced panicle development, ultimately leading to a lower yield compared to the control treatment. Nonetheless, the rice varieties in the alternate drought treatment were observed to have better grain numbers and yield/ha than in the continuous drought treatment (Figure 3.3). Similarly, Jing et al. (2022) also reported high grain yields under alternating drying irrigation. Thus, the findings have validated the initial hypothesis that grain yield under alternating drought stress was better than that under prolonged drought stress. Notably, alternate wetting and drying (AWD) in rice cultivation significantly enhances grain yields, shoot growth, and water use efficiency (WUE) (Jing et al., 2022; Zhang et al., 2013). Aside from being water-saving strategies, the AWD enhances the crop to produce effective tillers and develop stronger roots (Mote et al., 2020). Specifically, Isnawan and Ansori (2021) found that the root's length under AWD was longer under conventional irrigation, resulting in a higher yield. Nonetheless, it remains unclear how AWD increases rice yield. However, Charng et al. (2023) reported that plants that undergo intermittent abiotic stress have employed stress memory through acclimation. Therefore, additional studies are required to comprehend the physiological mechanism of roots under alternating drought stress.

During this extreme drought stress, the grain yield of all varieties was below 9 t/ha, with the highest yield seen in Merah Wangi (Figure 3.2). Previous studies show that the milking stage in rice was the most affected by drought stress (Zhao et al., 2023). The visual observation of the continuous drought stress proved that the water scarcity initiates a sequence of adverse events, including root drying, subsequent panicle collapse, and grain abortion (Ji et al., 2010). This phenomenon might be the cause of a low grain yield, in accordance with the report by Zhang et al. (2018). Furthermore, Ji et al. (2010) reported that a water deficit that occurs during the flowering stage can impact

pollen fertility. These collective findings demonstrated the importance of optimum irrigation during the late grain-filling stage.

3.4.2 Effect of Drought Stress on Harvest Index (HI)

Based on Figure 3.4, the results revealed that the MR297 had the highest HI under control irrigation with a value of 0.53, followed by Merah Wangi at 0.39. The average HI for the modern crop varieties ranged from 0.4 to 0.6, although these values may vary depending on the variety and external factors (Yang & Zhang, 2010). Furthermore, this study observed that alternate and continuous drought stress had no significant effect on the HI of rice varieties (Figure 3.4). In fact, none of the varieties in both drought treatments met the standard HI. This marginal effect of water stress aligns with the findings of Bwire et al. (2022). The results were also similar to Siangliw et al. (2022), who discovered that the HI during the water scarcity was reduced by 87% compared to the control condition. In addition, Elkheir et al. (2018) observed that several rice varieties exhibited similar HI due to concurrent flower initiation and grain filling rates, which may affect the final grain yields. Nonetheless, Merah Wangi consistently produced the highest HI in both drought treatments, highlighting its qualities as a high-yielding variety even under condition of water shortage. Therefore, Merah Wangi could be categorized as one of the most potential high-yielding varieties.

3.4.3 Effect of Drought Stress on Tillers Growth

The agronomic analysis delves into the impact of drought stress on rice growth by examining the tiller numbers. The plants in all treatments were subjected to drought after the booting stage, a developmental phase marked by the emergence of tillers primed for panicle and flower initiation. This study found that the number of tillers in the alternating and continuous drought stages was lower than that in the control condition (Figure 3.5). In the alternate treatment, the panicle growth reverted to its normative patterns upon reinstatement of irrigation, suggesting a limited impact of short-term drought on the tiller's number, as compared to the control condition. As seen in Figure 3.5, the tiller's number peaked at MR297, followed by Merah Wangi. Furthermore, it was shown that Nona Manis generated more tillering in the alternate

drought treatment than in the control condition. This observation was similar to Zhao et al. (2023), who found that short-term drought could enhance the photosynthetic activity in droughted plants, potentially facilitating increased tiller production in some varieties during water stress.

However, during the prolonged drought period, the water scarcity led to the degeneration of effective tillers, aligning with earlier studies (Yu et al., 2022; Zhao et al., 2023). All varieties experienced a significant loss of tillers compared to the control of MR297. In addition, Hu et al. (2023) also observed that severe water deficit and flooding suppressed the rate and quantity of tillers, consequently resulting in low rice output. The superior panicles that degenerated could be attributed to inadequate soil moisture levels necessary for sustaining the root system (Rotili et al., 2021). Despite the water stress, Merah Wangi and MR297 recorded high tiller numbers, suggesting that both varieties possess high-tillering characteristics (Figure 3.5). Nevertheless, it is notable that a robust tillering capacity did not invariably correlate with successful grain filling, as evidenced by the case of MR297 in this investigation. Despite its higher tillering capacity, MR297 demonstrated significantly lower grain yield under both alternate and continuous drought compared to Merah Wangi. It was unsurprising, as Huang et al. (2020) reported that the total grain yield was not significantly associated with the secondary tillers. Approximately 85% of the grain yield is contributed by the superior primary tillers (Huang et al., 2020). Thus, these findings suggest that high tillering capacity does not necessarily promise greater grain yield. In addition, tiller quantity varied among rice species, and it is regulated by environmental factors and genetic regulation (Hussien et al., 2014).

3.5 CONCLUSION

In summary, drought stress resulted in a variation in the agronomic traits of six rice varieties. The interaction between variety and treatment did not have a notable impact on grain yield. However, the individual treatments clearly showed a significant effect of drought stress on agronomic traits. Plants under alternate drought stress performed better than those under prolonged drought exposure. However, both drought stress resulted in a lower harvest index compared to the control condition. It is worth mentioning that Merah Wangi produced the highest grain yield under alternate drought

stress, signifying that it may have acclimated to drought stress. Similarly, the top grain achievers under prolonged drought stress, such as Merah Wangi, Moroberakan, and MR297 may possess drought tolerance. Collectively, it was suggested that these adaptative traits may correspond to the plasticity of roots which enables them to withstand water deficit conditions. Therefore, this agronomic study provides valuable insights on rice performance under drought stress as well as becoming a preliminary study for root adaptative traits.



CHAPTER FOUR

EFFECT OF ALTERNATE AND CONTINUOUS DROUGHT STRESS ON ROOT ANATOMICAL CHARACTERISTICS OF RICE

4.1 INTRODUCTION

According to Shafi et al. (2023), the development of robust root systems is vital to maintain good growth and development during drought conditions. The root system not only facilitates water absorption and uptake but also serves as a primary defense mechanism against the detrimental effects of drought stress (Daryani et al., 2022). Interestingly, grain yield has significant associations with root phenotypes under drought conditions (Kaysar et al., 2022; Sandhu et al., 2017; Sofi et al., 2021). Moreover, the preliminary study (Chapter 3) confirmed the detrimental effect of drought stress on the agronomic traits of rice. Nevertheless, the relationship between roots and grain yield remains relatively understudied.

Root plasticity refers to the ability of a root to alter this phenotype in response to rapid environmental changes (Sultan, 2000). The plasticity of root phenotypes could be reversible or irreversible, and it is correlated with adaptation and acclimation mechanisms, which could be adaptive traits to alleviate drought stress (Schneider & Lynch, 2020; Sjulgard et al., 2021; Uga et al., 2013). As discussed earlier, acclimation to an adverse condition involves reversible changes in root alterations, which could be observed through anatomical study. Root anatomy is a fundamental study that displays root structural adaptation to dynamic environmental conditions (Evert, 2006; Simpson, 2019).

Varieties that exhibit better root anatomical features while producing stable yields under drought stress are considered to be valuable breeding targets to mitigate climate change (Nicotra et al., 2010). Therefore, this root anatomical analysis aimed to identify the variation between six paddy varieties in response to different drought conditions. It was hypothesized that different conditions of drought stress could induce changes in root anatomical characteristics.

4.2 MATERIALS AND METHODS

4.2.1 Experimental Design

Referring to the previous methodology for agronomic analysis, the six rice varieties were re-planted with a slight modification. Unlike in agronomic analysis, the exposure of drought stress in this study was conducted at vegetative stage, in line with a study by Siangliw et al. (2022). This step ensured better root growth for sample collection. The vegetative stage involves a transition of juvenile to adult stage, in which rice actively develops tillering, shoots and roots via the organogenesis process (Dadras et al., 2020; Sluis & Hake, 2015). Thus, phenotyping the root under vegetative stage is a promising strategy to elucidate the functional role of roots under drought stress.

Based on Figure 4.1, the alternate drought treatment was initiated at 33rd DAS by allowing the soil to dry gradually for seven days and restored to normal water level for another seven days. This treatment was repeated twice. Meanwhile, the continuous drought treatment was conducted at 40th DAS, slightly delayed from alternate drought stress to allow better root development. The standing water was removed from the soil surface and maintained dry during the study period. As the tillering rate differs depending on the rice varieties, the period to initiate the drought treatment was different. The fertilizers NPK 15:15:15 was applied once every two weeks. Meanwhile, weed and pesticide management were conducted weekly.

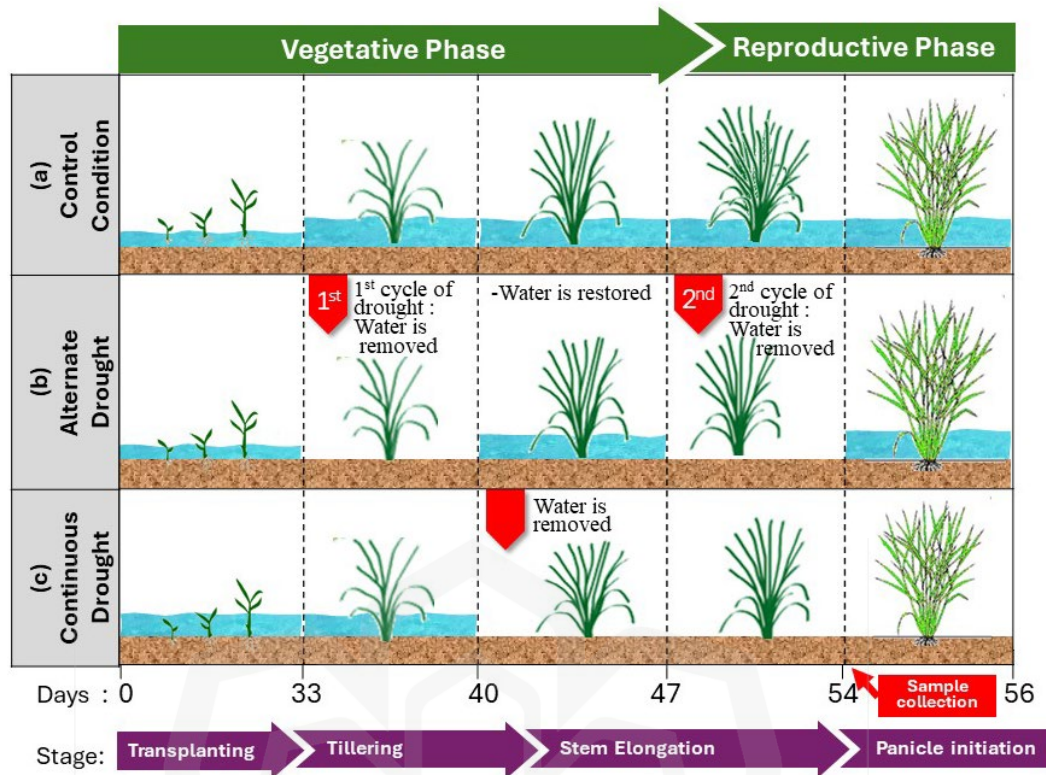


Figure 4.1: Infographic on the experimental design of anatomical analysis. The paddies were planted in the polybags with three replicates for each variety. Sample was collected on 54th DAS above. Control treatment involves normal watering throughout the experiment (a). Alternate drought stress involves two recurring drought phases. The first drought cycle started on 33rd DAS. The water was removed from the polybags. After seven days, the water was restored on 40th DAS. The second drought cycle started on 47th DAS (b). Continuous drought stress involves removing the water on 40th DAS and the soil was maintained dry during the study periods (c). The red arrow refers to the day when water was removed from the polybag, signaling the onset of the drought phase.

4.2.2 Sample Preparation

Based on Shafi et al. (2023), rice crown root has a functional role in the main pathway of water transportation. Thus, the crown root samples were collected starting from the 54th DAS. For the continuous drought treatment, the leaf scrolling score and drought score was used as visible reference for the samples collection. The root samples were excised once the leaf exhibited leaf scrolling score number 5 (O'Toole et al., 1979)

(Figure 4.2) and the drought score number 8 (Table 4.1) (Fen et al., 2015). The thick nodal in deep parts of the root system from three treatments were excised about 5 to 6 cm for further analysis. The freshly obtained root was washed with tap water to eliminate impurities and immediately kept in glass jars with fixative solution (methyated spirit 10% ethanol).

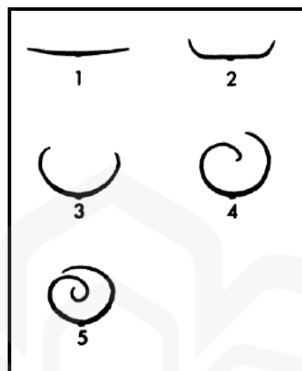


Figure 4.1 The Leaf Scrolling Score

Source: (O'Toole et al., 1979).

Table 4.1 The Corresponding Drought Score

Source: (Fen et al., 2015)

Drought Score	Description
1	No symptoms of stress effects
2	Slight tip drying
3	Tip drying extended to $\frac{1}{4}$ length in 25% of all leaves (normally the older leaves)
4	Tip drying extended to $\frac{1}{4}$ length in at most 50% of all leaves
5	Tip drying extended to $\frac{1}{4}$ length or more in 50% of all leaves with 25 % of leaves fully dried
6	50% of all leaves fully dried
7	More than 50% but less than 70% of all leaves fully dried

8	70% of all leaves fully dried
9	More than 70% of all leaves fully dried
10	All plants apparently dead

4.2.3 Cross Section and Cleaning

The root sample was cut approximately 7cm from the root tip. Cross sectioning was carried out using a sliding microtome at a thickness of 60-100µm. Polystyrene was used as a sample holder with a deep hole to fit the tiny size of the root. Then, the cut section located either on a razor blade or stick together with polystyrene were obtained using a wet paintbrush and immersed in the petri dish containing distilled water with five drops of Clorox with concentration of 7.4% sodium hypochlorite. After 4 minutes to allow surface sterilization, the samples were then cleansed thoroughly with distilled water.

4.2.4 Staining Process

The staining process was done using Safranin and Alcian blue solution. A few drops of Safranin were dropped into a petri dish containing distilled water with specimens and was left for 2 minutes. The sliced tissue was then rinsed with distilled water to remove the excess stain. Next, four to five drops of Alcian blue solution were dropped into the petri dish and left for another 4 minutes.

4.2.5 Dehydration and Mounting

The specimens were exposed to a series of ethanol concentrations (50%, 70%, 95%, and 100%) approximately for 3 minutes for rehydration. The dehydration process is vital to remove the remaining water residue in the specimen's tissue. The specimens were then placed on the microscope slide and mounted using Euparal mountant. Each microscope slide was labelled before drying in an oven with a temperature of 50°C for 1 week.

4.2.6 Observation

The root specimens were observed under a Nikon Eclipse E200 microscope captured by Leica LAS EZ Software. The root anatomical parameters such as root area (μm^2), root diameter (μm^2), cortex area (μm^2), stele area (μm^2), number of metaxylem, metaxylem diameter (μm) and metaxylem area (μm^2) were recorded for statistical analysis.

4.2.7 Statistical Analysis

The statistical test was performed using GraphPad Prism Ver. 9.0 software. The means value of the three replicates for six rice varieties were calculated for all root parameters. The data of root anatomical characteristics were subjected to a one-way and two-way ANOVA at $p \leq 0.05$ level to determine the significant difference among treatments. The mean values were compared with Fisher's LSD test at $p \leq 0.05$.

Principal Component Analysis (PCA) was performed to identify the relationship between the important variables and to evaluate its contribution in response to continuous drought stress. The root anatomical phenotypes such as root diameter, and the area of cross-sectional root, metaxylem, cortex and stele were selected, together with all the agronomic traits. The PCA was run using GraphPad Prism version 9.0, and two principal components that have high Eigen values were selected.

4.3 RESULTS

4.3.1 Effect of Drought Stress on Anatomical Root Area

The anatomical root area is one of the most common and significant parameters used to assess the effect of drought stress on the root system (Figure 4.3). It shows the capability of cells to grow and develop despite having lower water potential in the soil (Wang et al., 2020). Figure 4.4 shows the root parameter in six rice varieties under three treatments. A two-way ANOVA revealed that there was a significant interaction between varieties and the treatments applied to the plants ($p \leq 0.0023$). Under control treatment, Moroberekan recorded the largest root area, followed by Merah Wangi and Nona Manis. These top three varieties were significantly different from each other,

including the MR297. Meanwhile, Biris possessed the smallest root area and was less significant than MR297. In the context of alternate drought stress, it was observed that Moroberekan exhibited the highest root section area among the tested varieties, with the exception of Merah Wangi, that was not significantly different. When compared to control MR297, Merah Wangi, Apami and Nona Manis showed no significant difference. However, under prolonged drought stress, Merah Wangi, which had the second highest anatomical root area under well-watered and alternating drought conditions, performed the best of all other varieties, followed by Apami when exposed to continuous drought stress (Figure 4.3). These varieties were found to be not significantly different from the control variety, MR297, in terms of root anatomical area. In contrast, Biris exhibited significant differences from the control MR297, followed by Moroberekan and Nona Manis. These three varieties were among the varieties with the lowest root area.

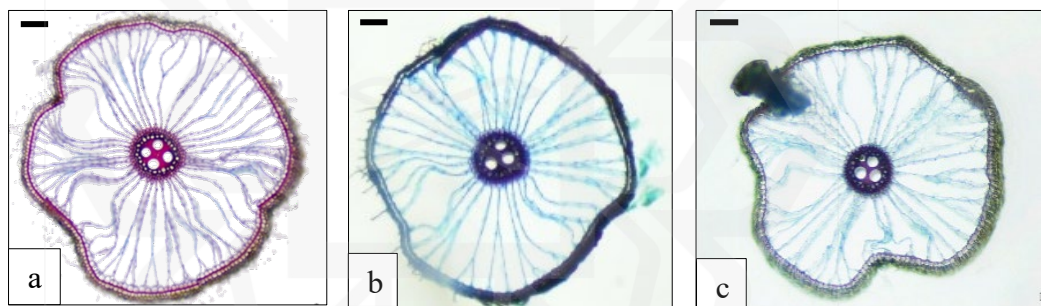


Figure 4.3: Cross section of anatomical root area of MR297 under normal watering (a) alternate drought stress (b) and continuous drought stress (c). The images were observed under a Nikon light microscope at 4X magnification (scale bar at 0.2 μ m) and captured using Leica LAS EZ software.

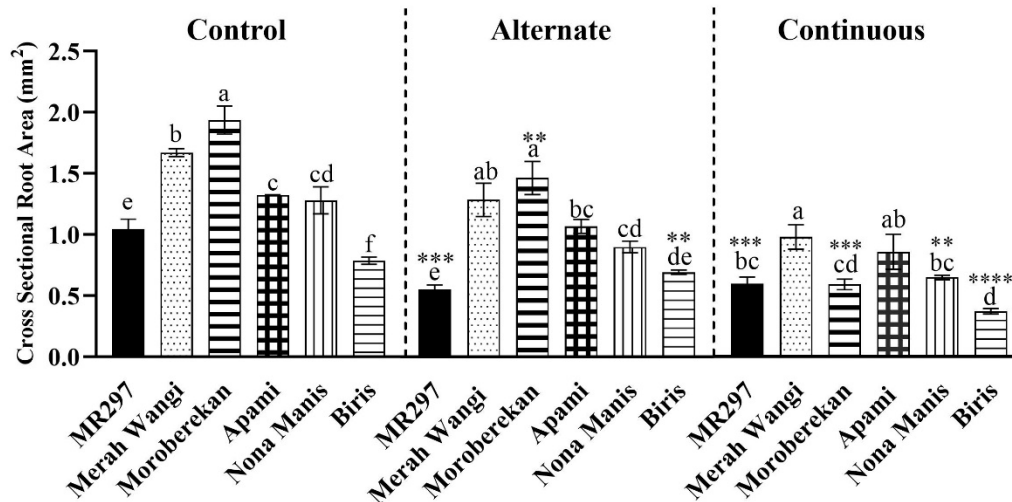


Figure 4.4: Cross sectional anatomical root area of six rice varieties in response to control, alternate drought and continuous drought treatment. Data were analysed using one-way ANOVA to test significant differences ($p \leq 0.05$) among the treatments means. Means with same letters are not significantly different. The data exhibited was the comparisons within the same water treatments. The stars (***) exhibited a significant level of variety as compared to the control MR297.

4.3.2 Effect of Drought Stress on Root Diameter

The root diameter is a useful indicator to analyse the drought-tolerance trait, as it also reflects the root thickness (Lozano et al., 2020). Based on a two-way ANOVA analysis, the alternate and continuous drought treatment of the rice variety had a significant effect on root diameter ($p \leq 0.05$). As shown in Figure 4.5, the root diameters for Merah Wangi and Moroberekan were significantly higher than MR297, while the lowest was seen in the Biris for the control treatment. Only Nona Manis was seen as not significant to MR297. After alternating two cycles of drought, the highest readings were observed in Moroberekan and Merah Wangi, which were significantly higher than Nona Manis, Biris and MR297. Comparing with MR297 under the control condition, it was found that Merah Wangi, Moroberekan, Apami and Nona Manis show no significant difference in root diameter. This implies that the alternate watering treatment was effective in achieving results comparable to those obtained with regular watering. As the continuous drought prolonged for more than 7 days, Apami recorded the highest

root diameter, which was not significantly different from Merah Wangi and Nona Manis. When compared to the control MR297, Apami had no significant difference, while the least significant was recorded in Merah Wangi. In contrast, Biris had the smallest root diameter affected by drought stress and was extremely different from the control MR297, suggesting that it was the most vulnerable variety in terms of root diameter.

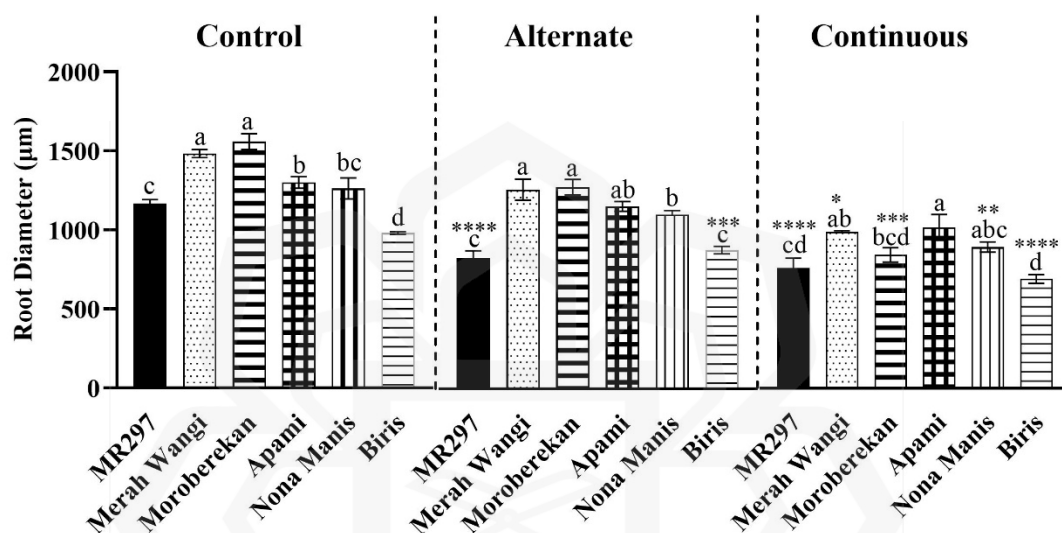


Figure 4.5: Root diameter of six rice varieties in response to control, alternate drought and continuous drought treatment. Data were analysed using one-way ANOVA to test significant differences ($p \leq 0.05$) among the treatments means. Means with same letters are not significantly different. The data exhibited was the comparisons within the same water treatments. The stars (****) exhibited a significant level of variety as compared to the control MR297.

4.3.3 Effect of Drought Stress on Cortex Area

The cortical size of drought-stressed crops has been extensively studied in previous research as it interacts with root diameter phenes (Henry et al., 2016; Sidhu et al., 2023). The cortex, which is in between the root epidermis and endodermis, serves as a secondary metabolite and photoassimilates storage (Lux et al., 2004). The cortex area was measured by subtracting the stele area from the root area. Based on Figure 4.6, a two-way ANOVA analysis revealed that there was a significant interaction ($p \leq 0.0036$)

between the treatments and varieties. The Moroberekan significantly recorded the largest cortex area under control treatment. This result tallied with the root area, as Moroberekan displayed the largest root area in the same treatment. Furthermore, the mean for all varieties except Nona Manis was significantly different from the MR297, with Biris being the lowest compared to the MR297.

Upon exposure to alternate droughts, there was a depletion in the cortex area for all varieties, resulting in a lower root area. Based on a one-way ANOVA, the cortex area varied significantly between varieties ($p \leq 0.002$). Moroberekan remained the variety with the largest cortex area compared to other varieties after two cycles of alternate droughts. In fact, it was significantly higher than MR297. In contrast, the cortex area of MR297 showed the lowest value and had no significant difference from Biris. The varieties that were not significant with the control MR297 were Merah Wangi, Apami and Nona Manis. The reduction pattern of cortex during the alternate drought was more obvious in continuous drought stress. The results showed that continuous stress significantly depressed the cortex area of all varieties ($p \leq 0.0004$). Merah Wangi recorded the highest cortex area when subjected to continuous drought conditions, indicating Merah Wangi could preserve the cortex area even under water deficit conditions. Furthermore, only the cortex areas of Merah Wangi and Apami were not significantly different from the MR297 under normal irrigation. The other varieties showed significant differences from the control MR297. However, under continuous drought treatment, Moroberekan showed no significant difference from the lowest varieties, Biris, Nona Manis, and MR297.

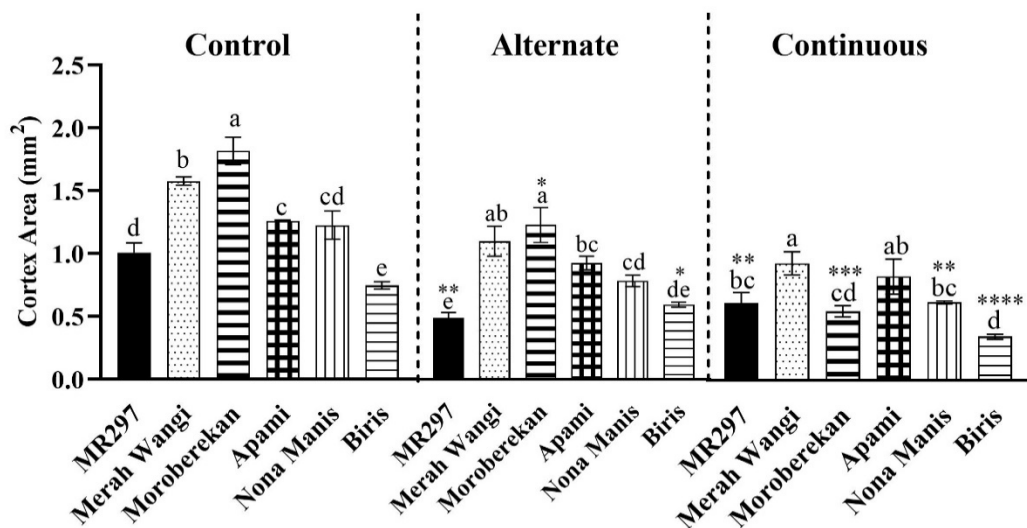


Figure 4.6: Cortex area of six rice varieties in response to control, alternate drought and continuous drought treatment. Data were analysed using one-way ANOVA to test significant differences ($p \leq 0.05$) among the treatments means. Means with same letters are not significantly different. The data exhibited was the comparisons within the same water treatments. The stars (****) exhibited a significant level of variety as compared to the control MR297.

4.3.4 Effect of Drought Stress on Stele Area

The stele area, the central tissue in the root, was measured as it encompassed the vascular tissue (Figure 4.7). The stele area varied between the varieties ($p < 0.0001$) and between treatments, indicating the interaction was highly significant. Generally, the stele areas for Moroberekan and Merah Wangi were significantly different from MR297 in all treatments. Referring to Figure 4.8, Moroberekan recorded the largest stele areas, both in control and alternate drought treatment. Merah Wangi and Apami were listed among the top three highest-ranked stele areas under control conditions. Both varieties were extremely different from MR297. In contrast, MR297 scored the smallest stele area of all treatments and had no significant difference from Nona Manis and Biris. Under alternate drought conditions, Apami, Nona Manis and Biris also had no significant differences from the control MR297. The stele area of all varieties became smaller with increasing drought stress, indicating the significant effect of continuous drought treatment ($p \leq 0.0003$). Despite the drought-induced condition, it was discovered that the Merah Wangi variety had the largest stolon area, outperforming the

domestically cultivated MR297 variety, followed significantly by Moroberekan and Apami. It might be posited that the bigger stele areas of those three varieties demonstrated better root acclimation to drought-induced stress in comparison to other varieties of rice. Nevertheless, Merah Wangi was the only variety shown to have a significantly higher stele area compared to MR297 grown under well-watered treatment.

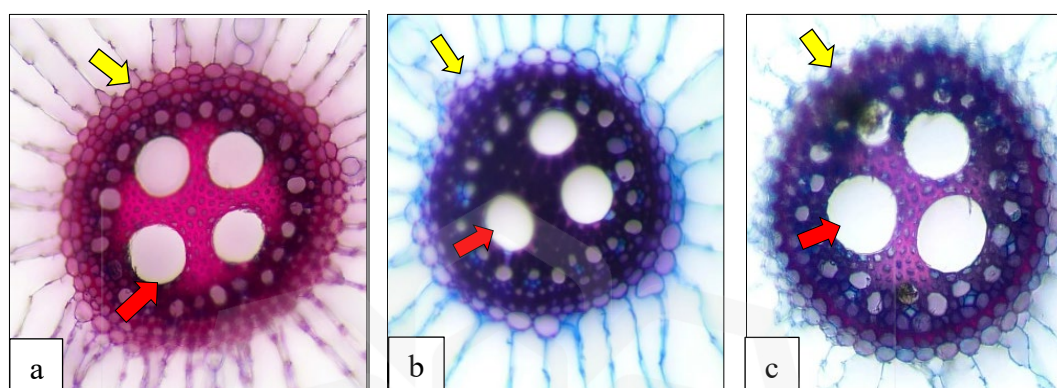


Figure 4.7: Anatomical root phenotype of MR297 under normal watering (a) alternate drought stress (b) and continuous drought stress (c). The images were observed under a Nikon light microscope at 400X magnification and captured using Leica LAS EZ software. Yellow arrow indicates the stele. Red arrow indicates the metaxylem in stele.

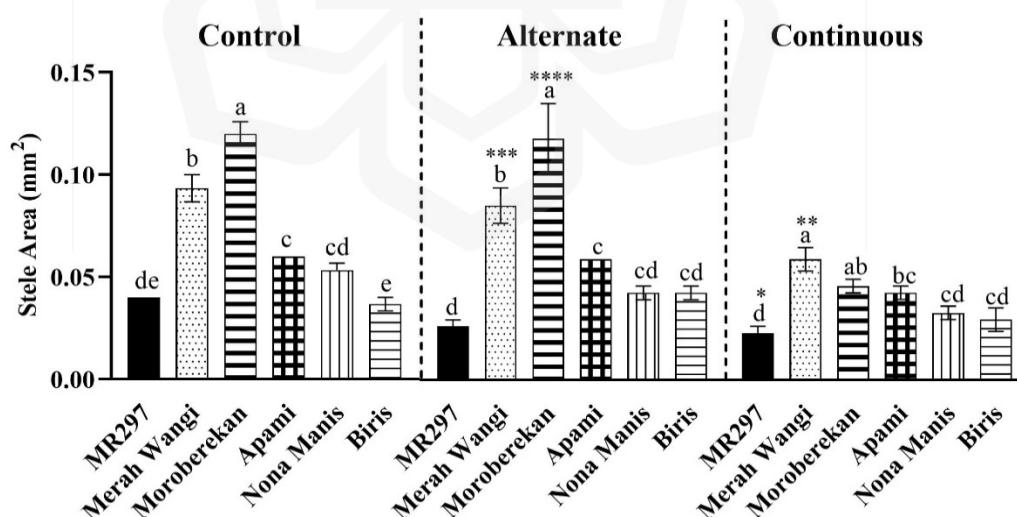


Figure 4.8: Stele area of six rice varieties in response to control, alternate drought and continuous drought treatment. Data were analysed using one-way ANOVA to test significant differences ($p \leq 0.05$) among the treatments means. Means with same letters

are not significantly different. The data exhibited was the comparisons within the same water treatments. The stars (****) exhibited a significant level of variety as compared to the control MR297.

4.3.5 Effect of Drought Stress on Metaxylem Number

The analysis of vascular tissues was carried out by calculating the number of metaxylems that are encompassed in the stele region. The xylem phenotype has been identified as a significant informative indication for assessing root structure and contributing to rice productivity under drought stress (Siangliw et al., 2022). Based on a two-way ANOVA of metaxylem numbers, it was discovered that the interaction between treatments and six rice varieties was not significant ($p \leq 0.0653$) in normal water conditions. The results showed that Moroberekan formed the highest metaxylem numbers, followed by Merah Wangi and Nona Manis (Figure 4.9). These three varieties had significant differences from the control, MR297. In contrast, the fewest metaxylem numbers were recorded in MR297 and Apami.

When plants were treated in an alternating drought, there was a significant variation of this treatment on the metaxylem numbers for six rice varieties ($p \leq 0.0042$). It was observed that the variety Moroberakan consistently exhibited the largest metaxylem numbers. This variety, together with Merah Wangi and Biris, were statistically significant when compared to the variety MR297 grown in watering conditions. However, the mean metaxylem numbers in Moroberakan were not found to be significantly different from those of the varieties Merah Wangi and Biris. Meanwhile, prolonged drought periods revealed that there was no significant difference in metaxylem number amongst Merah Wangi, Moroberekan, Apami, and Nona Manis, while Biris and MR297 were found to be significantly the lowest. When compared to MR297 in normal conditions, the result showed only continuous drought-induced Merah Wangi and Moroberakan exhibited a significant difference. Overall, the findings suggest that MR297, despite being commercially grown, did not develop a high number of metaxylems and performed poorly under alternate and continuous drought stress.

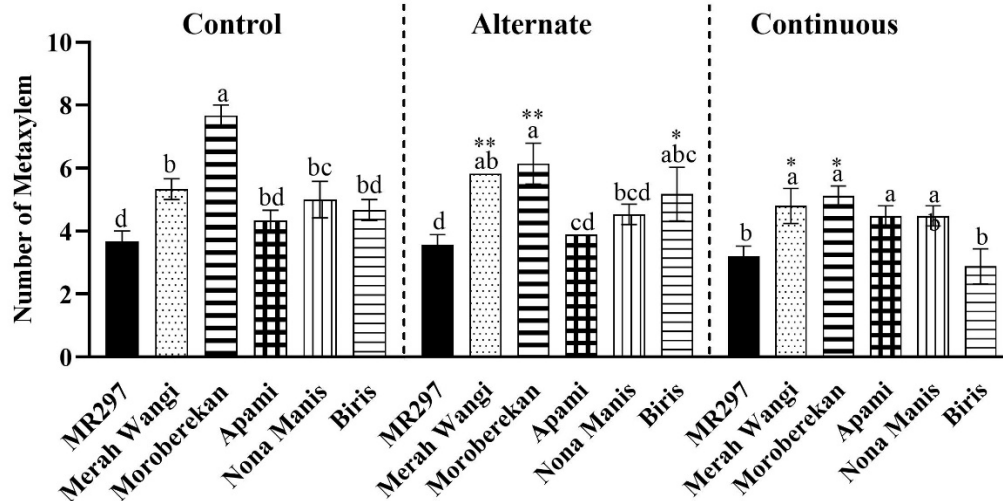


Figure 4.9: Number of metaxylem of six rice varieties in response to control, alternate drought and continuous drought treatment. Data were analysed using one-way ANOVA to test significant differences ($p \leq 0.05$) among the treatments means. Means with same letters are not significantly different. The data exhibited was the comparisons within the same water treatments. The stars exhibited a significant level of variety as compared to the control MR297.

4.3.6 Effect of Drought Stress on Metaxylem Area

The study on the effect of drought stress on metaxylem area provides useful information on drought tolerance, and it has been utilized in a study of Southeast Asian rice (Siangliw et al., 2022). In this study, it was found that the interaction between varieties and treatment was highly significant ($p \leq 0.003$). Moroberakan, naturally had the highest metaxylem area under normal watering, with no significant difference from Apami (Figure 4.10). However, both varieties showed a significant difference from MR297. Meanwhile, Biris and Nona Manis were among the varieties that had the smallest metaxylem area and were not significantly different from MR297. Based on a one-way ANOVA of alternating drought treatments, there was a significant difference in the metaxylem area amongst the six varieties ($p \leq 0.05$). Specifically, under alternate drought stress, Moroberakan had the largest metaxylem area and was statistically significant among the other varieties, followed by Merah Wangi, Apami and Nona Manis. In fact, the metaxylem area of Moroberakan exhibited a significant difference from the MR297 grown under normal conditions and had the highest in all the treatments. Nevertheless,

when the duration of drought stress exceeded 10 days, there was no statistically significant difference observed in the mean metaxylem areas between Moroberakan, Merah Wangi, Biris, and Apami. On the other hand, Merah Wangi developed the largest metaxylem area under continuous drought stress, indicating that this variety performed best under drought stress compared to other varieties. Indeed, the results also indicate that only Merah Wangi exhibited a significantly higher metaxylem area compared to MR297 under normal conditions, as observed in the case of metaxylem number.

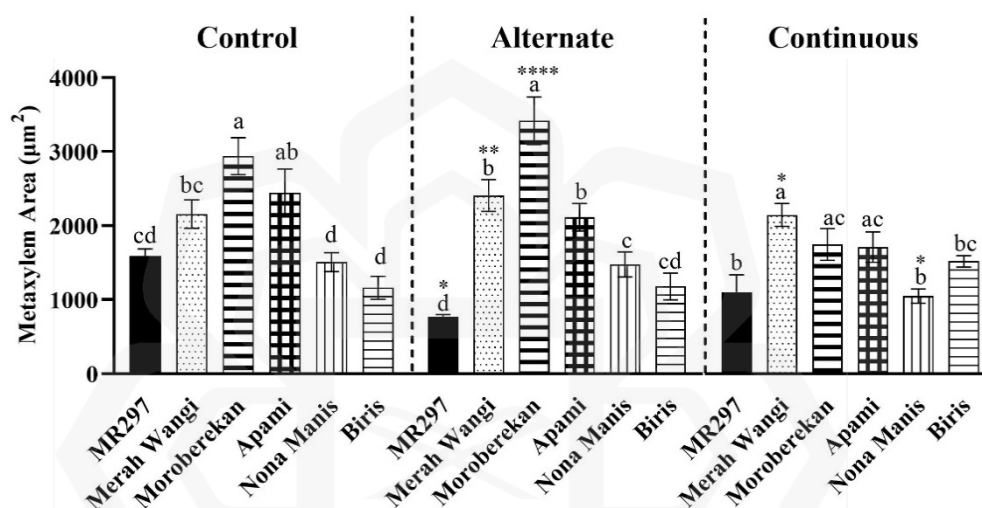


Figure 4.10: Metaxylem area of six rice varieties in response to control, alternate drought and continuous drought treatment. Data were analysed using one-way ANOVA to test significant differences ($p \leq 0.05$) among the treatments means. Means with same letters are not significantly different. The data exhibited was the comparisons within the same water treatments. The stars exhibited a significant level of variety as compared to the control MR297.

4.3.7 Effect of Drought Stress on Metaxylem Diameter

The analysis of metaxylem phenotype was continued with the measurement of diameter for each metaxylem under all treatments. The results of a two-way ANOVA displayed a significant interaction between the types of treatments and the rice varieties tested ($p \leq 0.0039$). Under normal watering, there was a significant effect of the treatment on the metaxylem diameter between six varieties ($p \leq 0.0003$). Moroberekan exhibited the largest metaxylem diameter, significantly differed from other varieties except Apami (Figure 4.11). Additionally, Apami and Merah Wangi were among the top three in average metaxylem diameters. Meanwhile, the lowest metaxylem diameter was recorded in Biris and it was significantly different from MR297. In contrast, Nona Manis and Merah Wangi had no significant difference from MR297. After two cycles of drought stress, a one-way ANOVA revealed that the alternate stress had a significant effect on the metaxylem diameter of six rice varieties ($p < 0.0001$). Moroberakan scored the longest metaxylem diameter, and it was the only variety that had a significant difference from the control MR297. In addition, Merah Wangi and Apami were among the top three varieties that had the highest diameter value under alternate drought treatments. As the drought severity progressed, the significant effect of continuous stress on the metaxylem of varieties was also noticed ($p \leq 0.038$). Notably, Merah Wangi displayed the highest metaxylem diameter under extreme drought stress, although not significantly different from Moroberekan and Apami. These three varieties had no significant difference in relative to the control MR297. Conversely, Nona Manis exhibited the lowest metaxylem diameters under the same treatment, with no significant difference from Biris and MR297.

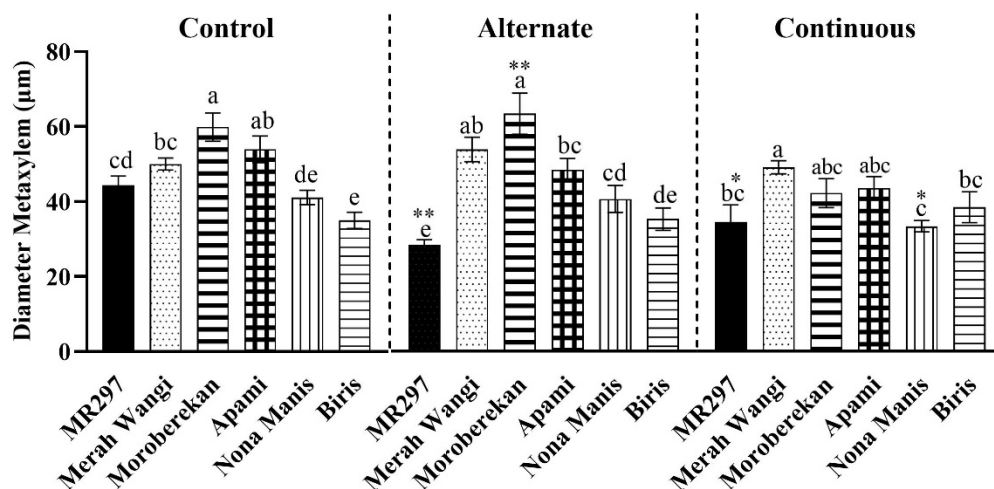


Figure 4.11: Diameter of metaxylem of six rice varieties in response to control, alternate drought and continuous drought treatment. Data were analysed using one-way ANOVA to test significant differences ($p \leq 0.05$) among the treatments means. Means with same letters are not significantly different. The data exhibited was the comparisons within the same water treatments. The stars exhibited a significant level of variety as compared to the control MR297.

4.3.8 Principal Component Analysis of Continuous Drought Stress

Principal Component Analysis (PCA) is a statistical technique that transforms numerous variables into smaller data sets. It aims to minimize the dimensionality of big data while retaining useful variables, thus providing simple but comprehensive information from the original variables (Mishra et al., 2017). PCA was constructed to identify the root anatomical and agronomic traits most associated with extreme drought stress. The results revealed that out of 9, the first three principal components exhibited eigenvalues >1 had 80.17% of the total variation among the traits studied under extreme drought stress. All root anatomical phenotypes contributed positively to PC1, accounting for 42.96%. Thus, PC1 represented the root anatomical phenotypes that were most affected by continuous drought stress (Figure 4.12). Conversely, agronomic traits such as the number of seeds and tillers negatively contributed to PC1. However, these two agronomic variables had strong contributions in PC2, which accounted for 23.96%. In addition, only the diameter and cortex area of the roots contributed

positively to the PC2. The other root anatomical phenotypes had a weak influence on PC2. Therefore, PC2 represented the agronomic traits.

Besides that, in PC1, it can be shown that all anatomical variables were closely clustered, indicating that these phenotypes were correlated. The vectors of root diameter and harvest index form a 90° angle, indicating these two variables were uncorrelated. A small angle between the harvest index and grain yield signifies that these two variables are positively correlated. Besides that, grain yield was also correlated with the area of the root, metaxylem, and stele. The result of both PCAs revealed that agronomic traits and anatomical root parameters such as the area of the root, cortex, metaxylem, and stele could be used as vital phenotypes associated with the drought stress response.

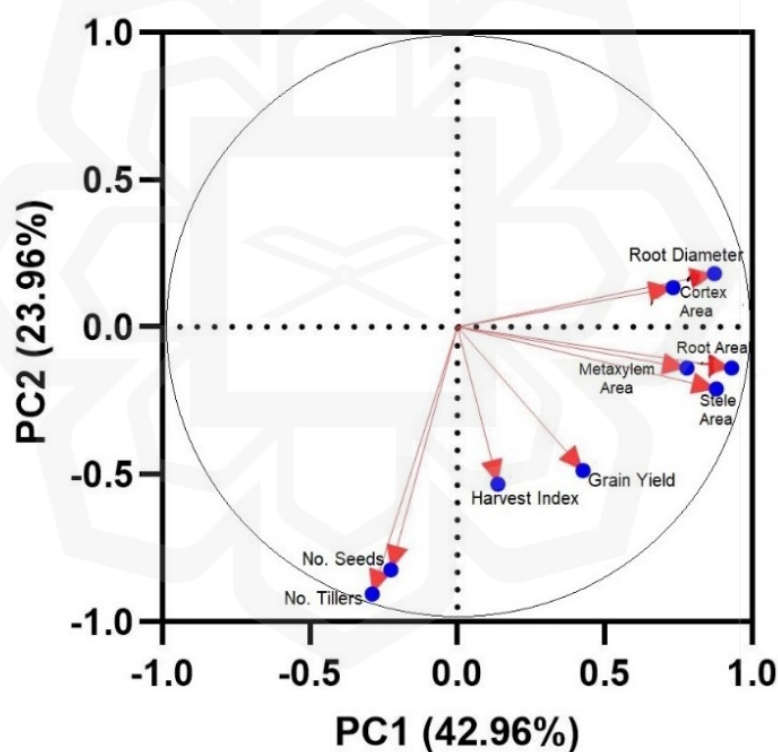


Figure 4.12: Principal Component Analysis (PCA) of agronomic traits and root anatomical phenotypes in continuous drought treatment. PC1(x -axis) represented the root anatomical traits and PC2 (y -axis) represented the agronomic traits.

4.4 DISCUSSION

4.4.1 Effect of Drought Stress on Root Area and Diameter

The variation in root area and diameter in all treatments provides beneficial knowledge for understanding the root architecture in response to drought stress. This study revealed that Moroberakan had the highest root area and diameter under alternate drought stress and was not significantly different from Merah Wangi (Figure 4.3, 4.4). Specifically, both varieties had a larger root area than control MR297. However, under progressive drought stress, the root anatomical area for all varieties differed significantly from the control MR297. These results indicate continuous drought stress showed negative shifts in the diameter and area of the crown root in all varieties. Despite that, varieties under alternating drought stress develop better than those under prolonged drought stress.

A similar observation was previously reported by Hazman and Brown (2018) and Henry et al. (2012), who discovered that severe drought severity caused poor root development, resulting in reduced root area for all plants. The inhibition of root growth was known to be affected by insufficient water and high soil resistance (Comas et al., 2012). In addition, Taiz and Zeiger (2006) reported that dry soil, driven by a prolonged drought condition, would cause a significant loss of cell turgor. When water is limited, the physiological cell growth attained by the series of cell divisions and differentiations is retarded, leading to root shrinkage (Fahad et al., 2017). This study observed that continuous drought treatment led to root shrinkage, which was manifested as a distorted root structure. Consequently, the decrease in the root's cross-sectional area resulted in reduced root diameter elongation and a simultaneous reduction in root thickness.

Nevertheless, other studies by Abdullah et al. (2010) found that drought stress could promote a larger root diameter in rice than in those subjected to regular watering. Root thickness is strongly related to higher water uptake, and a thicker root enhances the accumulation of non-structural carbohydrates (NSC) (Abdullah et al., 2010; Lozano et al., 2020; Yang et al., 2016). Under drought stress, the NSC, including sugar alcohol, enhances plant resilience to drought stress by conserving cell turgidity (Chen et al., 2002; Tomasella et al., 2019). This research indicates that the Merah Wangi exhibited the largest root anatomical area and diameter under continuous drought conditions, suggesting that Merah Wangi is more resilient and better acclimated to drought stress compared to other varieties. Previous studies by Lynch et al. (2021) and Ryser (2004)

have shown that roots with a larger diameter are more effective at penetrating compact, fine, and densely packed soils. Furthermore, an increased root area is necessary to provide hydraulic resistance in dry soils, as reported by Kim et al. (2020) and Ryser (2004). Therefore, thicker roots under water deficit conditions are valuable traits to promote drought tolerance.

4.4.2 Effect of Drought Stress on Cortex Area

In this study, the reduced cortical area observed across all varieties under continuous drought stress was linked to a decrease in root area. The performance of these rice varieties to drought stress aligns with findings from a recent study by Fonta et al. (2022), who compared the varieties 'IR64' and 'Azucena'. Azucena, identified as a drought-tolerant rice, showed a smaller root cortical area under drought conditions compared to the more drought-susceptible variety, IR64. This trait was suggested to have helped Azucena minimize the metabolic cost associated with root development (Gowda et al., 2011).

The findings of this study clearly demonstrated the ability of Moroberekan and Merah Wangi to maintain their cortical areas even under two cycles of drought stress (Figure 4.5). When subjected to continuous drought stress, Merah Wangi consistently had the largest cortical area. According to Chimungu et al. (2014), maize lines with a large cortical cell exhibited deeper roots, greater water status in plants, and better root exploitation for accessing deep soil water. Furthermore, the larger cortical size positively correlated with root diameter, allowing for greater anchoring and increasing the mechanical strength and rigidity of the root system during water scarcity (Chimungu et al., 2014). In alignment with this finding, several studies on rice suggest that deep rooting is advantageous for drought-tolerant varieties as it enables water uptake from deeper soil layers where moisture is still present (Gowda et al., 2011; Kitomi et al., 2015; Lynch, 2013). These results raised the question of whether Merah Wangi possessed deep roots, which could be beneficial traits under drought stress. Thus, further genetic analysis is required to validate these findings.

The cortical area of rice roots consists mainly of root cortical aerenchyma (RCA) and root cortical parenchyma (RCP) as the primary ground tissues (Chimungu

et al., 2015). The formation of RCA is a result of programmed cell death in parenchymatous ground tissues (Lynch et al., 2021). Although the RCA and RCP were not quantitatively analyzed in this study, they were visibly developed and present in all varieties across all treatments (Figure 4.13).

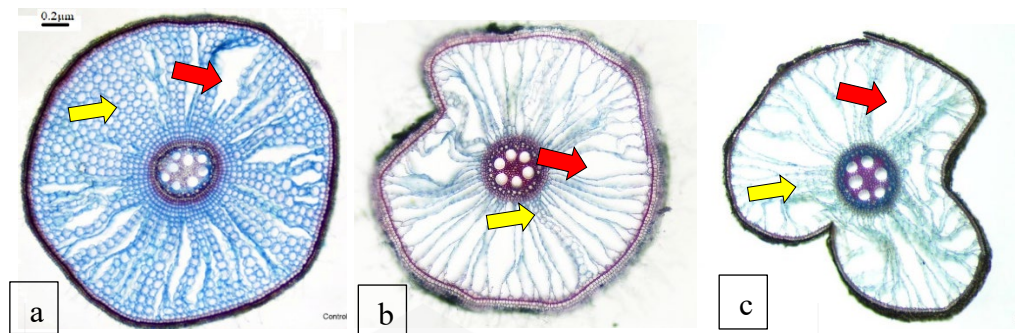


Figure 4.13: Cross section of anatomical root area of Merah Wangi under normal condition (a) alternate drought stress (b) and continuous drought stress (c). Red arrow indicates the root cortical aerenchyma. Yellow arrow indicates root cortical parenchyma. Scale bar at 0.2μm.

Chen et al. (2021b) investigated the longitudinal formation of aerenchyma in rice roots, while Sidhu et al. (2023) conducted similar research in maize studies. Studies have shown that RCA helps maize cope with drought stress by reducing the metabolic costs associated with soil exploration, although it does not enhance root penetration (Chimungu et al., 2015; Sidhu et al., 2023; Zhu et al., 2010). On the other hand, Basu et al. (2021) observed that the abundance of aerenchyma tissue reduces respiration rate due to fewer living cells, thereby promoting drought tolerance. Nevertheless, Henry et al. (2012) reported that aerenchyma's effect on water absorption during drought stress was insignificant as the formation of intercellular spaces primarily facilitated oxygen diffusion, especially in the control treatment where soils were flooded.

4.4.3 Effect of Drought Stress on Stele Area

The observation of stele area from this study contradicts the findings of Hazman and Brown (2022), Henry et al. (2012), and Siangliw et al. (2022), who found that stele areas were larger in drought treatment than well-watered conditions. However, Kadam

et al. (2015) reported that the tolerant rice cultivar N22 exhibited a high stele diameter under water deficit stress. The findings of this study showed that rice in well-watered conditions had a larger stele area than drought stress treatments (Figure 4.6). A comparison within the alternate drought treatment showed that Moroberakan and Merah Wangi formed the largest stele area. Although this result indicates that both root varieties acclimated to subsequent drought stress, Merah Wangi possessed better adaptation to prolonged water scarcity. Similar responses in a larger stele area were also observed in the drought-stressed Azucena, which shows a better root respiration rate than the IR64 (Fonta et al., 2022). Besides that, Manikanta et al. (2022) also discovered larger stele diameters in drought-tolerant rice, Karuthamodan, and Chuvannamodan. Therefore, it has been proposed that the thicker root with a larger stele in maize plants confers drought stress (Klein et al., 2020).

According to Phoura et al. (2020), a wider stele size confers multiple benefits to plants, including the enhancement of grain yield, maintenance of plant water status, and facilitation of water transportation under water scarcity. Henry et al. (2012) suggested that the greater stele size represents a conservative adaptive strategy aimed at optimizing water transport within the xylem during drought stress, thereby promoting grain productivity. This was in accordance with the finding of Siangliw et al. (2022), who discovered that 'Am Beus' rice accession, characterized by a larger stele diameter, exhibited the highest grain yield under drought. Taken together, these findings may validate the previous agronomic analysis (Chapter 3), which resulted in Merah Wangi as the top grain producer under extreme drought stress. In addition, fortification of the stele encircled by the lignified sclerenchyma constitutes a beneficial root trait facilitating root penetration under drought stress (Hazman & Brown, 2018). While the analysis of lignification and suberization is beyond the scope in this study, the presence of these structural formations was evident through the staining procedure. Therefore, studies delving into cortical areas provide insight into the drought tolerance mechanism, encouraging further research on root physiological.

4.4.4 Effect of Drought Stress on Metaxylem

The vascular tissue, specifically xylem vessels, has garnered attention in investigations concerning the impact of drought stress on root systems due to its pivotal role in water

transport. In line with other monocots, rice exhibits a circular arrangement of xylem vessels within the stele. The findings of this study significantly showed a poor development of metaxylem area and diameter under continuous drought treatments as compared to alternate drought stress (Figure 4.8,4.9). Previous studies by Henry et al. (2012) and Mostajeran and Rahimi (2008) reported a similar reduction in metaxylem dimensions in drought-exposed rice plants, positing that the decreased water supply offers advantages under drought stress by mitigating the risk of embolism and concurrently bolstering drought resistance (Lucas et al., 2013; Siangliw et al., 2022). Xylem vessels play a crucial role in cavitation processes (Mostajeran & Rahimi, 2008), with embolism accumulation accelerating during an advancing drought, potentially leading to hydraulic failure (Lucas et al., 2013). Consequently, the smaller metaxylem dimensions serve as a protective mechanism against cavitation, shielding the xylem from hydraulic failure (Hazman & Brown, 2018).

In the rehydration treatment following an alternate drought, the highest metaxylem area and diameter were observed in Moroberakan, followed by Merah Wangi (Figure 4.8,4.9). However, as the drought extended to seven days, Merah Wangi demonstrated the highest metaxylem area and diameter. Under continuous drought stress, although the metaxylem quantity in Moroberekan was the highest, its metaxylem area was lower than Merah Wangi. In the case of Moroberekan and Apami under continuous drought stress, despite having high metaxylem quantity, they were all relatively small in size (Appendix I). Collectively, this study elucidates that the competency of Merah Wangi to retain the metaxylem area in response to drought stress is considered a beneficial trait, as a wider xylem signifies a higher capacity to retain water within its living tissues, thereby potentially prolonging its viability under extreme drought conditions (Degraeve et al., 2021).

According to Abdullah et al. (2010), rapid development of metaxylem was found to be positively correlated with water transportation and concomitantly lead to enhanced drought tolerance (Gowda et al., 2011). Furthermore, the phenotype of xylem plays a pivotal role in modulating the metabolic expenditure associated with soil exploration and optimizing water use efficiency (Lynch et al., 2021). Meanwhile, Korner (2019) mentioned that as the severity of the drought worsens, plants will become completely reliant on their intrinsic water reservoir to feed the most vital tissues for

survival. Thus, a higher and wider metaxylem area serves to facilitate water uptake and transport processes, as evidenced by the higher grain yields observed under drought (Gowda et al., 2011).

4.4.5 Phenotypic Correlation Analysis

Various studies have reported significant associations between root phenotypes and grain yield under drought stress (Adeboye et al., 2021; Kaysar et al., 2022; Sandhu et al., 2017; Sofi et al., 2021). Generally, the PCA results showed that grain yield had a correlation with the number of tillers and grain yields (Figure 4.10). Under drought stress, it can be noted that the high yield of Merah Wangi during drought stress was driven by the high grain and tiller numbers. The lower grain number was also partially responsible for the lower grain yield achieved by Apami. In addition, Zhou et al. (2022) reported that primary tillers accounted for around 60% of grain yield, which was higher than secondary tillers. Thus, the variation in tiller number is essential as it contributes to grain yield (Hu et al., 2023).

Furthermore, the area of root, metaxylem, and stele contributed positively to PC1, with a positive correlation with grain yield. In other words, increases in root area cause an increase in grain yield, and vice versa. Based on the PCA conducted by Siangliw et al. (2022), anatomical parameters such as the number and diameter of metaxylem contributed to rice yield under drought stress. Zale rice variety, one of the top grain producers, possessed a high metaxylem number and a large xylem and stele size. Meanwhile, Comas et al. (2012) reported that large xylem diameters promote grain yields under water deficit conditions. Therefore, the large area of the root anatomical phenotypes of Merah Wangi and MR297 might correspond to the high yield of these rice varieties. As evident by the agronomic analysis, this study suggested that Merah Wangi may possess favourable anatomical root phenotypes, which allow it to withstand water deficit conditions. It is supported by Chu et al. (2014), who mentioned that superior root morpho-physiology positively impacts shoot growth, thus producing high grain quality. Collectively, these studies on root traits proved the importance of exploring the root phenotype in determining grain yield. In regard to the outstanding performance of Merah Wangi's roots under drought stress, molecular analysis would be beneficial to evaluate the genetic expression corresponding to drought tolerance.

4.5 CONCLUSION

In summary, the impact of alternate drought stress on root anatomical phenotypes was less compared to continuous drought stress. Similarly to our preliminary study, root performance under alternating drought stress has favourable root phenotypes compared to those under continuous drought stress. As a result, varieties with the ability to adapt and acclimatize their roots to drought stress can be directly utilized to evaluate their genetic traits. Moroberekan and Merah Wangi showed the highest value for all root parameters under alternating drought stress, signifying that both varieties acclimatized to drought stress better than other varieties. Biris and Apami were among the susceptible varieties, in terms of root anatomical phenotype. Conversely, the significant impact of continuous drought stress could be seen in the cortical area. Among all varieties, the drought-stressed Merah Wangi showed outstanding root performance compared to other varieties. This also confirms the earlier analysis on agronomic traits (Chapter 3) that Merah Wangi produced a stable yield despite drought stress. Although Moroberekan also demonstrated better anatomical traits, its agronomic performance was suboptimal. The phenotypic correlation between agronomic analysis and anatomical roots provides insights on root adaptations to drought stress. In addition to this phenotypic correlation, the favourable Merah Wangi variety requires further studies to evaluate the genetic basis underlying adaptive traits.

CHAPTER FIVE

TRANSCRIPTOMIC ANALYSIS ON ROOT OF RICE SUBJECTED TO DROUGHT STRESS

5.1 INTRODUCTION

Drought tolerance mechanisms require understanding of the molecular genetic basis. According to Goda et al. (2008), gene expression in response to drought stress involves the activation or repression of specific genes, which could be discovered using the RNA transcripts (Heintz-Buschart & Wilmes, 2018). This approach, known as transcriptomic has been shown to be effective in evaluating the adjustments of gene expression to abiotic stress (Wu et al., 2020; Xuhui et al., 2022). Quantitative gene expression using RNA sequencing (RNA-seq) allows greater coverage and comprehensive analysis while capturing the most subtle expression changes (Kukurba et al., 2015; Wang et al., 2010). The changes in gene expression in the leaf tissues of rice under drought stress have been well studied (Liang et al., 2021). However, transcriptome profiling of rice roots in response to different severity of drought phases is very limited. Thus, the elucidation of beneficial genes corresponding to root adaptation offers potential targets for developing more resilient rice under water deficit conditions.

The previous studies (Chapter 3 and 4) revealed that Merah Wangi was the highest-yielding variety while exhibiting the most outstanding root performance despite drought stress. Thus, it was assumed that this variety possessed adaptive traits, enabling it to acclimatize to recurring drought stress. However, such an assumption needs validation from the genetic basis of root. Interestingly, next-generation sequencing (NGS) offers rapid data generation, high sensitivity, and deep sequencing of numerous samples (Jian et al., 2022; Shendure et al., 2005). By leveraging this benefit, the goal of this study was to evaluate the transcriptomic analysis of Merah Wangi relative to the domestic grown rice MR297. While phytohormones play a crucial regulation in root development (Korver et al., 2018), the differentially expressed gene (DEGs) within drought treatment were screened down to elucidate the genetic expression underlying the plasticity of root under alternate and continuous drought conditions.

5.2 METHODOLOGY

5.2.1 Plant Material

In this study, two varieties were selected; MR297 as the positive control variety and Merah Wangi, identified as the best phenotype through the analysis of agronomic traits and root anatomical study (Chapter 4). These plants were re-planted with similar methodology as described in Chapter 4. The apical roots of the root system from three treatments were collected prior to RNA extraction. The root samples of the control and alternate drought treatment were taken on the 54th DAS. For the continuous drought treatment, the leaf scrolling score and drought score were used as visible references for the sample collection. The root samples were excised once the leaf exhibited leaf scrolling score number 5 (O'Toole et al., 1979) (Figure 4.2) and the drought score number 8 (Table 4.1) (Fen et al., 2015).

5.2.2 RNA Extraction

The preparation of the roots was based on the method by Okamura et al. (2021). The fresh roots of Merah Wangi and MR297 were gently removed from the polybag, separated from each other and roots were immediately washed with tap water for one minute. The washing time did not exceed two minutes. The soil was easily removed by submersion in water. The roots were subsequently dried on a dry tissue, and quickly ground using a mortar and pestle in the presence of liquid nitrogen.

Total RNA was extracted using a RNeasy Plant Mini kit (Qiagen, Hilden, Germany), according to the manufacturer's instruction. Extracted RNA was purified using RNase-free water. The yield and purity of the RNA were analyzed using a NanoDrop UV-Vis Spectrophotometer (Thermo Fisher Scientific Inc.) at Kulliyyah of Science, IIUM Kuantan. Each RNA precipitate (about 4µg) was transferred to Genewiz-RNA 0.5ml microtubes for further analysis. For concentrated samples, distilled water was added to a final volume of 30µl. The grouping samples were labelled as in the table below.

Table 5.1 Sample Labelling of the Treatment

Treatment	Sample Labelling
Control Merah Wangi versus control MR297	(CMW-VS-CMR)
Alternate Merah Wangi versus alternate MR297	(AMW-VS-AMR)
Continuous drought Merah Wangi versus continuous drought MR297	(DMW-VS-CMR)

5.2.3 mRNA Sequencing by Illumina HiSeq/Novaseq

Total RNA of each sample was quantified and qualified by Agilent 2100/2200 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), NanoDrop (Thermo Fisher Scientific Inc.). 1µg total RNA was used for the library preparation. Next generation sequencing (NGS) library preparations were constructed based on the manufacturer's protocol. The poly(A) mRNA isolation was performed using Poly(A) mRNA Magnetic Isolation Module or rRNA removal kit. The mRNA fragmentation and priming were performed using First Strand Synthesis Reaction Buffer and Random Primers. The first strand cDNA was synthesized using ProtoScript II Reverse Transcriptase and the second-strand cDNA was synthesized using Second Strand Synthesis Enzyme Mix.

The purified double-stranded cDNA was treated with End Prep Enzyme Mix to repair both ends, and a dA-tailing was added in one reaction, followed by a T-A Ligation to add adaptors to both ends. The size selection of adaptor ligated DNA was performed using beads, with fragments of ~400 base pairs (bp) recovered. Each sample was amplified using P5 and P7 primers. The polymerase chain reaction (PCR) products were cleaned up using beads, validated using an Qsep 100 (Bioptic, Taiwan, China), and quantified by Qubit 3.0 Fluorometer (Invitrogen, Carlsbad, CA, USA).

The libraries with different indices were multiplexed and loaded on an Illumina HiSeq/Novaseq instrument based on manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was carried out using a 2x150 paired-end (PE) configuration; image analysis, and base calling were conducted by the HiSeq Control Software (HCS) + OLB + GAPIipeline-1.6 (Illumina) on the HiSeq instrument, image analysis and base calling were conducted by the NovaSeq Control Software (NCS) + OLB + GAPIipeline-1.6 (Illumina) on the Novaseq instrument, image analysis and base calling were conducted by the Zebeacall on the MGI2000 instrument.

5.2.4 Data Analysis

5.2.4.1 *Quality Control*

To ensure high quality data, the pass filter data of fastq format were processed by Cutadapt (v1.9.1) by removing the technical sequences such as adapters, PCR primers and quality of bases lower than 20.

5.2.4.2 *Assembly*

The reads were assembled by Trinity (v2.2.0)[2], which combines three independent software modules; Inchworm, Chrysalis, and Butterfly and these were applied sequentially to process large volumes of RNA-seq reads. Then, the duplicated contigs were removed by cd-hit, to obtain the unigene sequence file.

5.2.4.3 *Expression Analysis*

RSEM[3](v1.2.23) estimated gene and isoform expression levels from the pair-end clean data with the unigene sequence file as a reference gene file.

5.2.4.4 *Differential Expression Analysis*

The analysis used the DESeq2 (v1.6.3) Bioconductor package, which was based on a model with a negative distribution model. The estimates of dispersion and logarithmic fold changes incorporate data-driven prior distributions. The differential expression was

based on the criteria of fold change greater than 2, and the Q value(fdr.padj) < 0.05 . The number of up- and downregulated genes were calculated.

5.2.4.5 Functional Annotation and Enrichment Analysis

The blast software was applied to annotate the unigene sequence. Gene ontology (GO) an international standardized gene functional system, was used to characterize the functions of the genes in each DEG group. Gene ontology (GO) consists of three ontologies: molecular function (MF), cellular component (CC), and biological processes (BP) (Wang et al., 2009a). The functional analysis was blasted to GOSeq(v1.34.1), which based on an extension of the hypergeometric distribution known as the Wallenius non-central hyper-geometric distribution (Young et al., 2010). This method allows to annotate a list of enriched genes with a significant $\text{padj} < 0.05$. The TopGO was used to plot directed acyclic graph (DAG).

For the Kyoto Encyclopaedia of Genes and Genomes (KEGG), the DEGs were blasted to KEGG pathway to gain insight into the biological role for better understanding of biological functions. KEGG is the primary public Pathway database (Kanehisa, 2008) used in this analysis. This database provides for a better understanding of high-level biological function (Kanehisa & Goto, 2000). The threshold used was Q value ≤ 0.05 . Cluster of Orthologous Group (COG), a protein databased were constructed based on the evolutionary relationship of the coding proteins in the complete genomes of eukaryotes.

5.2.4.6 Principal Component Analysis (PCA)

PCA was analyzed using R language software. The data points were projected onto the 2D plane such that the samples were spread out in the two directions that explain most of the differences. The x- axis reflects the first Principal Component (PC1), in which it separates the data points the most. Meanwhile, the y-axis is a direction that separates the data second most and the values are written as the second Principal Component (PC2).

5.2.4.7 Screening of Phytohormone Gene in Response to Drought Stress

All the significant DEGs in the three treatments were screened down to identify the gene regulation that corresponds to the phytohormone auxin, cytokinin and gibberellin. Besides that, the gene corresponds to DEEPER ROOTING 1 was also listed out. The determination of significant DEGs was according to the criteria of $[\log_2 \text{ fold change}] \geq 2.0$ and at a $p \leq 0.05$.

5.3 RESULTS

5.3.1 Unigene Annotation

De novo RNA sequencing was applied for the transcriptome analysis of *Oryza sativa*. The number of unigene annotations is listed in Table 5.2. A total of 479682 unigenes were annotated. Among them, the number of unigenes annotated in the non-redundant protein database (Nr) was 450102. Function annotation analysis revealed that 246341, 96171, and 213267 unigenes were annotated in the COG, KEGG, and Swissprot databases, respectively.

Table 5.2 Unigene Information Annotated in Different Database

Database	Number of Unigene
Annotated on Nr	450102
Annotated on Swissprot	213267
Annotated on COG	246341
Annotated on KEGG	96171
Total Annotated	479682
Total Unigene	849942

5.3.2 Identification of Differentially Expressed Genes (DEGs)

Figure 5.1 illustrates the number of DEGs, which refers to the change of Merah Wangi relative to MR297. The gene expressions were considered significant by the fold change ≥ 2.0 with a p-value ≤ 0.05 . In the control treatment (CMW-VS-CMR), out of 23062 DEGs, 5367 genes were upregulated, and 17695 genes showed repression relative to MR297. Meanwhile, 35257 genes were significantly expressed under alternating drought stress (AMW-VS-AMR), with 12700 and 22557 genes displayed up- and down-regulation, respectively. A total of 77115 DEGs were identified in response to the continuous drought treatment (DMW-VS-DMR). Specifically, 37588 genes in Merah Wangi were upregulated, while 39527 genes were downregulated in response to continuous drought stress.

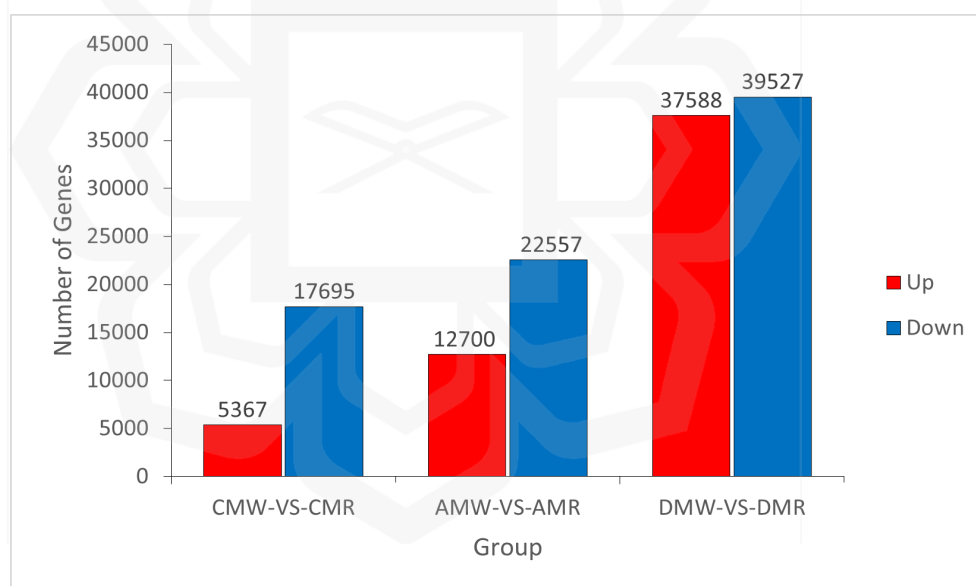


Figure 5.1: Bar graph of differentially expressed genes (DEGs) consisting of upregulation and downregulation in Merah Wangi relative to MR297. The determination of significant DEGs was according to the criteria of $[\log_2 \text{fold change [L2fc]}] \geq 2.0$ and at a $p \leq 0.05$.

Besides that, there were 8298 DEGs expressed in all treatments, and a notable proportion of DEGs were also unique to each group (Figure 5.2). Among the 17226 genes expressed in the control treatments, only 7246 transcripts were unique to control watering, whereas 2918 transcripts were similar to the alternate drought. Within the re-watering treatment, only 12690 genes were preferentially induced under alternate drought stress. The results also revealed that DEGs in the alternate drought groups overlapped more with continuous groups than the control groups. Additionally, 11351 genes from the alternate drought groups overlapped with the continuous drought group. Meanwhile, there were 52866 genes being specially expressed in severe drought stress, with similar 4600 DEGs also induced in the control condition. Furthermore, the results also revealed that DEGs uniquely expressed in continuous drought conditions were higher than in alternating drought and control condition.

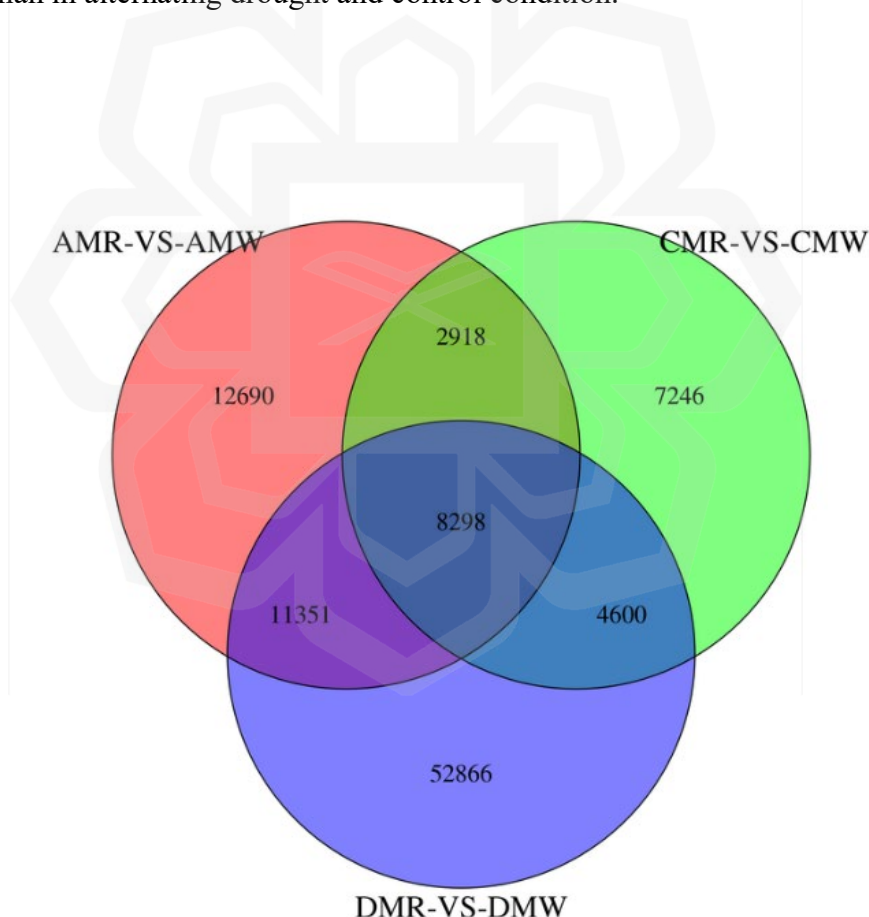


Figure 5.2: Venn diagram shows the number of expressed genes in the control conditions (CMR-VS-CMW), alternate drought treatments (AMR-VS-AMW) and continuous drought conditions (DMR-VS-DMW). Determination of significant DEGs was according to the criteria of $[\log_2 \text{ fold change } [L2fc]] \geq 2.0$ and at a $p \leq 0.05$.

5.3.3 Principal Component Analysis

Principal Component Analysis (PCA) is one of the most common classic methods to reduce the data's dimensionality and has been frequently applied in the analysis of gene expression (Yeung et al., 2001). The PCA displayed that replicates from control groups were clustered together with alternate drought treatments, indicating there was less variation in gene expression between these two groups (Figure 5.3). The first principal component (PC1) accounted for 16% of the variance, whereas the second principal component (PC2) accounted for 12.3% of the variance. In contrast with the control and alternate drought treatments, the continuous drought groups were clustered apart, which contributed to PC1. Furthermore, within the same continuous drought, the PCA plot also revealed a clear separation between the plotted drought MR297 (DMR) and drought Merah Wangi (DMW). Therefore, a remarkable genetic variation could be seen in both varieties in response to extreme drought stress.

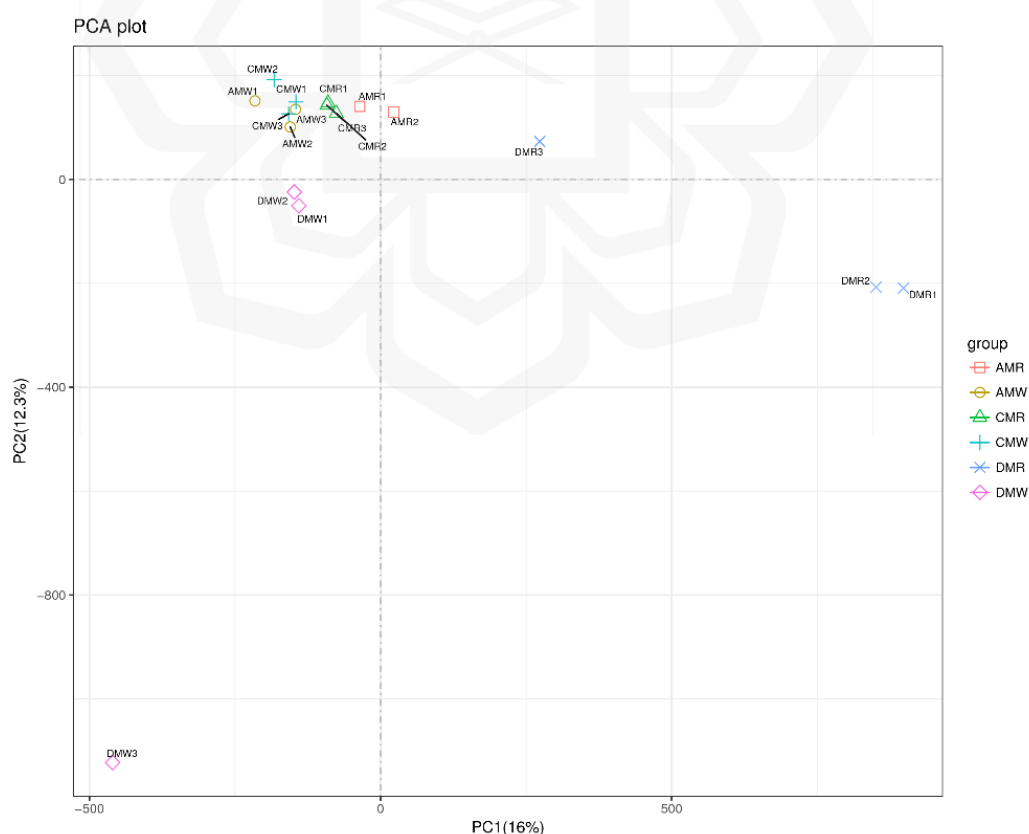


Figure 5.3: Principal component analysis of DEGs. X-axis corresponds for the first principal component (PC1), while Y-axis represents the second principal component (PC2). Determination of significant DEGs was according to the criteria of $[\log_2 \text{fold change } [L2fc]] \geq 2.0$ and at a $p \leq 0.05$.

5.3.4 Gene Ontology of DEGs

The top 30 most prominent gene ontology (GO) categories in Merah Wangi vs MR297 were selected and tabulated, as shown in Figure 5.4. According to the GO of control conditions, the majority of the transcripts were categorized in the MF, followed by CC and BP (Figure 5.4). Within the MF (10031 unigenes), ATP binding, was listed among the top four GO terms. Meanwhile, out of the 9022 total unigenes classified in the CC, the nucleus contained the most, with 3196 unigenes. Another top significant GO terms were the plasma membrane, cytoplasm, membrane, extracellular region, and nucleosome. In terms of BP, the GO category protein phosphorylation contained the most transcripts, with 1025 unigenes. The DAG belonging to BP can be seen in supplemental Figure 5.5. The most significant GO categories correspond to several responses, including response to biotic stress, defense response, response to other organisms, and response to external biotic stimulus.

A total of 34226 DEGs in response to alternate drought stress were annotated to Blast GO. Similarly to the GO from control condition, the MF comprised the majority of 15202 unigenes, with ATP binding being the most significant (Figure 5.6). Under the CC category, the largest number of unigenes were classified under the nucleus (4643 unigenes), followed by plasma membrane (3273 unigenes), cytoplasm (2751 unigenes), and membrane (2058 unigenes). Meanwhile, 5468 most prominent unigenes were annotated to the BP category, which further separated into response to stimulus and multi-organism process (Figure 5.7). Protein phosphorylation contains the dominant genes, with 1608 genes from the list followed by the defense response.

Under continuous drought stress, 24,425 unigenes belonged to MF, 39451 to CC, and 5802 to BP. In contrast with control and alternate drought stress, the majority of the unigenes in continuous drought stress were assigned to the CC category (Figure 5.8). Under this category, 9551 unigenes were significantly classified in the 'nucleus'

GO terms, followed by the ‘plasma membrane’, ‘chloroplast membrane’, and ‘plastid membrane’. Within the MF, most unigenes were assigned to the ‘binding’ category, such as ‘ATP binding’, ‘metal ion binding’, ‘DNA binding’, ‘ADP binding’, and ‘RNA’ and mRNA binding’. The least unigenes were categorized under BP, with 5802 unigenes. Further breakdown of this category has developed four GO terms, namely ‘metabolic process’, ‘cellular process’, ‘biological regulation’, and ‘response to stimulus’ (Figure 5.9). The top three significant terms according to the p-value and the darker colour of the box were ‘regulation of the RNA metabolic process’, ‘regulation of the nucleolus’, and ‘defense response’.

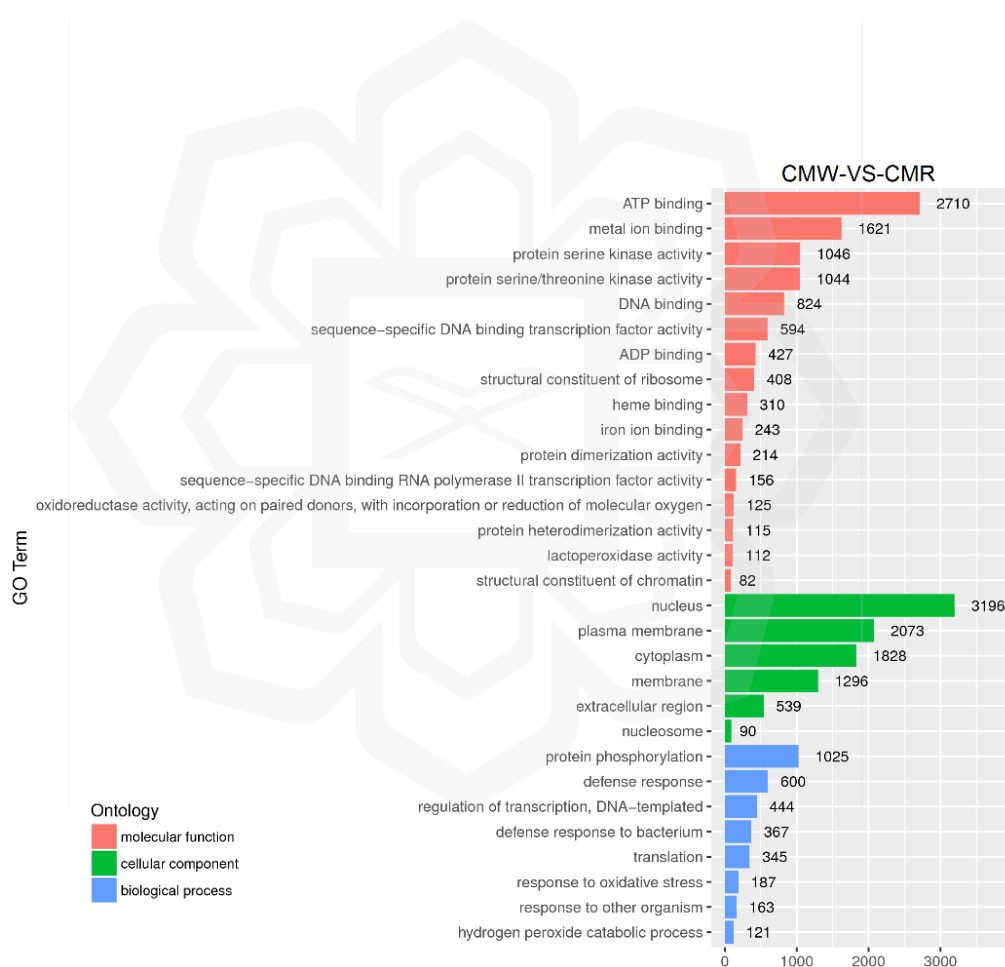


Figure 5.4: Gene ontology enrichment histogram of Merah Wangi vs MR297 in control condition. Color code is to distinguish the categories: molecular function, cellular component, and biological process. X- axis refers to the number of DEGs in the GO category. Y- axis refers to significantly enriched GO term. Determination of significant DEGs was according to the criteria of $[\log_2 \text{ fold change } [L2fc]] \geq 2.0$ and at a $p \leq 0.05$.

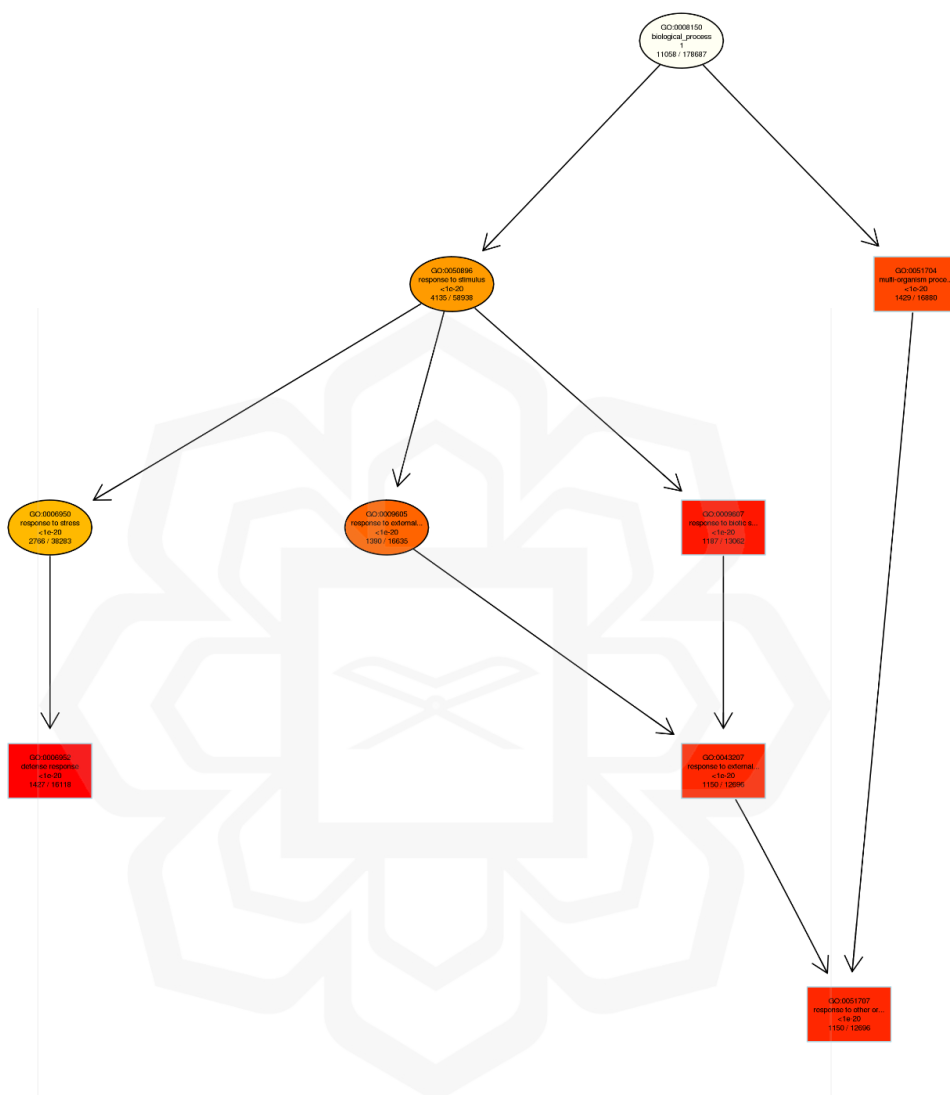


Figure 5.5: The directed acyclic graph (DAG) for the biological process in the control treatment between Merah Wangi vs MR297. The top 5 enriched GO terms were selected as primary nodes of the DAG. The associated GO terms are displayed by the inclusion relation. The box color represents the degree of significance, ranging from light yellow (least significant) to dark red (most significant). Inside the box of the significant terms, the information includes GO term, GO description, adjusted p-value, item number mapping the GO in the query list and total number of query list. Determination of

significant DEGs was according to the criteria of $[\log_2 \text{ fold change } [L2fc]] \geq 2.0$ and at a $p \leq 0.05$.

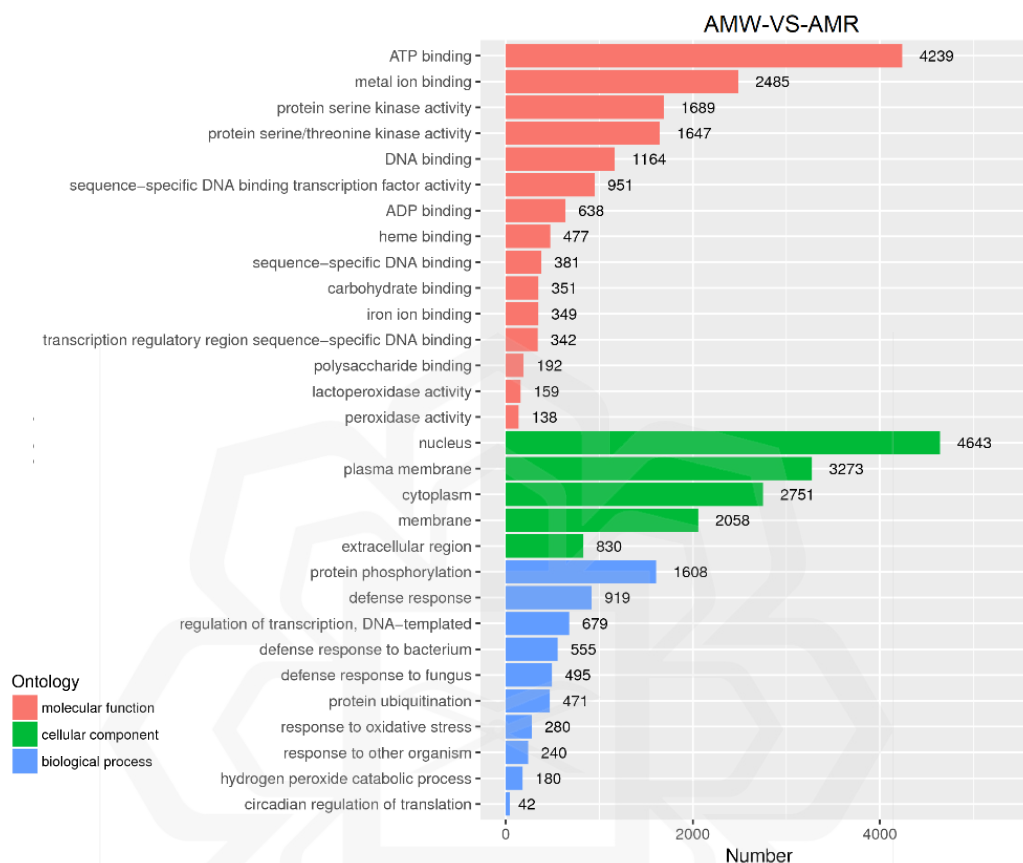


Figure 5.6: Gene ontology enrichment histogram of Merah Wangi vs MR297 in the alternate drought stress. Color code is to distinguish the categories: molecular function, cellular component, and biological process. X axis refers to the number of DEGs in the GO category. The Y axis refers to a significantly enriched GO term. Determination of significant DEGs was according to the criteria of $[\log_2 \text{ fold change } [L2fc]] \geq 2.0$ and at a $p \leq 0.05$.

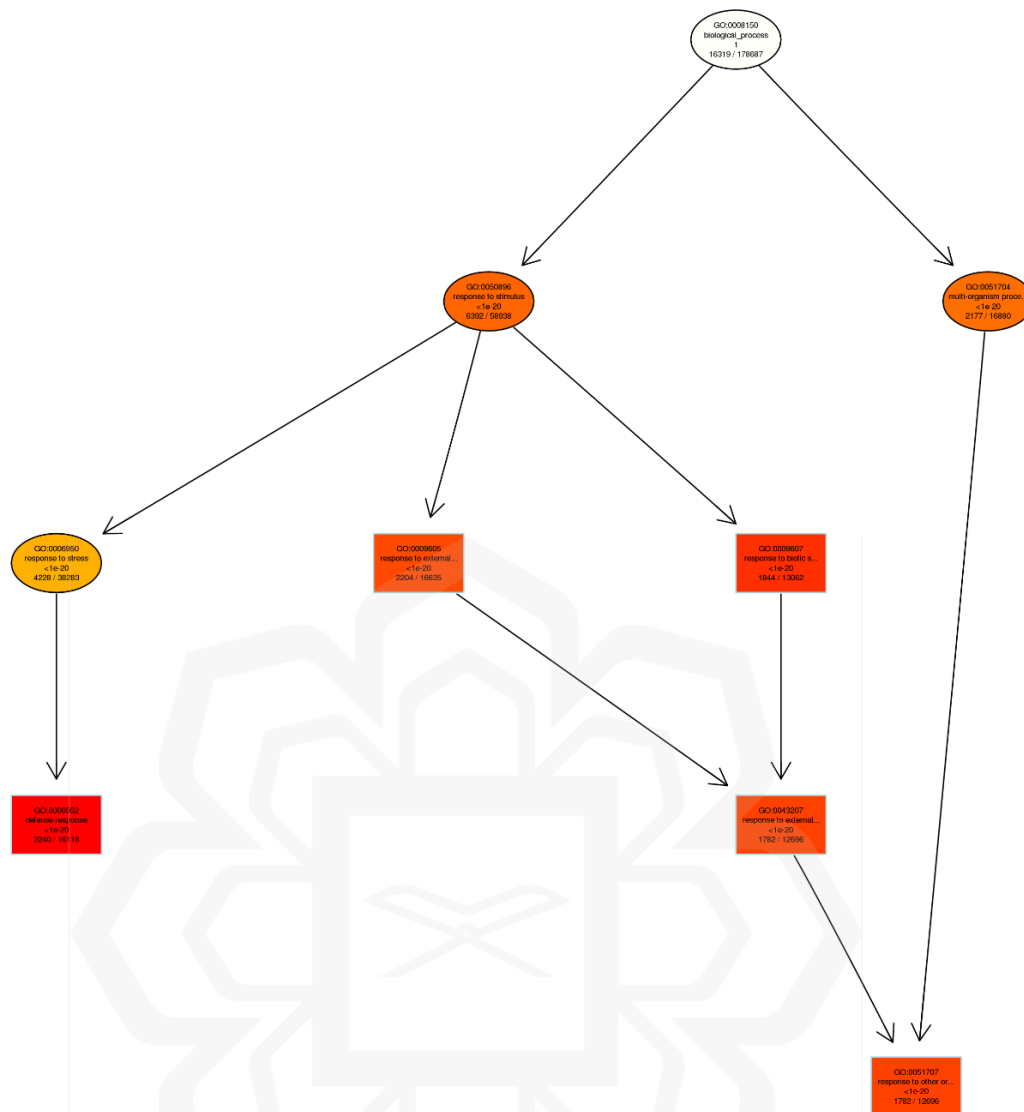


Figure 5.7: The directed acyclic graph (DAG) for the biological process in the alternate drought stress between Merah Wangi vs MR297. The top 5 enriched GO terms were selected as primary nodes of the DAG. The associated GO terms are displayed by the inclusion relation. The box color represents the degree of significance, ranging from light yellow (least significant) to dark red (most significant). Inside the box of the significant terms, the information includes GO term, GO description, adjusted p-value, item number mapping the GO in the query list and total number of query list. Determination of significant DEGs was according to the criteria of $[\log_2 \text{fold change [L2fc]}] \geq 2.0$ and at a $p \leq 0.05$.

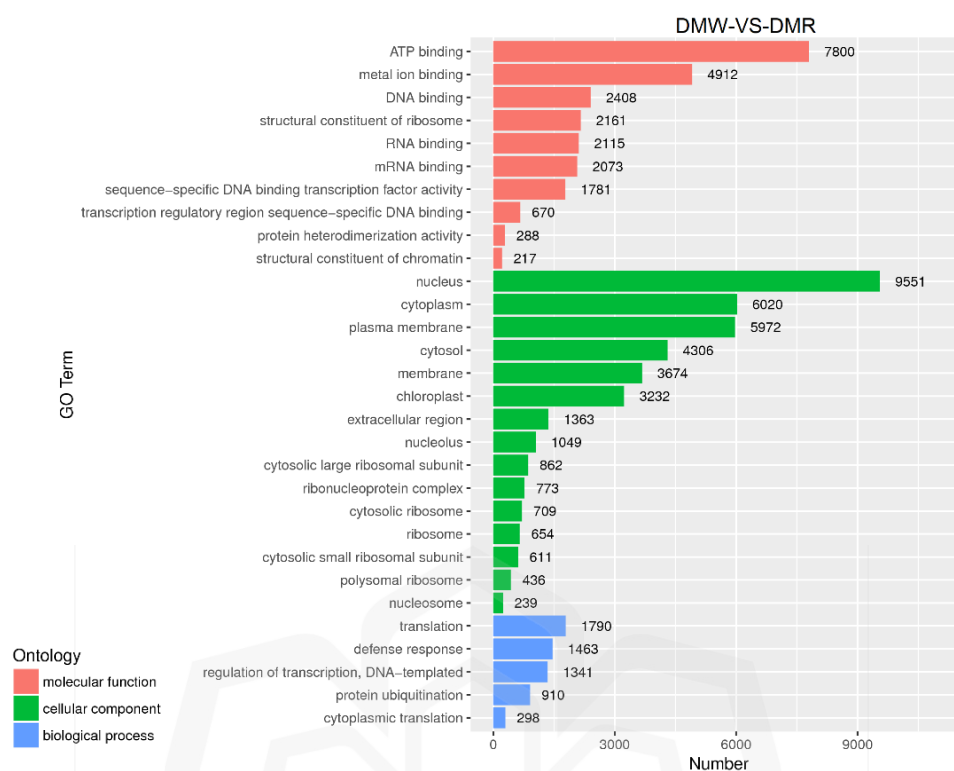


Figure 5.8: Gene ontology enrichment histogram of Merah Wangi vs MR297 in the continuous drought stress. Color code is to distinguish the categories: molecular function, cellular component, and biological process. X- axis refers to the number of DEGs in the GO category. Y- axis refers to a significantly enriched GO term. Determination of significant DEGs was according to the criteria of $[\log_2 \text{fold change}] \geq 2.0$ and at a $p \leq 0.05$.

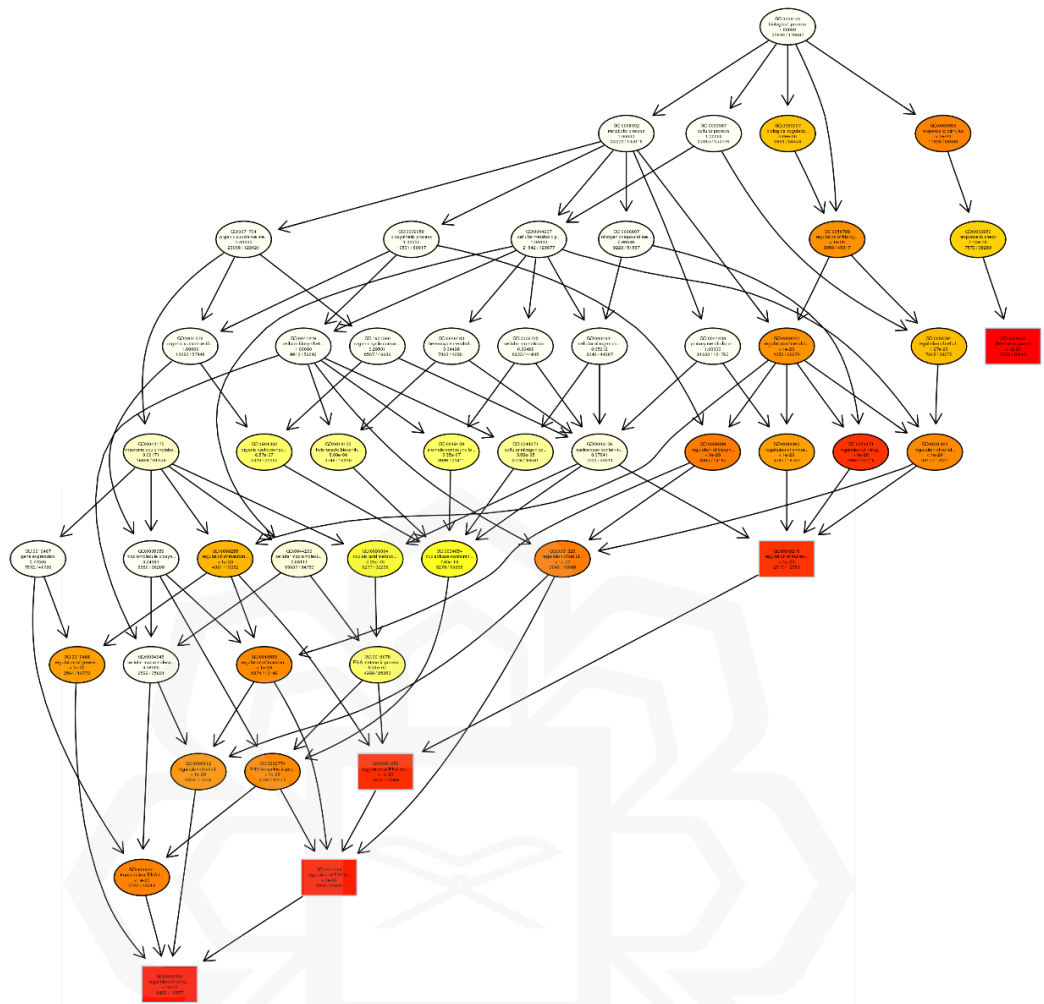
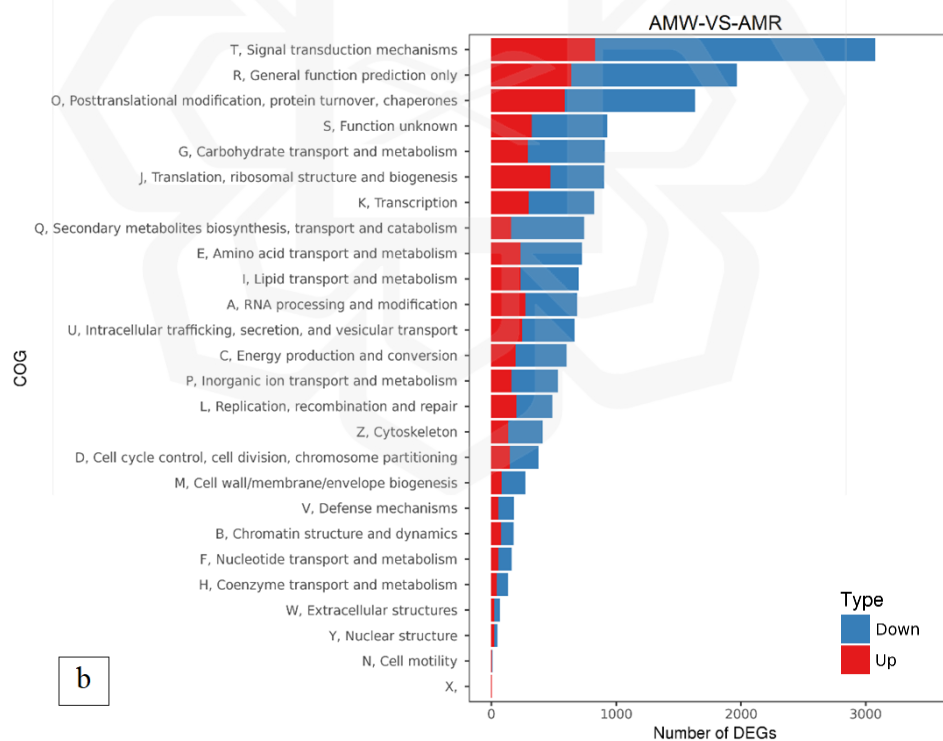
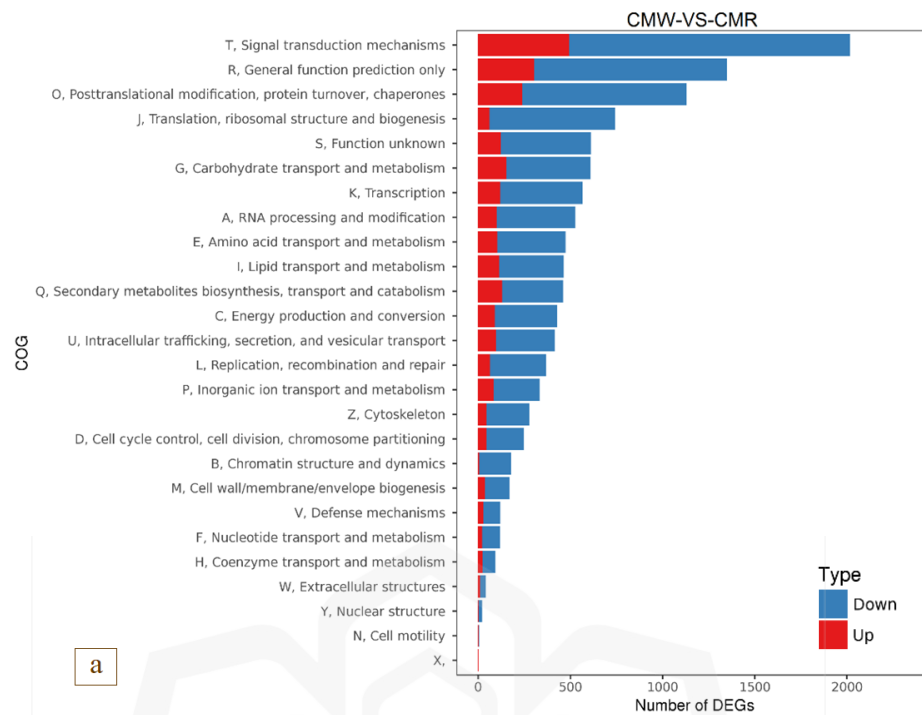


Figure 5.9: The directed acyclic graph (DAG) for the biological process in the continuous drought stress between Merah Wangi vs MR297. The top 5 enriched GO terms were selected as primary nodes of the DAG. The associated GO terms are displayed by the inclusion relation. The box color represents the degree of significance, ranging from light yellow (least significant) to dark red (most significant). Inside the box of the significant terms, the information includes GO term, GO description, adjusted p-value, item number mapping the GO in the query list and total number of query list. The determination of significant DEGs was according to the criteria of $[\log_2 \text{fold change [L2fc]}] \geq 2.0$ and at a $p \leq 0.05$.

5.3.5 Differential Gene Based on COG Analysis

Cluster of Orthologous Groups of proteins (COGs) is a protein database, that allows the representation of the evolutionary relationships among protein-coding genes from completely sequenced genomes (Galperin et al., 2017). Overall, 246341 unigenes were assigned to the COG classification between Merah Wangi vs MR297 (Table 5.1).

Among the 30 COGs categories, the cluster for ‘signal transduction mechanism’ represented the largest category across all treatments, followed by ‘general function prediction only’, and ‘posttranslational modification, protein turnover chaperones’. Within the ‘signal transduction mechanism’, the highest upregulated genes were found in continuous drought stress (Figure 5.10c), while the highest downregulated genes were observed in normal watering conditions (Figure 5.10a). Furthermore, most COGs under normal watering and alternate drought stress were represented by downregulated DEGs, whereas the ‘translation, ribosomal structure and biogenesis’ cluster had more upregulated genes than downregulated DEGs in response to two cycles of drought conditions (Figure 5.10b). Conversely, under continuous drought stress, most COGs were predominantly assigned by upregulated genes. Hence, prolonged drought stress induced the upregulation of DEGs involved in the ‘signal transduction pathway’.



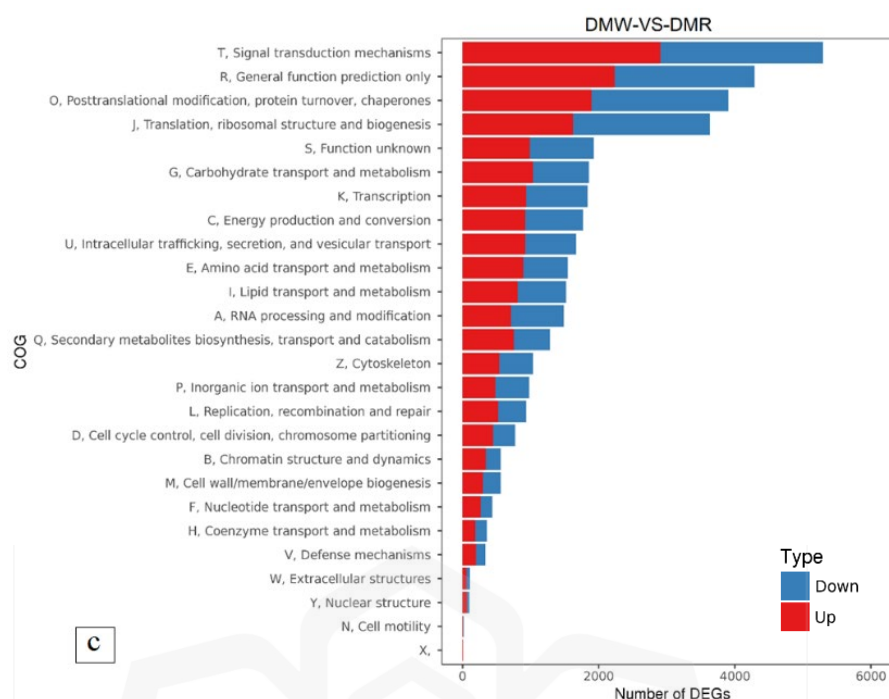


Figure 5.10: Histogram of differential gene COG functional classification in Merah Wangi vs MR297 in response to the control treatment (a), alternate drought stress (b) and continuous drought stress (c). The top 30 pathways that were most significantly enriched for the analysis were selected. Red bars are genes that are significantly upregulated and blue bars refer to downregulated genes. Determination of significant DEGs was according to the criteria of $[\log_2 \text{fold change } [L2fc]] \geq 2.0$ and at a $p \leq 0.05$.

5.3.6 Pathway Classification of DEGs

The unigenes of Merah Wangi vs MR297 were blasted to the Kyoto Encyclopaedia of Genes and Genomes (KEGG), a database that represents the network of genes in the genome with interacting molecules in the cell. A total of 9617 unigenes were annotated by the KEGG database. The top 30 (Q-value from small to large) pathways that were most significantly enriched were selected for analysis. Generally, under normal watering conditions, the Q-value for these top 30 pathways showed highly significant enrichment with ranges from 0.05 to 0.25 of RichFactor (Figure 5.11). The highest RichFactor was observed in ‘betalain biosynthesis’, indicating that DEGs under normal watering were significantly enriched in this shikimate pathway, although fewer genes have been reported in this pathway. Meanwhile, a large number of genes were involved in ‘metabolic pathways’, followed by biosynthesis of secondary metabolites.

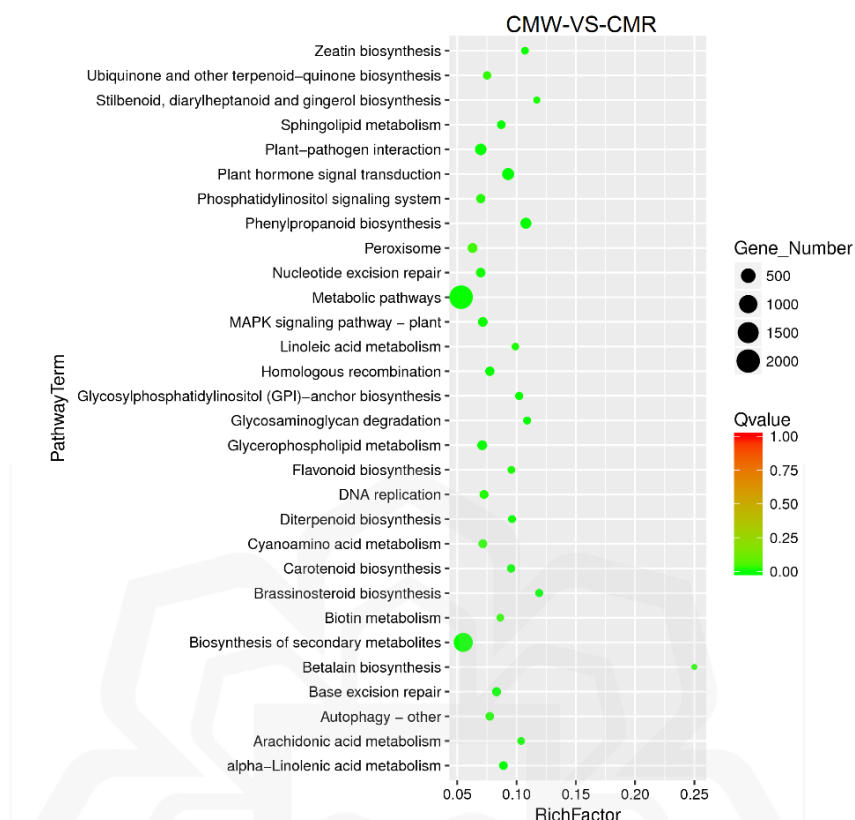


Figure 5.11: Scatter plot of differential gene KEGG enrichment in response to the control treatment between Merah Wangi vs MR297. The size of the dots reflects the number of differential genes in the pathway. The color code indicated the Q-value ranges. X-axis refers to the rich factor. Y-axis refers to pathway term.

In the alternate drought stress treatments, this study revealed that the dominant pathway terms were similar to the pathways under normal conditions. A relatively large group of DEGs were assigned to the ‘regulation of metabolic pathways’ and ‘biosynthesis of secondary metabolites’, with 3080 and 1733 genes, respectively (Figure 5.12). Based on the RichFactor, more than 10 pathways exhibited a RichFactor above 0.12, indicating a greater degree of enrichment. The significantly enriched pathway belongs to ‘flavone and flavanol biosynthesis’, ‘cutin, suberin and wax biosynthesis’, ‘phenylpropanoid biosynthesis’ and ‘brassinosteroid biosynthesis’. These pathways were more significantly impacted by the alternating two cycles of drought.



Figure 5.12: Scatter plot of differential gene KEGG enrichment in response to alternate drought stress between Merah Wangi vs MR297. The size of the dots reflects the number of differential genes in the pathway. The color code indicated the Q-value ranges. X-axis refers to the rich factor. Y-axis refers to pathway term.

In response to continuous drought stress, the study revealed that the majority of DEGs play a role in ‘plant hormone signal transduction’, which is a primary mechanism in the activation of drought resistance genes (Wei et al., 2016). Majority of DEGs were most associated with ‘plant-pathogen infection’ and ‘starch sucrose metabolism’ (Figure 5.13). According to the RichFactor, the DEGs were heavily enriched in metabolism pathways such as ‘benzaaxinoid biosynthesis’ and ‘stilbenoid and gingerol biosynthesis’. This KEGG enrichment analysis indicated that ‘plant hormone signal transduction’ and the ‘MAPK signalling pathway’ play important functions during

extreme drought stress. In addition, both environmental pathways contained the largest gene number with a high degree of enrichment in response to the prolonged drought stress.

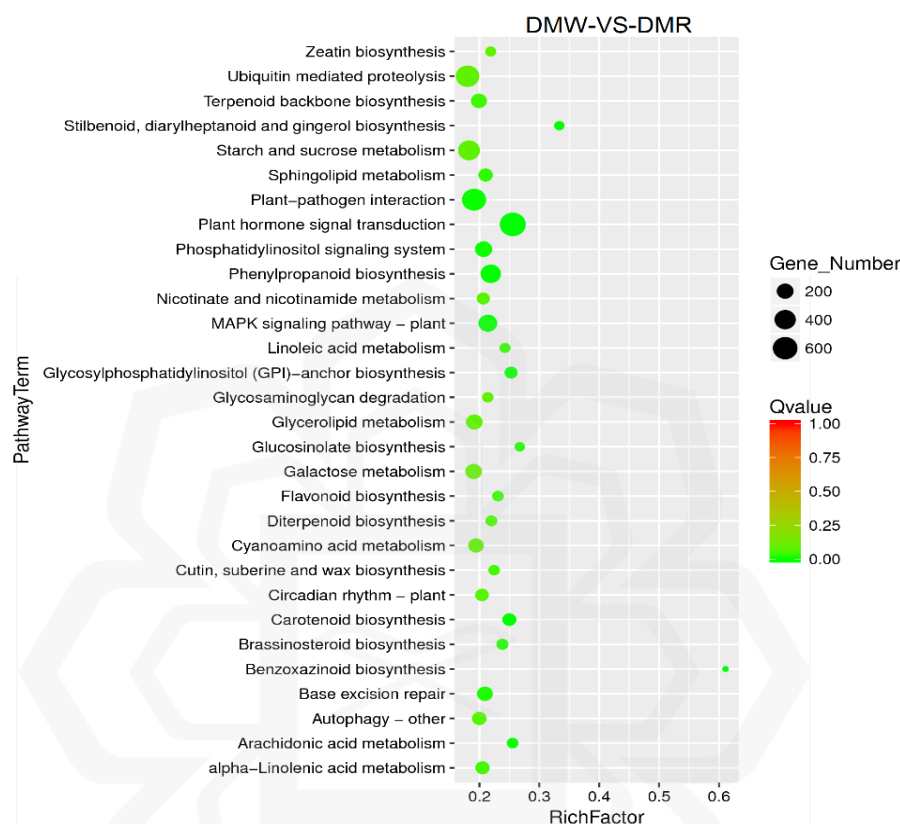


Figure 5.13: Scatter plot of differential gene KEGG enrichment in response to continuous drought stress. The size of the dots reflects the number of differential genes in the pathway. The color code indicated the Q-value ranges. X-axis refers to the rich factor. Y-axis refers to pathway term. Determination of significant DEGs was according to the criteria of $[\log_2 \text{ fold change } [L2fc]] \geq 2.0$ and at a $p \leq 0.05$.

5.3.7 The Screening of DEEPER ROOTING 1 Genes

Oryza sativa *DEEPER ROOTING 1* (*OsDRO1*) is the key regulator for rooting depth by controlling the growth angle of adventitious roots (Uga et al, 2013). Previous study reported this gene was mainly expressed in the meristematic of root tissues (Uga et al, 2013). The identification of unigenes encoding the protein DEEPER ROOTING 1

(DRO1) was analyzed in all treatments. The results found out only one transcript encoding DRO1, and it was downregulated under normal watering and alternate drought stress of Merah Wangi in relative to MR297 (Appendix II). In contrast, the continuous drought stress triggered the regulation of *DRO1* in Merah Wangi, which provide insights on the drought-responsive genes.

5.3.8 Phytohormones-Related Genes in Responses to Drought Stress

The expressions of phytohormone auxin, cytokinin and gibberellin (GA) in Merah Wangi vs MR297 were quantified for all the treatments. Under normal watering, the expression of downregulated genes for all the phytohormones were higher than upregulation of genes (Figure 5.14). Specifically, most of the major classes of auxin-responsive genes were downregulated. The genes include *Auxin response factor (ARF)*, *Auxin-induce protein*, *Auxin-responsive protein (Aux/IAA)*, SMALL AUXIN UP RNA (SAUR), and AUXIN-REGULATED GENE IN ORGAN SIZE (ARGOS). The upregulation of auxin genes consisted of similar expressions of activator and repressor genes (Figure 5.17a).

Meanwhile, the upregulation expression of cytokinin enzymes consists of two LONELY GUY (LOG) gene family and four different of *Cytokinin dehydrogenase (CKX)* gene family. In addition, the normal watering has abundantly downregulated LOG, which encodes the cytokinin riboside 5'-monophosphate phosphoribohydrolase, an enzyme that converts the inactive cytokinin nucleotides to the active form (Cheng et al., 2022). Similarly, for gibberellin, the activator GA genes were more downregulated in Merah Wangi vs MR297 (Figure 5.19a). The downregulation genes consisted of GA deactivating enzyme from the *Gibberellin dioxygenase (GA2ox)* and GA activator *Gibberellin 20 oxidase (OsGA20ox)*. Meanwhile the upregulation was dominantly controlled by the four types of *OsGA2ox* gene family. Besides that, only one transcript encoded the upregulation of GA activating gene, *Chitin-inducible gibberellin-responsive protein (CIGR)*.

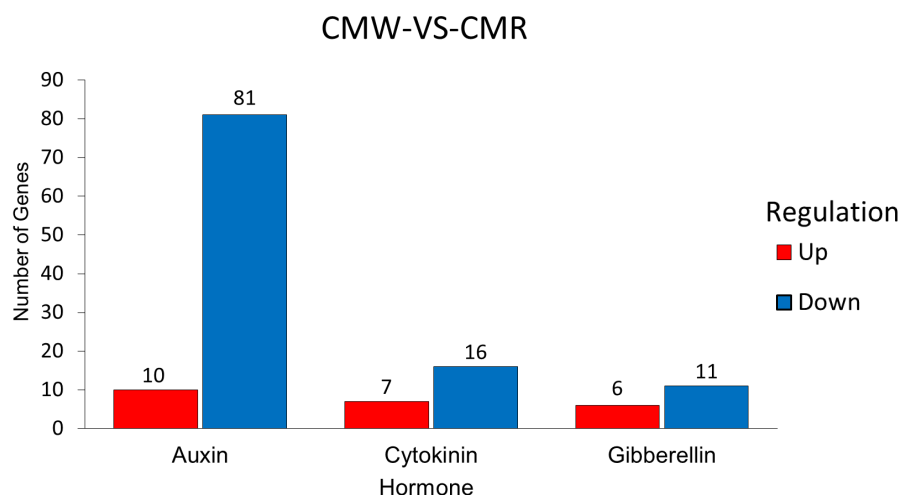


Figure 5.14: Histogram of the level of hormone expression in Merah Wangi vs MR297 under control condition. Red bars are genes that are upregulated and blue bars refer to downregulated genes. Determination of significant DEGs was according to the criteria of $[\log_2 \text{ fold change } [L2fc]] \geq 2.0$ and at a $p \leq 0.05$.

In the alternate drought stress, the DEGs encoding the selected hormones were abundantly downregulated, with auxin showing the highest level of both gene expression (Figure 5.15). Specifically, most of the auxin upregulated genes encoding the *Aux/IAA* gene family, followed by the *ARF* and *SAUR*. Besides that, most of the repressing enzymes, Indole-3-acetic acid amino synthetase GH3 (GH3) were downregulated in Merah Wangi. Meanwhile, in terms of cytokinin gene expression, the number of upregulated genes were encompassed by *LONELY GUY* cytokinin activating enzymes and repressors from *CKX* gene family, with five and four DEGS, respectively (Figure 5.17b). The results also revealed that most of the *LOG* was downregulated in response to the alternated drought treatments. Meanwhile, the upregulation of GA gene was primarily encoded by the GA activators such as *GA20ox*, Gibberellin-regulated protein, and Gibberellin receptor (Figure 5.18b). The results also revealed that most of the *GA20ox* were downregulated in response to the alternate drought stress. In addition, the Gibberellin-regulated protein 13 (GASA13) were equally upregulated and downregulated in the root of Merah Wangi vs MR297.

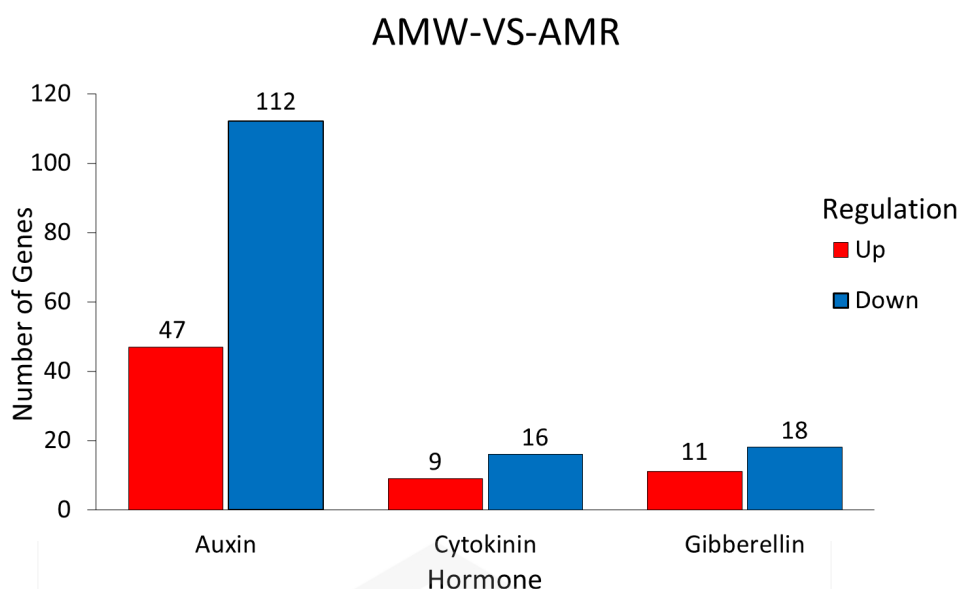


Figure 5.15: Histogram of the level of hormone expression in Merah Wangi vs MR297 under alternate drought conditions. Red bars are genes that are upregulated and blue bars refer to downregulated genes. Determination of significant DEGs was according to the criteria of $[\log_2 \text{ fold change } [L2fc]] \geq 2.0$ and at a $p \leq 0.05$.

This study revealed a distinct expression pattern of hormone-related gene under continuous drought stress, where the upregulated genes exhibited higher expression levels than the downregulated genes (Figure 5.16). The upregulation of auxin genes showed the highest genes numbers compared to other hormones, with auxin repressor *Aux/IAA*, followed by *GH3*, and *ARF* showed higher expression levels. Moreover, the continuous drought stress also upregulated the *SAUR* and *ARGOS* gene family. It was found out that the majority of the downregulation of auxin related genes was expressed by the auxin activator (Figure 5.18c). The expression of activators cytokinin and gibberellin exhibited the highest level compared to the repressor's gene (Figure 5.18c & Figure 5.19c). Specifically, the prolonged drought treatment has upregulated 34 DEGs encoded the LOG gene members and Gibberellin-regulated protein 14 (GASA14). The cytokinin repressors, CKX3 and CKX4 were also upregulated in the Merah Wangi vs MR297. Likewise in gibberellin, abundant GA activators were upregulated, including the *GA20ox* and *CIGR1* and *CIGR2* (Figure 5.19c). The findings also showed only eight DEGs were downregulated, primarily consisting of the repressors *GA20ox*.

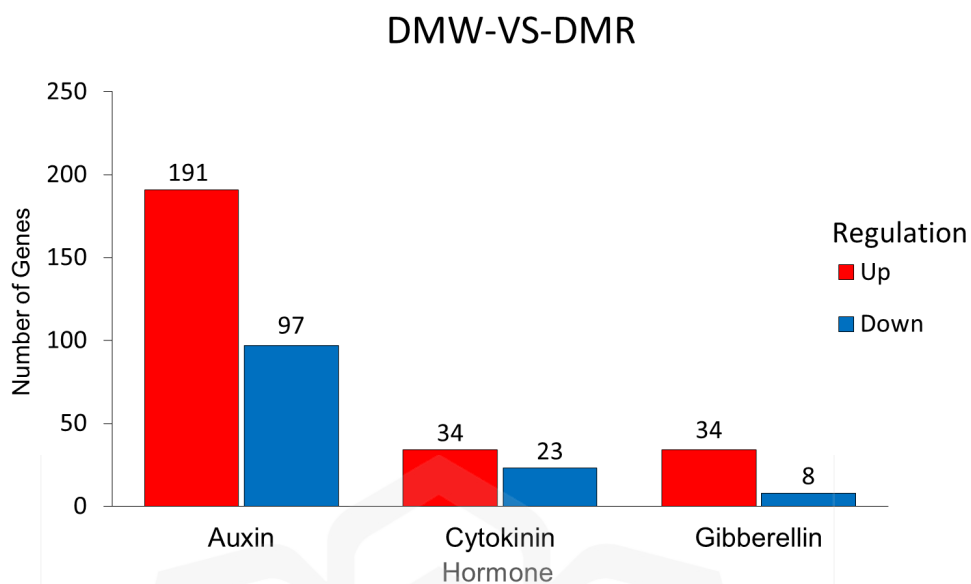


Figure 5.16: Histogram of the level of hormone expression in Merah Wangi vs MR297 under continuous drought conditions. Red bars are genes that are upregulated and blue bars refer to downregulated genes. Determination of significant DEGs was according to the criteria of $[\log_2 \text{ fold change } [L2fc]] \geq 2.0$ and at a $p \leq 0.05$.

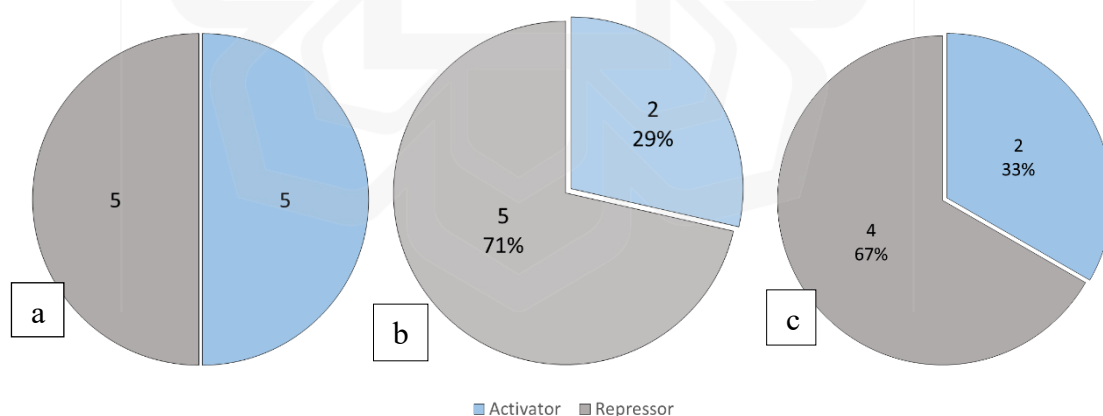


Figure 5.17: Venn diagram of regulators involved in the upregulation auxin (a) cytokinin (b) and gibberellin-related genes (c) of Merah Wangi vs MR297 under control condition. Blue represents the activator genes and grey refers to repressor genes. Determination of significant DEGs was according to the criteria of $[\log_2 \text{ fold change } [L2fc]] \geq 2.0$ and at a $p \leq 0.05$.

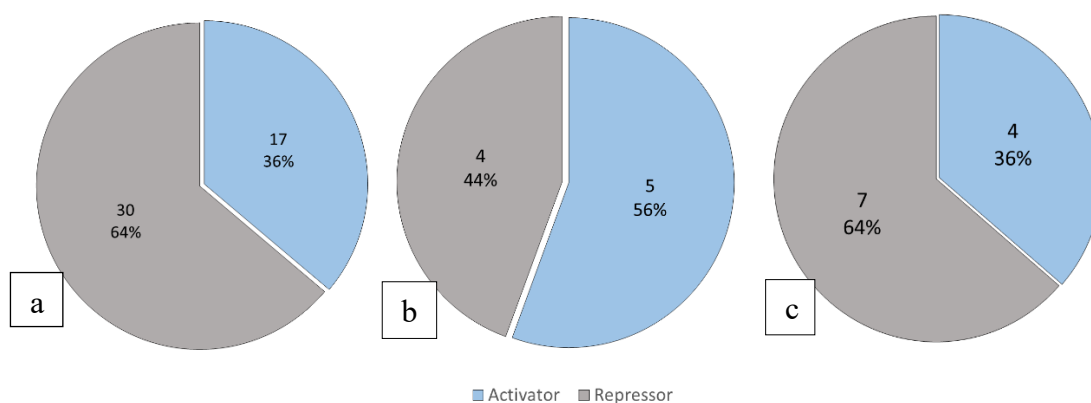


Figure 5.18: Venn diagram of regulators involved in the upregulation auxin (a) cytokinin (b) and gibberellin-related genes (c) of Merah Wangi vs MR297 under alternate drought conditions. Blue represents the activator genes and grey refers to repressor genes. Determination of significant DEGs was according to the criteria of $[\log_2 \text{ fold change [L2fc]}] \geq 2.0$ and at a $p \leq 0.05$.

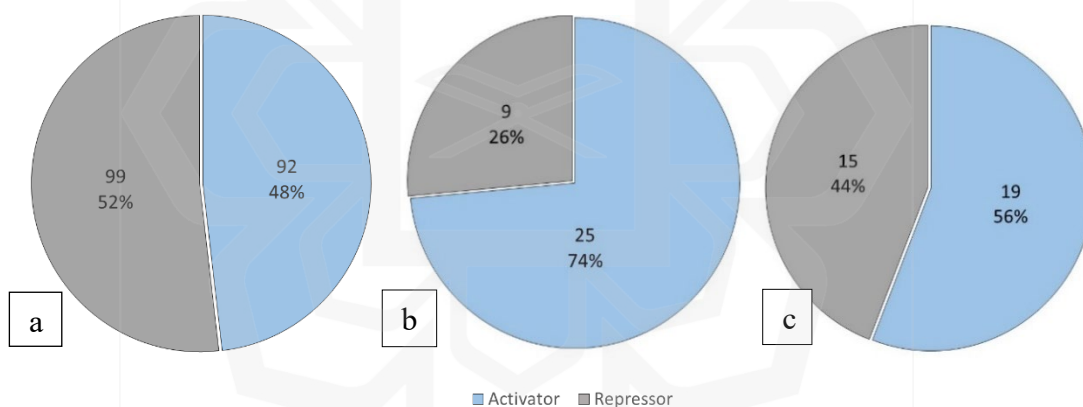


Figure 5.19: Venn diagram of regulators involved in the upregulation auxin (a) cytokinin (b) and gibberellin-related genes (c) of Merah Wangi vs MR297 under alternate drought conditions. Blue represents the activator genes and grey refers to repressor genes. Determination of significant DEGs was according to the criteria of $[\log_2 \text{ fold change [L2fc]}] \geq 2.0$ and at a $p \leq 0.05$.

5.4 DISCUSSION

5.4.1 The Transcriptome Data of Rice Root Differs under Different Form of Drought Stress

The effect of alternate and continuous drought stress resulted in higher gene upregulation in Merah Wangi compared to gene downregulation (Figure 5.1). Earlier studies reported that upon detecting drought stress, plants often upregulated genes as they activated various defense mechanisms to confer the stress (Golldack et al., 2014; Shinozaki et al., 2006). Moreover, Golldack et al. (2014) reported that stress tolerance relies on multi-component signalling networks that are aimed at restoring cellular homeostasis and enhancing survivability under stress conditions.

The COG analysis revealed that most DEGs were associated in ‘signal transduction pathway’ (STP) across all treatments. Interestingly, STP activity peaked during continuous drought stress (Figure 5.10c), followed by the alternate drought stress (Figure 5.10b). Specifically, only STP under continuous drought treatment was dominated by the upregulated genes. This finding suggests that the treatment of prolonged drought has triggered gene expression in the STP. According to Xiong et al. (2002), STP begins with signal detection and then produces secondary messengers. These secondary messengers initiate a protein phosphorylation, which eventually affects the proteins involved in cellular protection or regulatory proteins involved in signal transduction and the stress-responsive gene. Thus, drought-inducible genes may have a functional or regulatory role (Shinozaki et al., 2006). The hormones gibberellin, ethylene, abscisic acid (ABA), and salicylic acid are examples of gene products derived from regulatory molecules (Xiong et al., 2002). The high level of DEGs in the STP under continuous drought stress provides insights into drought-responsive genes.

KEGG enrichment analysis of control and alternating two cycles of drought revealed the majority of DEGs were mostly enriched in the ‘metabolites pathway’ and ‘biosynthesis of secondary metabolites’ (Figure 5.11 and Figure 5.12). This result aligns with a meta-analysis of a rice study under multiple abiotic stresses (Azad et al., 2024). Similarly, Kyndt et al. (2012) also reported that most genes in the rice root tips belonged mainly to the ‘metabolic pathway’ and ‘biosynthesis secondary metabolites’. However, in response to continuous drought stress, DEGs were mostly involved in the ‘plant hormone signal transduction’ and ‘ubiquitin proteolysis pathway’ (Figure 5.13). This

study suggested that gene expression in the hormone signaling pathway might be correlated to the drought response in Merah Wangi. Tang et al. (2022) also discovered ‘plant hormone signal transduction’ was the most enriched pathway under drought stress, consistent with these findings. Furthermore, Kou et al. (2022) highlighted the role of hormone signaling genes in determining the variability of root system architecture (RSA) under water deficiency. Therefore, understanding DEGs associated to root hormonal expression is crucial for studying drought tolerance.

5.4.2 The Regulation of *DEEPER ROOTING1* under Drought Stress

This study discovered the continuous drought treatment upregulated the protein DEEPER ROOTING1 (DRO1) in Merah Wangi against MR297. This rice-specific gene was only identified under continuous drought stress. Similar findings were reported by Singh et al. (2021) and Uga et al. (2013), who discovered that overexpression of *DRO1* in the drought-stressed root tips improved the root growth angle while causing roots to grow deeper downward. Previous research suggested that the great number of deep roots and the robust lateral root growth from deep nodal roots play a critical role in determining the capacity to access water from deeper soil horizon (Gowda et al., 2011; Hazman & Brown, 2018; Yuanyuan et al., 2022). The expression of deep rooting traits is controlled by the hormonal signal (Uga et al., 2011). Therefore, the elucidation of phytohormone-regulated genes in the root contributed to understanding the mechanism of drought tolerance.

In addition, *DRO1* aided the rice produce more grains under drought stress (Arai-Sanoh et al., 2014; Vinarao et al., 2023). The expression of *DRO1* in rice roots was considered a drought avoidance strategy, which is an initial strategy and significantly contributed to enhancing the plant’s drought resistance (Blum, 2005; Singh et al., 2021). This gene has been proposed as a beneficial gene for breeders aiming to generate stable grain yields even under drought conditions (Uga et al., 2013). Hence, the identification of *DRO1* in the drought-stressed root underscores the ability of Merah Wangi to tolerate severe drought conditions.

5.4.3 The Expression of Auxin in Response to Drought Stress

Phytohormones have a major role in adapting to environmental stress. Upon exposure to drought, plants incite a signalling cascade, leading to the accumulation of endogenous hormones and the activation of defense mechanisms (Osakabe et al., 2014). Generally, this study revealed the auxin genes were more highly expressed than cytokinin and gibberellin under all treatments (Figure 5.14). The three major early auxin-responsive genes, *Aux/IAA*, *SAUR* and *GH3* were all identified in all treatments. The highest expression of auxin was seen under continuous drought stress (Figure 5.14), which is consistent with a prior study that found drought stress triggers the expression of a wide array of auxin synthesis genes (Ke et al., 2015). The upregulated genes in Merah Wangi against MR297 were screened to investigate the regulation of auxin genes within the treatment. The severity of drought stress has resulted in different expressions of auxin gene family members.

This finding found that alternate and continuous drought stress has induced the upregulation of the repressor *Aux/IAA* higher than the activator *ARF* gene family members (Figure 5.17b,c). These results suggested the role of *Aux/IAA* in mediating the drought stress signaling pathways in Merah Wangi. Moreover, a few reports mentioned the role of *Aux/IAA* proteins in mediating drought stress tolerance (Kim et al., 2022b; Salehin et al. 2019). Furthermore, Zhang et al. (2020) discovered that the IAA was involved in mitigating plant dehydration by stimulating the relative water content.

Among the upregulated *Aux/IAA*, *IAA18* was abundantly expressed under continuous and alternate drought stress with 19 and 4 transcripts, respectively. Song et al. (2009) reported a similar finding, that drought stress has elevated the expression of *OsIAA8* gene in rice. In general, *OsIAA18* plays a role in mitigating the abiotic stresses in *Arabidopsis* (Li et al., 2020). In regulating the drought tolerance in rice, *OsIAA18* stimulates the expression of ABA biosynthesis genes, including the *OsNCED*, and upregulates the expression of ABA-responsive signaling pathway genes such as *OsRAB* family members (Wang et al., 2021). The role of *IAA18* was discovered to be associated with ABA biosynthesis, thus alleviating drought stress.

Additionally, the transcriptome data comparing Merah Wangi and MR297 under normal watering showed no DEGs encoded *IAA14* and *IAA15*. However, *IAA15* was abundantly upregulated under continuous drought stress. Kim et al. (2022b) emphasized

that *IAA15* regulates the inhibition of lateral root growth, thereby enhancing drought tolerance. Specifically, under drought stress, *IAA15* was primarily observed in the epidermis, while *IAA6* and *IAA9* were expressed in the stele (Shani et al., 2018). Meanwhile, *IAA17* was mainly expressed in the stele via root elongation (Fukaki et al., 2002). Notably, this study found that the repressor *OsIAA17* was only upregulated continuous drought stress, indicating that prolonged dehydration treatment triggered the expression of *OsIAA17* in the Merah Wangi roots. This suggests that the *OsIAA17* regulation plays a significant role in the plant's response to drought, contributing to the drought tolerance mechanism.

Besides that, the continuous drought stress had upregulated *OsIAA6*. A previous study by Jung et al. (2015) reported that *OsIAA6* was specially expressed in the veins and axillary meristems of rice tillers. However, the current study found that five transcripts encoding *OsIAA6* were detected in the drought-stressed root tips, while none were expressed under normal watering. Notably, *OsIAA6* is involved in mediating drought stress and controlling tiller outgrowth (Jung et al., 2015), although its regulatory role in the roots remains unknown.

Apart from Aux/IAA proteins, this study also identified the SAUR gene family. SAUR proteins correspond to the activator of early auxin-responsive genes, with 58 SAUR proteins that have been identified in rice (Guo et al., 2018; Jain et al., 2006). This study found that 7 out of 8 and 9 out of 13 SAUR genes were downregulated under normal watering and alternate drought stress, respectively. Meanwhile, under continuous drought stress, 14 out of 22 SAUR genes were upregulated, which primarily consisted of *SAUR36*. Remarkably, overexpression of *SAUR36* in *Arabidopsis thaliana* resulted in the reduction of cell expansion as well as inducing leaf senescence (Hou et al., 2013).

Studies of *SAUR36* on drought-stressed roots are limited. To date, some of the SAUR proteins that respond to abiotic stresses such as drought and salt stress are *SAUR41*, *SAUR75*, and *SAUR78*, suggesting that SAUR plays a role in regulating abiotic stress tolerance (Guo et al., 2018; Li et al., 2015; Qiu et al., 2020). Huang et al. (2023) reported that *OsSAUR* enhances the plant growth regulator by promoting auxin biosynthesis and polar transport. This study highlights the expression of *SAUR36* in the

plant's response to prolonged drought stress, suggesting its potential involvement in adaptive mechanisms in Merah Wangi.

Besides that, this study identified the upregulation of *OsGH3* under continuous drought stress. Interestingly, the expression of *OsGH3* under alternate drought stress was similar to that under normal growth conditions, where it was mainly downregulated. Zhang et al. (2009) also reported that *OsGH3* is inactive under control conditions but activated by drought stress. Thus, alternating two cycles of drought has acclimated the roots of Merah Wangi to drought stress. Moreover, some GH3 families were associated with plant defense responses (Singh et al., 2014). Specifically, *OsGH3.17* and the protein ARGOS, which influence cell division, expansion and enhance root elongation (Feng et al., 2011; Guo et al., 2022; Hu et al., 2006), were only upregulated under continuous drought stress. The upregulation of these genes in Merah Wangi under prolonged drought stress contributes to a better understanding of drought-responsive genes in rice roots.

The auxin-responsive gene, ARF, was found abundantly under drought stress. Conversely, under normal watering, ARF gene family members were primarily downregulated. It has been reported that this ARF regulates the adaptive response to drought stress (Verma et al., 2022). The results showed that 37 and 8 transcripts encoding ARF were upregulated in response to the continuous and alternate drought stress, respectively. Specifically, 13 family members of ARF proteins were upregulated in response to continuous drought stress, with *OsARF12*, *ARF14*, *OsARF22* and *ARF3* showing high induction. In addition, the upregulation of ARF proteins during both drought stress was remarkably higher than in normal watering plants, indicating the water deficit condition induces ARF expression in the rice roots. This finding aligns with research by Liu et al. (2022), who found out *ARF5*, *ARF10*, and *ARF16* genes in *Santalum* sp. were overexpressed under drought stress.

There are very few studies that reported the role of ARF in water deficiency. Previous research shows that ARF's role is closely associated with underground plant parts, rather than aboveground parts like foliage (Bouzroud et al., 2018). For instance, Goh et al. (2012) found that *ARF7* regulates lateral root formation in *Arabidopsis*, while *AtIAA12* and *AtARF5* induces meristematic root (Liu et al., 2020). In drought-stressed tomato roots, *ARF10* expression is triggered in lateral and primary roots (Bouzroud et

al., 2018). Okushima et al. (2007) reported that *ARF7* and *ARF19* together stimulate lateral root formation in the root stele. Taken together, this study suggests that ARF proteins are important for drought tolerance in Merah Wangi. Thus, the elucidation of ARF provides insights, with the precise mechanism of ARF-mediated stress in roots remains unclear.

5.4.4 Cytokinin Regulation in Response to Drought Stress

Further analysis was conducted to elucidate the cytokinin regulation in Merah Wangi against MR297 under different forms of drought stress. Similar regulatory patterns were observed under alternate drought stress and normal watering, with more downregulated cytokinin genes than upregulated ones (Figure 5.18a, b). In contrast, continuous drought stress led to the upregulation of cytokinin genes, primarily expressed by the LOG gene family. Previous studies reported that *OsLOGL9*, *LOGL9*, and *LOGL10* were widely expressed in shoots rather than roots (Tsai et al., 2012). However, this current finding shows that *LOGL9* and *LOG10* were abundantly expressed under drought-stressed roots.

The cytokinin repressor, *CKX* members were also detected across all treatments, with the highest transcript level identified under continuous drought stress (Figure 5.18c). Previous studies have reported that certain *CKX* genes, including *CKX1*, *CKX14*, and *CKX6*, contribute to a broad range of defense mechanisms and enhance drought tolerance (Khandal et al., 2020; Li et al., 2023; Pospisilova et al., 2016). In addition, higher expression of *CKX* results in a larger root system, which promotes drought tolerance (Ramireddy et al., 2018; Werner et al., 2010). For instance, incorporating *CKX* in transgenic barley enlarged the root system, leading to increased biomass without affecting shoot growth (Ramireddy et al., 2018). Simultaneously, these transgenic plants also showed less sensitivity to drought stress, indicating better drought tolerance. There were four and one transcripts encoding the upregulation of *OsCKX4* under continuous and alternate drought conditions, respectively. Interestingly, *OsCKX4* has been found to mediate rice crown root development without affecting shoot growth (Gao & Lynch, 2016). These findings suggest that cytokinin, particularly those from the *LOG* and *CKX* families, play crucial roles in mediating the root responses to drought.

5.4.5 Gibberellin Regulation in Response to Drought Stress

Gibberellin (GA) has a pivotal role in root meristem development (Shtin et al., 2022). Under normal watering and alternate drought stress, the expression of GA-activator genes, *GA20ox*, was mostly downregulated in Merah Wangi against MR297 (Figure 5.19a, 5.19b). However, prolonged drought treatment induced the expression of the deactivator genes *GA2ox3* and *GA2ox6*, higher than the activator *GA20ox* genes (Figure 5.19c). Similar findings were reported by Shohat et al. (2021), where drought stress reduced the expression of *GA20ox* and *GA20ox2* but stimulated *GA2ox7* in tomato leaf tissue. Specifically, the deactivating enzyme, *GA20ox* has been reported to be expressed in the root meristematic tissue of *Arabidopsis*, with the cortex and epidermis being the prominent sites (Barker et al., 2020). Furthermore, Barker et al. (2020) reported *GA20ox* is a necessary gene in both the meristematic and root elongation zones.

In contrast to the *Arabidopsis* findings, a recent root study by Fonouni-Farde et al. (2019) reported that GA controlled the size of the root apical meristem by negatively regulating cortical cell expansion, leading to inhibition of both primary and lateral root growth. Later, it was proposed that GA regulation has a distinct role in the root system, depending on the species and protein (Fonouni-Farde et al., 2019). Overexpression of GA deactivator, *GA2ox* has been suggested to prevent transpiration and enhance drought adaptation (Shohat et al., 2021). Furthermore, transgenic rice expressing *GA2ox* promoted drought tolerance by expanding bulliform cells, promoting the root system and water use efficiency, and producing more effective tillers (Lo et al., 2017). Therefore, the overexpression of *GA20ox* in drought-stressed root enhancing the drought tolerance, by improving the root architecture.

In addition, this study revealed that continuous drought stress upregulated five transcripts corresponding to *GASA12*, *GASA13*, and *GASA14*. In contrast, the alternate drought treatment upregulated only one transcript of *GASA13* genes, while none of *GASA* genes were found under normal watering. The role of *GASA* protein in stress tolerance and growth regulation has been discussed by Wu et al. (2022). Moreover, the expression of *GASA* in *Arabidopsis* has promoted root development while stimulating the plant's resistance to drought and salt stress (Wang et al., 2014). A recent study of the underground part found that some GA positively regulates cell proliferation in the

carrot (Wang et al., 2015). The *GASA* genes have been reported to influence root elongation (Trapalis et al., 2017; Wu et al., 2022).

The alternate drought stress led to the downregulation of *CIGR*, similarly to control condition. Conversely, continuous drought stress has triggered seven DEGs encoding *CIGR*, which belong to the GRAS family (Figure 5.19c). The identification of the rice gene *CIGR1* and *CIGR2* has been reported in the previous studies (Day et al., 2004; Kovi et al., 2011). While the functional role *CIGR* in root tissues remains to be elucidated, these rice genes have been linked to enhanced plant height and have been detected in the tissues of the tall rice variety, Pokkali (Kovi et al., 2011). Hence, the findings indicate the root growth and development under continuous drought stress are regulated by manipulating GA-related genes which can be beneficial for drought tolerance traits.

5.5 CONCLUSION

In conclusion, the root transcriptome study of Merah Wangi relative to MR297 showed a comprehensive regulatory mechanism involved in drought tolerance. The study revealed an abundance of DEGs in Merah Wangi, with more genes downregulated than upregulated, especially under alternating and continuous drought stress. The PCA displayed clustering patterns, with continuous drought stress exhibiting the most variation. The GO analysis indicated that most DEGs under alternate drought stress were involved in molecular function, while the prolonged drought stress induced DEGs in cellular process. Furthermore, the COG analysis highlighted that signal transduction was the largest category across all treatments, with upregulated genes being predominant under continuous drought stress. Besides that, KEGG pathway showed that the DEGs in the alternate drought stress had similar expressions to those under normal watering, with most DEGs enriched in the biosynthesis of secondary metabolites pathway. Conversely, continuous drought stress induced DEGs involving in plant hormone signalling pathway. This study also elucidated the role of *DEPPER ROOTING1* under continuous drought stress, suggesting its importance in drought avoidance. Phytohormone-regulation was crucial for adapting to drought conditions, with auxin genes showing the highest expression levels, followed by cytokinin and gibberellin genes. The treatment of alternating two cycles of drought has underscored

root acclimation, involving hormonal changes that enable Merah Wangi to better cope with prolonged drought stress. It was suggested that upregulation of hormone repressors such as *ARF*, *CKX* and *GA20ox* under prolonged drought stress contributed to drought tolerance by improving the root system. Therefore, these findings provide valuable insights into drought tolerance in rice roots, and the elucidation of candidate genes could be a resource in the breeding strategies for drought-resistant rice varieties.



CHAPTER SIX

GENERAL DISCUSSION

As a root has a direct role in water uptake, the aim of this research was to identify the root adaptive traits among the six rice varieties under alternating and continuous drought stress. The previous study emphasized the significance of selecting varieties that produce high yields while exhibiting strong drought tolerance (Xu et al., 2023). The experiment started with a preliminary analysis of agronomic traits. Six rice varieties, each with three replicates, were planted under alternating drought stress and continuous drought stress. Meanwhile, normal watering represents control treatment. Under alternating drought stress, the two cycles of drought were interspersed with phases of recovery with normal watering. Meanwhile, the continuous drought treatment, which represents extreme drought stress, involved removing water on the 40th day. The plants were then grown under water deficit conditions for about seven days. The integration of alternating drought stress provided valuable insights into rice's acclimation to drought stress.

The preliminary study hypothesized that the agronomic traits of rice would vary between drought-tolerant and susceptible varieties. The varieties with high yields may possess drought tolerance. By using the statistical analysis, all varieties within the treatments were compared to the control of domestically grown rice and elite variety, MR297. Based on the agronomic analysis, Merah Wangi produced the highest grain yield under alternate and continuous drought stress, followed by Nona Manis and MR297. In fact, the yield of Merah Wangi under both drought stresses was significantly higher than MR297. These findings showed the outstanding ability of Merah Wangi to produce a stable yield despite the water deficit condition. The impact of drought stress on grain productivity remains ambiguous when only evaluating the agronomic traits. The mechanism underlying the plant's adaptation to drought stress requires further investigation. To address this uncertainty, root anatomical analysis was incorporated into the study of drought tolerance mechanisms.

Previous studies reported the fundamental role of root architecture in determining plant productivity (Villordon et al., 2014). Consequently, a benchmark

was established, in which rice plants producing a higher grain yield while exhibiting superior root anatomy were considered the most drought tolerant. This also signified that roots under alternate drought stress undergo better acclimation to subsequent stress, whereas roots under continuous drought stress exhibit adaptive traits to drought stress. The experiment was repeated using similar rice varieties, with a slight modification in the drought exposure period and sample collection. The drought stress was exposed during the vegetative phase, and the root sample was collected at 54th DAS.

The results confirmed that root anatomical structure exhibited plasticity and varied among the rice varieties. These preliminary observations indicated the root structure under alternate treatments was similar to the roots under normal watering. The changes in root anatomical plasticity supported the hypothesis that varieties under alternating drought stress performed better than those under continuous drought stress. Under alternating drought stress, Moroberekan had the highest values for all root anatomical parameters, followed by Merah Wangi. In terms of cross-sectional root area, Merah Wangi was similar to the control MR297, while Moroberekan differed significantly from control MR297. Conversely, Biris and Apami were among the varieties with the lowest values for root anatomical phenotypes. Although the results indicated that the roots of Moroberekan and Merah Wangi acclimated well to the alternating drought stress, the validity of these findings has yet to be fully confirmed.

Conversely, the continuous drought stress significantly impacted the cross-sectional root and cortical areas of all varieties. The microscopic observations displayed distorted root structures due to root shrinkage, markedly different from those under control treatment. It was unsurprising, as extreme drought stress inhibited cell division and differentiation, resulting in poor root development (Fahad et al., 2017). As a result, all varieties except Merah Wangi and Apami, showed significant differences from MR297. Despite continuous drought stress, Merah Wangi developed the largest cross-sectional root, cortex, stele and metaxylem areas. Meanwhile, Biris and Moroberekan exhibited the lowest root anatomical phenotypes.

The findings have addressed one of the research questions while simultaneously introducing a new concept. Exploring root phenotypes is important in determining grain yield during drought stress. A previous study also mentioned that the resilience of grain production is determined by the root system architecture (RSA) (Adotey et al., 2021;

Brunner et al., 2015). Thus, it can be deduced that the high yield of Merah Wangi under both drought stress was driven by its superior root anatomy. However, in the case of Moroherakan, although the roots showed better performance under alternating drought cycles, its grain production was among the lowest. Taken together, it was hypothesized that the drought-stressed roots of Merah Wangi displayed better phenotypic plasticity than MR297 and Moroberekan.

The validity of these hypotheses remains uncertain unless the genetic expression underlying root adaptation is examined. It is noteworthy to mention that root acclimation is influenced by plasticity and regulated by several molecular responses, which enable plants to develop robust roots to withstand drought stress (Bandurska, 2022; Sjulgard et al., 2021). Therefore, with the aid of next generation sequencing tools, we conducted transcriptomic analysis to evaluate the gene expression controlling the root phenotype of Merah Wangi against MR297 under drought stress. We further assumed that Merah Wangi induced stress-responsive genes that have a specific role in controlling the RSA. Thus, Merah Wangi and MR297 were replanted for the extraction of RNA from their roots.

Based on the overall visualisation of DEGs (Figure 5.3.5 to Figure 5.3.), it became evident that a large number of genes were activated under continuous drought stress, followed by treatments with alternating drought stress. Interestingly, all treatments, including normal watering, showed a trend of downregulation genes outnumbering upregulated genes. Based on the gene ontology analysis, most DEGs under alternate drought stress were involved in molecular function, while those under prolonged drought stress were linked to cellular components. A COG analysis was conducted to study gene functional organization, revealing that most DEGs across all treatments were involved in signal transduction pathways (STP). Despite similar expression patterns among the treatments, the highest upregulation of STP was observed under continuous drought stress, indicating that the continuous drought stress affected the root's function.

Once the functional categories were identified, the analysis proceeded with KEGG analysis to delve into specific metabolic and signalling pathways. The findings revealed that DEGs under normal watering and alternate drought stress treatments were mainly involved in similar pathways, such as metabolite pathways and the biosynthesis

of secondary metabolites. Surprisingly, previous root studies under drought stress also showed an increase in metabolite accumulation in response to drought (Ghorbanzadeh et al., 2023; Wang et al., 2024). Specifically, under alternate drought stress, DEGs were enriched in flavone biosynthesis. The accumulation of phenolic compounds, including flavones, serves as a defence strategy by scavenging reactive oxygen species during drought stress (Agati et al., 2020; Nakabayashi et al., 2014). Moreover, Alves et al. (2020) also reported an increase in metabolites in response to recurring drought phases, suggesting that previous drought exposure helps plants acclimatize to subsequent water stress.

Nevertheless, the results revealed a distinct gene expression pattern under continuous drought stress. KEGG analysis revealed that most DEGs were associated with hormone signalling pathways while fewer were involved in secondary metabolites. This finding aligns with the theory proposed by Sharma et al. (2021) that phytohormone expression regulates root adaptative traits under drought stress. Furthermore, the benzoxazinoids pathway was also induced, which helps maintain cell turgor pressure (Sutour et al., 2024). The distinct result of COG and KEGG analysis between alternate and continuous drought stress has prompted a research question regarding the response of hormonal-related genes under extreme drought stress. Notably, phytohormones have a major role in adapting to environmental stress (Osakabe et al., 2014). In correlation to the root anatomical analysis, it was assumed that the regulation of phytohormone expression contributes to root development during water deficit conditions, in line with a study by Charng et al. (2022). This foundation paves the way for further exploration into the genetic expression underlying drought tolerance and root acclimation, with a focus on phytohormonal regulation.

With the goal of identifying candidate genes that are responsible for root adaptative traits, few hormonal-related genes from each treatment were screened out. Based on the results, although the total expression of auxin genes was higher than cytokinin in all treatments, it is worth mentioning that under normal watering and alternate drought treatments, most auxin-related genes were downregulated. This result aligns with the concept that optimal conditions induce cytokinin regulation for shoot growth while suppressing auxin to maximize carbon allocation (Kurepa & Smalle, 2022). Plants respond to moderate drought by sustaining their aerial growth. Therefore,

under normal watering and alternate drought stress, auxin genes were downregulated due to the water being readily available, reducing the need for plants to stimulate root elongation.

Additionally, the overall transcriptomic analysis revealed that the gene expression pattern under two cycles of drought stress was almost identical to that under normal watering, indicating that roots of Merah Wangi acclimated well during two cycles of drought. It became evident when the grain yields of all varieties under alternate drought stress were better than those under prolonged drought stress. Jing et al. (2022) and Zhang et al. (2013) also reported that alternate drying during irrigation significantly boosts grain yields, shoot growth, and water use efficiency (WUE). In addition, Kambona et al. (2023) extensively reviewed the concept of stress memory, suggesting that stress memory in plants improves drought stress tolerance. This study signifies that upon exposure to the first drought cycle, roots develop a stress memory, enabling plants to respond more effectively to recurring drought stress. Taken together, these findings proved that the adjustment of gene expression in Merah Wangi's root is a key survival for acclimating to recurring drought stress.

Conversely, the continuous drought stress has triggered the upregulation of auxin, cytokinin, and gibberellin, indicating the activation of the stress signalling network. These findings align with prior research that stated that, under long term water deficit condition, root growth favours overshoot growth, thus shifting to a larger root biomass for water uptake (Brunner et al., 2015; Fromm, 2019). Specifically, this study has successfully identified genes like *IAA18*, *IAA17*, *OsIAA6*, ARF 12, GH3, and ARGOS under both drought stress, but higher expression was found under continuous drought stress. IAA involves cell division and root elongation, thus contributing to drought tolerance (Feng et al., 2011; Fukaki et al., 2002; Guo et al., 2022). The higher expression of ARF12 has benefited the drought-stressed roots of Merah Wangi by stimulating meristematic root growth (Liu et al., 2020). In terms of tiller growth, Jung et al. (2015) reported that the repressor auxin *OsIAA6* controls tiller outgrowth, while GA activator *GA2ox* promotes effective tillering (Lo et al., 2017). Therefore, the high quantity of tillers in Merah Wangi under continuous drought stress may be attributed to this high gene expression.

Meanwhile, the upregulation of the cytokinin genes under continuous drought stress was predominantly regulated by *OsLOG* and *CKX*, which are crucial in mediating root responses to drought. Higher cytokinin levels, particularly those of *CKX* family, regulate root responses to drought by inducing a larger root system (Ramireddy et al., 2018). Furthermore, the GA activator gene *GA2ox*, *GA20ox*, and *GASA* also stimulate the meristematic cell, cell proliferation, and root elongation zones (Barker et al., 2020; Lo et al., 2017). Overall, identifying these hormonal-related genes and their expression level supports Merah Wangi's superior adaptability, as evidenced by its greater cortical and cross-sectional root areas under both forms of drought stress.

Besides that, deep rooting has been proposed as a root ideotype and a long-term solution for drought resistance (Uga et al., 2013). It is essential for enhancing water uptake from deeper soil layers. The *DRO1* gene positively regulates deeper root growth, leading to stable grain under water deficit conditions (Arai-Sanoh et al., 2014; Vinarao et al., 2023). Thus, the upregulation of *DRO1* in Merah Wangi under continuous drought stress likely contributed to its root adaptability and higher grain yields, as evidenced by agronomic analysis. Furthermore, the inhibition of lateral root growth in topsoil is an adaptive response that improves drought tolerance (Xiong et al., 2006; Zhang et al., 2015). Therefore, inhibition of the horizontal spread of lateral roots in the topsoil could shift more resources towards primary root growth (Xiong et al., 2006). Several gibberellin genes and *IAA15* play roles in suppressing lateral root growth (Fonouni-Farde et al., 2019; Kim et al., 2022b). The highest expression of these genes was also observed under continuous drought stress. Hence, this transcriptomic analysis has proven effective in identifying key traits that contribute to the adaptability of Merah Wangi under drought stress.

Previous studies have highlighted the importance of hormonal-crosstalk during vascular development (Dettmer et al., 2008; Ramachandran et al., 2020). This study also underscores the effect of drought stress on vascular development. Under alternating drought conditions, Moroberekan had the largest stele and metaxylem area and diameter, followed by Merah Wangi. However, after seven days of continuous drought stress, Merah Wangi developed the largest stele and metaxylem phenotypes. Notably, wider stele sizes improve water transportation and increase grain yield under drought stress (Phoura et al., 2020; Siangliw et al., 2022). Therefore, the indication that larger

stele and metaxylem areas are beneficial under drought stress was evident because Merah Wangi produced the greatest grain yield even under water deficiency. Specifically, auxin regulates the vascular differentiation of xylem and phloem through ATHB8 pathway, which encodes the thermospermine (Dettmer et al., 2008; Yoshimoto et al., 2016). The antagonistic role of auxin and thermospermine is crucial for proper xylem development. Although the molecular mechanism of xylem differentiation is not well studied, this xylem plasticity, together with the higher expression of auxin under drought stress, provide valuable insights into vascular differentiation, suggesting areas for future research.

In summary, drought stress significantly affected rice root anatomy and agronomic traits. This study confirms that root plasticity is vital to withstand drought stress. A detailed transcriptomic analysis comparing Merah Wangi against MR297 showed that the roots of Merah Wangi acclimate and adapt well to drought stress by activating hormonal-related genes, leading to higher grain yields. The similar pattern of gene expression under alternate drought stress and normal watering explains why alternating drought stress is less severe than prolonged drought. The high induction of upregulation of DEGs under continuous drought stress reflects a response mechanism to cope with adverse conditions. Therefore, understanding of root modification enhances our understanding on grain productivity under drought stress. Thus, it is important for plant breeders to select varieties that produce high yields while exhibiting high drought tolerance.

CHAPTER SEVEN

CONCLUSION

In conclusion, the impact of drought stress on agronomic traits and root anatomical phenotypes varied significantly between six rice varieties. The preliminary agronomic analysis results have answered the initial hypothesis, that the effect of drought stress is less pronounced under alternating two cycles of drought as compared to continuous drought stress. Thus, the alternate drought stress helps to explore the acclimation process, while prolonged drought enhances understanding on drought tolerance and resilience. Through agronomic analysis and root anatomical study, Merah Wangi was the top grain producer, while possessing better root performance, indicating its resilience to drought stress. Moreover, the identified genes are involved in various protective and regulatory processes, which collectively improve the root's ability to withstand and recover from stress. The variability of phytohormone-related genes probably reflects the regulation of the hormonal signaling pathway in adjusting root architecture under prolonged drought stress

Therefore, it was suggested that plasticity of root area, particularly vascular structure, cortex and stele might be useful resource to clarify the mechanism underlying drought tolerance. The integration of root anatomy and agronomic traits helps in pinpointing genetic expression, providing more detailed insights into the molecular mechanism governing the root development and stress response in Merah Wangi and MR297. The evidence from gene expression is crucial to prove the plasticity of root anatomical during acclimation and adaptation to drought stress. Although the functional role of the specific DEGs remains to be elucidated, the transcriptome data has laid the foundation for considering the crucial role of phytohormones such as auxin and cytokinin ratio during abiotic stresses. The limitations in root sampling time suggest the need for further research to validate the optimal sampling times to enhance the quantity and purity of RNA. Despite the limitations, this topic provides a valuable area for future research. It is important for plant breeders to select varieties that produce high yields while exhibiting high drought tolerance.

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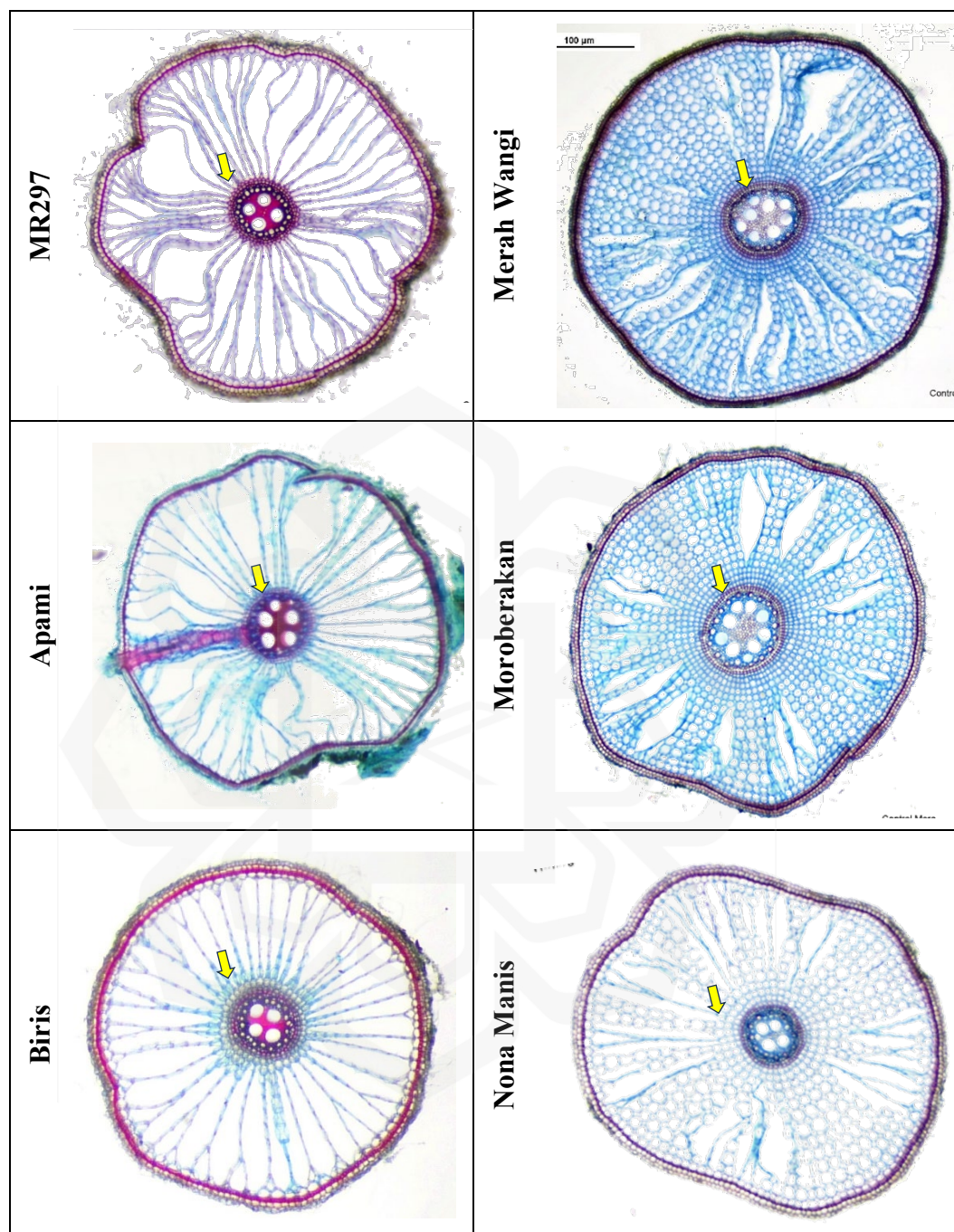
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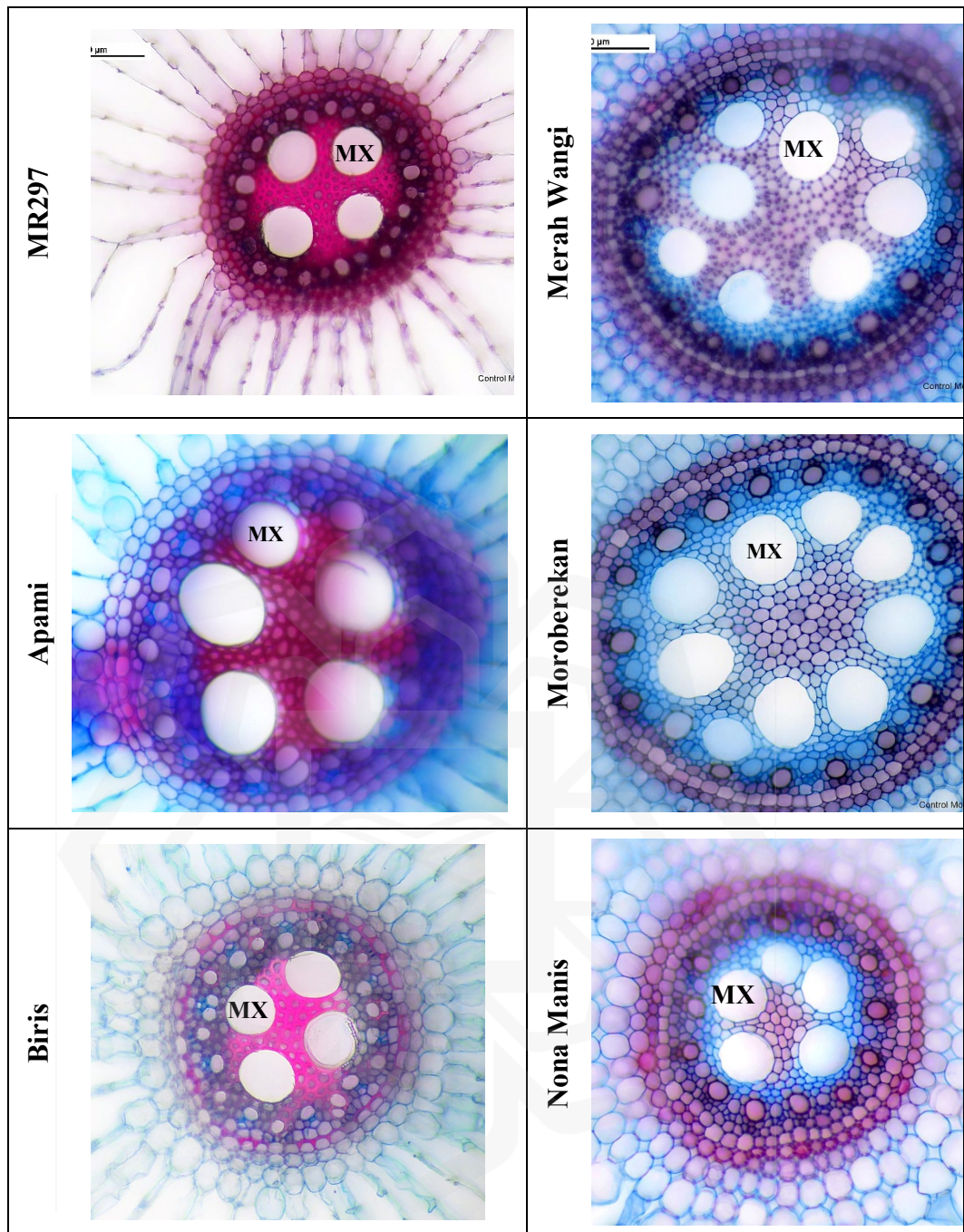
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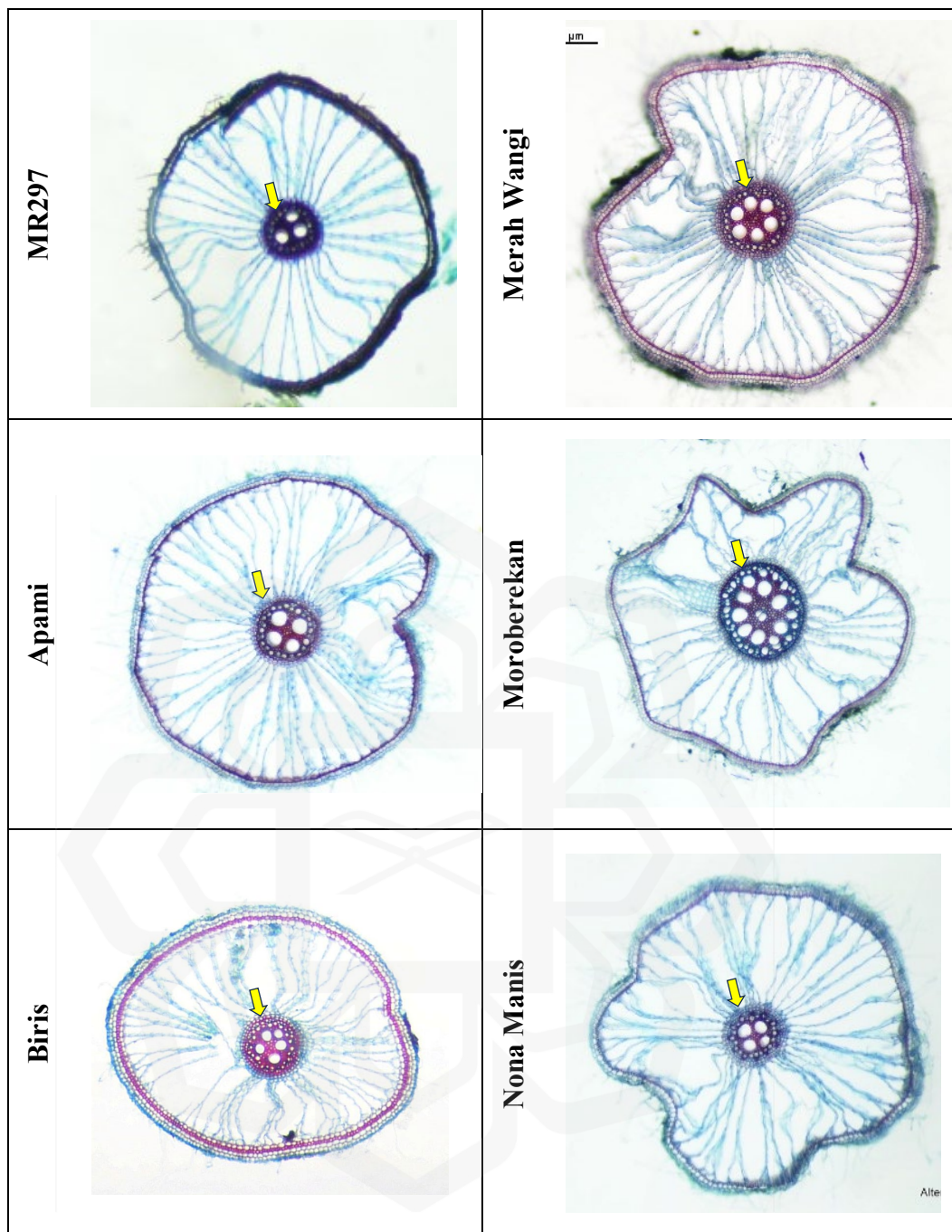
APPENDIX I: MICROSCOPIC OBSERVATION



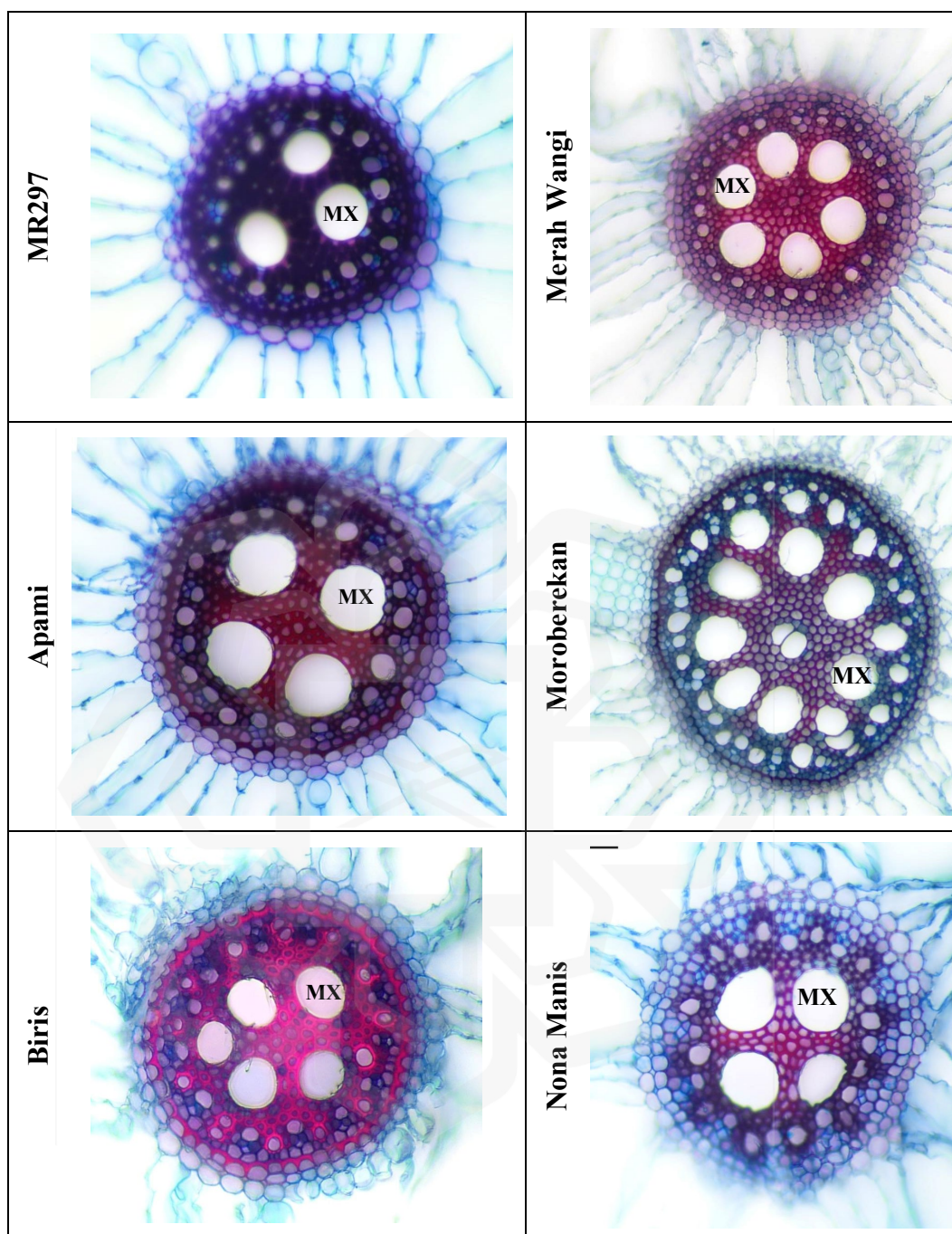
The effect of control treatments on the root anatomy of *Oryza sativa* L. varieties at tillering stage. Images of root anatomy were observed under a light microscope at 4X magnification and captured using Leica LAS EZ software. The yellow arrow indicates the stele region.



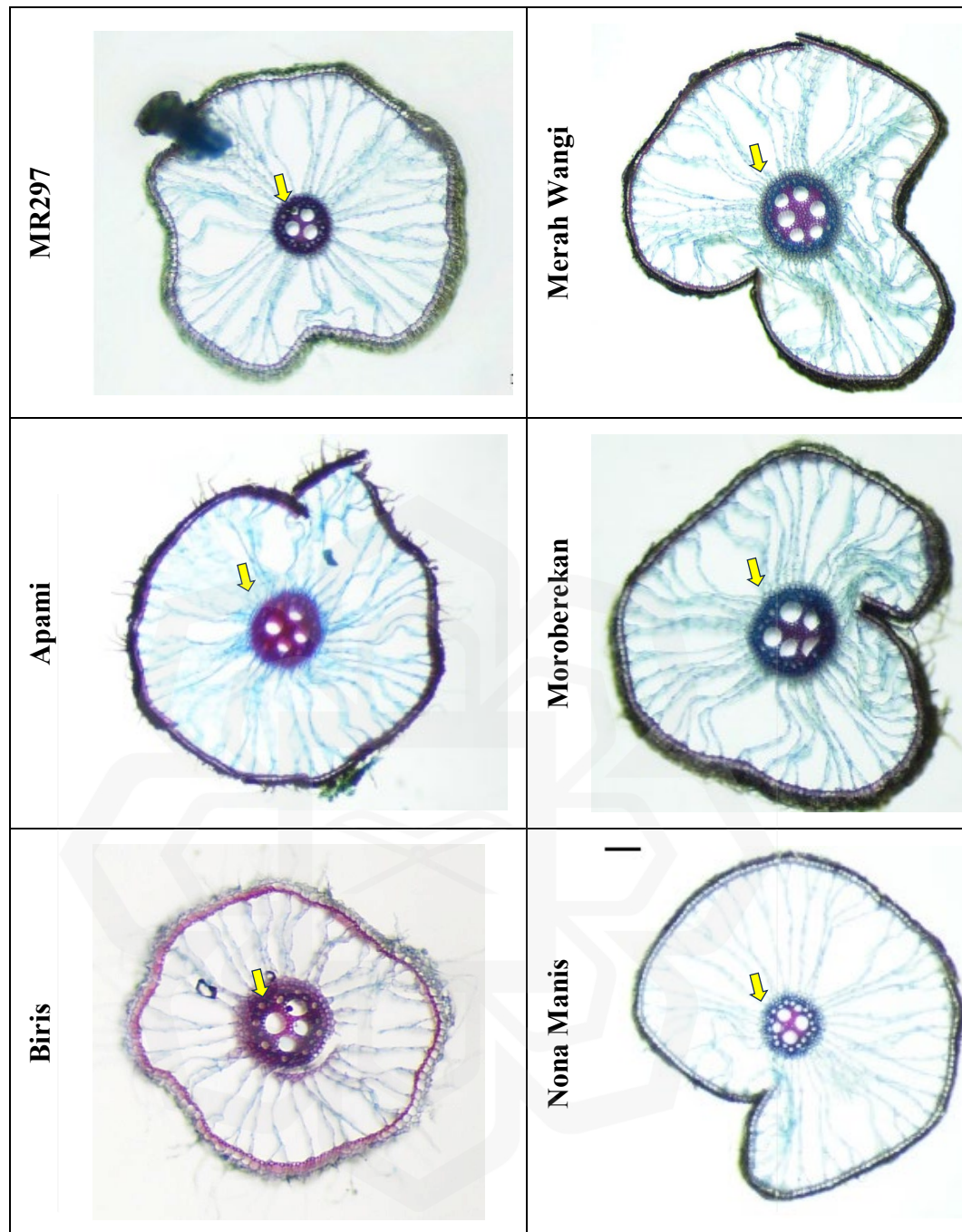
The effect of control treatment on the root stele area of *Oryza sativa* L. at tillering stage. The stele contains metaxylem (MX). The images were observed under Nikon Eclipse 50i microscope at 400X magnification and captured with Leica LAS EZ software. MX refers to the metaxylem in the stele.



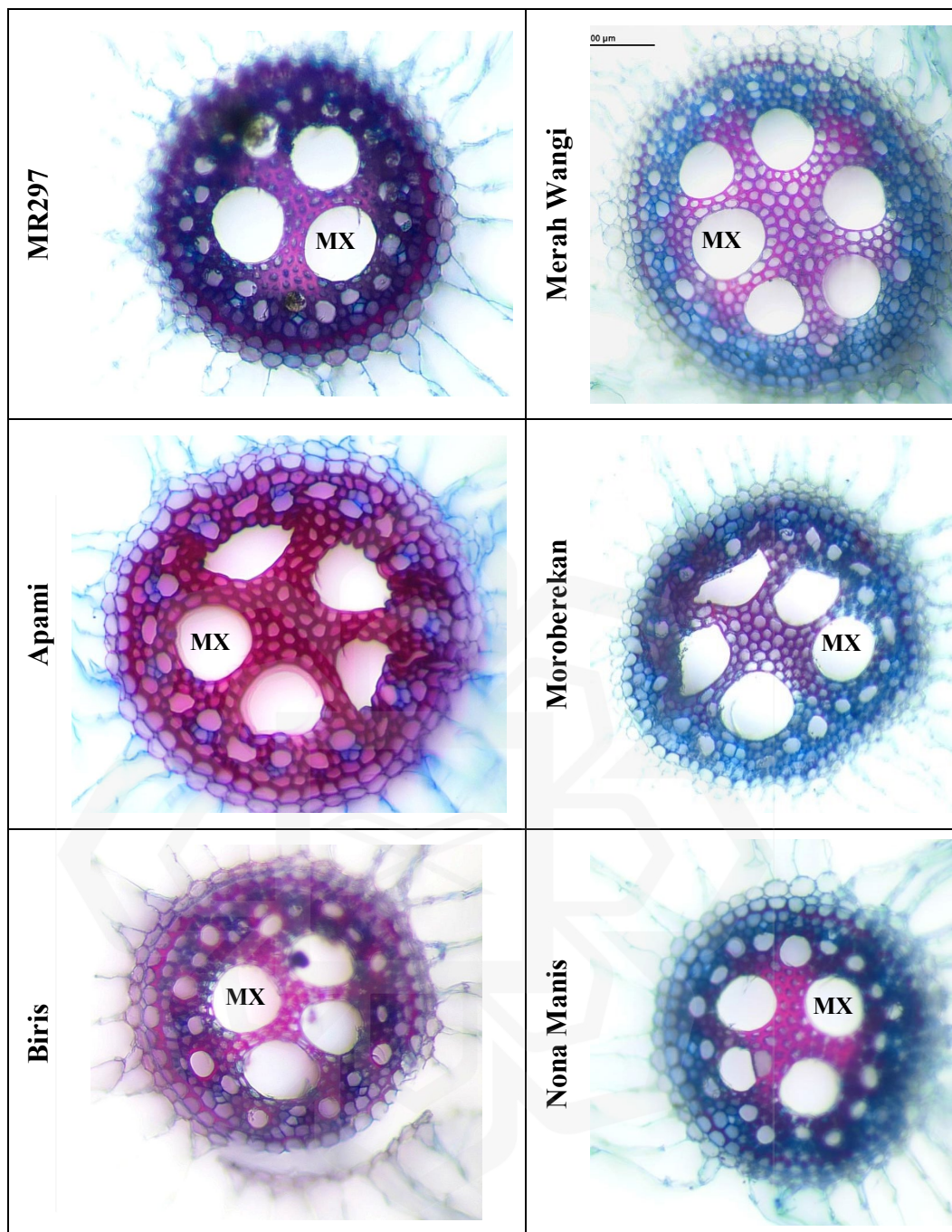
The effect of alternate drought treatment on the root anatomy of *Oryza sativa* L. varieties at tillering stage. Images of root anatomy were observed under a Nikon light microscope at 40X magnification and captured using Leica LAS EZ software. The yellow arrow indicates the stele region.



The effect of alternate drought treatment on the root stele area of *Oryza sativa* L. varieties at tillering stage. The stele contains metaxylem (yellow arrow). The images were observed under a Nikon light microscope at 400X magnification and captured using Leica LAS EZ software. MX refers to the metaxylem in the stele.



The effect of continuous drought treatment on the root anatomy of *Oryza sativa* L. varieties at tillering stage. Images of root anatomy were observed under a Nikon light microscope at 40X magnification and captured using Leica LAS EZ software. The yellow arrow indicates the stele region.



The effect of continuous drought treatment on the root stele area of *Oryza sativa* L. varieties at tillering stage. The stele contains metaxylem (yellow arrow). The images were observed under a Nikon light microscope at 400X magnification and captured using Leica LAS EZ software. MX refers to the metaxylem in the stele.

APPENDIX II:

REGULATION OF DRO1 GENES IN MERAH WANGI VS MR299

UNDER DIFFERENT TREATMENTS

Types of Regulation	Number of Transcripts Identified		
	Control	Alternate Drought	Continuous Drought
Upregulation	-	-	1
Downregulation	2	1	1