RESPONSE OF MEXICAN MINT (*PLECTRANTHUS AMBOINICUS*) PLANT TO VARIOUS WATERING REGIMES

by

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Dalhousie University is located in Mi'kma'ki, the
ancestral and unceded territory of the Mi'kmaq.
We are all Treaty people.

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ABSTRACT

Plants are sessile, and their ability to reprogram their metabolism to adapt to fluctuations in soil water level is crucial but not clearly understood. A study was performed to determine alterations in morpho-physiological, biochemical and volatile constituents following exposure of Mexican mint (*Plectranthus amboinicus*) to varying watering regimes. The water treatments were regular watering (RW), drought (DR), flooding (FL), dehydration after flooding (DHFL), or rehydration after drought (RHDR). The DR and FL treatments significantly ($p<0.01$) reduced plant fresh weight by 92% and 88%, respectively, when compared to RW. Net photosynthetic rate, transpiration rate, and stomatal conductance were significantly ($p<0.01$) reduced in DR and FL plants. A total of 68 key metabolites from the central carbon metabolites routes were found to be significantly ($p < 0.01$) impacted by water stress. Calvin cycle metabolites in FL plants, glycolytic metabolites in DR plants, and total tricarboxylic acid (TCA) cycle metabolites in DR and DHFL plants were significantly ($p < 0.05$) increased. Total Calvin cycle metabolites had a significantly ($p < 0.001$) strong positive association with TCA cycle ($r = 0.81$) and pentose phosphate pathway ($r = 0.75$) metabolites. D-Limonene was significantly more pronounced in DR plants by 256% compared to RW. In conclusion, the alterations in the morpho-physiological and biochemical parameters especially the volatile constituents of Mexican mint plants under different watering regimes were revealed. Future studies will use transcriptomic and proteomic approaches to identify genes and proteins that regulate metabolites.

LIST OF ABBREVIATIONS AND SYMBOLS USED

µg: Microgram
µL: Microliter
µM: Micromolar
AAA: Aromatic amino acids
ANOVA: Analysis of variance
ATP: Adenosine triphosphate
CCM: Central carbon metabolism
CBC: Calvin-Benson cycle
CO2: Carbon dioxide
DHFL: Dehydration after flooding
DNA: Deoxyribonucleic acid
DPPH: 1,1-diphenyl-2-picrylhydrazyl
DR: Drought
EC: Electric conductivity
ETC: Electron transport chain
FAD: Flavin adenine dinucleotide
FL: Flooding
FW: Fresh weight
GABA: Gamma-aminobutyric acid
H2O2: Hydrogen peroxide
NAD: Nicotinamide adenine dinucleotide
NADPH: Nicotinamide adenine dinucleotide phosphate
MAP: Medicinal and aromatic plants
MDA: Malondialdehyde
O2: Superoxide
ºC: Degrees Celsius
OPPP: Oxidative pentose phosphate pathway
PFES: Plant, food and environmental sciences
PSM: Plant secondary metabolites
PSII: Photosystem II
RHDR: Rehydration after drought
RFO: Raffinose family oligosaccharides
ROS: Reactive oxygen species
RNA: Ribonucleic acid
RWC: Relative water content
RW: Regular watering
SDH: Succinate dehydrogenase
TCA: Tricarboxylic acid
TSS: Total soluble solids
UV: Ultraviolet
WRI: World Resource Institute
wt: Weight

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CHAPTER 1: INTRODUCTION

1. Thesis background

Recent studies have identified abiotic stress due to climate change as a substantial threat to global food security in the twenty-first century (Ashmore et al., 2006; Battisti et al., 2009). These abiotic stresses include drought, flooding, high temperatures, soil salinity, tropospheric ozone, and excessive ultraviolet (UV) radiation, which are already causing significant reductions in agricultural productivity; and are anticipated to increase in frequency in the future due to global climate change (Ashmore et al., 2006; Ortiz et al., 2008; Battisti et al., 2009; Feng et al., 2009; Fuhrer, 2009). Tropical and subtropical regions in Asia and Africa are experiencing one of the fastest population growth, and are especially susceptible to food scarcity caused by these challenging abiotic stress factors (Lobell et al., 2008; Wassmann et al., 2009). Currently, global food security is being hindered by the rapidly increasing population coupled with extremely unpredictable weather circumstances (Hussain et al., 2018). According to the Intergovernmental Panel on Climate Change (IPCC), plants experience stress due to changes in climatic conditions that were identified as the primary factor determining agricultural production (Onyekachi et al., 2019). Water stress is the most significant constraint on crop yield and, consequently, food security in the face of changing climatic conditions (Iqbal et al., 2020). Fluctuating rainfall patterns and insufficient precipitation are causing frequent occurrences of drought and flooding conditions in the same location worldwide (Rajsekhar et al., 2017).

Water stress refers to a situation with either an excessive or insufficient amount of water for an extended period, leading to a significant imbalance in the water cycle and soil moisture conditions (Kopecká et al., 2023). Plants, being sessile organisms, undergo water stress because of inadequate or excessive water availability. The impact of drought stress is becoming increasingly serious due to the reduced supply of water and the growing demand for it. Currently, agriculture is responsible for 70% of the global requirement for clean water, and this proportion is projected to grow significantly in a short period (Boretti et al., 2019). The growth of plants is significantly impacted by severe water stress, which disrupts soil nutrient uptake, hinders the photosynthetic process, and ultimately, leads to a significant drop in agricultural output (Farooq et al., 2012). The main constraints impacting the food supply and ecological balance have driven numerous researchers to investigate efficient adaptation strategies for plants in unfavorable conditions. This involves creating robust plant species capable of withstanding the effects of climate change. The plant of the current research interest is *P. amboinicus* which is commonly known as Mexican mint.

P. amboinicus belongs to the Lamiaceae family, commonly known as the mint family. There are over 300 species of plants in this group, which can be either annual or perennial herbs or subshrubs (Arumugam et al., 2016). *Plectranthus* are succulents and encompass numerous species that have both commercial and medical significance (Arumugam et al., 2016). Mexican mint is an aroma-medicinal plant known for its leaves that have a unique smell and are covered in short, upright, silky hairs (Arumugam et al., 2016). Mexican mint extracts and essential oils have therapeutic properties and can be utilized as antiseptic, antibacterial, and spasmolytic agents (Sağdıç et al., 2002). The demand for *Plectranthus* products is rising and cannot be met by gathering from wild populations because of the deterioration of its natural environment and unpredictable rainfall patterns (Moradi et al., 2014). By improving the growing techniques of the Mexican mint, we can increase future production while protecting its natural biodiversity (Moradi et al., 2014). Stress-related factors disrupt the physiochemical balances in plants resulting in morphological, physiological, biochemical, and molecular alterations in plants, which affect their growth and productivity (Lisar et al., 2012). It is crucial to understand the plant regulation mechanisms in response to water stress to enhance plant productivity in unfavorable conditions (Wu et al., 2022).

The impact of water stress on plants is primarily observed in their leaves and roots (Lozano et al., 2020). The most accurate reflection of the plant's ability to adapt to unfavorable conditions can be observed through an analysis of the plant’s morphological traits and internal anatomical structure (Lozano et al., 2020; Pedersen et al., 2021). Water stress can restrict plant growth by impeding the division of cells in leaf meristematic tissue and the expansion of cells in elongation regions, while also producing intricate alterations in leaf thickness, palisade tissue, and spongy tissue as part of the adaptation process (Hilde Nelissen, 2018; Meng et al., 2021). Plants use a wide range of defense mechanisms to safeguard their photosynthetic organs from damage while adjusting to water stress, to sustain photosynthesis (Sharma, 2020; Yang et al., 2020). Photosynthesis is inhibited by both stomatal and nonstomatal mechanisms when plants are subjected to drought and waterlogging stress (Wu et al., 2022a). Water stress induces an elevation in free radicals and reactive oxygen species (ROS) like hydrogen peroxide (H_2O_2) and superoxide (O_2^-), which impact various plant functions such as the degradation of photosynthetic structures, impairment of cell membranes, denaturation of proteins, and overall suppression of plant growth (Kleinwächter & Selmar, 2014). Consequently, numerous plant species gather phenolic substances including tannins and flavonoids as a means of antioxidant defense to mitigate these detrimental impacts (Sousa Araújo et al., 2015).

The quality of medicinal and aromatic plants is contingent on the composition and concentration of plant secondary metabolites, which can be influenced by environmental conditions such as water stress (Nasrollahi et al., 2014; Kleinwächter et al., 2015). Secondary metabolites have significant functions in the ecological and physiological processes of plants (Albergaria et al., 2020). Water availability can influence the concentration of these compounds and affect the therapeutic qualities of medicinal plants (Akula et al., 2011) including *Plectranthus*. In general, water stress has been receiving increasing attention in the literature due to its significant impact on the growth of food and medicinal plants, particularly in arid and semi-arid regions characterized by chronic decreases in precipitation (McKiernan et al., 2014). However, previous studies have examined a few of the effects of water stress on morpho-physiological parameters of*Plectranthus* species, but there is a lack of research on the metabolic profile, volatile content, and therapeutic activities of *P. amboinicus* under various water levels.

1.2 Research Objective

The goal of this study is to advance our understanding of how *P. amboinicus* responds to different levels of water stress. This study comprehensively investigates the morphological, physiological, and biochemical aspects of *P. amboinicus* under varying water stress circumstances.

1.2.1 Specific objectives

1. To investigate the morpho-physiological and biochemical response of *P. amboinicus* under prolonged water stress conditions
2. To examine the impact of water stress on central carbon metabolism of *P. amboinicus*

3. To assess organic volatile compound alteration in leaves of *P. amboini*cus under water stress conditions

1.3 Thesis Organization

The thesis is organized into a manuscript format and comprised of a total of seven chapters. Chapter 1 is the introduction, research aim, and specific objectives of the thesis. Chapter 2 provides a comprehensive literature on an overview of (i) climate change and environmental stresses, and (ii) morpho-physiological, biochemical, and metabolic pathways of related plant members under water stress conditions. Study objectives 1 to 3 are presented under Chapters 3, 4, 5 and 6 respectively. Chapter 7 is the overall thesis conclusion with a contribution to knowledge and recommendations for further studies. It is important to note that Chapters 2, 3, and 4 have been published while Chapters 5 and 6 will be submitted for publication.

CHAPTER 2: LITERATURE REVIEW

A version of this chapter has been published in **Horticultural International Journal.** The citation is:

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2.1 Introduction

Climate change is posing a significant threat to life on Earth. In the present fluctuating climatic circumstances, fulfilling the increasing food demand and attaining a sustainable agri-food system for a growing population is becoming difficult (Salam et al., 2022). Among the abiotic stresses, water stress is the most detrimental one that hinders agricultural productivity to a great extent worldwide (Yahaya et al., 2022). Generally, plants can endure water stress, but at a significant cost to total biomass production. About half of the semi-arid and arid regions of the world are affected by water stress, which occurs owing to either water surplus or water scarcity. The entire area of the world's dry lands has expanded considerably in the last decade, and the extent of drought has resulted in USD 30 billion of global crop losses annually (Lesk et al., 2016a; Wu et al., 2022b). Additionally, the intensity and frequency of flooding disasters have been on the rise since the 1990s (Du et al., 2010; Shi et al., 2021). For instance, the floods caused a total crop loss of USD 5.5 billion between 1982 and 2016 (Kim et al., 2023). The World Resource Institute (WRI) Aqueduct water risk atlas reveals that approximately 25 countries experience exceptionally severe water stress annually, consistently depleting nearly all their accessible water resources. Approximately 4 billion individuals, which is equivalent to at least 50% of the global population, reside in areas characterized by severe water stress (Institute, 2023) (Figure 2.1).

Water stress interrupts the regular functions of plants leading to morphological, physiological, and biochemical alterations (Figure 2.2) (McDowell et al., 2022; Pepe et al., 2022). Previous research revealed that water stress induces oxidative stress, which negatively impacts biological membranes and macromolecules (Deoxyribonucleic acid (DNA), proteins, lipids, and photosynthetic pigments) (Sofy et al., 2021; Zandi et al., 2022). Water stress leads to nutritional imbalances, which have a large ecological impact on global agricultural output (Azeem et al., 2022). However, most plants possess a well-studied innate system for resisting water stress, which includes gene activation, as well as morphological, physiological, and biochemical adaptations (Ghafar et al., 2021).

This review focuses on the effects of water stress on plant morphology, physiology, and biochemical processes. Also, it explores the response of plants to alterations in central carbon metabolic pathways. These are crucial elements for understanding how plants respond to water stress. The understanding of these mechanisms under water stress is vital for boosting plantoutput under challenging conditions.

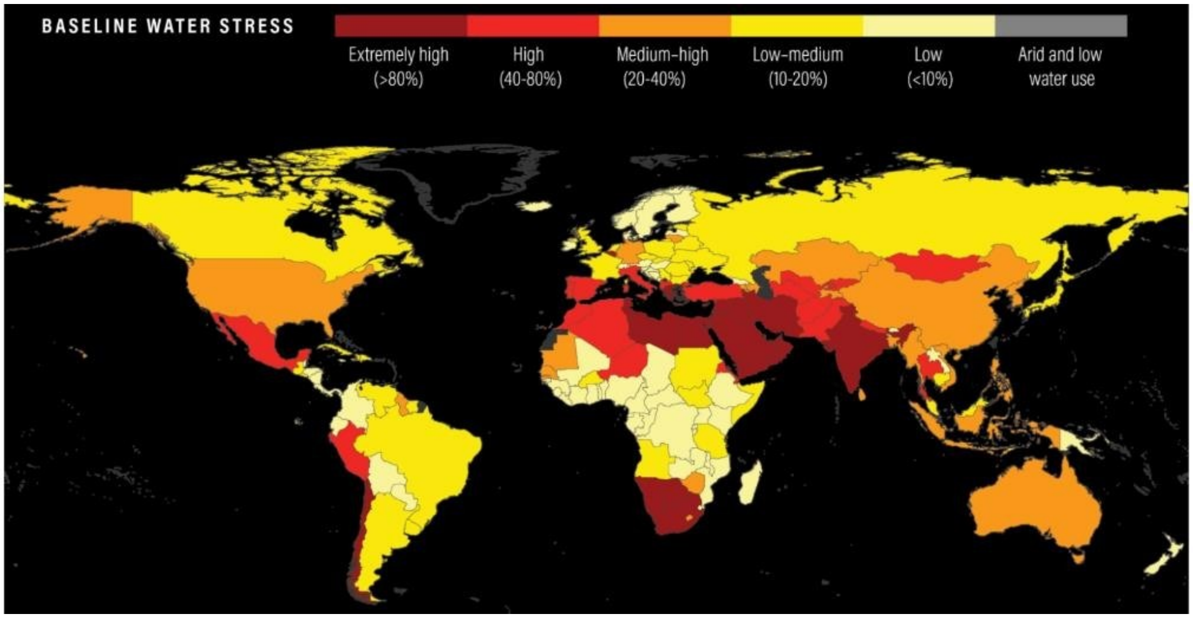

Figure 2.1. A world map depicting the data collected from the World Resource Institute (WRI) Aqueduct water risk atlas shows that about 25 countries accommodating one-quarter of the global population are highly under water stress conditions (Institute, 2023).

2.2 Factors contributing to water stress in plants

The changes in precipitation patterns observed currently can be attributed to global climate change, as demonstrated by increasing temperatures and heightened atmospheric CO_2 concentrations (Mishra et al., 2011; Dai, 2011; Nezhadahmadi et al., 2013; Arbona et al., 2013). The primary trigger for water stress on a global scale is sustained global climate change (Abdelmoneim et al., 2014). Nevertheless, water stress can be caused by other factors including elevated temperatures, intense sunlight, and arid winds, all of which contribute to the accelerated evaporation of soil moisture (Trenberth et al., 2014). Furthermore, these variables contribute to elevated water losses from plants, hence promoting plant vulnerability to water stress. Occasionally, drought does not arise solely from a scarcity of water in the soil (Salehi et al., 2016) but also, due to several soil conditions such as soil texture and structure, salinity, soil temperatures, barrier to absorption of water by roots, and diseases, despite the presence of sufficient water in the soil (Salehi et al., 2016). This particular form of drought is referred to as pseudo-drought or physiological drought, and it is not influenced by climatic conditions (Arbona et al., 2013). Floods on agricultural land typically result from either heavy or prolonged precipitation, however, they can also arise when water bodies overflow onto the land as well (Fukao et al., 2019).

2.3 Plant morphological response to water stress

The key indicators of how plants are affected by water stress can be observed in their leaves and roots, which exhibit apparent changes in their external morphological characteristics and interior anatomical structure. Water stress adversely affects early germination, plant height, relative root length, root diameter, the total biomass of leaves and roots, number of leaves and canopy size per plant.

2.3.1 Early seed germination and flowering

Seed germination is the initial and critical phase of plant development (Ghadirnezhad et al., 2023). Water is crucial for the process of seed germination because even if all other conditions are met, water stress prevents seeds from absorbing water and that can impede germination (Islam et al., 2018). Water stress directly impacts seed water imbibition, which results in decreased seed germination rate and inadequate seeding establishment (Achakzai, 2009). To achieve optimum seed germination, the soil must contain adequate moisture to enable the initiation of metabolic processes, the breaking of dormancy, and the transformation of stored nutrients in the seed into forms that can be utilized by the embryo (Aslam et al., 2015). The impact of water stress on plants might vary according to the duration, severity, and phenological stage. Once the seeds ingest water, the process of metabolic modifications is initiated (Ghadirnezhad et al., 2023).The availability of soil water and its water potential are the primary parameters that influence the absorption and utilization of water during the initial stages of germination (Anjum et al., 2017). Furthermore, dry conditions during the initial period greatly hinder the process of seed germination and seedling establishment. This is mostly due to a decrease in water absorption during the germination phase, a reduction in energy supply, and a weakening of enzyme activities. Seeds undergo a loss of viability and vigor throughout the process of dehydration (Ghadirnezhad et al., 2023). This phenomenon arises due to numerous metabolic alterations encompassing the generation of reactive oxygen species (ROS) that inflict harm upon RNA, DNA, and proteins leading to disruption of membrane integrity, diminished respiratory activity, and reduced ATP synthesis (Anjum et al., 2017).

2.3.2 Plant morphological structures exhibit specific alterations to water stress

The impact of water stress on plants is primarily evident in their root and shoot systems. The most accurate reflection of the ability of the roots and leaves to adjust to unfavorable conditions is through the assessment of their exterior morphological traits and internal anatomical structure (Liu et al., 2020; Lozano et al., 2020; Pedersen et al., 2021). Compared to roots, leaves exhibit the highest degree of variability in their long-term adaptation to the environment. Leaves exhibit similar responses to both drought and waterlogging stress, showing symptoms such as etiolation, atrophy, curling, senescence, and even abscission (Patharkar et al., 2019; Bhusal et al., 2020). Studies have shown that water stress can inhibit leaf growth and reduce leaf count and size (Wei et al., 2013; Fang et al., 2015; Wu et al., 2022b).

2.3.2.1 Shoot

A primary consequence of water stress is a reduction in plant growth because of less photosynthetic activity. Stem growth and in particular, leaf growth exhibit greater susceptibility towater stress compared to root growth (Kul et al., 2020). During periods of water stress, the plant reduces the rate of stem elongation and stimulates root growth as a response to seeking additionalwater resources (Kul et al., 2020)**.** Drought can restrict plant growth by impeding the division of cells in leaf meristematic tissues and the enlargement of cells in elongation regions. Additionally,it can trigger intricate alterations in leaf thickness, palisade tissue, and spongy tissue as part of the adaptation process(Binks et al., 2016; Hilde, 2018; Dong, 2021). The primary physiological consequences of leaves in response to waterlogging stress include leaf curling, yellowing, wilting, abscission, and decay. To achieve the desired outcome of the reduction of water loss and enhancement of water retention capacity, it is necessary to increase the amount of palisade and spongy tissues while simultaneously decreasing the size of leaves and stomata (Yin et al., 2012; Nawaz et al., 2014; Zúñiga et al., 2017). The latter occurs because leaves are unable to undergo normal morphogenesis as a result of insufficient water and nourishment (Xu et al., 2020). Consequently, certain plants adjust their leaves by reducing their thickness to enhance the capacity for CO_2 and inorganic nutrients to enter the leaves. This adaptation mechanism will facilitate improved gas exchange, enabling the plants to sustain respiration even in conditions of waterlogging stress (Lawson et al., 2017).

2.3.2.2 Root

Roots modify their morphology to enhance their ability to penetrate, spread, and make contact with the soil, hence facilitating more efficient absorption of water and nutrients in response to water stress conditions (Zhan et al., 2015; Rongsawat et al., 2021). These structural changes guarantee the essential intake of nutrition and water, hence sustaining plant physiological functions and productivity in times of water stress (Shoaib et al., 2022). During periods of drought, plants experience increased expenditure of photosynthates. Typically, plants distribute photosynthates to develop root systems to seek water and nutrients necessary for metabolic processes (Shoaib et al., 2022). It is important to allocate more resources towards root growth to enhance water absorption. Additionally, plants raise their respiration rate to sustain the roots in the drying soil. As a result, these mechanisms due to water stress compromise the overall productivity of the plant (Lynch et al., 2014; Colombi et al., 2019). The lack of water in the soil leads to an increase in mechanical resistance, which limits the ability of roots to penetrate the deeper layers of the soil. This hampers the plant's ability to utilize resources, resulting in a decrease in agricultural output (Lynch et al., 2022).

Hydrotropism, which refers to the directional growth of plant roots towards moisture, is crucial for plants to adapt to drought conditions. Root tips exhibit hydro patterning, wherein they counteract the effects of varying water potentials by adjusting their development direction to branch towards places with increased water availability regardless of gravitational forces (Shkolnik et al., 2016). Hydrotropic growth aids the movement of developing lateral roots towards water when they are surrounded by dry soil. Hydrotropism and hydro patterning work together to help plants adapt to drought conditions (Giehl et al., 2018).

2.3.3 The impact of water stress on plant growth and development

Water stress can significantly impair plant growth and development (Bhargava et al., 2013). Water stress is a complex form of stress for plants that can affect all aspects of their growth and development (Salehi et al., 2016). Furthermore, drought can have adverse effects on both the quantity and quality of plant development and productivity (Zlatev et al., 2012). Cell division, elongation, and differentiation are crucial factors that determine the growth and development of plants. Water stress leads to a decrease in plant water potential and turgor, hence, impairing the ability of plant cells to carry out their usual duties (Rahdari et al., 2012). Thus, water stress impacts all of the phases of plant growth and development starting from a decrease in cell turgor, disruption of enzyme activities, and a reduction in energy supply from photosynthesis (Osakabe et al., 2014a). Cell proliferation and enlargement are essential processes during the early stage of plant development and establishment (Salehi et al., 2016). Plant response to water stress and return to normalcy varies with the intensity of stress, and the length and timing of stress exposure in addition to the inherent health of the plant (Salehi et al., 2016).

2.4 Physiological responses of plants under water stress conditions

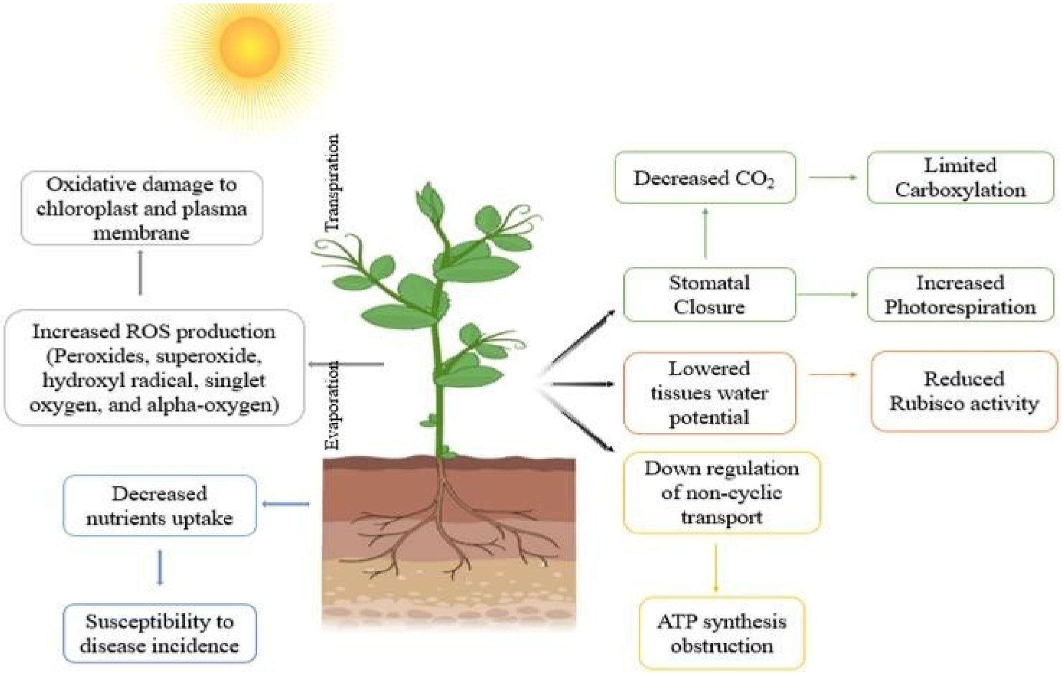

Figure 2.2. Impact of water stress on the structural, functional, and metabolic aspects of plant growth (modified from(Wahab et al., 2022))

Water stress has been reported to cause physiological stress in plants, and although different plants may respond differently, water stress frequently results in decreased leaf-relative water content, turgor loss, and stomatal closure (Figure 2.2) (Wahab et al., 2022).

2.4.1 Stomatal Aperture

Stomata closure is the foremost indicator of a plant's response to water stress. Plants adapt to water stress by controlling stomata movement, altering osmotic balance, and initiating an antioxidant defense system (Kamran et al., 2020; Zandi et al., 2022). In periods of intense water stress, stomata are completely closed in most plant species (Quraan et al., 2021; Hashmat et al., 2021). Water stress tolerance plants influence the rates of carbon fixation, photosynthesis, and water use efficiency since the stomatal system governs these processes (Wahab et al., 2022). Stomatal closure restricts CO$_2$ entry, allowing the electrons to produce more reactive oxygen species (Figure 2.2) (Yang et al., 2021). Studies have shown that water stress decreases stomatal conductance due to reduced aquaporin gene expression and also, because of anatomical traits that result in less chloroplast surface exposure to intercellular space per unit leaf area (Tosens et al., 2012). Stomatal closure, however, not only reduces water loss through transpiration but also reduces CO_2 and nutrient uptake and thereby, altering photosynthesis (Xiong, 2002). Dry-climate plants have evolved xeromorphic characteristics to minimize transpiration during water stress. Under water-stressed conditions, leaf shedding and reductions in leaf production, leaf size, and branching are additional ways to reduce transpiration loss (Basu et al., 2016). Another adaptation to counter water stress is sclerophylly, where plants develop hard leaves that will not suffer permanent damage from wilting and can be restored to full functionality once normal conditions return (Basu et al., 2016).

2.4.2 Photosynthesis

Water stress inhibits photosynthesis by reducing leaf area and the photosynthetic rate per unit leaf area (Basu et al., 2016). The primary causes of reduced photosynthetic rate are stomatal closure or metabolic impairment (Figure 2.2) (Tezara et al., 1999). During photosynthesis, CO_2 and H_2O in chloroplasts of plant cells manufacture sugars and O_2 as a by-product in the presence of light. Chlorophyll is a crucial part of chloroplasts, which are necessary for photosynthesis (Misson et al., 2010; Duursma et al., 2019). The photosynthetic system and its pigments including chlorophylla, b, and carotenoids are profoundly affected by water stress (Elansary et al., 2020) (Figure 2.2). Chlorophyll pigments are vital for photosynthesis and are affected by water stress, affecting the opening and closing of stomata in plant leaves (Hu et al., 2018). In many plants, water stress increased oxidative stress, degradation of chlorophyll pigment, and chlorophyll concentration (Allakhverdiev, 2020). Water sensitivity was mostly associated with a decrease in stomatal conductance, which lowered the transportation of CO_2 to lower net photosynthesis (Devi et al., 2011; Soares et al., 2019). The major factors responsible for slowing down photosynthesis might be stomatal closure (reduced stomatal CO_2 fixation) (Figure 2.2), non-stomatal (decreased photosynthesis activity in mesophyll tissues), or both (Tátrai et al., 2016). Water stress is a harmful abiotic stress that hinders photosynthesis by damaging the photosystems II (PS-II)because of their high susceptibility to external stimuli (Ings et al., 2013; Pourghasemian et al., 2020). Many studies on crops have demonstrated that carotenoids are less sensitive to water stressthan chlorophyll. It was shown that plants under water stress produce more xanthophyll pigmentssuch as zeaxanthin and antheraxanthin (Misra et al., 2000). In Japanese mint (*Mentha canadensis*), Misra et al., (2000) showed that severe water stress reduced the leaf solute potential, chlorophyll, and carotenoid contents with a greater loss of chlorophyll b than chlorophyll a.

2.4.3 Cell size and respiration

Plant growth is governed by the processes of cell division, cell expansion, and cell differentiation, as well as genetic, ecological, and physio-morphological mechanisms (Ahmad et al., 2018). Cell development is one of the physiological processes that is markedly affected by water stress when turgor pressure falls (Elansary et al., 2020). Water stress in higher plants can impede cellelongation by obstructing the flow of water from the xylem to adjacent elongating cells, ultimatelyleading to the mortality of the plant (Hasanuzzaman, 2020). The respiration rate is lowered in a broad range of plant parts during water stress including leaves, shoots, and the entire plant. On theother hand, a study conducted by (Wahab et al., 2022) showed that under water stress conditions, plant respiration rates remain unaltered or even elevated.

2.4.4 Leaf relative water content

The leaf relative water content (RWC) is a crucial regulator of physiological processes in plants. Leaf RWC is used to estimate the water condition in plants. It represents the equilibrium between the amount of water supplied to the leaf tissue and the rate at which water is lost through transpiration (Soltys et al., 2016). The decline in RWC is the primary symptom of water stress (Hussain et al., 2018) (Figure 2.2). The rate of growth and transpiration of leaf tissue are both substantially correlated with the relative water content of leaves (Kapoor et al., 2020). Lower RWC reduces the leaf water potential which enables the stomata to shrink. The main mechanism controlling leaf temperature is transpiration; decreasing the transpiration rate in leaves and raising leaf temperature are achieved through increasing stomatal resistance (Wu et al., 2022) (Figure 2.2). Under stress regimes, a decrease in relative water content resulted in a reduction in water content and osmotic potential in plants (Ashrafi et al., 2022). A reduced soil water potential during water stress hindered crop development and lowered plant osmotic potential, which results in inadequate nutrient uptake (Ashrafi et al., 2022).

2.5 Biochemical responses under water stress conditions

Accumulating biochemical compounds including proline, protein, sucrose, and glycine betaine (GB) improve crop productivity by minimizing ROS-generated oxidative stress (Perveen et al., 2020). Likewise, water stress affects physiological systems such as cellular respiration, photosynthetic rate, mineral nutrition, enzymatic activity, and cellular redox (oxidation/reduction) homeostasis. Consequently, biochemicals such as membrane lipo-proteins, DNA, and cellular protein are degraded in conditions of water deprivation (Khan et al., 2021). Accumulation of compatible solutes also known as osmo-protectants and organic acids in the cytoplasm was noticed in response to water stress. These solutes aid in scavenging ROS, improving water potential, and safeguarding biological molecules from lipid peroxidation (Wahab et al., 2022). Under water stress, plant cells aggregate soluble substances and thicken their cytoplasm. In a comparablepattern, noncyclic electron transport was increased to meet the requirements of decreased NADPH, ATP, and ROS synthesis (Figure 2.2) (Wahab et al., 2022).

2.5.1 Reactive oxygen species

Water-stressed plants have an increase in the formation of ROS, which occurs along their regular metabolic processes such as aerobic metabolism (Miller et al., 2010). The response of plants to water stress, either through photosynthesis or other means, results in oxidative damage of proteins, lipids, and nucleic acids. Since plants are sessile, they have evolved a plethora of techniques to assist them in adapting or surviving water stress (Caverzan et al., 2016). Enhanced ROS generation is inevitable in water stress conditions. However phytotoxic amounts of ROS are perilous (Hasanuzzaman et al., 2021), and can cause cellular damage and even cell death (Franchina et al., 2018). However, trace levels of ROS function as an essential signaling molecule that stimulates multiple stress-response pathways that commence crosstalk among them (Verma et al., 2019). The antioxidant system is comprised of enzymes that produce and scavenge ROS and also regulate the redox state of cells by reducing intracellular ROS levels (Verma et al., 2019).

2.5.2 Total phenolic content and osmotic adjustment

Phenolics are the most abundant and extensively dispersed phytochemical class in plants (King & Young, 1999). Plants produce many of these phenolic compounds that are involved in their responses to biotic and abiotic environments. Previous studies indicated a 100% increase in phenolic content under water stress conditions (Madzikane-Mlungwana et al., 2017; Franchina et al., 2018). For instance, drought-stressed tomatoes (*Solanum lycopersicum*) had more total phenolic than well-watered plants (Wahab et al., 2022). An osmotic adjustment occurs when the water potential in dividing cells is reduced, which maintains turgor pressure by accumulating solutes (Chaves et al., 2004). The osmotic adjustment has been linked to sustaining stomatal conductance, photosynthesis, leaf water volume, and growth under water stress (Figure 2.2) (Miranda et al., 2021). The recognized major solutes that are built up in response to water stress are inorganic cations, organic acids, carbohydrates, and free amino acids. Studies demonstrated that the build-up of suitable solutes such as proline and glycine betaine protects plants against the damaging effects of water stress not only through osmotic adjustment but also by detoxifying ROS, maintaining membrane integrity, and stabilizing enzymes or proteins (Basu et al., 2016). However, several plant sugars such as sucrose, trehalose, glucose, and fructose are primary osmolytes that are important in osmotic adjustment (Wahab et al., 2022).

2.6 Water Stress Alters Central Carbon Metabolism in Plants

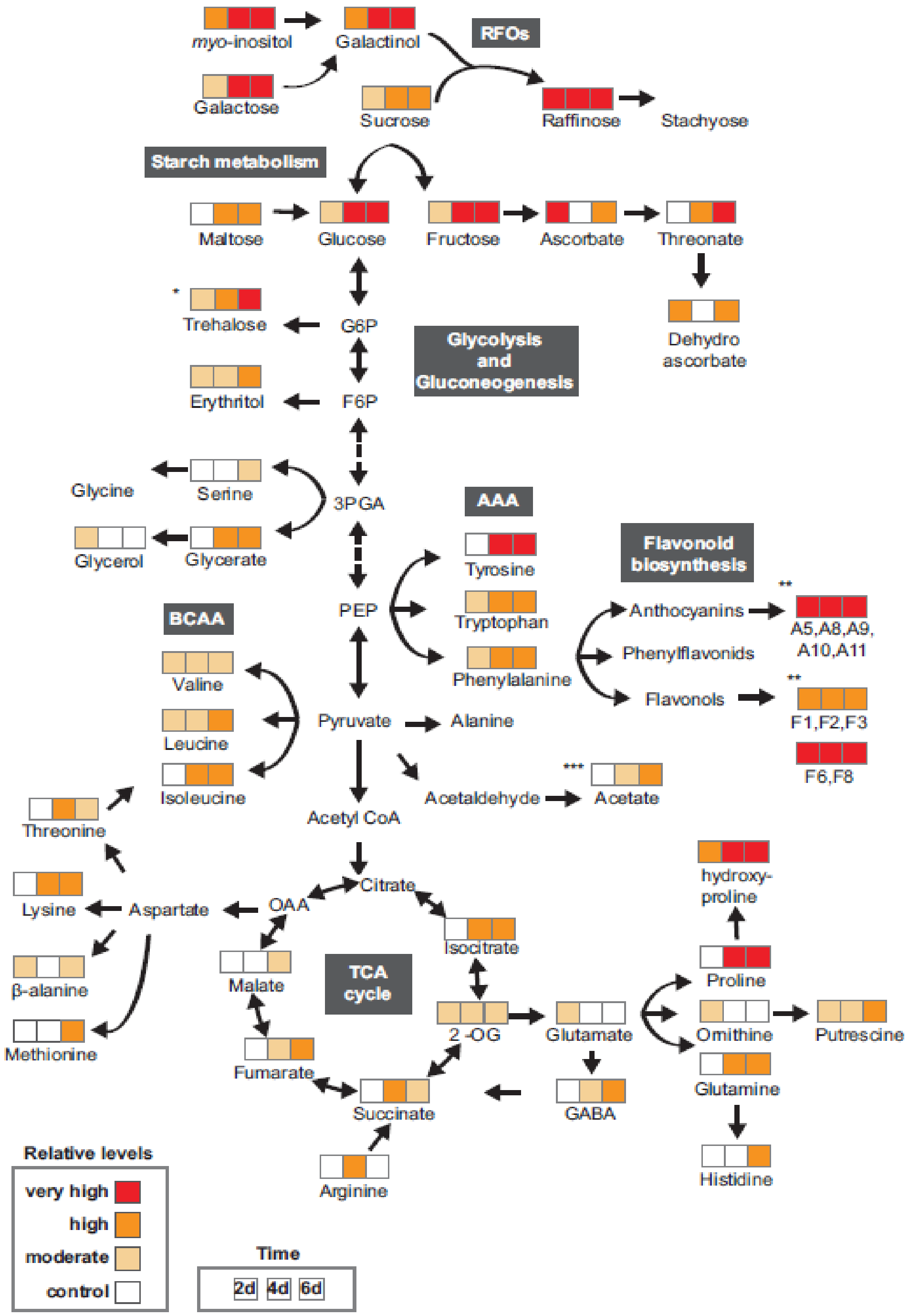

Figure 2.3. Metabolic response of wild-type Arabidopsis plants to water stress. The data for these images is extracted from (Fàbregas et al., 2018), (Pires et al., 2016a), (Kim et al., 2017; Nakabayashi et al., 2014). Boxes show the 2, 4, and 6-day water stress times (d). In comparison to 0 days of water stress, colours represent the relative accumulation levels of each metabolite: Red, extremely high, >4, orange, high, 2-4, pink, moderate, 1-2, and white, no appreciable changes from control. RFOs are raffinose family oligosaccharides. AAA stands for aromatic amino acids. BCAA stands for branched-chain amino acids.

2.6.1 Flooding stress

When soil is flooded, plants are most severely harmed by lack of oxygen, which negatively affects mitochondrial respiration (Bailey et al., 2008). Under severe flooding conditions, the impairment of oxidative phosphorylation of mitochondrial respiration occurs which eventually reduces the production of respiratory ATP (Bailey et al., 2012). To make more ATP and combat the energy crisis under water stress conditions, plants increase glycolytic flow, which causes sugar stores to be depleted more quickly (Bailey et al., 2012). Plants must produce enough ATP under these stressful conditions to maintain cellular processes and must replenish oxidized NAD+ to maintain the glycolytic flux. Pyruvate collected from glycolysis can be directed through fermentation pathways to rebuild the pool of NAD+ required for glycolysis under flooding conditions (Xu & Fu, 2022).

During stress, the tricarboxylic acid (TCA) cycle functions non-cyclically (Figure 2.3) (António et al., 2016). The production of 2-oxoglutarate, which can enter mitochondria to form succinate *via* 2-oxoglutarate dehydrogenase and succinate CoA ligase occurs simultaneously with the anaerobic accumulation of alanine. This extra ATP production helps to make up for the lack of energy caused by oxygen restriction due to flooding(Xu & Fu, 2022a). Malate dehydrogenase converts oxaloacetate to malate, which is used by the mitochondria to metabolise 2-oxoglutarate (Rocha et al., 2010a). It is typical for succinate to accumulate during hypoxic conditions brought following flooding since succinate dehydrogenase (SDH), a TCA cycle enzyme, requires oxygen to function (António et al., 2016). In various plant species, changes in other TCA cycle intermediates like citrate, malate, and fumarate take place under flooding stress (Figure 2.3) (Lothier et al., 2020).

2.6.2 Drought stress

The restricted water supply in drought-stressed plants inhibits photosynthesis and impairs plant growth and development (Figure 2.2) (Pinheiro et al., 2011). Due to stomatal closure and higher mesophyll diffusional resistance, less CO_2 is transported from the environment to the sites of carboxylation within chloroplasts during drought, which results in lower net CO_2 uptake. According to some research, photosynthesis is restricted more directly by CO_2 diffusional resistances under water deficits than by metabolic constraints (Pinheiro et al., 2011). Water-stressed leaves with reduced photosynthesis are exposed to surplus energy because photosynthesis is the primary sink for photosynthetic electrons, which generates ROS that can impede ATP synthesis (Killi et al., 2017). There is an indication that under drought, ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) activity declines, possibly connected to a reduction in ATP and Rubisco activase activity (Osakabe et al., 2014a). Photorespiratory flux in the leaves of C3 plants under drought improves substantially as CO_2 availability declines, causing electron sinks to contribute and leading to high rates of H_2O_2 production (Xu & Fu, 2022a). An imbalance between the supply and demand of ATP or NADPH could be the underlying cause of the alterations in the size of metabolic pools driven by drought stress (Walker et al., 2020).

One of the primary ways used by plants to maintain positive turgor pressure in water-limited situations is an osmotic adjustment, which involves the aggregation of solutes (Turner, 2018). Chemically, the osmolytes that build up during drought stress include soluble sugars (such as glucose, fructose, sucrose, and trehalose), raffinose family oligosaccharides (RFOs), amino acids (such as proline and GABA), and polyamines (e.g., putrescine and spermidine)(Kumar et al., 2021a). The accumulation of many of these osmolytes are also influenced by other abiotic factors like salinity, temperature, and flooding (Serraj et al., 2002). Soluble sugars have an important role in osmoregulation, as well as in maintaining a balance between the supply and consumption of carbon and energy in plants under water stress. They also act as signalling molecules that govern a multitude of physiological and developmental processes (Xu & Fu, 2022a).

2.7 Oxidative Stress in Plants: A Metabolic Signature

Among the plethora of environmental stresses, water stress leads to significant oxidative stress which causes a substantial overlap in plant physiological and molecular responses (Figure 2.2) (Savchenko et al., 2021). Consequently, measuring the amounts of specific metabolites can help determine the physiological status of a plant and the activities that are most probable to be affected by oxidative stress (Schützendübel et al., 2002). Many soluble sugars including sucrose, fructose, raffinose, sorbitol, mannitol, and fructans have been demonstrated to have antioxidant capabilities in plant tissues *in vitro*, and in certain model systems.

2.7.1 Sugar and sugar derivatives

Independent investigations have identified substantial changes in metabolic pathways under oxidative stress including alterations in glycolysis and the TCA cycle and activation of the oxidative pentose phosphate pathway (Figure 2.3) (Sipari et al., 2020). The redirection of glycolytic carbon flow into the oxidative pentose phosphate pathway is primarily credited for the changes in the sugar profile and changes in the quantities of sugar phosphates and soluble carbohydrates with antioxidant properties (Figure 2.3) (Lehmann et al., 2009). In Arabidopsis cell culture treated with H_2O_2, a rapid accumulation of sucrose and fructose followed by a rise in glucose level was observed (Chen et al., 2015). Multiple studies have consistently found that the stress caused by drought and flooding situations accelerates the process of glycolysis. This is a method used to provide energy for activating stress defense mechanisms and adapting to stressful conditions (Abbey et al., 2023a). Furthermore, water stress triggers "energy crises" by impairing the oxidative phosphorylation process in the mitochondrion, leading to a significant decrease in ATP generation (Bailey et al., 2008; Bailey et al., 2012). Scientists have demonstrated that the buildup of sugar is vital in determining how plants distribute their carbon resources and promote development (Koch, 2004). In order to endure the "energy crises" caused by stressful conditions, resilient plants enhance their glycolytic influx by collecting additional glucose (Abbey et al., 2023a). This allows them to create an adequate amount of ATP through glycolysis, which is essential for sustaining fundamental cellular processes and replenishing NAD+ to sustain the flow of glycolysis(Bailey et al., 2008).

Water stress leads to excess concentrations of ROS, which are accompanied by an accumulation of oligosaccharides as α-galactosyl extensions of sucrose (ElSayed et al., 2014; Nishizawa et al., 2008). These oligosaccharides had a higher degree of polymerization. Raffinose, one of these oligosaccharides, was identified in many plants while stachyose and verbascose, two with a higher degree of polymerization were only found in a few species and operate as osmo-protectants and stabilizers of cellular membranes (Janeček et al., 2011). Several lines of evidence suggested that these metabolites may enhance plants adaptation to oxidative stress (Smirnoff et al., 1989; Morelli et al., 2003; Nishizawa et al., 2008).

2.8 Metabolic alterations in central metabolites under water stress

A summarization of individual studies revealed that approximately 55 metabolites are affected by drought, and 46 metabolites are affected by flooding (Figure 2.3) (Xu & Fu, 2022). Interestingly, 23 metabolites showed common stress responses to various abiotic stresses such as cold, heat, drought, flooding, and salinity. Because photosynthesis and photorespiration are extremely sensitive to environmental changes, several steps in the Calvin-Benson cycle (CBC) and photorespiratory pathway respond to water stress. Induced stress in plants results in oxidative damage, which changes the core metabolism of the plant. The development of protective compounds such as appropriate solutes or osmolytes to shield plants from oxidative damage, and the prevention of ROS formation are common themes for metabolic changes in response to oxidative damage (Darko et al., 2019; Savchenko et al., 2021). Compatibility-promoting solutes such as sucrose, trehalose, raffinose, mannitol, sorbitol, inositol, and proline proliferate in response to water stress (Figure 2.3).

The shikimate pathway is activated by several abiotic stresses, which results in an accumulation of aromatic amino acids like tyrosine, phenylalanine, and tryptophan. These aromatic amino acids serve as the building blocks for the manufacture of antioxidant phytoalexins, alkaloids, and flavonoids (Vogt, 2010). Water stress can also trigger the production of sulfur-containing metabolites such as glutathione, methionine, and cysteine (Figure 2.3). These metabolites play crucial functions in the antioxidant systems of plants (Capaldi et al., 2015; Colovic et al., 2018). Oxidative damage brought on by water stress may increase sugar phosphates linked to glycolysis and the oxidative pentose phosphate pathway (OPPP), as well as a decline in TCA cycle intermediates and amino acids generated from the TCA cycle (Queval et al., 2009).

Variations in ethanol, acetaldehyde, and acetate, which are intermediates in the fermentation of ethanol are more specifically related to flooding stress. The ability of a plant to withstand flooding may be related to its ability to recover the carbon that would otherwise be lost as ethanol by turning it into acetate and then acetyl-CoA to keep the flooded plant's carbon metabolism running uninterrupted (Kreuzwieser et al., 2004). Acetate is a key metabolite that was recently found to be involved in a distinct drought-survival pathway (Kim et al., 2017). Plant drought tolerance is enhanced by the metabolic transition from glycolysis to acetate production *via* pyruvate decarboxylase and aldehyde dehydrogenase (Kim et al., 2017).

2.9 Alteration of essential oil compounds under water stress conditions

The economic importance of medicinal and aromatic plants is enormous because of the ongoing and expanding demand for their products in the domestic and international markets. The quality of medicinal and aromatic plants is contingent on the composition of plant secondary metabolites (PSMs), which are themselves influenced by environmental conditions such as water stress (Nasrollahi et al., 2014; Kleinwächter et al., 2015). Studies have shown that metabolite composition under stress depends on plant species, the compound being studied and the cultivation conditions. Studies have noticed alterations in the composition of essential oil extracted from aromatic plants under water stress conditions (Khalid, 2006). Japanese mint plant fresh and dry weights, nutritional content, and essential oil output were all remarkedly reduced as a result of water stress (Misra et al., 2000). For instance, linalool and methyl chavicol concentrations in sweet basil essential oil increased as drought stress intensified (Khalid, 2006). In comparison to the control treatment, the essential oil percentage of *Thymus carmanicus* rose by 12.5% and 44.9% under mild and severe water deficiencies, respectively (Bahreininejad et al., 2014). This observation can be ascribed to an increase in essential oil accumulation through a higher density of oil glands brought on by a diminution in leaf area under drought. A study on Thymus spp. found that water treatment at 67% field capacity led to a high essential oil production, but treatment at 33% field capacity resulted in a poor yield (Askary et al., 2018). It is critical to remember that the amount of essential oil does not always increase in proportion to the severity of water stress because, under more extreme conditions, the plant uses its assimilates to produce osmotic regulator compounds like proline and glycine-betaine as well as sugar compounds like sucrose, fructose, and fructan to provide the conditions it requires to thrive (Askary et al., 2018). Research conducted on basil highlighted that in response to water stress, the essential oil content per dry mass of the plant only slightly increased (Sharafzadeh et al., 2011). Some trace amounts of ß-myrcene and 2-octanone were absent in plants under regular watering but were found in water-stressed plants (Sharafzadeh & Zare, 2011). The amount of essential oil that aromatic plants contain depends on a variety of factors including species, environmental circumstances, and the degree and duration of water stress.

2.10 Conclusion

Water stress is a common threat, particularly in dry and semi-dry regions, and can impact all aspects of plant growth, development, and metabolism. The impact of water stress on plants is intricate due to its multifaceted nature. Global warming-related climate shifts are causing uncommon weather patterns around the world, most frequently in the form of drought and flooding. Plants have evolved intrinsic defenses against water stress through the process of evolution. The ability of all plants to respond to water stress varies. Even in highly tolerant plant species, tolerance is achieved by alterations in molecular and physiochemical processes that enable plants to adjust morphologically to water stress. However, they must pay a price for this tolerance in the form of decreased photosynthesis, which frequently results in lower biomass yields due to the cautious water management strategy used by plants. The genetic mechanisms that assist plants in producing enzymes, proteins, and chemicals suitable in various ways to fight water challenges are the reason for plant adaptation to stress conditions. Plants experiencing water stress typically exhibit increased concentrations of natural compounds. This rise may be attributed to a decrease in biomass production with a substantial increase in the overall content of secondary metabolites. Many biotechnological interventions were developed to improve the water stress adaptability of some high-value plants based on the understanding of the cause and effect of water stress on plants as well as the understanding of the responses plants make in different ways to become tolerant to such stress. But there are still a lot of gaps in our understanding of the causes and effects of water stress in plants, so we need to step up our efforts to gain a better understanding of the problem.

CHAPTER 3: MORPHO-PHYSIOLOGICAL AND BIOCHEMICAL RESPONSES OF *PLECTRANTHUS AMBOINICUS* TO VARYING WATERING REGIMES

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Abstract

Water stress is a detrimental event that adversely impacts crop productivity on a global scale. A study was carried out to examine the impact of fluctuating water stress conditions on Mexican mint plant morpho-physiological and biochemical indices. The treatments were regular watering (RW), drought (DR), flooding (FL), and resumption of regular watering after flooding (DHFL) orafter drought (RHDR) with three replications for each. The DR and FL treatments significantly ($p<0.01$) reduced plant fresh weight by 92% and 88%, respectively, when compared to RW. The relative water content (RWC) of DR plants showed a significant ($p<0.01$) reduction of 60% whencompared to the RW plants. Net photosynthetic rate, transpiration rate, and stomatal conductance were significantly ($p<0.01$) reduced in DR and FL plants, whereas sub-stomatal carbon dioxide was significantly ($p<0.01$) high in DR plants. Chlorophyll a and b were significantly ($p<0.05$) reduced in the DR and FL plants when compared to the RW plants. On the other hand, soluble sugar content in the DHFL plants was significantly (p<0.05) increased compared to that of the RWplants. FL plants showed a significant ($p<0.05$) increase in total 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging effect by 156%, total flavonoids content by 225%, total phenolics by 242%, and Malondialdehyde (MDA) content by 94% compared to RW. In conclusion, this studyhighlighted the morpho-physiological and biochemical changes in Mexican mint plants under varied regimes of watering. Mexican mint is an aroma medicinal plant and thus, further research should assess volatile organic compounds.

Keywords: Water stress, irrigation, flooding, drought, Mexican mint, abiotic stress

3.1 Introduction

The shift in climatic patterns presents a significant challenge to the survival of life on earth. This can be partly attributed to the increasing difficulty of meeting the escalating demand for food and establishing sustainable agricultural practices to support a growing population (Amna et al., 2021). The current state of changing climatic conditions, characterized by unexpected occurrences such as droughts, severe floods, earthquakes, and fluctuations in temperature destabilizes the hydrologic cycle (Zainab et al., 2021). In the last decade, there has been a significant increase in the expanse of arid regions worldwide. This expansion has been accompanied by a noticeable rise in the prevalence, severity, and frequency of drought and flooding events. Consequently, the cumulative impact of these droughts has led to an estimated global economic loss of almost $30 billion in agricultural production (Lesk et al., 2016b; Gupta et al., 2020). Flooding is the second most significant climate-related calamity following drought. The number, frequency, and severity of flood disasters have risen from the 1990s (Du et al., 2010; Shi et al., 2021). Plants exhibit adaptive responses to water stress (flooding, drought) through alterations in various plant morphological structures and biochemical processes. Such adaptation mechanisms include modifications in leaf and root morphology, adjustments in photosynthetic activity, and changes in antioxidant enzyme systems (Khaleghi et al., 2019; Ren et al., 2018). Currently, it is well acknowledged that drought stress primarily impacts the assimilation and translocation of nutrients from the root system to the foliage (Bista et al., 2020; Jiao et al., 2021).

Plants generate excessive amounts of reactive oxygen species (ROS) such as singlet oxygen, superoxide, and hydrogen peroxide in response to water stress. ROS exhibit a high reactivity and possess the ability to swiftly inflict damage on living tissues and macromolecules such as deoxyribonucleic acids, lipids, proteins, and carbohydrates (Cezary, 2018). Sugars such as glucose, fructose, sucrose and sugar alcohols like mannitol, and amino acids such as proline accumulate in many plant species when exposed to water stress. These compounds serve as osmolytes to aid in the regulation of osmotic balance and function as antioxidants. Their presence helps in the detoxification of ROS, protection of cellular membranes, and stabilization of enzymes and proteins. Ultimately, these compounds contribute to enhancing plant resilience against abiotic stress (Bartels et al., 2005; Ashraf et al., 2007; Seki et al., 2007). The influence of water stress on plants is contingent upon various factors including the extent and length of water supply, soil quality, soil water gradients, the plant species, and their developmental stages (Tardieu et al., 2018). Plants are sessile and have developed numerous adaption mechanisms that may improve their ability to thrive under both short- and long-term water stress conditions (Fu et al., 2001; C. Yin et al., 2005).

In this study, we used Mexican mint belonging to the Lamiaceae family with a diverse array of ethnobotanical benefits (Lukhoba et al., 2006). Mexican mint is a succulent Crassulacean acid metabolism plant whose growth and key metabolic pathways in the central carbon metabolism can be severely affected by different watering regimes (Abbey et al., 2023). However, it is not clear how varying watering regimes affect plant growth, photosynthesis rate, and stress response metabolites of Mexican mint. This knowledge can be utilized to enhance stress tolerance and ultimately enhance crop productivity (Reddy et al., 2004a). The present study aims to examine the growth, chlorophyll fluorescence indices, and biochemical composition of Mexican mint plants under different watering regimes.

3.2 Materials and Methods

Plant propagation and treatment application were modified from **Abbey et al. (2023).**

3.2.1 Location

The research was performed in the Plant Physiology Laboratory of the Department of Plant, Food, and Environmental Sciences, Faculty of Agriculture between December 2021 to July 2022.

3.2.2 Preparation and Rooting of Cutting

A healthy mother plant was chosen from the PFES greenhouse plant stock. The plant was well watered to relieve it from stress conditions. Stem cuttings were taken from branches from the youngest second and third nodes of soft tissues on the main stem. The stem cuttings were then pruned to 5-cm length leaving five pairs of corresponding leaves before placing in a moist perlite medium (Perlite Canada Inc., Montreal, QC, Canada) contained in a plastic flat tray (50cm length x 28cm width x 6.5cm depth). The planted trays were covered with a dome-shaped transparent cover to maintain ≥95% relative humidity for rooting, under 24-hour fluorescent lighting at a temperature of 22°C. The cuttings were sprayed with water twice a day until transplanting after three weeks.

3.2.3 Planting of rooted cutting and growing condition

The Mexican mint cuttings were transplanted into 15 cm diameter plastic pots filled with a mixture of 200 g of Promix-BX (Premier Tech Horticulture in Québec, Canada) and 150 g of vermicast. The cuttings were placed in a Biotranette Mark II Environmental growing chamber (Lab-Line Instruments Inc., ILL, USA) in a completely randomized design with three replications under 24°/20°C and a 12/12-hour day and night cycle. The plants were watered regularly for 3 months after which the five treatments were imposed.

3.2.4 Water stress treatment and experimental design

The experimental treatments used were previously reported by Abbey et al. (2023). In brief, to understand the morpho-physiological and biochemical response of the Mexican mint plant, the treatments used were regular watering (RW) at field capacity for 8 weeks; continuous drought (DR) for 8 weeks; continuous flooding (FL) for 8 weeks; rehydration, and resumption of regular watering for 4 weeks after a continuous drought for 4 weeks (RHDR); and dehydration for 4 weeks after continuous flooding for 4 weeks (DHFL). Regular watering was done every other day to maintain the field capacity of the growing medium. Flooding was simulated by complete submergence of the 15 cm diameter pot with the Mexican mint plant in water contained in a 20 cm diameter plastic pot. The experiment was arranged in a completely randomized design with three replications in the growth chamber.

3.2.5 Plant growth and physiological analysis

3.2.5.1 Total fresh weight

The total fresh weights of the plants were recorded using an Ohaus navigator portable balance (TTM Instruments Inc., Canada) at the end of the experiment i.e., 8 weeks after the treatment application.

3.2.5.2 Chlorophyll fluorescence and photosynthetic parameters

Water stress traits were assessed using a Chlorophyll fluorometer (Optical Science, Hudson, NH, USA) to obtain maximum quantum efficiency (Fv/Fm) and potential photosynthetic capacity (Fv/Fo). Other photosynthetic parameters i.e., sub-stomatal carbon dioxide concentration, net photosynthetic rate, and transpiration rate were determined from two pairs of healthy opposite leaves (n = 3) from each plant per treatment using LC*i* portable photosynthesis system (ADC BioScientific Ltd., Hoddesdon, UK).

3.2.5.3 Electrolyte leakage and leaf relative water content

Electrolyte leakage from disrupted cells were estimated by the electric conductivity using an EC 500 ExStik multi-purpose pH meter (EXTECH Instrument, Nashua, NH, USA). 1 cm diameter leaf discs were submerged in 20 mL deionized water and the electric conductivity was measured every 2 h for 12 h under room temperature and light conditions. Leaf relative water content was estimated by measuring the fresh, dry, and turgid weight of 1 cm diameter leaf discs with MXX-412 Denver precision electronic balance (Denver Instrument Company, CO, USA) from four opposite leaves in triplicate (n = 12). The turgid weight was determined by submerging the fresh leaf discs in 100 ml of deionized water for 48 h and weighed. The leaf discs were then dried in a Cole-Parmer mechanical convection oven dryer (Cole-Parmer Instrumental Company, Vernon Hills, IL, UK) at 65°C to constant weight for 48 h and weighed. The relative water content was calculated using the formula:

$$\text{Relative Water Content (RWC \%)} = \frac{\text{Fresh weight} - \text{Dry Weight}}{\text{Turgid Weight} - \text{Dry Weight}} \text{x} 100$$

3.2.6 Biochemical analysis

Healthy leaf samples were flash-frozen in liquid nitrogen. The samples were ground into fine powder and stored in a -80°C freezer until analysis.

3.2.6.1 Chlorophylls a and b, and carotenoid contents

Chlorophylls (Chls) a and b, and carotenoids were measured as described by Lichtenthaler (1987) with little modification. Ground leaf samples (0.2 g) were thoroughly mixed in 1 mL of 80% acetone followed by centrifugation at 15000 × *g* for 15 min. The absorbance of the supernatant was recorded at 646.8 nm, 663.2 nm, and 470 nm using a UV-Vis spectrophotometer (Jenway, Staffodshire, UK) against 80% acetone as a blank. The Chls and carotenoid contents were expressed as μg g^{-1} fresh weight (FW) of the sample.

3.2.6.2 Total soluble sugar (TSS)

Total soluble sugar was determined using the method of DuBois et al. (1956). Briefly, a 0.2 g of the ground leaf sample was homogenized in 1.5 mL of ice-cold freshly prepared 5% trichloroacetic acid (TCA). The mixture was vortexed for 2 min and centrifuged at 12000 × *g* for 3 min. An aliquot of 1 mL was transferred into a thick-walled glass test tube containing 1 mL of 5% phenol and mixed thoroughly. The reaction mixture was mixed with 5 mL of concentrated sulfuric acid, vortexed for 20 s, and incubated in the dark for 15 min. After the mixture had reached room temperature, the absorbance was measured at 490 nm against a blank. Total sugar was calculated using a glucose standard sugar curve and expressed as μg of glucose g^{-1} FW of the sample.

3.2.6.3 Total phenolics

Total phenolic content (TPC) was determined using the Folin-Ciocalteau assay as described by Ainsworth & Gillespie (2007) with little modification. A 0.2 g of the ground leaf sample was homogenized in 2 mL of ice-cold 95% methanol and incubated in the dark at room temperature for 48 h. The mixture was centrifuged at 13000 × *g* for 5 min before adding 100 μl of the supernatant to 200 μL of the 10% (*v/v*) Folin-Ciocalteau reagent. After vertexing for 5 min, the mixture was combined with 800 μL of 700 mM sodium carbonate (Na_2CO_3) and incubated in the dark at 25°C for 2 h. The absorbance of the supernatant was measured at 765 nm against to a blank. TPC was calculated using a gallic acid standard curve and expressed as mg of gallic acid equivalents g^{-1} FW of the sample.

3.2.6.4 Total flavonoid

Total flavonoid was measured following the colorimetric method described by Chang et al. (2020). A 0.2 g ground leaf sample was homogenized in 2.5 mL of 95% methanol followed by centrifugation at 15000 × *g* for 10 min. A reaction mixture was obtained by adding 1.5 mL of 95% methanol, 0.1 mL of 10% aluminum chloride ($AlCl_3$), 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water to 500 μL of the supernatant. The reaction mixture was incubated at room temperature for 30 min and the absorbance was measured at 415 nm against a blank lacking $AlCl_3$. Total flavonoid content was calculated using the quercetin equivalents and expressed as μg of quercetin g^{-1} FW using the formula below:

$$\text{Total flavonoid} = \frac{([\text{Flavonoids}](\frac{\mu g}{mL}) \text{ x total volume of methanolic extract(mL)}}{\text{mass of extract (g)}}$$

3.2.6.5 Lipid peroxidation concentration

The concentration of malondialdehyde (MDA) was used to determine the extent of lipid peroxidation following the method described by Hodges et al. (1999). A 0.2 g of the ground leaf sample was homogenized at 1 mL of 0.1% (w/v) trichloroacetic acid (TCA) followed by centrifugation at 17000 × *g* and 4°C for 10 min. 500 μL of the supernatant was mixed with equal volume of 0.5% thiobarbituric acid (TBA) in 20% TCA. The mixture was vortexed for 30 s, incubated at 95°C for 30 min and centrifuged at 10000 × *g* for 5 min. The absorbance of the reaction mixture was measured at 400 nm, 532 nm, and 600 nm against the blank of TBA solution. The MDA equivalent was expressed as nmol g^{-1} FW using the formula:

$$\text{MDA concentration} = [\frac{\text{Abs535} - \text{Abs600}}{155000}] \times 10^6$$

3.2.6.6 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging capacity

2,2-diphenyl-1-picrylhydrazyl radical scavenging capacity was measured using the method described by Fan et al. (2020). A 1.5 mL pure methanol was added to 0.2 g of the ground leaf sample and mixed thoroughly. The mixture was centrifuged at 12000 × *g* for 10 min and 100 μL of the supernatant was added to 2.9 mL of 60 μM fresh DPPH methanolic solution. The mixture was incubated in the dark at 22°C for 30 min and the absorbance was measured at 515 nm against a methanol blank. The following formula was used to calculate the scavenging capacity:

$$\text{Inhibition\%} = [\frac{\text{Ab} - \text{As}}{\text{Ab}}] \text{ x100\%;}$$

As is the absorption of treatments and Ab being the absorption of control samples.

3.2.6.7 Hydrogen peroxide accumulation

Hydrogen peroxide (H_2O_2) was determined according to the method described by (Patterson et al., 1984). A 0.2 g of the ground leaf sample was homogenized in 2 mL of cold acetone and the mixture was centrifuged at 10,000 × g for 10 min. A 0.4 mL Titanium (II) chloride and 0.2 mL of 17 M ammonia solution were added to 1 mL of the supernatant. The precipitate was washed five times with acetone by resuspension and dissolved in 2 mL of 1 M Sulfuric acid. The absorbance of the resultant mixture was measured at 410 nm against a blank. The H_2O_2 concentration was calculated according to a standard curve and expressed as mM g^{-1} FW.

3.2.7 Statistical analysis

Statistical analysis was performed using Minitab version 21. The values were expressed as a means of three replicates with standard deviations (SD). All the data were subjected to one-way analysis of variance (ANOVA). Tukey's honestly significant difference post-test was used to separate the means at 5% significance level.

3.3 Results and discussion

3.3.1 Morpho-physiological parameters

The results indicated that the different levels of water stress conditions significantly ($p<0.01$) affected plant growth (Figure 3.1). The DR and FL plants exhibited a substantial decrease of 92% and 86% respectively in total plant fresh weight, compared to the RW plants (Figure 3.1). Similarly, a previous study revealed that severe water stress showed a significant ($p<0.01$) reduction in the total fresh and dry mass accumulation in *Satureja hortensis* L (Cimbru Savory Summer) (Baher et al., 2002); DHFL and RHDR exhibited a significant ($p<0.01$) reduction in fresh weight by 43% and 33%, respectively, compared to that of the RW plants.

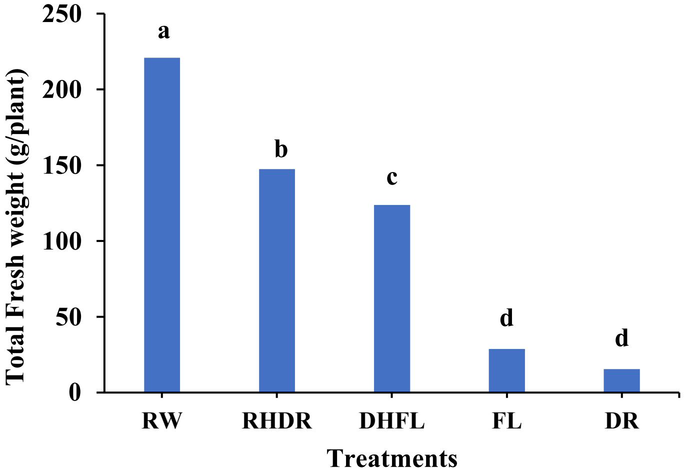

Figure 3.1. Total plant fresh weight as affected by varying watering regimes; Regular watering (RW), drought (DR), resumption of regular watering after drought (RHDR), flooding (FL), and resumption of regular watering after flooding (DHFL). Different alphabetical letters on the bars denote significant differences according to Tukey's honestly significant difference post-test analyses at $p \leq 0.05$.

Reactive oxygen species are elevated under situations of water stress, which leads to the disruption of the electron transport system in plants (Khorasani et al., 2023). This disruption results in oxidative activity occurring in both the chloroplasts and mitochondria, ultimately leading to a decrease in plant fresh weight (Khorasani et al., 2023). On the other hand, the lack of sufficient soil water leads to decreased nutrient uptake and distribution. As a result, the roots cannot absorb nutrients effectively and transport them to the shoot (Zarei et al., 2021).

The leaf RWC represents the equilibrium between the amount of water supplied to the leaf tissue and the rate at which transpiration loses water (Lugojan & Ciulca, 2011). In this study, the leaf RWC of the DR and FL plants significantly ($p<0.05$) reduced by 61% and 43%, respectively, compared to the RW plants (Figure 3.2).

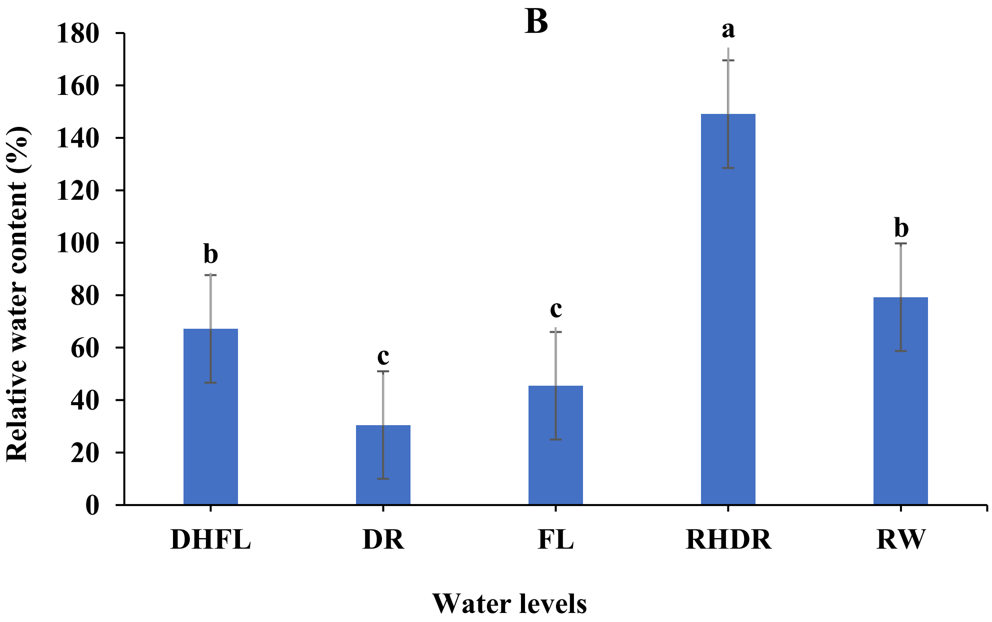

Figure 3.2. Relative water content (RWC) of the samples under various water stress conditions. The treatments include regular watering (RW), drought (DR), resumption of regular watering after drought (RHDR), flooding (FL), and resumption of regular watering after flooding (DHFL). Different alphabetical letters on the bars denote significant differences according to Tukey's honestly significant difference post-test analyses at a significant level of $p < 0.05$.

Moreover, the DHFL plants exhibited a non-significant ($p<0.05$) reduction of 61% in RWC compared to the RW plants while the RHDR plants exhibited a substantial increase of 88% compared to the RW plants (Figure 3.2). The application of water stress has a considerable impact on the physiology of plants and their water interactions (Alordzinu et al., 2021). The results of this experiment demonstrated that plants exhibited varying levels of leaf RWC in response to varied levels of water stress, indicating the impact of water stress on RWC. The outcome of this study validated the conclusions of Chen et al., (2015), Jaafari, (2000), Boutraa et al., (2010) who claimed that the leaf RWC declined as a result of increased water stress levels and enhanced resistance to water flow in stems and leaves (Alordzinu et al., 2021). A proficient xylem transport system that boosts plant capacity to absorb water could assist the plant in maintaining open stomata and high relative water content when soil water is reduced (Ezzine et al., 2023).

3.3.1.2 Electrolyte leakage

Electrolyte leakage is a characteristic feature of stress response in intact plant cells (Demidchik et al., 2014). This phenomenon is commonly employed to assess the damage caused by stress on plant tissues and as an indicator of a plant's ability to tolerate stress (Lee et al., 2010). In DR, FL and the DHFL plants, there were substantial reductions in cell electrolyte leakage compared to the RW plants (Figure 3.3). In contrast, a study conducted by Babaei et al., (2021) revealed that drought stress significantly ($p<0.05$) increases electrolyte leakage in plants. However, RHDR showed a significant ($p<0.05$) increase in electrolyte cell leakage compared to the RW plants (Figure 3.3).

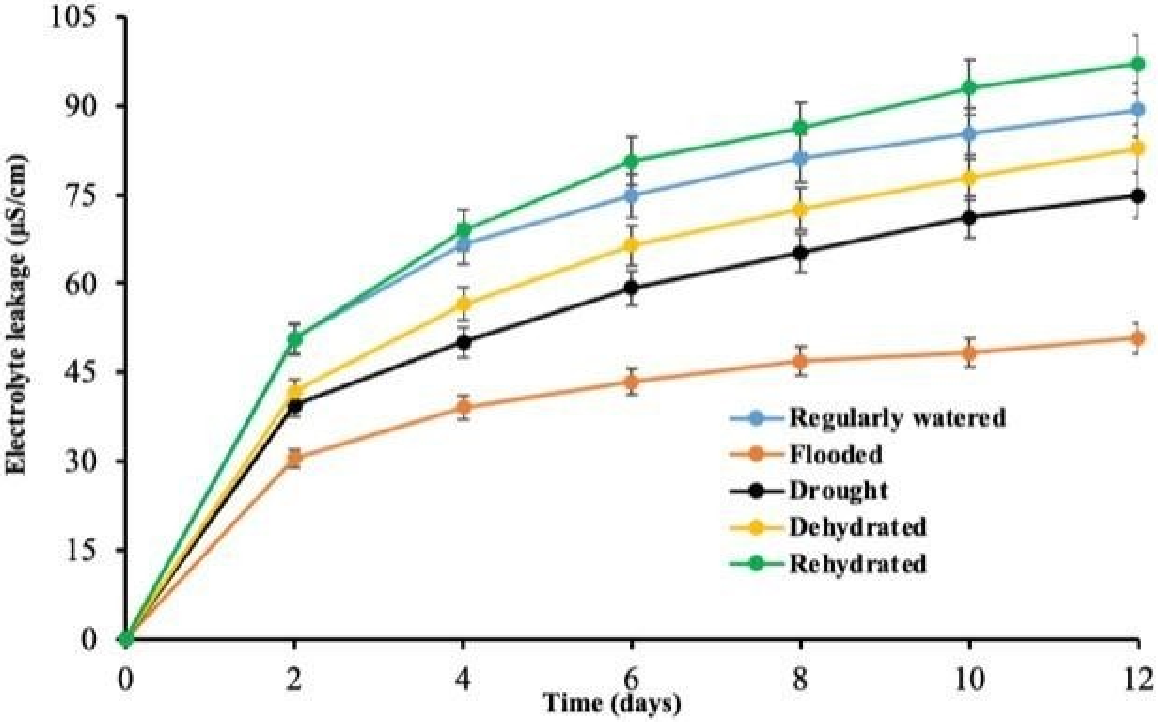

Figure 3.3. Electrolyte Leakage of the samples under various water stress conditions. The treatments include regular watering (RW), drought (DR), resumption of regular watering after drought (RHDR), flooding (FL), and resumption of regular watering after flooding (DHFL).

Generally, under extreme water stress conditions, plants typically experience cellular damage resulting in a high level of electrolyte leakage (Demidchik et al., 2014). Nevertheless, the decrease in electrolyte leakage during severe water stress conditions as observed in FL followed by DR may be attributed to the capacity of the plants to withstand stressful conditions. This finding is consistent with research conducted by Leopold et al. (1981). Furthermore, during periods of intense flooding, plant cells might have a dilution effect due to excessive water intake, which may lead to a decrease in the concentration of electrolytes. Furthermore, under drought conditions, plants primarily respond by closing their stomata, which might potentially restrict the exchange of gases and the absorption of ions (Pirasteh et al., 2016) leading to less concentration of electrolytes. This may be a contributing factor to the decline in electrolyte concentration in the FL and DR plants compared to their counterparts in the other treatments. Contrastingly, severe water stress led to a substantial increase in electrolyte leakage by 127% and 183% in Camarosa and Gaviota, respectively, compared to normal growth conditions (Zahedi et al., 2023).

3.3.1.3 Chlorophyll fluorescence indices and photosynthesis

Chlorophyll fluorescence indices are valuable parameters for investigating the impact of various environmental conditions on photosynthesis (Clijsters & Van Assche, 1985; Allakhverdiev & Murata, 2004). Leaf Fv/Fm is essential for assessing the condition and functionality of the internal chlorophyll system during periods of water-induced stress (Krause et al., 1991; Urban et al., 2017). Our findings indicate that Fv/Fm exhibited a modest decline of 7% and 10% under DR and FL conditions, respectively, as compared to RW plants.

Table 3.1. Chlorophyll fluorescence and photosynthesis response of Mexican mint under varying water stress conditions

Treatment	Fv/Fm	Fv/Fo	A (μmolm^{-2} s^{-1})	Ci (μmol mol $^{-1}$)	E (mol m^{-2}s-^{1})	gs (μmol mol $^{-1}$)	L*	a*	b*	c*	h*
RW	0.81^{ab}	4.48^{a}	0.48^{a}	341.80^{b}	0.22^{a}	0.01^{ab}	43.79^{ab}	10.89^{ab}	22.2^{a}	24.8^{a}	116.08^{a}
FL	0.73^{c}	2.68^{c}	0.30^{ab}	443.00^{b}	0.04^{c}	0.00^{b}	42.32^{b}	12.19^{a}	22.8^{a}	25.8^{a}	117.34^{a}
DHFL	0.82^{a}	4.76^{a}	0.46^{a}	357.81^{b}	0.28^{a}	0.01^{a}	42.51^{b}	12.45^{a}	23.9^{a}	26.9^{a}	117.48^{a}
DR	0.75^{bc}	3.10^{bc}	0.16^{b}	1075.0^{a}	0.06^{b}	0.00^{b}	50.84^{a}	9.82^{b}	27.8^{a}	29.5^{a}	109.93^{b}
RHDR	0.79^{ab}	4.12^{ab}	0.40^{ab}	380.40^{b}	0.23^{a}	0.01^{ab}	42.32^{b}	10.76^{ab}	24.7^{a}	27.1^{a}	114.57^{ab}
p-value	0.00	0.00	0.03	0.00	0.12	0.01	0.01	0.01	0.06	0.11	0.01

Fv/Fm, maximal photochemical efficiency; Fv/Fo, potential photosynthetic capacity; A (net photosynthetic rate), Ci (sub-stomatal CO_2 concentration), E (transpiration rate), gs (stomatal conductance), L* (lightness), a* and b* (chromaticity coordinates: -a*: green, +b*: yellow), c* (chroma), h* (hue angle) of Mexican mint affected by flooding (FL), drought (DR), dehydration after flooding (DHFL) and rehydration after drought (RHDR). Different alphabetical letters denote significant differences according to Tukey's honestly significant difference post-test analyses at a significant level of $p \leq 0.05$.

This suggests that drought stress may cause damage to the photosystem II (PSII) and limit the primary reaction of photosynthesis (Lichtenthaler et al., 1988). Concurrently, the decrease in Fm under stress conditions may be influenced by a decline in the functioning of the water-splitting enzyme complex that may be accompanied by a simultaneous cyclic electron transfer inside or surrounding PSII (Porcar et al., 2014). Consequently, the Fv/Fm ratio will diminish in response to drought-induced stress. The results of the present study were in line with a prior investigation, which showed that drought stress impeded the photochemical activity of PSII and reduced leaf Fv/Fm (Meng et al., 2016). A decrease in Fv/Fm signifies the inhibition of photosynthesis in response to water stress (Lichtenthaler et al., 1988; Zhuang et al., 2020). The photosynthetic electron transport chain is primarily influenced by the Fv/Fo component, which is the most sensitive part of the process (Mohammed et al., 2003; Kumar et al., 2020). The present study found that Fv/Fo was considerably decreased in DR, FL, and RHDR plants compared to RW (Table 3.2). However, the DHFL plants showed a non-significant ($p>0.05$) rise of 6% in Fv/Fo compared to the RW plants (Table 3.2).

Photosynthesis is the mechanism that provides the foundation for plant growth (Osakabe et al., 2014b) while stomatal conductance (Gs) represents the extent to which stomata are open. Stomata serve as openings in leaves that regulate the exchange of gases, hence controlling the processes of photosynthesis and transpiration in vegetation. Stomatal conductance was reduced significantly ($p<0.05$) with DR and FL by 74% and 100% respectively, compared to the RW plants. Stress reversal treatments, DHFL and RHDR, also showed a slightly significant ($p<0.05$) reduction in stomatal conductance compared to the RW plants. The transpiration rate reduced significantly ($p<0.05$) in the DR, FL, and DHFL plants whereas RHDR plants showed a moderate increase in the transpiration rate compared to the RW plants (Table 3.2). Interestingly, the intercellular carbon dioxide concentration was significantly ($p<0.05$) increased by all the treatments compared to RW (Table 3.2). It is well established that water stress in plants is characterized by stomatal closure and restricted gas exchange (Moradi et al., 2014a). Thus, plants often constrict their stomata in response to water stress to minimize water loss *via* transpiration. This might have restricted the intake of atmospheric CO_2, possibly causing a buildup of sub-stomatal CO_2 concentration (Ci).

Water stress can impede gas exchange and hence, impact the photosynthetic ability of plants (Subrahmanyam et al., 2006). The present study demonstrated that water stress substantially decreased net photosynthesis rate (A), transpiration €, and stomatal conductance (gs), which agrees with the findings of Ma et al. (2015) and Liu et al. (2016). Other studies demonstrated that water stress induces either stomatal restriction, non-stomatal restriction, or a combination of both during the process of photosynthesis (Izanloo et al., 2008; Carmo et al., 2010). The primary limiting factor for photosynthesis in this study was stomatal conductance, as it dropped significantly ($p<0.05$) under conditions of water stress (Table 3.2). Stomatal closure has been regarded as an initial plant response to water stress, aimed at regulating water loss (Harb et al., 2010; Yan et al., 2016). Therefore, plants usually exhibit stomatal closure and reduced photosynthesis as reactions to water stress (Bota et al., 2004).

3.3.2 Biochemical parameters under various stress conditions

3.3.2.1 Chlorophyll a and b, and carotenoids

Mexican mint plants exposed to prolonged periods of drought showed a non-significant ($p<0.05$) decrease in leaf chlorophyll content by 5%, compared to the RW plants (Table 3.2). At the same time, both prolonged periods of FL and DR showed a significant ($p<0.05$) reduction in chlorophyll b content by 12% and 25% respectively, compared to the RW plants (Table 3.2). In a similar study by Enneb et al. (2020), they reported a notable decrease in chlorophylls a and b contents in three assertions of *Vicia faba* L. under water stress conditions. This previous study reveals that drought stress leads to a reduction in total chlorophyll, which can lead to a diminished ability to capture light (Mafakheri et al., 2010).

Table 3.2. Biochemical responses of Mexican mint under varying water stress conditions

Treatment	**Chla** (μg g^{-1} FW)	**Chlb** (μg g^{-1} FW)	**Car** (μg g^{-1} FW)	**Sugar** (μg/g FW)	**ROS** (mM g^{-1} FW)	**MDA** (nmol g^{-1} FW)	**DPPH** (%)	**Total Flavonoid** (μg quecertin g^{-1} FW)	**Total Phenolics** (mg GAE/g FW)
RW	128.91^{d}	85.52^{c}	28.94^{d}	19.59^{ab}	157.30^{ab}	1.04^{c}	6.80^{b}	245.50^{c}	215.3^{bc}
FL	159.04^{c}	75.24^{cd}	52.49^{b}	25.39^{ab}	216.05^{a}	2.03^{a}	17.47^{a}	800.20^{a}	725.38^{a}
DHFL	249.53^{a}	147.87^{a}	111.45^{a}	42.92^{a}	144.50^{ab}	1.53^{b}	4.49^{c}	265.80^{c}	185.10^{c}
DR	121.26^{d}	63.61d	40.32^{c}	31.32^{ab}	232.20a	0.99^{c}	8.36^{b}	386.00^{b}	319.30^{b}
RHDR	190.39^{b}	125.82^{b}	112.26^{a}	11.52^{b}	110.50^{b}	0.69^{d}	7.56^{b}	198.50^{c}	304.09^{b}
p-value	0.00	0.00	0.00	0.02	0.01	0.00	0.00	0.00	0.00

Chla (chlorophyll a), Chlb (chlorophyll b), Car (carotenoid), Sugar (soluble sugar determination), Total flavonoid, Total phenolics, DPPH (ROS Scavenging effect), MDA (malondialdehyde concentration), ROS (reactive oxygen species determination) of Mexican mint affected by flooding (FL), drought (DR), dehydration after flooding (DHFL) and rehydration after drought (RHDR). Different alphabetical letters denote significant differences according to Tukey's honestly significant difference post-test analyses at a significant level of $p < 0.05$.

Additionally, water stress slows down the rate of carbon fixation by reducing the amount of CO_2 influx into leaves, which directly affects overall plant metabolism (Farooq et al., 2009; Universiti Malaysia Terengganu et al., 2021). Thus, a reduction in Ribulose bisphosphate carboxylase oxygenase (Rubisco) activity, photosynthetic pigments, and photosynthetic electron transport components may cause a drop in molecular oxygen that may lead to the accumulation of reactive oxygen species (ROS) that can cause oxidative stress and harm the photosynthetic apparatus (Reddy et al., 2004b; Basu et al., 2016; Universiti Malaysia Terengganu et al., 2021)). RHDR and DHFL showed significant ($p<0.05$) increases in chlorophylls a and b compared to RW plants (Table 3.2).

Both DR and FL plants showed a significant ($p<0.05$) increase in carotenoid by 39% and 81% respectively, compared to RW plants (Table 3.2). The above findings were also in agreement with a study conducted by Mohammadkhani & Heidari, (2007) where carotenoid contents were increased in drought-stressed *Origanum vulgare* L. In RHDR plants, the carotenoid content significantly ($p<0.05$) increased by 287% compared to that of the RW plants. Similarly, a study conducted by Deng et al. (2003) revealed a notable increment in the carotenoid content in *Boea hygrometrica* following a sudden drop in the initial hours of desiccation and subsequently,returned to its original level upon rehydration. On the contrary, the pigment-protein complexes inthe leaves of *Chirita heterotrichia* were destroyed after being dehydrated for 8 days and did not recover after being rehydrated (Deng et al., 2003b). The results of the present study indicated thatDHFL plants showed a massive increase of 285% in carotenoid content compared to RW plants (Table 3.2). Plants usually intensify their defense by maintaining information from previous stress events (Jacques et al., 2021), this stress memory could be one of the possible reasons for the abilityof RHDR and DHFL plants to withstand future stress was enhanced compared to other treatments. Carotenoids are believed to have a protective function against photoinhibition. Xanthophyll is a vital component of carotenoids and plays a significant role in safeguarding photosynthesis. Therefore, carotenoids are essential for enhancing the ability of plants to tolerate water stress (Deng et al., 2003a). It is reasonable to postulate that Mexican mint may have acquired an adaptive strategy to preserve membrane integrity and antioxidant defense by elevating the carotenoid content during periods of water stress.

3.3.3.2 Phenols and flavonoids

Total phenolics was significantly ($p<0.05$) affected by differences in watering regime. There was a tremendous increase in the FL plants phenolics compared to that of the RW plants. The DR plants also showed a significant ($p<0.05$) increase in total phenolics by 43% compared to the RW plants (Table 3.2). The RHDR plants showed a non-significant ($p<0.05$) increase in the total phenolic concentrations compared to the RW plants (Table 3.2). Interestingly, DHFL plants showed a significant ($p<0.05$) decrease in phenolics compared to that of the RW plants.

Flavonoids, which possess a significant antioxidant capacity, have gained interest due to their favorable impact on human health and their ability to safeguard plants against oxidative damage caused by water stress (Qiu et al., 2013). In this study, the production of total flavonoids was increased significantly ($p<0.05$) by 225% in FL plants compared to the RW plants. Also, DR plants showed an increase in total flavonoids by 57% compared to that of RW plants. Interestingly the stress reversals i.e., DHFL and RHDR showed a non-significant ($p<0.05$) alteration in the total flavonoid content (Table 3.2). The concentration of total flavonoids in DHFL plants exhibited a marginal increase of 8% when compared to RW plants. However, in contrast, plants treated with RHDR had a reduced flavonoid concentration by 19% when compared to the plants treated with RW.

Under conditions of water stress, polyphenols tend to accumulate in response to an increase in ROS (Sarker et al., 2018b). Polyphenols can chelate transition metal ions and may inhibit lipid peroxidation by capturing the lipid alkoxyl radical (Sarker et al., 2018b). Moreover, peroxidase oxidizes flavonoids, which then participate in H2O2-scavenging (Sarker et al., 2018b). Similarly, Khalil et al. (2018) conducted a study that demonstrated that the concentration of flavonoids was enhanced during drought circumstances. A study conducted by de Lima et al., (2023) in *Croton floribundus* and *Croton urucurana* revealed that the antioxidant activity was higher in reaerated plants after continuous flooding. It is crucial to understand that the enhancement of secondary metabolite production is not consistent across all plant tissues and organs. However, it may alter various parts or stages of development within the same plant species. For instance, in a study conducted by Pirbalouti et al., (2017) the amount of phenolic compounds was increased in the shoots of two types of basil (*Ocimum basilicum* L.) plants when they received less water (30%

FC) compared to when they were irrigated normally. In contrast, an investigation conducted by Caser et al., (2019a) demonstrated that leaves of *Salvia dolomitica* Codd. when exposed to intense water scarcity, showed a notable reduction in the overall concentrations of phenols and flavonoids.

3.3.3.3 Reactive oxygen species

Water stress triggers the buildup of reactive oxygen species (ROS) (Per et al., 2017; Tang et al., 2017). High amounts of reactive oxygen species (ROS) are extremely reactive and toxic, causing oxidative stress and molecular and cellular damage (Atkinson et al., 2012). Additionally, they have the potential to induce cellular apoptosis (Lehmann et al., 2009). In the present study, there was a significant increase of 155% in ROS concentration in FL plants compared to RW plants. In DR plants, there was a significant ($p<0.05$) accumulation of ROS by 22% compared to RW plants. RHDR showed a slightest increase of 11% in ROS compared to RW plants (Table 3.2). In contrast, the DHFL plants showed a significant ($p<0.05$) decrease in ROS compared to the RW plants. Chloroplast is the primary producer of ROS in green plant parts. This occurs when the photosynthetic electron transport system becomes too active, resulting in an overflow of reduced power. This excess reducing power is responsible for the conversion of oxygen into various ROS (Foyer et al., 1994; Foyeret al., 2003). While ROS is formed under normal plant growth conditions, it is significantly amplified under stress conditions (Dat et al., 2000) as observed in the present study. Additionally, mitochondrial electron transport can generate ROS, such as superoxide and H_2O_2, especially during periods of water stress (Elstner et al., 1994). The increase in ROS concentration under different water stress conditions was also consistent with various studies conducted. For instance, a study conducted by Radwan et al., (2017) revealed thatwhen *Thymus vulgaris* L. plants when exposed to 30-40% of water holding capacity, there was a significant ($p<0.05$) increase in the concentration of ROS compared to those plants that received 70-80% of water.

Malondialdehyde (MDA) is a well-known biochemical marker that indicates an increase in the activity of ROS and oxidative stress in plant tissues during unfavourable conditions (Farag, 2019). MDA is regarded as the ultimate outcome of lipid peroxidation and a key signal of oxidative damage that may occur in cellular membranes under water stress conditions (Farag, 2019). In the present study, MDA concentration was significantly ($p<0.05$) high i.e., 94% more in FL plants and

47% in DHFL compared to RW plants. In contrast, RHDR plants had significantly ($p<0.05$) reduced concentrations of MDA compared to those of RW plants. In a similar, Khaleghi et al., (2019) reported comparable findings of reduced levels of MDA with rehydration of drought-stressed Osage orange (*Maclura pomifera)* plants. Unexpectedly, the DR plants showed a significantly ($p<0.05$) the least concentrations of MDA of 5% compared to the RW plants although several studies under similar conditions have reported otherwise (Sarker & Oba, 2018a; Khaleghi et al., 2019; Farag et al., 2019). These findings might demonstrate that Mexican mint has a distinctive adaptive strategy, as well as a strong antioxidant defense system and effective cellular repair.

3.3.3.4 Total soluble sugar

The cellular buildup of osmolytes such as sugars in response to water stress is frequently linked to mechanisms for tolerating water stress (Pirzad et al., 2011). Total soluble sugar (TSS) contents in DR and FL were non-significantly ($p<0.05$) increased by 60% and 30%, respectively, compared to the RW plants (Table 3.2), which is similar to previous observations that reported a substantial rise in TSS concentrations in plants under severe water stress Farag et al., (2019). The DHFL plants exhibited a significant ($p<0.05$) increase of about 119% in TSS concentration compared to the RW plants (Table 3.2). The regulation of TSS in plants experiencing water stress is influenced by various mechanisms that impact the production and transport of soluble sugars inside the leaves (Arabzadeh, 2012). Furthermore, soluble sugars have a crucial function in regulating osmotic adjustment in plants (Zhou et al., 2009). For instance, the RHDR plants exhibited a notable reduction in TSS of about 41% compared to the RW plants. In contrast, a study conducted on orchids (*Dendrobium moniliforme*) revealed that TSS increased in rehydrated plants (Wu et al., 2016). In general, it is widely acknowledged that plants respond to water stress by accumulating organic molecules such as sugars, which are known as osmolytes or compatible solutes (Oraki & Aghaalikhana, 2012; Ibrahim, 2014), which are crucial for plants' adaptation to water stress (Farag, 2019). In addition, it can safeguard crucial enzymes and various components of plant cells from harmful oxidation by scavenging reactive oxygen species (ROS)(Farooq et al., 2012).

3.4 Conclusion

The occurrence of global climate change and extreme weather patterns has resulted in water stress emerging as a significant constraint to the production of agriculture. The current study provides evidence that water stress has a detrimental effect on plant growth, physiological processes, and chemical composition. The timing, duration, and amount of growing medium water level determine the severity of water stress that influences a plant's growth and development performance. The study also suggests that water stress severely affects the photosynthetic machinery of Mexican mint which may lead to reduced plant growth and development. The results demonstrated a swift recovery process upon rehydration of drought-stricken plants or dehydration of waterlogged plants, returning them to their typical growth circumstances. To deepen our understanding of these plant adaptations and clarify the precise metabolic responses that enable water stress tolerance, we suggest conducting additional experiments that encompass a broader spectrum of water stress levels. This will facilitate a more extensive investigation of plant response and provide more profound insights into the underlying plant physiologic and metabolic alterations associated with water stress tolerance.

CHAPTER 4: HOW CENTRAL CARBON METABOLITES OF MEXICAN MINT (*PLECTRANTHUS AMBOINICUS*) PLANTS ARE IMPACTED UNDER DIFFERENT WATERING REGIMES

A version of this has been published in the **Metabolites**. The citation is:

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Abstract

Plants are sessile, and their ability to reprogram their metabolism to adapt to fluctuations in soil water level is crucial but not clearly understood. A study was performed to determine alterations in intermediate metabolites involved in central carbon metabolism (CCM) following exposure of Mexican mint to varying watering regimes. The water treatments were regular watering (RW), drought (DR), flooding (FL), and resumption of regular watering after flooding (DHFL) or after drought (RH). Leaf cluster formation and leaf greening were swift following the resumption of regular watering. A total of 68 key metabolites from the CCM routes were found to be significantly ($p < 0.01$) impacted by water stress. Calvin cycle metabolites in FL plants, glycolytic metabolites in DR plants, total tricarboxylic acid (TCA) cycle metabolites in DR and DHFL plants, and nucleotide biosynthetic molecules in FL and RH plants were significantly ($p < 0.05$) increased. Pentose phosphate pathway (PPP) metabolites were equally high in all the plants except DR plants. Total Calvin cycle metabolites had a significantly ($p < 0.001$) strong positive association with TCA cycle ($r = 0.81$) and PPP ($r = 0.75$) metabolites. Total PPP metabolites had a moderately positive association with total TCA cycle metabolites ($r = 0.68$; $p < 0.01$) and a negative correlation with total glycolytic metabolites ($r = -0.70$; $p < 0.005$). In conclusion, the metabolic alterations of Mexican mint plants under different watering regimes were revealed. Future studies will use transcriptomic and proteomic approaches to identify genes and proteins that regulate the CCM route.

4.1 Introduction

A universal shift in weather patterns due to sustained global climate change has resulted in fluctuations in edaphic stress conditions, particularly drought, flooding, soil salinity, and soil impedance (Vaccaro et al., 2009). Plants are subject to such harsh and variable edaphic stress conditions because they are sessile. Typically, plant response to the onset of edaphic stress includes inhibition of plant root hydraulic conductance, reductions in stomatal conductance and photosynthesis, reprogramming of affected metabolic pathways, and ultimately, cessation of plant growth and cell death (Reddy et al., 2004a; Moradi et al., 2014a; Klassen et al., 2017; R. Guo et al., 2018). The initial biochemical response to soil water stress is the production of free radicals and reactive oxygen species such as hydrogen peroxide and superoxide, which adversely affect plant morpho-physiological functions and interrupt metabolic processes to the detriment of the plant (Cramer et al., 2011). According to Stoychev et al., (2013), such stress conditions lead to alteration in energy metabolism for survival and adaption.

The Calvin cycle occurs in the stroma of the chloroplast after photolysis (light reaction) and so, it is referred to as the dark reaction phase of photosynthesis. From the light reaction, energy in the form of ATP and NADPH are generated and later used to produce glucose and downstream carbohydrate molecules in the Calvin cycle Sharma et al., (2020). The carbohydrates from the Calvin cycle enter the central carbon metabolism (CCM) route via the glycolytic pathway (Fernie et al., 2004; Timm et al., 2021). The CCM is a complex series of enzymatic steps for the conversion of sugars into metabolic precursors in cells (Cramer et al., 2011). The CCM route, which consists of the glycolytic pathways, the pentose phosphate pathway (PPP) for sugar interconversion, and the tricarboxylic acid (TCA) cycle for final complete oxidation (Cramer et al., 2011; Fuchs & Berg, 2014) provides 12 metabolites that are the basic carbon precursors of all biosynthetic pathways (Ataman et al.,, 2017). PPP is divided into an oxidative phase that produces ribose-5-phosphate for the synthesis of nucleic acids and a non-oxidative phase that produces NADPH (Ge et al., 2020). The latter is an important reducing power for the synthesis of fatty acids, nucleotides, and non-essential amino acids for numerous metabolic pathways. So far, the impact of drought, flooding, or water stress recovery on CCM is understudied but there are few reports on the individual pathways.

Cellular respiratory metabolism in plants via the TCA cycle occurs in the matrix of the mitochondrion, and it is essential for energy supply to different organelles for the maintenance of various physiological functions. A study conducted by Moradi et al., (2014) revealed that drought disrupted the thyme (*Thymus* sp.) TCA cycle and also, reduced the synthesis of amino acids. In contrast, drought-induced metabolic reprogramming in Arabidopsis increased TCA cycle intermediate metabolites (Pires et al., 2016b; Fàbregas et al., 2018). Kumar et al., (2021) examined inbred maize (*Zea mays*) plants under water stress conditions and revealed that TCA cycle metabolites like succinate, α-ketoglutarate, and fumarate were reduced in drought-tolerant varieties while citrate and isocitrate were increased in drought-sensitive varieties. However, none of the studies reported on flooding or drought-flooding cycles. The ability of a plant to tolerate water stress is dependent on cellular concentrations of metabolic solutes like proline, betaine, fructose, and sucrose (Wang et al., 2012). Research conducted using drought-tolerant wheat (*Triticum aestivum*) varieties showed that under severe water stress conditions, pyruvic acid, phenylpyruvate, fructose-6-phosphate, glucose, sucrose, and fructose were remarkably increased via the glycolytic pathway (Guo et al., 2018; Kumar et al., 2021a) suggesting an increase in sugar synthesis under water stress conditions. Nucleotide metabolism is the most critical cellular component for plant growth that affect many metabolic processes (Witte et al., 2020). Purine and pyrimidine nucleotides are the building blocks for nucleic acid biosynthesis, which provide the required energy for the biosynthesis of carbohydrates, proteins, lipids, and secondary metabolites known to be central to all cellular metabolisms (Zrenner et al., 2006).

Water is crucial for plant growth and development and globally, increasing numbers of farm operations are impacted by drought or flooding conditions because of changes in precipitation patterns caused by global climate change and competition with rising global population and manufacturing industries' requirements for water (Ashrafi et al., 2018a). Therefore, a fundamental understanding of plant response to fluctuations in water stress will be highly valuable for the development of stress-tolerant crops and the management of crop water requirements. Mexican mint is a herbaceous perennial plant that belongs to the Lamiaceae family with a diverse array of ethnobotanical characteristics, culinary properties, and aroma-medicinal compounds (Lukhoba et al., 2006; Moradi et al., 2014a). Like most other plants, these properties can be affected by drought or flooding to a varying degree (Moradi et al., 2014a). Although Mexican mint can survive drought conditions to some extent, its growth and chemical composition may be remarkably altered in prolonged stress conditions.

Mexican mint is physiologically a Crassulacean acid metabolism (CAM) plant that fixes CO_2 into C4 acids at night (Paul, 2012). Because CAM plants absorb CO_2 at night, their stomata are closed much of the day to conserve water and can tolerate limited drought conditions. However, the biochemical and physiological mechanisms underpinning the Mexican mint plant's response to drought or flooding are not reported. Based on current knowledge, it was hypothesized that exposure of Mexican mint plants to prolonged drought or flooding will cause extensive disruption to CCM, but the plant will recover as soon as regular watering resumes. Therefore, the present study determined variations in metabolites of the different CCM routes following prolonged exposure of Mexican mint plants to drought or flooding and reversals to regular watering. This knowledge may be extended to other plants under the current dispensation of climate change and global warming.

4.2 Materials and methods

4.2.1. Location

This research was conducted in the Plant Physiology and Stress Laboratory of the Department of Plant, Food, and Environmental Sciences, Faculty of Agriculture, Dalhousie University, and targeted metabolite quantitation was performed at the University of Victoria Genome BC (UVic GBC)—Proteomics Centre of The Metabolomics Innovation Centre, Canada, between December 2021 and July 2022.

4.2.2. Preparation and rooting of cuttings

Cuttings of soft tissue branches from the youngest second and third nodes on the main stem of healthy and well-watered Mexican mint mother plants were collected from the PFES greenhouse plant stock. Each cutting was trimmed to 5 cm in height with four pairs of corresponding leaves. Mexican mint is an easy rooting plant, so rooting hormone was not used. Four nodes on the stem part of the cuttings were embedded in moist perlite (Perlite Canada Inc., Montreal, QC, Canada) contained in a plastic flat tray of dimensions 50 cm length × 28 cm width × 6.5 cm depth. The planted trays were covered with a dome-shaped transparent plastic cover to maintain ≥95% relative humidity environment to induce rooting. The trays were placed on a planting shelf with 24 h fluorescence light at 22 °C. The cuttings were watered every other day, but the leaves were finely sprayed with water twice a day with no addition of fertilizer. The rooted cuttings were ready for transplanting after three weeks.

4.2.3. Planting of rooted cuttings and growing condition

Uniformly rooted cuttings were transplanted into 15 cm diameter plastic pots with saucers underneath, and each pot contained 200 g of Promix-BX mixed with 150 g of vermicast. The Promix-BX potting medium (Premier Horticulture Inc., Quakertown, PA, USA) contained 75–85% sphagnum peat moss, horticultural grade perlite, vermiculite, and dolomitic and calcitic limestone, and the vermicast was produced by Red Wiggler (*Eisenia fetida*) worms purchased from Growing Green Earthworm Castings (Lower Wedgeport, NS, Canada). The potted plants were arranged in a completely randomized design with three replications in a Biotronette Mark II Environmental Chamber (Lab-Line Instruments Inc., Melrose Park, IL, USA). The growth chamber was set at a 24°/20 °C day/night temperature cycle and a 12/12 h day/night light cycle. The plants were grown for 120 days, during which they were regularly watered to field capacity every three days prior to the imposition of water stress treatment. Pots were rearranged weekly to offset unpredictable occurrences due to variations in the environment.

4.2.4. Water stress treatment

To understand how the central carbon metabolism of plants recovering from water stress after reversal of drought (i.e., ≤10% field capacity) or flooding (i.e., oversaturation) is altered, the treatments used were regular watering (RW) at field capacity; continuous drought (DR); continuous flooding (FL); rehydration and resumption of regular watering after continuous drought for 8 weeks (RHDR); and dehydration and resumption of regular watering after continuous flooding for 8 weeks (DHFL). Regular watering was done every other day to maintain the field capacity of the growing medium. Flooding was simulated by complete submergence of the 15 cm diameter pot with the Mexican mint plant in water contained in a 20 cm diameter plastic pot. The experiment was arranged in a completely randomized design with three replications in the growth chamber.

4.2.5. Central carbon metabolites

4.2.5.1. Sample preparation

The green leaves of the potted Mexican mint plants were harvested after 8 weeks of water stress treatment and immediately dipped in liquid nitrogen (N), ground into a fine powder, and stored at −80 °C until analysis. The ground leaf tissue samples on ice were shipped by overnight courier to UVic GBC for targeted metabolite quantitation. Triplicate samples (50 mg) of ground leaf tissue per treatment were separately added to 500 L of 80% methanol and homogenized using a MM400 mill mixer (Retsch, Haan, Germany). The mixture was then sonicated for 5 min in an ice-water bath, followed by centrifugation at 21,000× g for 20 min at 5 °C. A quantity of 250 µL of the supernatants was removed and combined with 150 µL of dichloromethane and 150 µL of water. After centrifugal clarification of the mixture and vortex mixing for 30 s, triplicate samples of 80 µL aliquots of the supernatant were dried in a N gas flow. There were three biological replicates and two technical replicates. The obtained residues were used for the assays described below.

4.2.5.2. Tricarboxylic acid cycle assay

Standard stock solutions were prepared based on the method description provided by Han et al. (2013). The standard stock solutions of all targeted carboxylic acids were prepared in 80% methanol at concentrations of 200–1000 µM. A total of 50 µL of each standard solution or the supernatant of each sample was mixed and reacted with 50 µL of a 200 mM 3-nitrophenylhydrazines (NPH) solution at 30 °C. Following the reaction, 450 µL of water was added to each solution, and 10 µL of the resulting solution was injected into a C18 liquid chromatography (LC) column (2.1 × 100 mm, 1.8 µm) for quantification of the carboxylic acid by ultrahigh LC-multiple reaction monitoring/mass spectroscopy (UPLC-MRM/MS), and ion detection was performed on an Agilent 1290 UHPLC coupled to a Sciex 4000 QTRAP MS instrument (AB Sciex, Concord, ON, Canada).

4.2.5.3. Glucose and selected sugar phosphates

An 80 L aliquot of the dried residue of each sample was mixed with 50 L of 50% methanol. After that, 50 mL of standard solutions of glucose, ribose, ribose-5-phosphate, glucose-6-phosphate, and mannose-6-phosphate were serially diluted before being combined and reacted with 100 mL of 25 mM 3-amino-9-ethylcarbazole (AEC) solution, 50 mL of 50 mM sodium cyanoborohydride ($NaCBH_3$) solution, and 20 mL of LC/MS grade acetic acid for 70 min at 60 °C. A quantity of 300 µL of chloroform and 200 µL of water were added during the reaction period, after which each supernatant was centrifuged at 12,500× g for 5 min and vortexed for 15 s before adding 50 mL of water. Finally, 10 µL of the mixture was injected into a pentafluorophenylpropyl (PFP) LC column (2.1 × 150 mm, 1.7 µm) to run UPLC-MRM/MS on an Agilent 1290 UHPLC coupled to an Agilent 6495B QQQ instrument (Conquer Scientific, Poway, CA, USA) with positive-ion detection as previously described by (Han et al., 2013).

4.2.5.4. Other metabolites

In 50% methanol, a solution known as an internal standard (IS) was created that contained 25 isotope-labelled metabolites including adenosine monophosphate (AMP), adenosine-5-triphosphate (ATP), uridine monophosphate (UMP), uridine triphosphate (UTP), uridine diphosphate (UDP)-glucose, fructose-6-phosphate (fructose-6P), fructose-bisphosphate, glycerol-3-phosphate, nicotinamide adenine dinucleotide (NAD), NADH, glucose-1-phosphate, ribose-5-phosphate, and others. Moreover, all the targeted metabolites were produced as serially diluted standard solutions in the IS solution at concentrations ranging from 0.00002 to 10 M. Eighty-liter aliquots of dried residue were dissolved in 100 L of the IS solution. Ten microliters of each sample solution or each standard solution was injected onto a C18 LC column (2.1 × 100 mm, 1.9 µm) to run UPLC-MRM/MS and negative ion detection on a Waters Acquity UPLC system coupled to a Sciex QTRAP 6500 Plus MS instrument using tributylamine acetate buffer-acetonitrile/methanol (1:1, v/v) as the mobile phase with a gradient of 0.25 mL/min (from 10% to 50% B over 25 min) and 60 °C.

4.2.6. Calculation and statistical analysis

Concentrations of the detected analytes in the above assays were calculated with IS calibration by interpolating the constructed linear regression curves of individual compounds using the analyte-to-internal standard peak area ratios measured from injections of the sample solution. All data obtained were subjected to one-way ANOVA using Minitab version 21 (Minitab, Inc., State College, PA, USA). Tukey’s honestly significant difference post-test was used to separate treatment means at $p \leq 0.05$. A multivariate statistical analysis of grouped compounds, two-dimensional principal component analysis (PCA), and hierarchical clustering for differential metabolism per group were constructed with Euclidean distance using XLSTAT version 2022.3 (Addinsoft, New York, NY, USA).

4.3 Results and discussion

4.3.1 Plant growth

The severity of prolonged drought and flooding on plant growth and productivity are well documented (Xiong et al., 2018; B. Yadav et al., 2021), but not much has been reported on stress reversal. The results of the present study revealed that although Mexican mint morphological recuperation through RHDR and DHFL plants can be fast and occured within 4 hrs, the overall recovery of total plant size and leaf greening similar to regularly watered (RW) plants can delay (Fig. 1A). This was proven by the resumption of leaf cluster formation on RH and DHFL plants compared to the stressed i.e., drought (DR) and flooded (FL) plants, respectively (Figure 4.1).

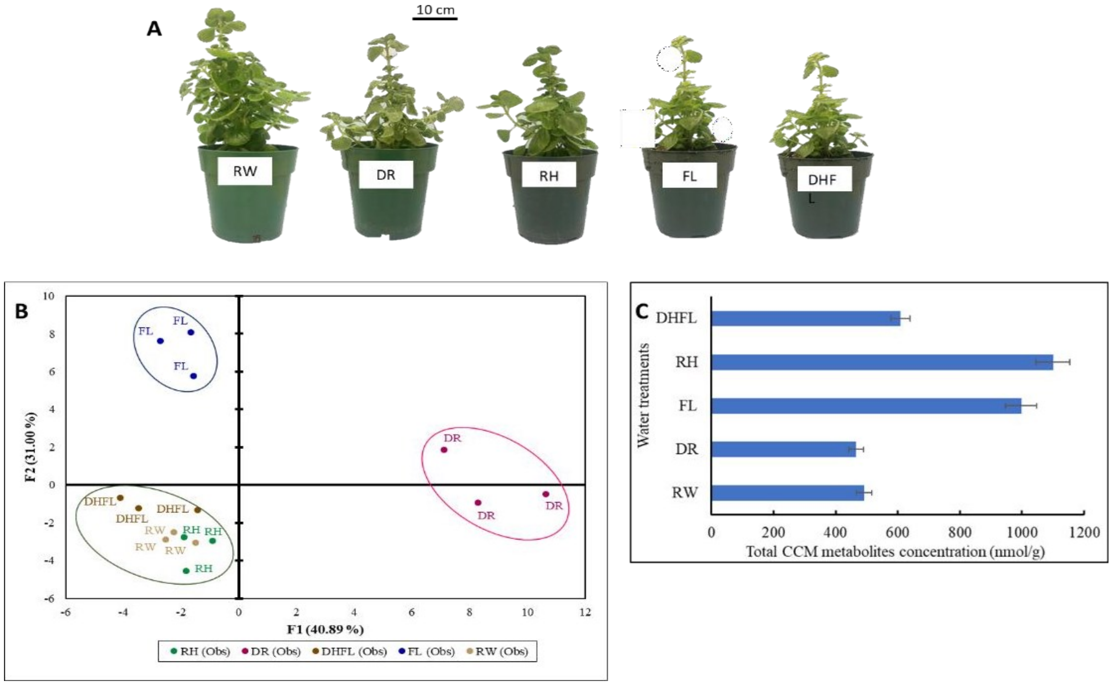

Figure 4.1. Response of Mexican mint plants to varying watering regimes. (A) Plant growth, (B) 2-dimension principal component analysis of metabolic profiles, and (C) bar chart of total concentrations of intermediate metabolites of the central carbon metabolic (CCM) routes with error bars. Regular watering (RW), drought (DR), flooding (FL), resumption of regular watering after flooding (DHFL) or after drought (RHDR).

4.3.2. Total CCM metabolites

Plants produce large quantities of specialized metabolites, which are end-products of cellular regulatory activities (S. Wang et al., 2019), but the compositions of these metabolites can be highly modulated by stress conditions (Moradi et al., 2014a; Klassen et al., 2017; M. Kumar et al., 2021b) as demonstrated in the present study (Figure 4.1B-C). Anecdotally, Mexican mint as a CAM plant is known to tolerate some extent of water deficit stress (Paul, 2012), but its tolerance level and the mechanistic response to prolong and continuous water stress conditions are unknown up to now. To understand this mechanism, changes in metabolites involved in CCM under different watering regimes were assessed. A 2-dimensional PCA biplot presented in Figure 4.1B explained *ca*. 72% of the overall variations in total metabolites. The first (F1) and second (F2) factors represent *ca*. 41% and 31%, respectively. The PCA revealed three distinct clusters that discriminated between the different water treatments concerning the total metabolites of the CCM routes. Based on the cluster formation, it seemed the metabolism of RH and DHFL plants were adjusted after 8 weeks of stress recovery and resumed normal growth and metabolism that was similar to the RW plants. These results suggested that irrespective of the prolonged drought and flooding, the Mexican mint plants underwent metabolic reprogramming when regular watering was resumed as previously reported for intermediate metabolites in flood-stressed clover (*Trifolium* spp.)(Stoychev et al., 2013), and drought-stressed Arabidopsis (Pires et al., 2016b) and corn (Kumar et al., 2021b). Drought or flooding causes remarkable degradation of chlorophyll and impairment of photosynthesis in addition to increased osmotic adjustment and MDA content (Cramer et al., 2011; Xiong et al., 2018). These may explain the yellowing and stunting of the DR and FL plants compared to the RW plants.

4.3.3 Metabolites profile of the different CCM routes

The results showed that the abundance of intermediate metabolites involved in the CCM routes was significantly ($p < 0.05$) altered due to water stress (Table 4.1). During water stress, plants go through rapid metabolic adjustment to maintain proper metabolism as an adaptation mechanism (Timm et al., 2021). The process of metabolic adjustment requires several regulatory mechanisms to mediate signaling between multiple metabolic pathways, and to initiate alterations in the composition of metabolites throughout the various CCM routes (Cramer et al., 2011). Metabolic analysis in the present study revealed the presence of 68 putative metabolites in the Mexican mint plants, which were categorized into five carbon-mediated metabolic groups for Calvin cycle, glycolysis, TCA cycle, PPP, and nucleotide biosynthesis (Fuchs & Berg, 2014).

Table 4.1. Total metabolites involved in specific central carbon metabolic pathways in Mexican mint under water stress.

Treatment	Calvincycle (nmol/g)	Glycolysis (mmol/g)	TCA (mmol/g)	PPP (nmol/g)	Nucleotide biosynthesis (nmol/g)
DR	31.33^{b}	29.42^{a}	59.86a	56.67^{b}	288.65^{b}
FL	67.75^{a}	9.04^{b}	36.62^{bc}	182.62^{a}	701.27^{a}
DHFL	47.61^{ab}	2.57^{c}	51.62^{ab}	196.32^{a}	310.62^{b}
RH	41.06^{b}	2.07^{c}	27.59c	149.38^{a}	879.76^{a}
RW	36.95^{b}	1.90^{c}	37.83^{bc}	147.73^{a}	267.60^{b}
p-value	0.002	0.00	0.002	0.000	0.000

PPP, pentose phosphate pathway; TCA, tricarboxylic acid. Regular water, RW; drought, DR; flooding, FL; rehydration after drought, RH; dehydration after flooding, DHFL. Values are means of three replications and different alphabets denote significant differences according to Tukey's HSD post-test analyses at a significance level of $p < 0.05$.

Remarkable shifts in total concentrations of intermediate metabolites and energy generation were noticed with variations in the watering regime (Table 4.1). It is reported that plant response to water stress begins with rapid production of reactive oxygen species that act as an alarm signal that triggers acclamatory-defense responses by specific signal transduction pathways (Cramer et al., 2011; Kleinwächter et al., 2014). Consequently, these reactions result in a series of metabolic reprogramming in the plant. This can explain the significant ($p < 0.05$) alterations in total metabolites of the different CCM routes and energy generation in the Mexican mint plants following water stress imposition and water stress recovery as shown in Table 4.1 and explained further below.

4.3.3.1 Calvin cycle intermediates

The production of downstream carbohydrate molecules including glucose in the Calvin cycle were significantly ($p < 0.05$) influenced by the different watering regimes (Table 4.1, Figure 4.2). Interestingly, the concentrations of total metabolites involved in the Calvin cycle was reduced non-significantly ($p > 0.05$) by *ca.* 15% in Mexican mint plants exposed to DR compared to those under RW (Table 1). On the contrary, the concentrations of total Calvin cycle metabolites were significantly ($p < 0.001$) increased by *ca*. 83% in FL plants compared to RW plants (Table 1). Plants that recovered from water stress i.e., RH and DHFL plants had their total Calvin cycle metabolites slightly increased by *ca.* 11% and 29%, respectively, although not significantly ($p > 0.05$) different from that of the RW plants (Table 1). This means that the water-stressed Mexican mint plants recovered their photosynthetic capabilities upon rehydration after a period of drought or upon dehydration after a period of flood and their photosynthetic capabilities approached that of the RW plants.

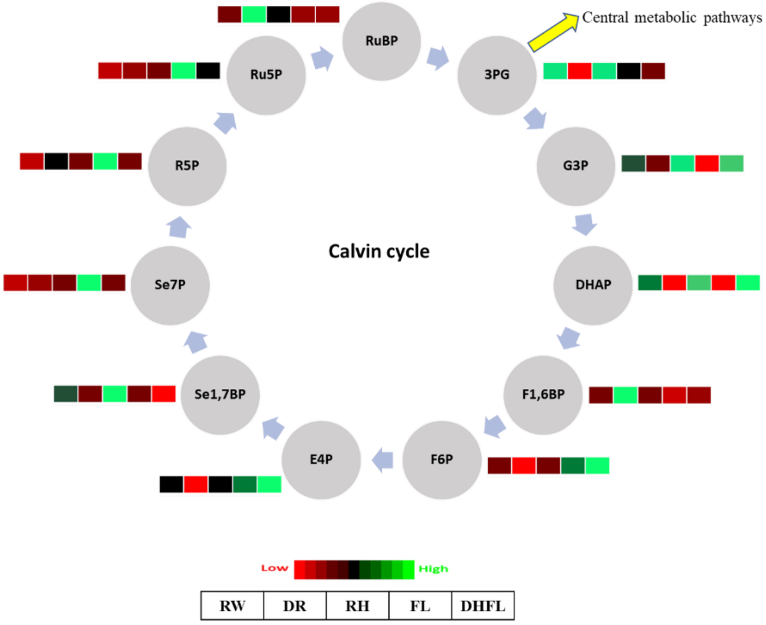

Figure 4.2. Heat map depicting the individual concentrations of intermediate metabolites of the Calvin cycle in Mexican mint plants under varying watering regimes (n=3). 3-phosphoglyceric acid (3PG), glyceraldehyde-3-phosphate (G3P), dihydroxyacetone phosphate (DHAP), fructose-1,6-bisphosphate (F1,6BP), fructose-6-phosphate (F6P), erythrose-4-phosphate (E4P), sedoheptulose-1,7-bisphosphate (Se1,7BP), sedoheptulose-7-phosphate (Se7P), ribose-5-phosphate (R5P), ribulose-5-phosphate (Ru5P) and ribulose-1,5-bisphosphate (RuBP). Metabolite concentrations in each block compartment are normalized across all data for an individual compound such that similar colour intensities between compounds can represent widely differing concentrations. The red colour represents a lower concentration, and the green colour represents the higher concentration of a particular metabolite. The compartments were arranged from left to right as regular watering (RW), drought (DR), resumption of regular watering after drought (RH), flooding (FL), and resumption of regular watering after flooding (DHFL).

The ANOVA shows that the individual intermediate metabolites involved in the Calvin cycle significantly ($p < 0.05$) varied with variation in watering regime. Specifically, fructose-1,6-bisphosphate (F1,6BP) and ribulose-1,5-bisphosphate (RuBP) were significantly ($p < 0.05$) highest in DR plants. But the overall reduction in total Calvin cycle metabolites in DR plants (Table 4.1) can be attributed to low concentrations of sedoheptulose-7-phosphate (Se7P), erythrose-4-phosphate (E4P), F1,6BP, dihydroxyacetone phosphate (DHAP), 3-phosphoglyceric acid (3PG), and ribulose-5-phosphate (Ru5P) as shown by the heatmap in Figure 4.2. The result suggested a possible reduction in the rate of photosynthesis of the DR plants, which was consistent with previous reports for other plants where drought reduced the abundance of numerous Calvin cycle proteins and other associated enzymes involved in photosynthesis (Todaka et al., 2017; Ben Abdallah et al., 2018; Brito et al., 2019). Although enzymatic activities were not investigated, Wingler et al., (2000) suggested that the reduction in Calvin cycle metabolites can be ascribed to impairment in photorespiratory enzyme activities, which can affect photosynthesis under drought stress. In soybean (*Glycine max*), Chen et al., (2016) revealed that drought stress repressed the expression of genes involved in the Calvin cycle, and resulted in a substantial reduction in photosynthetic abilities.

On the other hand, the increase in Calvin cycle metabolites in FL plants can be ascribed to increased concentrations of Se7P, E4P, F6P, ribose-5-phosphate (R5P) and Ru5P (Figure 4.2). Previous studies demonstrated that the ability to maintain photosynthesis was strongly associated with flood tolerance in several plant species (Irfan et al., 2010). It was found that under flooding conditions, most flood-tolerant plants switch from aerobic to anaerobic respiration to increase photosynthetic carbon production. Such an flood adaptation mechanism could have been one of the reasons for increased Calvin cycle metabolites in FL plants as reported for *Kandelia candel* by Pan et al.,(2018). Furthermore, Se7P produced from sedoheptulose-1,7-bisphosphate (Se1,7BP) through the action of the enzyme, sedoheptulose-bisphosphatase, is critical for RuBP regeneration. Therefore, the increase in Se7P concentration in FL plants, probably indicated an increase in photosynthetic rate and carbohydrate synthesis as previously noticed by (Parry et al., 2013) . RH and DHFL plants had high concentrations of DHAP and Glyceraldehyde-3-phosphate (G3P) similar to concentrations found in RW plants (Figure 4.2). Collectively, G3P and DHAP are termed as triosephosphate and can interconvert via the catalyzation of isomerase (Mostofa et al., 2018). G3P and DHAP are important intermediates for photosynthesis and glycolysis.

Studies have shown that during glycolysis, *ca*. 0.05 – 0.3% of glucose is non-enzymatically transformed into methylglyoxal, which can be increased following plant exposure to stressful conditions (P. Singh & Dhaka, 2015; M. A. Hossain et al., 2016). The present finding suggested that G3P, a key end-product of photosynthesis, was increased in plants under regular watering (i.e., RW) or plants that resumed regular watering after stress (i.e., RH and DHFL) compared to DR or FL plants. Sugar accumulation in response to drought and flooding has been reported by many authors (Polacik, 2010; Z. Hossain et al., 2014; Tamang et al., 2021). Glucose is produced at the end of the Calvin cycle, and therefore, the increase in concentrations of glucose in both DR (high) and FL (moderate to high) plants (Figure 4.3) can be considered as a biochemical strategy to supply substrates for high-energy generation through glycolysis (Fernie et al., 2004) as the stressed-plants switched to a survival mode (Stoychev et al., 2013).

4.3.3.2 Glycolytic pathway intermediates

The 3C sugar from the Calvin cycle enters the glycolytic pathway. In this study, we observed that the enzymatic breakdown of glucose *via* glycolysis was significantly ($p < 0.001$) higher in DR plants followed by FL plants when compared to RW plants. Total glycolytic metabolites were increased by *ca.* 1448% in DR plants and *ca*. 376% in FL plants compared to that of the RW plants (Table 4.1). Specifically, the increase in glycolytic metabolites in DR plants can be attributed to high concentrations of glucose, F1,6BP, phosphoenolpyruvic acid (PEP), pyruvic acid (PA), NADH and adenosine triphosphate (ATP) (Figure 4.3). Also, the increase in glycolytic metabolites in FL plants can be attributed to high concentrations of F6P, PEP, and PA. These observations suggested that PEP and PA were increased in Mexican mint plants under both drought and flood conditions. Because total glycolytic pathway metabolites were not significantly ($p > 0.05$) altered in RH and DHFL plants, we suggested that glucose metabolism was altered by DR and FL conditions but was normalized after the plants were exposed to regular watering conditions as portrayed by the RH and the DHFL plants, respectively.

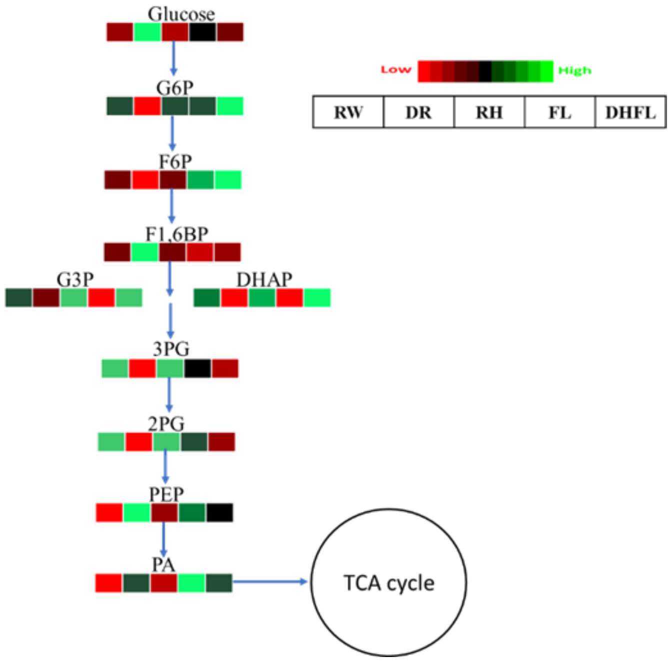

Figure 4.3. Heat map depicting the individual concentrations of intermediate metabolites of the glycolysis pathway in Mexican mint plants under varying watering regimes (n=3). Glucose-6-phosphate (G6P), fructose-6-phosphate (F6P), fructose-1,6-bisphosphate (F1,6BP), glyceraldehyde-3-phosphate (G3P), dihydroxyacetone phosphate (DHAP), 3-phosphoglyceric acid (3PG), 2-phosphoglyceric acid (2PG), phosphoenolpyruvic acid (PEP), and pyruvic acid (PA). Metabolite concentrations in each block compartment are normalized across all data for an individual compound such that similar colour intensities between compounds can represent widely differing concentrations. The red colour represents a lower concentration, and the green colour represents the higher concentration of a particular metabolite. The compartments were arranged from left to right as regular watering (RW), drought (DR), resumption of regular watering after drought (RH), flooding (FL), and resumption of regular watering after flooding (DHFL).

Consistently, numerous studies have reported that drought and flooding stress stimulate glycolysis as a strategy for providing energy during stress defense activation. For instance, in wheat, Guo et al. (2018) reported that glucose, pyruvate, and PEP content were considerably increased in drought-tolerant genotypes. Similarly, glucose and other glycolysis intermediates accumulate in *Pinus painaster* (de Miguel et al., 2016), *Thymus vulgaris* and *T. Kotschyanus* (Ashrafi et al., 2018) and *Lotus japonicus* (Sanchez et al., 2012) in response to drought stress. Moreover, water stress instigates "energy crises" through the impairment of oxidative phosphorylation of the mitochondrion resulting in a drastic reduction of ATP production (Bailey-Serres et al., 2012; Bailey-Serres & Voesenek, 2008). Sugar (e.g. glucose) accumulation plays a crucial role in carbon resource allocation and plant growth (Koch, 2004). To survive the energy crises under such stressful conditions, tolerant plants increase their glycolytic influx by accumulating more glucose to produce sufficient ATP *via* glycolysis to maintain basic cellular functions and regenerate NAD^+ to maintain the glycolytic flux (Bailey-Serres & Voesenek, 2008). Sugar accumulation in response to drought and flooding has been reported by serval authors (Hossain & Komatsu, 2014; Polacik & Maricle, 2013; Tamang et al., 2021). Thus, the increased accumulation of glucose content in both DR and FL plants could be considered a biochemical strategy to supply substrates for high-energy generation through glycolysis (Fernie et al., 2004). This resulted in increased ATP production in DR plants. Furthermore, it has been reported that pyruvate metabolism plays a critical role in water stress tolerance in plants (Good & Zaplachinski, 1994; Guo et al., 2018; Pan et al., 2018). In response to FL, Pan et al. (2018) showed that *Kandelia candel* accumulated high pyruvate contents in the leaves due to increased activities of 6-phosphofructokinase (PFK) and pyruvate kinase (PK). Also, pyruvate production from glycolysis can be directed to recycle a pool of NAD+ through the fermentation pathway and/or serves as a hub for amino acid, fats, and sugar biosynthesis (Ricoult et al., 2006; Xu et al., 2022). In poplars, Peng et al. (2018) revealed that pyruvate accumulation in flooded plants was strongly linked to amino acid biosynthesis and associated with flood tolerance. Although the activities of these enzymes were not examined in this study, the increased pyruvate content in DR and FL is consistent with previous findings suggesting that the glycolysis pathway is crucial for modulating energy metabolism and could result in increased amino acid production in *Plectranthus* under water stress. Nevertheless, further studies are required to examine these effects.

4.3.3.3 Pentose phosphate pathway intermediates

Changes in cellular abundance of PPP intermediate metabolites have been reported to strongly affect several other metabolic pathways (Das et al., 2017). In the present study, it was found that the total concentration of PPP metabolites were not significantly ($p > 0.05$) different amongst FL, DHFL, RH, and RW plants and their average of 168.93 nmol/g was ca. 189% more than that of the DR plants (Table 4.1). Comparatively, the concentration of total PPP metabolites in DHFL plants was non-significantly ($p > 0.05$) the highest followed by FL plants and moderate in RW and RH plants. The low concentration of total PPP metabolites in DR plants can be associated with significant ($p < 0.001$) reductions in G6P and E4P, although the concentrations of R5P and xylulose-5-phosphate (Xu5P) were significantly ($p < 0.001$) enhanced compared to the RW plants (Figure 4.4). On the contrary, there was an increase in total PPP metabolites concentration in the

FL plants (Table 4.1) that can be ascribed to a significant ($p < 0.001$) increase in the concentrations of R5P, Ru5P, Se7P, E4P, and F6P (Figure 4.4).

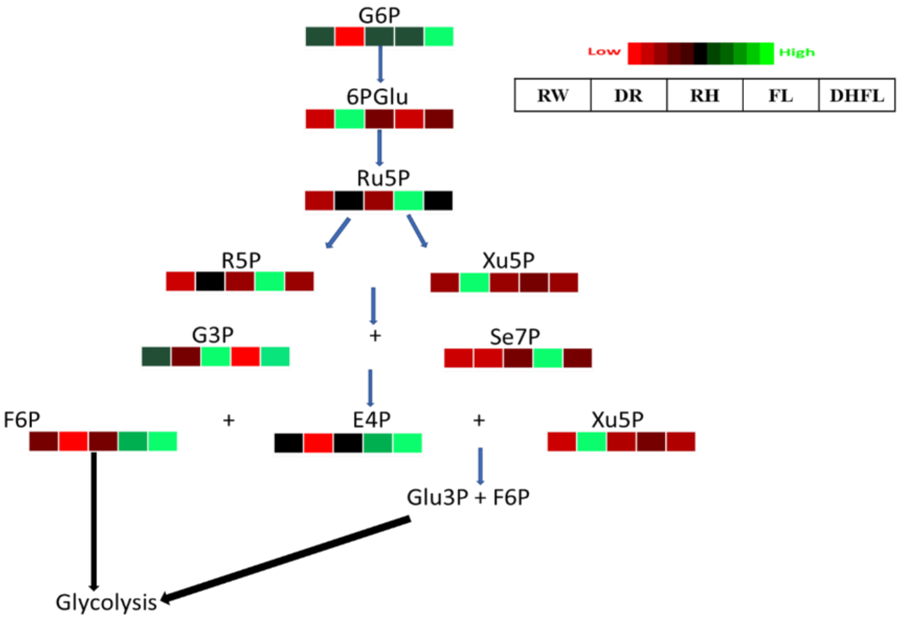

Figure 4.4. Heat map depicting the individual concentrations of intermediate metabolites of the pentose phosphate pathway in Mexican mint plants under varying watering regimes (n=3). Glucose-6-phosphate (G6P), 6-phosphogluconate (6PGlu), ribulose-5-phosphate (Ru5P), ribose-5-phosphate (R5P), xylulose-5-phosphate (Xu5P), glyceraldehyde-3-phosphate (G3P), sedoheptulose-7-phosphate (Se7P), fructose-6-phosphate (F6P), erythrose-4-phosphate (E4P), and glucose-3-phosphate (Glu3P). Metabolite concentrations in each block compartment are normalized across all data for an individual compound such that similar colour intensities between compounds can represent widely differing concentrations. The red colour represents a lower concentration, and the green colour represents the higher concentration of a particular metabolite. The compartments were arranged from left to right as regular watering (RW), drought (DR), resumption of regular watering after drought (RH), flooding (FL), and resumption of regular watering after flooding (DHFL).

Similarly, increased concentrations of G6P, E4P, G3P and F6P accounted for the high concentrations of total PPP metabolites in DHFL plants (Figure 4.4). It was established that PPP is primarily responsible for the major supply of NADPH for several biosynthetic pathways in cells and also, contributes to antioxidant production (Singh et al., 2012). The increased NADPH in DR plants possibly indicated an enhancement of the antioxidant system as reported for drought-stressed soybean plants by Liu et al., (2013). This suggested that maintaining redox potential could be a necessity for Mexican mint plant protection against drought-induced oxidative stress (Juhnke et al., 1996). Overall, the RH plants had similar PPP metabolites profile to the RW plants, and the abundance of G6P, 6PG, R5P, Xu5P, G3P, and Se7P in RW, RH, and DHFL plants were similar

(Figure 4.4). Consequently, the concentration of the resultant product i.e., glucose in RW, RH, and DHFL plants entering the glycolytic pathway were similar (Figure 4.3) since PPP runs parallel to glycolysis in the cytosol. Interestingly, the concentration of NADPH in FL and DHFL plants were similarly low as found in RW plants. The increase in R5P, E4P, and F6P concentrations in FL plants as a flooding stress tolerance mechanism can lead to enhanced production of downstream compounds including nucleotides, aromatic amino acids, and fatty acids (Huang et al., 2003; Kruger & von Schaewen, 2003; Ge et al., 2020). Overall, Mexican mint plants increased PPP metabolic flux under flooding stress to achieve energy homeostasis – a mechanism reported for *K. candel* by (Pan et al., 2018).

4.3.3.4 Tricarboxylic acid cycle intermediates

It was found that both total and individual TCA cycle intermediate metabolites were significantly ($p < 0.002$) affected by the different watering regimes (Table 4.1; Figure 4.5). Total TCA cycle metabolites were significantly ($p < 0.001$) increased by *ca*. 58% in DR plants and *ca*. 36% in DHFL plants compared to their RW counterparts (Table 1). In contrast, drought stress reduced TCA cycle metabolites in maize, rice, and sesame (*Sesamum indicum*) plants (Todaka et al., 2017;Yang et al., 2018; Di et al., 2019). This contrasting report could be attributed to differences in plantgenotype i.e., Mexican mint versus maize, rice, and sesame. The least total TCA cycle metaboliteswere found in RH plants and were not significantly ($p > 0.05$) different from those of the RW andFL plants. When compared to RW plants, flooding stress did not alter the total concentration of TCA metabolites of the Mexican mint plants. This conformed with previous reports for flood- tolerant soybean(Komatsu et al., 2011; Wang et al., 2017), *Acanthus ilicifolius* (Liu & Zheng, 2021), *Lotus japonicus* (Rocha et al., 2010b), and rice (Locke et al., 2018) plants. According to Menezes-Silva et al., (2017), plants can intensify their defense against stress by maintaining information from previous stress events. This “stress memory” in plants involves physiochemicalprocess such as photosynthesis, maintenance of water status and osmotic adjustment (Jacques et al., 2021). These can explain the results of the present study, which showed that the removal of flooding (i.e., DHFL plants) increased total TCA cycle metabolites compared to FL or RW plants(Table 4.1). It therefore seemed obvious that the ability of DHFL plants to withstand future stresswas enhanced compared to FL and RW plants. Although the reductions in TCA cycle metabolitesdid not significantly ($p > 0.05$) impact ATP production in this study, such reduction could likely alter TCA-mediated amino acids production (Sweetlove et al., 2010), which must be investigated in future studies.

More specifically, there was a remarkable increase in 8 out of 9 of the individual TCA cycle intermediate metabolites in the FL plants compared to the other treatments. The concentrations of incoming PA from glycolysis and the TCA cycle intermediates metabolites – isocitric acid, α-ketoglutaric acid, succinic acid, fumaric acid, and malic acid – were particularly highest in FL plants compared to all the other treatments (Figure 4.5). This suggested greater mitochondrial activity in FL plants to generate carbon skeletons for amino acid biosynthesis (Sweetlove et al., 2010). The highest concentrations of isocitric acid, aconitic acid and citric acid were noticed in DR plants, which had the least concentrations of oxaloacetic acid, acetyl-CoA, malic acid and α-ketoglutaric acid.

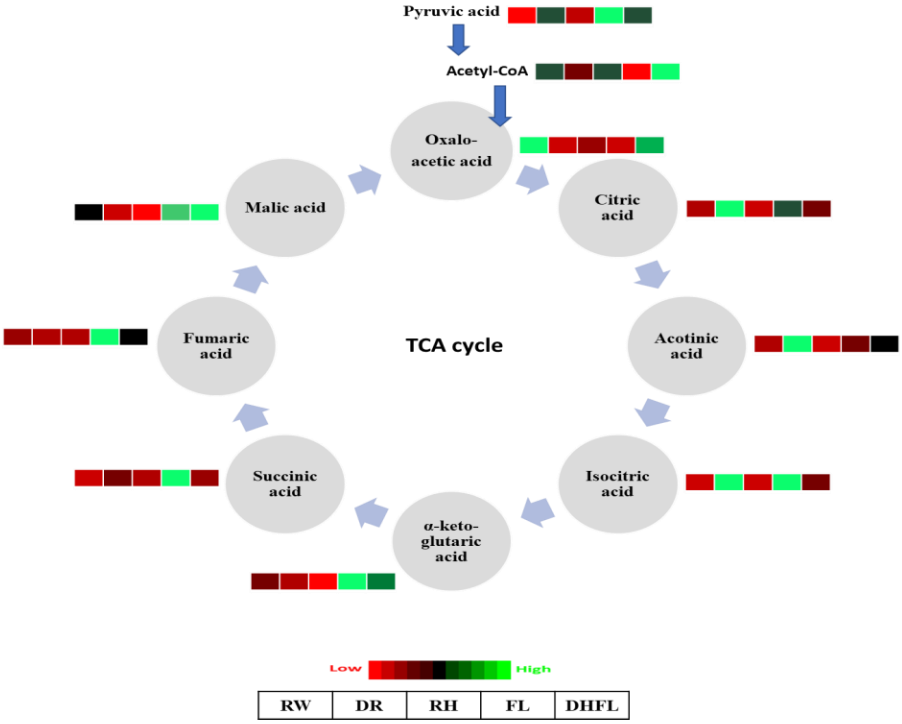

Figure 4.5. Heat map depicting the individual concentrations of intermediate metabolites of the tricarboxylic acid cycle in Mexican mint plants under varying watering regimes (n=3). Metabolite concentrations in each block compartment are normalized across all data for an individual compound such that similar colour intensities between compounds can represent widely differing concentrations. The red colour represents a lower concentration, and the green colour represents the higher concentration of a particular metabolite. The compartments were arranged from left to right as regular watering (RW), drought

(DR), resumption of regular watering after drought (RH), flooding (FL), and resumption of regular watering after flooding (DHFL).

Apart from oxaloacetic acid that was comparatively highest in RW plants, all the other metabolites were low. The low concentrations of succinic acid, isocitric acid, acotinic acid and citric acid in RW plants were similar to RHDR and DHFL plants (Figure 4.5). Particularly, the RH plants had low concentrations of all the individual TCA cycle intermediate metabolites. For the DHFL plants, the increase in TCA cycle metabolites can be ascribed to high levels of acetyl-CoA, oxaloacetic acid and malic acid. According to Sweetlove et al. (2010), TCA cycle intermediate metabolites do not have similar flux, and the activities of the different enzymes in the different steps are independent of each other. As such, suppression of one enzyme within the cycle does not alter the activity of the other enzymes. Therefore, the high concentrations of the TCA cycle metabolites in the FL and DHFL plants can be attributed to the link between TCA cycle and other associated pathways including ammonium assimilation, and biosynthesis of amino acids, nucleotides and secondary metabolites that contribute to plant stress tolerance (Sweetlove et al., 2010; Jacques et al., 2021).

Studies showed that changes in malate, citrate, α-ketoglutaric acid and fumarate during flood stress occurred in many different plants (Komatsu et al., 2011; Rocha et al., 2010b; Wang et al., 2017). The accumulation of succinate is obvious during flood-induced hypoxia conditions since succinate dehydrogenase requires oxygen (Xu et al., 2022b). Also, both drought and flood stresses stimulate the accumulation of citrate in plants (Wang et al., 2017; Ashrafi et al., 2018a). Besides, citrate is not only involved in amino acid metabolism but also serves as an antioxidant and intermediate in respiratory metabolisms to generate energy for defense pathways in stress adaptation mechanisms (Zhao et al., 2015). Furthermore, α-ketoglutaric acid plays an important role in respiration and N assimilation for the biosynthesis of proline, glutamate, glutamine, and arginine. These amino acids function in regulating osmotic potential and act as osmolytes to maintain protein integrity, and to mediate water stress tolerance in plants (Guo et al., 2018; Xu et al., 2022b). Therefore, like many other plants, the accumulation of these metabolites can be essential for water stress tolerance in Mexican mint plants.

4.3.3.5 Nucleotide biosynthetic pathway intermediates

Apart from guanine monophosphate (GMP), all the determined nucleotide pathway intermediate metabolites were significantly ($p < 0.05$) influenced by variation in the watering regime (Table 4.1). Nucleotide metabolism is the most critical cellular component for plant growth that affects several metabolic processes (Witte et al., 2020). These nucleotides are essential for information storage and recovery of dividing and expanding tissues. The results revealed that total nucleotide intermediates concentrations were significantly ($p < 0.001$) highest in RH (*ca*. 229%) and FL (*ca.* 162%) plants compared to RW plants (Table 1). Total concentrations of nucleotide intermediates in DR, DHFL and RW plants were not significantly ($p > 0.05$) different, and ranged from 267 to 311 nmol/g. Evidence from several studies revealed that nucleotide biosynthesis increased drought tolerance in plants such as Arabidopsis (Watanabe et al., 2014), orchid (*Dendrobium* sp.) (Zhao et al., 2019; Zhang et al., 2021), pitaya (*Hylocereus undatus*) (Fan et al., 2014), drought-tolerant wild wheat (*Triticum boeoticum*) (Liu et al., 2015) and soybean (Das et al., 2017). Based on these previous reports, we surmised that nucleotide metabolism will be a critical mechanism for water stress tolerance in Mexican mint. These nucleotides may also allow for the repair and maintenance of water stress-induced cellular damages which as a result, might have promoted water stress tolerance in RH and DHFL plants.

Furthermore, the *de novo* biosynthesis of purine is characterized by the formation of adenosine monophosphate (AMP) and GMP (Witte et al., 2020). AMPs are synthesized from activated ribose (5-phosphoribosyl-1-pyrophosphate), key amino acids such as glutamine and glycine, and 10-formyl tetrahydrofolate (Witte et al., 2020). GMPs are obtained from the deamination of AMP or direct transport of inosine 5′-monophosphate (IMP) in the chloroplast (Witte et al., 2020). The results of the present study showed that the relative concentrations of the individual nucleotide intermediates i.e., guanine diphosphate (GDP), GTP, AMP, and adenosine diphosphate (ADP) known to be involved in purine biosynthesis were significantly ($p < 0.001$) increased in DR plants except for GMP (Figure 4.6). Conversely, these metabolites were significantly ($p < 0.05$) reduced in the FL plants except for GMP, which was highest in both FL and RW plants.

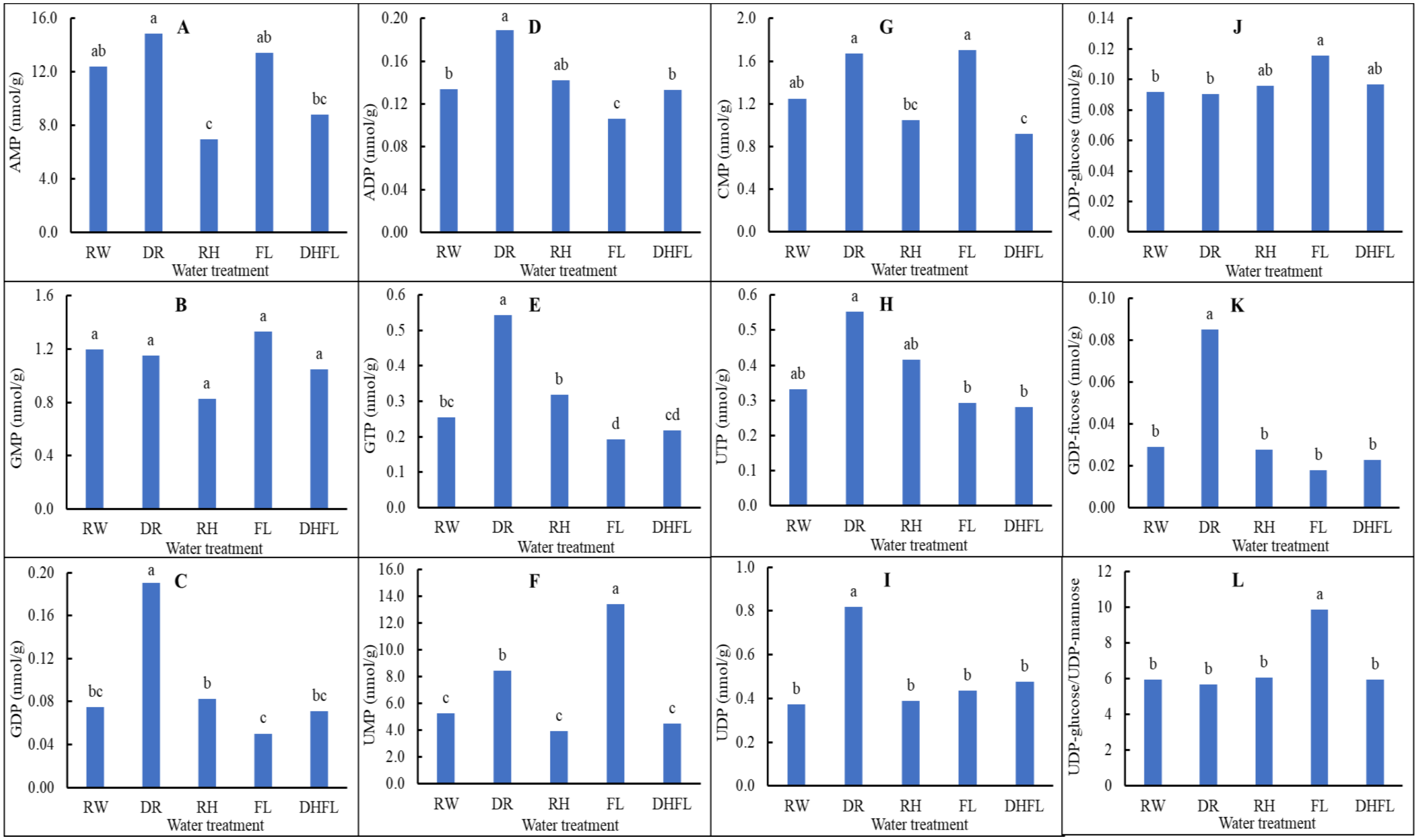

Figure 4.6. Changes in nucleotide biosynthetic pathway metabolites in Mexican mint plants in response to varying watering regimes (n=3). A: adenosine monophosphate (AMP), B: guanine monophosphate (GMP), C: guanine diphosphate (GDP), D: adenosine diphosphate (ADP), E: guanine triphosphate (GTP), F: uridine monophosphate (UMP), G: cytosine monophosphate (CMP), H: uridine triphosphate (UTP), I: uridine diphosphate (UDP), J: ADP-glucose, K: GDP-fucose and L: UDP-glucose/UDP-mannose. Different alphabetical letters on the bars denote significant differences according to Tukey's honestly significant difference post-test analyses at a significant level of $p < 0.05$.

During the *de novo* biosynthesis of pyrimidine, uridine monophosphate (UMP) is typically formed from phosphoribosyl diphosphate (PRPP), aspartate and carbamoylphosphate to play a critical role in cytosine monophosphate (CMP) biosynthesis (Zrenner et al., 2006; Witte et al., 2020). The results of the present study indicated that concentrations of UMP, uridine triphosphate (UTP), uridine diphosphate (UDP), and CMP metabolites were significantly ($p < 0.001$) increased in DR plants compared to RW plants (Figure 4.6). Ultimately, UMP, UDP, and CMP concentrations were increased in FL plants while UTP was slightly reduced. Intriguingly, purine and pyrimidine nucleotides may also function as co-substrates for carbohydrate metabolism. For example, ADP-glucose is an activated precursor for starch synthesis while UDP and UTP are directly involved in the synthesis, degradation, and transportation (i.e., in the form of UDP-glucose or UTP-glucose) of various forms of carbohydrates including sucrose, sugar components of glycoproteins and cell wall matrix polysaccharides (Lim et al., 2004; Zrenner et al., 2006). UDP-glucose act as a glucosyl donor for the biosynthesis of hormones and secondary metabolites (Lim et al., 2004). Furthermore, ADP-glucose and UDP-glucose/UDP-mannose concentrations in the Mexican mint plants were significantly ($p < 0.05$) elevated in FL plants but were not altered in DR plants compared to the RW plants (Figure 4.6). These findings suggested that carbohydrate metabolism and secondary metabolites syntheses can enhance flood tolerance. This provides a new insight into drought and flooding tolerance in Mexican mint plants.

4.3.4 Association between central carbon metabolites in response to water stress

A two-dimensional (2-D) principal component analysis and Pearson correlation coefficient (r) were used to further assess the association amongst the central carbon metabolites in response to water stress (Figure 4.7&4.8; Table 4.2). The PCA-biplot showed a projection of response variables in the factor spaces and explained 96.28% of the total variation in the data set (Figure 4.5). Our results showed that the total metabolite in the Calvin cycle had a significantly ($p < 0.001$) strong positive association with that of the TCA cycle ($r = 0.814$) and PPP metabolites ($r = 0.749$), while the total PPP metabolites had a significantly ($p < 0.01$) moderate positive association with TCA metabolites ($r = 0.684$) and a negative correlation with total glycolysis metabolites ($r = -0.702$, $p < 0.005$) (Figure 4.7; Table 4.2). Moreover, since both PPP, Calvin cycle and glycolysis pathways have some metabolites in common, the correlation test between the various individual metabolites of each pathway showed distinct significant associations (Figure 4.8; Table 4.2).

Table 4.2. Pearson correlation coefficients (r) amongst the specific central carbon metabolic pathways in Mexican mint under water stress and their significance at $p \leq 0.05$.

	TCA	**Glycolysis**	**Calvin Cycle**	**PPP**
Glycolysis (nmol/g)	r = -0.268 P = 0.335			
Calvin cycle (nmol/g)	r = **0.814** P **= 0.000**	r = -0.215 P = 0.442		
PPP (nmol/g)	r = **0.684** P = **0.005**	r = **-0.702** P = **0.004**	r = **0.749** P = **0.001**	
Nucleotide metabolism (nmol/g)	r = 0.380 P = 0.162	r = **0.645** P = **0.009**	r = 0.501 P = 0.057	r = -0.168 P = 0.549

PPP, pentose phosphate pathway; TCA, tricarboxylic acid

The results confirm that most Calvin cycle metabolites had a significant positive association with major TCA metabolites (Figure 4.8; Table 4.2). For example, sedoheptulose-7P, ribose-5P and ribulose-5P levels correlated with fumaric acid and succinic acid levels whereas ribulose-bisP and xylose-5P exhibit a moderate and strong positive association with aconitic acid and citric acid respectively.

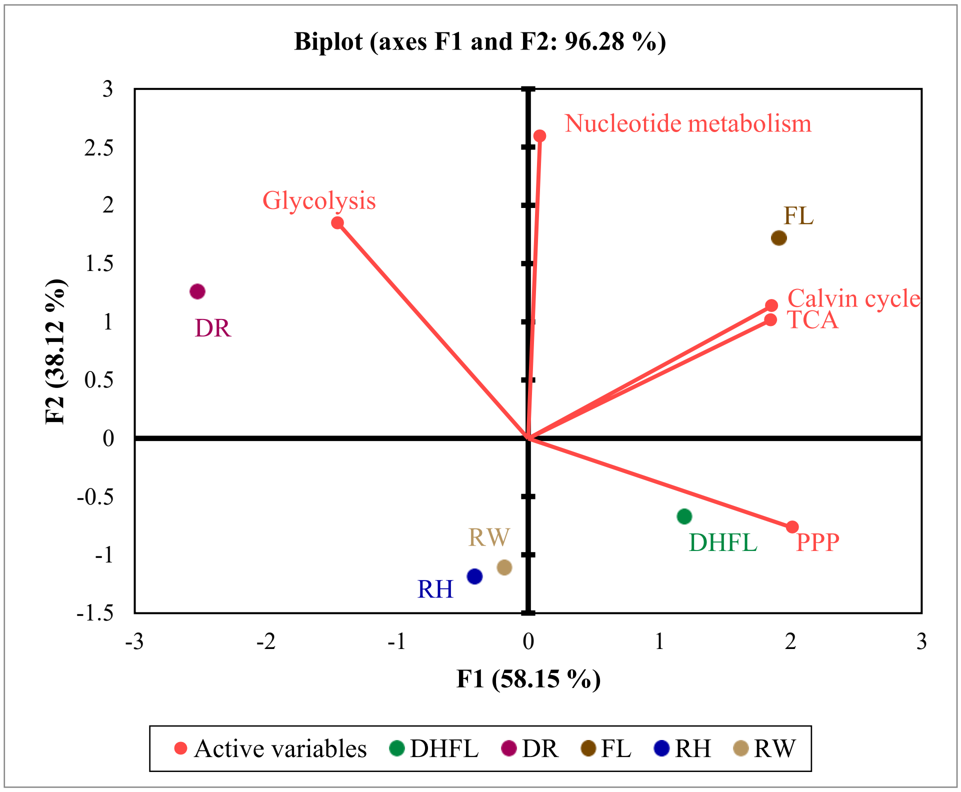

Figure 4.7. A two-dimensional principal component analysis (2-D PCA) biplot showing relationships amongst the explanatory variables (total metabolites involved in specific central carbon metabolic) Calvin cycle, glycolysis, pentose phosphate pathway (PPP), tricarboxylic acid (TCA) cycle and nucleotide metabolism of Mexican mint under water stress. Projection of the variables in the 2-D factor space (F1 and F2) explained a total of 96.28% of the variations in the dataset. Variables that are closely located are not different compared to variables located at a distance within a quadrant or between quadrants. Regular water, RW; Drought, DR; Flooding, FL; Rehydration after drought, RH; Dehydration after flooding, DHFL.

Also, PPP metabolites exhibited a positive correlation with most TCA metabolites (Figure 4.8; Table 4.2). In addition to sedoheptulose-7P, ribose-5P and ribulose-5P, 6-phosphogluconate exhibited a strong association with Aconitic acid citric acid (Figure 4.8; Table 4.2). Intriguingly, while glucose had a strong positive association with Isocitric acid, aconitic acid and citric acid, pyruvic acid, a key product of glycolysis had a strong association with fumaric acid, succinic acid, α-ketoglutaric acid, isocitric acid and citric acid metabolites (Figure 4.8; Table 4.2) suggesting that the product of glucose metabolism feeds the TCA cycle probably for energy generation and production of other metabolic compounds (Rocha et al., 2010). Additionally, ATP and NADPH which are essential for energy production during water stress adaptation had a moderate negative correlation with malic, fumaric, and succinic acid and a moderate positive association with aconitic and citric acid (Figure 4.8). Furthermore, the total nucleotide biosynthesis metabolites had a significant ($p < 0.01$) moderate positive correlation with glycolysis metabolites (r = 0.646) and a moderate association with Calvin cycle metabolites (r = 0.501) although this was not significant ($p > 0.05$) (Table 4.2). This was reflected in glucose's moderate positive association with AMP, ADP, GDP, GTP, CMP and UTP and strong positive association with UDP and UDP-glucose (Figure 4.8). Pyruvic acid had a moderate association with ribose, AMP, GMP, CMP, ADP-glucose, and UDP-glucose. Similarly, key individual Calvin cycle metabolites including ribulose-bisP, ribulose 5P and 5ibose-5P showed a strong association with different nucleotide biosynthetic metabolites (Figure 4.8). Overall, the coordinated association between the various pathway metabolite confirms that water stress instigates metabolic reprogramming of *Plectranthus* to regulate energy production and biosynthesis of essential compounds for stress adaptation and tolerance.

Additionally, the PCA biplot showed that flooding had a strong influence on the Calvin and TCA cycle and moderate regulation of nucleotide biosynthesis and PPP (Figure 4.7). On the contrary, drought stress tends to strongly affect glycolysis and moderately influence nucleotide metabolism with less influence on Calvin and TCA cycle. This is consistent with previous studies which revealed that plants stimulate glucose metabolism during drought as a key strategy for energy production to maintain basic cellular functions thereby mediating drought tolerance (Ashrafi et al., 2018; de Miguel et al., 2016; Guo et al., 2018). Also, flooding-tolerant plants undergo anaerobic respiration and drive an increase in photosynthetic carbon production via the Calvin cycle (Pan et al., 2018). Besides, the function of the TCA cycle is to increase to support ATP output for tolerance. Moreover, dehydration after flooding showed a strong influence on PPP and a moderate effect on Calvin and TCA cycles. Nevertheless, rehydration after the drought had only a moderate influence on PPP and no effect on the other central carbon metabolic pathways (Figure 4.7).

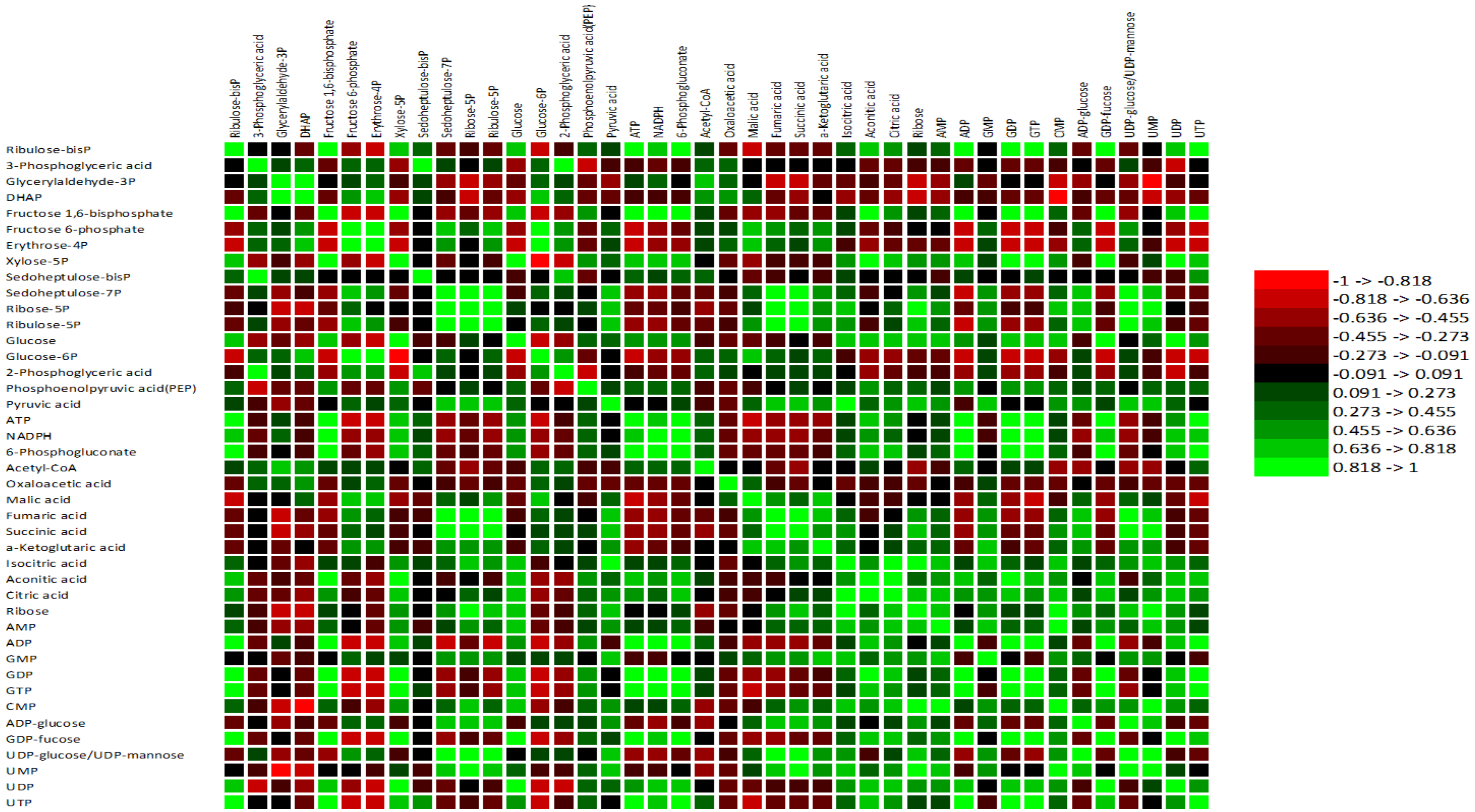

Figure 4.8. Correlation matrix among individual metabolites of the central carbon metabolic pathway in Mexican mint response to water stress. The red color represents a strong negative association, and the green color represents a strong positive association.

4.4 Conclusion

Water stress is one of the major limiting factors of crop growth and productivity worldwide. In this study, we demonstrated that water stress affects the central carbon metabolic pathways that influence plant growth and development. The results revealed that 68 key metabolites involved in the Calvin cycle, glycolysis, tricarboxylic acid (TCA), pentose phosphate pathway (PPP) and nucleotide biosynthesis were significantly affected by both flooding and drought. This indicates that such metabolite accumulation is crucial for energy production and provides carbon skeletons for further macromolecule biosynthesis, thereby promoting water stress tolerance. However, rehydration after drought and dehydration after flooding enhanced fast recovery to normal conditions and had no obvious effect on the central carbon metabolic profile of *Plectranthus*. The finding of this study for the first time revealed the metabolic alteration of *Plectranthus* to flooding and drought stress and elucidates the mechanism of water stress tolerance within this genus. Additionally, further analysis using transcriptomics and proteomics approaches is required to identify genes and proteins that regulate these central carbon metabolic pathways in response to water stress.

CHAPTER 5: SHIKIMATE, HEXOSAMINE, CARBOHYDRATE BIOSYNTHESIS, AND ELECTRON TRANSPORT CHAIN IN MEXICAN MINT VARY WITH VARIATIONS IN THE WATERING REGIME

Abstract

Plants are sessile and their ability to reprogram their metabolism to adapt to fluctuations in soil water content is crucial, but not clearly understood. A study was performed to determine alterations in intermediate metabolites involved in plant central carbon metabolism following exposure of Mexican mint (*Plectranthus amboinicus*) to varying watering regimes. The water treatments included regular watering (RW), drought (DR), flooding (FL), and resumption of regular watering after flooding (DHFL) or after drought (RH). This investigation mainly focused on shikimate, starch and sucrose biosynthesis, and the electron transport chain (ETC). The DHFL resulted in a notable increase in metabolite concentration within the shikimate pathway and the synthesis of sucrose and starch compared to RW plants. The results indicated that DR plants exhibited a significant ($p < 0.05$) increase in metabolite accumulation in the ETC compared to the other pathways. Furthermore, a negative correlation was observed between the ETC pathway and the remaining pathways. In conclusion, the metabolic alterations of DR and FL plants and their reversals i.e., DHFL and RHDR were revealed. Future studies will use transcriptomic and proteomic approaches to identify genes and proteins that regulate plant metabolic routes.

5.1 Introduction

The fluctuations in global climatic conditions pose a threat to the survival of life on Earth. Importantly, achieving sustainable agriculture to meet the increasing food demand for a growing population poses significant challenges, particularly in light of the current state of climate conditions (Salam et al., 2022). Unpredictable climate conditions are largely caused by environmental pressures, which include both biotic and abiotic stresses. Water stress is the primary abiotic stress that reduces crop growth, yield and impedes global food security. The occurrence can be ascribed to a variety of environmental conditions including temperature changes, fluctuations in light levels, drought, flooding, and their combinations. The availability of water can remarkably impact crop physiological responses resulting in diminished crop yields and compromised harvest quality (Moradi et al., 2014b).

In reaction to the commencement of edaphic stress, plants often reduce root hydraulic conductivity, diminish stomatal conductance and photosynthesis, reprogramming metabolic pathways and eventually, stop growing (Reddy et al., 2004b). During periods of stress, it is necessary to restructure the plant metabolic network to maintain crucial metabolic equilibrium and produce defensive metabolites that enhance stress resilience. In such cases, the central metabolism of plants functions acts as a central hub that expeditiously adjusts metabolic demands (Xu et al., 2022). More specifically, the shikimate pathway facilitates the transfer of carbon from central carbon metabolism to the production of aromatic amino acids (AAs) such as L-tyrosine (Tyr), L-phenylalanine (Phe), and L-tryptophan (Trp), as well as various other aromatic natural compounds (Yokoyama et al., 2021). The aromatic AAs are particularly important in plants as they serve as crucial building blocks to produce various natural aromatic compounds, which is a distinctive characteristic of plants (Maeda et al., 2012; Tohge et al., 2013; Maeda et al., 2021). The shikimate process converts phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate (E4P) derived from glycolysis and the pentose phosphate pathways, respectively, into chorismate, which serves as the ultimate shared precursor for all aromatic AAs (Yokoyama et al., 2021). These aromatic compounds are crucial for plant growth and survival. They encompass phytohormones, defense compounds, and phenolic polymers like lignin, the second most important biopolymer after cellulose (Vanholme et al., 2010; Kliebenstein, 2012; Maeda et al., 2021). Hence, plants need to precisely regulate the functioning of the shikimate pathway to manage the synthesis of various aromatic compounds and to coordinate intricate developmental and physiological functions (Yokoyama et al., 2022). The aromatic AAs are also key protein precursors and building blocks for other secondary metabolites in plants (Kleinwächter et al., 2015). Secondary metabolites are generated by plants when required for adaptation under stressful conditions (Dixon, 2003).

Sucrose and starch are recognized as important molecules in coordinating plant reactions to abiotic stresses (Nemati et al., 2018). The abundance of key enzymes in the metabolism of sucrose such as sucrose synthase and sucrose phosphate synthase is directly correlated with the amount of sucrose present (Liu et al., 2018). Moreover, sucrose is distributed through numerous intracellular routes because most plants shed starch and acquire sucrose during water stress. Thus, starch concentration varied greatly in water-stressed plants (Cui et al., 2019). Research into starch and sucrose gene expressions in response to drought has gained popularity in recent years. Most studies demonstrated up-regulation of sucrose-related genes; however, transporter gene down-regulation may lead to a reduction in plant biomass (Cui et al., 2019).

Photosystems I (PSI) and II (PSII) are primarily responsible for mediating light-driven reactions of photosynthesis in the thylakoid membranes (Rochaix, 2011). Proton pumping into the thylakoid lumen is coupled with an electron transfer reaction, and the resulting proton gradient is used to generate ATP. The Calvin-Benson cycle, used for CO_2 fixation and other assimilative processes, is fueled by both ATP and NADPH (Rochaix, 2011). Reactive oxygen species (ROS) are excessively generated under water stress conditions, which disrupts the photosynthetic apparatus (Asada, 1999; Chang, 2017). According to studies, P700 oxidation was shown to be increased in response to water stress, along with an increase in nonphotochemical chlorophyll fluorescence quenching (NPQ) and a decline in the quantum efficiency of PSII, plastoquinone pool, and the quantum efficiency of PSI (Kohzuma et al., 2009; Huang et al., 2013) These findings imply that the combined response of the photosynthetic electron transport (PET) reactions from PSI to water stress protects P700 against over-reduction.

In plant systems, factors that affect plant physiological and developmental programs have a direct impact on the synthesis of secondary metabolites (Mahajan et al., 2020). Therefore, it is anticipated that water stress alters plant central carbon metabolism. Understanding the role of different metabolites and pathways in water stress tolerance of *P. amboinicus* is essential for uncovering the processes behind its ability to withstand water stress, as the metabolite composition has a direct connection to plant physiology. This study presents the observed changes in metabolites linked to the shikimate pathway, electron transport chain, starch, sucrose biosynthesis, and hexosamine pathway under varying water stress conditions. This study will provide the necessary information to enhance water stress tolerance in crops through genetic engineering and breeding methods.

5.2 Materials and methods

The planting and experimental treatments were previously published in Abbey et al. (2023).

5.2.1 Location

This research was conducted in the Department of Plant, Food and Environmental Sciences (PFES), Faculty of Agriculture, Dalhousie University between December 2021 and July 2022

5.2.2 Preparation and rooting of cuttings

A healthy parent of Mexican mint was chosen from the PFES greenhouse plant stock. The plant was well watered before stem cuttings were taken from the healthy plant chosen by cutting the soft tissue branches from the youngest second and third nodes on the main stem. The stem cuttings were then pruned to 5 cm height leaving four pairs of corresponding leaves. All the stem cuttings were placed in a moist perlite medium (Perlite Canada Inc., Montreal, QC, Canada) in a plastic flat tray of dimensions 50 cm length x 28 cm width x 6.5 cm depth. To maintain the ≥95% relative humidity to induce the rooting, the trays were covered with a dome-shaped transparent cover. The trays were positioned on a planting shelf under continuous 24-hour fluorescent lighting at a temperature of 22°C. The cuttings were sprayed with water twice a day and were ready for transplanting after a period of three weeks.

5.2.3 Transplanting and growing conditions

Healthy and uniform seedlings were transplanted into a 15-cm plastic pot containing 200 g of Promix BX and 150g of vermicast mix medium (75: 25 (*w/w*)) and placed in a saucer. The Promix-BX potting medium (Premier Horticulture Inc., Quakertown, USA) comprised of 75–85% sphagnum peat moss, horticultural grade perlite, vermiculite, dolomitic and calcitic limestone (Lower Wedgeport, NS, Canada). The potted plants were placed in a Biotronette Mark II Environmental Chamber (Lab-Line Instruments Inc., Melrose Park, IL, USA), with a 24°/20°C day/night temperature cycle and a 12/12 hr day/night light cycle with a relative humidity of 75%. The plants were watered to field capacity once every three days for 120 days before the treatment application.

5.2.4 Experimental design and water treatment

The experimental treatments were regular watering (RW), drought (DR), flooding (FL), and drought followed by rehydration (RHDR), and flooding followed by dehydration (DHFL) which were arranged in a completely randomized design with three replications. The RW plants were watered every other day to maintain the field capacity of the growing medium. The DR plants were subjected to continuous dehydration and the FL plants were subjected to continuous flooding by immersing the Mexican mint pots (15 cm diameter) in a larger pot (20 cm diameter) filled with water. Both DR and FL treatments were imposed for 8 weeks. The stress reversals were dehydration and resumption of regular watering after continuous flooding for 4 weeks (DHFL); and rehydration and resumption of regular watering after continuous drought for 4 weeks (RHDR).

5.2.5 Plant carbon metabolites

5.2.5.1. Sample preparation

The green leaves were then harvested, flash-frozen in liquid nitrogen, and pulverized into fine powder. All the ground leaf samples were placed on dry ice and were sent to UVic GBC for analyses of targeted metabolite quantification. In brief, 500 µl of 80% methanol was added to each 50-mg sample and homogenized using an MM 400 mill mixer for 1 min and repeated three times. This process was carried out at a shaking frequency of 30 Hz with the support of two metal balls. The homogenized samples were sonicated for 5 min in an ice-water bath, followed by centrifugal clarification at a force of 21,000 x g and 5 ºC for 20 min. A 250-µL of the supernatants was extracted and combined with 150 µL of water and 150 µL of dichloromethane and vortexed for 30 s. The mixture was centrifugated and three separate aliquots (80 µL) of the supernatant were subjected to drying using a nitrogen gas flow before analyzing the residue.

5.2.5.2 Glucose and selected sugar phosphates

The dried residue (80 µL) was added to 50 µL of 50% methanol. The solution or 50 µL of each of the serially diluted standard solutions of glucose, ribose, ribose-5P, glucose-6P, and mannose-6P was mixed with 100 µL of 25 mM 3-amino-9-ethylcarbazole (AEC) solution, 50 µL of 50 mM $NaCBH_3$ solution, and 20 µL of LC/MS grade acetic acid. The mixtures were allowed to react at 60°C for 70 min. After the reaction, 200 µL of water and 300 µL of chloroform were added and vortexed for 15 s followed by centrifugation for 5 min. Each supernatant (50 µL) was mixed with an equal volume of water. Subsequently, 10 µL of the resulting mixture was injected into a penta fluorophenyl propyl (PFP) LC column (2.1 x 150 mm, 1.7 µm) to run UPLC-MRM/MS on an Agilent 1290 UHPLC coupled to an Agilent 6495B QQQ instrument with positive-ion detection, as previously described by (Han et al., 2013).

5.2.5.3 Other metabolites

An internal standard (IS) solution containing 25 isotope-labelled metabolites (adenosine monophosphate (AMP), adenosine-5-triphosphate (ATP), uridine monophosphate (UMP), uridine triphosphate (UTP), fructose-6P, fructose-bisP, UDP-glucose, glycerol-3P, NAD, NADH, glucose-1P, ribose-5P) was prepared in 50% methanol. Serially diluted standard solutions of all the targeted metabolites were prepared in the IS solution at concentrations of 2 x 10^{-5} to 10 µM. The dried residue of the 80-µL aliquot of each sample was dissolved in 100 μL of the IS solution. Each sample solution or standard solution 10 μL was injected into a C18 column (2.1 x 100 mm, 1.9 μm) for UPLC-MRM/MS analysis, using (-) ion detection on a Waters Acquity UPLC system coupled to a Sciex QTRAP 6500 Plus MS instrument, with the use of a tributylamine acetate buffer and – acetonitrile/methanol (1:1, v/v) was for gradient elution (10% to 50% B over 25 min) at 0.25 mL/min and 60ºC.

5.2.6 Calculation and statistical analysis

The process involved interpolating the linear regression curves of each compound by using the analyte-to-internal standard peak area ratios obtained from injections of the sample solution. The collected data were analyzed using Minitab version 21 (Minitab, Inc., State College, PA, USA) with a one-way ANOVA. The treatment means were separated using Tukey's honestly significant difference post hoc test at a 5% significance level.

5.3 Results and discussion

5.3.1 Plant metabolic network

Secondary metabolites play key roles in cellular signaling, energy storage, membrane development and scaffolding, and the allocation of resources across organs under stress conditions (Wen et al., 2015). In Mexican mint, DR plants have a significant ($p < 0.05$) impact on all the metabolic pathways that were studied (Figure 5.1). However, the response of each metabolite to water stress depends on various factors including the intensity and duration of the stress. In this context, the metabolites involved in the carbon pathway such as shikimate, sucrose, starch biosynthesis, electron transport chain, and hexosamine are discussed.

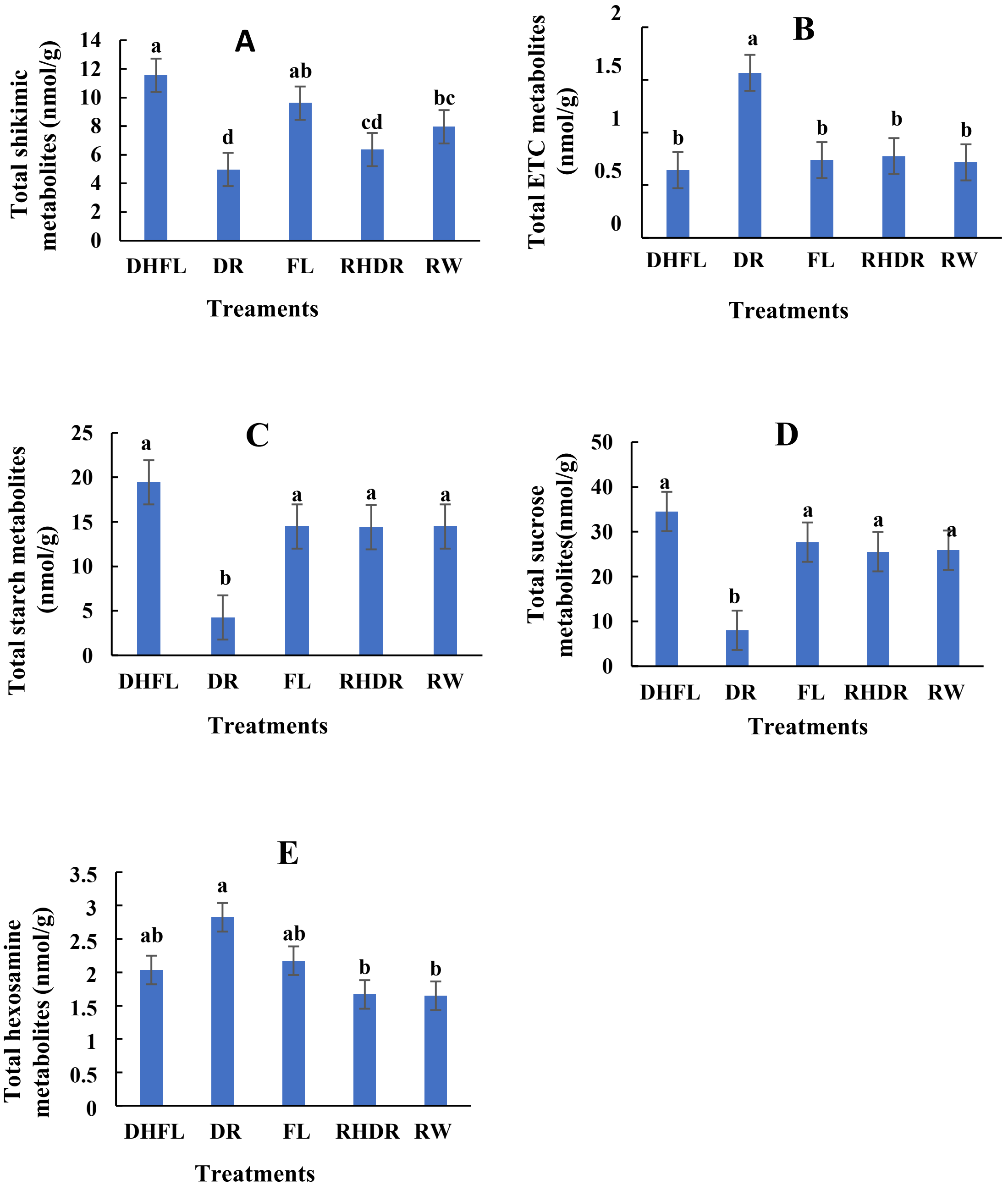

Figure 5.1. Alterations of metabolites involved in various carbon metabolic pathways in Mexican mint in response to varying watering regimes (n=3). (A) shikimic pathway; (B) electron transport chain (ETC); (C) starch synthesis; (D) sucrose; (E) hexosamine biosynthesis pathway

5.3.1.1 Shikimate pathway

In response to water stress, many intermediate metabolites were repressed in the plastid-based shikimic acid pathway in Mexican mint. There was a significant ($p < 0.05$) variation in the concentration of shikimic acid metabolites among DR, FL, RHDR, and DHFL when compared to the RW plants (Figure 5.1). The concentration of the shikimate pathway metabolites was significantly ($p < 0.05$) higher in DHFL plants by 44% compared to the RW plants (Figure 5.1).

The increased concentration of the total metabolites in DHFL plants was associated with the high concentration of shikimate-3-phosphate and erythrose-4-phosphate (Figure 5.2). Total shikimate metabolites were decreased by 37% in DR plants compared to the RW plants. The decrease in the total metabolites in DR-stressed plants was associated with the minimal accumulation of erythrose 4 -phosphate and shikimate-3phosphate (figure 5.2).

The shikimate pathway converts phosphoenolpyruvate (PEP) and D-erythrose 4-phosphate (E4P), intermediates from glycolysis and the pentose phosphate pathways respectively, to chorismate, the universal precursor for all aromatic amino acids (AAA) and many other downstream metabolites (Maeda & Dudareva, 2012). The concentration of PEP was highest in DR and FL plants, although not significantly ($p > 0.05$) different from RW plants. This can be understood when compared to the previous works that indicate PEP is synthesized *via* glycolytic processes (Ting, 1985). Studies have reported that drought and flooding stress triggers glycolysis which generates energy for stress defense activation and adaptation (Hossain et al., 2014; Polacik et al., 2013; Tamang et al., 2021). For instance, Guo et al. (2020) observed that *Triticum aestivum* with drought tolerance had significantly ($p < 0.05$) higher levels of PEP. Also, DHFL and RHDR plants showed non-significant ($p > 0.05$) moderately higher concentrations of PEP compared to the RW plants. As a result, it is evident that drought and flooding stress triggered PEP production, but the return to normal following these stresses (i.e. DHFL and RHDR plants) led to the return of PEP to normal levels. The concentration of erythrose-4-phosphate was not significantly ($p > 0.05$) higher in FL, DHFL, and RHDR plants compared to RW plants. However, DR plants showed a significantly ($p < 0.05$) reduced concentration of erythrose-4-phosphate compared to RW plants. Shikimate kinase, the fifth enzyme in the shikimate pathway, phosphorylates the C3 hydroxyl group of shikimates with ATP as a co-substrate to form shikimate-3-phosphate (Maeda & Dudareva, 2012). The concentration of the shikimate-3-phosphate is significantly ($p < 0.05$) reduced in DR and RHDR plants compared to the RW plants. This can be compared with the study conducted by Correia et al. (2018), which revealed that drought stress downregulated the concentration of shikimate and phenylalanine when compared to RW plants. On the other hand, the concentration of the shikimate-3-phosphate was significantly ($p < 0.05$) higher in FL and DHFL plants compared to the RW plants.

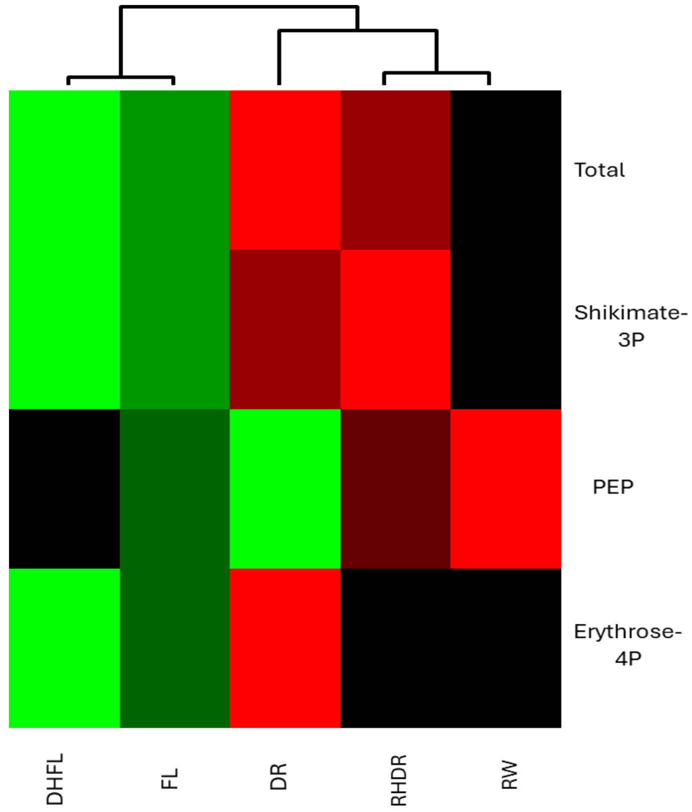

Figure 5.2. Heat map of metabolites profile involved in shikimate biosynthesis in leaves of Mexican mint under water stress. Regular water, RW; Drought, DR; Flooding, FL; Rehydration after drought, RHDR; Dehydration after flooding, DHFL. Metabolite concentrations in each block compartment are normalized across all data for an individual compound such that similar colour intensities between compounds can represent widely differing concentrations. The red colour represents a lower concentration, and the green colour represents the higher concentration of a particular metabolite.

5.3.1.2 Electron transport chain (ETC)

A notable disparity was observed in the intermediate compounds of ETC when subjected to different water stress conditions (Figure 5.3). Total ETC intermediates were significantly ($p<0.05$) increased in DR plants by 115%. The concentration of total ETC metabolites were increased non-significantly ($p>0.05$) in RHDR plants by 8% compared to the RW plants. This increase in total ETC metabolites in DR plants can be attributed to a corresponding increase in concentrations of ATP, ADP, NADH, FAD, and NAD (Figure 5.3). In contrast, a study conducted by Wang et al. (2018) revealed that there was a decrease in the concentrations of ATP in young apple leaves under drought stress conditions. The DHFL plants exhibited a non-significant ($p > 0.05$) reduction in total ETC metabolites by approximately 9% compared to the RW plants. The decline observed in the DHFL plants can be attributed to lower concentrations of FAD, NADH, and ATP (Figure 5.3).

Furthermore, water stress exerts detrimental effects on the performance of PS I and II, as well as on the process of the photosynthetic electron transport chain. During stress conditions, the intake of CO_2 is influenced by various factors such as stomatal closure, changes in CO_2- fixing enzyme activities, disruption of membranes, and low synthesis of ATP (Sharma, et al., 2020). These factors can hinder the activity of RuBisCO and impact the regeneration of RuBP.A study conducted by Sharma et al. (2020) reported a reduction in photosynthetic activity as aresult of water-stress-induced negative impacts on PS II, leading to a decline in the quantum yield of PS II in plant species. Thus, the complexity of the compounds may be attributed to many factors. The alterations in the concentrations of the metabolites are contingent upon the duration, severity, and rate of stress imposition.

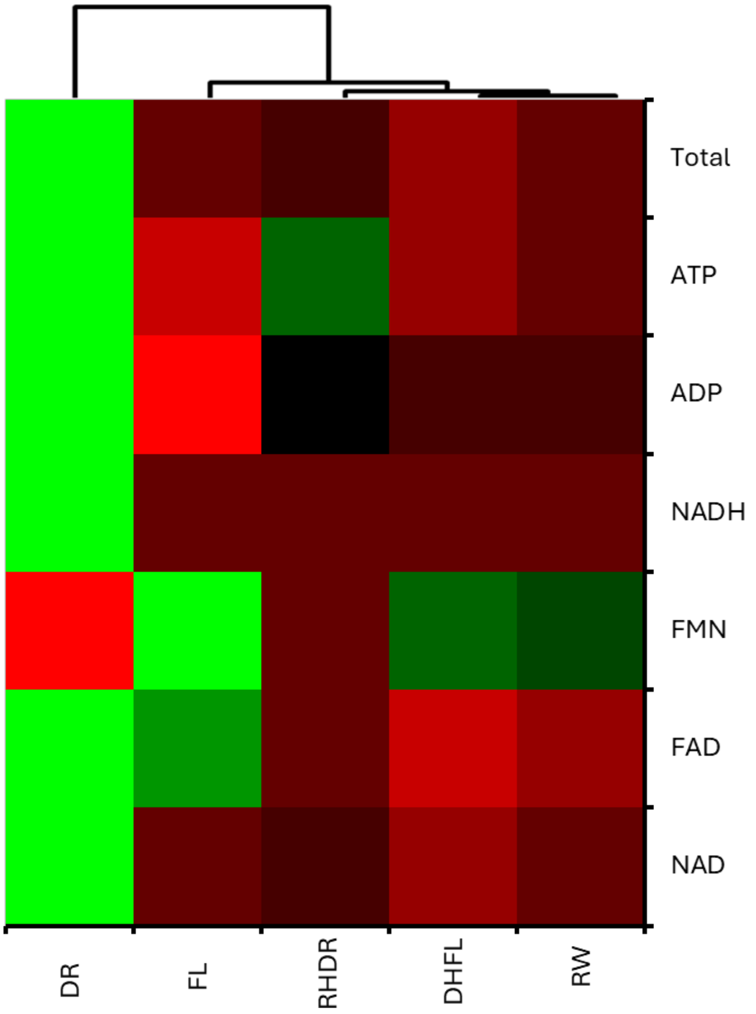

Figure 5.3. Heat map of metabolites profile involved in ETC in leaves of Mexican mint under water stress. Regular water, RW; Drought, DR; Flooding, FL; Rehydration after drought, RH; Dehydration after flooding, DHFL. Metabolite concentrations in each block compartment are normalized across all data for an individual compound such that similar colour intensities between compounds can represent

widely differing concentrations. The red colour represents a lower concentration, and the green colour represents the higher concentration of a particular metabolite.

5.3.1.3 Starch and sucrose biosynthesis

Sucrose is the predominant carbohydrate transported in the phloem of most plant species (Stein et al., 2019). Starch is a storage carbohydrate often produced in plants which is composed of glucose homopolymers (Streb et al., 2012).

In this study, it was observed that DHFL plants increased the total concentration of starch metabolites by approximately 34% (Figure 5.1) compared to RW plants. The spike observed in the overall metabolite concentration of starch in DHFL plants can be attributed to high levels of glucose-1-phosphate, glucose-6P, fructose-6P, DHAP, and glyceraldehyde-3-phosphate. As compared to RW plants, the results showed that DR and RHDR plants declined in their total starch metabolite concentration by about 70% and 0.6%, respectively (Figure 5.1). Thedecrease in metabolite concentration in DR plants is attributed to a robust negative associationof glucose 1-phosphate, glucose-6P, fructose-6P, DHAP, and 3-phosphoglyceric acid (Figure 5.4). The DR plants showed a higher concentration of fructose 1,6-biphosphate and ribulose- bis phosphate, compared to RW plants (Figure 5.4). However, DR plants showed significantly($p < 0.05$) lower concentration of starch synthesis total metabolites compared to RW plants (Figure 5.4). According to a study conducted by Prathap et al. (2020), drought stress led to a decrease in the temporary starch content in leaves. For instance, drought stress resulted in the reduction of starch synthesis in cotton (Zahoor et al., 2017). Similarly, Cuellar-Ortiz et al., (2008) found that starch levels in broad bean leaves plummeted while starch levels in pods increased under drought stress. These suggest that the impact of drought on starch biosynthesisis dependent on the plant tissue examined.

Conversion of glucose 1-phosphate and ATP to ADP-glucose and PPi is the first committed step in starch synthesis in the plastid, catalyzed by ADP-glucose pyrophosphorylase (AGPase) (Kolbe et al., 2005). Starch synthases and branching enzymes use the ADP-glucose as a substrate to enlarge the glucan chains within the starch granule (Kolbe et al., 2005). Findings of the current investigation indicated that there was a significant ($p < 0.05$) rise in the ADP-glucose concentration in FL plants, while there was a non-significant ($p > 0.05$) decline in DR plants, compared to the RW plants (Figure 5.1).

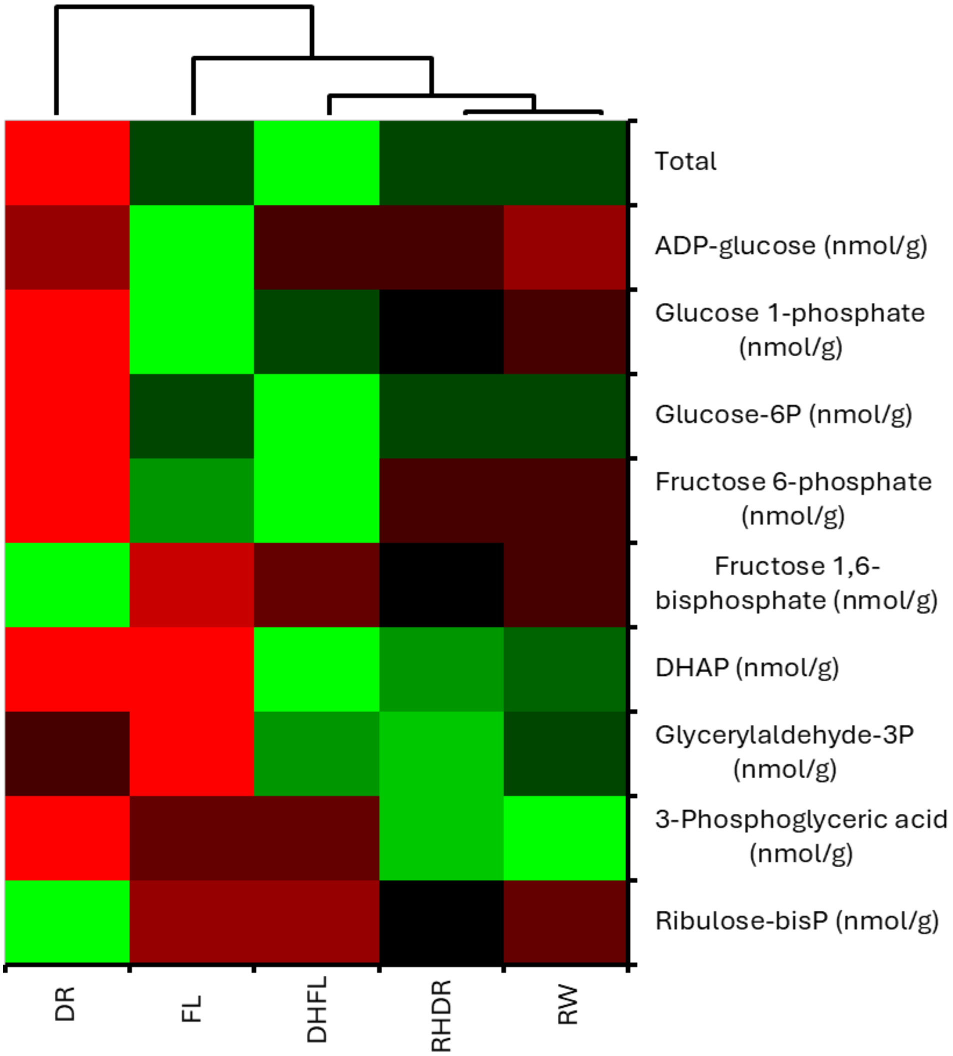

Figure 5.4. Heat map of metabolites profile involved in Starch biosynthesis in leaves of Mexican mint under water stress. Regular water, RW; Drought, DR; Flooding, FL; Rehydration after drought, RHDR; Dehydration after flooding, DHFL. Metabolite concentrations in each block compartment are normalized across all data for an individual compound such that similar colour intensities between compounds can represent widely differing concentrations. The red colour represents a lower concentration, and the green colour represents the higher concentration of a particular metabolite.

Sucrose is the end product of photosynthesis and the main type of sugar that is carried through the phloem (Stein et al., 2019). As plants are sessile they require an intricate and adaptable photosynthetic apparatus to keep their internal environment stable during stress conditions (Rolland et al., 2006).

The investigation uncovered that the concentration of total sucrose metabolites significantly ($p<0.05$) declined by 70% in DR plants compared to RW plants (Figure 5.5). The decrease in total metabolic concentration in DR plants can be attributed to low levels of glucose 1-

phosphate, glucose-6P, fructose 6-phosphate, and UDP-glucose (Figure 5.5). However, the concentration of fructose 1,6-bisphosphate was found to be the highest in DR plants compared to all the other treatments investigated (Figure 5.5). However, prior research has shown that cotton *(Gossypium spp)*, wheat *(Triticum spp),* and cassava *(Manihot esculenta)* upregulated sucrose production in response to drought stress (Cui et al., 2019). The FL plants also showed a non-significant (*p*>0.05) increment in the total concentration of sucrose synthesis by 7% compared to the RW plants. The present findings in FL plants agree with the previous study, which indicates that the disparity between sugar utilization and its synthesis leads to the buildup of sugars in diverse plant tissues (Sami et al., 2016). The concentration of total sucrose metabolites was non-significantly (*p*>0.05) increased by 30% in DHFL plants compared to RW plants. The increased concentration of metabolites in DHFL plants was attributed to the high levels of glucose 1-phosphate, glucose-6P, and fructose 6-phosphate. Furthermore, RHDR and DHFL exhibited a non-significant (p > 0.05) effect compared to the RW plants, which indicates the ability of the stress reversals to recuperate from water stress. Therefore, this study suggests that sugar signaling is crucial for maintaining proper metabolic function, stress tolerance, growth, and development.

Sucrose-P synthase is a crucial component of the plant system that regulates the production of sucrose and the distribution of carbon between starch and sucrose synthesis in the presence of light (Rufty et al., 1983). Although the sucrose-P synthase activity was not investigated, Wang et al., (2022) suggests that, drought stress significantly affects the activity of sucrose-P synthase in plants. This could explain the noticed reduction in total starch and sucrose metabolites in DR plants (Figures 5.4 and 5.5). Further studies can be conducted for the complete understanding of the results obtained.

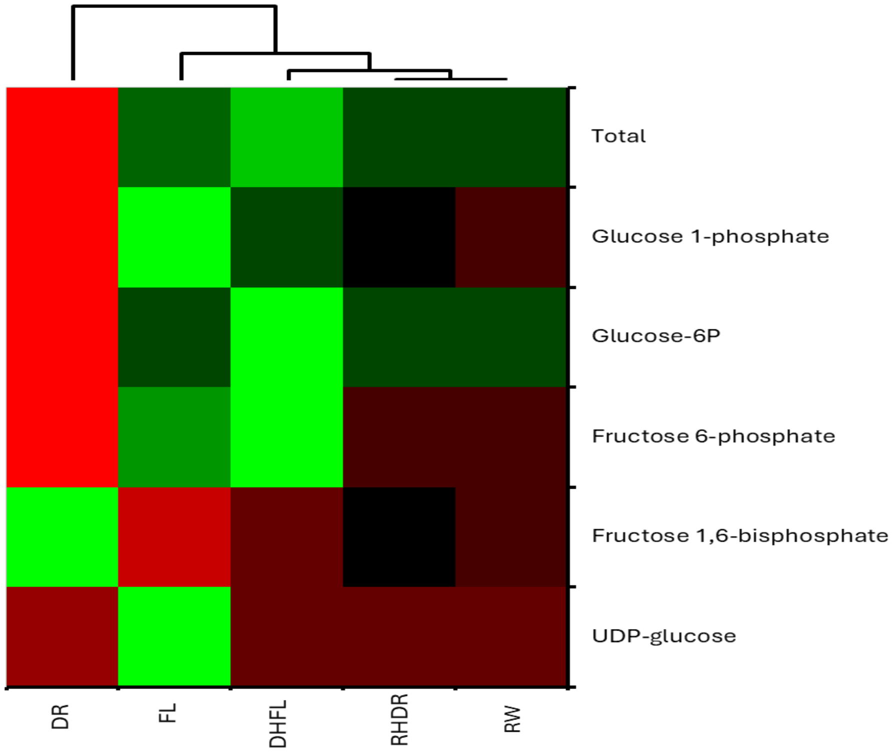

Figure 5.5. Heat map of metabolites profile involved in Sucrose biosynthesis in leaves of Mexican mint under water stress. Regular water, RW; Drought, DR; Flooding, FL; Rehydration after drought, RH; Dehydration after flooding, DHFL. Metabolite concentrations in each block compartment are normalized across all data for an individual compound such that similar colour intensities between compounds can represent widely differing concentrations. The red colour represents a lower concentration, and the green colour represents the higher concentration of a particular metabolite.

5.3.1.4 Hexosamine biosynthesis pathway

The hexosamine biosynthetic pathway (HBP) is a critical pathway that utilizes a variety of energy sources including glucose, acetyl-CoA, glutamine, and UTP to generate uridine diphosphate N-acetylglucosamine (UDP-NAcGlc), an active nucleotide sugar that contains GlcNAc (Chiaradonna et al., 2018).

Diverse water stress conditions elicit various modifications in the hexosamine pathway. Notably, DR plants showed a significant ($p < 0.05$) variation and exhibited a 72% increase in the total hexosamine metabolites compared to the RW plants. After prolonged drought stress, there is an association between the rise in metabolite concentration and UDP-NAcGlc, N-acetyl glucosamine-6-phosphate, fructose-6-phosphate, and Glucosamine 6P concentrations (Figure 5.6). N-acetyl glucosamine-6-phosphate is an essential amino sugar component that plays a critical role in protein glycosylation, glycolipid synthesis, and cell wall composition (Chen et al., 2023). Therefore, the tremendous increase of N-acetyl glucosamine in DR and RHDR plants could indicate the plant survival strategy under stress conditions. A study conducted by Chen et al., (2023) has indicated that higher levels of UDP-NAcGLc are required for plants to effectively cope with detrimental environmental stresses. The FL and DHFL plants significantly ($p < 0.05$) increased their total hexosamine metabolites by approximately 32% and 24% respectively, compared to the RW plants. An increase in the metabolic concentration in FL plants is attributed to an increase in glucosamine-6-phosphate and fructose-6-phosphate compared to the RW plants (Figure 5.6). The physiological response of RHDR plants appeared to be comparable to that of the RW plants. There is a moderate to substantial negative association between the metabolic concentration in RHDR compared to RW plants (Figure 5.6). Extensive research is required to fully understand the broad implications of the hexosamine-signaling pathway and its role under water stress conditions.

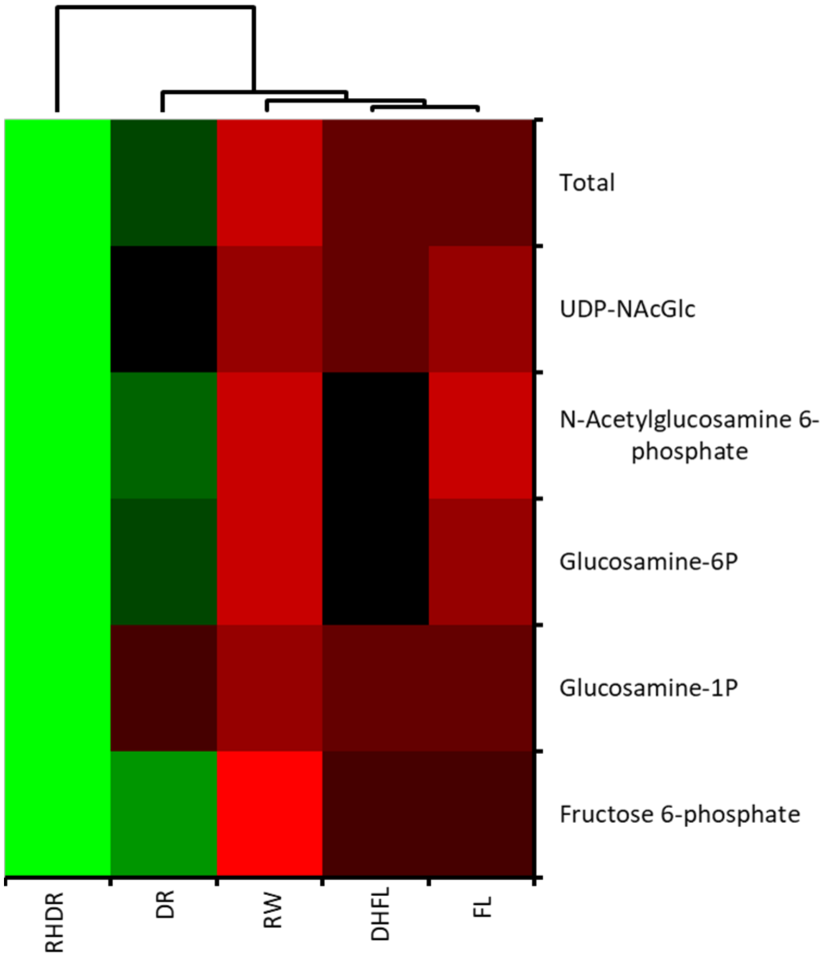

Figure 5.6. Heat map of metabolites profile involved in hexosamine biosynthesis in leaves of Mexican mint under water stress. Regular water, RW; Drought, DR; Flooding, FL; Rehydration after drought, RH; Dehydration after flooding, DHFL. Metabolite concentrations in each block compartment are normalized across all data for an individual compound such that similar colour intensities between

compounds can represent widely differing concentrations. The red colour represents a lower concentration, and the green colour represents the higher concentration of a particular metabolite.

5.4 Conclusion

The current study examined the primary carbon metabolic pathways that undergo modifications in response to water stress. The metabolites associated with the shikimate pathway, starch and sucrose synthesis, the electron transport chain, and the hexosamine pathway exhibit significant alterations under diverse water stress conditions. The total shikimate metabolites showed a significant increase in FL & DHFL plants compared to RW plants. Whereas total ETC metabolites showed a significant elevation in DR and FL plants. Surprisingly, total sucrose and starch metabolites were reduced in DR plants compared to RW plants. The metabolites in the hexosamine pathway showed a tremendous increase under all varying water stress conditions studied compared to RW.

Current research investigates the behavior of central carbon metabolites in *P. amboinicus* under different water stress circumstances. Also, revealed multiple methods implemented and adapted by the plant to endure harsh conditions, which is crucial for understanding the plant's tolerance mechanism. The findings effectively demonstrated the plant's ability to recover from such challenges. Researchers are concentrating on developing resilient plant species for challenging environmental circumstances, requiring a thorough understanding of plant behavior. Understanding the characteristics of these metabolites in crucial plant pathways during stressful situations is essential for enhancing breeding programs to develop better plant species. It is advised to perform further investigation using transcriptomic and proteomic methods to pinpoint the genes and proteins that control plant metabolic pathways during water stress.

CHAPTER 6: ALTERATIONS IN THE VOLATILE COMPOUNDS IN MEXICAN MINT UNDER VARYING WATER STRESS CONDITIONS

Abstract

Water stress is an environmental factor that has a substantial effect on the alterations of secondary metabolites. A study was performed to investigate the alterations in the essential oil compounds in Mexican mint under varying water stress conditions. The water treatments were regular watering (RW), drought (DR), flooding (FL), and resumption of regular watering after flooding (DHFL) or after drought (RHDR). D-Limonene was significantly ($p < 0.05$) more pronounced in DR treatment by 256% when compared to RW. Cymene significantly ($p < 0.05$) increased in DR plants by 37% whereas FL plants had the least by 7% when compared to RW plants. Both Caryophyllene and Eucalyptol were significantly ($p <0.05$) increased in DR plants by 68% and 87% when compared to RW plants. All the compounds analyzed were significantly ($p < 0.05$) increased under DR conditions but least under FL conditions when compared to the RW. In conclusion, water stress prompted researchers to concentrate more on essential oil compounds. Future studies must analyze the quality of the essential oils generated under varied water stress conditions to have a deeper understanding.

6.1 Introduction

The impact of climate change on the overall ecosystem has been substantial, particularly on crop cultivation (Onyekachi et al., 2019). The Intergovernmental Panel on Climate Change (IPCC) has determined that alterations in climatic conditions impose marked pressures on plant life, particularly in low-lying regions predominantly inhabited by developing nations. This has primarily influenced agricultural output (Andy, 2016). Throughout their life cycle, plants are exposed to varying environmental conditions that can impede their growth and development. However, they have evolved distinct mechanisms to adapt to these challenges (Baidya et al., 2022). Stresses can be either biotic, which is imposed by the organisms, or it can be abiotic arising from an excess or deficit in the physical or the chemical environment. Water stress is one of the most detrimental abiotic stresses and hinders global agricultural productivity (Yadav et al., 2021). Climatic changes, such as the rise in evaporation and the decline in regional precipitation caused by global warming are setting the stage for future severe droughts (Azhar et al., 2018).

Water stress is a major global issue that has a detrimental impact on the cultivation of various crops (Azhar et al., 2018). Water stress has the potential to disrupt numerous physiological and biochemical processes, thereby impeding the growth and development of plants (Muhammad Aslam et al., 2022; Yahaya et al., 2022). Water stress is severe in approximately 50% of the global semi-arid and arid regions. Water stress conditions can interrupt essential plant physiological and biochemical activities, eventually reducing plant growth and productivity (Pepe et al., 2022).

In the present study, the test plant is *Plectranthus amboinicus*, which is also known as Mexican mint. It is a prominent member of the Lamiaceae family, which is the most diversified and extensive herbal family in the pharmaceutical industry (Arumugam et al., 2016). Mexican mint can be found in many parts of the world, and it is renowned for its high essential oil output and valuable phenolic compounds (Arumugam et al., 2016). The utilization of this plant is prevalent among indigenous populations residing in tropical rainforest regions, primarily for traditional medicinal practices or as an ingredient in culinary applications (Arumugam et al., 2016). The primary reason can be attributed to the inherent ability of the plant to produce a notable quantity of essential oil containing a substantial concentration of bioactive compounds including carvacrol, thymol, β-caryophyllene, α-humulene, γ-terpinene, p-cymene, α-terpineol, and β-selinene (Lukhoba et al., 2006). Previous research has mainly focused on the effects of drought stress on essential oil content in different plant species. This study aims to investigatethe impact of flooding and drought stress on the chemical components of plants, as well as howthese components modify under different water levels. It is hypothesized that the wateravailability in plants can enormously regulate the chemical composition of essential oils and influence their concentrations. Therefore, this study aims at advancing our understanding of the intricate relationship between the presence of water and the specific chemical compostion of essential oils. This will provide valuable insights necessary for enhancing the efficiency andexcellence of plant growth and quality, whether in natural or agricultural environments.

6.2 Materials and Methodology

6.2.1. Location

This research was conducted in the Department of Plant, Food and Environmental Sciences (PFES), Faculty of Agriculture, Dalhousie University between November 2022 and June 2023.

6.2.2 Preparation of plant material

A healthy parent Mexican mint was chosen from the PFES greenhouse plant stock. The plant was well watered before stem cuttings were taken from the healthy plant chosen by cutting the soft tissue branches from the youngest second and third nodes on the main stem. The stem cuttings were then pruned to 5 cm height leaving four pairs of corresponding leaves. All the stem cuttings were placed in a moist perlite medium (Perlite Canada Inc., Montreal, QC, Canada) in a plastic flat tray of dimensions 50 cm length x 28 cm width x 6.5 cm depth. To maintain the ≥95% relative humidity to induce the rooting, the trays were covered with a dome-shaped transparent cover. The trays were positioned on a planting shelf under continuous 24-hour fluorescent lighting at a temperature of 22°C. The cuttings were sprayed with water twice a day and were ready for transplanting after three weeks.

6.2.3 Transplanting and growing condition

Subsequently, the healthy seedlings were moved to plastic pots with a diameter of 15 cm, with a saucer underneath. These pots were filled with a potting medium consisting of a blend of 200 g of Promix (Premier Horticulture Inc., Quakertown, PA, USA) and 150g of vermicast. The vermicast used in the study was obtained from Red Wiggler (*Eisenia fetida*) worms (Growing Green Earthworms Castings, Lower Wedgeport, NS, Canada). The potted plants were set up in a Biotronette Mark II Environmental Chamber (Lab-Line Instruments Inc., Melrose Park, IL, USA). The growth chamber was set to a 12/12 h day/night light and a 24°/20°C day/night temperature cycles. Before applying the water stress treatment, the plants were left to grow for 120 days under regular watering to field capacity every three days.

6.2.4 Water treatment

There were five different water level treatments i.e., regular watering (RW), prolonged drought (DR); prolonged flooding (FL); rehydration after drought (RHDR), and dehydration after flooding (DHFL). The RW plants were watered every other day to maintain field capacity of the growing medium. The DR plants were subjected to continuous dehydration and the FL plants were subjected to continuous flooding by immersing the Mexican mint pots (15 cm diameter) in a larger pot (20 cm diameter) filled with water. Both DR and FL treatments were imposed for 8 weeks. The stress reversals were dehydration and resumption of regular watering after continuous flooding for 4 weeks (DHFL); and rehydration and resumption of regular watering after continuous drought for 4 weeks (RH). The experimental treatment was arranged in a completely randomized design (CRD) with three replications.

6.2.5 Essential oil extraction from fresh leaves of Mexican mint using a non-polar hexane solvent

Essential oils from Mexican mint were extracted from the fresh leaves as described by Mohd-Hairul (2010) using a non-polar hexane solvent. After 8 weeks of continuous treatment application, all the green leaves were harvested and dipped immediately in liquid nitrogen (N). Later, the leaves were ground into fine powder and were stored at -80°C until further analysis. A 5 g of ground leaf powder was soaked in 40 mL of hexane. After 72 hours of soaking the samples, they were filtered using 125 mm Whatman filter paper to remove the residues. Subsequently, the concentrated extract solution was obtained by removing n-hexane through the rotary evaporator at 65°C. The colorless n-hexane was separated from the sample and was collected in the receiving flask. The resulting extract concentrate was dissolved and made up to 4 mL volume using non-polar hexane.

6.2.6 Gas chromatography-mass spectrometry analysis of the extracted essential oil

The analysis of volatile compounds was conducted using gas chromatography-mass spectrometry (GC-MS) as described by Ashaari et al., (2021). A 100µL volume of the sample was diluted to a final volume of 2mL by adding non-polar hexane and was filled into the vials. The essential oil was injected into the GC injection port with a 1:50 split mode and separated on a nonpolar Rxi – 624 silicon MS capillary column (30 m × 0.25 mm) with a 1.4 µm df film (Restek) fitted to an Auto System XL GC-MS. A universal fused Silica press tight connector (RK20429) was used to connect the guard column (RK10029-Rxi Guard Column (5mx0.25mm)) with the column. The injector temperature was 260°C and the oven temperature was programmed at an initial temperature of 80°C for 2 min, rising from 25°C per min to 175°C and maintained at that temperature for 1 min. The temperature subsequently increased by 45°C per min to 250°C and was maintained at that temperature for a further 4.6 min. The carrier gas was helium at a constant pressure of 5 kPa. Data acquisition for a specific mass of interest was conducted using selected ion monitoring (SIM) mode in GC-MS. The Scion 456 gas chromatography was directly interfaced with an Auto System Triple Quadrupole (TQ) mass spectrometer. After exiting the GC column, the components underwent ionization and fragmentation in the mass spectrometer using electron or chemical ionization sources. Ions were separated at this location according to their distinct mass-to-charge (m/z) ratios. The components exit the GC column and enter the MS by a transfer line with a temperature of 260°C. The source temperature was also 260°C. Sample ionization was 70 eV electron impact and was analyzed in positive mode. The areas of the peaks were directly proportional to the amount of the relevant molecule. GC-MS analysis of complex samples resulted in the generation of several peaks in the gas chromatogram. Each peak corresponded to a distinct mass spectrum, which was utilized for the identification of individual compounds. Structural determination was a comparison of mass spectral patterns to databases.

6.2.7 Biochemical analysis

6.2.7.1 Total flavonoids

Total flavonoids were determined as described by Chang et al. (2002) with some modifications. A 0.2 µL of essential oil extract was homogenized with 2.5 ml of 95% methanol. The mixture was centrifuged at 13000 *g* for 10 min and 500 µL of the supernatant will be transferred into a new tube. To each tube, 1.5 mL of 95% methanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water were added. The mixture was vortexed and incubated for 30 min at room temperature and absorbance was measured at 415 nm against a blank. Flavonoids content was estimated using a quercetin standard curve. Total flavonoids content will be calculated using the formula:

Flavonoid (%) = (flavonoids)(µg/mL) x total volume of methanolic extract (mL)/ mass of the extract (µg) x 100

6.2.7.2 Total phenolics

Total phenolic content (TPC) was determined by the Folin–Ciocalteu assay described by Ainsworth & Gillespie, (2007b) with little modification. A 0.2µl of essential oil extract per treatment was homogenized in 2 ml of ice-cold 95% methanol and incubated in the dark at room temperature for 48 hrs. The mixture was centrifuged at 13000 g for 5 min and 100 µl of supernatant was transferred into a new microfuge tube. 200 µl of 10% Folin-Ciocalteau reagent was added and vortexed for 5 min. 800 µL of 700 nM sodium carbonate (Na_2CO_3) was added, vortexed for a min and incubated at room temperature (25°C) for 2 h. The absorbance of the resultant mixture was measured at 765 nm. Total phenolic content was estimated using gallic acid equivalents standard curve and was expressed as mg gallic acid equivalents per g of the sample.

6.2.8 Calculation and statistical analysis

Statistical analysis was performed using Minitab version 21. The values were expressed as a means of three replicates with standard deviations (SD). All the data were subjected to a one-way analysis of variance. Tukey's honestly significant difference post-test was used to separate the means at a 5% significance level.

6.3 Results and discussion

The present study showed a detailed characterization of the chemical constituents of volatile oils isolated from Mexican mint. Five specific chemical compounds, namely limonene, linalool, cymene, eucalyptol, and caryophyllene.

6.3.1 Monoterpenes

D-Limonene, a monocyclic terpene, is synthesized by over 300 plant species (Burdock, 2016). D-Limonene was the major compound that was detected in the GC-MS analysis of the essential oil extracted from the aerial parts of the Mexican mint (Figure S6.1). D-Limonene was more pronounced in DR plants compared to all the other stress treatments studied (Figure S6.1). The DR plants reported a significant ($p < 0.05$) increase of 256% in the concentration of D-limonene in comparison to the RW plants. These findings were in line with studies by Kulak, (2019), whose findings showed that rosemary (*Rosmarinus officinalis*) had a higher concentration of D-limonene during drought conditions than it did during regular watering. The results of the research on summer savory (*Satureja hortensis* L.) indicated that the quantity and potency of the essential oil indicated an increase in response to extreme drought conditions (Baher et al., 2002b). Under drought stress, fennel (*Foeniculum vulgare*) showed a substantial rise in D-limonene concentrations (Zali et al., 2018). Similarly, FL plants reported a non-significant ($p > 0.05$) increase of 16% in the concentration of D-limonene compared to RW plants. Plants that recovered from stress conditions i.e., DHFL and RHDR plants had their D-limonene concentrations increased by 74% and 56% respectively (Figure 6.1). Based on the ANOVA, the RHDR and DHFL plants exhibited no significant change ($p > 0.05$) compared to RW plants. A study conducted by Kulak, (2019) had similar outcomes, whereas the stress recovery method of withholding water for a specific duration followed by reintroducing plants to normal conditions resulted in increased quantities of D-limonene. Studies have consistently reported that plants exposed to water stress accumulate high concentrations of secondary metabolites, which include all different classes of natural products (Ghorbanpour et al., 2017). Under stress conditions, there was a notable increase in the amounts of secondary metabolites, including simple and complex phenols, as well as various types of terpenes (Ghorbanpour et al., 2017). A study conducted by Nowak et al. (2010) revealed that water stress results in a massive increase in the concentration of monoterpenes. The biosynthesis of monoterpenes exhibits an increase in response to water stress conditions(Ghorbanpour et al., 2017). Hence, this phenomenon may provide a plausible explanation for the observed increase in D-limonene concentrations in response to water stress circumstances. Monoterpenes are secondary metabolites that are present in plants (Anandakumar et al., 2021). Monoterpenes have a range of pharmacological activities, including antibacterial, antifungal, antioxidant, anticancer, vasorelaxant, hypotensive, and antispasmodic actions (Tan et al., 2016; Vieira et al., 2018). D-Limonene is a widely recognized monoterpene compound that is frequently utilized as a constituent of aroma in essential oils (Suh et al., 2017; Ravichandran et al., 2018). Additionally, it is effective in treating a variety of diseases and disorders. Given D-limonene's diverse range of beneficial characteristics, it can be utilized efficiently to enhance human well-being (Anandakumar et al., 2021). Therefore, under water stress conditions, increasing the concentration of D-limonene could improve the essential oil's quality by enhancing its beneficial characteristics.

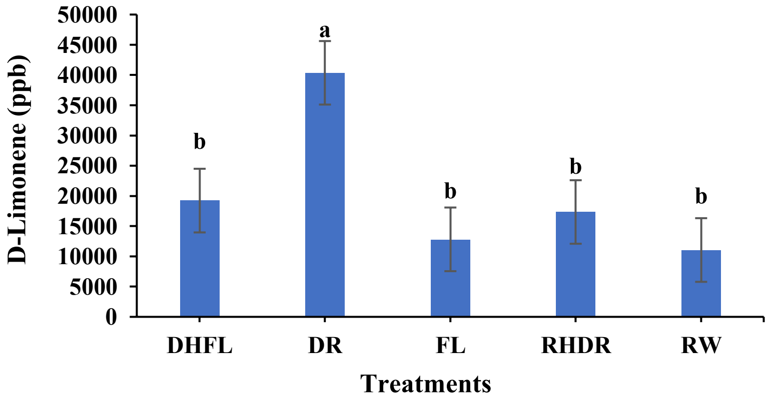

Figure 6.1. Response of D-limonene compound in Mexican mint plants under varying water stress conditions; dehydration after flooding (DHFL), drought (DR), flooding (FL), rehydration after drought (RHDR), regular watering (RW). Different alphabetical letters on the bars denote significant differences according to Tukey's honestly significant difference post-test analyses at a significant level of 5%.

p-Cymene is characterized by the presence of a benzene ring with a methyl and an isopropyl group as substituents (Marchese et al., 2017). The DR plants showed a significant ($p < 0.05$)

variation and reported an increase of 37% in the concentrations of *p*-cymene (Figure 6.2). A similar trend has been noticed in rosemary (*Rosmarinus officinalis*) (Kulak, 2019). The study conducted by (Baher et al., 2002b) indicated that there was an increase in the quantity of essential oil in Summer savory (*Satureja hortensis* L.) in response to water stress when compared to regularly watered plants. The summer savory (*Satureja hortensis* L.) samples that were exposed to a vigorous drought treatment had higher levels of volatile oil in comparison to samples treated with a modest drought treatment (Baher et al., 2002b). Similarly, drought remarkably increased the concentrations of *p*-cymene and essential oil components infennel (*Foeniculum vulgare*) (Zali et al., 2018). The FL plants exhibited a marginal elevation in the content of *p*-cymene by 7% compared to RW plants. The stress reversals, specifically DHFL and RHDR plants, increased the concentration of *p*-cymene by 18% and 7% respectively compared to RW plants. There was no significant (p>0.05) difference observed between the FL, DHFL, and RHDR plants, in comparison to the RW plants (Figure 6.2). The observation here possibly indicates that the stress reversals aided plants to retrieve back to normal conditions. In a similar vein, a research investigation carried out by Kulak, (2019) demonstrated a decline in the concentration of *p*-cymene in the recovery phase after periods of drought. Therefore, this could mean that when plants were in drought conditions the concentrations for *p*-cymene were high, and when re-watered the plant was brought back to regular conditions the concentration started to decline. The *p*-cymene is a naturally occurring alkyl-substituted aromatic hydrocarbon (Marchese et al., 2017). This compound is widely recognized as the most prominent monoterpene compound found in aromatic plants (Marchese et al., 2017). Various research has proven the pharmacological characteristics of *p*-cymene monoterpenes, which comprise an antioxidant, anti-inflammatory, antiparasitic, antidiabetic, antiviral, anticancer, antibacterial, and antifungal capabilities (Balahbib et al., 2021). Therefore, in conditions of water stress, increasing *p*-cymene concentrations could boost its therapeutic properties and possibly result in an improvement in the quality of the essential oil.

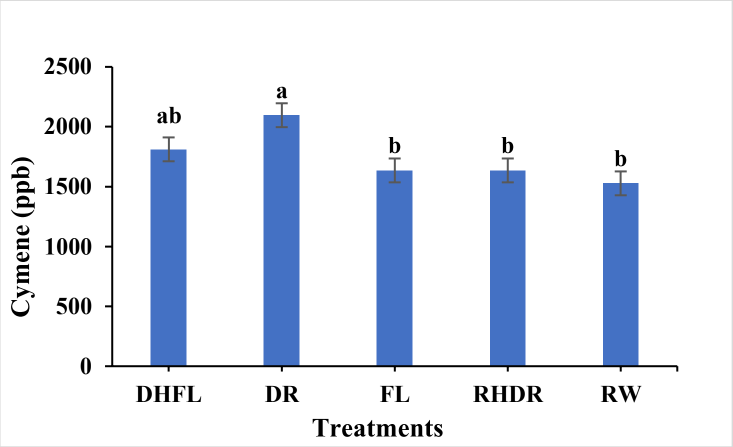

Figure 6.2. Response of cymene compound in Mexican mint plants under varying water stress conditions; dehydration after flooding (DHFL), drought (DR), flooding (FL), rehydration after drought (RHDR), regular watering (RW). Different alphabetical letters on the bars denote significant differences according to Tukey's honestly significant difference post-test analyses at a significant level of $p < 0.05$.

Linalool is a prominent component found in numerous essential oils. Linalool is a monoterpene alcohol, that exhibits antibacterial and antifungal properties (Pattnaik et al., 1997), along with antioxidant (Liu et al., 2012), anti-inflammatory (Peana et al., 2002), and anticancer effects (Jana et al., 2014). In the present study, it was shown that DR plants demonstrated a significant ($p<0.05$) increase in the concentration of linalool, with a notable rise of 38% compared to RW plants (Figure 6.3). Similarly, a study conducted on sweet basil (*Ocimum basilicum*) has revealed that the concentration of linalool has increased under drought stress (Simon et al., 1992). Contrastingly, a study conducted by Ashrafi et al., (2018b) revealed that basil plants regularly watered at 100% field capacity were reported to show the highest concentrations of linalool with no significant ($p >$ 0.05) variation when compared to plants treated with 50% field capacity. The stress reversals, DHFL and RHDR plants, exhibited a significant ($p<0.05$) increase in the concentration of linalool by 33% and 35% respectively compared to the RW plants. It was also shown that DR plants, as well as the stress reversals DHFL and RHDR plants, do not exhibit significant ($p < 0.05$) variance among the treatments. However, when compared to RW plants these treatments exhibit a significant ($p < 0.05$) variation. The FL plants have exhibited a marginal elevation in the concentration of linalool by 6% compared to RW plants. Plants that experience stressful conditions exhibit modifications in both their primary and secondary metabolism, which can have potential effects on several quantitative and qualitative traits (Salam et al., 2023). It is well known that a variety of environmental factors, including the availability of water, can affect the build-up of naturally occurring compounds in plants (Rioba et al., 2015; Szabó et al., 2017; Manukyan, 2019). Abiotic stress-inducing situations have been demonstrated to increase the exceptional defensive functions of monoterpenes and other volatile isoprenoids in plants (Khakdan et al., 2021). The release of notable monoterpenes such as linalool, is an indication of oxidative defense mechanisms in reaction to water stress. These processes are of utmost importance in protecting plants from oxidative stress (Khakdan et al., 2016). This could be one of the reasons for the increase in the concentrations of the monoterpenes such as linalool under water stress conditions. The widespread study of linalool is attributed to its abundant presence in nature and its significant incidence as a key ingredient in plants that have a well-documented history of use. Therefore, in situations of increased stress, the increase in levels of linalool may play a role in enhancing its advantageous characteristics, potentially leading to an enhanced overall quality of the essential oil.

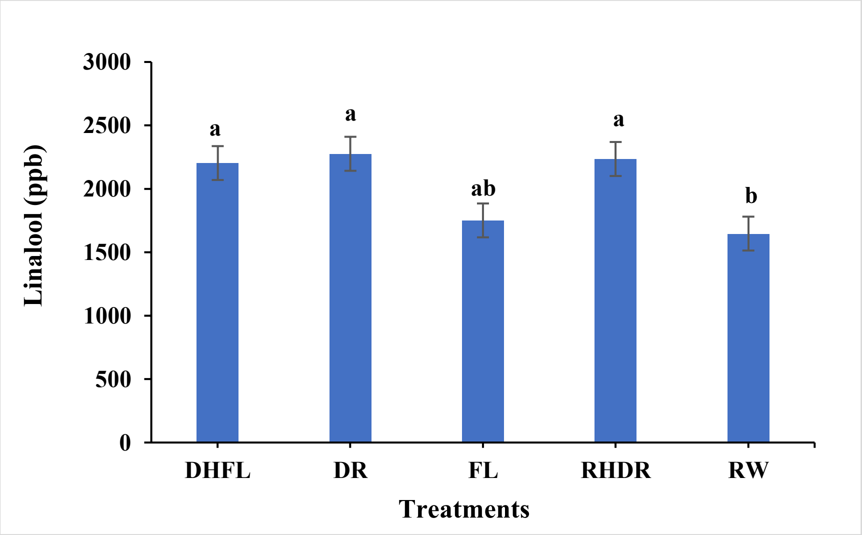

Figure 6.3. Response of linalool compound in Mexican mint plants under varying water stress conditions; dehydration after flooding (DHFL), drought (DR), flooding (FL), rehydration after drought (RHDR), and regular watering (RW). Different alphabetical letters on the bars denote significant differences according to Tukey's honestly significant difference post-test analyses at a significant level of $p < 0.05$.

Eucalyptol is a naturally occurring substance present in the essential oil component of several plant sources such as eucalyptus, rosemary, and camphor laurel (Seol et al., 2016). In the present study, the DR plants have shown a significant ($p < 0.05$) increase in the concentration of eucalyptol by 87% compared to the RW plants (Figure 6.4). In contrast, a study conducted by Kulak (2019) on rosemary (*Salvia Rosmarinus*) found that the percentage of eucalyptol was not significantly ($p > 0.05$) altered under water stress conditions. A study conducted by Kulak, (2020) found that the amount of eucalyptol in basil (*Ocimum basilicum)*, sage (*Salvia Officinalis*), and lavender (*Lavandula officinalis)* increased as the plants matured and encountered drought stress. The stress reversals i.e., DHFL and RHDR plants have shown a significant ($p < 0.05$) increase in the concentration of eucalyptol by 41% and 16% respectively compared to RW plants. Similarly, plants exposed to regular watering after a period of drought showed the highest percentage of eucalyptol compared to the regularly watered plants (Kulak, 2019). The FL plants have shown a slight increase in the concentration of eucalyptol by 6% but shown no significant ($p > 0.05$) variation to the RW plants. The antioxidant characteristics of eucalyptol are of significant importance in its therapeutic capacity, since it demonstrates efficacy in the mitigation of reactive oxygen species (ROS) and enhancement of cellular defense systems (Reuter et al., 2010). Eucalyptol has been shown to possess the capacity to reduce lipid peroxidation (Rodrigues et al., 2020). Consequently, an increase in eucalyptol concentrations may be a stress-reduction mechanism used by Mexican mint. The medicinal benefits of eucalyptol have been recognized since ancient times and continue to hold importance in modern circumstances.

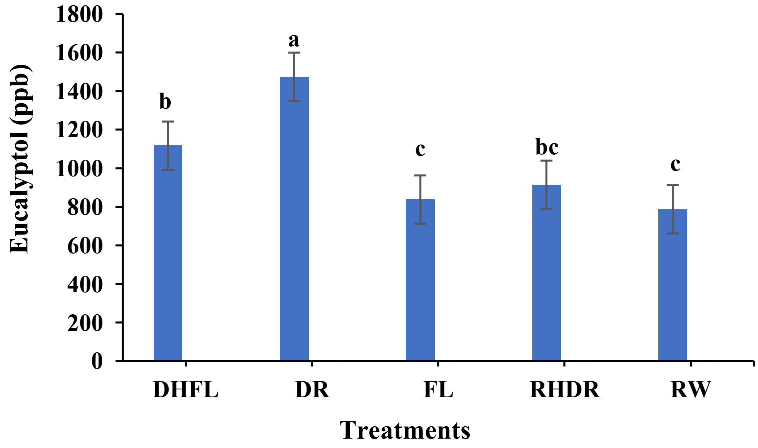

Figure 6.4. Response of Eucalyptol compound in Mexican mint plants under varying water stress conditions; dehydration after flooding (DHFL), drought (DR), flooding (FL), rehydration after drought (RHDR), regular watering (RW). Different alphabetical letters on the bars denote significant differences according to Tukey's honestly significant difference post-test analyses at a significant level of $p < 0.05$.

The significant emissions of important monoterpenes, such as linalool and limonene, are associated with protection against water-induced stress, indicating a strong capacity to quench oxygen and act as antioxidants (Loreto et al., 2010). Research indicates that sesquiterpenes may have a specialized role in plant cells as stress response agents, helping to counteract the harmful effects of stress through antioxidant mechanisms, as demonstrated by their effectiveness in scavenging ROS (Loreto et al., 2010). Moreover, recent research suggests that excessive production of monoterpenes and sesquiterpenes may possess comparable antioxidant characteristics, serving as protective agents against H_2O_2 (Khakdan et al., 2021). They are considered a standard for oxidative defense systems during water stress to shield plants from oxidative stress (Khakdan et al., 2021). Multiple studies have consistently demonstrated that the presence of drought and flooding circumstances induces an acceleration of glycolysis as a mechanism to generate energy for the activation and adaption of stress defense (Abbey et al., 2023a). For instance, (Guo et al., 2018) observed a significant ($p < 0.05$) increase in glucose, PA, and PEP concentrations in drought-tolerant genotypes of wheat. In reaction to dry conditions, *Pinus painaster* (de Miguel et al., 2016), *Thymus vulgaris*, *T. Kotschyanus* (Ashrafi et al., 2018b), and *Lotus japonicus* (Bailey-Serres et al., 2012) exhibited an accumulation of glucose and other glycolytic intermediate metabolites. Therefore, the increase in glycolysis may lead to an increase in the mevalonate pathway which eventually increases the production of monoterpenes and sesquiterpenes under water stress conditions.

6.3.2 Sesquiterpene

Caryophyllene, a bicyclic sesquiterpene molecule, is a constituent of the essential oils found in various plants. In the current research, DR plants exhibited a statistically significant variation ($p<0.05$) in the content of β-caryophyllene. The concentration of β-caryophyllene exhibited a 68% increase in DR plants compared to RW plants. Similarly, a study conducted by Morshedloo et al., (2017) revealed that the concentration of β-caryophyllene in *O. vulgare subsp. virens* was shown to be elevated in response to water stress, suggesting a potential beneficial effect in the regulation of water stress. Contrastingly, a research investigation conducted on rosemary (*Rosmarinus officinalis*) has demonstrated a decrease in the concentrations of β-caryophyllene in response to drought-induced stress (Kulak, 2019). The FL plants showed a slight increase in the concentration of β-caryophyllene by 8% compared tothe RW plants (Figure 6.5). Also, the stress reversals i.e., DHFL and RHDR plants showed an increase in the concentration of β-caryophyllene by 23% and 4% (Figure 6.5) respectively compared RW plants. According to the ANOVA FL, DHFL and RHDR plants showed no significant ($p > 0.05$) difference in the concentration of β-caryophyllene compared to RW plants (Figure 6.5). In contrast, a study conducted by (Kulak, 2019) demonstrated that rosemary *(Rosmarinus officinalis)* exhibited a decrease in the concentration of β-caryophyllene when subjected to stress recovery through rehydration following a period of drought. This decline in rosemary was found to be statistically significant ($p < 0.05$) when compared to the control group that received regular watering. Several studies have demonstrated that sesquiterpenes can carry out distinct stress response functions within plant cells, thereby mitigating the adverse effects of stress through antioxidant-mediated mechanisms (Yadav et al., 2014). These findings suggest that sesquiterpenes exhibit notable efficacy in scavenging ROS (Yadav et al., 2014).

Additionally, β-caryophyllene being a natural sesquiterpene may exhibit comparable antioxidant characteristics, serving as protective agents against H2O2. Non-enzymatic oxidative defense systems are regarded as an essential requirement in the context of water stress, as they play a significant role in safeguarding plants against oxidative stress (Khakdan et al., 2016).

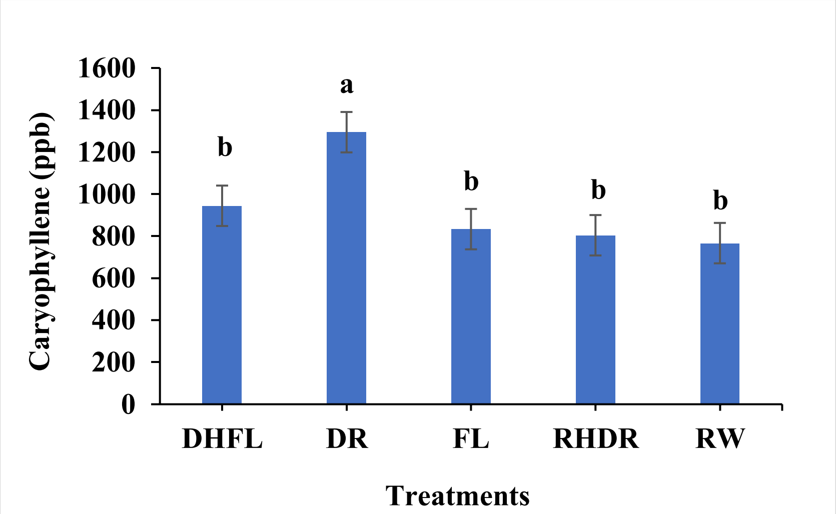

Figure 6.5. Response of β-Caryophyllene compound in Mexican mint plants under varying water stress conditions; dehydration after flooding (DHFL), drought (DR), flooding (FL), rehydration after drought (RHDR), and regular watering (RW). Different alphabetical letters on the bars denote significant differences according to Tukey's honestly significant difference post-test analyses at a significant level at 5%.

Sesquiterpenes are a vast and diverse collection of natural chemicals. Considering the crucial function of these chemicals in plant defense response, their rise during water stress aligns with the carbon balance theory (Caser et al., 2019b). The above-mentioned theory suggests that plants allocate more resources to defensive mechanisms when their development is restricted. For instance, significant quantities of sesquiterpenes were detected in *Inula Montana* plants exposed to varying levels of water stress (Caser et al., 2018). The study conducted by Caser et al., (2019b) found that plants exposed to drought experienced a decrease in hydrocarbon sesquiterpenes and an increase in oxygenated sesquiterpenes. These dynamics can be seen as a protective strategy against a hostile environment, such as severe water scarcity.

6.4 Antioxidant properties of the essential oil extracted from Mexican mint under water stress conditions

6.4.1 Phenolics and flavonoids

Phenolic chemicals, known for their antioxidant characteristics that ensure plant survival, have garnered interest for their significant role in preserving human health (Bettaieb et al., 2011). The present study aimed to determine the overall phenolic content in the essential oil derived from Mexican mint. The DR plants experienced a significant ($p < 0.05$) increase in the phenolic concentration by 340% compared to RW plants (Figure 6.6A). The study conducted by Mirniyam et al (2022) had revealed that the concentration of polyphenolics has increased under drought stress in ahowan (*Trachyspermum ammi* L.). Conversely, a study conducted by Albergaria et al., (2020) on 43 species of medicinal plants found that the frequently held belief that there is a general rise in the concentration of phenolic compounds in response to water stress is frequently inaccurate. In practical terms, these plants may experience a decline or no change in concentration when exposed to water stress. Drought-stressed plants of *Labisia pumila* (Jaafar et al., 2012), *Salvia officinalis* (Radwan et al., 2017), and *Salvia sinaloensis* (Caser et al., 2018) have exhibited an enhancement in the production of phenolic compounds. The concentration of phenolics significantly increased by 161% in FL plants compared to RW (Figure 6.6 A).

Flavonoids are a widely occurring class of chemicals found in nature (Wang et al., 2018). Their wide range of profitable bioactive effects, including antiviral, antibacterial, anti- inflammatory, cardioprotective, antidiabetic, anticancer, and anti-aging properties,has consistently garnered significant attention and has been successfully substantiated byvarious research (Krych et al., 2013; Ragab et al., 2014; Tian et al., 2014). In this study, we assessed the total flavonoid content extracted from Mexican mint's essential oil. The DR plantsexhibited a significant ($p < 0.05$) increase in total flavonoid concentration by 324% comparedto RW plants (Figure 6.6 B). The study conducted by Caser et al., (2019) has revealed that water deficit stress in sage (*Salvia dolomitica Codd*) led to a decrease in the overall flavonoidcontent in the leaves under moderate and severe drought conditions, as compared to regular watering. The FL plants exhibited a significant ($p < 0.05$) increase in total flavonoid content by 212% compared to the RW plants. The stress reversals i.e. DHFL and RHDR plants have also shown a significant ($p < 0.05$) increase in the total flavonoid concentration by 111% and 68% respectively compared to RW plants (Figure 6.6 B).

Flavonoids and phenolics are plant polyphenols that are produced via distinct processes, such as malonic acid and shikimic acid (Kamalizadeh et al., 2019). The latter refers to the primary route by which aromatic amino acids are synthesized from basic carbohydrate substrates (Kamalizadeh et al., 2019). The equilibrium between the production and consumption of carbohydrates significantly influences the increase in flavonoid and phenolic compounds (Gharibi et al., 2016). Phenolic concentration is believed to increase in a high CO2 environment mostly because of the rise in the source or sink ratio (Peñuelas et al., 1998). Moreover, the modification of phenolic compounds during water stress may be attributed to variations in leaf structure and metabolic components that serve to protect against oxidative damage (Hernández et al., 2004). Each plant species may employ a distinct strategy for the distribution of flavonoid compounds inside the subcellular compartment, which could vary depending on the species (Winkel et al., 2002). Water stress can impede the movement of soluble sugar inside cells, potentially leading to an increase in flavonoids and phenolic compounds during periods of water deficit. This rise may be attributed to higher levels of soluble carbohydrates within plant cells (Jaafar et al., 2012). Water stress triggers oxidative stress in plants, leading to the generation of ROS (Munné et al., 2003). Polyphenols and flavonoids are very adaptable organic molecules that allow plants to effectively scavenge ROS (Di Ferdinando et al., 2014). Water-stressed plants of *Labisia pumila* (Jaafar et al., 2012), *Salvia officinalis* (Radwan et al., 2017), and *Salvia sinaloensis* (Caser et al., 2018) have exhibited an enhancement in the production of phenolic compounds under stress conditions.

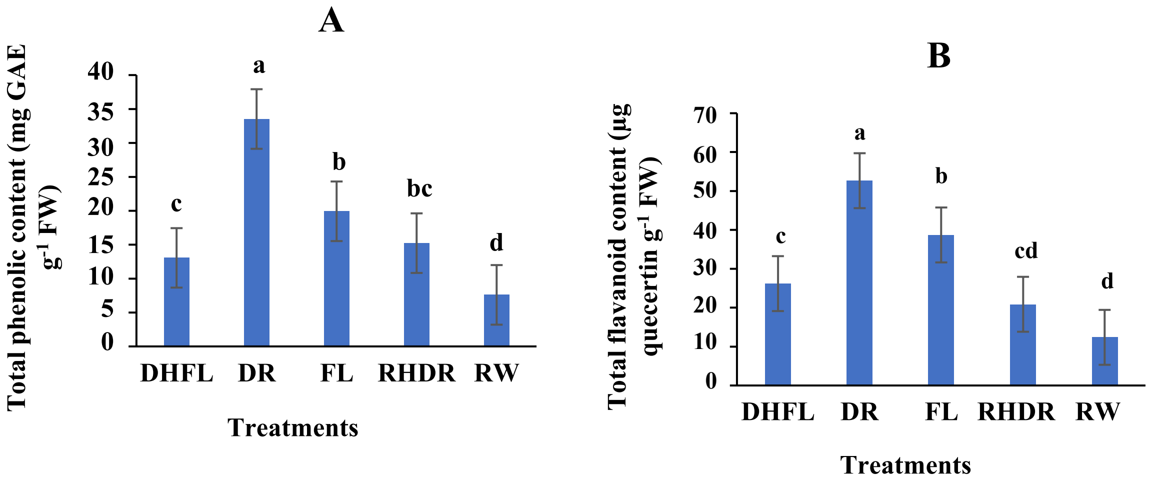

Figure 6.6. Total **(A)** phenolic and **(B)** flavonoid content in Mexican mint plants under varying water stress conditions; Dehydration after flooding (DHFL), Drought (DR), Flooding (FL), Rehydration after drought (RHDR), and Regular watering (RW). Different alphabetical letters on the bars denote significant differences according to Tukey's honestly significant difference post-test analyses at a significant level of $p < 0.05$.

6.5 Conclusion

The results of the study have revealed that water stress causes significant alterations in the chemical constituents of Mexican mint. D-limonene showed the highest concentration among all the compounds that were analyzed. The study uncovered that the chemicals limonene, cymene, linalool, eucalyptol, and caryophyllene consistently exhibited a significant increase in their concentration in DR plants compared to RW plants. As anticipated earlier drought stress can increase the production of secondary metabolites in Mexican mint as a response to environmental stresses. Also, this study offers a thorough summary of the variations in active ingredients and antioxidant qualities in Mexican mint. The study also emphasizes how critical it is to incorporate these superior plant kinds into domestication initiatives and the food industry, especially in areas with limited water resources. Additionally, further investigation is required to gain an in-depth knowledge of the alterations occurring in the plant's components in response to water stress conditions.

CHAPTER 7: CONCLUSION

7.1 Thesis findings

Current global climatic conditions and weather patterns cause serious threats to agricultural production. These adverse climatic conditions lead to various environmental stresses which could be both biotic and abiotic. Among these abiotic stresses, water stress causes a detrimental effect on crop yield. Adverse rainfall patterns caused by climate change make water stress a major concern in today's agriculture. Water stress is one of the major constraints for the food supply and a balanced environment. The overall aim of the thesis is to advance our knowledge of the response of *Plectranthus amboinicus* to varying water levels.

Chapter 3 revealed that water stress has a detrimental effect on plant growth, physiological processes, and chemical composition. This objective provides evidence that timing, duration, and amount of growing medium water level determine the severity of water stress that influences a plant's growth and development performance. Water stress impacts the photosynthetic machinery of Mexican mint. The reduced photosynthetic capacity of the plant eventually leads to reduced plant growth and productivity. Additionally, under water stress conditions, the relative water content of the leaves decreases, which is a significant factor because leaves are primarily responsible for the essential oil production in Mexican mint. Water stress triggers the buildup of reactive oxygen species (ROS) which leads to oxidative stress and cellular damage.

Chapter 4&5 showed that 68 key metabolites involved in the Calvin cycle, glycolysis, tricarboxylic acid (TCA), pentose phosphate pathway (PPP), and nucleotide biosynthesis were significantly affected by both flooding and drought. Additionally, there was an observed increase in the concentration of metabolites. These metabolites play a vital role in energy production and serve as carbon building blocks for the synthesis of larger molecules, hence improving the plant's ability to tolerate water stress. Several studies have investigated the impact of water stress on morpho-physiological characteristics in this genus. However, there is a lack of extensive research on the metabolic changes in Mexican mint. The finding of this study is the first to reveal the metabolic alteration of *Plectranthus* to flooding and drought stress and elucidates the mechanism of water stress tolerance within this species. Furthermore, the study provided valuable insights into the water stress tolerance mechanisms of Mexican mint under various water stress conditions and has effectively demonstrated the plant's capacity for recovery from these types of stresses.

Chapter 6 showed the changes in chemical components of essential oil produced from Mexican mint leaves exposed to water stress conditions. A significant increase in the concentration of essential oil compounds was observed in water stress conditions. The primary components that were investigated include D-limonene, linalool, p-cymene, eucalyptol, and caryophyllene. Notably, the concentration of D-limonene showed a significant increase under drought condition. Under water stress conditions, the content of antioxidants was significantly increased, which may indicate a boost in the therapeutic properties of Mexican mint. Overall, this objective elucidates the influence of water stress on the essential oil components of Mexican mint.

7.2 Thesis contributions

The findings presented in this thesis addressed various knowledge gaps and enhanced our understanding of how Mexican mint responds to different levels of water stress. Multiple studies have investigated the morpho-physiological responses of several *Plectranthus* plant species under drought stress. The current research prioritized gaining an extensive grasp of the Mexican mint's responses to different water conditions. Gaining insight into the metabolic profile of a plants subjected to water stress is crucial for understanding the plant's mechanism of stress tolerance. This study is the first to reveal the metabolic response of Mexican mint to different levels of water stress. Prior research has documented the presence of essential oil constituents within the *Plectranthus* genus (Sabra et al., 2018; Ngo-Mback et al., 2019; Ashaari et al., 2021). However, limited investigation has been conducted on the impact of different levels of water stress on the composition of these essential oil compounds. This study also filled the gap by uncovering plant reactions to flooding, as well as drought-flooding cycles. Furthermore, this study revealed a distinct understanding of the changes in concentrations of antioxidants during water stress, potentially illuminating on the therapeutic benefits of Mexican mint essential oil.

7.3 Future recommendations

The goals established for carrying out this study were attained. The purpose of this thesis was accomplished effectively in terms of increasing our knowledge and understanding of how the Mexican mint responds and functions under various water stress conditions. However, there are a few experimental recommendations that future studies can explore. For instance, as this study was carried out solely on a small scale in growth chambers and greenhouses, the results may differ when performed under field conditions. Additionally, it is necessary to conduct field trials to examine the plants' response to the prevailing climate conditions. This study has mostly focused on investigating the response of plants to water stress in solitude. However, further research is necessary to explore the effects of combination stresses, as this poses a substantial challenge within the existing limitations of agriculture. Further analysis using transcriptomic and proteomic approaches is required to identify genes and proteins that regulate these central carbon metabolic pathways in response to water stress. This study has evaluated the concentrations of a limited number of vital components found in essential oils. However, to accurately evaluate the quality of the oil, a comprehensive knowledge of the maximum number of chemical compounds is necessary.

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APPENDIX

Figure S6.1 The GC-MS chromatograms of the identified volatile compounds in the essential oil of Mexican mint under varying water stress conditions. The retention times of each compound are shown on the chromatograms.

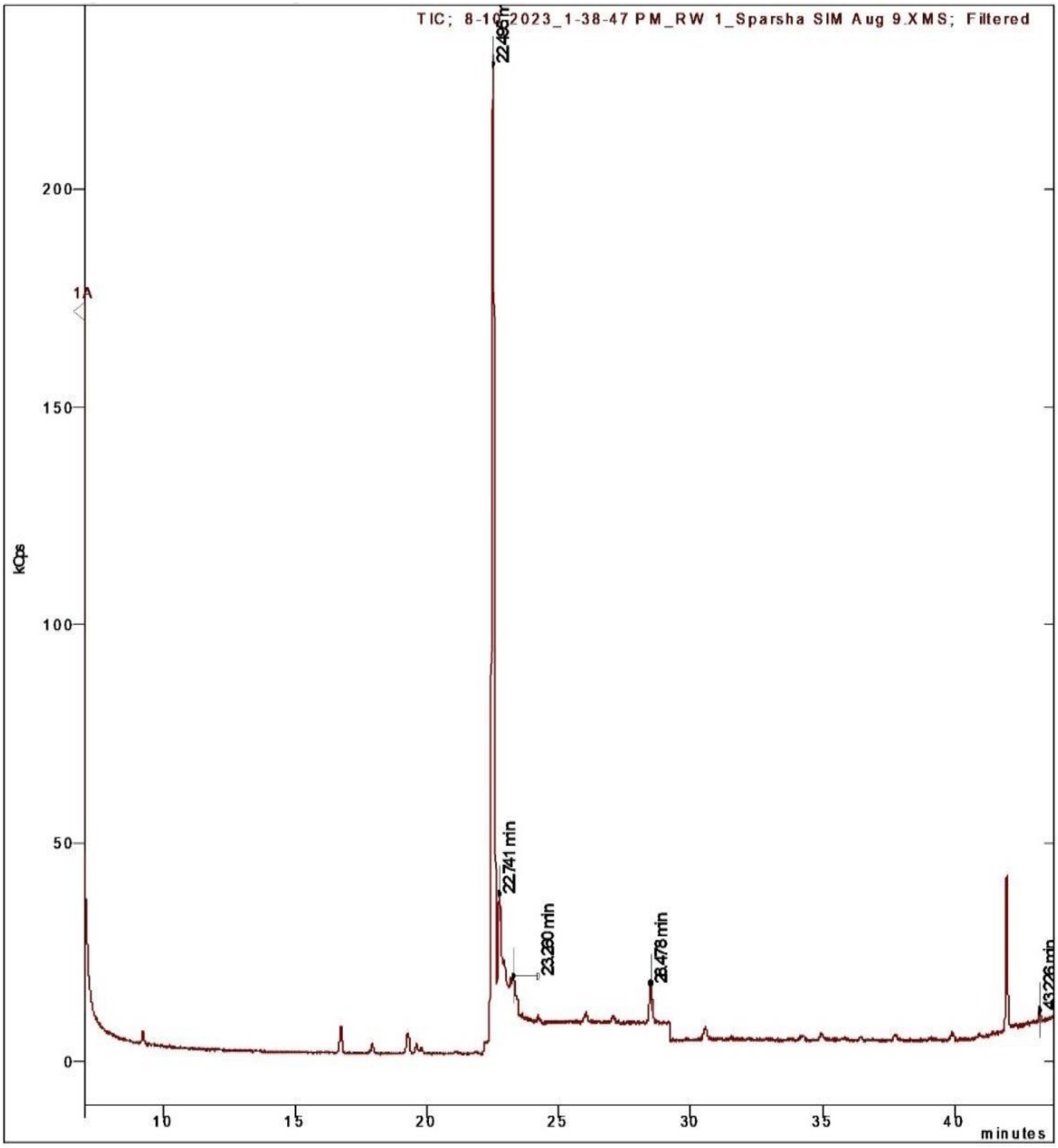

Table S6. 2. The retention time of the volatile compounds identified in the GC-MS analysis of essential oil extracts from Mexican mint. The chromatogram of the compounds is presented in (Figure S6.1)

Compounds	**Retention time**
Linalool	28.47
Caryophyllene	43.21
Cymene	22.72
Eucalyptol	23.24
Limonene	22.47