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The Effect of Carbamazepine 'CBZ' on the Expression of Eight Stress-Related Genes in a Palestinian Local Tomato Cultivar 'RAM'

By

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In Partial Fulfillment of the Requirements for the Degree

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'The Effect of Carbamazepine 'CBZ' on the Expression of Eight Stress-Related Genes in a Palestinian Local Tomato Cultivar 'RAM'

by

Nelly Basem Mitri

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in biotechnology.

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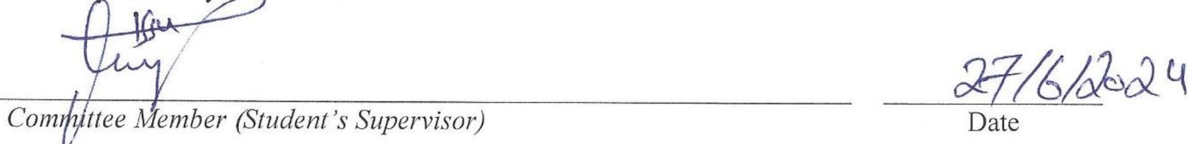

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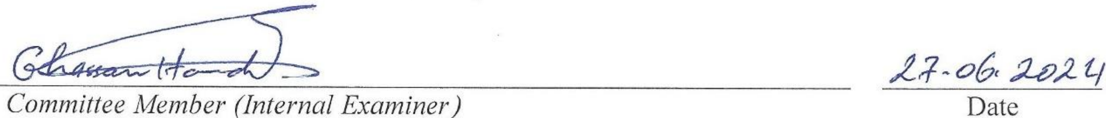

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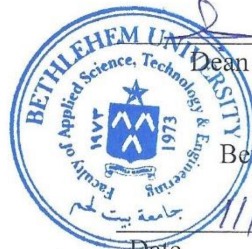

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by Nelly Basem Mitri

ABSTRACT

Tomatoes, scientifically known as *Solanum lycopersicum*, hold significant agricultural value globally, as well as in Palestine. Whether enjoyed fresh or processed into various forms, tomatoes serve as a vital crop. In Palestine, as well as in most other countries, tomato cultivation consumes relatively enormous amount of irrigation water. is water-dependent. As part of the Middle East region, Palestine suffers from water scarcity which necessitates searching for new approaches to supply the agricultural sector with irrigation water. One of these approaches is the use of reclaimed wastewater (RWW). Indeed, several countries in the Middle East have been already utilizing RWW in agriculture including Egypt, Jordan and Israel. One of the challenging obstacles of using RWW, as shown in several recent studies, is the presence of different pharmaceuticals and their secondary metabolites in RWW. One of these drugs is carbamazepine (CBZ) which has been found in tertiary RWW in Israel. This study aimed to investigate the effect of CBZ on the gene expression of eight stress-related genes in plants.

A local tomato cultivar known as RAM was grown in a hydroponic system with two different solutions of CBZ concentrations (10nM and 200nM). Plants irrigated with tap water served as control treatment. RNA was extracted from leaves and roots samples every 24 h up to 72 hours of exposure to CBZ. cDNA was synthesized by reverse transcription and gene expression was measured using the real time rt-PCR technique.

The results revealed distinct patterns of gene expression in tomato leaves and roots following CBZ treatment. In leaves, a consistent upregulation of gene expression was observed after 48 hours of exposure, indicating an initial stress response followed by a return to baseline levels. Conversely, the roots exhibited no clear pattern of gene expression changes. This study is the first of its kind in Palestine to examine the impact of CBZ on the local cultivar RAM. We recommend further studies on a larger scale to understand the effect of other pharmaceuticals found in RWW on tomato and other important plants.

" تأثير كاربامازيبين "CBZ" في التعبير الجيني لثمانية جينات مرتبطة بالإجهاد في صنف بندورة محلي فلسطيني" رام""

نلي باسم متري

ملخص

البندورة، المعروفة علمياً باسم Solanum lycopersicum، تحمل قيمة زراعية كبيرة عالمياً، وكذلك في فلسطين. سواء تمت استهلاكها طازجة أو تم معالجتها إلى أشكال مختلفة، تعتبر البندورة محصولاً مهماً. في فلسطين، وكذلك في معظم الدول الأخرى، تستهلك زراعة البندورة كمية كبيرة نسبياً من مياه الري. كون فلسطين جزءاً من منطقة الشرق الأوسط، تعاني من ندرة المياه مما يستلزم البحث عن طرق جديدة لتوفير مياه الري للقطاع الزراعي. أحد هذه الطرق هو استخدام مياه الصرف الصحي المعالجة. بالفعل، استخدمت عدة دول في الشرق الأوسط مياه الصرف الصحي المعالجة في الزراعة بما في ذلك مصر والأردن وإسرائيل. احدى التحديات لاستخدام مياه الصرف الصحي المعالجة، كما أظهرت عدة دراسات حديثة، هو وجود مختلف أنواع الأدوية ومخلفاتها الثانوية في مياه الصرف الصحي المعالجة. واحدة من هذه الأدوية هي الكربامازيبين (CBZ) التي تم العثور عليه في مياه الصرف الصحي المعالجة في إسرائيل. هدفت هذه الدراسة إلى الاستقصاء تأثير CBZ على تعبير الجينات الثمانية المرتبطة بالإجهاد في النباتات. تم زراعة سلالة بندورة محلية تعرف باسم RAM في نظام مائي بدون تربة باستخدام محلولين مختلفين من تراكيز CBZ (10 نانو مول و200 نانو مول). خدمت النباتات التي تم ريها بمياه الصنبور كعامل سيطرة. تم استخراج الحمض النووي (RNA) من عينات الأوراق والجذور كل 24 ساعة حتى 72 ساعة من التعرض لـ CBZ. تم تصنيع الحمض النووي (cDNA) بواسطة الترجمة العكسية وتم قياس تعبير الجين باستخدام تقنية الـ rt-PCR الحقيقية الزمنية. كشفت النتائج عن أنماط متميزة لتعبير الجينات في أوراق البندورة والجذور بعد التعرض ل CBZ. في الأوراق، لوحظ تعبير مستمر للجينات بعد 48 ساعة من التعرض، مما يشير إلى استجابة إجهادية أولية تلتها عودة إلى مستويات الأساس. على النقيض، لم تظهر الجذور أي نمط واضح لتغييرات تعبير الجينات. تعتبر هذه الدراسة الأولى من نوعها في فلسطين لفحص تأثير CBZ على السلالة المحلية RAM.كما ونوصي بإجراء دراسات إضافية على نطاق أوسع لفهم تأثير الأدوية الصيدلانية الأخرى الموجودة في مياه الصرف الصحي على البندورة والنباتات الهامة الأخرى.

DECLARATION

I declare that the Master Thesis entitled "dissertation title" is my own original work, and hereby certify that unless stated, all work contained within this thesis is my own independent research and has not been submitted for the award of any other degree at any institution, except where due acknowledgment is made in the text.

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Abbreviations

APX 1	Ascorbate peroxidase
ATG	Autophagy related genes
CBZ	Carbamazepine
cDNA	Complementary Deoxyribonucleic acid
CECs	Contaminants of emerging concern
CTD	C-terminal domain
Ct	Cycle threshold
DBD	DNA binding domain
Δ	Delta
DNA	Deoxyribonucleic acid
dsDNA	Double strand Deoxyribonucleic acid
ECs	Emerging contaminants
GABA	Gamma aminobutyric acid
GAD1	Glutamic acid decarboxylase 1
GAD2	Glutamic acid decarboxylase 2
HS	Heat stress
HSFs	Heat stress transcription factors
HSPs	Heat shock proteins
HSR	Heat shock response
Kg	Kilogram
L	Liter

Mg	Mega bite
Mg	Milli gram
mRNA	Messenger ribonucleic acid
Ng	Nano gram
NLR	Nucleotide binding leucine rich repeat
nM	Nano molar
NTC	No template control
OD	Oligomerization domain
PCs	Pharmaceutical contaminants
PCR	Polymerase chain reaction
PPCPs	Pharmaceutical and personal care products
RE	Relative expression
RNA	Ribonucleic acid
ROS	Reactive oxygen species
Rt PCR	Reverse transcription polymerase chain reaction
RWW	Reclaimed wastewater
Sgt 1	Suppressor of G2 allele
Ug	Micro gram
Ul	Micro litter
uM	Micro molar
WWTP	Wastewater treatment plants

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CHAPTER 1

1.0 Introduction

Tomatoes, scientifically known as *Solanum lycopersicum*, are one of the most significant vegetable crops that are widely cultivated and are economically significant worldwide, ranking second to potatoes in the world's vegetable production (Aldubai et al.,2022).

Scientists' interest in the tomato as a model plant has significantly increased, particularly with the sequencing of its genome (The Tomato Genome Consortium, 2012). The significance of tomatoes lies in their role as an essential model for studying various aspects of plant biology, including plant diseases, fruit development, ripening processes, cell wall biosynthesis, salinity tolerance, and more (Kimura, S., and Sinha, N. 2008).

Tomatoes possess various characteristics which enhance their usage as model plants and these are; a short life cycle which lasts about three months, compact genome size (950 Mb), efficient seed production, high self-fertility and homozygosity, capacity for asexual propagation through grafting, the potential to regenerate entire plants from various explants and last is their ability to grow under different cultivation conditions (Gerszberg et al.,2015).

Both the production and consumption of tomatoes are on the rise, driven by the nutritional and economical significance of tomatoes in human lives (Gerszberg et al.,2015). Tomatoes offer versatility, as they can be consumed fresh, added to salads, or processed into paste, soups, sauces, juices, or powdered concentrates. As this highly demand on tomatoes makes it an unreplaceable crop worldwide.

In Palestine, tomatoes rank fourth with an area of 15456 dunum according to Ministry of Agriculture in year 2021-2022 (PCBS and MoA, 2023). As for the production of tomatoes in Palestine it is demonstrated in Fig 1.1 where it was up growing and then started to decline.

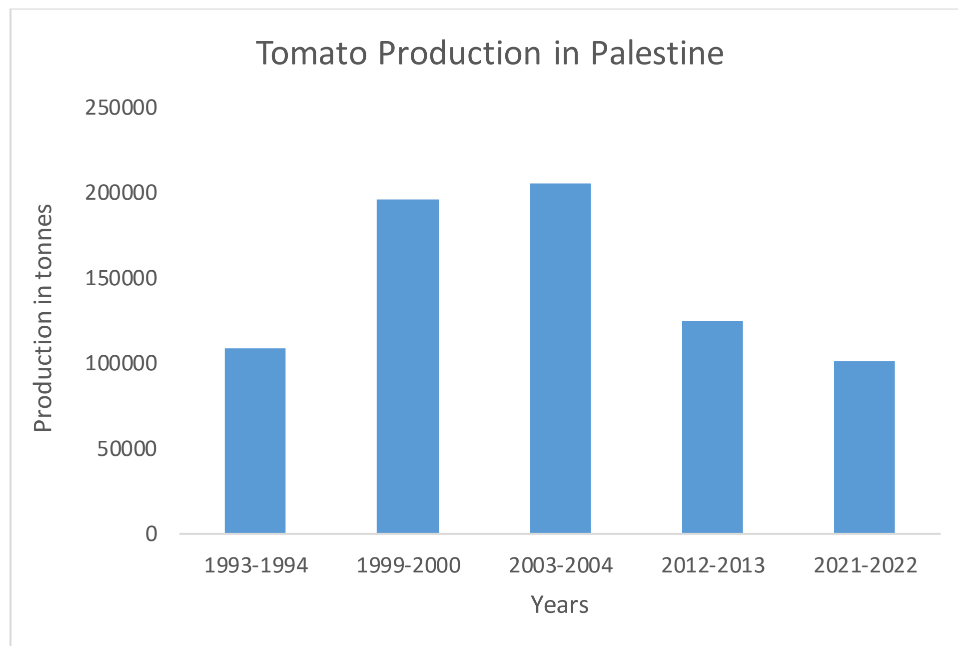

Fig 1.1 Tomato production in Palestine from 1993 until 2022 (PCBS & MoA,2023)

Tomatoes are classified as fleshy fruits due to their high-water content, which gives them their juicy texture. Consequently, tomatoes require sufficient water to support their growth. However, with modern agriculture increasingly relying on irrigation rather than rainfed systems, water availability poses a challenge. In regions like the Middle East, many countries, including Palestine, suffer with water scarcity (Craddock et al.,2021), making it one of the most critical issues in agricultural practices.

Water resources in Palestine are limited, predominantly dependent on groundwater, surface water, and rainwater harvesting (Alsayeda et al.,2020). However, these resources face many challenges including political conflicts, where the Israeli occupation controls most of the

available water resources, hyperbolic population growth and climate changes (Alsayeda et al.,2020).

Concerning this situation, the utilization of Reclaimed Waste Water (RWW) for agricultural irrigation emerges as a solution currently employed by several countries including Israel (Craddock et al.,2021). In Israel, RWW use constitutes over 80% of reclaimed wastewater used for agricultural irrigation. However, in Palestine, the utilization of RWW is not widely accepted due to a lack of scientific knowledge and cultural context. Indeed, recent studies are concerned about the biosafety of RWW especially due to the presence of pharmaceuticals (Mordechay et al.2021).

1.1 Reclaimed Wastewater

The field of agriculture requires significant amounts of water, with agriculture standing out as the foremost consumer of water, constituting 70% of total water usage (Ingrao et al.,2023). This consumption places considerable pressure on freshwater resources, a concern highlighted by the World Health Organization (WHO, 2019). Population growth, urbanization, climate change, and high food demand are contributing to this escalating pressure. (Paltiel et al.,2016). These trends contribute to water scarcity, limiting the availability of freshwater use for agricultural purposes.

Water scarcity is a worldwide issue that is predominant and acute especially in arid and semi-arid areas including the Middle East, North Africa, Australia and parts of the South Western United States (Martinez et al.,2018). These areas experience limited precipitation and high evaporation rates, leading to challenges in maintaining sufficient water resources for various uses including agriculture, industry, and domestic consumption. In this regard, the necessity to explore new approaches for acquiring water for irrigation purposes, has emerged.

Reclaimed wastewater emerged as the innovative approach (Dolnicar and Schafer,2009).

Reclaimed wastewater (RWW) offers an alternative water source, relieving the pressure on traditional water resources and promoting sustainable agricultural practices (Biel et al.,2018). By utilizing RWW, farmers can reduce their dependency on freshwater supplies, decreasing the pressure on water sources while maintaining productivity in agricultural activities (Ingrao et al.,2023). This approach helps save water and preserve it so to minimize the water shortages, which ultimately supports the sustainability of both the environment and the economy on the long term.

1.2 Usage of Reclaimed Wastewater in Agriculture

RWW is primarily utilized for crop irrigation, including various vegetables and fruits (Schapira et al.,2020). Approximately 10% of the world's agricultural land relies on RWW for irrigation purposes (Intriago et al.,2018). Moreover, RWW serves as a crucial water source for agricultural irrigation, particularly prevalent in regions having water scarcity issues. Countries such as Spain, France, Italy, Cyprus, Malta, the USA, Israel, and Jordan commonly employ RWW for irrigation (Bacerra et al.,2015)

In Europe, approximately 2% of treated wastewater is utilized for irrigation purposes. However, in Malta and Cyprus, this percentage exceeds 50%, indicating a significant reliance on reclaimed wastewater for agriculture. Conversely, countries like Greece, Spain, and Italy have lower percentages of treated wastewater used for irrigation (Chojnacka et al.,2020).

Israel stands out as a leader in the utilization of reclaimed wastewater for agriculture, with over 95% of wastewater being treated and more than 80% of it reused for irrigation (Orlofsky et al., 2016). This accounts for approximately 50% of the total water used in Israel's agricultural sector.

1.3 Biosafety of Reclaimed Wastewater

Utilizing reclaimed wastewater for irrigation conserves water and enhances the Agro-environment with organic matter and nutrients (Paltiel et al.,2016). Nonetheless, there are concerns regarding the safety of using reclaimed wastewater for crop irrigation due to the contamination of various pollutants in the reclaimed wastewater (Wu et al.,2014).

Traditional concerns have predominantly focused on disease-causing pathogens and heavy metals. However, the emergence of contaminants of emerging concern (CECs), such as pharmaceutical and personal care products (PPCPs), has emerged as a new issue due to their potential impact on both the environment and public health (Montemurro et al.,2019).

Inefficient removal of CECs within wastewater treatment plants (WWTPs) can result in unpredictable long-term environmental consequences. Specifically, these CECs may be introduced into agricultural fields through repeated irrigation with RWW, potentially accumulating in soils and subsequently translocating into crops intended for human consumption as shown in Fig 1.2 (Fernandes et al.,2021).

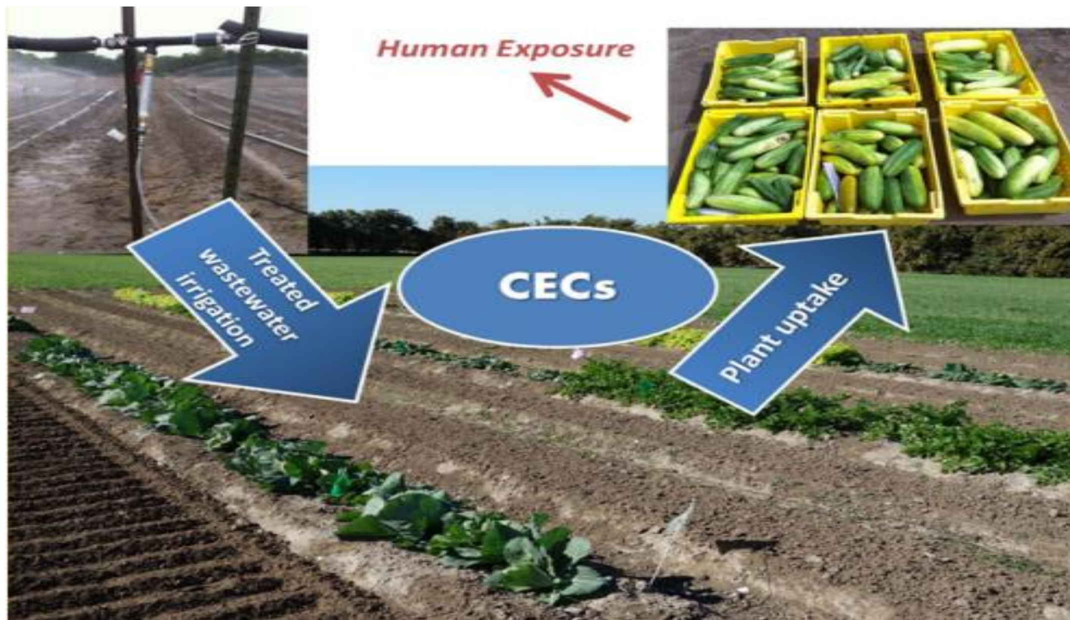

Fig 1.2 The process of CEC transfer from RWW to humans through uptake of plants. (Wu et al.,2014)

The behavior and persistence of CECs in the environment are influenced by a range of factors, including their physical-chemical properties, adsorption characteristics, conjugation form, and charge within the soil-compound system, as well as soil properties and agricultural practices (Martinez et al.,2018).

Given the diverse properties of CECs commonly found in RWW and the increase in utilizing RWW highlights the need for the development of highly sensitive techniques to measure organic contaminants originating from wastewater in soil so to accurately assess the potential risks associated with this irrigation method (Montemurro et al.,2019).

Consequently, it is essential to gather data on the occurrence and accumulation of CECs in agricultural soils, as well as their potential translocation into food products, to ensure the safe utilization of RWW and subsequent acceptance by consumers.

1.4 Pharmaceuticals in Reclaimed Wastewater

Recently, aquatic environments have become increasingly affected by Emerging Contaminants, posing a threat to the ecosystem and human health (Fernandes et al.,2021). Pharmaceutical contaminants (PCs), derived from pharmaceutical industries, are of particular concern due to their composition of biologically active compounds (Biel et al.,2018). These pharmaceuticals comprise a wide range of biological compounds utilized for disease prevention, cure, or treatment. PCs exhibit diverse structures and are engineered to be target-specific, enabling absorption and distribution within the human body.

PCs possess the ability to interact with and be absorbed by living organisms, posing a potential hazard to the entire ecosystem. These contaminants, found in various forms such as hospital effluents, industrial discharges from pharmaceutical industries, agricultural runoff containing pesticides and fertilizers, as well as human and animal excreta from households and sewers, enter the environment and contribute to ecosystem damage (Montemurro et al.,2019).

Pharmaceuticals are continually introduced into the agroecosystem through RWW irrigation and are detected in measurable quantities in all irrigation water, soil, and plants, comprising 99.6% of the samples (Mordechay et al., 2021).

Pharmaceuticals are categorized into various classes or groups based on factors such as their mechanism of action (how they bind and act against their biological target), mode of action, chemical structures, and intended treatment of diseases (Samal et al.,2022). When classified according to their curative or remedial use, these pharmaceuticals are grouped into therapeutic classes or groups. Examples of such classifications include analgesics and anti-inflammatories,

antidepressants, antibiotics, antiviral medications, anticoagulants, sedatives, and cardiovascular drugs, anticonvulsants.

Anticonvulsants are medications primarily used to prevent or treat seizures and epilepsy. Like other pharmaceuticals, anticonvulsants can enter wastewater through various routes, including disposal via urine and feces, improper medication disposal, and incomplete removal during wastewater treatment processes. Due to their potential persistence and bioaccumulation, anticonvulsants in wastewater pose concerns for environmental and human health. Examples of anticonvulsants include: Phenytoin (Dilantin), Valproic acid (Depakene), Lamotrigine (Lamictal), Gabapentin (Neurontin), Pregabalin (Lyrica), Topiramate (Topamax), Levetiracetam (Keppra), Carbamazepine (Tegretol). (Samal et al.,2022)

1.5 Carbamazepine (CBZ)

Carbamazepine (CBZ) is a medication commonly prescribed as an anticonvulsant and mood stabilizer for the treatment of epilepsy and bipolar disorder (FDA,2009).

Carbamazepine is a dibenzoazepine derivative with the chemical formula C15H12N2O. Its structure is characterized by a tricyclic framework known as 5H-dibenzo[b,f]azepine (Paltiel et al., 2016). This structure, which is demonstrated in fig 1.3, consists of three fused rings:

1. A di-benzene ring system (dibenzo), which refers to two benzene rings fused together.

2. An azepine ring (5H-azepine or 5-membered ring with one nitrogen), which is fused to the di-benzene ring system.

Additionally, carbamazepine has a carbamoyl substituent (-CO-NH-) attached to the nitrogen atom in the azepine ring. This substituent is crucial for its pharmacological activity as an anticonvulsant and mood stabilizer (National Center for Biotechnology Information, 2024).

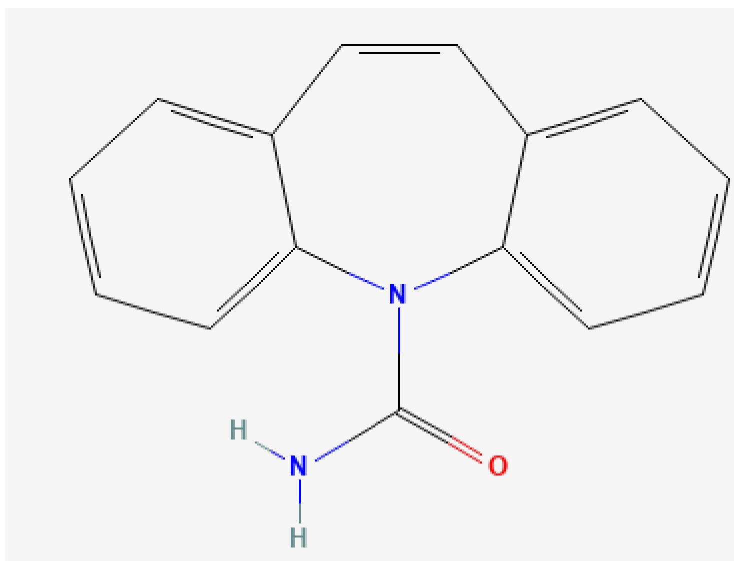

Fig 1.3 Carbamazepine Structure (National Center for Biotechnology Information, 2024).

In children, CBZ has a half-life ranging from 3 to 32 hours, whereas in adults, it ranges from 18 to 55 hours after a single dose. The therapeutic dosage typically falls between 400 and 1200 mg per day, which corresponds to plasma concentrations of 4-12*10^6 ng/L. CBZ is primarily metabolized in the liver, with less than 3% of the compound excreted in urine (Schapira et al.,2020).

In vivo, CBZ undergoes almost complete metabolism to form 10,11-epoxy CBZ, which is an active metabolite. This metabolite is further metabolized by epoxide hydrolase into 10,11-dihydro CBZ, which is the major metabolite detected in urine (Paltiel et al., 2016). This drug possesses specific physio-chemical properties that makes it resistant to microbial decomposition, thus it can accumulate in the soil and be absorbed by the roots until it reaches the leaves of the

plants, where it is highly concentrated, which proposes an involuntary route for CBZ intake by humans (Schapira et al.,2020).

As documented, CBZ is one of the most commonly prescribed antiepileptic drugs in Israel (Berman et al.,2016). In recent studies carried out in Israel, the concentration of CBZ and its derivatives in reclaimed wastewater ranged from 1.3 to 2.2 μg/l before being applied to the soil. After approximately 100 days of irrigation with these reclaimed wastewaters, the concentration in the leaves of tomatoes grown in different soils was found to be between 100 to 150 ng/g (dry weight) (Mordechay et al.,2018). Another study in Israel showed that CBZ concentration in reclaimed wastewater from Teva Kefar Saba, which is a pharmaceutical formulation facility, was 0.84 mg/l which corresponds to discharge loads of around 0.25 Kg CBZ per day. (Lester et al.,2013).

1.6 Biosafety of Carbamazepine

The accumulation of pharmaceutical contaminants (PCs) in soil poses a potential risk to human health, as these contaminants can be taken up by crops during growth. Specifically, carbamazepine (CBZ), one of the most commonly detected drugs in soil (Schapira et al.,2020). It has been reported that CBZ translocate from soil into plant tissue. This highlights the potential pathway for human exposure to PCs through the consumption of contaminated crops (Montemurro et al., 2019)

Carbamazepine exhibits resistance to biodegradation (Keen et al.,2012), this suggests that CBZ is resistant to breakdown or degradation during wastewater treatment (WWT) processes, making it challenging to effectively eliminate from wastewater processes (Keen et al.,2012).

Following conventional treatment methods, approximately 30% of CBZ undergoes degradation, while the remaining portion is released into recipient water bodies (Faria et al.,2020). This persistence of CBZ in wastewater effluents highlights the challenges associated with effectively removing pharmaceutical contaminants during treatment processes

Leafy greens exhibit the highest number and concentration of PCs (Mordechay et al., 2018). Given the elevated likelihood of PCs being present in leafy greens, RWW irrigation should be restricted to crops with minimal risk of PC transfer to the food chain. Additionally, to reduce the human exposure to CBZ and other PCs, wastewater treatment should be enhanced, and RWW irrigation should be limited to non-edible crops or fruit-bearing crops where the edible part is protected from direct contact with wastewater (Mordechay et al., 2021).

Research has indicated that Carbamazepine can affect plant metabolic pathways and cellular functions, leading to disruptions in normal growth and development. Studies have shown that Carbamazepine exposure can alter various physiological processes in plants. For example, Desbiolles et al., 2020 has observed that CBZ interfere with photosynthesis by affecting chlorophyll content and photosynthetic efficiency in aquatic plants like duckweed (Lemna minor). These responses indicate that plants may perceive Carbamazepine exposure as a stressor, triggering adaptive mechanisms to cope with its presence. Furthermore, studies have highlighted the potential for Carbamazepine to accumulate in plant tissues, posing risks of bioaccumulation and biomagnification through the food chain. Understanding these impacts is crucial as plants possess a repertoire of stress-related genes that enable them to respond to environmental stressors, including pharmaceutical pollutants. As the next section gives a brief description on the roles of the major stress- related genes in tomatoes.

1.7 Major Stress-Related genes in Tomato

1.7.1 Heat Stress Transcription Factors (Hsfs)

In every eukaryotic organism, the reaction to heat stress (HS) relies on the functionality of heat stress transcription factors (Hsfs) (El shershaby et al.,2019). Plants, in particular, have evolved large number of Hsfs families, as there are 27 Hsfs families in *S. Lycopersicum* (Scharf et al., 2012).

Plant Hsfs are categorized into three classes, namely A, B, and C, based on distinctive structural features within the following domains:

1- An N-terminal DNA-binding domain (DBD)

2- A leucine-zipper oligomerization domain (OD).

3- C-terminal region with class specific domains. (CTD)/ the presence of class-specific motifs/ (Scharf et al., 2012).

Hsfs are responsible for genes associated with maintaining protein homeostasis. These includes genes responsible for molecular chaperones, such as heat shock proteins (Hsps), proteins involved in signal perception and transduction, protection against reactive oxygen species (ROS), regulation of posttranslational modifications (Ikeda et al., 2011), and they play a role in regulating the response to abiotic stresses, such as drought and salinity (Huang et al., 2016).

1.7.1 a HsfA1

Class A Hsfs exhibit a specific C-terminal activator domain that confers transcriptional activator functionality (Ikeda et al., 2011). The HsfA class is recognized as a positive regulator of the heat stress (HS) response, and among its four members, which are: (HsfA1a, HsfA1b, HsfA1c and HsfA1e), HsfA1a stands out as the most crucial regulator of HS response and thermotolerance in plants. HsfA1a is continuously expressed yet remains inactive by being bound to cytosolic chaperons mainly Hsp90. As it regulates the initial response to HS (Yoshida et al., 2011).

1.7.1 b HsfB1

Class B Hsfs exhibit a C-terminal region that includes motifs that function as repressors, negatively influencing the transcription of heat shock proteins (Hsps) (Ikeda et al., 2011). Thus, HsfB1 serves as a transcriptional repressor but can also function as a co-activator for HsfA1a. Therefore, HsfB1 have a dual function and it works as a co-activator of HsfA1a for various Hsps, while simultaneously serving as a transcriptional repressor for other Hsfs, such as HsfA1b and HsfA2 (Fragkostefanakis et al.,2019). This dual role highlights its involvement in activating chaperones to protect and regulate homeostatic levels when there is a constantly changing environmental conditions. Notably, HsfB1 levels are maintained under control by the interaction of HsfB1 with Hsp90.

1.7.2 Heat Shock Protein (Hsps)

Plants undergo physiological changes during a heat stress (HS); moreover, they have evolved anti-stress mechanisms, referred to as a heat shock response (HSR), to protect themselves against both biotic and abiotic stress (Zai et al.,2015).

The HSR network involves activating Hsfs and the synthesis of the Hsps which are molecular chaperons that are important in regulating the cellular protein homeostasis (Tilman et al.,2015). Hsps are involved in the folding of intracellular proteins, regulating protein metabolic activities, and facilitating the protein transport into different organelles (Lang et al.,2021).

1.7.2a Heat Shock Protein 90 (Hsp90)

Hsp90 is a chaperon protein produced in plants when under stressful conditions especially abiotic ones. In tomato, the Hsp90 has at least 7 Hsps genes. This Hsp90 has multi-functions in which are related to regulating the protein homeostasis such as stress signal transduction, assisting in protein folding and stabilizing proteins during a HS (Zai et al.,2015).

1.7.3 Suppressor of G2 allele (Sgt 1)

Plants have an intricate defense mechanism featuring intracellular nucleotide-binding leucine-rich repeat (NLR) receptors, constituting the largest group of resistance genes. Upon activation, these NLR receptors stimulate an immune response (Chen et al.,2021).

The proper folding and maintenance of NLR proteins are crucial, as any misfolded proteins can disrupt cellular function. To ensure the integrity of NLRs, Sgt1 has been identified as a key player (Shirasu.K., 2009). Sgt1 interacts with various protein complexes, particularly with Hsp90, forming a chaperone complex essential for regulating NLR protein levels and stabilizing them.

1.7.4 Ascorbate Peroxidase (APX 1)

The plant antioxidative system responds to the formation of reactive oxygen species (ROS) triggered by various abiotic stresses like high salinity, low temperatures, or drought.

Among the crucial enzymes in this system is ascorbate peroxidase (APX), which acts to reduce ROS, such as hydrogen peroxide, by utilizing ascorbate as an electron donor (Leng et al., 2021). This process serves to protect the plant from oxidative damage arising from the accumulation of excess hydrogen peroxide, thereby preventing any damage and maintaining normal plant growth.

1.7.5 Autophagy Related Gene (ATG 18)

In times of stress, plants rely on autophagy to maintain cellular homeostasis by degrading unnecessary or dysfunctional proteins. This degradation process, facilitated by autophagy-related (ATG) genes, involves various stages, and ATG18 is one of the ATG genes. (Quezada-Rodríguez et al.,2021).

ATG18 plays a significant role in regulating vacuolar shape and facilitating the recruitment of the ATG8 and ATG16 complex, essential for phagophore formation. Additionally, research indicates that HSfA1 acts as a positive regulator of autophagy induced by drought stress in which HSfA1 binds to the promoters of ATG10 and ATG18, promoting the formation of autophagosomes under adverse environmental conditions, thereby aiding in plant adaptation to stress (Wang et al.,2015).

1.7.6 Glutamic Acid Decarboxylase (GAD)

Glutamic Acid Decarboxylase (GAD) plays a key role in synthesizing gamma-aminobutyric acid (GABA) through the decarboxylation of glutamic acid, making it a crucial enzyme in GABA production. GABA, a widely distributed non-protein amino acid, is integral to plant stress responses, aiding in reducing stress (Takayama et al.,2015). The accumulation of GABA assists plants in coping with various stressors. Moreover, GABA synthesized by GAD serves as a signaling molecule in response to abiotic stress, regulating calcium levels to activate downstream pathways crucial for stress adaptation

GAD1→ typically refers to Glutamate Decarboxylase 1, which is one of the isoforms of the enzyme GAD.

GAD2→ typically refers to Glutamate Decarboxylase 2, which is one of the isoforms of the enzyme GAD.

1.8 Relative Quantification using Real-time Polymerase Chain Reaction

1.8.1 Real-Time Polymerase Chain Reaction

Real-Time Polymerase chain reaction stands out as a highly potent molecular technique due to its quantitative, precise, sensitive, and rapid characteristics (Valones et al.,2009). Researchers utilize real time PCR for gene expression analysis, detection and quantification of pathogens, genetic variation studies, and diagnostics. Its ability to generate quantitative data efficiently has made rt-PCR an essential tool in molecular biology research and has contributed significantly to advancements in different fields (Artika et al.,2022).

Real-Time PCR is used for both amplification and quantification of DNA, giving the chance of monitoring the amplification process in real time; which means as the reaction is happening, the data is being collected (Wong et al.,2005). Thus, providing quantitative information about the initial amount of target DNA in the sample and providing results shortly after the amplification process, without the need of performing gel electrophoresis, so allowing for faster data analysis. This is possible as a result of using a fluorescent reporter molecule into the reaction. This fluorescent can be in the form of a fluorescent labeled probe or a DNA binding dye such as SYBR Green.

SYBR Green Assay depends on SYBR Green I dye, a nonspecific fluorescence dye, which binds to double-stranded DNA by attaching itself between the base pairs (Dragan et al., 2012). The PCR reaction is performed where the DNA template is amplified through repeated cycles of denaturation, annealing of primers and extension by DNA polymerase (Cao et al.,2020). As the DNA amplification proceeds, the SYBR Green dye binds to the newly synthesized dsDNA

molecules, leading to an increase in fluorescence intensity shown in Fig.3. The fluorescence at each cycle is monitored in real-time using a fluorescence detection system and is proportional to the amount of DNA, allowing for the quantification of DNA amplification (Dragan et al., 2012).

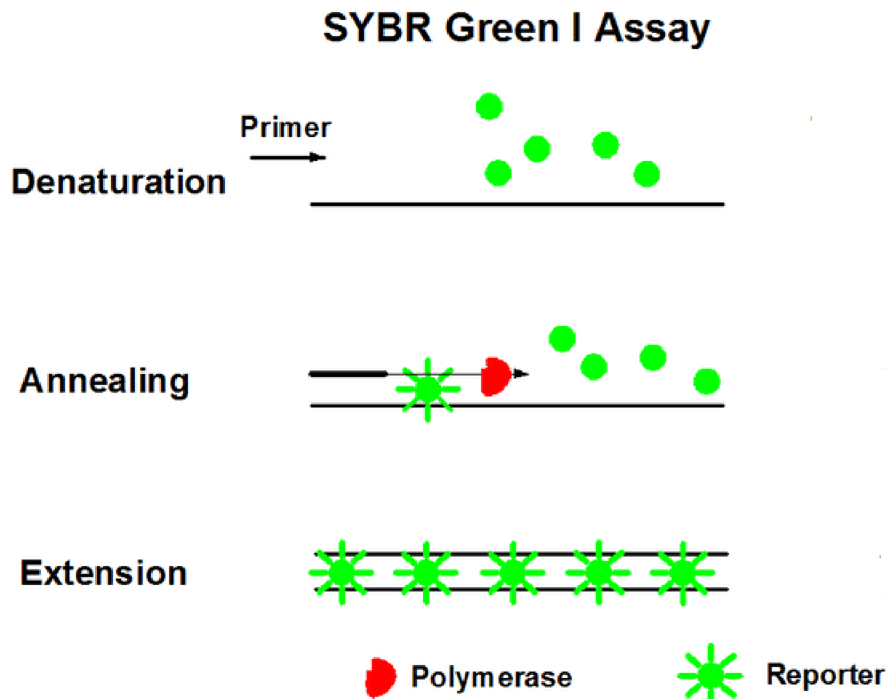

Fig 1.4 SYBR Green mode of action (Cao et al.,2020).

1.8.2 Relative Quantification

Real-time quantitative PCR is a pivotal tool in molecular and genomic studies (Valones et al.,2009). This technique serves prominently in quantifying genes and analyzing their expression through two primary approaches: relative and absolute quantification. Relative quantification compares gene expression of one sample to another, while absolute quantification relies on a

standard curve of known template concentrations for precise quantitation (Harshitha, R., & Arunraj, D. R., 2021).

In relative quantification, alterations in gene expression of the experimental samples are assessed using either an external standard or a reference sample, commonly referred to as a calibrator (Wong et al.,2005). As the obtained results are analyzed through different mathematical methods, and one of these methods is called the Comparative Ct method.

The comparative Ct method, also known as the ΔΔCt method, is a mathematical model used to quantify changes in gene expression by comparing the relative fold difference between an experimental sample and a calibrator sample (Wong et al.,2005).

CHAPTER 2

Problem Statements and Objectives

2.1 Problem Statement

RWW has been greatly used in agricultural irrigation, with its utilization increasing globally in many countries including the Middle Eastern countries like Egypt, Jordan, and Israel. Israel is considered as a global leader in RWW implementation (Orlofsky et al., 2016). However, in Palestine, efforts to establish RWW facilities are still ongoing, with hopes that RWW will be utilized on a larger scale in the near future. Nonetheless, the effect of pharmaceuticals, which might be present in RWW, on the plant health and consequently on human health has not been thoroughly investigated (miarov et al., 2020).

In this study, CBZ was investigated as a pharmaceutical agent potentially inducing a stress response in tomato seedlings, thereby influencing the plant's gene expression. Eight specific genes, namely HSFA1, HSFB1, HSP90-1, GAD1, GAD2, ATG18, Sgt1, and Apx1, were analyzed in this context. These genes play crucial roles in stress response mechanisms of the plant.

2.2 Specific Objective (s)

1- Establishing a mini-hydroponic system to grow tomato seedlings.

2- Planting tomato seedlings under different CBZ regime.

3- mRNA extraction from leaves and roots followed by mRNA assessment.

4- cDNA synthesis of several genes involved in plant stress response.

5- Relative Quantification of gene expression using real time rt-PCR.

6- Analysis of the real time rt-PCR results.

CHAPTER 3

Methodology

3.1 *Solanum lycopersicum* Cultivar RAM

A local tomato cultivar called RAM (originally from Ramallah District), which was obtained from the UNESCO Biotech Center at Bethlehem university, was used in this experiment. RAM seeds were placed on a petri-dish lined with water-wetted cotton at room temperature. After 10 days, seedlings were transferred to HR2 soil containing peat, coconut fiber, ventilation material and fertilizers. They were incubated at 25℃-28 ℃ for three weeks at a 16- hour photo period and they were irrigated every other day as shown in figure 3.1

Fig 3.1 Three weeks old RAM cultivar grown on HR2 soil.

3.2 Carbamazepine Solution Preparation

-A stock solution of 100uM was prepared by dissolving 0.0236 grams of CBZ in 1000 ml distilled water.

-Then two working solutions were prepared each with a different concentration; 10nM and 200nM.

3.3.1 Hydroponic Experimental Setup

Tomato seedlings were removed from the nursery tray, and the roots were gently washed from any soil particles using running water. The seedlings transferred to a hydroponic system, figure 3.2. The system was made using a 50 ml Falcon tube containing 50 ml of either the10nM CBZ solution, the 200nM CBZ solution or tap water, and the solutions were replaced every 2 days to keep a sufficient supply of oxygen and CBZ. The seedlings were incubated at 25-28℃ up to 72 hours. For each experimental point (time vs solution), three replicates were used, each in a separate tube.

As a control, 3 seedlings were incubated in tap water under the same conditions.

Fig 3.2 Hydroponic systems used to grow RAM cultivar under two CBZ concentrations; 10nM and 200nM.

3.3.2 Sample collection

For each treatment, three young leaves were collected and placed in an Eppendorf tube, from three plants (replicates), and stored at -20ºC. The same collection procedure was applied for the roots. The plant samples were incubated till the end of the experiment (72h) and were then used for RNA extraction as explained in Section 3. 5. The Eppendorf tubes were labeled as described in table 3.1

Table 3.1 Labeling of the collected samples

Label	Treatment description
0A	0=water solution A= at 24 hours
0B	0=water solution B= at 48 hours
0C	0=water solution C= at 72 hours
1A	1=10nM CBZ solution A= at 24 hours
1B	1=10nM CBZ solution B= at 48 hours
1C	1=10nM CBZ solution C= at 72 hours
2A	2=200nM CBZ solution A= at 24 hours
2B	2=200nM CBZ solution B= at 48 hours
2C	2=200nM CBZ solution C= at 72 hours

3.4.1 RNA Extraction

-RNA extraction was performed using the RNeasy Plant Mini Kit protocol following the manufacturer instructions.

-Briefly, the steps of the RNA extraction are:

A. Grinding, lysing and homogenization:

1. Disrupt a maximum of 100 mg plant material, using a mortar and a pestle.

Immediately place the plant tissue in liquid nitrogen. Grind thoroughly. Decant tissue powder and liquid nitrogen into RNase-free, liquid-nitrogen–cooled, 2 ml microcentrifuge tube. Allow the liquid nitrogen to evaporate, but do not allow the tissue to thaw.

2. Add 450 µl Lysis Buffer RLT to a maximum of 100 mg tissue powder. Vortex vigorously.

3. Transfer the lysate to a QIA shredder spin column (lilac) placed in a 2 ml collection tube. Centrifuge for 2 min at full speed. Transfer the supernatant of the flow-through to a new microcentrifuge tube without disturbing the cell-debris pellet.

4. Add 0.5 volume of ethanol (96–100%) to the cleared lysate, and mix immediately by pipetting.

B. RNA binding step:

5. Transfer the sample, with any precipitate, to an RNeasy Mini spin column (pink) in a 2 ml collection tube. Close the lid, and centrifuge for 15 s at ≥8000 x g (≥10,000 rpm). Discard the flow-through.

C. Washing steps:

6. Add 700 µl Buffer RW1 to the RNeasy spin column. Close the lid, and centrifuge for 15 s at ≥8000 x g. Discard the flow-through.

7. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid, and centrifuge for 15 s at ≥8000 x g. Discard the flow-through.

8. Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid, and centrifuge for 2 min at ≥8000 x g. Optional: Place the RNeasy spin column in a new 2 ml collection tube. Centrifuge at full speed for 1 min to dry the membrane.

D. Elution step:

9. Place the RNeasy spin column in a new 1.5 ml collection tube. Add 30–50 µl RNase-free water directly to the spin column membrane. Close the lid, and centrifuge for 1 min at ≥8000 x g to elute the RNA.

3.4.2 Assessment of the extracted RNA by Nanophotometer

The quantity and quality of RNA was assessed using nanophotometer were 2 ul were placed and measured. Absorption was measured at 230, 260 and 280 nM. Quality was determined based on 260/280 and 260/230.

3.5 cDNA synthesis

- cDNA was synthesized using the qPCRBIO cDNA Synthesis Kit (PCR Biosystems)

-The reaction setup was prepared based on manufacturer instructions as follows:

Reagent	*20ul reaction*	*Final concentration*
5X cDNA synthesis mix	*4ul	1X
20X RTase	1	1X
RNA	-**	500 ng
PCR grade dH2O	Up to 20 ul final volume	

**contains random hexamers.*

***For each sample, appropriate amount of RNA was added to obtain a final concentration of 500 ng per reaction.*

3.6 Primers preparation

Primers were obtained from the literature as mentioned in table 3.2. Working solutions of 10uM were prepared in ddH2O and used in the real time PCR. The sequences for each primer are shown in table 3.2

Table 3.2 List of primers

Gene Name	Primer Sequence 5'- 3'	Reference
HsfA1	F: GGTCAGGCGAAGAAACAATGC R: TCATATCGGGTGCAACCTTCACG	(Mishra et al., 2022)
ATG18	F: CCGAAGCAGAACTCCAAAT R: AACCTCAGCCTCTCCACGAC	(Mishra et al., 2022)
GAD1	F: AAACTTCCCATTTCCCAACC R: CGATTGATCGGAGGAGAAAA	(Takayama et al., 2015)
GAD2	F: CTTTGATCTTCTCCGTCGTTG R: ATATCGAGACGCGAAAGTCG	(Takayama et al., 2015)

Hsp90-1	F: AATTTATGGAGGCCATTGCT R: TTCATCAACCTCCTCTACCTTT	(Moshe et al., 2016)
Sgt1	F: ATGGCGTCCGATCTGG R: TTGACAGTTGGTTGAGCATCT	(Moshe et al., 2016)
Apx1	F: ACGATGATATTGTGACACTCTTCCA R: AAGCGATGAAACCACAAAAACA	(Moshe et al., 2016)
HsfB1	F: GGTCAGCGGAAGAAACAATGC R: TCATATCGGGTGCAACCTTCACG	(Mishra et al., 2022)
B-actin	F: TGGAGGATCCATCCTTGCATCAC R: TCGCCCTTTGAAATCCACATCTGC	(Zhang et al., 2021)

3.7 Real Time PCR

The PCR thermal cycling were performed using API 7300 (Biosystems).

3.7.1 Conditions

qPCRBIO SyGreen Blue Mix kit (PCR Biosystems) was used in the relative quantification PCR.

The master mix was performed following the manufacturer instructions as follows:

Reagent	*20ul reaction*	*Final concentration*
2X SYBR mix	10 ul	1X
Forward primer	0.8	0.4uM
Reverse primer	0.8	0.4uM
cDNA	2 ul	50ng
PCR grade dH2O	Up to 20 ul final volume	

3.7.2 96-well PCR plate setup

-The study was performed using eight Micro Amp Optical 96-well reaction plates with barcodes (Applied Biosystems). Four of the plates were used to study the expression of the eight genes (Section 1.7) in leaves at two different CBZ concentrations, and another four plates for the same study using root tissues.

-Each gene was studied at time-points (24h,48h, and 72h) after growing tomato seedlings on CBZ on in tap water. The expression of the genes in tomato grown in tap water at each time-point was used as a calibrator (reference sample). For each time-point the PCR was conducted in three replicates. The expression of B-actin gene was used as an internal control.

-Each gene and treatment were given a code to facilitate programing the plate-map (table 3.3)

Table 3.3 Coding system for real time rt-PCR plates' treatments.

Code	Code Description
G1	HsfA1 in CBZ concentration
G2	HsfB1 in CBZ concentration
G3	Hsp90-1 in CBZ concentration
G4	Sgt1 in CBZ concentration
G5	APX1 in CBZ concentration
G6	ATG18 in CBZ concentration
G7	GAD1 in CBZ concentration
G8	GAD2 in CBZ concentration
W	Water

NTC	No control template
For example: - G1 72 means the HsfA1 gene after 72 hours at CBZ concentration. -GW1 72 means the HsfA1 gene after 72 hours in water solution.	

Table 3.4 An example for the plate-map for gene 1 to gene 4 at 10nM CBZ concentration for the leaves.

	1	2	3	4	5	6	7	8	9	10	11	12
A CBZ-24h	G1-24h	G1-24h	G1-24h	G2-24h	G2-24h	G2-24h	G3-24h	G3-24h	G3-24h	G4-24h	G4-24h	G4-24h
B CBZ-48h	G1-48h	G1-48h	G1-48h	G2-48h	G2-48h	G2-48h	G3-48h	G3-48h	G3-48h	G4-48h	G4-48h	G4-48h
C CBZ-72h	G1-72h	G1-72h	G1-72h	G2-72h	G2-72h	G2-72h	G3-72h	G3-72h	G3-72h	G4-72h	G4-72h	G4-72h
D Water-24h	GW1-24h	GW1-24h	GW1-24h	GW2-24h	GW2-24h	GW2-24h	GW3-24h	GW3-24h	GW3-24h	GW4-24h	GW4-24h	GW4-24h
E Water-48h	GW1-48h	GW1-48h	GW1-48h	GW2-48h	GW2-48h	GW2-48h	GW3-48h	GW3-48h	GW3-48h	GW4-48h	GW4-48h	GW4-48h
F Water-72h	GW1-72h	GW1-72h	GW1-72h	GW2-72h	GW2-72h	GW2-72h	GW3-72h	GW3-72h	GW3-72h	GW4-72h	GW4-72h	GW4-72h
G	Actin-24h	Actin-24h	Actin-24h	Actin-48h	Actin-48h	Actin-48h	Actin-72h	Actin-72h	Actin-72h	Actin-W24h	Actin-W24h	Actin-W24h
H	Actin-W48h	Actin-W48h	Actin-W48h	Actin-W72h	Actin-W72h	Actin-W72h	NTC-1	NTC-1	NTC-1	NTC-2	NTC-2	NTC-2

-Similar maps were done for G1-4 at 200nM CBZ concentration in the leaves and roots as well as for G5-8 at both CBZ concentrations in leaves and roots.

3.7.3 Thermal Cycling Conditions

There were three stages (as shown in fig 3.4), where the first stage also known as the initial denaturation stage was at 95ºC for 2 minutes, then the second stage of 40 repeated cycles at 95ºC for 5 seconds then at 60ºC for 30 seconds. The third stage was the dissociation curve which was employed for results validation.

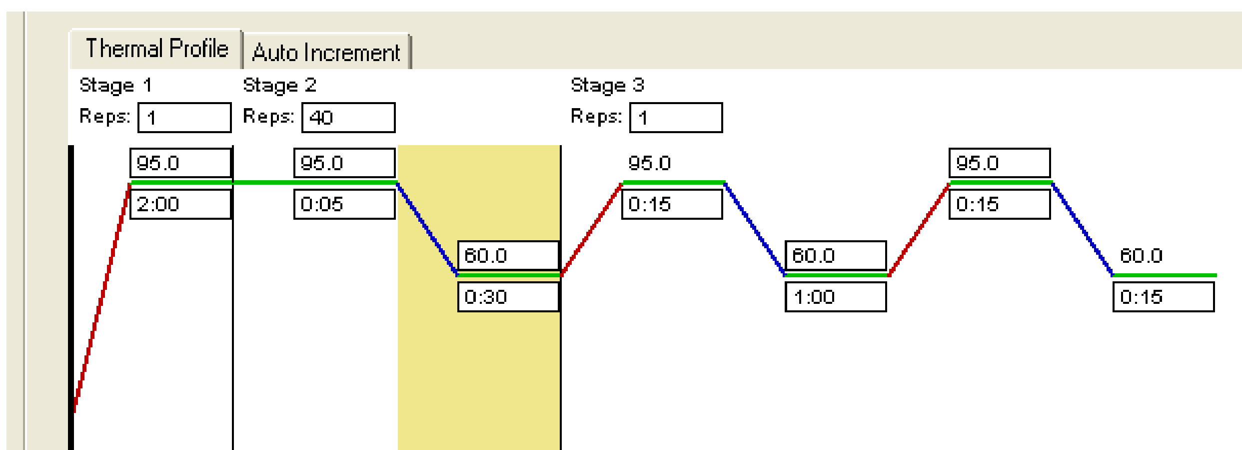

Fig 3.3 Thermal cycling conditions.

3.8 Data analysis using Delta-Delta Ct Method (ΔΔ Ct)

For this method, the cDNAs' threshold cycle (Ct) values, acquired via RT-qPCR, are assessed using the comparative ΔΔCT method (Giulietti et al., 2001). This method compares the Ct value of the test samples with that of the reference sample. Expression Ct values for each target gene are normalized to the values of the housekeeping gene(s). The relative expression of target genes in different samples is calculated using the formula:

$$\Delta Ct = Ct\ (\text{target gene}) - Ct\ (\text{housekeeping gene})$$

where Ct (target gene) represents the threshold cycle value for the gene of interest and Ct (housekeeping gene) represents the threshold cycle value for the housekeeping gene used for normalization (B-actin gene) Subsequently, the relative expression of all samples in comparison with the control was calculated using the formula

$$RE = 2^{-\Delta\Delta Ct}, \text{ where } \Delta\Delta Ct = \Delta Ct\ (\text{sample}) - \Delta Ct\ (\text{control})$$

Where RE indicates the relative expression, ΔCt (sample) is the difference between the Ct values of the target gene and the housekeeping gene(s) for the test sample, and ΔCt (control) is the difference between the Ct values of the target gene and the housekeeping gene(s) for the control sample (samples in water).

CHAPTER 4

Results

4.1 RNA Extraction and Assessment

RNA was extracted using the RNeasy Plant Mini Kit from both leaves and roots. As shown in table 4.1 The quantity of RNA extracted from the leaves ranged from 92 ng/ul to 180 ng/ul, while the quality, which is measured at concentration of 260/280, ranged from 1.5 to 2.2. As for the RNA extracted from the roots (table 4.2); the quantity ranged from 100 ng/ul to 950 ng/ul, while the quality at 260/280 ranged from 1.4 to 1.5. The 260/230 concentration shows the content of polysaccharides present, which was acceptable and ranged from 1.2 to 12.0 in the leaves, while in the roots it ranged from 1.4 to 1.7.

A. Table 4.1 Assessment of RNA quality and quantity extracted from RAM leaves:

Sample Name	RNA Concentration	260/280	260/230
1. 0A	180ng/ul	2.0	2.0
2. 0B	138ng/ul	2.0	1.9
3. 0C	142ng/ul	2.2	1.2
4. 1A	92ng/ul	2.1	1.6
5. 1B	126ng/ul	2.1	1.5
6. 1C	160ng/ul	2.2	1.7
7. 2A	100ng/ul	2.0	1.8
8. 2B	105ng/ul	1.5	1.7
9. 2C	100ng/ul	1.8	1.9

B. Table 4.2 Assessment of RNA quality and quantity extracted from RAM Roots:

Sample Name	RNA Concentration	260/280	260/230
1. 0A	616ng/ul	1.4	1.6
2. 0B	770ng/ul	1.4	1.4
3. 0C	700ng/ul	1.5	1.6
4. 1A	616ng/ul	1.4	1.4
5. 1B	740ng/ul	1.5	1.5
6. 1C	770ng/ul	1.4	1.5
7. 2A	100ng/ul	1.5	1.5
8. 2B	950ng/ul	1.5	1.7
9. 2C	500g/ul	1.4	1.5

4.2 Relative Quantification Result Analysis

In this section, we present the findings of our study investigating the effects of CBZ on the gene expression of eight genes related to stress response in the leaves and roots of the plants. Two different CBZ concentrations were used 10nM and 200nM, and water was used as a control treatment.

4.2.1 HsfA1 gene

For the HsfA1 gene expression in the leaves, at 10nM concentration there was an eight-fold increase in its expression after 48 hours (Fig 4.1 A). However, at 200nM, a similar trend was seen yet the increase was 2.5-folds at 48 hours (Fig 4.1 B). As for the same gene expression in the roots, at both concentrations there was a 2-fold increase after 3 days, interestingly after one day, the 10 nM resulted in a down regulation (Fig 4.2 A), while at 200nM the gene expression was upregulated (3times) (Fig 4.2 B).

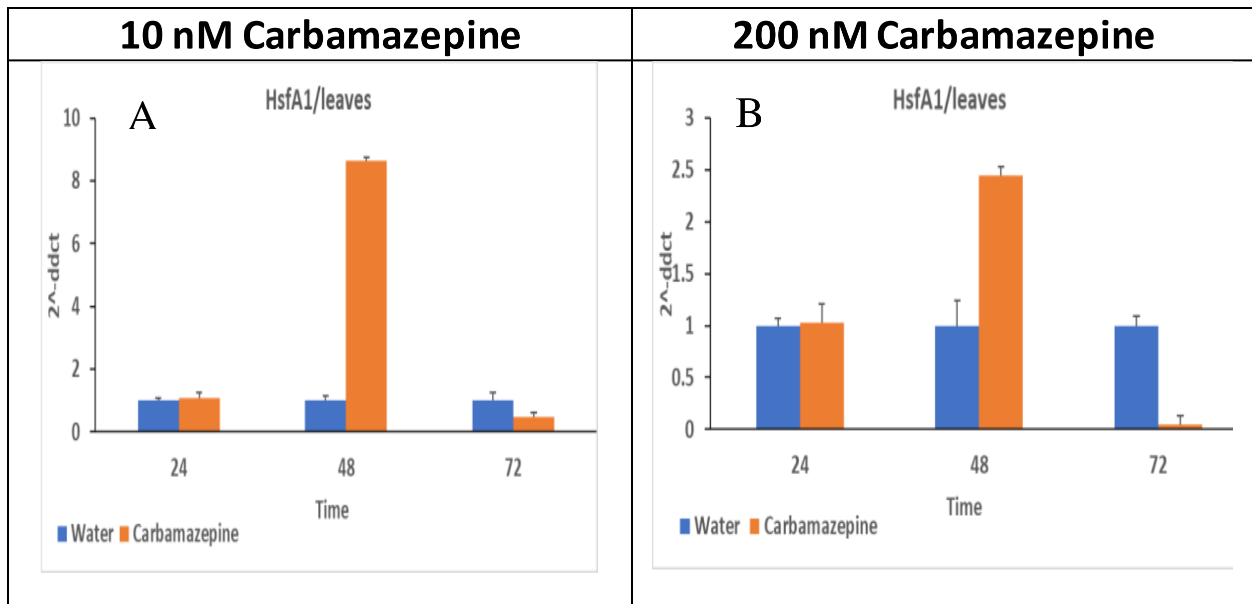

Fig 4.1 The effect of CBZ on the expression of the HsfA1 gene on RAM cultivar leaves, at concentration of 10nM (A) and 200nM (B). Bars represent standard deviation.

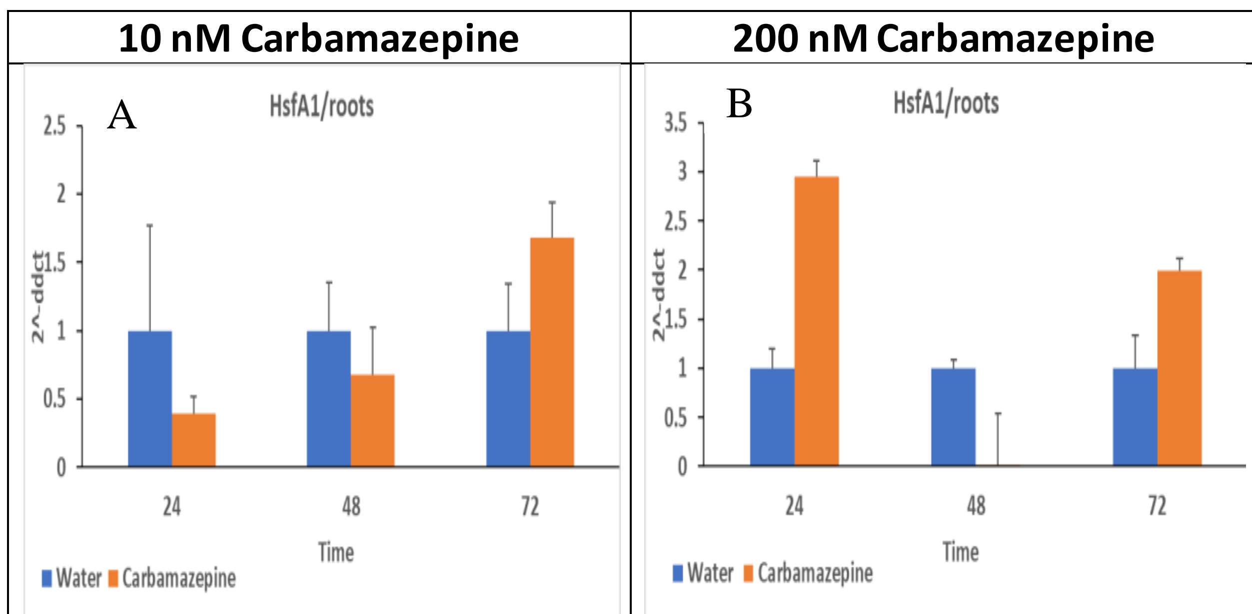

Fig 4.2 The effect of CBZ on the expression of the HsfA1 gene on RAM cultivar roots, at concentration of 10nM (A) and 200nM (B). Bars represent standard deviation.

4.2.2 HsfB1 gene

The HsfB1 gene expression in the leaves, at 10nM concentration there was an increase in its expression (5 times) after 48 hours (Fig 4.3 A). Yet, at 200nM concentration, a similar trend was seen yet the increase was 2.5 times at 48 hours (Fig 4.3 B). As for the same gene expression in the roots, at 200 nM concentration, the HsfB1 gene needed three days and a high CBZ concentration to be upregulated (20 times) (Fig 4.4 B).

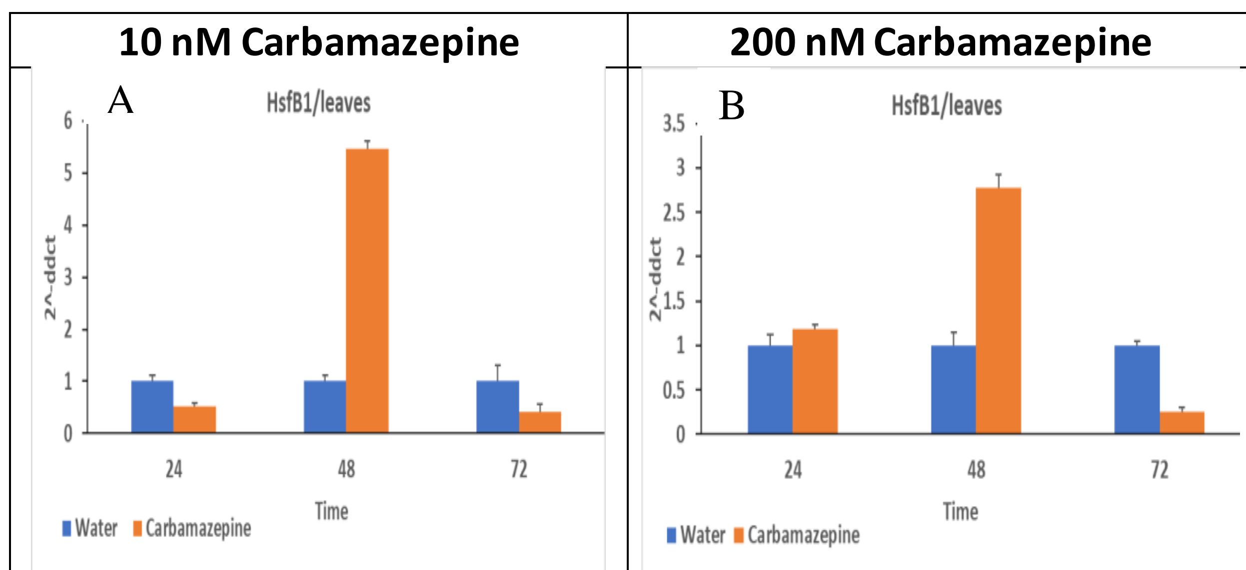

Fig 4.3 The effect of CBZ on the expression of the HsfB1 gene on RAM cultivar leaves, at concentration of 10nM (A) and 200nM (B). Bars represent standard deviation.

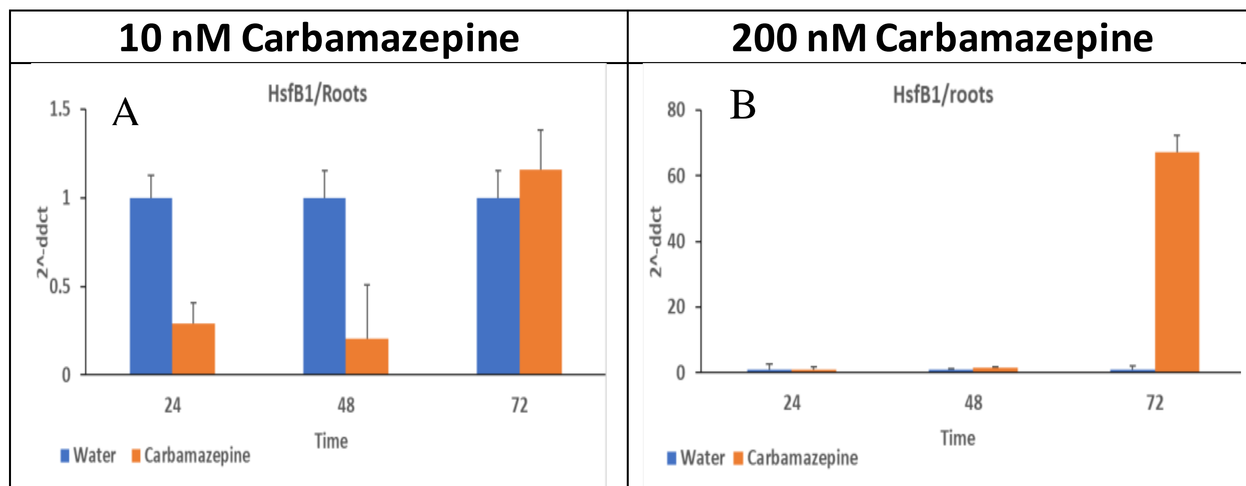

Fig 4.4 The effect of CBZ on the expression of the HsfB1 gene on RAM cultivar roots, at concentration of 10nM (A) and 200nM (B). Bars represent standard deviation.

4.2.3 Hsp90-1

The expression of the Hsp90-1 gene in leaves, at 10nM concentration there was a 2-fold increase in its expression after 48 hours (Fig 4.5 A). However, at higher concentration (200nM), there was a 4-fold increase after 24 hours (Fig 4.5 B). Though at 200 nM concentration, there was no obvious change on the gene expression (Fig 4.6 B). At the lower concentration (10nM), the expression of Hsp90-1 gene increased 4 and 6-folds after 48h and 72 h, respectively (Fig 4.6 A)

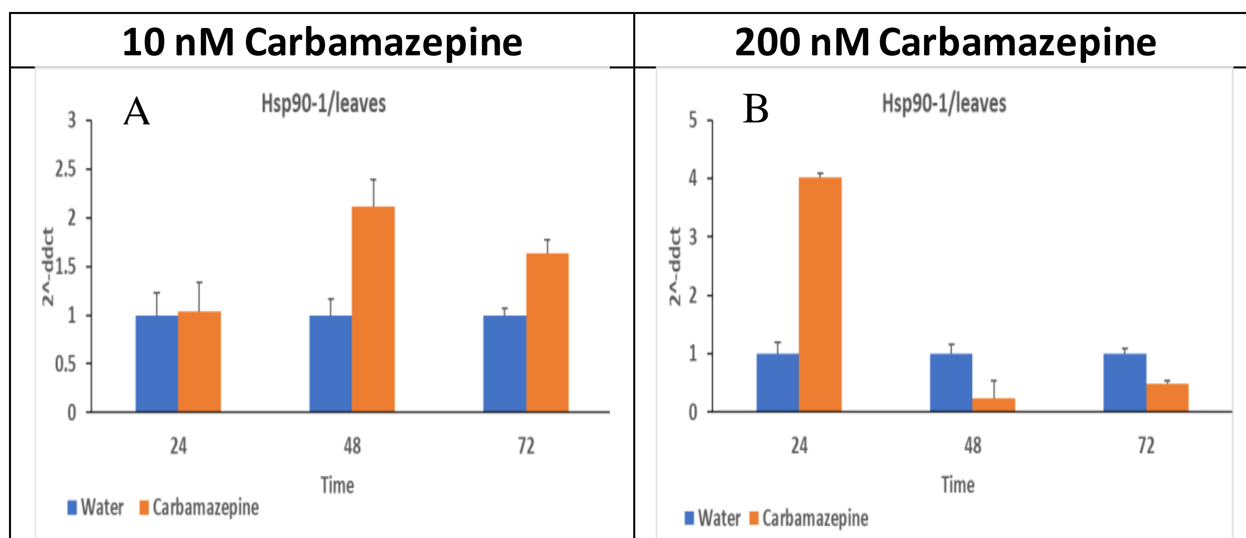

Fig 4.5 The effect of CBZ on the expression of the Hsp90-1 gene on RAM cultivar leaves, at concentration of 10nM (A) and 200nM (B). Bars represent standard deviation.

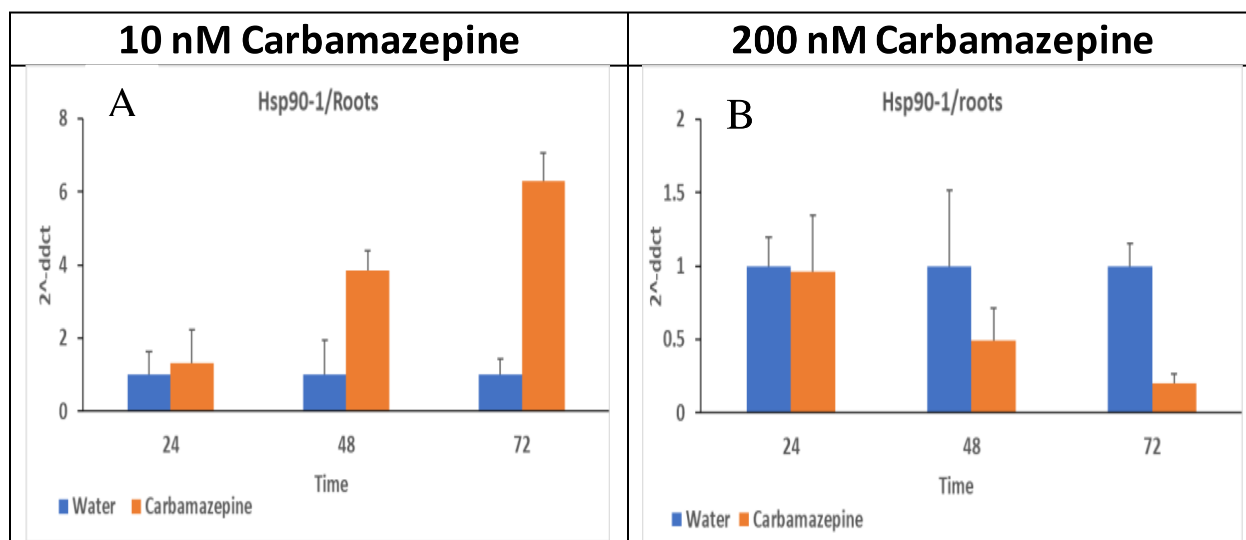

Fig 4.6 The effect of CBZ on the expression of the Hsp90-1 gene on RAM cultivar roots, at concentration of 10nM (A) and 200nM (B). Bars represent standard deviation.

4.2.4 Sgt 1 gene

As shown in Fig 4.7, the Sgt1 gene expression in the leaves, at 10nM concentration, increased 3-folds after 48 hours (Fig 4.7 A), while at 200nM concentration, a 4-fold increase was seen after 24h (Fig 4.7 B). As for the same gene expression in the roots, there was no significant difference in the gene expression of Sgt1 gene at the 10 nM concentration (Fig 4.8 A), while at 200nM concentration and after 48 hours there was a 5-folds increase in its expression (Fig 4.8 B).

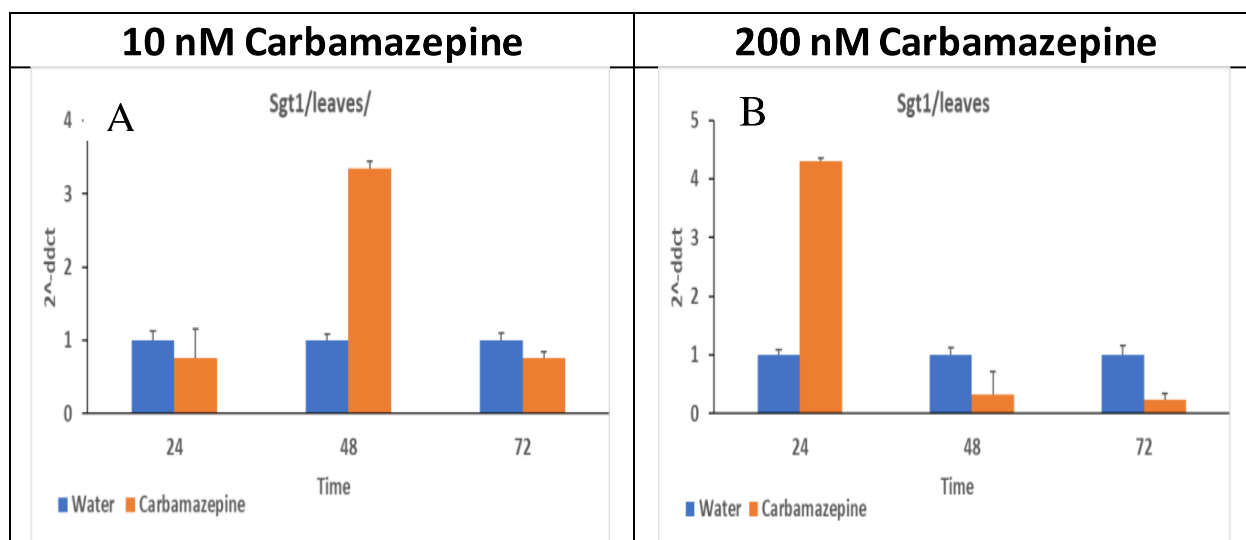

Fig 4.7 The effect of CBZ on the expression of the Sgt1 gene on RAM cultivar leaves, at concentration of 10nM (A) and 200nM (B). Bars represent standard deviation.

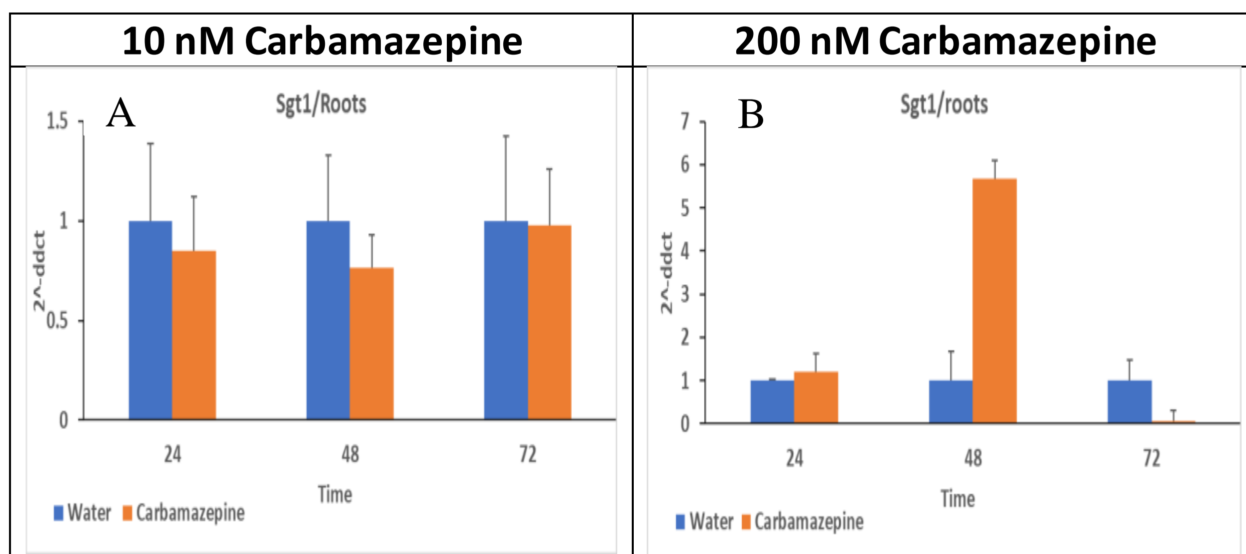

Fig 4.8 The effect of CBZ on the expression of the Sgt1 gene on RAM cultivar roots, at concentration of 10nM (A) and 200nM (B). Bars represent standard deviation.

4.2.5 Apx 1 gene

The Apx1 gene expression, at both concentrations, showed no significant difference between the control and the treatment samples in the leaves (Fig 4.9). While expression of Apx1 gene was increased by 2.5 -folds after 48 hours at 10 nM concentration (Fig 4.10 A), it was significantly upregulated (20-folds) after the same time at 200 nM concentration (Fig 4.10 B)

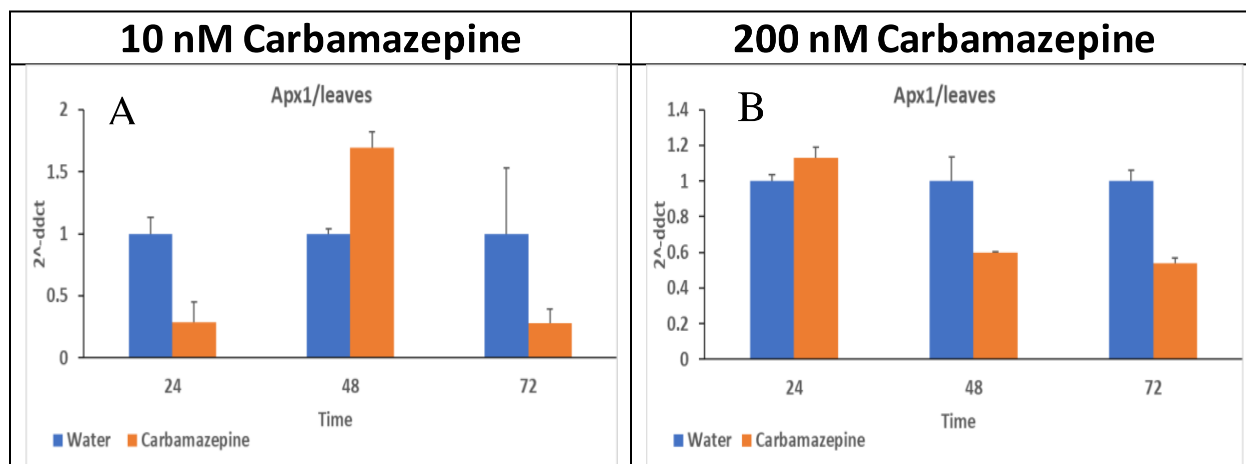

Fig 4.9 The effect of CBZ on the expression of the Apx1 gene on RAM cultivar leaves, at concentration of 10nM (A) and 200nM (B). Bars represent standard deviation.

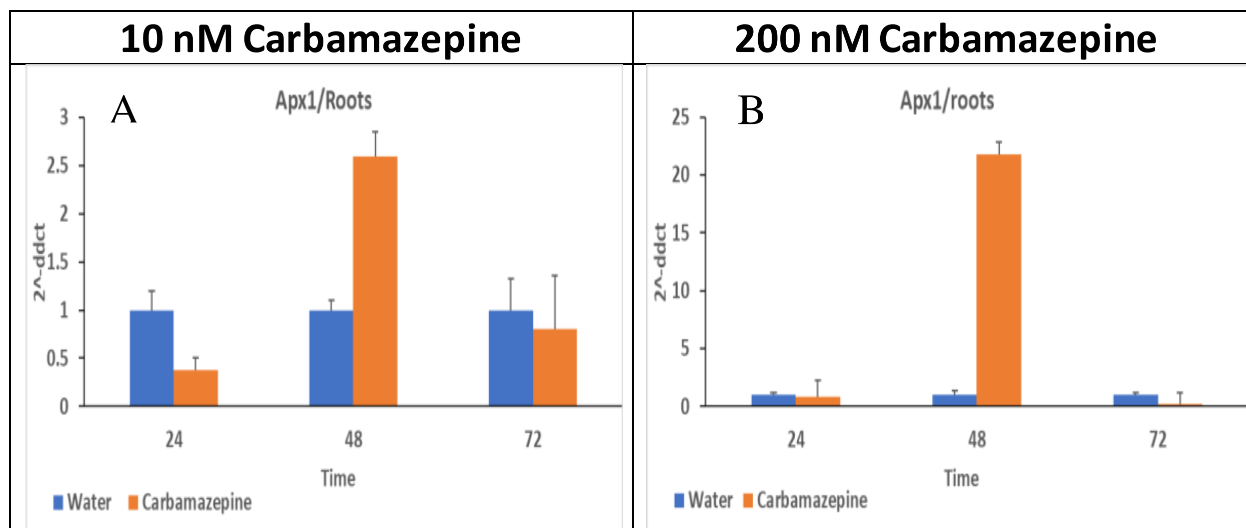

Fig 4.10 The effect of CBZ on the expression of the Apx1 gene on RAM cultivar roots, at concentration of 10nM (A) and 200nM (B). Bars represent standard deviation.

4.2.6 ATG 18 gene

The expression of ATG18 in the leaves, at 10nM concentration there was an 8- fold increase in its expression after 48 hours (Fig 4.11 A), yet a lower increase (2.5-folds) was observed after the same time at 200 nM concentration (Fig 4.11 B). While the expression of ATG18 in the roots significantly increased (8-folds) after 24h at 10 nM concentration (Fig 4.12 A), it was downregulated after 48h at 200 nM concentration (Fig 4.12 B).

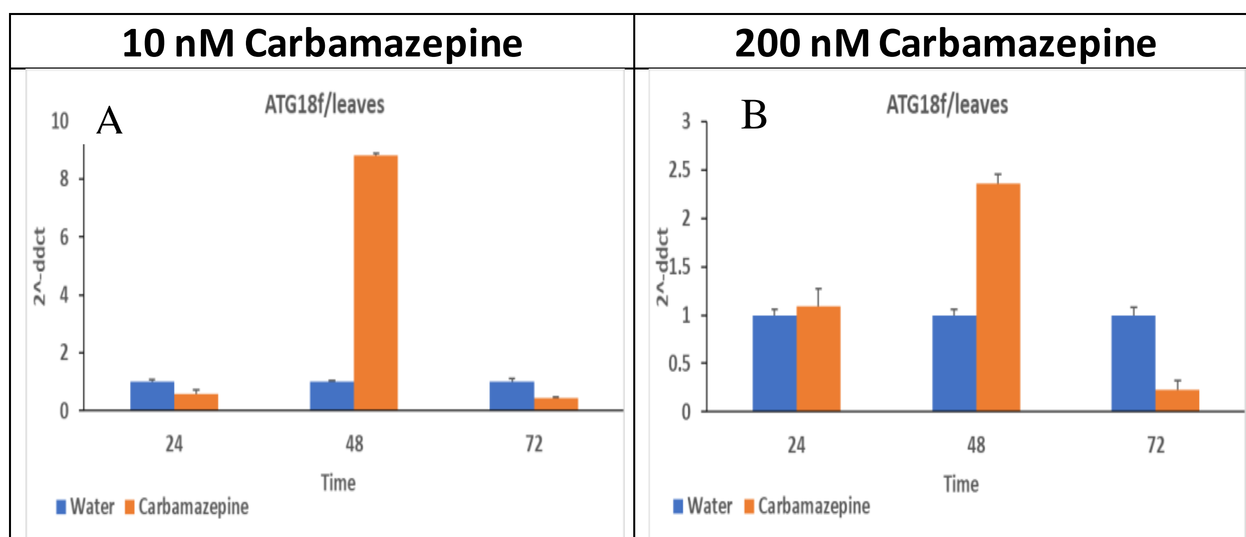

Fig 4.11 The effect of CBZ on the expression of the ATG18 gene on RAM cultivar leaves, at concentration of 10nM (A) and 200nM (B). Bars represent standard deviation.

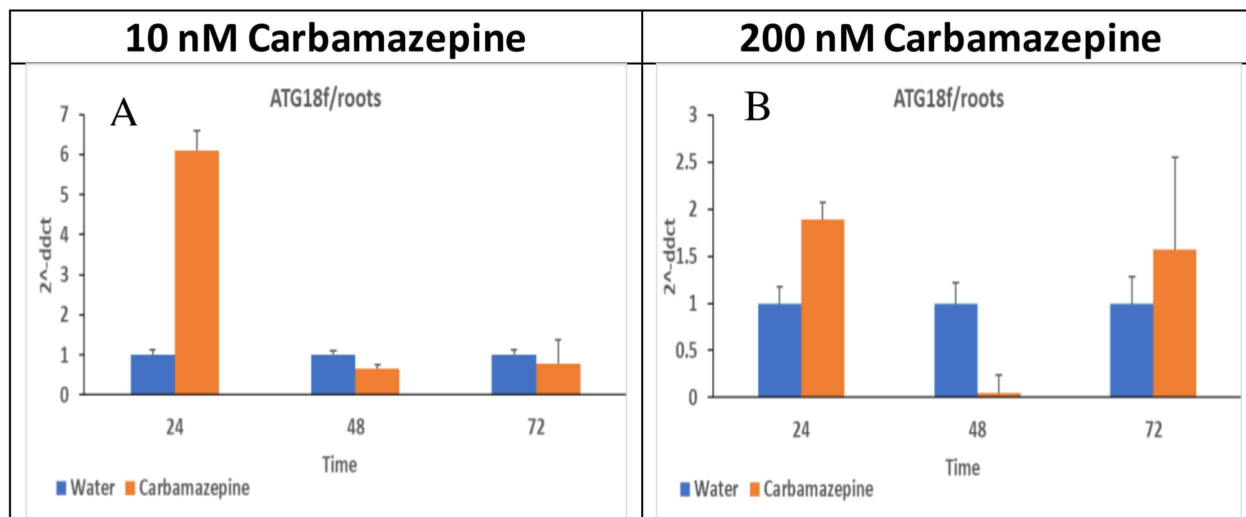

Fig 4.12 The effect of CBZ on the expression of the ATG18 gene on RAM cultivar roots, at concentration of 10nM (A) and 200nM (B). Bars represent standard deviation.

4.2.7 GAD1 gene

At 10 nM CBZ concentration, GAD1 was upregulated (9-folds) after 48h (Fig 4.13 A). However, such upregulation started after 24h of exposure to CBZ (4-folds) and continue to be upregulated up to 48h at 200nM concentration (Fig 4.13 B). In the roots, GAD1 gene was mildly upregulated after 72h at 200nM concentration (Fig 4.14 B).

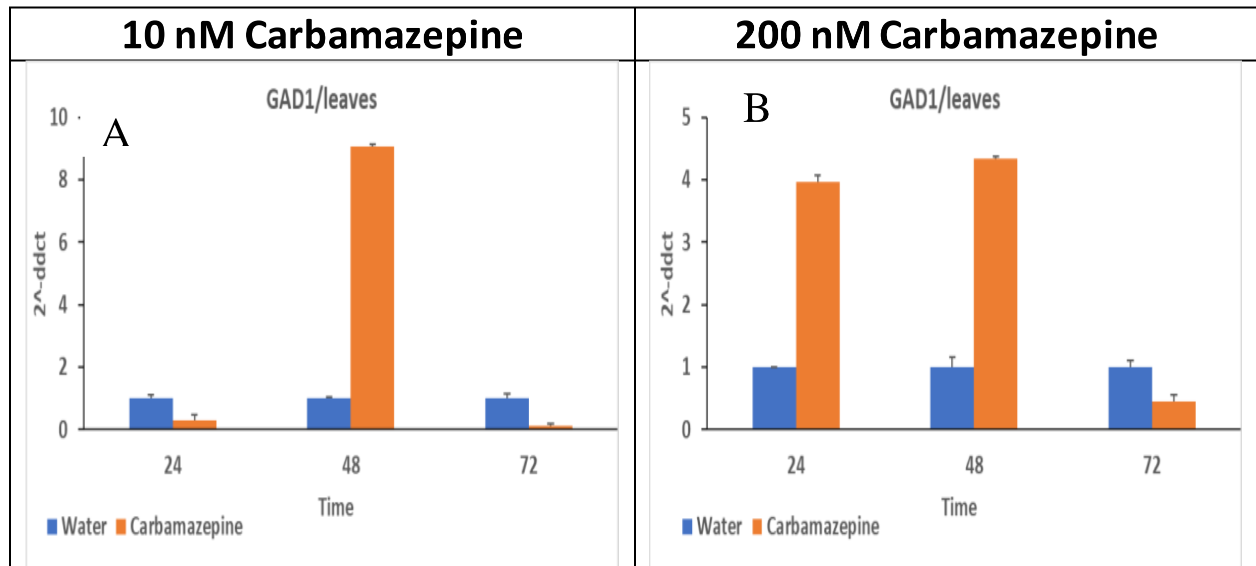

Fig 4.13 The effect of CBZ on the expression of the GAD1 gene on RAM cultivar leaves, at concentration of 10nM (A) and 200nM (B). Bars represent standard deviation.

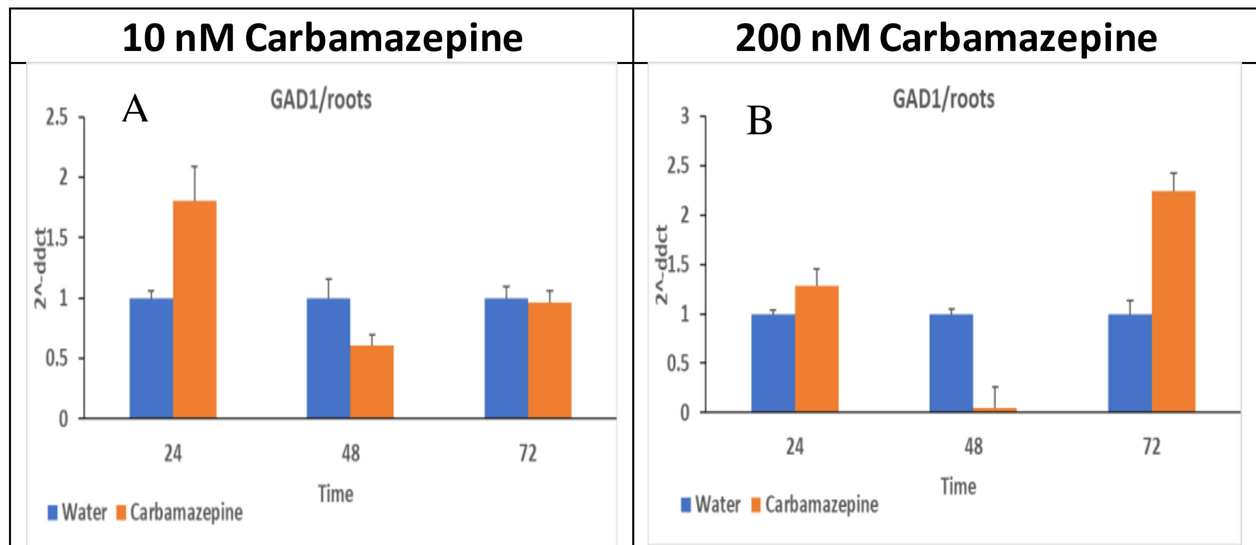

Fig 4.14 The effect of CBZ on the expression of the GAD1 gene on RAM cultivar roots, at concentration of 10nM (A) and 200nM (B). Bars represent standard deviation.

4.2.8 GAD2 gene

As shown in Fig 4.15 A, GAD2 gene expression was increased 8-folds after 48h of exposure to 10 nM of CBZ. However, only 3-fold increase was observed for this gene at 200nM after 24h (Fig 4.15 B). In the roots, exposure to 200nM of CBZ increases GAD2 expression by 4-folds after 24h (Fig 4.16 B). At the lower concentration (10 nM), no significant change in GAD2 expression was observed (Fig 4.16 A).

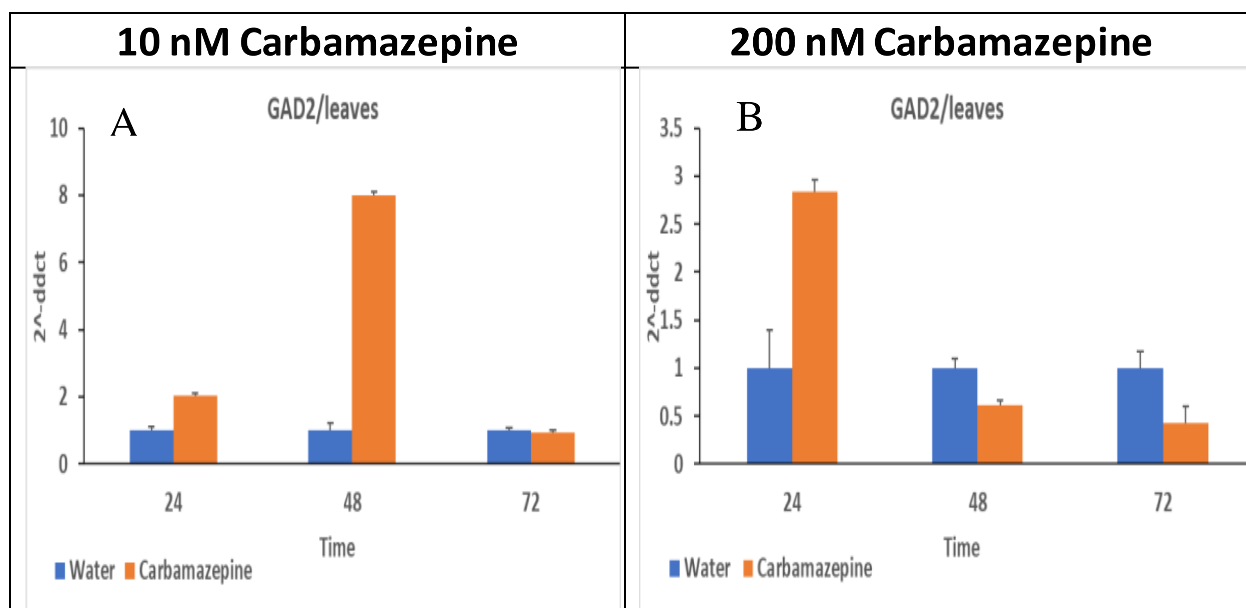

Fig 4.15 The effect of CBZ on the expression of the GAD2 gene on RAM cultivar leaves, at concentration of 10nM (A) and 200nM (B). Bars represent standard deviation.

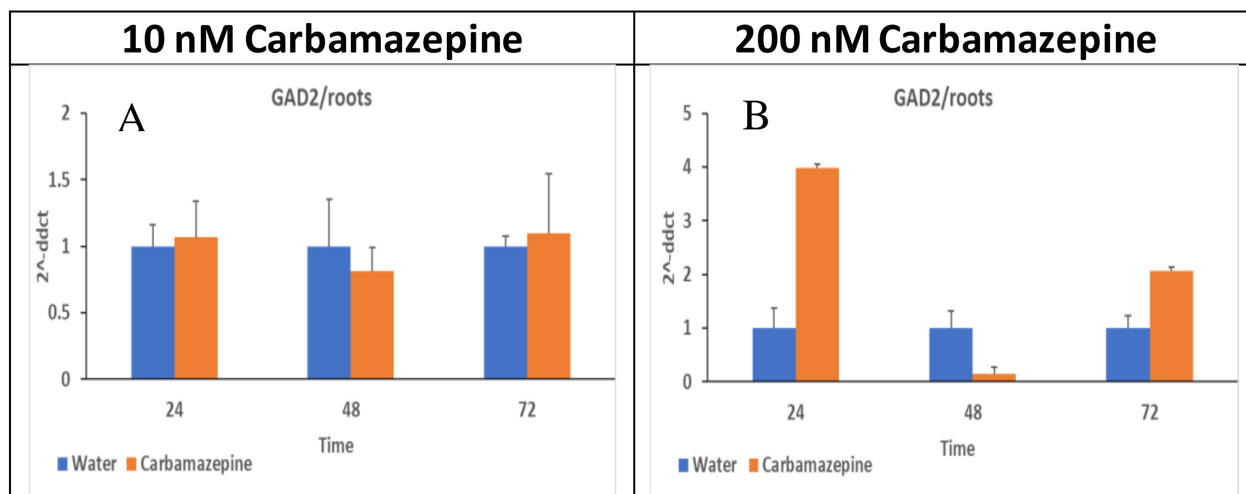

Fig 4.16 The effect of CBZ on the expression of the GAD2 gene on RAM cultivar roots, at concentration of 10nM (A) and 200nM (B). Bars represent standard deviation.

4.3 Real time RT-PCR graphs

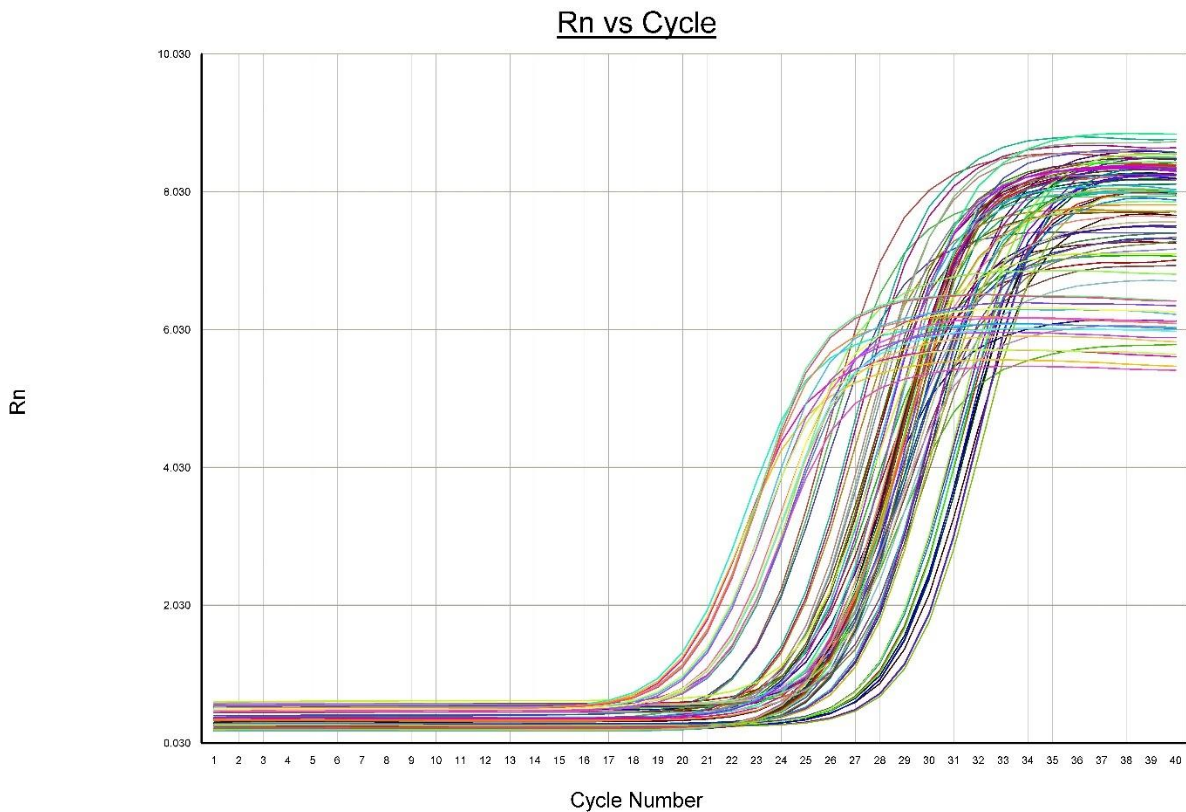

Fig 4.17 qPCR amplification plot (Rn vs. cycle number) of 4 genes (HsfA1, ATG18, GAD1, GAD2) from the leaf's samples. cDNA from tomato plants irrigated with CBZ or water were run in triplicate. B-actin served as an internal control. NTC (no template control) was negative and showed no amplification in 40 cycles.

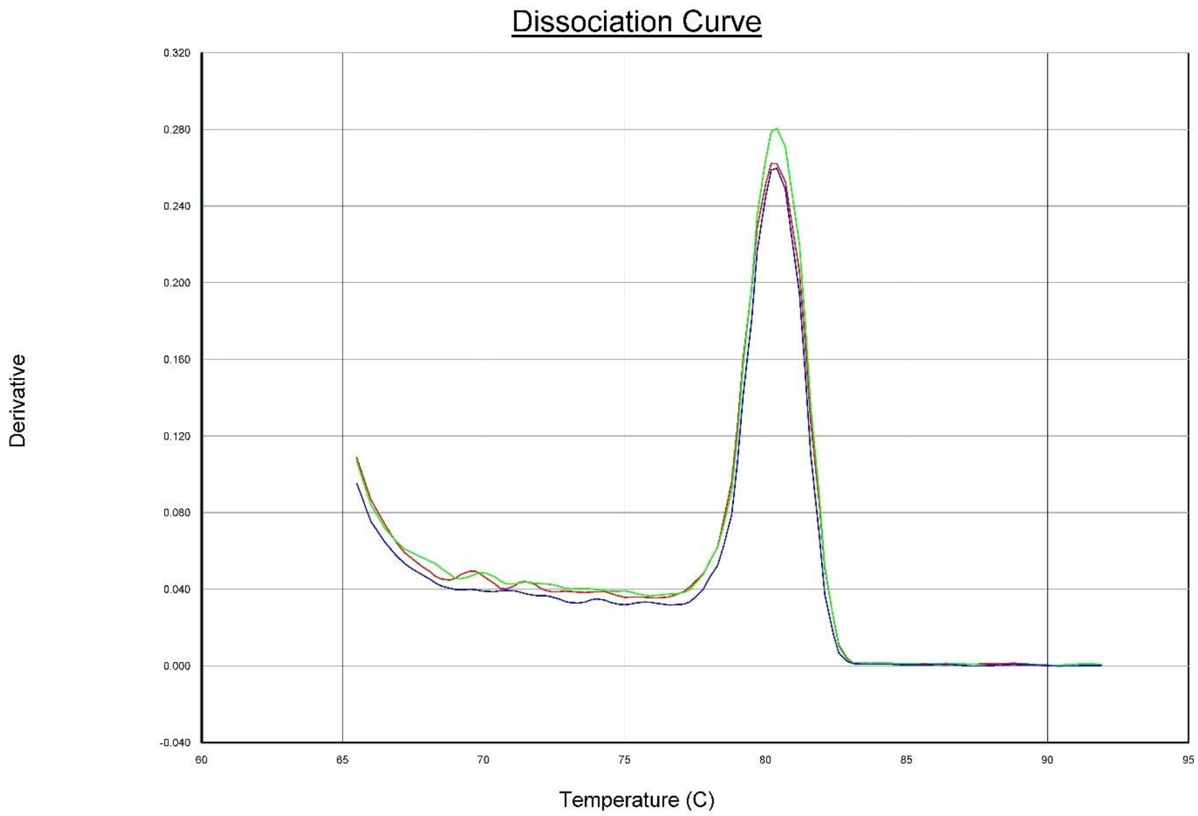

Fig 4.18 Melt curve analysis to ensure assay specificity and different PCR products can be differentiated by their melting features.

CHAPTER 5

Discussion

In this study, we investigated the potential impact of carbamazepine (CBZ), a pharmaceutical compound ubiquitous in RWW, on stress-related gene expression in the Palestinian local tomato cultivar 'RAM'. This research is particularly important given the increasing use of RWW in many countries, driven by water scarcity concerns, and the associated risks posed by pharmaceutical contamination.

Recent studies have highlighted the presence of various pharmaceuticals, including CBZ, in RWW, raising concerns about their potential impact on both plants and human's health ((Mordechay et al.2021); (Christou et al., 2017)). Our study builds upon this knowledge by investigating how CBZ affects the expression of stress-related genes in tomato plants, which are essential for their adaptation and survival under different environmental conditions.

Two different concentrations of CBZ were employed in this study: 10nM and 200nM. The 10nM concentration equates to approximately 2.358 ug/l of CBZ, which is comparable to the concentration of 1.3 to 2.2ug/l recently detected in RWW utilized for irrigating tomato plants in Israel (Mordechay et al., 2018). The 200nM concentration was employed to investigate the impact of a significantly higher CBZ concentration, approximately 20 times greater than that detected in reclaimed wastewater (RWW), on the tomato plant.

For conducting this experiment, the hydroponic system was chosen in which plants are grown without soil by immersing their roots in a nutrient-rich water (Son et al.,2020). Hydroponic systems represent a relatively novel approach to agriculture, by overcoming the constraints associated with soil-based systems. Hydroponic systems offer various advantages such as

enhanced water availability, accelerated growth rates, and higher crop yields (Gashgari et al.,2018). In addition, hydroponic systems have the ability to provide precise control over growing conditions, thereby minimizing external influences and allowing for accurate assessment of CBZ effects. This approach is particularly advantageous for studying pharmaceutical-plant interactions, as it enables the isolation of specific variables, such as CBZ exposure, while maintaining optimal plant growth conditions.

When plants encounter stressful conditions such as drought, extreme temperatures, nutrient deficiencies, or toxic metabolites, they activate specific stress-related genes as part of their response mechanism (Zai et al.,2015). These genes encode for proteins that help the plant adapt and survive under adverse conditions. For example, some stress-related genes may produce enzymes involved in the synthesis of protective compounds like antioxidants, which help maintain cellular integrity and prevent damage from reactive oxygen species (Sun et al., 2018). Other genes may regulate the expression of water transporters or ion channels to optimize water uptake and retention in drought-stressed plants. Additionally, stress-related genes can also trigger signaling pathways that activate stress-responsive transcription factors, leading to changes in gene expression patterns throughout the plant.

The selection of eight stress-related genes for analysis was based on their crucial roles as key regulators during plant exposure to stress conditions. These genes encode proteins involved in various protective mechanisms which are vital for plant survival. By focusing on these genes, we aimed to gain a deeper understanding of gene expression patterns in response to CBZ treatment.

Our results revealed distinct patterns of gene expression in tomato leaves and roots following CBZ treatment. In leaves, a consistent upregulation of gene expression was observed after 48 hours of exposure, indicating an initial stress response followed by a return to baseline levels.

Moreover, this pattern was further emphasized by the observation that this upregulation occurred in seven out of eight genes studied, indicating a robust and widespread response to CBZ exposure. Furthermore, among the upregulated genes, GAD1, GAD2, HsfA1, and ATG18 (which corresponds to Fig 4.1, Fig 4.11, Fig 4.13, Fig 4.15 respectively) emerged as the most prominently upregulated. This highlights the potential importance of these genes in the tomato plant's response to CBZ-induced stress, suggesting their involvement in key defense pathways or regulatory mechanisms. As this pattern was observed at the 10nM CBZ concentration.

The differential expression of genes in response to CBZ treatment reflects the dynamic interplay between stress perception, signal transduction, and activation of defense mechanisms. GAD1 and GAD2, for instance, are involved in the synthesis of γ-aminobutyric acid (GABA), a non-protein amino acid known for its roles in stress tolerance and signaling.

HsfA1 belongs to the heat shock transcription factor family, which orchestrates the expression of heat shock proteins (HSPs) crucial for protein folding and stress adaptation. In contrast, ATG18 participates in autophagy, a cellular recycling process vital for maintaining cellular homeostasis under stress. Moreover, their expression levels could be related, as according to (Mishra et al., 2022), their study explored the intricate relationship between environmental (drought) and biological (virus) factors in influencing the health and resilience of tomato plants, both of which induce stress in tomatoes. The results indicated that although drought only mildly increased HSP induction in leaf and root tissues, the pivotal role of tomato HsfA1a in drought tolerance involves

activating ATG genes and inducing autophagy. Specifically, ATG10 and ATG18f are genes regulated by HsfA1. Several assays demonstrated that HsfA1a directly binds to the promoters of ATG10 and ATG18f. Silencing ATG10 and ATG18f not only reduces HsfA1-dependent drought tolerance but also inhibits autophagosome formation in plants overexpressing HsfA1. Thus, the induction of HsfA1a under drought stress leads to increased transcripts of ATG10 and ATG18f, highlighting the critical role of HSFA1 and ATG18f in regulating autophagy and enhancing plant resilience to drought.

APX1 is one such enzyme involved in scavenging hydrogen peroxide, a type of ROS. Interestingly, despite the significant alterations in the expression levels of several genes, the gene APX1 did not show any significant difference in its expression levels. This suggests that other stress-induced mechanisms might be involved in such the response to CBZ-induced stress.

However, at the 200nM CBZ concentration, this upregulation pattern started after 48 hours as in the 10nM concentration or started earlier after 24 hours of exposure to CBZ. This temporal pattern suggests that tomato plants activate specific defense mechanisms upon encountering CBZ, potentially to mitigate its adverse effects.

Conversely, the roots exhibited a less profound pattern of gene expression changes, where at the lower concentration (10nM), only three genes were upregulated (Hsp90-1, ATG18, APX1). While at the higher concentration (200nM), more genes were involved with the APX1 being upregulated up to 20 times. In the roots, the trend of gene expression was less obvious, which may be attributed to differences in CBZ accumulation between leaves and roots. The transpiration process in leaves could have led to a higher CBZ accumulation compared to roots, influencing the observed gene expression patterns. As in a study conducted by Shenker et al.

(2011) on cucumber plants grown in hydroponic systems, in which the uptake of and bioaccumulation of CBZ was greater in the leaves (comprising 76-84% of the total uptake). This indicates that CBZ is primarily transported through the transpiration stream. Further investigation into the mechanisms governing CBZ uptake and distribution within plant tissues is needed to better understand its effects on root physiology.

The results showed significant alterations in the expression levels of stress-related genes in response to CBZ treatment, demonstrating the ability of pharmaceutical compounds to modulate gene expression in plants. As a similar previous study was conducted on the tomato seedling, which had their roots bathing in different CBZ concentrations;10nM,100nM,1000nM respectively (Gorovits el al.,2020). The study showed that plants develop a typical stress response as several stress biomarkers, such as HSPs, GABA shunt enzymes and glutamate decarboxylase (GAD1 and 2), reacted noticeably to the presence of CBZ in both leaves and roots. Furthermore, for this study, we employed the Real Time rt-PCR analysis technique for the relative quantification of specific mRNAs. In the abovementioned study, (Gorovotis et al. (2020)), explored the effects of CBZ on gene expression using the Western blot technique, which assesses protein levels rather than mRNA transcription levels as evaluated by Real time rt-PCR.

To the best of our knowledge, there has been insufficient research conducted on the impact of CBZ on the expression of stress-related genes in tomatoes. The current study represents the first attempt to investigate how CBZ in RWW affects gene expression in a local Palestinian tomato cultivar known as 'RAM'. As Palestine grapples with increasing water scarcity, the use of reclaimed wastewater for agricultural purposes is likely to become more prevalent. Our study

underscores the importance of assessing the potential risks associated with pharmaceutical contamination in RWW and its impact on crop plants and may subsequently affect human health.

Despite the insights provided by this study, several limitations must be acknowledged. Our experimental design focused on a single tomato cultivar under controlled conditions, limiting the generalizability of our findings to other plant species and environmental contexts. Additionally, the concentrations of CBZ used in our experiments may not fully reflect environmental exposure levels, warranting further investigation into dose-response relationships.

In conclusion, our study investigated the effects of carbamazepine, a prevalent pharmaceutical in reclaimed wastewater, on stress-related gene expression in the Palestinian local tomato cultivar 'RAM'. Given the increasing use of RWW, understanding the risks of pharmaceutical contaminants is crucial. Our research showed that CBZ exposure leads to distinct gene expression patterns in tomato leaves and roots, indicating an initial stress response, particularly in leaves. This study highlights the ability of pharmaceutical compounds to significantly alter plant gene expression. Despite focusing on a single tomato cultivar under controlled conditions, our findings underscore the need for further research to generalize these results to other plant species and real-world environments, considering the growing reliance on RWW in agriculture and its potential implications for crop health and human safety.

CHAPTER 6

Recommendations

- Further studies are recommended to reveal the effect of other drugs that are present in RWW on the health of tomato and other important plants.
- Human consumption of plants irrigated with RWW should be assessed based on the possible effects of such plants on the human health. This necessitates more studies to be conducted to reveal whether RWW irrigated plants have negative impact on human health.
- Such research requires cooperation between the academic researchers and the Ministry of Agriculture.

CHAPTER 7

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